Determining lineage fate, survival and proliferation of differentiating thymocytes: interplay between Notch, TCR, PI3K and MAPK pathways

by

Gladys W. Wong

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Immunology
University of Toronto

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Doctor of Philosophy
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2012

Abstract

A common bipotent thymocyte precursor gives rise to both lineages of T cells, αβ and γδ. This thesis addresses how the interplay between intrinsic T cell receptor (TCR) signals and cell extrinsic signals provided by Notch and TCR ligands help to assign and support a final lineage fate decision. Emerging data supports a model in which differential TCR signaling capacity plays an instructional role in specifying lineage fate, particularly through induction of the ERK - early growth response gene (Egr) - inhibitor of DNA binding 3 (Id3) pathway. In particular, Id3 expression serves to regulate adoption of the γδ fate. Moreover, Id3 is both necessary and sufficient to enable γδ-lineage cells to differentiate independently of Notch signaling and become competent interferon (IFN)-γ-producing effectors. These findings identify Id3 as a central player that controls both adoption of the γδ fate and their maturation in the thymus. While loss of Notch signaling in γδTCR-expressing CD4-CD8- (DN)3 cells does not affect development, Notch signals are critical for pre-TCR-bearing cells to transition to the CD4+CD8+ (DP) stage of αβ T cell development. Notch signals affect the activation of the PI3K/Akt pathway, which is required for pTα/TCRβ (pre-TCR)-induced survival, differentiation and proliferation of developing αβ-lineage thymocytes. Here, I identify the key molecular players responsible for the interaction between the Notch and PI3K pathways at this critical developmental stage. Notch
induction of *Hes1* expression is necessary to repress the expression of the PI3K/Akt pathway inhibitor, PTEN, which in turn facilitates pre-TCR-induced differentiation. *c-Myc*, another critical target of Notch, is required for proliferation during β-selection. Lastly, I find that the majority of DN3 cells expressing both pre-TCR and γδTCR follow the signal strength model for lineage development, and commit and mature along the γδ-lineage. However, manipulation of signal strength, through γδTCR ligand availability or Id3 expression, can skew this development outcome. Taken together, the results from this thesis provide a detailed examination of the molecular mechanisms that are instrumental in determining lineage fate, survival, and proliferation of differentiating thymocytes. Central to these outcomes is the interplay between the Notch, TCR, PI3K, and MAPK signaling pathways.
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To my best friends, Loretta and Michael, who know everything about me and still love me despite this. You have been with me to celebrate life’s incredible ups and mourn life’s bitter downs. I would be lost without you. Sis, you are the most courageous, generous, and beautiful person I know. I love you so much, and I am so proud to be your sister. You are the source of my most cherished memories. Michael, you are patient, loving, kind, and generous, and you are by far the smartest person I know. You have shown me the world in its true light, and inspired me to be a better person, to aspire to heights I never imagined possible before I met you. I am grateful for your love, and return it whole-heartedly.

To my grandfather and grandmother, I hope you know how much I love you and miss you. I strive to be the best person I can be so that I can make you proud.

To Bullet and the Boos, you were the best pets in the whole world. I love you, and hope you are doing well in Pet Heaven.

This thesis is dedicated to all of you.

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<tr>
<td>β2M</td>
<td>β2-microglobulin</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic helix-loop-helix</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>DETC</td>
<td>Dendritic epidermal T cell</td>
</tr>
<tr>
<td>Dhh</td>
<td>Desert hedgehog</td>
</tr>
<tr>
<td>DL1</td>
<td>Delta-like 1</td>
</tr>
<tr>
<td>DN</td>
<td>Double-negative</td>
</tr>
<tr>
<td>DP</td>
<td>Double-positive</td>
</tr>
<tr>
<td>Egr</td>
<td>Early growth response</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FL</td>
<td>Fetal liver</td>
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<td>FTOC</td>
<td>Fetal thymic organ culture</td>
</tr>
<tr>
<td>HES-1</td>
<td>Hairy and enhancer of split 1</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>ICN</td>
<td>Intracellular domain of Notch receptor</td>
</tr>
<tr>
<td>Id</td>
<td>Inhibitor of DNA binding</td>
</tr>
<tr>
<td>Ihh</td>
<td>Indian hedgehog</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>LSK</td>
<td>Lineage⁻ Stem cell antigen 1 (Sca1)⁺ CD117⁺</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>PDK</td>
<td>phosphoinositide-dependent kinase</td>
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</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
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<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<td>ZAP70</td>
<td>Zeta-chain-associated protein kinase 70</td>
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Chapter 1

Introduction

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1 Hematopoiesis and lymphocyte development

All mature blood cells are generated from a self-renewing, multipotent hematopoietic stem cell (HSC). The first definitive site of hematopoiesis is the dorsal aorta. Some reports indicate that the hemangioblast, a mesodermal progenitor with bipotent endothelial and hematopoietic potential (1, 2), generates a phenotypically endothelial intermediate, the hemogenic endothelium, which retains hematopoietic potential and subsequently gives rise to HSCs within the dorsal aorta (3, 4). Others have suggested that the aortic floor itself is hemogenic. In this case, HSCs develop directly from the aortic floor, in a process whereby contractions in the floor of the dorsal aorta expel endothelial cells into the sub-aortic space, which subsequently leads to their transformation into hematopoietic cells (5). The hematopoietic cells generated during mid-gestation within the dorsal aorta enter the circulation and migrate to the placenta and the fetal liver (FL) (6, 7). In the FL, HSCs reside and expand until just prior to birth, when they migrate to their permanent home and major site of adult hematopoiesis, the bone marrow (BM) (8-10).

The most immature hematopoietic progenitors in the bone marrow are termed LSK, owing to the fact that they express cell antigens CD117 (c-Kit) and Sca-1, but lack expression of other lineage markers associated with more differentiated cell types (11, 12). Within this heterogeneous LSK population, the expression of recombination-activating genes, Rag1 and Rag2, as well as CD135 (Flt3) cytokine receptor, mark these cells as lymphoid-primed early during hematopoiesis (13, 14). Recent studies show that the earliest T-cell progenitors in the adult thymus do not retain B-cell potential (15, 16), but myeloid potential is retained in these cells (17-19). These conclusions lend support to a model of lineage divergence (Fig. 1) which proposes that the myeloid lineage is the default lineage, and that gene expression must be altered from this baseline state to specify blood cells into other respective cell lineages (20).

2 T cell development

The thymus is seeded with progenitor cells as early as embryonic day 11.5 (E11.5) in mice (21). Hematopoietic progenitors migrating into the thymus have the potential to initiate a T cell
Figure 1. The model for myeloid-based hematopoiesis as it compares to the classical model. The myeloid-based model proposes that the myeloid lineage is the default lineage, and that gene expression must be altered from this baseline state to specify blood cells into other respective cell lineages. MPP = multipotent progenitor, CMEP = common myeloid/erythroid progenitor, CLP = common lymphoid progenitor, CMLP = common myeloid/lymphoid progenitor. E, M, T, B represent erythroid, myeloid, T and B cells, respectively. Figure adapted from Kawamoto et al. (20).
program, characterized by discrete stages of extensive proliferation, increasing lineage commitment, and selection (Fig. 2). This highly ordered process is governed by a multitude of molecular signals and environmental cues. CD4−CD8− double-negative (DN) cells, which can be further characterized based on the expression of CD44, CD117, and CD25, constitute the earliest T cell progenitors residing in the thymus (22-25). The most primitive CD44+CD117+CD25− DN1 cells are multipotent (18, 19, 26, 27), and expression of CD25 marks their entry into the T cell lineage specified DN2 stage (28, 29). At this time, recombination of the T cell receptor (TCR)β, TCRγ, and TCRδ loci commences with the expression of Rag1 and Rag2 (30). Recombination continues into the subsequent CD44−CD117−CD25+ DN3 stage, wherein thymocytes are irreversibly committed to the T cell lineage and subjected to the first developmental checkpoint (24, 31). DN3 cells that express productively rearranged TCRβ chain with its partner, invariant pre-TCRα, in a pre-TCR complex with CD3 molecules signal through this complex and up-regulate key downstream molecules which allow them to survive β-selection and differentiate to the subsequent CD4+CD8+ double positive (DP) stage (32-34). DN3 cells which express productively rearranged TCRγ and TCRδ chains are also signaled to survive, and while they mature from a CD24hi to CD24lo phenotype, they remain CD4−CD8− double-negative (35-38). In addition to rescue from apoptosis, pre-TCR and γδTCR at the DN3 stage of development also drive cellular expansion and allelic exclusion (33, 34). After the DP stage, the generation of an αβTCR, and successful passage across two more selection processes, termed positive and negative selection, leads to the maturation of DP cells into CD4 or CD8 single positive (SP) cells, which leave the thymus and enter the periphery upon receiving the appropriate exit signals (32, 39).

2.1 Generation of the pre-TCR

DN3 cells that differentiate along the αβ-lineage must form a functional pre-TCR complex in order to successfully traverse the β-selection checkpoint and mature into DP cells (32-34). Prior to β-selection, the majority of DN3 cells are in the process of rearranging their TCRβ chain, or have generated out-of-frame rearrangements (40). These cells are small, in comparison to the 15% of DN3 cells which have productively rearranged their TCRβ chain, expressed the pre-TCR, and entered S-phase following successful β-selection (40). Pre-TCR is similar to the αβ TCR in that it is composed of two components: a β-chain and an α-component. In the αβ TCR,
Figure 2. Schematic of the discrete stages of T cell development within the thymus. CD4⁻CD8⁻ double-negative (DN)1-4 cells are the most primitive subset. These cells can develop along the αβ-lineage and give rise to the subsequent CD4⁺CD8⁺ double positive (DP) cells that mature into CD4 or CD8 single positive (SP) cells. Alternatively, DN cells can develop into γδ T cells.
the TCRα chain is the protein product of a successful and productive TCRα locus rearrangement, while in the pre-TCR, the α-component is a germline-encoded surrogate of the TCRα chain, termed the pre-Tα. The pre-Tα is unique and not interchangeable with TCRα during T lymphocyte development, as it contains unique features that are responsible for optimal proliferation, survival, differentiation, and commitment in developing DN3 cells (41). These differences allow the pre-TCR to signal cell-autonomously (42) at a lower threshold, as well as target spontaneously to lipid rafts (43), unlike its more mature counterpart, the αβ TCR, which requires engagement with peptide-Major Histocompatibility Complex (pMHC). These abilities are due to a unique extracellular domain of pre-Tα that contains specific charged residues that promote spontaneous dimerization (42), leading to its autonomous signaling capacity.

Genes that encode the variable region of T cell receptors are assembled during the early stages of lymphocyte differentiation from germline variable (V), diversity (D), and joining (J) gene segments by a process referred to as V(D)J recombination. Rag1 and Rag2 are evolutionarily conserved genes in vertebrates that encode proteins central to the process of V(D)J-recombination (44-46). Appropriately, Rag1 and Rag2 are highly expressed only in lymphocyte tissue (46, 47), and are expressed at low levels in other organs (48, 49). In the absence of Rag1 or Rag2, mice are unable to undergo V(D)J rearrangement, and consequently lack mature lymphocytes (44-46). Expression of a TCRβ transgene rescues the thymocyte developmental block found in mice with a Rag2-deficiency, up until the DP stage of development (50). Similarly, in mice which are TCRβ- or pre-Tα -deficient, a pre-TCR cannot be formed, and this leads to impaired T cell development and a pronounced arrest at the DN3 stage (44, 45, 51, 52). Together, these data demonstrate that successful rearrangement and subsequent expression of the TCRβ in the context of a pre-TCR is essential for developmental progression of DN3 cells.

Many knockout and over-expression studies have been instrumental in deciphering the key signaling molecules downstream of the pre-TCR. Similar to what has been found for the TCR, the pre-TCR has also been shown to require CD3 components, as well as TCR-associated tyrosine kinases, Lck and Fyn, and scaffolding proteins, SLP-76, and LAT, and other downstream molecules in order to signal differentiation at the β-selection checkpoint (53-58) (Fig. 3).
Figure 3. Pre-T-cell receptor (pre-TCR) signaling depends on a multitude of signaling and adaptor molecules, including Lck, Fyn, ZAP70, and SLP-76. Downstream of pre-TCR signaling, activation of pathways such as phospholipase C and MAPK leads to survival, proliferation and differentiation of immature T cells, as well as allelic exclusion of TCRβ. Figure adapted from von Boehmer et al. (59).
2.2 ERK/MAPK signaling

E proteins E2A (E12, E47) and HEB are indispensable transcription factors early in T cell development. E proteins are basic helix-loop-helix (bHLH) family members (60) that bind the consensus E-box DNA sequence (CANNTG) with their basic region, and interact with proteins via their helix-loop-helix domain (61, 62). In thymocytes, E2A and HEB work together as heterodimers to promote the expression of genes critical for thymocyte development and differentiation (63, 64). In E2A- or HEB-deficient mice, thymocyte development is partially arrested, with αβ-lineage cells more severely affected than cells of the γδ-lineage (65, 66). Mice with a double-deletion of E2A and HEB experience a severe developmental block before pre-TCR expression and a dramatic reduction of pre-Tα expression (67). The arrested thymocytes also have increased proliferation and expansion, suggesting a role for these E proteins that is broader than T cell differentiation, and encompasses cell cycle arrest before pre-TCR expression as well. E proteins play an important role as regulators of thymocyte selection, functioning as gatekeepers at critical checkpoints during thymocyte development (67-69). The developmental block imposed on Rag-deficient and SCID thymocytes is removed upon loss of E2A, suggesting a crucial role for E proteins in preventing differentiation of these cells to the DP stage in the absence of a pre-TCR (65, 69).

Signaling through the pre-TCR activates the ERK-MAPK pathway, leading to the expression of early growth response family members Egr1-4 (70). Egr factors are required for the development of DN3 cells across the β-selection checkpoint to the DP stage. Inhibiting Egr function impairs this progression, while ectopic expression allows DN3 cells to bypass β-selection without a functional pre-TCR (70). Among the earliest transcriptional changes induced by pre-TCR signals is the expression of Egr3 (71, 72). Studies of this pathway have suggested that Egr3 expression is important at this stage because it serves to control thymocyte proliferation in response to pre-TCR signals (73), as well as induce the expression of inhibitor of DNA binding 3 (Id3) (69). Egr1 has also been shown to induce Id3 expression in collaboration with NF-ATc1, which is activated downstream of calcium mobilization by pre-TCR signaling (74). Id proteins (1-4 in mammals) are HLH-only proteins (75) that heterodimerize with E proteins and prevent them from binding DNA. Id protein expression is induced upon pre-TCR signaling-mediated activation of the ERK-MAPK pathway (68), leading to elevated Id3 gene
expression levels at β-selection (76). This critical step allows Egr proteins to mediate DN3 differentiation across the β-selection checkpoint. By inducing Id3 expression, the developmental arrest imposed by E2A function is removed as a result of the Id3/E2A heterodimerization (77) (Fig. 4).

3 The phosphoinositide-3-kinase pathway

The phosphoinositide-3-kinase (PI3K) family of conserved lipid kinases can be subdivided into two classes, both of which are activated following stimulation of transmembrane surface receptors. Class IA are activated by receptor tyrosine kinase engagement and Class IB are activated by the βγ subunit of G-proteins, downstream of G-protein coupled receptors (78, 79). PI3K activity leads to the production of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) from its precursor, phosphatidylinositol 4,5-bisphosphate (PIP2). Upon generation, PIP3 functions to activate downstream molecules, most notably the Serine/Threonine kinase Akt. Increased levels of PIP3 promote the translocation of Akt to the membrane, which results in its conformational change, phosphorylation by phosphoinositide-dependent kinase 1 (PDK1), and subsequent activation (80). Akt itself has phosphorylation targets that influence cellular translation and apoptosis. These include BAD, a Bcl-2 family member and pro-apoptotic factor, forkhead box family member FoxO1 (81), and 4E-BP1, a repressor of mRNA translation (82, 83). By phosphorylating and inactivating these targets, the PI3K pathway, through Akt, mediates cell proliferation and survival.

Considering the potential danger of unregulated proliferation and survival, PI3K signaling is kept in check by a number of mechanisms (84). For example, the regulatory subunit of PI3K, p85, is controlled by molecules SHP-1 and Cbl-b (85), while (Src homology 2)-containing inositol phosphatase (SHIP)1 and SHIP2 have been shown to terminate PI3K signaling (86). SHIP1 and SHIP2 are lipid phosphatases, which hydrolyze the 5' phosphate of the inositol ring from its substrates (87, 88). SHIP is predominantly expressed in hematopoietic cells and its phosphatase activity has been shown to be critically required for repressing signaling pathways which are up-regulated in response to cytokine and growth factor stimulation (89), such as Bruton tyrosine kinase and Akt (90, 91). In this way, SHIP regulates cell proliferation and survival (88, 92). However, SHIP does not appear to regulate basal PIP3 levels, but rather the magnitude and duration of increases in PIP3 and Akt upon stimulation (93, 94). Conversely, PTEN, another
Figure 4. A schematic overview of how signaling from the pre-TCR/CD3 complex leads to induction of Id3. In turn, Id3 heterodimerizes with E2A and inhibits its activity. In this way, the developmental and proliferative block imposed by E2A is alleviated in differentiating thymocytes by pre-TCR signaling.
lipid phosphatase involved in the regulation of PI3K/Akt signaling, can affect basal PIP3 levels, and this is discussed in more detail in a subsequent section.

3.1 PI3K and T cell development

In thymocyte development, PI3K signaling is activated in response to a variety of environmental cues, including TCR engagement, cytokine or chemokine receptor signaling, and co-stimulatory molecules. Mice with a deficiency in the p110γ catalytic subunit of PI3Kγ experience a multitude of symptoms including decreased cellular proliferation, increased sensitivity in response to apoptotic stimuli, and reduced splenic CD4+ SP cell numbers (95). Similarly, p110δ−/− mice have a normal thymus in terms of CD4 versus CD8 distribution, but suffer from impaired proliferation of CD4+ T cells in response to anti-CD3 stimuli. However, because p110γ−/− and p110δ−/− T cells have residual PI3K due to isoform redundancy (96), development in these mice is not impaired. A more striking defect was seen in mice with a deletion of both p110γ and p110δ. p110γ−/−δ−/− thymocytes have a stronger blockage at the β-selection checkpoint, markedly reduced thymic cellularity, and reduced survival of DP cells (97). Most recently, the p110δ subunit of PI3K, but not p110γ, was found to be required for the phosphorylation of Akt downstream of pre-TCR-mediated PI3K signaling (98, 99).

T cells with reduced expression of PI3K effector PDK1 are able to transit from DN to DP, albeit with a 10-fold reduction in thymic cellularity, while a more complete deletion of PDK1 prevents these cells from traversing the β-selection checkpoint and developing into DP cells (100). Additionally, expression of a constitutively active form of p85α in T cells leads to lymphoproliferation, autoimmune disease, an increase in T cell numbers, particularly CD4+ cells, and development of T cell lymphomas when backcrossed to the p53−/− background (101). However, T cell development is unaffected in mice with a deficiency in the p85α regulatory subunit, but a partial block early in B cell development is observed (102, 103). Transgenic mice in which Akt is constitutively activated in T cells similarly develop lymphoma and autoimmune disease, but thymic selection is not impaired in these animals (104, 105). Increasing active Akt in thymocytes enhances their viability in culture, and allows them to resist apoptosis in response to a variety of stimuli. This is likely due to elevated levels of Bcl-XL associated with higher Akt activity (104). Combining the results of these studies, the role of the PI3K pathway in T cell
proliferation, survival, and differentiation is clear, as is the need to keep its signaling tightly regulated to prevent uncontrolled growth and tumorigenesis.

### 3.2 Phosphatase and tensin homologue, PTEN

Loss of chromosome 10 in brain, bladder, and prostate cancer was observed for a number of years before PTEN, the phosphatase and tensin homolog deleted from chromosome 10, was isolated in a study of human breast tumours in 1997 (106). Since then, Pten has been identified as one of the most commonly mutated genes in human cancer. From sequence analysis of its open reading frame, Pten was found to contain a protein tyrosine phosphatase domain, which specifically catalyzes position D3 of phosphatidylinositol (3,4,5) triphosphate, a direct product of PI3K (107). Other inositol phosphates without a phosphate at position 3 of their inositol ring are not dephosphorylated by PTEN. In addition to the N-terminal phosphatase domain, the crystal structure of PTEN shows a tightly associated C-terminal C2 domain (108). Together, the two domains form the catalytic unit of PTEN. The C-terminal tail of the protein is not required for its enzymatic activity, but is rather used for accessing the substrate in the lipid membrane (108, 109), and for positioning the catalytic domain on the membrane.

### 3.3 Effects of PTEN deletion in mouse models

Shortly after it was discovered that a variety of human tumours exhibited an altered expression pattern for PTEN, mouse models were generated to investigate the function of PTEN in regulating basic cellular processes and development, as well as to elucidate its role in lymphocyte and T cell development. Considering PTEN was first discovered in cancer tissues, it was no surprise that the protein was found to be required for cells to maintain their sensitivity to apoptosis (110). In a breast cancer cell line, expression of PTEN induces a G1 arrest followed by apoptosis (111). Genome-wide loss of Pten is embryonic lethal at E9.5, due to overgrowth of the cephalic and caudal regions and defective chorio-allantoic development. Furthermore, Akt is hyper-phosphorylated in resting Pten<sup>−/−</sup> cells, while co-expression of dnAkt and PTEN do not have an additive effect on death, indicating that these proteins act in the same pathway to influence cell survival (107). Phosphorylation of Akt depends on PIP3 levels, which are elevated in the Pten<sup>−/−</sup> cells. Heterozygous Pten mice have an increased propensity to develop cancer, ranging from cancer of the breast, thyroid, endometrium, prostate, as well as T cell lymphomas (112). Loss of heterozygosity of the wildtype Pten allele also results in increased levels of
phospho-Akt. Together, these results proved a functional connection between PTEN and Akt in tumour development.

3.4 PTEN and T cell development

PI3K is coupled to a number of important pathways in the developing T cell, including T cell receptors, TCR co-stimulatory molecules, chemokine receptors, and the cytokine receptor γc chain. Considering the function of PTEN is primarily to inhibit PI3K signaling, and knowing the importance of the PI3K/Akt pathway in promoting T cell survival and proliferation, the role of PTEN in developing thymocytes quickly became an important area of research.

Pten" mice suffer from a lethal polyclonal autoimmune disorder similar to what was previously described in Fas-deficient mice (113). These pathologies, typical of autoimmune disorders, include an enlarged spleen and hyperplastic lymph nodes, inflammatory infiltration of most other organs especially the lung, and glomerulopathy. Activation-induced cell death of CD3ε-enriched splenic cells is impaired, indicating that PTEN is crucial for Fas-mediated elimination of activated lymphocytes. Jurkat T cell lines, which are Pten-deficient, are hyper-responsive to T-cell receptor stimulation, as measured by Itk kinase activity and activation of ERK. PTEN expression in these cells decreases their viability, but the effect can be overcome with co-expression of myristoylated Akt (114). As homozygous Pten mutant mice are embryonic lethal (112), mice containing T cell-specific Pten deletions have been generated via the Cre-loxP system. Pten" mice that have been bred to mice expressing cre-recombinase under the control of the proximal-lck promoter/enhancer (Pten"/Lck-Cre) are hyperproliferative and autoreactive (spontaneous activation of CD4+ T cells, auto-antibody production), secrete an increased level of Th1 and Th2 cytokines, resist apoptosis, have increased levels of phospho-Akt and phospho-ERK, and suffer impaired peripheral tolerance to superantigens (115). Furthermore, T cell requirement for CD28 co-stimulation is lost with Pten-deletion, suggesting that TCR signals are normally negatively regulated by PTEN. Naïve Pten-deleted T cells can activate the PI3K pathway and induce IL-2 production upon TCR stimulation alone, without the requirement for CD28 co-stimulation (116), while Pten-deleted CD4+ T cells do not require CD28 co-stimulation for cellular expansion, nor do they undergo anergy in response to TCR stimulation alone without co-stimulation. Furthermore, mice with T cell-specific Pten deletions also suffer premature death due to CD4+ T cell lymphomas, as Pten-deficiency leads to constitutive phosphorylation of
Akt, as well as constitutive membrane association and phosphorylation of Itk, a T-cell specific pleckstrin homology domain-containing kinase that activates ERK (117). CD4/8 lineage commitment is influenced by ERK signaling, with CD4+ T cell numbers increasing with increased ERK activity (118). Since ERK phosphorylation is increased in Pten-deleted mice, T cell differentiation in these mice is skewed to the CD4+ T cell lineage at the expense of the CD8 lineage.

### 3.5 PTEN and T cell β-selection

It is well established that one of the pathways activated by pre-TCR signaling is the PI3K pathway (97). Signal transduction from the PI3K pathway leads to the phosphorylation, activation, and mobilization of a variety of downstream effectors which regulate cell survival, proliferation, and differentiation. Induction of PTEN is one mechanism by which T cells regulate this pathway. Analysis of E16 thymi from Pten$^{0/0}$Lck-Cre mice shows accelerated generation of DP during ontogeny, and tumours arise in these mice by the 14th week of development (119). Prior to the onset of cancer, phospho-Akt protein levels are increased compared to Pten-sufficient mice, but other characteristics such as thymus weight and cellularity are similar. Later in development Pten$^{0/0}$Lck-Cre mice suffer from defects in peripheral and central tolerance, increased phosphorylation of Akt and ERK, and increased thymic cellularity (115). Interestingly, breeding Pten-deficient mice with those deficient for interleukin-7 (IL-7) receptor or pre-TCR signaling reveals a significant regulatory role for PTEN in the developing T cells, as Pten-deletion is sufficient to mimic a pre-TCR or an IL-7 receptor signal in DN3 cells, respectively. Both of these receptors activate the PI3K signaling pathway as one of their downstream effectors. More interestingly, deletion of Pten can substitute for both IL-7 and pre-TCR signals at the same time, as Pten$^{-/-}$γc$^{-/-}$Rag2$^{-/-}$ triple-knockout mice can bypass the β-selection checkpoint, generate DPs, and populate the thymus in similar numbers compared to wildtype cells (119).

### 4 Notch signaling

Another critical signaling pathway induced with thymic stromal-lymphocyte interactions is the Notch signaling pathway. The Notch pathway is highly conserved and regulates many aspects of development and differentiation in vertebrate and invertebrate organogenesis (120, 121). Notch signaling can affect cell proliferation and survival, and influence cell fate decisions by activating
or inhibiting certain developmental programs. Vertebrates express four different Notch receptors (Notch 1–4), which can engage five known ligands (Delta-like 1, 3, 4, Jagged 1 and 2) (122, 123). The method by which Notch receptor transmits signal to the nucleus is unique and distinct from other conserved signaling pathways. The intracellular domain of Notch receptor (ICN) itself localizes into the nucleus and forms a transcriptional complex that modifies expression of downstream target genes. This is accomplished by sequential proteolytic cleavages to the Notch receptor following ligand engagement, resulting in the liberation of ICN (124). Subsequently, ICN is targeted to the nucleus via its nuclear localization signal sequence, where it interacts with co-factor CSL (CBF1/RBP-Jκ in mammalian cells, Su(H) in drosophila, and LAG-1 in C. Elegans) and co-activator mastermind-like (MAML) to form a transcriptional complex that activates downstream Notch target genes (125-129) (Fig. 5). In this way, the Notch signaling pathway has been found to interact with a number of other signaling pathways including the Ras and the PI3K/Akt pathways, both of which transduce growth factor signals (130, 131).

4.1 Notch signaling in T vs. B lineage commitment

The role of Notch signaling in T vs. B lineage commitment is well established (132-134). Notch signals promote the T cell fate in BM precursors entering the thymus, as precursors which are deficient in Notch1 expression develop into the B lineage instead (132). Similarly, cells which are deficient in the RBP-Jκ gene, which is crucial to the formation of the active Notch transcriptional complex, also disrupts T cell fate adoption in the thymus (135). Conversely, ectopic expression of constitutively active Notch1 (ICN domain) leads to the generation of T cells within the BM at the expense of B cell development (134).

4.2 Notch signaling in T cell development

At later stages, Notch signaling is essential for the generation and survival of early DN thymocytes, as well as their transition to the DP stage (133, 136, 137). Mice with T cell specific RBP-Jκ- or Notch1-deficiency have decreased numbers of DP cells, resulting in a smaller thymus and higher percentage of DN cells (137-139). Notch signals are required for the expression of pre-TCR(137), and are further necessary as a concurrent signal with pre-TCR at the β-selection checkpoint (140-142).
Figure 5. The Notch signaling pathway. The intracellular domain of Notch receptor (ICN) is released from the membrane via sequential proteolytic cleavages upon receptor-ligand interactions. ICN localizes into the nucleus and forms a transcriptional complex with co-factor CSL (CBF1/RBPJ in human/mouse cells, Su(H) in drosophila, and LAG-1 in C. Elegans) and co-activator (CoA) to form a transcriptional complex which modifies expression of downstream target genes. CoR represents a co-repressor.
Initially, it was thought that Notch signaling at the β-selection checkpoint was required solely to induce pre-TCR expression, via cooperation with E2A to turn on *Rag1* and *Rag2* genes (137). However, Notch signaling was still required in *Rag2*−/− DN3 cells undergoing β-selection with a virally-transduced and functional TCRβ (140). In addition to inhibiting cellular differentiation, loss of Notch signaling leads to rapid cellular apoptosis and atrophy, and loss of glucose metabolism in DN3 cells. This suggests that Notch signaling requirements in differentiating DN3 cells is more complex than simply inducing pre-TCR expression, and points to a more instrumental role for this signaling pathway in mediating survival, proliferation, and differentiation of mouse DN thymocytes to the DP stage of development, independent of the pre-TCR signaling pathway, at the β-selection checkpoint (140). Notch-ligand interactions maintain cell size, glucose metabolism, and survival of DN3 cell by activating PI3K signaling and inducing phospho-Akt expression (141). PDK1 is a key intermediate in this pathway, as *Pdk1*-deficient pre-T cells are unresponsive to the trophic effects of Notch signaling, leading to differentiation arrest at the β-selection checkpoint and significantly decreased proliferation and survival.

### 4.3 OP9-DL1 in vitro system

Initially, it was believed that a thymic 3-dimensional architecture was required for the generation of T cells from hematopoietic precursors, so fetal thymic organ cultures (FTOC) were the only way researchers could study T cell development in vitro (143, 144). However, as mentioned above, differentiation of T cells from multipotent hematopoietic precursors requires, among other things, Notch signaling. Monolayers from various stromal cell lines, which had been used for B cell differentiation, could be made to support T lineage development if they allowed for Notch signaling. Notch signaling can been provided by ectopically expressing Notch ligand, Dll1 (DL1), in a stromal cell line on which the developing T cells are cultured (145). A particularly good stromal cell line to use is the OP9 cells, which were initially harvested from the BM of a newborn op/op mouse (146). op/op mice have a mutation leading to the absence of macrophage colony-stimulating factor, which preferentially allows for differentiation of hematopoietic cells away from the monocyte and macrophage cell lineages (147) that would otherwise overwhelm the in vitro culture and prevent differentiation into other cell types. The development of the OP9-DL1 stromal cell line, in which the Notch ligand, Dll1, is constitutively
expressed via a retroviral vector, makes it possible to culture T cells from their hematopoietic progenitors with ease (148). This technology also allows for manipulation of gene expression within developing T cells via retroviral transductions, thereby decreasing the temporal and monetary costs of generating a genetically modified Tg mouse (149).

4.4 c-Myc

Thirty years ago, the cellular homolog of the retroviral \( v\)-myc oncogene was discovered (150). Named \( c\)-myc, the gene was subsequently found to participate in multiple mammalian tumours (151). c-Myc is a transcription factor which recognizes consensus E box binding motifs (152). Its transactivation and transcription repression domains are located at the N-terminus (153, 154), the sequence-specific DNA binding domain is within the basic region, and the helix-loop-helix leucine zipper (HLH/LZ) domain in the C-terminus mediates protein-protein interactions (60).

Deletion of \( c\)-myc is embryonic lethal, and manipulations of the gene have shown that this is likely due to its wide influence in regulating key cellular processes including proliferation, differentiation, and apoptosis (155-157). Over-expression or conditional activation of \( c\)-myc increases Cyclin A and Cyclin E expression (158, 159), and c-Myc has also been shown to regulate expression and function of CDK inhibitors CDKN1A (160, 161) and CDKN1B (162, 163). Additionally, c-Myc regulates apoptosis in a context- and cell-dependent manner. In myeloid differentiation, c-Myc-induced apoptosis is dependent on the Fas/CD95 death receptor pathway (164). In lymphoid cell cultures, c-Myc activation leads to down-regulation of anti-apoptotic factor, Bcl-X\(_L\) (165). However, the ability of c-Myc to induce apoptosis is dependent on the balance of survival and death inputs from multiple cellular pathways. Ras-mediated PI3K activation is one such pathway (166). Akt activation downstream of PI3K signaling can up-regulate Bcl-2, which inhibits c-Myc-mediated apoptosis but not proliferation in some cell types (155, 167). Interestingly, c-Myc has been shown to bind the promoter of \( Pten \) and up-regulate its activity (168), suggesting another potential mechanism by which c-Myc induces apoptosis.

c-Myc expression and function is regulated in response to a variety of mitogenic signals, such as growth factors and Wnt (169). c-Myc has also been identified as a downstream target of Notch in T-ALL cell lines and primary DN3 cells (170, 171). c-Myc contains two potential RBP-Jκ binding sites that are conserved between humans and mice, and ICN1 binds directly to one of
these (170, 172). This relationship between c-Myc and Notch is likely important for the pro-growth effects induced by Notch signaling during thymocyte development.

4.5 HES1

HES1 is a member of the \textit{Hes} family (Hes1-7), which are mammalian homologs of the \textit{Drosophila} genes \textit{hairy} and \textit{Enhancer of split} (173). HES proteins contain 3 conserved domains that are required for its transcription factor activity: the bHLH, Orange, and WRPW domains (174). The basic region of the bHLH domain contains a proline which confers non-canonical N box sequence (CACNAG) binding abilities to HES. The WRPW carboxyl terminal domain recruits Groucho (175), which in turn recruits histone deacetylases to the chromatin complex (176) and allows HES to actively mediate transcriptional repression (177, 178). The Orange domain, a protein interaction motif, has been proposed to mediate a similar recruitment of a co-repressor, or stabilize or regulate the WRPW-mediated repression in an intra- or intermolecular interaction (179). Additionally, HES can mediate repression via dominant-negative regulation. Most bHLH factors bind to the canonical E box sequence (CANNTG) to activate gene transcription. HES can form non-functional heterodimers with these bHLH transcription factors and inhibit their activity (177, 178). Known targets of dominant-negative repression by HES include E47 and Mash1.

HES1 maintains cells in a proliferative state (180), and directly promotes cellular proliferation through transcriptional repression of the cyclin-dependent kinase (CDK) inhibitor \textit{Cdkn1a}, and to a lesser extent, \textit{Cdkn1b} (181). HES1 binds the 5’ flanking basal enhancer region of \textit{Cdkn1a} and inhibits promoter activity. While moderate levels of HES1 can reduce \textit{Cdkn1a} and \textit{Cdkn1b} expression and accelerate the growth rate of these cells, too much HES1 leads to an apparent growth inhibition due to decreased viability of the cells. This was reported in a previous study whereby toxicity associated with HES1 over-expression was observed (182).

4.6 HES1 regulation

The half-life of HES1 mRNA and HES1 protein are 23.1 ± 1.7 min and 22.3 ± 3.1 min, respectively (183). The short half-lives explain the ability for \textit{Hes1} transcript to oscillate during somite segmentation in 2 hour cycles (184, 185). The ubiquitin-proteasome pathway is responsible for the degradation of HES1, which remains in its ubiquinated form in the presence
of proteasome inhibitors (183). Accumulation of HES1 protein leads to suppression of Hes1 mRNA expression, and loss of serum-induced Hes1 mRNA oscillatory patterns. This is due to the ability of HES1 to repress its own expression by binding its promoter. In support of this, constitutive expression of HES1 leads to low levels of Hes1 mRNA, while dominant-negative HES1 leads to sustained Hes1 mRNA.

Hes1 and Hes5 are downstream targets of Notch signaling. Transiently transfected reporter gene assays with ICN mRNA show increased promoter activity for Hes1, Hes5, and Hes7 (186, 187). Furthermore, Hes1 and Hes5 are upregulated by ICN in neural precursor cells, and necessary to inhibit their neuronal differentiation (188). Much of the work addressing the interaction between Notch signaling and HES proteins has focused on the nervous system, where Notch and subsequently, HES, play a significant role. Other cell types in which the Notch-HES pathway play a critical role include blood cells, inner ear hair and support cells, somites, and endocrine-exocrine cells (173, 189). RBP-Jκ is essential for Hes1 expression in response to Notch stimulation. Expression of dominant-negative mutants of RBP-Jκ in mammalian culture cells or RBP-Jκ-deficient OTII cells failed to induce Hes1 expression in the presence of ICN, suggesting the importance of the ICN transcriptional complex in mediating Hes1 expression (190, 191). Hes1 can also be regulated independently of the canonical Notch pathway signaling (192, 193). In mesodermal and neural cells, Hes1 is the target of sonic hedgehog (Shh) and desert hedgehog (Dhh). Transfection of full length Shh and Dhh, and to a lesser extent Ihh (Indian hedgehog), leads to a large increase in Hes1 mRNA post-stimulation, while inhibition of the hedgehog pathway leads to a significant decrease in Hes1 mRNA (193). Smoothened function is necessary for this interaction, but Notch and RBP-Jκ are not, since inhibition of Notch pathway with γ-secretase inhibitors, or with a dominant negative RBP-Jκ, does not affect Shh’s ability to induce Hes1 mRNA.

### 4.7 HES1 and T cell commitment and differentiation

HES proteins are effectors of Notch signaling in a variety of cell types, including blood cells. In the hematopoietic system, expression of ICN1 in the bone marrow leads to the up-regulation of Hes1 and Hes5 mRNA (194). Since Notch signaling was found to be crucial to the development of T cells within the thymus, studies were undertaken to determine the role of HES1 in T cell differentiation, expansion, and survival. Expression of Hes1 is readily detectable as early as
embryonic day 15 (E15) in both the lymphocyte and stromal subsets of the developing thymi (195). Within the thymocyte subsets, Hes1 expression varies markedly (196), and follows the pattern of Notch1 mRNA expression. Hes1 expression is highest in the DN subset, and this expression is rapidly down-regulated 100-fold upon differentiation to the DP stage. CD4 thymocytes have slightly higher expression of Hes1 mRNA compared to DP cells, and CD8 thymocytes have slightly higher Hes1 mRNA levels than CD4 thymocytes.

The thymi of Hes1-deficient fetuses are severely hypocellular, and retroviral over-expression of Hes1 increases thecellularity of developing thymocytes in FTOC (197). HES1 is required for early T cell precursor proliferation, both at pre-TCR-independent and pre-TCR-dependent expansion stages (195). Hes1 mutation severely decreases proliferation in immature thymocytes which have yet to initiate TCR gene rearrangement. While fetal liver from Hes1−/− mice can reconstitute B cell development in irradiated Rag2−/− mice, the absolute number of thymocytes is greatly reduced compared to wildtype (195). Some groups have reported that Hes1-deficiency in hematopoietic precursors leads to arrest of T cell development at the early DN stage, much like Notch1-deficiency (195, 198). Developing Hes1-deficient T cells are arrested at the DN stage of development, specifically at the DN3 stage (199).

Experiments have shown that expression of a transgenic ICN in the bone marrow leads to the development of T cells in this organ at the expense of B cells, as well as the development of T cell leukemias and lymphomas (134, 200). In mice with a T cell-specific deletion of Notch1, the reverse is true: B cells mature in the thymus at the expense of T cells (132). In these studies, however, no changes to myeloid activity are determined, and no mechanism for this effect is elucidated. With Hes1 or Hes5 over-expression in mouse BM, accumulation of early immature B cell precursors is observed along with a suppression of B lineage cells in a dose-dependent manner. This, however, is not a full inhibition of the B cell lineage like what is seen with transgenic ICN expression in the BM (194). Myeloid lineage cells in these mice have reduced maturation potential, retain an undifferentiated immature phenotype, and maintain colony forming capabilities which are not observed with wildtype mice (194). While one study did not observe changes in T cell maturation or in the thymic CD4 versus CD8 profile in Hes1- or Hes5-over-expressing mice, another study found that over-expression of Hes1 led to N-box-dependent repression of the CD4 promoter and down-regulation of CD4 expression in CD4+ CD8− T H cells (201). Although HES1 is an important downstream effector of Notch signaling in T cell
development, it is insufficient to drive T cell programming in human hematopoietic stem cells (199). *Hes1* expression in these cells induces a partial block in B-cell development in the absence of Notch receptor–ligand interactions, but it does not inhibit monocyte development, nor does it promote T or NK lineage outcomes (199). Thus, HES1 cannot substitute for Notch signaling to induce T cell differentiation from hematopoietic stem cells. However, HES1 may play a key role downstream of Notch signaling at later stages of T cell development, and the ability for HES1 to substitute for Notch signaling at these critical time-points, such as T cell commitment, remained to be addressed.

Most recently, a study looking at the effect of inactivating HES1 in adult mouse bone marrow showed that T cell development was severely impaired, while HES1 seemed to be dispensable for other Notch-mediated effects, such as hematopoietic stem cell maintenance and self-renewal, and marginal zone B cell development (198). Specifically within T cell development, HES1 is required at the early stage of lineage commitment. Competitive intra-thymic transfer experiments show that *Hes1*-deficient progenitors are largely outcompeted by wildtype cells to generate T cells, but those which do differentiate along the T lineage are able to mature into DP, CD4+ and CD8+ thymocytes. Most of the *Hes1*-deficient cells remain CD4−CD8− DN, and the majority of these are immature B cells, not T cells. Experiments done in vitro with *Hes1*-deficient progenitor cultured on OP9-DL1 cells showed a similar result. In the absence of HES1, differentiating progenitors accumulate at the DN1 stage. These results suggested that *Hes1*-deficient progenitors are unable to fully respond to Notch1 signals, and this impairs their ability to commit to the T cell fate. Once committed however, they are able to differentiate fully and generate mature CD4+ and CD8+ thymocytes.

Importantly, this study concluded that HES1 is no longer required in developing thymocytes beyond the β-selection checkpoint. This result is not unexpected, considering that *Hes1* expression has been shown to drop dramatically following the DN stage (196), following the decline of Notch signaling. However, an interesting question arises from this conclusion: is HES1 required during the phase of β-selection, and if so, in what context and for what purpose? While Wendorff *et al.* employ *HES1*^{fl/fl;Lck-Cre} mice and observe that HES1 is dispensable through and beyond β-selection (198), it is likely that the timing of their genetic manipulation, as well as the timing of their analysis, prevented them from observing the effect of HES1-deletion at this checkpoint. Considering that HES1 is an effector of Notch signaling, and considering Notch
signals plays a critical role in differentiation of DN3 cells across the β-selection checkpoint, it is imperative that the requirement for HES1 at the β-selection checkpoint be more thoroughly examined.

5  The thymic microenvironment

The thymic microenvironment provides a multitude of signals that make it uniquely suited to support and promote T lineage differentiation (202). Production of cytokines such as IL-7 by the thymic stroma has been appreciated for many years as being essential to thymocyte development (203-207). Similarly, chemokines secreted by the thymic stroma are well-recognized as influential factors of T cell migration into appropriate thymic microenvironments, but only recently have their roles in directly influencing T cell differentiation been elucidated (Fig. 6) (39, 98, 208).

5.1  Cytokines

The thymic stroma, as well as other thymic resident cells, provides soluble factors such as cytokines that help to generate a favourable environment for T cell proliferation, differentiation and development. One family of cytokines, known as the common cytokine receptor γc family, encompasses interleukin-2 (IL-2), IL-4, IL-7, IL-9, IL-15 and IL-21. These cytokines share γc as a component of their receptors (209-211). Transmission of signal from the γc family of cytokines is accomplished through engagement of their respective receptors, leading to receptor association with the Janus Kinase (JAK)-Signal Transducers and Activators of Transcription (STAT) pathway. JAKs are tyrosine kinases that are activated upon a cytokine receptor binding its ligand. JAKs in turn activate members of the STAT family through tyrosine phosphorylation (212, 213). Upon activation, STATs dimerize and translocate to the nucleus, where they induce transcriptional changes by binding to specific elements within the promoter regions of target genes (212, 213). Different cytokines can recruit different JAKs and STATs, which helps to explain how these cytokines, which activate the same JAK-STAT pathway, give rise to different downstream effects. The specificity of STAT activation is partially mediated by the SH2-domain, which selectively binds to cytoplasmic tyrosines of particular receptors (214, 215). JAKs can also phosphorylate cytokine receptors to generate a docking site for other SH2-containing adapter proteins. This is a mechanism by which JAK activation by cytokine signaling...
Figure 6. Cytokine and chemokine signaling requirements during T cell development in the thymus. The thymus is organized into two discrete areas: the cortex and medulla, with early T cell differentiation occurring in the cortex. After entry into the thymus via postcapillary venules at the cortico-medullary junction, thymus seeding progenitors (TSPs) require cytokines SCF and IL-7 to commit to the T cell lineage, as well as additional support from chemokines CCL12, CCL25, CCL19 and CCL21.
can initiate signaling of other pathways, such as the Ras and PI3K pathways (216, 217). Most γc family cytokines play important roles outside of the thymus, in later stages of lymphocyte cell development. For example, IL-2 is important in regulating proliferation and apoptosis of activated T cells, as well as the development of regulatory T cells (218, 219), while IL-4 plays a role in specifying a mature T cell as a T helper 2 (220). Within the thymus, however, IL-7 plays an indispensable role early in T cell development (209).

5.2 IL-7 and T cell development

Critical to thymocyte development is the cytokine IL-7, which signals through its receptor, IL-7R, found on the surface of the developing thymocyte. The IL-7R is composed of a unique IL-7Rα chain, which associates with JAK1, and the γc chain, which associates with JAK3 (221, 222). Together, these initiate downstream signal transduction that includes activation of STAT5 and PI3K (221, 223-225). IL-7R signaling induces proliferation in developing thymocytes (226), as well as promotes the survival and development of these cells (227-229). In terms of development, IL-7 is required for normal T cell differentiation (204, 205, 230-232), as well as production of γδTCR cells (231, 233, 234). γδTCR cells are absent in IL-7R-deficient mice (231), due to the requirement for IL-7 in inducing TCRγ locus rearrangement (234, 235). IL-7-mediated activation of PI3K and STAT5 play distinct roles with respect to T cell development. IL-7-induced STAT5 activation is important for regulating differentiation (236), while IL-7 promotes T cell survival through activating the PI3K signaling pathway. PI3K signaling increases the expression of anti-apoptotic factors such as B cell lymphoma 2 (Bcl-2), and inhibits the expression of the pro-apoptotic factors BAX and BAD (237, 238).

5.3 Chemokines

Chemokines are 8-10 kDa highly basic proteins that bind G-protein coupled receptors (239). Upon engagement of its receptor, the receptor-associated G protein subunits dissociate to initiate a signaling cascade that involves the activation of tyrosine kinases Src and Fak, MAPKs, and phospholipases (240). Another critical pathway activated by chemokines is PI3K, which promotes actin polymerization and cytoskeleton rearrangement.
5.4 Chemokines and entry into the thymus

Chemokines are important for directing the migration of leukocytes to sites of inflammation and injury, as well as for surveillance trafficking to secondary lymphoid organs (241). Chemokines are effectors of inflammation, and were originally studied for this reason. They have since been shown to play a more diverse role in the immune system, by regulating processes such as leukocyte extravasation, degranulation, cellular differentiation and development (209, 220, 242-253). Chemokines have well-established roles in directing the movement of T cells during their development within the thymus and in the periphery to position the cells to the site of an immune response (241, 254). In the thymus, chemokines are produced by thymic stromal cells. Prior to thymus vascularization, chemokines CCL21 and CCL25 play a critical role in attracting lymphoid progenitors into the thymic primordium, such that in the absence of their function, thymocyte cell numbers are markedly reduced until E14.5 (255-257), when circulation allows for vasculature-dependent colonization of the fetal thymus (256) and cells immigrate into the cortico-medullary junction, which is enriched in vasculature. Seeding of the post-natal thymus by circulating progenitors is mediated by the cell adhesion molecule Platelet (P)-selectin, expressed on thymic endothelium, which binds P-selectin glycoprotein ligand 1 (PSGL1) (258).

5.5 Chemokines and thymocyte migration

Once within the thymus, lymphoid progenitors differentiating along the T cell lineage are directed by chemokines to travel through distinct microenvironments, where they receive further differentiation and migration cues. Developing thymocytes regulate their ability to receive chemokine signaling by changing the types and levels of chemokine receptors expressed on their cell surface (259), while microenvironments within the thymus recruit specific thymocyte populations by exhibiting differential expression patterns and generating a chemokine gradient to encourage their directional motion (260, 261). Chemokine receptors CXCR4, CCR7, and CCR9 have all been reported to play a role in the migration of immature thymocytes from the cortico-medullary junction into thymic cortex (262-265).

DN2 and DN3 thymocytes that have a deletion in CXCR4, the receptor for chemokine CXCL12, are unable to migrate efficiently to the subcapsular region of the thymic cortex (263, 266). Similarly, a deficiency in CCR7, the receptor for CCL19 and CCL21, partially arrests developing thymocytes at the cortico-medullary junction (264). In both cases, the impaired chemokine
signaling impedes T cell development and reduces thymic cellularity (263, 264, 266). CCR9, the receptor for CCL25, is important for normal thymocyte development, as well as proper localization of DN2 and DN3 cells. CCR9 is normally expressed by late DN thymocytes and DP cells, and confinement of its expression to this developmental window is important, as constitutive expression of CCR9 in earlier DN subsets partially blocks thymocyte development at the DN3 stage, and results in mislocation of DN2 and DN3 cells throughout the cortex instead of defined within the subcapsular region (265, 267). Thus, chemokines CCL19, CCL21, CCL25, and CXCL12 are crucial, in a context and time dependent manner, for proper intrathymic migration of these early progenitors to the outer thymic cortex, which subsequently allows these cells to receive the necessary environmental cues to allow for their differentiation beyond the DN stage.

5.6 Chemokines and T cell development

Interestingly, T cell differentiation has been found to be also directly regulated by chemokines. CXCR4 was recently shown to associate with the pre-TCR signaling complex and act as a co-stimulator during thymic β-selection (98, 99). In vitro, where CXCR4's role on differentiation could be assessed independent of its function in regulating T cell migration, pre-β-selected CXCR4-deficient DN3 cells did not generate as many DN4 and DP cells in terms of percentage and absolute cell numbers compared to wildtype DN3 cells (98). This was not due to the lack of CXCR4 expression impairing other critical signals such as Notch, but rather a requirement for CXCR4 in addition to these other signals at the β-selection checkpoint. Furthermore, CXCR4 was found to promote the survival of DN thymocytes by up-regulating Bcl-2A1 expression, and promote proliferation in coordination with the pre-TCR (98). The mechanism by which CXCR4 regulates Bcl-2A1 mRNA expression came to light in a more recent study. Again done in vitro to concentrate on CXCR4's role on development and not migration, CXCR4 was observed to mediate PI3K signaling along with pre-TCR and Notch signaling at the β-selection checkpoint (99). Using a pharmacological antagonist of CXCR4, decreasing CXCR4 signaling led to reduced proliferation and survival in DN3 cells, as well as reduced levels of phosphorylated Akt. Additionally, in vitro stimulation of CXCR4 with its ligand, CXCL12, increased phosphorylated forms of Akt and ERK, with the former dependent on the PI3K catalytic subunits p110δ and p110γ (99). Together, these studies suggest an important role for CXCR4 in mediating the development of DN3 cells across the β-selection checkpoint by activating the PI3K pathway.
6 αβ versus γδ lineage development

6.1 Stochastic versus instructional model in determining αβ- or γδ-lineage fate

In an early attempt to resolve the question of how the αβ- versus γδ-lineage decision is made, a timing-dependent model was proposed, based on observations that γδ T cells were found in the fetal thymus prior to any detectable αβ T cells (268). The model predicted that during ontogeny, developing T cells are first given an opportunity to develop along the γδ-lineage (Fig. 7). However, if no productive γ- and δ-chains are generated, cells can attempt to develop along the αβ-lineage by successfully rearranging their β- and α-gene loci. Thus, all αβ T cells would be considered failed γδ T cells. In contradiction with this model, KN6 γδTCR transgenic (Tg) mice contained normal numbers of αβ T cells, indicating that productively rearranged γ- and δ-chains did not block αβ T cell generation (269). This was found to be due to a cis-acting DNA silencer present in the regions flanking the γ gene, which made the transgenic γ and δ chains transcriptionally silent (269). Upon removal of this silencer, development of αβ T cells was severely blocked in KN6 γδTCR Tg mice, suggesting that factors that activate the silencer play an important role in lineage decisions (270). In light of these findings, a second model of lineage specification was proposed, whereby commitment to the αβ T cell lineage occurs prior to TCR expression, in precursor T cells programmed to repress the expression of the γ-chain by inducing factors that interact with the silencer element. Unlike the timing-dependent model, in which developing T cells are thought to become the lineage that matches the TCR they express, this model proposed that specification is predetermined (269). Despite some of the findings in these studies now being dated by more recent discoveries, their importance is maintained, as they pioneered the ideas for two opposing models for αβ- versus γδ-lineage bifurcation, and initiated a longstanding debate on a topic that still remains relevant today.

These two proposals to explain the underlying mechanisms governing αβ- and γδ-lineage specification have evolved to the present day as the stochastic model and the instructional model. Evidence has emerged in support of both (271-275). The stochastic model states that early thymocytes are pre-committed to a lineage prior to TCR rearrangement and expression (Fig. 7). In this case, the role of the TCR is only to provide survival signals to cells whose TCR matches the lineage pre-selected by the cell, and not to direct lineage choice. A broader interpretation
Figure 7. Various models addressing the question of how the αβ- versus γδ-lineage decision is made. In the timing-dependent model, developing T cells are first given an opportunity to productively rearrange their γ- and δ-chains to become γδ T cells. If no productive rearrangements are made, the cell subsequently attempts to develop along the αβ-lineage by successfully rearranging their β- and α-gene loci. The stochastic model states that early thymocytes are pre-committed to a lineage prior to TCR rearrangement and expression, and the cell only receives survival signals if its TCR matches the lineage pre-selected by the cell, the cell survives. The instructive model suggests the lineage adopted by the developing T cell is dependent on the TCR expressed, while the quantitative signal strength model specifies that fate decision is based on the amount of signal delivered to cells at the point of lineage commitment, and not the identity of the TCR per se.
posits that pre-commitment is enforced by factors that favor γ and δ over β gene rearrangements and/or expression. Compared to the stochastic model, the instructive model suggests a far more involved role for the TCR, whereby the lineage adopted by the developing T cell is dependent on the TCR expressed (Fig. 7). Specifically, the instructive model proposes that distinct signals are produced from the γδTCR and pre-TCR, which differentially instruct cells to adopt the appropriate cell fate: the fate which matches the TCR from which the signal is derived.

6.2 Point of lineage divergence

To begin deducing which of the two proposed models is correct, and to further elucidate the key molecular factors involved in fate decision, the precise point of αβ- versus γδ-lineage divergence needed to be determined before the experimental data could be accurately interpreted. αβ- and γδ-lineage cells arise from a common DN T cell progenitor (275-277), in which TCRβ, TCRγ and TCRδ-chain rearrangements have been initiated (278, 279). DN cells that successfully rearrange their TCRβ chain, and express it together with pTα to generate a pre-TCR complex (280, 281), progress along the αβ-lineage to become CD4+CD8+ double-positive (DP) and subsequently CD4+ or CD8+ single-positive (SP) cells (52). Alternatively, DN cells that produce a γδTCR, remain as DN but down-regulate CD25 expression, and progress along the γδ-lineage (35, 36). In this maturation process, γδ T cells decrease CD24 expression and express a set of genes, termed the γδ gene expression profile, not found in αβ-lineage DP cells (37, 38, 282).

If the TCR plays a role in lineage commitment, successful rearrangement and expression of the appropriate TCR needs to precede the point of lineage divergence. In accordance with this, the αβ versus γδ divergence point was found to be after TCRβ, but before TCRα, recombination (283). To more precisely define the stage of αβ- versus γδ-lineage bifurcation, the lineage potential of DN2 and DN3 cells was recently assessed using an in vitro clonal assay (145, 148), in which single cells were deposited into individual wells containing OP9-DL1 cell monolayers (284). Lineage divergence was observed to occur between the late DN2 to DN3 developmental stages, as many DN2 cells were bipotent, while few DN3 cells gave rise to both progeny (284). Interestingly, a subsequent study looking at the effect of γδTCR expression on lineage decision noted that TCRγδ+CD25+ thymocytes differentiated into DP cells quite efficiently, while only a few TCRγδ+CD25+CD24hi cells and no TCRγδ+CD25+CD24lo cells adopted the αβ-lineage fate (285). This indicates that γδTCR expression does not prevent immature thymocytes from
adopting the αβ-lineage fate, but maturation of γδTCR-bearing cells, marked by CD24 down-regulation, does mark cells as γδ-lineage cells. Considering that decreased levels of CD24, and not γδTCR expression, marks irreversible γδ-lineage commitment, the stochastic and instructional model as initially proposed both become invalidated. Consequently, a third model which encompasses this and other similar findings has been proposed, and is discussed in more detail in the next section.

6.3 Pioneering the signal strength model

Attempts to characterize the unique signals produced by the TCR of opposing lineages initially led to a proposal that the difference is qualitative in nature, based on distinct usage of downstream signaling molecules (43). However, in cases where γδTCR expression or signal strength was reduced (272, 273), differentiation along the αβ-lineage to the DP stage was seen to occur. Additionally, γδTCR has also been shown to promote development of a small number of DP cells in TCRβ- or pTα-deficient mice (35, 51, 286). Conversely, premature expression of a transgenic αβTCR in developing thymocytes was shown to support the development of DN cells with γδ-lineage characteristics (287, 288). As these findings conflict with both the stochastic model and the qualitative instructive model, a quantitative model came into favor. The quantitative signal strength model rectifies the apparent dichotomy that one TCR can direct development into either lineage, as fate decision is based on the amount of signal delivered to cells at the point of lineage commitment, and not the identity of the TCR per se (Fig. 7). Cells adopting the αβ- versus γδ-lineage fate receive quantitatively different signals from the TCR they express, with γδTCR signaling generally being thought of as a strong signal, and pre-TCR a weak one. The designation of “weak” and “strong” signaling comes from several lines of evidence, some of which will be discussed here.

The αβTCR and γδTCR signaling complexes are composed of different subunits. Surface-biotinylation of the γδTCR signaling complex of Vγ6Vδ1 transgenic (Tg) mice revealed that it did not include the CD3δ subunit, due to failure of CD3δ dimers to associate with the γδTCR and be transported to the surface (289). This is in stark contrast to the αβTCR, where CD3δ is a vital component of the signaling complex. αβTCR+ cells that do not express the CD3δ subunit fail to phosphorylate TCRζ and LAT, and thus fail to mobilize calcium and activate the ERK/MAPK pathway (290, 291). Compared to the αβTCR signaling complex, γδTCR signaling induced
calcium mobilization with greater magnitude and kinetics, greater ERK phosphorylation, and stronger proliferative responses, without CD28 co-stimulation and in the absence of the CD3δ subunit (289). From this observation, it was proposed that the γδTCR was a better signal-transduction complex than the αβ-TCR. However, the potential relevance of these findings to αβ- versus γδ-lineage bifurcation remained unclear, as did the role of pre-TCR and its signaling potential in comparison to that of the γδTCR. Incidentally, the same TCR-Tg mice were used to resolve these outstanding issues, as these animals expressed a γδTCR-Tg, but contained both αβ-lineage DP cells and γδTCR+ DN thymocytes, making them a perfect model to assess how differential fate decisions can be made and induced by the same TCR.

In a follow-up study, modulation of surface γδTCR levels through TCR/CD3ζ expression was shown to alter the balance of lineage fate decisions. CD3ζ is an important component of the TCR signaling complex, and it has been shown to play a role in promoting TCR expression (292, 293). In γδTCR-Tg mice, reduction in CD3ζ expression led to a corresponding decrease in surface TCR levels (273). This hindered γδ-lineage development, and instead favored the generation of αβ-lineage DP cells. Conversely, when a full-length CD3ζ Tg was introduced in γδTCR-Tg mice, γδTCR surface expression increased, resulting in a decreased number of αβ-lineage DP cells, and a concomitant increase of γδTCR DN cells. Regulation of TCR signaling by modulation of CD5, a negative effector of TCR signaling (294-296), also affected lineage choice. Removing or decreasing the expression of CD5 led to decreased numbers of DP cells, and increased γδ-lineage specification (273). Taken together, the presence of stronger TCR signals, either through higher surface TCR expression or lowered inhibition of signaling, favored the γδ-lineage fate at the expense of αβ T cell generation. Furthermore, strength of signal could convincingly be measured as a differential induction of downstream signaling molecules ZAP-70 and ERK. Ex vivo γδ-lineage T cells expressed higher levels of phosphorylated ZAP-70 and ERK1/2, compared to αβ-lineage cells (273), and also induced higher levels of CD5 expression, which is an indicator of TCR signal strength.

6.4 Ligand or no ligand

Despite these advancements addressing the issue of αβ- versus γδ-lineage bifurcation, the mechanistic basis mediating differential signal strength remained unclear. Putative candidates included inputs from other signaling pathways, such as Notch (297) and IL-7 (271), as well as
molecules downstream of the TCR itself. One attractive hypothesis proposed signal strength outcome was dictated by TCR ligand-engagement. Efforts directed at determining the precise role of pTα at the β-selection checkpoint had lead to mounting evidence in favor of the notion that pTα chain was not simply a surrogate of the TCRα, but a specialized subunit with distinct functions and characteristics. The discovery that β-selection proceeded normally in major histocompatibility complex (MHC)-I and MHCII-deficient mice (298, 299) indicated that, unlike the αβTCR, the pre-TCR did not engage ligand, or at least did so without dependence on the conventional MHC molecules. In agreement with the ligand-independent hypothesis, pre-TCR was found to localize to membrane rafts and activate downstream signaling molecules such as Lck and ZAP-70 in the absence of ligation, a unique ability not observed with either the αβTCR or γδTCR (43). Pre-TCR also constitutively localized in lysozymes and spontaneously formed oligomers using specific charged residues in its extracellular domain (42, 300), events that otherwise would require recognition of peptide-MHC to induce the activation of αβTCR-bearing T cells.

Unlike the αβ-lineage, γδ T cell development does not involve a pre-TCR checkpoint, but rather a single selection point with a fully rearranged γδTCR (301), and in contrast to the pre-TCR, ligands have been characterized for some γδTCR subsets (302, 303). These include positive selection and maturation of cutaneous intraepithelial γδTCR dendritic epidermal T cells (DETCs) on a thymic stromal determinant (302), as well as positive selection of a proportion of γδ T cells on non-classical MHCI molecules T10 and T22 (37, 304), although this last point is still a source of some controversy (305). However, antigen recognition by the γδTCR is not MHC-restricted (306), unlike the αβTCR, and allows for direct recognition of a diverse array of antigens. Previous studies have shown that stable cell surface expression of T10 and/or T22 can occur in the absence of additional peptide or nonpeptide antigens, but requires heterodimerization with β2-microglobulin (β2m) (306, 307). In support of a role for γδTCR-mediated positive selection, KN6 and G8 Tg γδTCR T cells, which are specific for T10 and T22, are absent in β2m-deficient mice (304). Ligand-mediated negative selection has also been observed with these Tg T10- and T22-specific γδ T cells, which fail to develop in C57BL/6 mice that express both T10 and T22, but developed normally in BALB/c mice that only express T10 (37).

With respect to αβ- versus γδ-lineage bifurcation, ligand-engagement was found to be a decisive factor for the positive selection, differentiation, and maturation of γδ T cells. In line with
previous observations (273), reduction of TCR signaling in KN6 Tg γδTCR RAG-deficient mice, via removal of ligand (β2m-deficient), or critical signaling effector Lck (308), impaired γδ-lineage development and maturation of γδ T cells, and simultaneously promoted αβ-lineage commitment to the DP stage (272). The provision of exogenous β2m to β2m−/− KN6 Tg γδTCR+ thymocytes reverted lineage fate back to γδ T cells (272). As γδTCR-Tg T cells could be manipulated into either lineage based on ligand availability, this suggested a lack of pre-commitment in these cells to one lineage versus another. However, the data does not completely invalidate the stochastic signal strength model, as it is possible that ligand engagement exclusively promotes the survival of cells pre-committed to the γδ-lineage, because strong TCR signaling match the signal requirements of γδ-pre-committed cells, while αβ-lineage cells fail to receive a weak signal that matches their pre-commitment status. Characterization of KN6 Tg γδTCR DP cells revealed a down-regulation of surface γδTCR expression, lowered CD5 levels, decreased ERK1/2 phosphorylation, and reduced transcription of ERK-MAPK-pathway-induced Egr1 and Egr3, compared to cells that matured along the γδ T cell pathway. These findings are consistent with the signal strength model and suggest that, in the absence of ligand-engagement, developing γδTCR-Tg T cells receive quantitatively less TCR signal and mature along an alternate lineage, i.e., the αβ-lineage.

6.5 Evidence for the instructional role of TCR via signal strength

Strong TCR signals were found to result in greater induction of the ERK-MAPK pathway (272), which suggested that over-expression of downstream effector, Egr, could mimic the strong signal induced by ligand engagement. In agreement with this hypothesis, ectopic Egr1 expression in KN6 Tg γδTCR RAG−/−β2m−/− thymocytes returned the lineage potential of these cells in favor of γδ T cell development (272). Similarly, ectopic Egr1 expression promoted γδ T cell lineage development in non-Tg fetal thymocytes at the expense of αβ-lineage development. Thus, the molecular basis for TCR signal strength stems from the level of ERK-MAPK pathway induction, with stronger γδTCR signals mediating heightened expression of Egr proteins. Again, this study seems to suggest a lack of lineage specification in developing T cells prior to TCR signaling, as signal strength modifications readily affect fate decisions. However, changes in fate adoption with altered TCR signal strength could be due to selective survival of pre-committed T cell precursors receiving TCR signals at strengths appropriate for their lineage identity, instead of each individual bipotent cell favoring one lineage over another. This unresolved issue was
directly addressed in an in vitro study where ligand engagement, and therefore strong signaling induction, was mimicked by engaging γδTCR with cross-linking antibodies (285). The single cell clonal analysis of γδTCR+ DN3 cells revealed a minority of immature γδTCR T cells adopted the αβ-lineage in the absence of antibody. However, γδTCR antibody treatment removed this lineage potential entirely, with all γδTCR cells from antibody-treated wells developing and maturing along the γδ-lineage in the absence of detectable αβ-lineage development. As this was done at the single cell level, the study definitively concluded that lineage decisions are not predetermined prior to TCR expression, as commitment to one lineage can be reverted to another with alterations in signal strength.

6.6 The role of Id proteins in αβ/γδ lineage bifurcation

Evidence in support of the signal strength model revealed that strong γδTCR signals correlated with higher ERK/MAPK pathway activation (273), which leads to a higher induction of downstream signaling effector Egr and its target, Id3 (309). A more detailed analysis of this molecular pathway revealed that expression of Id3 was necessary but not sufficient to promote the γδ-lineage fate in fetal thymocytes (272), suggesting that Id3 is an important target through which strong ERK/MAPK signals could mediate αβ- versus γδ-lineage decisions (Fig. 8). However, over-expression of Id3 in thymocytes prior to the time when the αβ- versus γδ-lineage choice is made may perturb T-lineage differentiation (310). A deficiency in E2A prior to the onset of TCR gene rearrangement leads to a profound block at the DN stage of T cell development (65), as E proteins regulate the expression of the pre-Tα and Rag genes, as well as promote VDJ rearrangements within the TCRβ, γ and δ loci (65, 311-313). Post-rearrangement and pre-TCR or γδTCR expression, however, the induction of Id3 expression allows thymocytes to successfully transition from the DN to the DP stage (77), as the developmental arrest imposed by E2A function is removed as a result of the Id3/E2A heterodimerization. However, over-expression of Id3 in T cell progenitors does not favor αβ T cell development (311). Instead, it has the opposite effect of disrupting αβ, but not γδ-lineage differentiation, making it a potential candidate that influences the αβ versus γδ decision. In terms of the signal strength model, strong TCR signals may be detrimental to the development of αβ T cells, by decreasing E protein activity through heightened ERK activation and Id3 expression.
Figure 8. A schematic overview, based on the strength of TCR signal, of the αβ versus γδ-lineage fate decision made by a DN3 thymocyte. Differentiation into an αβ or γδ T cell is dictated by TCR signal strength delivered to DN3 cells at the point of lineage commitment, as measured by the level of ERK/MAPK pathway activation and Id3 induction. The requirement for Notch signaling at this stage of development depends on the magnitude by which the TCR signals can inhibit E2A. Strong signals produced by the TCRγδ induce high levels of Id3 that allows for further maturation in the absence of Notch signals, while weak signals produced by the pre-TCR require help from the Notch signaling pathway to inhibit E2A to a level necessary for successful β-selection. Furthermore, TCR-ligand engagement is not required for pre-TCR signaling, and while it may not be necessary for all TCRγδ selection, it may affect the polarization of γδT cells to IFNγ secretion versus IL-17 production. Figure is from Wong et al. (314).
6.7 The requirement for Notch signaling in αβ and γδ T cells

Several studies have addressed the role of Notch signaling in γδ T cell development, especially in the context of its potential effects on the αβ- versus γδ-lineage decision. Notch1+/− hematopoietic cells were shown to favor γδ T cell development compared to wild-type precursors in mixed bone marrow reconstitution chimeras (297), and a T cell specific deletion of RBP-Jκ allowed for the generation and emigration of γδ T cells, while severely disrupting αβ T cell development (138). Similarly, fetal thymus organ culture experiments using pharmacological inhibition of Notch signaling (315), and conditional expression of a dominant-negative form of MAML (dnMAML) (142), both pointed to a differential requirement for the Notch pathway in αβ- versus γδ-lineage development.

In vitro analyses further support the notion for the requirement of Notch signaling as critical for αβ-lineage development, whereas γδ T cells can mature in the absence of further Notch–ligand interactions once γδTCR expression is achieved (284). By using γ-secretase inhibitors to reduce the strength of Notch signaling, or by removing Notch ligand from the cultures, it was found that Notch signals were indispensible for development of T cell precursors along the αβ-lineage to the DP stage, independent of whether this transition was mediated by a pre-TCR or Tg γδTCR (284). In contrast, γδ-lineage development in γδTCR+ T cells could proceed in the absence of Notch signaling. Collectively, these results suggested that weak pre-TCR signals promoting the adoption of the αβ-lineage fate require continuous Notch signals, while strong TCR signals that promote the adoption of γδ-lineage fate do not. Importantly, the necessity for Notch signaling in αβ, but not γδ, T cell lineage specification beyond TCR expression should not be misconstrued as a role for this pathway in influencing lineage decisions. Rather, Notch–ligand interactions do not appear to direct a lineage choice, but promote the survival and differentiation of cells that have received their lineage identity on the basis of the strength of TCR signals. What remains to be addressed is the mechanistic connection by which pre-TCR and γδTCR are able to instruct differential requirements for Notch signaling.

7 Thesis objectives

T cell development requires a multitude of signaling pathways to participate in crosstalk, cooperation, and antagonism. At the DN3 stage of development, communication between signaling pathways is particularly critical, as these cells are committed to continue along the T
cell lineage, whether it be αβ or γδ, and must receive the appropriate environmental cues to allow transition to the next developmental stage, or face certain death. It is here that the pre-TCR or γδTCR acts in concert with Notch to promote survival, differentiation, and proliferation signals via activation of downstream targets such as HES1, c-Myc, PI3K and Id3.

In this thesis, the primary goal is to examine the molecular players and processes that participate in differentiating DN3 cells to the next developmental stage, whether it be the DP cell or the immature γδ T cell. By employing the in vitro T cell differentiation system, OP9-DL1 cells (145), availability of Notch ligand to developing T cells can be easily manipulated. Furthermore, the use of Rag2-deficient progenitors allows for the transduction of functional and productive TCR chains. This allows for the precise timing of the expression of pre-TCR or γδTCR, circumvents the possibility of non-productive TCR gene rearrangements, and permits for the study of the rare γδ T cell population in greater numbers.

In Chapter II, I examine the role of Notch signaling in mediating survival, differentiation, and proliferation in DN3 thymocytes committing to the αβ T cell lineage. Previous results from the Zúñiga-Pflücker laboratory show that Notch signaling maintains cell size and promotes glucose metabolism and survival of DN3 cells, and the mechanism by which it achieves this is through activation of the PI3K pathway, leading to induction of phospho-Akt (141). Despite these studies establishing Notch’s critical role in activating PI3K signaling in thymocyte development, the identity of the relevant downstream Notch targets bridging the two pathways remained unclear. To this end, Chapter II investigates the mechanism by which Notch signaling promotes activation of the PI3K pathway, identifying c-Myc, HES1 and PTEN as key players in this cascade. In agreement with previous findings, Notch signaling drives survival, differentiation, and proliferation of thymocytes along the T cell lineage. Specifically at the DN3 stage, Notch induction of HES1 is required for repression of PTEN and maintenance of PI3K signaling, while c-Myc expression is necessary to drive proliferation.

Because of a discrepancy in the signal strength model, transduced DN3 cells that express the γδTCR and differentiate along the γδ-lineage do not require concurrent input from the Notch pathway, in contrast to pre-TCR-bearing DN3 cells, which must receive signals from both Notch and pre-TCR in order to survive and differentiate (140). Previous reports show that elevated ERK-MAPK pathway signaling, which leads to heightened Id3 induction, is both necessary and
sufficient to drive γδ-lineage adoption, and correspondingly decrease the number of αβ-lineage cells. Chapter III extends the theme of examining the molecular players involved in DN3 differentiation and addresses the differential signaling cascades activated downstream of pre-TCR and γδTCR that lead to their differential lineage fates and requirement for Notch signaling. Over-expression of Id3 in DN3 cells was found to be sufficient to promote γδ-lineage development, in the absence of both TCR expression and Notch signaling. Furthermore, requirement for Notch signaling at this selection checkpoint depends on the magnitude by which the TCR signals can down-regulate E2A expression. Strong TCR signals leading to a high induction of Id3 could successfully suppress E protein activity to a level that allowed for passage through selection, independently of Notch signaling, while weak signals such as those produced by the pre-TCR were unable to independently down-regulate E2A to levels that allowed for successful traversal of the β-selection checkpoint, and thus require concurrent Notch signaling to achieve this successfully.

Considering that αβ- and γδ-lineage cells arise from a common progenitor, and considering that the TCRβ, γ and δ chains are simultaneously rearranged in the DN3 cell, the question arises: for the small percentage of DN3 cells that complete rearrangement and assembly of a fully functional pre-TCR and γδTCR and signal through these at the same time, how would these cells interpret the combined TCR signals to determine their lineage fates? Chapter IV looks at the lineage fate outcome of a DN3 cell which simultaneously expresses a TCRβ and γδTCR, and determines whether this decision can be manipulated by the availability of extrinsic and intrinsic molecular factors. Here, I show that DN3 cells simultaneously expressing functional TCRβ, γ and δ chains have a high propensity to develop along the γδ lineage. However, manipulation of Notch signals, γδTCR ligand availability, and strength of signal via Id3 expression levels, can affect this outcome. Specifically, loss of Id3 in γδTCR-bearing DN3 cells does not increase their propensity to differentiate along the αβ-lineage in the presence of Notch signals, but it does inhibit maturation along the γδ-lineage and, surprisingly, also leads to the generation of DP cells in the absence of Notch signals. Furthermore, manipulation of γδTCR ligand strength does not divert the lineage choice of γδTCR-expressing DN3 cells to the αβ-lineage. Instead, weak ligand inhibits Notch-independent development and maturation along the γδ-lineage.

Together, this thesis addresses the underlying mechanisms by which a DN3 cell expressing its respective T cell receptor is able to survive, differentiate, and proliferate to the next stage of
development. A developing T cell's choice to commit to either $\alpha\beta$ or $\gamma\delta$ T lineage is ultimately decided by its TCR, but requires a multitude of molecular factors and ligands that interact at this stage to influence how a developing thymocyte's fate is achieved.
Chapter 2
HES1 opposes a PTEN-dependent check on survival, differentiation and proliferation of TCRβ-selected thymocytes

The work described in this chapter has been submitted for publication: Gladys W. Wong, Gisele C. Knowles, Tak W. Mak, Adolfo A. Ferrando, and Juan Carlos Zúñiga-Pflücker. HES1 opposes a PTEN-dependent check on survival, differentiation and proliferation of TCRβ-selected thymocytes. 2012. *Blood*, in press.

GWW performed all the experimental results shown in this Chapter. GCK performed cell sorting and provided technical expertise, TWM supplied critical reagents, AA Ferrando provided reagents and scientific advice. JCZP provided scientific and technical advice and expertise.
8 Introduction

In the thymus, incoming lymphocyte progenitors encounter an environment known to support intrathymic T cell development including factors such as the Notch ligand Delta-like 4 (Dll4) (316, 317), the cytokine interleukin-7 (IL-7) (203-207) and the chemokine CXCL12 (98, 99). However, how signals derived from these factors are integrated by a developing thymocyte to realize the T cell differentiation program remains to be elucidated.

T cell development is a highly ordered process typically characterized by the expression of CD4 and CD8. The earliest T cell subset is contained among CD4^+CD8^-, double-negative (DN), cells (22, 23), which can be further defined based on the expression of CD44, CD117, and CD25. The most primitive CD44^+CD117^+CD25^- DN1 cell subset contains multipotent progenitors (18, 19, 26, 27) and expression of CD25 marks entry into the T-lineage specified DN2 stage (28). Here, expression of recombination-activating gene-1 (Rag1) and Rag2 induces TCRβ, TCRγ, and TCRδ gene loci to rearrange V(D)J gene segments (30), which continues into the subsequent CD44^+CD117^+CD25^+ DN3 stage, wherein thymocytes irreversibly commit to the T-lineage and are subjected to their first developmental checkpoint, β-selection (24, 31). DN3 cells expressing a productively rearranged TCRβ chain with its partner pTα and CD3 form the pre-TCR complex that mediates passage across β-selection, resulting in rescue from apoptosis, cellular proliferation, TCRβ gene allelic exclusion, and differentiation of DN3 cells to the subsequent CD4^+CD8^+, double positive (DP), stage (32-34).

Notch signaling is initiated when the Notch receptor (Notch1) engages its ligand (Dll4), which leads to the transcriptional activation of Notch target genes (122-128, 318). Notch signals induce adoption of the T cell fate in progenitors that enter the thymus (132-134), and are essential for the survival, proliferation, and differentiation of DN thymocytes along the αβ-lineage, to the DP stage (136-139). Previously, our findings revealed that Notch receptor-ligand interactions are crucial for maintaining cell size, glucose metabolism, and survival of DN3 cells prior to the initiation of β-selection (141). This was due to Notch signals supporting the activation of the phosphatidylinositol-3-kinase (PI3K) pathway, leading to Akt/PKB phosphorylation. In support of this notion, pre-T cells deficient in phosphoinositide-dependent kinase 1 (PDK1), an enzyme which phosphorylates and activates AGC serine kinases, including Akt (319), were found to be
unresponsive to trophic effects of Notch signaling. Despite these studies establishing the critical role for Notch in activating PI3K signaling in developing T cells, the identity of relevant targets downstream of Notch responsible for bridging the two pathways remained unclear. Additionally, other signaling pathways mediated by IL-7R and CXCR4 are known to promote PI3K/Akt activation (98, 99, 320, 321).

Recent studies examining the role of Notch in T-ALL have implicated HES1 and c-Myc as critical targets of Notch signaling in leukemic cells (170, 171, 322). Furthermore, PTEN (Phosphatase and Tensin homolog), an inhibitor of the PI3K pathway, was found to be an indirect target of activated Notch1 in T-ALL cells, via a HES1-mediated repression of the Pten promoter (168). Together, these results suggested a potential mechanism for developing thymocytes by which Notch signaling supported the activation of the PI3K pathway, involving HES1 and PTEN as likely candidate genes.

Here, we investigate the role of HES1, PTEN and c-Myc downstream of Notch signaling in DN3 thymocytes. Using the OP9-DL1 T-cell differentiation system (145, 148), we show that loss of Notch-ligand interactions in DN3 cells led to the down-regulation of Hes1 transcription with a concomitant rise in Pten mRNA expression. DN3 cells with reduced HES1 function exhibited a phenotype similar to loss of Notch signaling, including elevated levels of PTEN expression even in the presence of Notch signaling, supporting the previous report identifying HES1 as a transcriptional repressor of the Pten promoter (168). This was accompanied by impaired proliferation and differentiation along the αβ-cell lineage to the DP stage. Thus, HES1 plays an important role in mediating PI3K regulation and trophic effects by Notch at the β-selection checkpoint. In support of this connection, restoration of PI3K signaling in pre-T cells, through the loss or down-regulation of PTEN, was sufficient to mediate β-selection in the absence of Notch signaling. However, without Notch signals, ectopic expression of c-Myc was critical to also ensure cellular proliferation. Taken together, these findings suggest that Notch signals at β-selection serve to promote PI3K-mediated survival and differentiation through HES1 repression of PTEN, as well as induce c-Myc expression to drive proliferation of thymocytes as they reach the CD4⁺ CD8⁺ stage of T cell development, at which point Notch signaling ceases, to avoid an otherwise inevitable path to leukemic transformation.
9 Experimental Procedures

9.1 Mice

Rag2-deficient mice were bred and maintained in the animal facility of Sunnybrook Research Institute (Toronto, Ontario, Canada) in specific pathogen-free conditions. Pten\(^{ff}\) mice and Lck-cre mice were kindly provided by Dr. T.W. Mak (University Health Network, University of Toronto, Canada). Rag2\(^{-/-}\) Pten\(^{ff};Lck-cre+\) mice were generated by crossing Rag2\(^{-/-}\) mice with Pten\(^{ff};Lck-cre+\) mice. C57Bl/6 and CD1 timed-pregnant mice were obtained from Charles River Laboratories (Montreal, Canada). All animal procedures were approved by the Sunnybrook Health Science Centre Animal Care Committee (Toronto, Ontario, Canada).

9.2 Generation of HES1 and PTEN shRNA constructs and viral-producing cells

shRNA sequences were ordered from Sigma-Aldrich, or obtained from the RNAi consortium and oligonucleotides were ordered from Invitrogen. shRNA sequences were subsequently digested with XhoI and EcoRI and ligated with the Rapid DNA Ligation Kit (Roche) into the LMP vector digested with the same enzymes. Bacterial clones were screened for insert by PCR. GP+E.86 cells transfected for each of these shRNA constructs were generated.

9.3 OP9 co-culture and retroviral transduction

HES1 and dominant-negative HES1 constructs were kindly provided by Dr. R. Kageyama (Kyoto University, Japan) and Dr. A. Strom (Karolinska Institute, Sweden), respectively. Retroviral constructs were generated by subcloning cDNA into MigR1, and stable retroviral-producing GP+E.86 packaging cell lines were generated for each construct. OP9-DL1, OP9-DL4 and OP9-Ctrl cells were produced and maintained as previously described (323), and cultures were supplemented with 1 ng/ml mouse IL-7 and 5 ng/ml human recombinant Flt-3L (Peprotech). Fetal liver was obtained from timed-pregnant Rag2\(^{-/-}\) or CD1 female mice on d 14 of gestation, and bone marrow was harvested from 6-8 week old Rag2\(^{-/-}\) Pten\(^{ff};Lck-cre+\) or Rag2\(^{-/-}\) Pten\(^{+/+};Lck-cre+\) mice. Single-cell suspensions were generated by disruption through a 40-mm nylon mesh screen using a syringe plunger. Bone marrow cell suspensions were purified for Lin\(^{-}\) CD117\(^{+}\) Sca1\(^{+}\) HPCs by cell sorting before culture with OP9-DL1 cells. For retroviral transduction of HPCs, CD24\(^{lo/-}\) CD1 fetal liver cells were enriched for HPCs by complement-
mediated lysis with CD24 antibody, and transduced by overnight culture with stable retrovirus-producing GP+E.86 packaging cells. Transduced (GFP+ or GFP+YFP+) or non-transduced Lin– CD117+ Sca1+ HPCs were purified by cell sorting cultures as previously described (140) and returned to culture with OP9-DL4 cells for T-lineage differentiation. For retroviral transduction of DN3 cells, d 7 HPC OP9-DL1 co-cultures were passaged onto an overnight culture with stable retrovirus-producing GP+E.86 packaging cells. Transduced (GFP+ or GFP+YFP+) or non-transduced CD44– CD25+ DN3 cells were purified by cell sorting from day 8 cultures as previously described (140).

9.4 Flow cytometry and cell sorting

All single-cell suspensions were stained with commercially available antibodies (BD Pharmigen and e-biosciences) and analyzed with a BD-LSRII flow cytometer, using Flowjo software (Treestar, Inc.). Dead cells were excluded from the analyses using DAPI gating.

9.5 Quantitative Real-Time PCR

Thymocyte populations were purified by flow cytometry or selection using magnetic anti-CD45 beads (Miltenyi Biotech). Total RNA was extracted using TRIzol (Invitrogen) and converted to cDNA using Quantitect Reverse Transcription Kit (Qiagen). Expression of the indicated genes was measured by quantitative real-time PCR using SYBR GreenER (Invitrogen). Primer sequences used are listed in Table 1. β-actin was used to normalize cycle thresholds.

9.6 Immunoblots

Whole cells lysates were prepared by resuspending cell pellets in RIPA lysis buffer with protease inhibitors. Protein concentrations were determined by Bradford Assay. Equal amounts of protein from each sample were loaded and resolved using 10% SDS-PAGE, and transferred onto polyvinylidifluoride membranes (Amersham Biosciences). PTEN (Cell Signaling Technology), phospho-GSKβ (Ser9) (Cell Signaling Technology) and GAPDH (Millipore) specific antibodies were used to probe the immunoblots.

9.7 PTEN-luciferase reporter assays

293T cells were transfected with a PTEN-luciferase reporter construct (pGL3 PTEN HindIII-NotI) (168) along with plasmids encoding HES1 (pcDNA3-HES1, generously provided by Dr.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>β-actin</td>
<td>F: ATGGTGGGAATGGGTCAAGAA R: TCTCCATGTGCCAGATTGAAGTTG</td>
</tr>
<tr>
<td>cdkn1a</td>
<td>F: GTCTGAGCGCGCTGAAAGATT R: TCTGCGCTTTGGAATGATAGAAA</td>
</tr>
<tr>
<td>cdkn1b</td>
<td>F: AGCCTGGAGCGGATGGA R: AGTCCCGGTTAGCTCCTCATG</td>
</tr>
<tr>
<td>cdkn1c</td>
<td>F: AGAACCCTGGGACTTCAACT R: GTGAGAAGGCACGACACAGA</td>
</tr>
<tr>
<td>Myc</td>
<td>F: ATCCCGGAGTTGGAAACAAT R: TGAGCTTTTTGCTCCTCTGCTT</td>
</tr>
<tr>
<td>Deltex1</td>
<td>F: TCTGGAGGTACATCGAGGTGTCAAGAGT R: TCGGAGCAGCCTGCCTCATAG</td>
</tr>
<tr>
<td>Hes1</td>
<td>F: TCCTGACGGGCAATTGGC R: GGAAGGGTGCACGTGCTTNGG</td>
</tr>
<tr>
<td>Pten</td>
<td>F: GGGGAAGTAAGGACCAGAAGACAAA R: CCACGGGTCTGTAATCCAGGT</td>
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**Table 1.** Primer sequences used for quantitative real-time PCR. β-actin was used to normalize cycle thresholds.
A. Strom) and/or dnHES1 (pcDNA3-dnHES1, generously provided by Dr. A. Strom) and/or shHES1. PTEN reporter activity was normalized to pRL-CMV Renilla-luciferase expression plasmid. PTEN reporter activity and Renilla luciferase levels (normalization control) were measured 48 h after transfection with the Dual-Luciferase Reporter Assay kit (Promega).

10 Results

10.1 Loss of Notch signaling in DN3 cells leads to cellular atrophy, but not decreased levels of CD127 and CXCR4 expression

As cytokine and chemokine-driven responses in developing T cells are often regulated at the level of receptor expression (266, 324-326), we sought to address whether Notch signals target the PI3K pathway directly, or indirectly by affecting the expression of these receptor-mediated pathways. In particular, we compared the expression levels of CD127 (IL7Rα) and CXCR4 in Rag2−/− DN3 cells cultured in the presence or absence of Notch signaling. To this end, hematopoietic progenitor cells (HPCs) from Rag2−/− fetal livers (E14) were cultured for 8 days on OP9-DL1 cells to allow for T cell lineage commitment and differentiation to the DN3 stage (140). Co-cultures were subsequently sorted for DN3 cells, returned to OP9-DL1 or OP9-Ctrl cells, and analyzed 2 days later (Fig. 9A). As IL-7 is supplemented at 1 ng/ml, and SDF-1α is endogenously expressed in OP9 cells (99), IL-7 and SDF-1 (CXCL12) levels are equivalent in OP9-DL1/Ctrl co-cultures. Importantly, Rag2−/− cells were used to circumvent the confounding effects of pre-TCR signaling on survival, proliferation, and differentiation. As expected, Rag2−/− DN3 cells remained CD44+CD25+ on both OP9-DL1 and OP9-Ctrl cells, albeit CD25 expression appeared to be reduced in the absence of Notch signals, consistent with the report of Notch signaling targeting CD25 gene expression (327). Additionally, Rag2−/− DN3 cells experienced accelerated cell death and atrophy in the absence of Notch ligand, demonstrated by decreased cell numbers (141) and cell size (FSC) (Fig. 9). This was accompanied by a slight decrease in surface expression of CD127 and CXCR4. However, these changes in surface expression were likely due to the observed cellular atrophy.
Figure 9. Notch signaling in developing thymocytes inversely affects the expression of HES1 and PTEN. A) Rag2⁻/⁻ E14 fetal liver (FL)-derived hematopoietic progenitor cells (HPCs) cultured with OP9-DL1 cells for 8 d are used to give rise to CD44⁻ CD25⁺ DN3 cells, which are then sorted and returned to either OP9-DL1 or OP9-Ctrl cells for 2 d prior to analysis. Flow cytometric analysis of CD44, CD25, CD127 and CXCR4 expression is shown for Rag2⁻/⁻ DN3 cells cultured for 2 d in the absence (Ctrl) or presence (DL1) of Notch signaling. Overlay histograms showing cell size (FSC), and surface expression of CD127 and CXCR4 of DN3 cells, cultured as indicated, are shown on the right. Data are representative of at least three independent experiments. B) QRT-PCR analysis of mRNA expression, normalized to β-actin, of Notch downstream target genes (Deltex1, Hes1 and c-Myc) and Pten is shown for Rag2⁻/⁻ DN3 cells cultured for 1 or 2 d as indicated. Data are representative of at least three independent experiments, with standard deviation of the mean shown as error bars. C) QRT-PCR analysis of mRNA expression (as above) is shown for C57BL/6 ex vivo-isolated DN4 and DP thymocyte subsets. Data are representative of at least three independent experiments.
10.2 Transcriptional changes in DN3 cells upon loss of Notch signaling

As loss of PI3K signaling in Rag2−/− DN3 cells cultured in the absence of Notch signals was not likely due to alterations in CD127 and CXCR4 expression, we sought to identify relevant downstream Notch targets responsible for Notch interaction with PI3K at the β-selection checkpoint. To this end, we identified and measured by QRT-PCR the changes in transcript levels that occur upon Notch signaling withdrawal, using mRNA from Rag2−/− DN3 cells cultured for 24 and 48 hours on OP9-DL1 or OP9-Ctrl cells. As expected, loss of Notch signaling in Rag2−/− DN3 cells was accompanied by decreased transcript levels for known Notch target genes Deltex1, c-Myc and Hes1 (Fig. 9B). Recently, HES1 was found to bind and repress the promoter of Pten, an inhibitor of the PI3K pathway (168). A gene expression reporter assay confirmed that HES1 expression decreased Pten promoter activity (Fig. 10). Furthermore, Notch signaling withdrawal in Rag2−/− DN3 cells resulted in decreased Hes1 mRNA and was coupled with increased Pten transcripts (Fig. 11B), making HES1 and PTEN likely candidates for bridging upstream Notch signals to downstream effects on the PI3K pathway. In support of this, analyses of ex vivo thymocytes revealed that, upon loss of Notch signaling associated with traversing β-selection (202), similar changes in transcript levels are observed. Specifically, upon the down-regulation of Notch signaling from the DN4 to the DP stage of development, transcript levels of Notch target genes Deltex1, c-Myc and Hes1 are decreased, concomitantly with increased Pten transcripts (Fig. 9C). Considering these data together, particularly the inverse relationship between HES1 and PTEN levels observed with loss of Notch signaling, we sought to determine whether HES1 played a critical role in the Notch-mediated activation of the PI3K pathway in DN3 cells at the β-selection checkpoint.

10.3 Transcriptional changes, decreased cellularity and DP development following inhibition of HES1 function

HES1 is critically required for proliferation in early T cell progenitors (195, 196, 198, 328). Here, we retrovirally co-transduced DN3 cells to express two key Notch target genes, Hes1 and c-Myc, and assessed whether these cells could traverse the β-selection checkpoint in the absence of Notch signaling (OP9-Ctrl cultures). Flow cytometric analysis revealed that HES1 and c-Myc over-expression in DN3 cells did not overcome the need for Notch-mediated signals at this checkpoint, as seen by the failure to give rise to DP cells (Fig. 12). Interestingly, HES1/c-Myc-
Figure 10. Ectopic expression of HES1 inhibits *Pten* promoter activity. Luciferase reporter construct (pGL3) containing a ~2.7 Kb 5’ upstream region of the *Pten* promoter (pGL3-PTEN) was used to measure the effects of HES1 and/or dnHES1, and/or shHES1 encoding constructs on *Pten* promoter activity. A) 293T cells were transfected with pGL3-PTEN, HES1, dnHES1, and pSV40-Renilla-luciferase, as indicated, and subsequently lysed and assessed for luciferase activity 48 h later. B) 293T cells were transfected with pGL3-PTEN, HES1, shHES1, and pSV40-Renilla-luciferase, as indicated, and subsequently lysed and assessed for luciferase activity 48 h later. Data are representative of three independent experiments.
Figure 11. Expression of a dominant negative form of HES1 (dnHES1) leads to up-regulation of *Cdkn1* and *Pten* expression, and impaired T cell development. A) QRT-PCR analysis of mRNA expression of HES1 target genes (*Cdkn1a,b,c* and *Pten*) in *Rag2*^-/-* DN3 cells retrovirally-transduced to express dnHES1 and/or GFP (MigR1), and then cultured with OP9-DL1 cells for 2 d prior to analysis. QRT-PCR results are normalized to β-actin expression levels, and the data are representative of three independent experiments. B-C) Analysis of PTEN and phosphorylated GSK3β (Ser9) protein expression in B) BWZ.36 cells or C) DN3 cells retrovirally-transduced to express dnHES1, shHES1, and/or GFP (MigR1) is shown as immunoblots of whole cell lysates probed with antibodies specific for PTEN, GSK3β (Ser9), or GAPDH. Data are representative of three independent experiments. D-F) Developmental progression of FL-derived HPCs transduced to express dnHES1 and/or GFP (MigR1) and subsequently cultured for 10 d with OP9-DL4 cells. Flow cytometric analysis of D) CD44 and CD25; and, E) CD4 and CD8, surface expression is shown for GFP^+* gated cells on days 4, 7, and 10 of co-culture, as indicated; while, F) shows the corresponding cellularity of DP cells present in the cultures, as indicated. DP cellularity was obtained by multiplying the total cellularity by the percentage of DP cells present in the cultures of each independent experiment. Data are representative of three independent experiments.
**Figure 12.** Expression of HES1 and c-Myc is insufficient to promote differentiation of DN3 cells across the β-selection checkpoint in the absence of Notch signals. DN3a cells were retrovirally co-transduced to express HES1 and/or GFP (MigR1) and c-Myc (YFP⁺) and cultured for 6 days on OP9-Ctrl cells. A) Flow cytometric analysis of CD4 and CD8 cell surface expression is shown for GFP⁺, YFP⁺, CD45⁺ gated cells; while B-C) shows the corresponding cell size and CD71 expression, respectively, as indicated. Results are representative of three independent experiments.
transduced DN3 cells were smaller in size, as compared to c-Myc only (MigR1/c-Myc) transduced cells, but expressed higher levels of CD71, an indicator of increased PI3K/Akt pathway activity (319).

To more clearly evaluate the role of HES1 in early T cell development, we expressed a dominant-negative version of HES1 (dnHES1) (329) in DN3 cells. Importantly, a gene expression reporter assay confirmed that dnHES1 expression functionally repressed HES1 activity (Fig. 10). HES1 has been proposed to influence proliferation through inhibition of cyclin-dependent kinase inhibitors (181, 330). In agreement with this notion, expression of dnHES1 in Rag2−/− DN3 cells cultured on OP9-DL1 cells increased Cdkn1a, Cdkn1b, and Cdkn1c transcript levels (Fig. 11A). GSK3β is an important downstream target of PI3K/Akt signaling (140-142), and its phosphorylation at Ser9 is mediated by Akt. In support of our hypothesis that decreased HES1 function decreases PI3K/Akt pathway activity, dnHES1-expressing cells show increased PTEN mRNA and protein levels (Fig. 11A-C), corresponding to decreased phosphorylated GSK3β protein levels (Fig. 11B). Together, these results confirm HES1’s role as a repressor of Pten expression in DN3 cells, and further support a mechanism by which Notch signaling influences PI3K pathway activation in thymocytes undergoing β-selection.

We addressed the role of HES1 during T-lineage differentiation by retrovirally transducing fetal liver-derived HPCs to express dnHES1 and/or GFP (MigR1), and using flow cytometry to assess their ability to respond to Notch signals, in co-culture with OP9-DL4 cells (323). Figure 11D-F shows that dnHES1-transduced HPCs displayed a reduced efficiency in T cell differentiation that is particularly noticeable by day 10 of culture, when over 6-times more control (MigR1)-transduced HPCs differentiated to the DP stage than dnHES1-transduced cells (Fig. 11E, F). To circumvent the possibility of non-specific interference by dnHES1 on other bHLH or HES-family transcription factors, shRNA targeting Hes1 was used. A gene expression reporter assay confirmed that shHES1 expression functionally repressed HES1 activity (Fig. 10B). Consistent with results from dnHES1-transduced cells, shHES1-transduced DN3 cells showed increased PTEN protein levels (Fig. 11C), and shHES1-transduced HPCs showed reduced efficiency in differentiation along the T cell lineage (Fig. 13A-C). Together, these findings are consistent with previous reports showing that Hes1-deficiency in hematopoietic precursors is associated with an arrest of T cell development (195, 198).
Figure 13. Expression of an shRNA targeting *Hes1* (shHES1) leads to impaired T cell development. A) Developmental progression of FL-derived HPCs transduced to express shHES1 and/or GFP (MigR1) and subsequently cultured for 10 d with OP9-DL4 cells. Flow cytometric analysis of A) CD44 and CD25; and, B) CD4 and CD8, surface expression is shown for GFP⁺ gated cells on days 4, 7, and 10 of co-culture, as indicated; while, C) shows the corresponding cellularity of DP cells present in the cultures, as indicated. DP cellularity was obtained by multiplying the total cellularity by the percentage of DP cells present in the cultures of each independent experiment. Data are representative of three independent experiments.
10.4 Inhibition of HES1 function allows for non-T lineage differentiation in the presence of Notch signals

The apparent delay and impairment of T cell development in dnHES1-transduced HPCs is evident before and during β-selection. Previous studies using human hematopoietic cells showed that over-expression of HES1 could induce a partial block on B cell development, but could not impose T cell differentiation (199). Here, we find that loss of HES1 function with dnHES1-expression did not promote B cell development of HPCs in OP9-DL1 cultures, but instead increased myeloid-lineage (CD11b+) cell potential (Fig. 14), based on absolute numbers (data not shown) and percentages. While HES1 does not appear to play a critical role in Notch-mediated T versus B lineage bifurcation, it is involved downstream of Notch signaling in the divergence away from myeloid lineage outcomes.

10.5 HES1 function is required for efficient β-selection

To examine the specific role of HES1 in T cell differentiation at the β-selection checkpoint separately of its role in early proliferation and differentiation, HES1 function was manipulated at the later DN3 stage of development. To this end, DN3 cells were isolated and transduced to express dnHES1 and/or GFP (MigR1), and cultured on OP9-DL4 cells. Expression of dnHES1 resulted in a marked reduction in the number of cells reaching the DP stage of differentiation (Fig. 15). Additionally, DN3 cells transduced to express an shRNA targeting Hes1 when cultured on OP9-DL4 cells showed a similar decrease in their ability to develop to the DP stage, as compared to GFP (MigR1)-only transduced DN3 cells (Fig. 15C, D). Furthermore, to more precisely examine the effect of interfering with HES1 function at the β-selection checkpoint, Rag2−/− DN3 cells were co-transduced to express dnHES1 and/or a rearranged TCRβ chain, and pre-TCR-induced differentiation was analyzed. As expected, in the absence of a TCRβ chain, after 6 days of culture with OP9-DL4 cells, MigR1/MIY- and dnHES1/MIY-transduced Rag2−/− DN3 cells remained at the DN stage, while MigR1/TCRβ-expressing cells underwent differentiation from the DN to DP stage (Fig. 16A). In contrast, dnHES1/TCRβ-expressing Rag2−/− DN3 cells showed a marked decrease in differentiation to the DP stage and failed to expand, while MigR1/TCRβ-transduced cells proliferated extensively in response to pre-TCR-
Figure 14. Expression of a dominant negative form of HES1 (dnHES1) leads to CD11b+ non-T-lineage cell differentiation even in the presence of Notch signals. Developmental progression of FL-derived HPCs transduced to express dnHES1 and/or GFP (MigR1) and subsequently cultured for 10 d with OP9-DL4 cells. Flow cytometric analysis of CD19 and CD11b surface expression is shown for GFP+ gated cells on days 4, 7, and 10 of co-culture, as indicated. Data are representative of three independent experiments.
Figure 15. Expression of a dominant negative form of HES1 (dnHES1) in DN3a cells impairs T cell differentiation across the β-selection checkpoint. A) Developmental progression of DN3a cells transduced to express dnHES1 and/or GFP (MigR1) and subsequently cultured with OP9-DL4 cells for 2-4 d. Flow cytometric analysis of CD4 and CD8 surface expression is shown for GFP+ gated cells on days 2, 3, and 4 of co-culture as indicated with the cellular fold expansion (total cellularity at each time point divided by the number of cells used at the start of the culture, input) observed in the cultures shown for the indicated times and conditions. C) Developmental progression of DN3a cells transduced to express shHES1 and/or GFP (MigR1) and subsequently cultured with OP9-DL4 cells for 1-3 d. Flow cytometric analysis of CD4 and CD8 surface expression is shown for GFP+ gated cells on days 1, 2 and 3 of co-culture as indicated with D) the total cellularity observed in the cultures shown for the indicated times and conditions. Data are representative of three independent experiments, with standard deviation of the mean shown as error bars.
Figure 16. Expression of a dominant negative form of HES1 (dnHES1) impairs T cell differentiation across the β-selection checkpoint. Developmental progression of in vitro-derived Rag2−/− DN3 cells co-transduced to express a TCRβ (TCRβ) and/or YFP (MIY) and dnHES1 and/or GFP (MigR1), and cultured with OP9-DL4 cells for 4 d. A) Flow cytometric analysis of CD4 and CD8 surface expression is shown for GFP+ and YFP+ gated cells after 4 d of co-culture, as indicated. B) The cellular fold expansion (total cellularity at each time point divided by the number of cells used at the start of the culture, input) observed in the cultures is shown for the indicated times and conditions. Data are representative of three independent experiments, with standard deviation of the mean shown as error bars. C) Corresponding histograms of cell size and CD71 expression for GFP+ YFP+ gated cells are shown, as indicated. Results are representative of at least three independent experiments.
derived signals (Fig. 16B). Additionally, dnHES1/TCRβ-expressing Rag2<sup>−/−</sup> DN3 cells showed lower levels of CD71 surface expression, but differences in cell size were not observed (Fig. 16C). In keeping with the results from HPCs or DN3 cells transduced to express dnHES1, Rag2<sup>−/−</sup> DN3 cells co-transduced with dnHES1 and TCRβ developed beyond the DN3 stage but exhibited defects in differentiation and decreased cell numbers compared to controls, suggesting that the Notch-dependent differentiation and survival of DN3 cells at the β-selection checkpoint is at least partially mediated by HES1 and likely due to its ability to repress the PI3K inhibitor, PTEN.

10.6 PTEN enforces the Notch-dependent survival and differentiation of DN3 cells across the β-selection checkpoint

PTEN dephosphorylates phosphatidylinositol-3,4,5-triphosphate (PIP3), thus opposing the activity of PI3K. Deletion of PTEN in pre-T cells was found to substitute for IL-7 and pre-TCR signals, both of which lead to downstream activation of the PI3K pathway, and mediate survival and differentiation to the DP stage (119). As Notch signals also provide trophic effects at the β-selection checkpoint via PI3K pathway activation, and considering previous (168) and above findings showing that HES1 represses PTEN expression, we sought to define the relationship between PTEN and Notch in early thymocytes. To this end, we made use of Pten<sup>flox/flox</sup> (Lck-cre<sup>+</sup>) mice, in which deletion of Pten in Pten<sup>f/f;Lck-cre+</sup> T cells begins at the DN3 stage and is complete by the DP stage (Fig. 17A). To test whether the absence of PTEN allows DN3 cells to survive and differentiate across the β-selection checkpoint without Notch signals, bone marrow-derived HPCs from of Pten<sup>f/f;Lck-cre+</sup> and Pten<sup>+/+;Lck-cre+</sup> mice were cultured with OP9-DL1 cells for 14 days. From these cultures, DN3a (prior to β-selection) (76) cells were sort-purified, placed back in culture in the presence or absence of Notch signals, and analyzed 5 days later (Fig. 17B, C). Consistent with our hypothesis, DN3a cells from Pten<sup>f/f;Lck-cre+</sup> mice were able to differentiate into DP cells, while Pten<sup>+/+;Lck-cre+</sup> DN3a cells failed to survive and differentiate in the absence of Notch signals (Fig. 17B,C).

DN3a cells cultured on OP9-Ctrl cells simultaneously experience the absence of two critical signals: Notch and pre-TCR. To circumvent this issue, and to more precisely time the induction of a concomitant gain of pre-TCR signals and a reduction of PTEN expression, we employed Rag2<sup>−/−</sup> DN3 cells co-transduced to express TCRβ and PTEN-shRNAs, respectively. This
Figure 17. Conditional *Pten* deletion in DN3 cells allows for T cell differentiation across the β-selection checkpoint in the absence of Notch signals. A) Deletion of exons 4 and 5 of the *Pten* allele in *PTEN*<sup>ff</sup>;Lck-cre<sup>+</sup> mice is initiated at the DN3 stage of development and completed by the DP stage. DNA from whole thymus of *PTEN*<sup>ff</sup>;Lck-cre<sup>+</sup> or *PTEN*<sup>ff</sup> mice, and from sorted DN2, DN3, DN4 and DP thymocyte subsets of *PTEN*<sup>ff</sup>;Lck-cre<sup>+</sup> mice was extracted and amplified by PCR. Agarose gels with the PCR products corresponding to the deleted and floxed alleles are shown, as indicated. Data are representative of at least three independent experiments. B) Developmental progression of culture-derived *PTEN*<sup>ff</sup>;Lck-cre<sup>+</sup> or *PTEN*<sup>+</sup>+/Lck-cre<sup>+</sup> DN3 cells cultured for 6 d with OP9-DL1 or OP9-Ctrl cells. Flow cytometric analysis of CD4 and CD8 cell surface expression is shown for CD45<sup>+</sup> gated cells; while C) shows the corresponding fold expansion and DP cellularity, as indicated. Lin<sup>−</sup> c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells sorted from BM of *PTEN*<sup>ff</sup>;Lck-cre<sup>+</sup> or *PTEN*<sup>+</sup>+/Lck-cre<sup>+</sup> mice were cultured with OP9-DL1 cells for 14 d, sorted for DN3a cells, and returned to OP9 stroma for 6 d. Fold expansion was obtained from the total cellularity divided by the number of cells used at the start of the culture (input), and DP cellularity by multiplication of the total cellularity by the percentage of DP cells present in the cultures. Results are representative of three independent experiments.
approach not only coordinates the timing of pre-TCR expression, but ensures that any death of DN3 cells is not due to a lack of pre-TCR signals (140). Two PTEN-shRNA lentiviral constructs (LMP-HP_522, LMP-HP_524) were tested in Rag2\(^{-/-}\) DN3 cells, with HP_522 and HP_524 shRNA constructs knocking down Pten mRNA by 50% and 75%, respectively (Fig. 18A). A similar decrease was seen in PTEN protein expression (Fig. 18B). While PTEN-shRNA transduced TCRβ\(^{+}\) Rag2\(^{-/-}\) DN3 cells bypassed the requirement for Notch signals at the β-selection checkpoint, as measured by the presence of DP cells on OP9-Ctrl cells (Fig. 18C,D), control firefly luciferase-shRNA (LMPff)-transduced TCRβ-expressing cells failed to traverse the β-selection checkpoint in the absence of Notch signals. The survival and differentiation of PTEN-shRNA-transduced TCRβ\(^{+}\) Rag2\(^{-/-}\) DN3 cells was PTEN-dose dependent, as greater knockdown of PTEN with HP_524 shRNA led to a greater differentiation capacity in the absence of Notch signals. Similar to what was seen with Pten\(^{ff/Lck-cre+}\) cells, PTEN-knockdown in TCRβ\(^{+}\) Rag2\(^{-/-}\) DN3 cells cultured without Notch signals had dramatically reduced proliferative potential compared to those receiving Notch signals.

10.7 c-Myc induction is required for Notch-mediated cellular proliferation

While conditional Pten-deleted DN3a cells survive and differentiate to DP cells in the absence of Notch signaling, they fail to undergo cellular proliferation. This may be due to the loss of additional Notch-mediated proliferation mechanisms that cannot be compensated by the absence of PTEN. To address this, we generated Rag2\(^{-/-}\) Pten\(^{ff/Lck-cre+}\) mice, which allowed for a more precise definition of the interactions between pre-TCR, Notch, and PTEN at the β-selection checkpoint, as the timing of pre-TCR expression, loss of PTEN, and Notch availability can be manipulated by TCRβ-transduction, Lck-mediated Pten deletion and Delta-like availability in OP9 cell cultures. In vitro-derived DN3 cells from Rag2\(^{-/-}\) Pten\(^{ff/Lck-cre+}\) and Rag2\(^{-/-}\) Pten\(^{+/+Lck-cre+}\) mice were transduced to express TCRβ and YFP (MIY) and cultured in the absence or presence of Notch signals (Fig. 19). As seen before, proliferation of TCRβ-transduced Rag2\(^{-/-}\) Pten\(^{ff/Lck-cre+}\) DN3 cells receiving Notch signals was dramatically higher than that of the other groups (Fig. 19C). Also in agreement with the previous experiments, in the absence of Notch signals, TCRβ-transduced Rag2\(^{-/-}\) Pten\(^{+/+Lck-cre+}\) DN3 cells could not survive or differentiate, while Pten-deleted cells overcame this block (Fig. 19), albeit still with much reduced cellularity compared to cells receiving Notch signals.
Figure 18. Knockdown of \textit{Pten} expression in \textit{Rag2}\textsuperscript{−/−} DN3 cells with TCR\textbeta allows for T cell differentiation across the \textbeta-selection checkpoint in the absence of Notch signals. A) QRT-PCR analysis (normalized to \textbeta-\textit{actin}) of \textit{Pten} mRNA expression in \textit{Rag2}\textsuperscript{−/−} DN3 cells transduced to express PTEN shRNA (LMP-HP\textsubscript{522} or LMP-HP\textsubscript{524}) or firefly luciferase shRNA (LMPff). Data are representative of three independent experiments. B) Analysis of PTEN protein expression in NIH3T3 cells retrovirally-transduced to express the indicated shRNAs is shown as immunoblots of whole cell lysates probed with antibodies specific for PTEN or GAPDH. Data are representative of three independent experiments. C-D) Developmental progression of culture-derived \textit{Rag2}\textsuperscript{−/−} DN3a cells transduced to express shRNAs, as indicated, and subsequently cultured for 6 d with OP9-DL1 or OP9-Ctrl cells. C) Flow cytometric analysis of CD4 and CD8 surface expression is shown for GFP\textsuperscript{+} (shRNAs) and YFP\textsuperscript{+} (TCR\textbeta or MIY) gated cells; while, D) shows the corresponding cellularity of DP cells present in the cultures, as indicated. DP cellularity was obtained by multiplying the total cellularity by the percentage of DP cells present in the cultures of each independent experiment. Data are representative of three independent experiments.
Figure 19. Conditional Pten deletion and ectopic expression of c-Myc allows for survival, differentiation and proliferation of DN3 cells across the β-selection checkpoint in the absence of Notch signals. Developmental progression of culture-derived Rag2<sup>−/−</sup> PTEN<sup>+/+;Lck-cre<sup>+</sup></sup> or Rag2<sup>−/−</sup> PTEN<sup>f/f;Lck-cre<sup>+</sup></sup> DN3 cells retrovirally co-transduced to express TCRβ (GFP<sup>+</sup>) and MIY or c-Myc (YFP<sup>+</sup>) and cultured with OP9-Ctrl or OP9-DL1 cells for 6 d. A) Flow cytometric analysis of CD4 and CD8 cell surface expression is shown for GFP<sup>+</sup>, YFP<sup>+</sup>, CD45<sup>+</sup> gated cells; while B-C) shows the corresponding DP cellularity and fold expansion, respectively, as indicated. Lin<sup>−</sup> c-Kit<sup>+</sup> Sca-1<sup>+</sup> cells sorted from BM of PTEN<sup>f/f;Lck-cre<sup>+</sup></sup> or PTEN<sup>+/+;Lck-cre<sup>+</sup></sup> Rag2<sup>−/−</sup> mice were cultured with OP9-DL1 cells for 14 d, retrovirally-transduced, then sorted for YFP<sup>+</sup> and GFP<sup>+</sup> DN3 cells, and cultured as indicated. Fold expansion was obtained from the total cellularity divided by the number of cells used at the start of the culture (input), and DP cellularity by multiplication of the total cellularity by the percentage of DP cells present in the cultures. Results are representative of three independent experiments.
Considering c-Myc transcript levels are reduced in the absence of Notch signals (Fig. 4B), we examined whether restoring c-Myc expression in DN3 cells could allow for cellular proliferation in the absence of Notch signals. To this end, we co-transduced Pten<sup>+/+Lck-cre+</sup> or Pten<sup>−/−Lck-cre+</sup> Rag2<sup>−/−</sup> DN3 cells to express c-Myc and TCRβ. In the absence of Notch signaling, only TCRβ/c-Myc-expressing Pten<sup>−/−Lck-cre+</sup>, but not Pten<sup>+/+Lck-cre+</sup>, DN3 cells traversed the β-selection checkpoint and differentiated into DP cells (Fig. 19A, B). Furthermore, Rag2<sup>−/−</sup> Pten<sup>−/−Lck-cre+</sup> DN3 cells proliferated appreciably, generating DP cells at percentages comparable to TCRβ<sup>+</sup> Rag2<sup>−/−</sup> Pten<sup>+/+Lck-cre+</sup> cells cultured on OP9-DL1 (Fig. 19). In the presence of Notch signaling, TCRβ/c-Myc co-transduced DN3 cells from mice of both genotypes differentiated across β-selection and proliferated extensively.

Together, these results indicate that in lieu of Notch signals that induce HES1-mediated repression of PTEN expression, loss of PTEN partly substitutes for the required Notch receptor-ligand interactions, with an added requirement for ectopic c-Myc expression to restore the Notch-induced proliferative burst associated with cells traversing the β-selection checkpoint.

11 Discussion

In this study, we addressed the mechanism by which Notch signals mediate trophic effects at the β-selection checkpoint. In mice, loss of Notch signaling completely blocks development of DN cells to the DP cell stage (133, 136, 137), even upon expression of a functional TCRβ chain (140-142, 284, 331). Our lab recently showed that Notch-ligand interactions were crucial for maintaining PI3K/Akt pathway activity, leading to survival and glucose metabolism in DN3 cells (140, 141). Despite these studies demonstrating a relationship between Notch signaling and the PI3K pathway, the precise mechanism for this interaction was unknown. Here, we find HES1, PTEN, and c-Myc as key molecular players downstream of Notch for the regulation of survival, differentiation and proliferation at the β-selection checkpoint (Fig. 20).

PI3K signaling and downstream Akt/PKB activation, is essential for the survival and metabolism of proliferating pre-T cells (100, 141). In the thymus, known upstream inducers of this pathway include IL-7R (CD127) (119, 332) and CXCR4 (98, 99). However, whether these signaling pathways or their molecular intermediates are the targets of Notch, and the means by which Notch regulates activation of the PI3K pathway remained unclear. Although cytokine and chemokine-based responsiveness of developing T cells is often regulated at receptor expression
**Figure 20.** A model of the effects Notch signaling via HES1 on PI3K/Akt pathway activity at the β-selection checkpoint. The figure depicts a thymus epithelial cell expressing Delta-like engaging and activating the Notch receptor (via the generation of intracellular Notch, ICN) on a DN3 thymocyte leading to the induction of HES1 and c-Myc expression. HES1, a transcriptional repressor, down regulates PTEN expression, thus decreasing PTEN’s inhibition on PI3K/Akt pathway activity, occurring downstream of CXCR4 and/or IL-7R signaling. Although repression of PTEN by Notch, via HES1, is sufficient to induce survival and differentiation in pre-TCR⁺ DN3 thymocytes, c-Myc is additionally responsible for the proliferative effects downstream of Notch during β-selection.
levels (266, 324-326), no changes in CD127 and CXCR4 expression were found with the loss of Notch signaling, suggesting that decreased PI3K signals are not due to receptor down-regulation. Additionally, we previously showed that IL-7R function, as measured by STAT5 phosphorylation, is retained following the loss of Notch signals (141). Recently, IGF1R expression was reported to be regulated by Notch signals in T-ALL (333), providing a potential link to PI3K pathway activation in leukemic cells. However, a gene expression microarray analysis from Rag2−/− DN3 cells cultured on OP9-DL1 or OP9-Ctrl cells failed to show changes in Igf1r expression upon loss of Notch signals (unpublished results). These results suggest that receptors that can activate PI3K are operationally available for DN3 cells, but that concomitant Notch signals are required to ensure that this signaling pathway becomes active and responsive to external cues.

The importance for Notch signaling at the DN3 stage and its ability to support PI3K activation is additionally critical when considering the dual signaling properties of CXCR4 (334), namely its ability to promote either cell survival or apoptosis via PI3K/Akt or p38/MAPK, respectively. Under circumstances where Notch signaling is discontinued and CXCR4 continues to operate with a diminished capacity to induce PI3K signals, the observed loss of DN3 cells could be due to CXCR4-induced p38 signaling, which has been shown to disrupt early thymocyte differentiation (335). In this regard, we previously showed that inhibition of p38 signaling leads to increased survival and proliferation of β-selected DN3 cells cultured on OP9-DL1 cells (140).

A key question that arises is how a signaling pathway like Notch, which directly regulates gene transcription within the nucleus, can affect the activity of receptor proximal signals that engage the PI3K pathway. Here, we establish a mechanism of interaction between Notch and the PI3K pathway, whereby Notch-dependent transcriptional activation of HES1 mediates the down-regulation of PI3K pathway inhibitor, PTEN (168). This interplay explains how Notch is able to influence the signaling outcomes from cell surface receptors that employ the PI3K pathway. The functional interaction among these players was also observed in T-ALL (168), highlighting the need for tight regulation of these interactions during normal T cell development. This is achieved by the temporal regulation of Notch receptor expression after the β-selection checkpoint (336), as well as the auto-inhibitory loop of HES1, which limits the heightened level of PI3K responsiveness in DN cells.
Our findings point to HES1 as the key executor of Notch signals that ultimately affects PI3K activity. HES1 appears to not only repress the expression of various cyclin-dependent kinase inhibitors (181, 330), but also represses Pten promoter activity, ensuring that β-selected cells can maximize their ability to proliferate and respond to the growth-promoting cues provided by the pre-TCR and present within the thymus microenvironment. Experimentally, interfering with HES1 function in DN3 cells increases PTEN expression, resulting in decreased ability to differentiate and progress across β-selection. However, a complete blockage in T cell development is not seen, and this is likely due to incomplete inhibition of HES1 function by dnHES1, or compensation by Hes1 related genes, e.g., Hey genes (337). Although HES1 was previously observed to promote proliferation of thymocytes (181, 195, 328), we found that HES1 over-expression, even with ectopic c-Myc expression, was unable to compensate for Notch signal withdrawal at the β-selection checkpoint. This is likely due to the strong transcriptional repressor activity of HES1 (182), which becomes unchecked with a retroviral expression system. Nonetheless, HES1 over-expression in DN3 cells led to increased CD71 expression, an indicator of PI3K/Akt pathway activity in thymocytes (319), supporting the proposed regulatory gene network.

A recent report using Hes1ff mice revealed a critical role for HES1 in early T-lineage commitment, proliferation, and differentiation (198). Our findings that interfering with HES1 function affected early T cell development and expansion, and led to an increase in non-T lineage cells are consistent with that report. However, our results point to a mechanistically important role for HES1 at the β-selection checkpoint, which was not seen in the Hes1ff mice. Several potential explanations exist for the apparent discrepancies. Key differences exist between the approaches used, including the stage of T cell development at which HES1 function is manipulated, and the timing for when the developmental effects are interrogated. For example, we used Rag2−/− DN3 cells to more precisely pinpoint the developmental stage at which the requirement for HES1 function was examined, either before or after DN3 cells are induced to receive signals from the pre-TCR or Notch receptors. With this approach, we were able to discern a clear role for HES1 in β-selecting DN cells to down-regulate PTEN expression. However Wendorff et al. (198) did not offer a potential mechanism of action for HES1 in T cell development, and did not see a role for HES1 in regulating PTEN expression. This last conclusion was based on an analysis of DP leukemic cells, and not normal DN cells, and as such,
it is likely that DP cells, which typically do not express HES1, would not show a change in PTEN expression when the Hes1 gene is deleted. Additionally, we found that Pten deletion efficiently restored DN3 survival and differentiation to the DP stage upon Notch signal withdrawal.

While conditional loss of PTEN in DN3a cells allows for their survival and differentiation to the DP stage in the absence of Notch signals, their proliferative capacity is greatly diminished, indicating that other pathways downstream of Notch are responsible for this outcome. Several studies of T-ALL have implicated c-Myc as a direct downstream target of Notch signaling, and as a critical component in transformation and cell growth (170, 171, 338). In T cell development, c-Myc is reported as a mediator of proliferation, but not developmental progression (339, 340). In agreement with these reports, we find that Notch-induced c-Myc expression at this stage of development is responsible for promoting cellular expansion, leading to a large DP cell pool, and proliferation within the DN subset. While we showed that loss of HES1 function leads to increased expression of cell cycle inhibitors, ectopic expression of c-Myc in the absence of Notch signals would likely counter the loss of HES1-mediated Cdkn gene repression (341), thus enabling cell cycle progression and bypassing the need for Notch signaling to support the proliferative burst typically associated with pre-TCR signaling. Collectively, our findings show that more than one downstream effector is responsible for the trophic effects of Notch signaling at the β-selection checkpoint.

Notch signaling is required by pre-T cells to traverse the β-selection checkpoint. Here, we identify key signaling intermediates downstream of Notch that are responsible for T-lineage differentiation, proliferation, survival and cellular metabolism. HES1 and PTEN are largely responsible for coordinating differentiation, survival and metabolism of pre-T cells at the critical β-selection checkpoint by bridging Notch signals to the activation of the PI3K/Akt pathway, while Notch induction of c-Myc expression drives the proliferation of β-selected cells that reach the DP stage of T cell development, at which point Notch signaling ceases to avoid an otherwise inevitable path to leukemic transformation.
Chapter 3
Marked Induction of the Helix-Loop-Helix Protein Id3 Promotes the γδ T Cell Fate and Renders Their Functional Maturation Notch Independent

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*Contributed equally to this work.

GWW performed the experimental results shown in figures 29, 34-36, and table 3 of this Chapter. JPHL, SYL and JML performed the experimental results shown in figures 21-28 and 30-33. MC performed the experimental results shown in figure 34A, and MR provided technical support. DJK, JCZP and DLW provided scientific and technical advice and expertise.
12 Introduction

T cells comprise two major lineages that arise from a common progenitor and are identified by the T cell receptor (TCR) complex they express, αβ and γδ (277, 284). Nevertheless, the molecular processes that underlie specification of the αβ and γδ fates remain poorly understood. Two models have been advanced to explain the role of the TCR in this fate decision: stochastic and instructional (342). The stochastic model stipulates that fate adoption is independent of TCR signals, which serve only to rescue survival of the appropriately matched, predetermined fate. Conversely, the instructional model posits that TCR signals direct adoption of the fate that matches the TCR from which the signal is derived (i.e., pre-TCR for the αβ fate and γδTCR for the γδ fate). While existing data do not easily fit either model, as originally proposed, emerging evidence supports an instructional model in which TCR signal strength dictates fate adoption (272, 273, 284). Indeed, recent single-cell progenitor analyses defined the CD4−CD8−CD44−CD25+ (DN3) stage as the developmental step at which T-lineage commitment is completed (284). These studies further demonstrated that ectopic expression of a pre-TCR or γδTCR in Rag2-deficient DN3 thymocytes was capable of instructing commitment to the αβ and γδ lineages, respectively (284). Moreover, it was recently shown that the linkage between specific TCR expression and fate selection can be severed genetically in that a single TCR complex could induce the differentiation into either fate when the resulting signals were modulated (272, 273). These findings support the hypothesis that the strength of the TCR signal is a critical, lineage-determining factor and that this determination can be made irrespective of the TCR complex from which the signals arise.

Our previous analyses suggested that the stronger signals that promote adoption of the γδ-fate involve pronounced activation of the extracellular signal-related kinase (ERK)-early growth response gene (Egr)-inhibitor of DNA binding 3 (Id3) pathway (ERK-Egr-Id3 pathway) (272, 273). Nevertheless, it remained unclear how differential ERK-Egr-Id3 signaling might function in regulating fate choice or how it might be integrated with signaling input from other pathways, such as Notch. One attractive target of strong TCR signals is E proteins, which are basic helix-loop-helix transcription factors that bind DNA at E-box motifs (CANNTG) (343). Mice in which E protein genes are ablated (e.g., mice lacking E2A, HEB, or both) exhibit severely perturbed development of αβ T cells but show relatively mild effects on γδ T cell differentiation (66, 67,
Accordingly, strong TCR signals might selectively impair αβ T cell development by phenocopying E protein deficiency, either through marked induction of Id3, which can heterodimerize with E proteins and block their function, or by increased ERK activation, which can induce E protein degradation through a phosphorylation-dependent mechanism (344, 345).

E proteins may also serve as a common downstream focal point for TCR and Notch signaling. Notch molecules are surface receptors involved in a wide variety of cell-fate decisions, including αβ and γδ lineage commitment (346, 347). Recently, it was determined that αβ-lineage precursors are dependent upon Notch signaling throughout their pre-TCR-induced differentiation program to the CD4+CD8+ DP stage; however, γδ-lineage thymocytes become Notch-independent upon expression of the γδTCR complex (284). Like TCR signaling, Notch signaling can suppress E protein function through ERK activation or Id3 induction (344, 345, 348). We suggest a model in which the lineage-specific requirement for Notch signaling is determined by the relative strength of TCR signals, through effects on E protein activity. Accordingly, we hypothesize that Notch-independent differentiation is conferred on γδTCR-expressing cells through strong TCR signals, which are capable of repressing E protein activity below the threshold required for γδ differentiation without Notch signals. In contrast, the pre-TCR signals that promote the αβ fate are too weak without the assistance of Notch to suppress E protein activity sufficiently to surmount the E protein mediated checkpoint at the DN3 stage (68, 69). Because of the ability of Id3 to regulate E protein activity, our model suggests that Id3 would be a key molecular mediator that facilitates γδ-T cell commitment and differentiation.

Here, we show that Id3 is required for strong TCR signals to both promote adoption of the γδ fate and oppose the αβ-fate outcome. Moreover, although in most cases Id3 is required for the differentiation of the broader repertoire of non-autoreactive γδ-T cells, in certain cases when γδ-TCR affinity for ligand was very high, Id3 appears to restrict the differentiation potential of that particular TCR-Vγ subset. Id3 also plays a critical role in conferring Notch-independence to developing γδ-lineage cells and in arming them to proliferate and produce IFNγ in response to stimulation. This function of Id3 requires its ability to interact with and suppress E protein targets. Taken together, these findings implicate Id3 as an important molecular-determining factor in enforcing T-cell-fate decisions, integrating Notch and TCR signals, and enabling functional maturation.
13 Experimental Procedures

13.1 Mice

All mice were maintained in the fully accredited animal facilities at either the Fox Chase Cancer Center or the Sunnybrook Research Institute, and their use was approved by the Institutional Animal Care and Use Committee. All mouse strains used were described previously (272). Id3−/− mice were kindly provided by Drs. R. Benezra (Memorial Sloan-Kettering Cancer Center) and Y. Zhuang (Duke University) (349) and used both on the 129 background and after backcrossing to C57BL/6 for three generations. Egr1Tg mice were provided by Toru Miyazaki (University of Tokyo) (350).

13.2 Retroviral transduction and culture

LZRS-pBMN-linker-IRES-eGFP (LZRS) and LZRS-Egr1 vectors have been previously described (70). Murine Id3 was cloned by PCR using primers that spanned the start and stop codons. Serine 49 of Id3 was mutated to P (S49 → P) by standard methodology and, following sequence verification, was subcloned into pMiY using standard methodology. Viral supernatants were produced as described and used to spin-infect progenitors prior to seeding fetal thymic lobes (Il2rg−/−Rag2−/−) or plating on OP9 stromal layers as described (284, 351). The effect of ectopically expressed genes on thymocyte development was assessed flow cytometrically by gating on cells positive for the fluorescent indicator protein. DN3 cells (d7 HSC OP9-DL1 cultured) were transduced by overnight coculture using stable retrovirus-producing GP+E.86 packaging cells as previously described, followed by coculture on OP9 monolayers in OP9-medium (α-MEM supplemented with 1 ng/ml mouse IL-7 [Peprotech] and 5 ng/ml human recombinant Flt-3 ligand [Peprotech]) (284).

13.3 Flow cytometry

Skin preparations were performed as described (352). All single-cell suspensions were stained with commercially available Ab (BD PharMingen, eBiosciences, and Caltag) and analyzed on either a FACS Vantage SE (Becton Dickinson) or BDLSR using Flowjo software (Treestar, Inc.). Anti-Vγ Ab were generously provided by Dr. P. Pareira (Pasteur Institute). BrdU staining was performed according to manufacturer's specifications following a 4 hr pulse with BrdU (i.p.
injection of 100 μg/mouse). Where applicable, dead cells were excluded from the analyses using propidium iodide (PI) or DAPI gating.

13.4 Real-time PCR

Thymocyte populations were purified by flow cytometry, where necessary, following depletion using magnetic anti-CD8 beads (Miltenyi Biotec). RNA was extracted using the RNeasy kit according to manufacturer's specifications (QIAGEN) and converted to cDNA using the SuperscriptII kit (Invitrogen). Expression of the indicated genes was measured by real time PCR using stock Taqman probes purchased from Applied Biosystems, Inc., as described, on a ABI Prism 7500 Real Time PCR machine (74). Some of the quantitative PCR analyses were performed using SYBR GreenER (Invitrogen). Primer/probe set order numbers are given in Table 2. β-Actin expression was used to normalize cycle thresholds, which were then converted into fold difference. All samples were analyzed in triplicates or quadruplicates for +RT or duplicates for −RT, and a nontemplate control was added to each PCR reaction.

13.5 Immunoblotting

Cells lysates and nuclear extracts were prepared as previously described (68, 140), resolved by SDS-PAGE through 10% acrylamide gels, and transferred onto PVDF membranes (Amersham Biosciences, Baie d'Urfé, QC). Immunoblotting was performed with antibodies specific for E47 (N-649), HEB (A-20), Tata Binding Protein (ITBP-18), and GAPDH, all from Santa Cruz.

13.6 Proliferation and cytokine production

DN thymocytes prepared by anti-CD8 bead depletion were stimulated on plate bound anti-CD3 Ab and subjected to MTT assay as described (272). DN thymocytes were labeled with CFSE according to manufacturer's directions (Molecular Probes, Invitrogen) and stimulated for 48 hr with plate-bound anti-CD3 Ab (5 μg/ml). CFSE labeling was detected by flow cytometry. For detection of IFNγ-producing cells, DN thymocytes were stimulated for 24 hr with anti-CD3 Ab. For the last 4 hr of stimulation, GolgiPlug (Benton Dickinson) was added to block protein secretion. The cells were permeabilized and stained for intracellular IFNγ using the BD Cytofix/Cytoperm Solution Kit, and the percentage of IFNγ producing cells was determined by flow cytometry.
Table 2. Primer sequences used for quantitative real-time PCR. β-actin was used to normalize cycle thresholds.
13.7 PMA and ionomycin stimulation

CD45^GFP^ cells were sorted from transduced DN3 cultured for 6 days on either OP9-Cntl or OP9-DL1 cells. Sorted cells (5 × 10^4 cells/well of a 96-well plate) were subsequently cultured with 50 ng/ml PMA (Sigma) and 500 ng/ml Ionomycin (Sigma) in OP9 medium supplemented with 50 U/ml IL-2 (Peprotech). Supernatants were harvested after 36 hr, and IFNγ levels were quantified using the DuoSet ELISA Development System (R&D Systems) according to the manufacturer's protocol.

14 Results

14.1 Id3 is required for Egr1-mediated regulation of fate adoption

We have previously shown that the ERK-Egr1-Id3 pathway plays an important role in the specification of T-lineage fates (272). Expression of Egr1, Egr3, and Id3 mRNA was found to be higher in thymic γδ cells than in DN3 and DN4 precursors, representing a mixture of αβ and γδ lineage progenitors (Fig. 21A (277, 284)). Moreover, as reported previously, retroviral transduction of Egr1 into C57BL/6 E13.5 fetal thymocytes markedly increased the frequency and number of mature γδ T cells (i.e., CD24^lo γδTCR^+) at the expense of αβ-lineage-committed DP thymocytes (Fig. 21B, C) (272). The ability of ectopically expressed Egr1 to simultaneously promote γδ development and oppose the development of αβ lineage cells was dependent on the Egr target, Id3, as these effects are markedly diminished in Id3-deficient cells (Fig. 21B, C). Id3-deficiency did not completely eliminate the ability of Egr1 transduction to alter cell fate, perhaps because of partial compensation by other Egr targets, such as Id2 (Fig. 22). Taken together, these data implicate the ERK-Egr-Id3 pathway as a key component of the strong signals that modulate αβ-γδ lineage fate in the thymus.

14.2 Id3 is required for development of the normal repertoire of γδ T Cells

Because Id3-deficiency impaired the Egr-induced development of fetal γδ T cells, we asked whether γδ cell development was similarly perturbed in adult mice. Surprisingly, we found that Id3-deficiency resulted in an increased number of CD24^lo γδ-lineage cells in the thymus (Fig. 23A). Consistent with a recent report, the expansion appeared to be restricted to a subpopulation
Figure 21. Id3 is required for Egr1-mediated promotion of γδ development. (A) Expression levels of Egr1, Egr3, and Id3 mRNA in thymocyte subsets. Egr and Id mRNA expression was quantified in triplicate in the indicated cell populations by real-time PCR, standardized using β-actin, and normalized to expression in DN3. Results ± SD are depicted graphically. (B and C) Induction of γδ development by enforced expression of Egr1 is dependent upon Id3. E13.5 Id3+/− and Id3−/− fetal thymocytes were transduced with empty vector (LZRS) or Egr1 (LZRS-Egr1), seeded into thymic lobes, and placed in fetal thymic organ culture for 2 days. Developmental progression was assessed by flow cytometry on electronically gated transduced (GFP+) cells. The absolute number of γδ-lineage cells (γδTCR+ or γδTCR+CD24lo) and αβ-lineage DP thymocytes per lobe is depicted graphically in (C). Results are representative of three experiments performed.
Figure 22. *Id2* expression is slightly elevated in *Id3*−/− γδTCR+ thymocytes. γδTCR expressing thymocytes were isolated from the indicated mice, following which the indicated mRNAs were quantified by real time PCR using Taqman probes. mRNA levels were normalized to that of GAPDH and standardized to that expressed in γδ cells from C57BL/6 mice. Error bars represent SD.
Figure 23. Id3 deficiency disrupts the development of γδ T Cells.  (A and B) Id3 deficiency results in expansion of the Vγ1.1+ subset.  Id3−/− mice were backcrossed to the C57BL/6 background for three generations before tissues from mice of the indicated genotypes were analyzed by flow cytometry.  Results are depicted as histograms and summarized graphically on the right.  Error bars represent SD.  (C and D) Id3-deficiency rescues deletion of autoreactive γδ precursors. Thymocyte preparations were produced from Rag2+/− KN6 Tg mice expressing positively selecting ligand (T-10d) or higher-affinity negatively selecting ligand (T-10d/b) and from KN6 Tg mice expressing high-affinity ligand but lacking Id3. Preparations were analyzed by flow cytometry and gated DN cells were displayed as histograms as above. Differences in absolute number of the indicated DN cells are marked by asterisks are statistically significant (p < 0.05). Results are representative of three experiments performed with a minimum of two mice per genotype in each experiment. Error bars represent SD.
of Vγ1.1⁺ γδ T cells (353), as other γδ cells, including the Vγ2 and Vγ3 dendritic epidermal T cell (DETC) subsets, were reduced (Fig. 23A, B). Unlike the conventional Vγ2 and Vγ3 subsets, Vγ1.1⁺ γδ cells have been suggested to represent so-called NK γδ T cells, which are thought to be autoreactive and selected on high-affinity ligands (354-356). We reasoned that such cells might normally be deleted by excessively strong signals but are allowed to survive and expand in the absence of Id3. Since the putative selecting ligand(s) for the Vγ1.1⁺ subset have not been identified, we sought to test this possibility by using the KN6 γδ TCR transgenic (Tg) mice in which KN6 γδ TCR Tg thymocytes are positively selected in the thymus on the nonclassical MHC class Ib molecule, T10d, but are deleted by the 10-fold higher-affinity T-10b (and/or T-22b) ligand (Fig. 23C) (37, 357). Our hypothesis predicts that deletion of KN6 Tg thymocytes by T-10b ligand should be prevented by Id3-deficiency. Indeed, Id3-deficiency blocked deletion of KN6 Tg thymocytes by T-10b ligand, resulting in a marked expansion of KN6 Tg thymocytes with a mature CD24lo phenotype (Fig. 23D). These data suggest an apparent dichotomy of Id3 function, with Id3 restricting the development of TCR-Vγ subsets bearing high-affinity γδTCRs while at the same time being required for development of the broader repertoire of non-autoreactive γδ-T cells.

14.3 Regulation of lineage fate by the Egr-Id3 pathway does not require alterations in TCR repertoire

The selective inhibition of particular Vγ subsets by Id3-deficiency could result from effects on the processes of lineage commitment, selection and maturation, or gene rearrangement (353). Consistent with the latter possibility, the E protein targets, whose functions are antagonized by Id3, have been shown to be involved in regulating TCR gene rearrangement (313, 358). Accordingly, it was possible that ectopic expression of Egr1 and Id3-deficiency were modulating lineage fate indirectly by altering the TCR repertoire. To test this possibility, we revisited the role of superinduction of Egr1 and Id3 on T cell fate in a model with a fixed TCR repertoire. For this purpose, we employed KN6 γδ TCR Tg Rag2-deficient mice in which lineage choice can be manipulated by modulating access to ligand or signaling output from a single TCR complex (37, 272). Thymocytes from KN6 Tg mice expressing T10d ligand adopt the γδ fate (CD4⁺CD8⁻ γδ TCR⁺ CD24lo), whereas those lacking ligand (i.e., β2M deficient) commit to the αβ lineage, undergo proliferative expansion, and develop into DP thymocytes (Fig. 24). Accordingly, we
**Figure 24.** Schematic of αβ/γδ lineage commitment in the KN6 γδ TCR transgenic model. The signal strength hypothesis posits that weak signals promote commitment to the αβ lineage while comparatively strong signals promote commitment to the γδ lineage, irrespective of the TCR complex from which they originate. Accordingly, the signal strength model predicts that a single TCR should be capable of supporting development of both αβ and γδ lineages depending upon the nature of the resulting signals. This was tested by manipulating the signals transduced through the KN6 γδ transgenic TCR and then assessing the developmental consequences. When expressed on a *Rag2<sup>−/−</sup>* background to prevent expression of other receptor forms, the KN6 γδ transgenic TCR directs adoption of the γδ fate in response to ligand engagement (T-10d). γδ lineage cells are defined phenotypically as γδTCR<sup>+</sup>CD24<sup>lo</sup> DN thymocytes. Removal of ligand diverts KN6 transgenic thymocytes to the αβ lineage, which is characterized by robust proliferative expansion, differentiation to the DP stage, and downregulation of TCR expression.
employed the KN6 Tg model to assess the ability of Egr1 and Id3 to modulate lineage fate independently of alterations in the TCR repertoire. To determine whether ectopic expression of Egr1 would divert cells from the αβ lineage to the γδ fate, we ectopically expressed Egr1 in KN6 Tg mice lacking ligand (i.e., committing to the αβ lineage; Fig. 25A, left panel) (350). Indeed, we found that the Egr1 Tg both antagonized αβ-lineage development, as indicated by the reduced number of DP thymocytes, and promoted development of mature CD24lo γδ-lineage cells (Fig. 25A). We next investigated whether the Egr1 target, Id3, was required for strong signals (generated by γδTCR-ligand interactions) to promote γδ development and simultaneously oppose development of αβ-lineage cells. In agreement, Id3-deficiency interfered with the ligand-induced development of mature CD24lo γδ-lineage cells while increasing the number of αβ-lineage DP thymocytes (Fig. 25B). Importantly, Id3-deficiency caused proportional changes in αβ-lineage DP and CD24lo γδ-lineage cells, consistent with an effect on lineage commitment. These data suggest that the Egr-Id3 pathway plays a critical role in lineage assignment that does not require alterations in the TCR repertoire. Id1 deficiency had no effect on lineage assignment in this model (data not shown). Notably, the ability of strong signals to promote the γδ lineage was associated with markedly reduced expression of E proteins (E47) relative to that observed in cells committing to the αβ fate in the absence of ligand (Fig. 26).

14.4 Effects of Id3 deficiency on fate adoption are associated with changes in survival and proliferation

We next wished to determine how strong signals employ Id3 to both promote development of γδ-lineage cells and oppose αβ-lineage fate. Previous studies indicated that the E proteins can control cell growth and survival (68, 69, 73). Accordingly, we reasoned that repression of E proteins by Id3 might differentially influence growth and survival of developing αβ and γδ T-lineage cells. Interestingly, the impaired generation of mature CD24lo γδ-lineage cells in ligand-expressing Id3−/− KN6 Tg mice was associated with increased Annexin V staining, suggesting that Id3 is required to promote the survival of these cells (Fig. 27A). The CD24lo population also exhibited increased BrdU incorporation. Perhaps this contributes to the increased apoptosis among these cells, since cycling cells are more sensitive to apoptotic stimuli (95). Effects on survival and proliferation also appear to be associated with the ability of Id3 to oppose αβ-lineage development (Fig. 27B). Indeed, Id3-deficiency caused an increase in survival among
**Figure 25.** Egr1 and Id3 can modulate lineage choice without affecting the TCR repertoire. (A) Enforced expression of Egr1 diverts KN6 Tg thymocytes to the γδ fate. Thymocytes from mice of the indicated genotypes were analyzed by flow cytometry: (1) Lig−, KN6 Tg Rag2+/β2m−; (2) Lig−Egr1Tg, KN6 Tg Rag2+/β2m−/Egr1Tg. (B) The strong TCR signals that promote adoption of the γδ fate require Id3. Mice of the indicated genotypes were analyzed by flow cytometry: (1) Lig+, KN6 Tg Rag2+/β2m−; (2) Lig+Id3−/−, KN6 Tg Rag2+/β2m−/Id3−/−. (A and B) Results are depicted as histograms and summarized graphically on the right with each symbol corresponding to an individual mouse. Differences between the absolute number of DP and CD24lo DN thymocytes between these mice are statistically significant (asterisk indicates p < 0.05).
Figure 26. Effect of signal strength on E protein expression. High salt nuclear extracts were prepared from DN thymocytes of the indicated genotypes, following which equal quantities of protein were immunoblotted with the indicated Ab. The immunoblotting of Tata Binding Protein (TBP) serves as a loading control.
Figure 27. Id3 promotes the γδ fate and opposes the αβ fate, and these effects are associated with alternations in growth and survival. (A and B) Id3 promotes survival of mature CD24lo γδ-lineage cells and impairs growth and survival of CD8ISP and αβ lineage DP. Apoptosis and proliferation of the indicated populations were evaluated flow cytometrically by Annexin staining and BrdU incorporation, respectively. Each symbol represents an individual experiment involving at least three animals, with a line connecting the animals with a different genotype contained within a single experiment (upper panels). The difference in percent (%) of apoptotic cells between Lig+ and Lig+Id3−/− populations was significant (p < 0.05) for the following populations: (1) γδTCR+CD24lo; (2) ISP; and (3) DP. The mean percent BrdU incorporation ± SD is presented graphically (bottom panels) with a minimum of ten mice per condition. *p < 0.05; **p < 0.01. (C) Effects of Id3 on growth and survival are associated with differential regulation of Bcl2 family members. Expression of the indicated genes was measured by real-time PCR on thymocyte subsets purified by flow cytometry. Expression levels were standardized using β-actin and normalized to levels in Rag2−/− DN3 thymocytes. Data are representative of two different experiments. Error bars represent SD.
cells that differentiate to the αβ-lineage DP stage (i.e., through a CD8 immature single positive or ISP intermediate) and accumulate in Id3−/− mice-expressing ligand (Fig. 27B). Moreover, Id3-deficiency increased the fraction of BrdU+ ISP cells, suggesting that Id3, in the context of strong signals, normally suppresses their growth. Taken together, these findings indicate that following strong TCR signals, Id3 functions to promote the production of CD24lo γδ-lineage cells and opposes the development of αβ lineage, and this is associated with effects on growth and survival.

To gain insight into the molecular basis for the differences in survival and proliferation observed in Id3-deficient mice, we examined the expression levels of molecular effectors of these pathways. An attractive set of candidates was the RORγt-BclXL axis, shown recently to require the E protein targets of Id3 for expression (73). In accord with this model, expression of RORγt and the prosurvival factor BclXL was elevated in Id3−/− ISP cells relative to that in Id3+/+ ISP cells, consistent with their increased survival (Fig. 27C). Interestingly, expression of RORγt and BclXL was not decreased in Id3−/−CD24lo γδ lineage and, thus, did not correlate with the increased apoptosis of these cells (Fig. 27C). However, Bcl-2 expression was substantially reduced in Id3−/−CD24lo γδ-lineage cells, consistent with their increased apoptosis (Fig. 27C). These data suggest that Id3 induction in the context of a strong signal is associated with the increased survival of γδ-lineage cells and decreased survival of αβ-lineage cells. Further, these fates correlate with differential, cell-context-dependent effects on the expression of the prosurvival factors Bcl-2 and BclXL, respectively. Expression patterns of other E protein targets, CDK6 and Bim, were not consistent with alterations in growth and survival (Fig. 28).

Because Id3-deficiency is associated with changes in growth and survival of αβ and γδ lineage cells occurring under the influence of strong TCR signals, we thought it possible that Id3 may regulate lineage selection prior to TCR signaling by acting on precommitted progenitors. Lineage fate has been shown to be complete in DN3 progenitors and can be controlled by ectopic expression of TCR complexes in Rag2−/− DN3 thymocytes (284). To determine whether Id3 induction might be functioning by selectively inducing apoptosis among precommitted αβ-lineage precursors, we coexpressed Id3 (to mimic a strong signal) with TCRβ (pre-TCR-induced αβ-fate) in Rag2−/− DN3 thymocytes. Our results showed that Id3-transduction did not result in selective loss of αβ-lineage precursors, suggesting that strong signals through Id3 are acting on
Figure 28. Expression patterns of E protein targets CDK6 and Bim in KN6 Tg thymocytes. The indicated populations of thymocytes were isolated by flow cytometry following which the levels of mRNA encoding CDK6 and Bim were quantified by real time PCR. Results from triplicate measurements were normalized to GAPDH and depicted graphically relative to the expression level in Rag2\(^{-/-}\) DN3 thymocytes. CDK6 levels are decreased in all Id3\(^{-/-}\) populations and so the change in CDK6 expression levels is not consistent with the observed increase in proliferation in Id3\(^{-/-}\) ISP cells. Likewise, the increases in Bim expression in all populations of Id3\(^{+/+}\) cells fail to explain the decreased apoptosis among Id3\(^{+/+}\) ISP, although the slightly higher Bim levels in CD24\(^{lo}\) Id3\(^{-/-}\) thymocytes are consistent with the increased apoptosis among that cell population. Error bars represent SD.
**Figure 29.** *Id3*-transduction does not selectively kill αβ lineage progenitors. *Rag2*−/− DN3 were retrovirally-transduced with the indicated constructs and cultured on OP9 monolayers for 3 days following which apoptosis was measured using Annexin 5 staining. *Id3*-transduction did not induce the selective death of TCRβ-expressing αβ lineage progenitors as measured on day 3 or day 2 (not shown). Error bars represent SD.
the specification or commitment process to promote γδ-lineage differentiation and oppose the αβ fate (Fig. 29). Conversely, to determine whether strong signals promote the generation of CD24<sup>lo</sup> γδ-lineage cells by preferentially expanding a small precommitted γδ-lineage population, we ectopically expressed the KN6 γδTCR in Rag2<sup>−/−</sup> thymocytes, which lack precommitted γδ-lineage cells, and assessed effects on fate adoption in the presence and absence of ligand (Fig. 30). Importantly, KN6 γδTCR-expressing precursors adopted the γδ-fate in the presence of ligand (at the expense of the αβ-fate) and, conversely, were diverted to the αβ fate (at the expense of the γδ fate) when ligand expression was suppressed by shRNA (Fig. 30). Altogether, these data suggest that strong signals and Id3 are able to influence lineage commitment per se. The effects on ISP and DP survival that we observed in vivo likely occur in a minority of cells that escape ligand engagement early in development. Recent observations suggest that ligand engagement of uncommitted DN thymocytes induces commitment to the γδ lineage; however, cells that escape ligand engagement and commit to the αβ fate appear to become susceptible to deletion by ligand as they differentiate to the ISP or DP stages (285).

14.5 γδ-biased profile gene Rgs1 is closely linked with γδ T Cell function

Because Id3-deficiency interfered with γδ cell maturation in response to strong TCR signals, we investigated whether effector function was similarly compromised. Id3-deficiency impaired both TCR-induced proliferation and IFNγ production (Fig. 31A-C). Additionally, this effect was largely restricted to γδ-lineage cells because the TCR-induced proliferation of αβ-lineage CD4 T cells was not affected by Id3-deficiency, although proliferation of Id3<sup>−/−</sup> CD8<sup>+</sup> T cells was somewhat delayed following TCR activation (Fig. 32) (77, 349). Of note, both proliferation and IFNγ production occur only in mature γδ-lineage cells that are CD24<sup>lo</sup> (Fig. 33) (37).

Recent reports have suggested that full functional maturation of γδ-lineage cells requires trans presentation of lymphotokin-β (LTβ) to developing γδ-lineage cells by DP thymocytes (38, 282). This induces a set of genes termed the “γδ-biased gene profile,” whose expression is enriched in
Figure 30. shRNA knockdown of T10/22 redirects γδTCR-expressing cells to the αβ lineage. A) Scid.adh thymic lymphoma cells were retrovirally transduced with the MSCV-based LMP vector driving expression of the indicated MiR30-based shRNAs. Knockdown was assessed by performing real time PCR on T10/22 mRNA and normalizing the expression level to β-actin. Error bars represent SD. B) The KN6 TCRγ and TCRδ subunits were cloned into pMiY as a fusion protein linked by the 2A Tescovirus linker peptide. Retroviral infection of the Scid.adh thymic lymphoma conferred expression of the KN6γδ TCR as indicated by anti-TCRδ surface staining by FACS and by identifying the TCRγ subunit through blotting detergent extracts with the anti-2A Ab. C) The KN6 γδTCR complex was transduced into Rag2−/− thymocytes as a TCRγ-2A-TCRδ fusion protein, following which transduced cells were cultured on OP9 stromal cells that were transduced with control shRNA (Lig+) or with shRNA-1092 (Lig−). Developmental progression was monitored on the indicated days by flow cytometry with the indicated Ab. γδ cells were defined as γδTCR+/CD24lo. αβ lineage cells were defined as CD4+CD8+. Absolute numbers on each day are graphed below.
Figure 31. Induction of Id3 by strong signals during γδ development is required for functional competence. (A–C) Id3-deficiency impairs TCR-dependent proliferation and production of IFNγ. DN thymocytes from KN6^+Lig^+ and KN6^+Lig^+Id3^-/- mice were labeled with CFSE, stimulated for 48 hr on plate-bound anti-CD3 (10 μg/ml), and analyzed by FACS (A). DN thymocytes from the same mice as in (A) were stimulated as above, and cellular expansion was evaluated on triplicate wells by MTT assay. (B). DN thymocytes were stimulated with plate-bound anti-CD3 Ab for 24 hr, following which the proportion of IFNγ producing cells was determined by intracellular staining and flow cytometry. (B and C) Mean ± SD is presented graphically. (D) Expression of the γδ-biased gene profile and functional competence can be separated. Expression of the indicated genes was measured on flow cytometrically isolated cell populations by real-time PCR. Mean ± SD is presented graphically.
Figure 32. *Id3*-deficiency impairs the TCR-mediated proliferation of CD8SP, but not CD4SP, thymocytes. CD4 and CD8 SP thymocytes were purified by flow cytometry from mice with the indicated genotypes, labeled with CFSE and stimulated with plate-bound anti-CD3 and anti-CD28 for the indicated times. *Id3*-deficiency had no effect on proliferation of CD4SP, but did delay the proliferation of a subpopulation of CD8SP thymocytes.
Figure 33. CD24\textsuperscript{lo} \(\gamma\delta\text{TCR}^+\) thymocytes are phenotypically and functionally mature. CD24\textsuperscript{hi} and CD24\textsuperscript{lo} thymocytes from KN6 Ligand\textsuperscript{+} mice were isolated by flow cytometry and stimulated for 48h with plate-bound anti-CD3 Ab. Following this stimulation only CD24\textsuperscript{lo} cells were capable of IFN\(\gamma\) production.
γδ cells and linked to function: ICER, Nurr1, Nor1, Nurr77, and Rgs1 (38, 282). Because ligand-selected KN6-Tg γδ-lineage cells are functional (Fig. 31A-C), yet develop in the near absence of DP thymocytes (Fig. 25B), we examined whether these cells express the γδ-biased gene profile. Surprisingly, KN6 Tg γδ cells from Id3-sufficient, ligand-expressing mice exhibited increased expression for all of the γδ-biased genes examined (Fig. 31D), indicating that DP-mediated transconditioning may not be absolutely required for their induction. These findings support the notion that elaboration of the γδ-biased gene profile is associated with γδ function (38, 282); however, Id3-deficiency appeared to sever this linkage. Indeed, despite the fact that proliferation and IFNγ production in response to TCR engagement were impaired in γδTCR+ cells from Id3-deficient mice, these cells displayed elevated expression of a subset of the γδ-biased profile-genes (Fig. 31D), with the notable exception of Rgs1 expression, which was reduced in these cells. One potential explanation for this observation is that particular members of the γδ-biased profile may be more closely linked to γδ-function than others. Specifically, Rgs1 expression correlated well with γδ cell function in that it was highly induced in functional KN6 Lig+ γδ cells and reduced in those lacking Id3 (Fig. 31D); expression of the Nr4a family members (Nurr1, Nor1, and Nur77) was not. The elevated expression of the Nr4a genes in the nonfunctional, Id3-deficient γδTCR+CD24lo cells may relate to their role in regulating cell death in this misdirected population exhibiting increased apoptosis (Fig. 27) (359, 360). Our data indicate that induction of some genes within the γδ-biased profile, such as Rgs1, is tightly linked to γδ T cell function, whereas others appear to be separable.

14.6 Id3 induction by strong signals is necessary and sufficient to promote Notch-independent maturation

The above findings provide support for Id3 as an important molecular effector of the strong signals that dictate γδ fate choice and maturation. However, it remained unclear whether other aspects of γδ-lineage development might rely on Id3. We wished to determine whether Id3 induction in the context of strong signals was required to confer Notch-independent maturation upon γδTCR-expressing cells (284). To test this possibility, we ectopically expressed either a pre-TCR or γδTCR in Rag2−/− DN3 cells and then assessed the role of Id3 in determining the Notch-dependence of their development in vitro (Fig. 34). As reported previously (284), pre-TCR (TCRβ-transduced) expressing Rag2-deficient DN3 cells proliferated robustly and
**Figure 34.** Id3 induction by strong signals is necessary and sufficient to confer Notch independence on γδ-lineage cells. (A) Notch independence of γδ-lineage cells is impaired by Id3-deficiency. *Rag2*" or *Rag2"Id3" DN3 cells were transduced as indicated and cultured on OP9-DL1 or OP9-Cnt1 cells for 5 days, and the expression of CD4, CD8, and CD24 was analyzed by flow cytometry. Bar graphs on the right show the fold increases (relative to control transduced cultures) in absolute numbers of DP or γδ+ CD24lo DN cells from the indicated transductions, culture conditions, and genotype. (B) Notch signals reduce E protein expression. Equal quantities of protein from detergent extracts of cells cultured as in (A) were immunoblotted with the indicated Ab. (C and D) Ectopic expression of Id3 is sufficient to confer Notch independence on TCR− thymocytes. *Rag2"Id3" DN3 cells were transduced as indicated, cultured on OP9-DL1 or OP9-Cntl cells for 6 days, with the fold increases in cellularity over input shown in (C) and the expression of CD24 and forward-light scatter (FSC) analysis by flow cytometry are shown in (D). These data are representative of at least three independent experiments. Error bars represent SD.
developed to the DP stage (Fig. 34A). This differentiation was independent of Id3, as it was not impaired by Id3-deficiency but was dependent upon Notch signaling (i.e., required a Delta-Notch ligand, DL1; Fig. 34A). Even the αβ-lineage DP cells that developed in the γδTCR-expressing cultures were dependent upon Notch signals (Fig. 34A) (284, 331). In contrast, γδ-lineage commitment and development into mature DN CD24lo γδ cells was not dependent upon Notch signals but was dependent upon Id3. Indeed, Id3-deficiency markedly reduced the numbers of CD24lo γδ-lineage cells, instead diverting the γδTCR-expressing cells to the αβ-lineage DP stage (Fig. 34A), as we observed in vivo (Fig. 25). These data indicate that Id3 function is particularly important for elaboration of the γδ lineage, but is dispensable for adoption of the αβ fate. Interestingly, expression of the E protein target of Id3, E47, was reduced by Notch signaling, even in the absence of TCR (pre-TCR or γδTCR) expression (Fig. 34B). However, consistent with the requirement of Notch signals for differentiation of pre-TCR expressing Rag2-deficient DN3 cells, we observed that E47 levels were dramatically reduced only when both pre-TCR and Notch signals are present (Fig. 34B, left side), indicating that the ERK-Notch-mediated degradation of E proteins may be critically important for reducing E protein activity at the β-selection checkpoint (68, 69, 361). This finding provides a potential explanation for why the generation of αβ-lineage DP cells is clearly dependent upon Notch, yet independent from Id3 (Figures 34A, B) (344, 345, 361). Alternatively, Rag2-deficient DN3 cells transduced to express the KN6 γδTCR showed a strong reduction in E47 levels irrespective of Notch signals (Fig. 34B, right side). Because these data suggest that Id3 plays an important role in conferring Notch independence to γδ-lineage cells, we next examined whether Id3 induction was sufficient. In fact, ectopic expression of Id3 in Rag2-, Id3-double-deficient DN3 cells led to increased cellular expansion (Fig. 34C) and maturation (e.g., CD24 downmodulation; Fig. 34D and Table 3), which were both independent of Notch signaling. Moreover, the ability of Id3 to confer these capabilities on Rag2-, Id3-double-deficient DN3 cells was abrogated by a mutation that eliminated the ability of Id3 to interact with and suppress E protein function (S → P; Fig. 34C, D) (362). Id3 expression following transduction was approximately 10-fold higher than is expressed endogenously by CD24lo γδ cells (Fig. 35). These results show that different mechanisms are responsible for mediating the reduction in E protein activity that is necessary for DN3 cells to differentiate into either γδ or αβ lineage cells, with the γδ option relying solely on Id3 induction, while the αβ option requires additional input from Notch signals.
Table 3. Comparison of the phenotypic markers on transduced $Rag^{2-/-}Id^{3-/-}$ DN3 thymocytes to those expressed by $CD24^{lo} \gamma\delta$ lineage cells. After 6 days of culture on either OP9-DL1 or OP9-Ctrl stromal layers, transduced cells were stained with Ab reactive to the indicated proteins. $Id^{3-}$-transduced cells are very similar phenotypically to $CD24^{lo} \gamma\delta TCR^+$ thymocytes.
**Figure 35.** Comparison of the ectopic expression level of Id3 to endogenous levels in γδ lineage cells. Id3 levels in the indicated populations were compared by real time PCR. Triplicate measurements were normalized to GAPDH and then expressed graphically relative to the levels in CD24\(^{hi}\) γδ lineage cells. Error bars represent SD.
14.7 Id3 induction is necessary and sufficient to promote functional competence of γδ lineage cells

A recent report implicated ligand “experience” in the thymus as an important determinant of effector function, arming such γδ cells as IFNγ-producing effectors (363). Since we have found that ligand-mediated induction of Id3 was necessary to arm KN6 Tg γδ cells for TCR-induced proliferation and IFNγ production (Fig. 31C), we asked whether Id3 was sufficient to promote the functional maturation of Rag2-deficient DN3 cells. Indeed, ectopic expression of Id3 in Rag2-, Id3-double-deficient DN3 cells enabled these cells to become responsive to stimulation. In response to mitogenic stimulation, Id3-transduced cells both increased in size (Fig. 36A) and produced IFNγ at amounts comparable to mature CD24lo γδ cells (Fig. 36B). Furthermore, Id3 transduction conferred these capabilities to Rag2-, Id3-double-deficient precursors in the absence of Notch receptor-ligand interactions (Fig. 36B). As above, the ability of Id3 to promote Notch-independent acquisition of effector function was lost when the Id3 mutant construct was used (Fig. 36A, B). The Id3-induced acquisition of effector function was accompanied by the induction of ICER and Rgs1, a γδ-biased gene profile member that we have found to be closely linked to γδ function (Fig. 36C), but not induction of Nurr1, whose expression appears to be separable from γδ cell function (Fig. 31). Sox13 is another recently described marker of γδ lineage cells (364); however, while Sox13 expression was not detected in DP cells, it was also not found to be elevated in CD24lo γδ-lineage cells, in agreement with recent evidence suggesting it may not reliably mark all γδ cells (285). Taken together, these data suggest that Id3 is both necessary and sufficient for adopting the γδ-lineage fate and for arming γδ T cells to produce IFNγ in response to ligand experience.

15 Discussion

The present report not only provides new insights into the role of Id3 in regulating T-lineage fate assignment in response to strong signals, but also into the role of Id3 in rendering γδ-lineage cells independent of Notch signals and functionally competent. Id3 is required for strong signals to promote adoption of the γδ lineage and oppose adoption of the αβ fate. We recently reported that γδ-lineage cells are far less dependent on Notch signaling than αβ-lineage cells (284). Based on our present findings, it is now clear that Id3 is both necessary and sufficient for Notch independence of γδ-lineage cells and that this independence requires a functional helix through
Figure 36. Ectopic expression of Id3 is able to confer functional competence on TCR−thymocytes. (A) Id3-transduced cells increase in size upon PMA + ionomycin stimulation. (B) Id3-transduction confers competence to produce IFNγ on Rag2−, Id3-double-deficient DN3 cells. (A and B) Sorted CD45+ GFP+ cells from transduced DN3 cells (as indicated) were stimulated for 36 hr with PMA and Ionomycin (PMA + Iono) or placed in culture without stimulation (Unstim) and (A) analyzed by flow cytometry to determine cell size by forward-light scatter (FSC). (B) Levels of IFNγ in culture supernatants were quantified by an antibody-capture ELISA. (C) Id3-transduction confers expression of some γδ-biased genes. RNA transcript expression of Rgs1, ICER, Nurr1, and Sox13 was measured by real time PCR as in Figure 31 from sorted CD45+ GFP+ cells from transduced DN3 cells cultured on OP9-Cntl or OP9-DL1 cells as indicated. Error bars represent SD.
which Id3 heterodimerizes with, and suppresses the function of, E proteins. Finally, Id3 is also necessary and sufficient to arm γδ-lineage cells for effector function defined by TCR- or mitogen-induced proliferation and IFNγ production. These findings place Id3 as a central molecular mediator of the strong signals that influence T cell fate, as well as their developmental and functional characteristics.

It has been suggested for some time that αβ- and γδ-lineage precursors are differentially dependent upon Notch signaling, though this has been controversial until recently (139, 297, 346, 365). Indeed, we showed that while αβ-lineage precursors remain Notch dependent during their pre-TCR induced transition to the DP stage, γδ precursors become independent of Notch signaling upon expression of the γδTCR (284). Nevertheless, it was unclear how γδTCR signaling permitted Notch-independent differentiation of γδTCR-expressing DN cells. We now show that Id3 is an important molecular effector of this process. The molecular basis for this effect has not been established; however, recent evidence from the Murre lab (as well as genetic analysis from Drosophila) suggests interplay between E proteins and the Notch pathway (64, 366). Moreover, numerous reports support the notion that suppression of E protein function (at least transiently) is required for early thymocyte differentiation (69, 74, 343). We now propose a model whereby lineage commitment and Notch dependence are determined by graded reductions in E protein activity mediated by differences in TCR signal strength (Fig. 37). DN3 thymocytes are prevented from further development by E protein-mediated enforcement of the β-selection checkpoint, as evidenced by the ability of E2A deficiency to enable pre-TCR-deficient thymocytes to traverse the β-selection checkpoint and differentiate to the DP stage (67, 69). Paradoxically, E protein deficiency blocks the development of pre-TCR-expressing cells beyond the β-selection checkpoint to the DP stage, suggesting that the induction of αβ-lineage development by pre-TCR signals is dependent upon partial or temporally restricted suppression of E protein activity (68). Accordingly, pre-TCR signals may suppress E protein activity, in part by induction of Id3 but are unable by themselves to suppress E protein activity beyond the threshold required for αβ-lineage development and, thus, require Notch-ligand interactions to do so. Notch signaling has been reported to suppress E protein function both by ERK-dependent induction of E protein degradation (344, 345) and through induction of Id3, which can directly repress E protein function (348). Unlike the weak signals that promote αβ-lineage development, the strong signals that confer Notch-independent differentiation upon γδ-lineage cells are
Figure 37. Model by which strong TCR signals render γδ lineage cells Notch independent. Development beyond the β-selection checkpoint requires suppression of E protein function. Our model posits that T lineage fate and developmental characteristics are determined by graded suppression of E protein function by TCR signals of differing strength. Pre-TCR signals are too weak by themselves to suppress E proteins beyond the threshold required for the αβ lineage differentiation program. They require assistance from Notch to do so, providing an explanation for the Notch-dependence of αβ lineage differentiation to the DP stage. γδ lineage commitment, in contrast, is dictated by strong TCR signals capable of suppressing E protein function beyond the threshold required for γδ lineage commitment, and do so without assistance from Notch.
dependent upon Id3 induction and are sufficient to suppress E protein activity beyond the threshold required for γδ-lineage commitment and development without assistance from Notch activity.

Because effects on growth and survival are associated with the regulation of lineage fate by Id3 in the context of strong TCR signals, it is possible that Id3 is not affecting lineage fate per se, but rather is affecting the growth and survival of precommitted αβ or γδ lineage progenitors. Nevertheless, a number of lines of evidence argue against this perspective and for a role of Id3 in influencing lineage commitment. If strong signals were simply eliminating αβ-lineage cells, then Id3-transduction would be predicted to preferentially eliminate pre-TCR-expressing αβ-lineage progenitors; however, Id3 transduction does not do so. Regarding the promotion of the γδ fate by strong signals (or Egr transduction), the converse argument could be made that strong signals promote the generation of CD24lo γδ-lineage cells by preferentially expanding a small precommitted population. However, we demonstrated that ectopic expression of the KN6 γδTCR in Rag2−/− thymocytes (which lack pre-committed γδ-lineage cells) promoted the γδ fate and opposed the αβ fate in the presence of ligand and produced the converse effect in the absence of ligand (Fig. 30). It should be noted that this represents the first example where the expression of a specific γδTCR ligand was demonstrated to be required for selection and development of γδ-lineage precursors. Altogether, these data suggest that strong signals and Id3 drive lineage commitment.

While we had previously reported impaired development of γδ T cells in fetal Id3-deficient mice, the role of Id3 as a critical molecular effector in γδ cell specification and development is also revealed by the perturbation of γδ development in adult Id3-deficient mice. A recent report suggested that Id3 functions to restrain the development of γδ-lineage cells, as γδ T cell numbers were increased in adult Id3-deficient mice (353). The expansion was accompanied by enhanced V(D)J recombination at the TCR loci (γ, δ, and β) and an outgrowth of the Vγ1.1+ γδ cell subset. We observed similar effects of Id3-deficiency, but propose an alternative interpretation. We suggest that Id3 serves to terminate V(D)J recombination following TCR expression (either pre-TCR or γδTCR expression), thereby limiting the developmental time window during which TCR rearrangement can occur and explaining why TCR rearrangements were more extensive in Id3-deficient mice. We further demonstrated that while γδ T cell numbers were increased in Id3-
deficient mice, the expansion appeared to be restricted to the Vγ1.1+ subset, as the Vγ2 and Vγ3 γδ subsets were severely reduced. We think these differential effects on particular Vγ subsets relate to their respective affinities for selecting ligands, with Id3-deficiency impairing selection of those with intermediate affinity, while allowing those of high affinity to escape deletion. This interpretation is supported by our demonstration that Id3-deficiency impairs selection of KN6 γδTCR Tg thymocytes on the intermediate affinity T-10d ligand, while rescuing them from deletion by T-10b and T22b, for which their affinity is 10-fold higher (357). Indeed, at least some Vγ1.1+ γδ cells, which are expanded in Id3-/− mice, belong to an NK γδ T cell lineage thought to be selected on high-affinity ligand (354-356). Taken together, these findings suggest an apparent dichotomy of Id3 function, restricting production of γδ cells whose TCR affinity for ligand is very high, while promoting differentiation of the broader repertoire of nonautoreactive γδ T cells.

Along with its role in promoting the Notch independence of developing γδ T cells, Id3 plays an important role in the acquisition of effector function by γδ lineage cells. We found that Id3-deficiency impairs the ability of KN6 γδTCR Tg+ cells to proliferate and produce IFNγ in response to TCR ligation. Conversely, ectopic expression of Id3 in the absence of TCR expression conferred the ability to proliferate and produce IFNγ in response to mitogenic signals. The Hayday laboratory has reported that developing γδ T cells require presentation of LTβ in trans by DP thymocytes for both functional competence and the expression of a number of genes termed the “γδ-biased gene profile” (38, 282). DP thymocytes are not present in appreciable numbers in either the ligand-expressing KN6 γδTCR Tg mice or among Id3-transduced DN3 cells cultured on OP9-monolayers in vitro. Nevertheless, despite the absence of DP thymocytes, the γδ T cells that developed in these models were functional and expressed several of the genes comprising the “γδ-biased gene profile” (38, 282). Interestingly, we found that Id3-deficiency separated γδ function from expression of the γδ-biased gene profile in that the Id3-deficient γδ cells expressed a subset of the profile genes, with the notable exception of Rgs1, and yet were not functional. One possible explanation is that only a subset of the profile genes is uniquely associated with γδ function. For example, although the function of Rgs1 in T cells is unknown, our data suggest that Rgs1 expression is tightly correlated with γδ T cell function, raising the interesting possibility that Rgs1 plays an active role in promoting the functional competence of γδ T cells.
The role of Id3 in promoting functional competence also provides an important mechanistic insight into a recent report regarding the role of ligand in shaping γδ T cell effector function. The report contends that ligand-mediated selection in the thymus does not play a significant role in shaping the γδ TCR repertoire but does influence the nature of effector function (363). That is, γδ T cells that purportedly did not encounter a selecting ligand during development (antigen-naive) are programmed to produce IL-17, whereas antigen-experienced γδ T cells produce IFNγ. While ruling out the possibility of ligand-mediated selection in the thymus is extremely difficult, we would agree that ligand-mediated selection arms γδ T cells to produce IFNγ. Indeed, our data supports the notion that the induction of Id3 following TCR-ligand engagement plays a critical role.

Taken together, our findings place Id3 as a central molecular mediator of the strong signals that influence T cell fate, enable developing γδ T cells to gain Notch-independent maturation, and shape their functional attributes. Although it is likely that Id3 (perhaps with assistance from Id2) mediates these effects by suppressing the function of E proteins, this interpretation remains to be fully characterized. Future efforts will be directed at determining how the ERK-Egr-Id3 pathway, as well as other mediators of strong signals, is differentially employed to specify T-lineage fates in the thymus.
Chapter 4
Enforcement of the $\gamma\delta$ T cell fate by the pre-TCR in developing T cells with attenuated Id3- and $\gamma\delta$TCR ligand-signaling

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GWW performed all the experimental results shown in this Chapter. PZ designed and produced critical reagents, and offered scientific advice. DLW provided reagents and scientific advice. JCZP provided scientific as well as technical advice and expertise.
16 Introduction

T cells can differentiate along distinct αβ or γδ cell lineages, but bifurcate from a common bipotent precursor. In mice, the earliest subset of T cells are CD4+CD8- double negative (DN) thymocytes, and this can further be divided into four subgroups (DN1-4) based on the expression of CD25 and CD44 (23). At the DN3 stage, rearrangements of the TCRβ, γ, and δ genes are complete and cells are subjected to their first developmental checkpoint to ensure proper generation and expression of the pre-TCR or γδTCR. This is a pivotal moment in T cell development, as single-cell progenitor analyses have identified this as the point of T-lineage commitment, and also the stage at which a DN cell specifies its lineage fate as αβ or γδ (284).

The αβ or γδ lineage choice decision is governed by a multitude of factors. Two competing models have been proposed for this process: the stochastic and instructional models. The stochastic model suggests that the TCR does not dictate fate adoption, and that signaling from the receptor is required solely to rescue those DN3 cells which have adopted the lineage that matches the receptor they express. In contrast, the instructional model proposes that the TCR does dictate fate adoption, such that the DN3 cell differentiates into the lineage which matches its TCR.

While evidence exists to support either model, recent studies support a version of the instructional model which posits that the strength of signal transduced by the TCR expressed by the DN3 cell dictates its lineage specification (272, 273). The apparent connection between a cell’s lineage choice and the TCR it bears on its surface can be detached with manipulations to its signal strength. Several studies note that promoting stronger signals favours the γδ-lineage fate and opposes the αβ one (272, 273), and molecularly speaking, the “strength” of TCR signal translates as a differential induction of downstream signaling pathway ERK/MAPK. Overexpression of ERK-MAPK downstream effector, Egr, promotes development along the γδ-lineage by mimicking the strong signal induced by the γδTCR (272). Furthermore, expression of Egr target Id3 is both necessary and sufficient to promote the γδ-lineage fate in developing DN3 cells in the absence of TCR expression (367), suggesting a critical role for Id3 in mediating αβ-versus γδ-lineage decisions at this developmental checkpoint.
Several studies have shown that ligand-engagement highly influences the αβ- versus γδ-lineage decision due to its affects on γδTCR signal strength. In the absence of ligand, the ERK/MAPK pathway and other indicators of signal strength, such as CD5, are decreased in expression or activity (272). Since ligand availability alters γδTCR signal strength, it can also dissociate the apparent connection between lineage adoption and the TCR expressed. γδTCR-expressing DN3 cells develop along the αβ-lineage and become DP cells in the absence of ligand engagement (272), while provision of the ligand allows these cells to adopt the γδ-lineage fate. An in vitro single-cell analyses where γδTCR was cross-linked with antibodies to mimic ligand engagement similarly revealed that all γδTCR-expressing DN3 cells adopt the γδ-lineage in the presence of antibody, whereas some develop along the αβ-lineage in its absence (44). Together, these findings suggest a pivotal role for ERK/MAPK in dictating signal strength and, in accordance with the signal strength model of lineage adoption, developing γδTCR-Tg T cells which cannot engage ligand receive quantitatively less TCR signal and alternatively mature along the αβ-lineage or may give rise to unique subclass of IL-17-producing γδ T cells (272, 273, 362, 375).

Several reports have indicated a differential requirement for Notch signaling in αβ versus γδ lineage fate (138, 142, 284, 297, 315). Recently, we showed that γδTCR-bearing thymocytes adopting the γδ lineage do not require concurrent signals from Notch to mature past the DN3 stage, while their pre-TCR-expressing counterparts must receive Notch signaling along with pre-TCR-mediated signals in order to successfully differentiate to CD4+CD8+ DP stage (284). The differential Notch requirement for the two lineages is a consequence of the TCR signal strength at the DN3 stage. Pre-TCR and Notch both signal to activate the ERK/MAPK pathway to modify E2A activity (344, 345, 348). We have proposed a model in which weak pre-TCR signals must seek collaboration with Notch at the β-selection checkpoint to successfully down-regulate E2A and allow for developmental progression, while signals stemming from the γδTCR are strong and promote high levels of Id3, which are sufficient to repress E2A and allow for differentiation in the absence of aid from Notch signals (367). In support of this, Id3 over-expression is sufficient to drive DN3 cells to proliferate and differentiate along the γδ lineage without the requirement for Notch signaling.

Considering the common origin of αβ and γδ-lineage cells from a bipotent immature T cell precursor, it is possible for a DN3 cell to simultaneously express and transmit signals through a functional pre-TCR and a functional γδTCR, especially considering that TCRβ, γ and δ chains
are all rearranged at the DN3 stage. In a previous study looking to address the consequences of simultaneously expressing a TCRβ and γδTCR in vivo using transgenic (Tg) mice, it was found that the numbers of αβ and γδ-lineage cells in TCRβ/γδ-expressing cells were both high, and comparable to TCRβ- and γδTCR-Tg mice, respectively (368). In this case, however, the TCR chains were expressed earlier than physiological for T cell development, and premature expression of TCR transgenes has been shown to lead to aberrant developmental progression (369, 370).

Here, we attempt to definitively answer the question of lineage choice by simultaneously expressing TCRβ and γδTCR in Rag2−/− DN3 cells via retroviral transduction followed by in vitro coculture. We find that Rag2−/− DN3 cells expressing both pre-TCR and γδTCR follow the signal strength model for lineage development, and develop along the γδ-lineage. These cells downregulate CD24 surface marker, and express γδ-biased genes such as ICER, Nurr1, and Rgs1, but not αβ-lineage gene Tcra. Furthermore, these cells are functionally competent, as they produce IFNγ in response to stimulation. With Id3-deficiency, Rag2−/− DN3 cells expressing both pre-TCR and γδTCR do not increase their propensity to differentiate along the αβ-lineage in the presence of Notch signals, likely due to compensatory mechanisms by other Id proteins, but surprisingly generate DP cells in the absence of Notch signals. Moreover, these cells express higher transcript levels of TCRα, show impaired down-regulation of CD24, and display reduced functional competence to generate IFNγ, particularly in the absence of Notch signals. Together these results suggest a requirement for strong signals, as measured by Id3 induction, to differentiate and mature as IFNγ-producing γδ T cells. Lastly, manipulation of γδTCR ligand strength does not divert the lineage choice of γδTCR-expressing DN3 cells to the αβ-lineage, nor does it decrease expression of γδ-biased genes in these cells. However, we find that in the presence of a weak ligand, the level of γδTCR signaling is sufficient only to promote the γδ T cell choice, but not to induce CD24 downregulation or Notch-independence. Nevertheless, in the presence of additional pre-TCR signals, we observed a more effective differentiation towards the γδ-lineage, indicating that the pre-TCR can serve to enforce to a γδ-lineage choice in the case of weaker γδTCR signals. Taken together, these findings further cement the view that the cumulative signal strength sensed by developing DN cells dictates its lineage choice.
17    Experimental Procedures

17.1 Mice

Rag2-deficient mice (44) were bred and maintained in the Sunnybrook Research Institute animal facility in specific pathogen-free conditions. Id3<sup>−/−</sup> mice were kindly provided by Dr. Y. Zhuang (Duke University) (349) and backcrossed to C57BL/6 Rag2-deficient mice. All animal procedures were approved by the Sunnybrook Health Science Centre Animal Care Committee (Toronto, Ontario, Canada).

17.2 Retroviral Transduction and Culture

Retroviral constructs were generated by subcloning the cDNA of interest into the MigR1 or MIY plasmids, upstream of the internal ribosomal entry site, and stable retroviral-producing GP+E.86 packaging cell lines were generated for each construct. OP9-DL1, OP9-DL4 and OP9-Ctrl cells were produced and maintained as previously described (323), and cultures were supplemented with 1 ng/ml mouse recombinant IL-7 and 5 ng/ml human recombinant Flt-3L [Peprotech]. Fetal liver was obtained from timed-pregnant Rag2<sup>−/−</sup> or Rag2<sup>−/−</sup>/Id3<sup>−/−</sup> female mice on d 14 of gestation. Single-cell suspensions were generated by disruption through a 40-mm nylon mesh screen using a syringe plunger. For retroviral transduction of DN3 cells, cells from d 7 HPC/O9-DL1 co-cultures were passaged for an overnight co-culture with stable retrovirus-producing GP+E.86 packaging cells. Following this, the transduced (GFP<sup>+</sup>YFP<sup>+</sup>), or mock non-transduced, CD44<sup>−</sup>CD25<sup>+</sup> DN3 cells were purified by cell sorting and placed back onto OP9 co-cultures, as previously described (140).

17.3 Flow cytometry

All single-cell suspensions were stained with commercially available antibodies (BD Pharmigen and e-biosciences) and analyzed with a BD-LSRII flow cytometer, using Flowjo software (Treestar, Inc.). Dead cells were excluded from the analyses using DAPI gating.

17.4 Quantitative Real-Time PCR

Thymocyte populations were purified by flow cytometry or selection using magnetic anti-CD45 beads (Miltenyi Biotech). Total RNA was extracted using TRIzol (Invitrogen) and converted to cDNA using Quantitect Reverse Transcription Kit (Qiagen) according to manufacturer’s
instructions. Expression of the indicated genes was measured by quantitative real-time PCR using SYBR GreenER (Invitrogen). Primer sequences are listed below. β-actin was used to normalize cycle thresholds. Primers used: β-actin F: ATGGTGGGAATGGGTCAGAA, R: TCTCCATGTGTCGCCAGTTG; ICER F: CATGGCTGTAACTGGAGATG, R: AGCTCGGATCTGGTAAGTTG; Id2 F: ATGAAAGCCTTCAGTCCGGT, R: GTGGTCCGACAGGCTGTTTT; Nurr1 F: CATGGACCTCACCAACACTG, R: ACAGGGGCAATTTGGTACAAG; Rgs1 F: CACATCTGGAATCTGGGATG, R: TAGTCCCTCACAAGCCAACCAG; TCRα-C F: ACATCCAGAACCCAGAACCT, R: TGAGACCGAGGATCTTTTAA.

17.5 Generation of BALB/c stromal cells

Ear fibroblasts from BALB/c mice were isolated by incubating the minced and exposed epidermis layer with 0.3% trypsin/PBS. The minced pieces were treated at 37°C for 60 min, cut into smaller 2-3 mm squares, and subsequently incubated at 37°C, in a 5% CO₂ incubator in medium (DMEM supplemented with 10% FBS) for 3-4 days, or until fibroblast outgrowth became confluent. The cells were then harvested, pelleted by centrifugation, and resuspended in media.

17.6 PMA and ionomycin stimulation

CD45⁺GFP⁺ cells were sorted from transduced DN3 cultured for 6 days on OP9-Ctrl, OP9-DL1, or BALB/c-DL4 cells. Sorted cells (5 x10⁴ cells/well of a 96-well plate) were subsequently cultured with 50 ng/ml PMA (Sigma) and 500 ng/ml Ionomycin (Sigma) in OP9 medium supplemented with 50 U/ml IL-2 (Peprotech). Supernatants were harvested after 36 hr, and IFNγ or IL-17 levels were quantified using the DuoSet ELISA Development System (R&D Systems) according to the manufacturer's protocol.

18 Results

18.1 DN3 cells simultaneously expressing productive TCRβ and γδTCR chains develop and mature along the γδ-lineage

Previously, we found that TCRβ- and γδTCR-expressing DN3 cells have a high propensity to develop along their respective lineages (284). Furthermore, the pre-TCR and γδTCR produced
quantitatively different signals at the DN3 selection checkpoint, and this signal strength differential was responsible for directing lineage fate outcomes (272, 367). Considering these results, we next sought to determine the lineage choice of a DN3 cell that simultaneously expresses a pre-TCR (TCRβ) and a γδTCR. Specifically, we wanted to address whether strong signals transmitted from a functional γδTCR would be sufficient to override developmental signals emitted from an equally functional pre-TCR. To test this possibility, we ectopically expressed TCRβ, γδTCR, or both in Rag2−/− DN3 cells and assessed the role of each TCR in determining lineage fate in the presence of Notch signals by culturing them on OP9-DL1 cells. As reported previously (284), pre-TCR- (TCRβ-transduced) expressing Rag2-deficient DN3 cells proliferated robustly, developed along the αβ-lineage to the DP stage, and maintained high expression of CD24 (Fig. 38). In contrast, γδTCR-expressing DN3 cells developed along the γδ-lineage and matured into DN CD24lo γδ cells, with only a small proportion of cells differentiating along the αβ-lineage to become DP cells. Of note, Rag2−/− DN3 cells which did not bear any TCR (MigR1/MIY-transduced) remained DN and CD24hi, indicating their inability to progress along either lineage (Fig. 38A, B).

Strikingly, DN3 cells simultaneously expressing both TCRβ and γδTCR followed a developmental path much like γδTCR-only cells, in that they progressed to become DN CD24lo γδ cells, with only a small proportion of cells differentiating to become DP cells (Fig. 33A, B). Interestingly, TCRβ expression with γδTCR slightly increased the level of CD24-downregulation compared to γδTCR alone (Fig. 38B). Previously, we observed large proliferation differences between cells adopting the αβ vs. γδ lineage fates (284). Here, we find that, in agreement with these findings, TCRβ-bearing DN3 cells proliferate extensively after successful preTCR expression (β-selection), while γδTCR-expressing DN3 cells proliferate to a much lower extent (Fig. 38C). Interestingly, TCRβ/γδ-expressing DN3/OP9-DL1 cocultures had a markedly higher cellularity compared to their γδTCR-only-expressing counterparts, but still much lower than that of TCRβ-only/OP9-DL1 co-cultures (Fig. 38C).

We previously showed a differential requirement for Notch signaling between DN3 cells expressing the pre-TCR and committing to the αβ lineage, and those expressing a γδTCR and differentiating along the γδ-lineage. Considering this, we sought to address the role of Notch signals in the developmental progression of DN3 cells simultaneously expressing both TCRβ and
Figure 38. γδTCR-expressing DN3 cells predominantly differentiate along the γδ-lineage, irrespective of TCRβ co-expression and the availability of Notch signals. A, B) Developmental progression of culture-derived Rag2−/− DN3 cells cultured for 6 d with OP9-DL1 or OP9-Ctrl cells. Flow cytometric analysis of A) CD4 vs. CD8 and B) CD24 cell surface expression is shown for GFP+YFP+ gated cells; while C) shows the corresponding fold expansion. Fold expansion was obtained from the total cellularity divided by the number of cells used at the start of the culture (input).
γδTCR by culturing them without Notch signals on OP9-Ctrl stroma. As expected, Rag2-deficient cells that did not express a TCR, or those expressing a pre-TCR (TCRβ), were unable to develop. These cells remained DN, and apoptosed in the absence of the trophic signals provided by Notch signaling, as indicated by the complete loss of cellularity in these cultures (Fig. 38A, C). Conversely, both γδTCR- and TCRβ/γδ-expressing Rag2+/− DN3 cells were able to differentiate along the γδ-lineage, remain DN, and mature to become CD24lo, albeit with reduced cell numbers compared to the same cells cultured with Notch signals (Fig. 38C), in agreement with previous findings (284). Further in agreement with previous findings, DP cells that developed in the γδTCR-expressing cultures were also dependent upon Notch signals, and absent from OP9-Ctrl co-cultures (Fig. 38A) (284, 331, 367).

18.2 Strong Id3 induction downstream of γδTCR is necessary to induce maturation along the γδ-lineage, both in the presence and absence of Notch signaling

Id3 is an important molecular effector of the strong signals that dictates γδ fate choice and maturation, but it is dispensable for adoption of the αβ fate (367). Considering this, we sought to determine whether the loss of Id3 in TCRβ/γδ-expressing DN3 cells would drive lineage choice away from the γδ-lineage towards an αβ one. To test this possibility, we ectopically expressed either a pre-TCR, γδTCR, or both in Rag2+/− or Rag2+/−Id3−/− DN3 cells, cultured them on OP9-DL1, or OP9-Ctrl, cells and assessed the role of Id3 in lineage fate decisions. As reported previously (367), differentiation of pre-TCR-expressing DN3 cells was not impaired by Id3-deficiency, and these cells were able to proliferate robustly and develop to the DP stage (Fig. 39A, C). In the presence of Notch signals, Id3-deficiency doubled the percentage of γδTCR-only DN3 cells differentiating to the αβ-lineage, but did not affect the differentiation of TCRβ/γδ-expressing cells in terms of the percentage of DP cells generated, in contrast to our previous findings (Fig. 39A) (367). However, in striking contrast with γδTCR-expressing Rag2+/− DN3 cells, a small percentage of γδTCR- and TCRβ/γδ-expressing Rag2+/−Id3−/− DN3 cells seemed to bypass the requirement for Notch signaling in αβ-lineage development by differentiating to the DP cell stage on OP9-Ctrl cells (Fig. 39A). Notably, the maturation potential of γδTCR-only and TCRβ/γδ-expressing DN3 cells along the IFNγ-producing γδ T cell lineage was severely affected in the absence of Id3, as neither population could downregulate CD24 expression.
Figure 39. Loss of Id3 inhibits the maturation of \( \gamma \delta \) TCR-expressing T cells as IFN\( \gamma \)-producers.

A,B) Development of culture-derived \( \text{Rag}2^{-/-} \) DN3 or \( \text{Rag}2^{-/-} \text{Id}3^{-/-} \) DN3 cells retrovirally-transduced to express TCR\( \beta \), \( \gamma \delta \) TCR, neither or both, and cultured for 6 d with OP9-DL1 or OP9-Ctrl cells. Flow cytometric analysis of A) CD4 vs. CD8 and B) CD24 cell surface expression for GFP\(^+\)YFP\(^+\) gated cells; while C) shows the corresponding fold expansion. D) QRT-PCR analysis of \( \gamma \delta \)-biased genes ICER, Nurrl, and Rgs1, and \( \alpha \beta \)-gene TCR\( \alpha \)-C in transduced \( \text{Rag}2^{-/-} \) DN3 or \( \text{Rag}2^{-/-} \text{Id}3^{-/-} \) DN3 cells cultured for 6d on the indicated stroma, with mRNA levels normalized to \( \beta \)-actin. E) Sorted CD45\(^+\)GFP\(^+\) cells from transduced DN3 cells (as indicated) were stimulated for 36 hr (PMA+Iono.) or placed in culture without stimulation (Unstimulated). Levels of IFN\( \gamma \) in culture supernatants were quantified by ELISA.
Consistent with previous reports, proliferation is reduced in αβ-lineage and γδ-lineage DN3 cell cultures in the absence of Notch (284) (Fig. 39C). However, cells receiving Notch signals seem to proliferate more extensively in the absence of Id3, regardless of the TCR chain they expressed (Fig. 39C).

The CD24hi DN phenotype is shared between pre-selection DN3 thymocytes and cells that have committed but not yet matured along the IFNγ-producing γδ T cell lineage. Similarly, the CD24lo DN phenotype is shared between mature IFNγ-producing γδ T cells and apoptosing immature DN thymocytes which have not received a TCR signal. Considering this ambiguity associated with using the CD24 marker, we sought to verify the identity of CD24hi and CD24lo DN cells present in the cocultures by determining their gene expression profiles. Interestingly, γδTCR- and TCRβ/γδ-expressing Rag2−/−Id3−/− DN3 cells expressed similar or greater transcript levels of γδ-biased genes ICER, Nurr1, and Rgs1 when compared to their Rag2−/− DN3 counterparts (Fig. 39D). This did not seem to be due to a consequence of increased Id2 expression in Id3-deficient cells, as the transcript for Id2 was not elevated in Rag2−/−Id3−/− DN3 cells compared to their Rag2−/−DN3 counterparts (Fig. 39D). However, despite a retention of γδ-identity in the absence of Id3, Rag2−/−Id3−/− DN3 cells showed a greater induction of germline Tcra transcripts, particularly in the presence of Notch signals (Fig. 39D). Furthermore, while γδTCR- and TCRβ/γδ-expressing Rag2−/− DN3 cells are able to produce IFNγ in response to stimulation with and without Notch signals, only TCRβ/γδ- but not γδTCR-expressing Rag2−/−Id3−/− DN3 cells are capable of the same functionality (Fig. 39E). In the absence of Notch signals, the ability of TCRβ/γδ-expressing Rag2−/−Id3−/− DN3 cells to produce IFNγ is also quite weak (Fig. 39E).

18.3 γδTCR ligand availability affects the maturation status but not differentiation potential of γδTCR-bearing Rag2−/− pre-T cells

Results from our previous work (367), and Figure 39, highlight the necessity for Id3 in inducing strong signals downstream of γδTCR strong signals and mediating maturation along the γδ-lineage. However, the role of ligand involvement in mediating strong signals remains controversial, particularly in light of recent evidence supporting a ligand-independent developmental pathway for IL-17-producing γδ T cells in the thymus (363). Even without ligand engagement, IL-17 γδTCR cells appear to be γδ-lineage cells due to the strong signals delivered
through their TCR, which drive high induction of ERK1 and ERK2 in a manner similar to IFNγ-producing γδ T cells, which have encountered ligand. In light of this, we sought to further examine the mechanistic basis behind how weak and strong signaling are induced, by addressing whether ligand engagement plays a necessary role for the induction of a signal strength differential between pre-TCR and γδTCR. In H-2b mice, KN6 γδTCR recognizes the closely related non-classical MHC class I molecule T22, in association with β2-microglobulin. In H-2d mice, the T22d gene is known to be defective (371), thus another β2M-associated molecule, T10, which is closely related to the T22 gene, interacts weakly with KN6 in these mice. T10d and T22b proteins differ by four residues in the a1I and a2 domains (371), and while T10 is able to mediate positive selection of KN6 cells, it is characterized as a "weak" ligand that fails induce negative selection as well as activation of mature KN6 cells (270, 371-373). Considering this, we generated Balb/c (H-2d) stromal cell lines expressing a weak KN6 γδTCR ligand to determine whether it could affect αβ vs. γδ-lineage fate decisions.

H-2d stromal cells, with or without ectopic Dll4 expression, BALB/c-DL4, or BALB/c-Ctrl, respectively, were used for co-cultures with Rag2-/- DN3 cells transduced to express a pre-TCR, γδTCR, or both. As expected, the differentiation of pre-TCR (TCRβ-transduced) expressing DN3 cells was not impaired by the loss of T22b on BALB/c-DL4 cells, as these cells were able to proliferate robustly and developed to the DP stage (Fig. 40A, C). The observed difference in percentage of DP cells between TCRβ-transduced cells on OP9-DL1 and BALB/c-DL4 simply reflects the superior nature of OP9 cells to support lymphocyte development (374). Interestingly, in the presence of weak ligand, γδTCR-expressing DN3 cells did not divert their lineage choice to become αβ-lineage DP cells (Fig. 40A). In fact, fewer γδTCR-expressing DN3 cells developed into DP cells on BALB/C-DL4 than on OP9-DL1 cells, with this difference again likely due to the increased effectiveness of OP9 cells to support lymphopoiesis. However, maturation along the IFNγ-producing γδ-lineage was affected by decreased ligand strength. Notably, CD73, a marker found to correlate with γδ-lineage commitment, and upregulate on maturing IFNγ-lineage γδ T cells (Wiest, unpublished observations) was unaffected, but CD24 expression failed to be downregulated on BALB/c-DL4 co-cultures (Fig. 40B).

On BALB/c-Ctrl cells, γδTCR-expressing cells behave much like their TCRβ-transduced counterparts in that they cannot downregulate CD24, nor can they survive in the absence of
Figure 40. Provision of a weak γδTCR ligand does not promote αβ-lineage choice in γδTCR-expressing DN3 cells, both in the presence and absence of Notch signals. A,B) Development of culture-derived Rag2−/− DN3 cells retrovirally-transduced to express TCRβ, γδTCR, neither or both, and cultured for 6 d with OP9-DL1, BALB/c-DL4, or BALB/c-Ctrl cells. Flow cytometric analysis of A) CD4 vs. CD8 and B) CD24 vs. CD73 cell surface expression is shown for GFP+YFP+ gated cells; while C) shows the corresponding fold expansion. D) QRT-PCR analysis of γδ- biased genes ICER, Nurr1, and Rgs1, and αβ-gene TCRα-C in transduced Rag2−/− DN3 cells cultured for 6d on the indicated stroma, with mRNA levels normalized to β-actin.
Notch signaling, as seen by the absence of cells in the culture (Fig. 40B, C). We previously put forth a model that suggested that the strong signals downstream of γδTCR, mediated by strong induction of Id3, is required for Notch-independence at the DN3 developmental stage. Here, we find that in the presence of a weak ligand, the level of γδTCR signaling is sufficient only to promote the γδ T cell choice, but not to induce CD24 downregulation or Notch-independence (Fig. 40A,B). To further elucidate whether the CD24<sup>hi</sup>CD4<sup>-</sup>CD8<sup>-</sup> population, which make up the majority of cells within γδTCR-expressing Rag2<sup>-/-</sup> DN3/BALB/c-DL4 cocultures, are γδ-lineage cells, we assessed their expression of γδ-lineage biased genes. Provision of weak ligand did not affect γδTCR-transduced DN3 cells from inducing expression of ICER, Nurr1, and Rgs1, nor did it lead to increased transcription of TCRα-constant region the way Id3-deficiency did (Fig. 39C, 40C). Taken together, these data suggest that a weak ligand is not sufficient to drive γδTCR-expressing Rag2<sup>-/-</sup> DN3 cells away from a γδ T cell lineage fate towards an αβ one, but it affects the maturation potential of these cells along the γδ T cell lineage as determined by CD24 and CD73 expression.

18.4 γδTCR ligand availability affects the maturation status but not differentiation potential of γδTCR-bearing Rag2<sup>-/-</sup>Id3<sup>-/-</sup> pre-T cells

Our results from Figure 40 suggest that the provision of a weak ligand, via BALB/c stromal cells, to γδTCR-expressing DN3 is insufficient to divert their lineage choice to αβ DP cells, but appears to affect its maturation as IFNγ-producers, as assayed by CD24 expression. Considering this outcome, we sought to address whether combined inhibition of strong signaling from the γδTCR, with reduced ligand strength and Id3-deficiency, could reveal an αβ-lineage potential in γδTCR- and in particular TCRβ/γδ-expressing DN3 cells. We have already observed that, in the presence of Notch signaling, Id3-deficiency doubles the percentage of γδTCR-only DN3 cells that can differentiate along the αβ-lineage, but does not affect the differentiation of TCRβ/γδ-expressing cells in terms of the percentage of DP cells generated (Fig. 39A). However, these cells remain able to induce a number of genes which are members of the γδ-biased gene profile while remaining CD24<sup>hi</sup>.

As expected, in the absence of Id3 and strong γδTCR ligand, differentiation of pre-TCR-expressing DN3 cells was not impaired (Fig. 41A). These cells proliferated robustly in their
developmental progression to the DP stage (Fig. 41C). Again, a difference in percentage of DP cells was observed between TCRβ-transduced cells on OP9-DL1 and BALB/c-DL4, reflecting the higher efficiency of OP9 cells as a stromal cell for lymphogenesis (374). Interestingly, in the presence of weak ligand (Balb-DL4), γδTCR-expressing Id3-deficient DN3 cells did not divert their lineage choice to become αβ-lineage DP cells than the same cells on OP9-DL1 (Fig. 41A). However, maturation along the IFNγ-producing γδ-lineage was more severely affected by loss of both Id3 and strong ligand, as CD24 expression was even higher on γδTCR-expressing Id3-deficient DN3 cells cultured with BALB/c-DL4 than with OP9-DL1 cells, while CD73 also failed to upregulate (Fig. 41B). As with Rag2−/− DN3 cells, on BALB/c-Ctrl cells, γδTCR-expressing Rag2−/−Id3−/− DN3 cells behave much like their TCRβ-transduced counterparts in that they did not downregulate CD24, upregulate CD73, or survive in the absence of Notch signaling, as seen by the absence of cells in these culture (Fig. 41B, C). This supports the notion that Notch is required for the differentiation of γδTCR-expressing DN3 cells in the absence of Id3 and/or strong γδTCR ligand. Thus, in agreement with our findings in Figure 40, weak ligand appears to be adequate enough to induce sufficiently strong signals in γδTCR-expressing DN3 cells to promote a γδ-lineage choice, but not to induce maturation to IFNγ-producers (CD24 downregulation and CD73 upregulation) nor Notch-independence (Fig. 41).

To further characterize the identity of CD24hiCD4−CD8− cells within the γδTCR-expressing Rag2−/−Id3−/− DN3/BALB/c-DL4 coculture, we assessed cells for the expression of γδ-lineage biased genes. Surprisingly, we found that the provision of weak signal in combination with Id3-deficiency did not, for the most part, prevent γδTCR-transduced DN3 cells from inducing expression of ICER, Nurr1, and Rgs1, albeit Nurr1 and Rgs1 levels were highly reduced in γδTCR+ Rag2−/−Id3−/− DN3 cells cultured with weak γδTCR ligand versus strong ligand (Fig. 41D). Interestingly, TCRβ/TCRγδ-transduced Rag2−/−Id3−/− DN3 cells appeared to express higher levels of γδ-biased genes than γδTCR-expressing Rag2−/−Id3−/− DN3 cells, regardless of ligand strength. Taken together, these data suggest that a weak ligand in combination with Id3-deficiency is still not sufficient to drive γδTCR-expressing DN3 cells away from a γδ T cell lineage fate, and that the addition of preTCR signals helps to ensure that these cells adopt a γδ-lineage choice. This last conclusion, albeit surprising in view of the instructional model, it is consistent with the signal strength model for T cell lineage choice.
Figure 41. Provision of a weak γδTCR ligand with loss of Id3 does not promote αβ-lineage choice in γδTCR-expressing DN3 cells, both in the presence and absence of Notch signals. A,B) Development of culture-derived Rag2−/− Id3−/− DN3 cells retrovirally-transduced to express TCRβ, γδTCR, neither or both, and cultured for 6 d with OP9-DL1, BALB/c-DL4, or BALB/c-Ctrl cells. Flow cytometric analysis of A) CD4 vs. CD8 and B) CD24 vs. CD73 cell surface expression is shown for GFP+YFP+ gated cells; while C) shows the corresponding fold expansion. D) QRT-PCR analysis of γδ- biased genes ICER, Nurr1, and Rgs1, and αβ-gene TCRα-C in transduced Rag2−/− Id3−/− DN3 cells cultured for 6d on the indicated stroma, with mRNA levels normalized to β-actin.
19 Discussion

This current report addresses the question of how a DN3 cell chooses an αβ or γδ-lineage fate when it receives signals directing it to either or both lineages through dual and simultaneous expression of a pre-TCR and γδTCR. We also provide novel insights into the role of TCR signal strength in determining αβ versus γδ lineage choice. The ERK/MAPK pathway, via Egr and Id proteins, has a central role in mediating the strength of signal emitted from the TCR. Recently, we solidified this understanding by observing that Id3 was necessary and sufficient for the strong signals downstream of γδTCR that promote γδ T-lineage fate and oppose the αβ one (367). Id3 was also found to be necessary for providing γδ T cells with Notch-independence and functional competence. Here, we find that the majority of DN3 cells which simultaneously express a TCRβ and γδTCR choose a γδ T cell fate, in agreement with the principles of the signal strength model. We also confirm the importance of Id3 for the maturation along the IFNγ-producing γδ lineage.

Manipulating factors involved in regulating the strength of TCR signal, such as Id3 expression or TCR-ligand strength, does not seem to skew lineage choice towards an αβ fate, unlike our previous observations (367), but has an impact on maturation status of a cell committing to the γδ-lineage as IFNγ-producers, as well as its functional competence, as measured by its ability to produce IFNγ in response to stimulation. This observed difference from our previous results may simply be a consequence of increasing the efficiency of γδTCR-expression through encoding the γδTCR chains within a single retroviral vector for transduction, instead of two different vectors. Furthermore, an increase of Id2 transcript is not seen in TCRγδ-expressing Rag2−/− Id3−/− DN3 cells differentiating along the γδ-lineage (data not shown), which may indicate that other Id proteins are involved to compensate for the Id3-deficiency.

Previously, Pereira and colleagues found that the αβ versus γδ choice was highly independent of the TCR, as TCRβ/γδ transgenic mice generated similar numbers of αβ cells as those transgenic for TCRβ only, and similar numbers of γδ cells as those transgenic for γδTCR only (368). However, the timing of TCR expression in the thymocytes of these transgenic mice was premature, and it has been well characterized that such temporally dysregulated expression can affect developmental outcomes (287, 288). Furthermore, the study used a Vγ transgene that included the flanking DNA sequences encoding a putative silencer element that has been shown to shut off TCRγ chain mRNA expression, leading to the loss of surface γδTCR chain expression
While the silencing effect on the TCRγ locus has been linked to pre-TCR-induced proliferation (375), the finding that the majority of T cells in the γδTCR Tg mice used in Pereira’s study are of the αβ-lineage indicates that the presence of the silencer hampers the ability of γδTCR-expressing DN3 cells from realizing their true γδ-lineage potential, since the majority of T cells in γδTCR Tg mice without the flanking DNA regions mostly develop along the γδ-lineage (270). To address these issues, we made several modifications in our own study. The use of the in vitro T cell differentiation system, OP9-DL1, allows us to temporally regulate TCR expression precisely through timed T cell development kinetics, in vitro retroviral transduction, and prospective isolation of the population of interest (DN3 cells) by cell sorting. Furthermore, the TCRγ gene used in this study was cloned without flanking sequences to prevent the inclusion of the putative silencer element, which would otherwise hinder γδ T cell development in favor of the αβ-lineage in γδTCR-expressing DN3 cells. Lastly, to prevent the expression of TCR variants before, with, or instead of, the rearranged TCRs provided by retroviral transduction, we used Rag2−/− cells.

Here, we find that αβ- versus γδ-lineage choice in γδTCR-expressing DN3 cells is independent of TCRβ co-expression, in agreement with the findings published by Pereira’s group (368). However, unlike this previous study, we find that the majority of γδTCR-expressing DN3 cells develop along the γδ T cell lineage and remain DN, instead of along the αβ-lineage to gain expression of CD4 and CD8. This difference is an important distinction, as it affects how the results are interpreted. The finding that the majority of γδTCR-expressing DN3 cells differentiate along the γδ-lineage is in agreement with previously published results from other groups (284, 285), and reflects the idea that strong signals, such as those transmitted from the γδTCR in the presence of ligand, promote the γδ lineage fate and oppose an αβ choice.

In the context of the signal strength model for αβ versus γδ lineage bifurcation, one would predict that co-expression of a TCRβ in γδTCR-expressing DN3 cells would not impact lineage choice. In principle, the addition of TCRβ could only increase the total strength of TCR signal received by the DN3 cell at the bifurcation point, and drive lineage choice to the γδ-fate. In support of this notion, instead of preventing differentiation and maturation along the γδ-lineage, TCRβ expression with γδTCR increases CD24-downregulation. Additionally, no change in the percentage of DP cells development is seen for TCRβ/γδ-expressing Rag2−/−Id3−/− DN3, while
γδTCR--expressing Rag2\(^{-}\)Id3\(^{-}\) DN3 cells differentiate into twice the percentage of DP cells when compared to their Id3-sufficient counterparts.

Importantly, differentiation is not the only consequence of TCR-selection at the DN3 developmental checkpoint. Another critical outcome is proliferation, and this could, in theory, be differentially regulated in TCRβ, γδTCR, and TCRβ/γδ-expressing DN3 cells. Here, we find that TCRβ/γδ-expressing Rag2\(^{-}\) DN3 cells proliferate more extensively than their γδTCR-only-expressing counterparts. This increase in proliferation may indicate that, while the TCRβ chain cannot drive differentiation along the αβ-lineage, it can promote some αβ-like proliferation, and suggests that the different roles of a TCR to promote survival, differentiation, and proliferation can be separated and distinctly driven by different TCRs.

Recent insights into the molecular basis for αβ versus γδ- lineage choice have culminated in support of a signal strength model to dictate lineage fate. However, it remains unclear whether differential signal initiation mechanisms lead to changes in quantitative strength. It is now widely accepted that the pre-TCR is capable of cell-autonomous signaling (42, 43, 300), which fits into the model of it emitting a weak signal. While ligands have been characterized for some γδTCR subsets (302, 303), the role of ligand engagement for γδ T cell development is much less clear. Removal of γδTCR ligand has been shown to decisively influence the αβ versus γδ lineage choice by reducing transcription of Egr1 and Egr3, impairing γδ-lineage development and maturation, while simultaneously promoting αβ-lineage commitment to the DP stage (272). These findings suggested that strong signals transduced from the γδTCR was dependent on ligand-engagement, and in the absence of ligand, γδTCR-expressing DN3 cells would receive quantitatively weaker signal, and mature along the αβ-lineage. However, recent evidence suggests that IL-17-producing γδ T cells develop independently of ligand availability, and continue to receive signals through their TCR and induce similar levels of ERK1 and ERK2 as IFNγ-producing γδ T cells, which have been shown to require ligand (363, 376, 377). Here, we attempt to clarify the role of γδTCR ligand at the αβ versus γδ bifurcation point by providing KN6 γδTCR-expressing T cells with a weak ligand, via culture with BALB/c stromal cells. Our results show that provision of weak γδTCR ligand does not lead γδTCR-expressing DN3 cells to abandon a γδ T cell fate and develop as αβ-lineage DP cells, but does appear to affect the ability of TCRγδ-bearing cells to mature into the CD24\(^{lo}\) stage. Weak ligand may be sufficient to drive
differentiation into the \( \gamma\delta \)-lineage as retroviral transduction of \( \gamma\delta \)TCR leads to the overexpression of \( \gamma\delta \)TCR on DN3 cells, and weak signaling through each individual \( \gamma\delta \)TCR may additively equate to a sufficiently strong signal received by the cell that is capable of inducing \( \gamma\delta \)-lineage commitment and expression of \( \gamma\delta \)-biased genes.

It remains to be determined whether differences in CD24 and CD73 expression can accurately reflect the maturation status of a \( \gamma\delta \) T cell, or mark the bifurcation between cells committing to the \( \alpha\beta \) versus \( \gamma\delta \)-lineage. Expression of \( \gamma\delta \)-biased genes in CD24\(^{hi}\) \( \gamma\delta \)TCR\(^{+}\) \( \text{Rag2}^{-/-} \text{Id3}^{-/-} \) DN3 cells, or DN3 cells from the BALB/c-DL4 coculture suggests that these cells have indeed selected the \( \gamma\delta \) lineage. Thus, CD24 expression may not be a reliable marker of lineage commitment, unlike CD4 and CD8. CD24\(^{hi}\) CD4\(^{+}\)CD8\(^{-}\) always marks \( \alpha\beta \)-lineage DP cells, but to define the lineage of CD24\(^{hi}\)CD4\(^{-}\)CD8\(^{-}\) and CD24\(^{lo}\)CD4\(^{-}\)CD8\(^{-}\) cells requires further analysis of their gene expression profile. Moreover, unpublished data from our own work suggests that CD24\(^{hi}\)CD4\(^{-}\)CD8\(^{-}\) \( \gamma\delta \) T cells may not be immature, as originally thought, but rather fully functional \( \gamma\delta \) T cells that are capable of secreting cytokine upon stimulation. Specifically, we speculate that weaker \( \gamma\delta \) T cell receptor signaling induces IL-17 \( \gamma\delta \) T cell differentiation, and stronger \( \gamma\delta \) T cell receptor signals induce IFN\( \gamma \)-producing \( \gamma\delta \) T cells. Future attempts to more definitively address the role of \( \gamma\delta \) T cell ligand in \( \alpha\beta \) versus IL-17 \( \gamma\delta \) versus IFN\( \gamma \) \( \gamma\delta \) lineage fates will require the generation of stromal cell lines with graded levels of TCR-ligands, including with the ligand completely removed via targeted deletion of the T10 and T22 genes.

TCR-selection at the DN3 stage can be Notch-dependent or -independent, and this decision is based on lineage choice, which is ultimately a reflection of TCR signal strength. Recent work in this area has inspired a model for Notch signaling requirements at the DN3 stage of development, which is based on the strength of TCR signals, as measured by Id3 induction (367). In this model, strong TCR signals which induce high levels of Id3 are able to suppress E protein activity beyond the threshold required for passage across selection, independently of Notch signaling. Conversely, the weak signals which emanate from a pre-TCR require concurrent Notch signals to achieve downregulation of E2A to levels that allow for successful traversal of the \( \beta \)-selection checkpoint. Here, we find that loss of Id3 in \( \gamma\delta \)TCR-bearing DN3 cells does not make these cells Notch-dependent, but rather diverts lineage choice to \( \alpha\beta \)-lineage DP cells in the absence of Notch signals. This finding is surprising, considering DP cells do not conventionally
develop without Notch signals, but it makes sense in the context of signal strength. No DP cells appear in γδTCR-expressing Rag2\(^{-}\) DN3 cocultures without Notch signaling. It is only when Id3 and Notch signaling are removed that total signal strength seems to be lowered to a point where lineage potential shifts from γδ to αβ, but perhaps because of compensation by other Id proteins, the signal strength is not lowered enough to require concurrent Notch signaling. Interestingly, we also find that provision of a weak γδTCR ligand alters the requirement for Notch signaling in γδTCR-expressing cells. In the presence of a weak ligand, γδTCR-expressing DN3 cells can no longer survive, differentiate, and proliferate in the absence of Notch signaling (BALB/c-Ctrl stromal cells), in support of the notion that Notch-independence at this stage of development requires strong signals from the TCR.

Altogether, these data confirm previous reports that the strength of TCR signal dictates lineage choice, and strength of signal can be measured as a function of Id3 induction, but not ligand strength in cells expressing a high amount of TCR. More importantly, these data provide novel insights into the lineage decisions of a DN3 cell that expresses both a TCRβ and γδTCR. In this case, TCRβ co-expression with γδTCR appears to provide additive TCR signal strength effects, and further promote γδ-lineage selection, maturation and function. A refined limiting dilution assay may help to determine the frequency of αβ versus γδ-lineage commitment in pre-TCR, γδTCR, and TCRβ/γδ-expressing DN3 cells and more conclusively assess whether minute differences occur between these populations that cannot be revealed in bulk cultures, especially with respect to conditions in which signal strength is impaired through Id3-deficiency or weaker ligand availability. Further work in this area is also needed to address the differential ligand, differential maturation (as determined by CD24 and CD73), and differential TCR signal strength requirements of IFN\(γ\) vs. IL17-producing γδ T cells.
Chapter 5

Discussion and Future Directions
20 The OP9-DL1 stromal cell line

The Notch signaling pathway plays a central role in a variety of developmental systems. In T cell development, it is indispensable for promoting survival, differentiation, and proliferation of immature thymocytes (133, 136, 137, 378). In light of this requirement, the development of the OP9-DL1 stromal cell line has revolutionized our ability to study T cell differentiation in vitro (145). Chapters II, III, and IV demonstrate the ease with which T cells can be cultured from their hematopoietic precursors on OP9-DL1 monolayers supplemented with Flt-3L and IL-7. Manipulation of Notch signaling availability in these developing T cells is easily achieved by provision (OP9-DL1) or removal (OP9-Ctrl) of the Notch ligand, Dll1. In this way, the differential requirement for Notch signaling in αβ versus γδ T cell lineage development can be addressed with precision. The ability to grow T cells in vitro allows for manipulation of their gene expression with overnight retroviral transductions, which overcomes the time-consuming, costly, and cumbersome generation of genetically modified Tg mice, as well as bypasses complications of gene manipulations resulting in embryonic or premature adult lethality. Using the in vitro retroviral transduction technique, I was able to over-express, knock-down, and employ dominant-negative constructs in a developmental stage-specific manner to manipulate gene expression. I also made use of genetically modified mouse models. Together, these genetic manipulations helped to identify the molecular pathways and players involved in differentiating a DN3 cell to its next stage of development, whether it be an immature γδ T cell or a DP cell.

21 Regulating αβ versus γδ lineage bifurcation

21.1 The signal strength model and its molecular characteristics

Chapter III and IV bring new insights into the factors which mediate the decision of αβ versus γδ lineage fate. In Chapter III, I examine the role of Id3 in driving the strong signals required to promote γδ-lineage specification, as well as relieve cells of Notch-requirement. Strong Id3 expression is found to be necessary and sufficient for DN3 cells to adopt the γδ-lineage fate, in the absence of both Notch and TCR signals, as well as direct them into IFNγ-producing effector cells upon stimulation. These findings place Id3 as a central molecular mediator of the strong signals that influence T cell fate, as well as their developmental and functional characteristics. In Chapter IV we further extend these findings to address the lineage outcome of a DN3 cell that simultaneously expresses a pre-TCR and a γδTCR. We find the signaling capacity of the γδTCR
continues to drive lineage selection. In line with the signal strength model, in cases when γδ TCR signal strength is dampened with Id3-deficiency or decreased γδ TCR ligand availability, concurrent signaling by the pre-TCR does not drive commitment to the αβ-lineage, but rather provides a cooperative and additive signal to induce strong γδ-lineage development, maturation, and functional competence. This confirms the results obtained in Chapter III, which determines that the strength of TCR signal dictates lineage choice and strength of signal can be measured as a function of Id3 induction. Interestingly, the results in Chapter IV suggest that γδ TCR-ligand strength and Id3-deficiency do not affect a DN3 cell’s decision to develop along the γδ T cell lineage when it expresses high amounts of γδ TCR, but rather affects the CD24 expression of these cells, which may translate to differences in maturation status or effector function of these cells.

Considering the critical role of Id3 downstream of strong γδ TCR signals, the finding that \( Id3^{-/-} \) mice have dramatically increased numbers of γδ T cells in the thymus and spleen (353) is surprising, and seemingly contradictory to our work and previously published reports (272, 311). A more detailed analysis of the expanding γδ T cell population in \( Id3^{-/-} \) mice revealed no differences in fetal γδ T cell development compared to wild-type mice, but rather increased numbers of a specific \( V\gamma1.1^{+} \gamma\delta \) T cell subset. It may be possible to reconcile these opposing observations by appreciating that the \( V\gamma1.1^{+} \) subset of γδ T cells is a self-reactive subset that selects with high affinity for its ligands (379). In agreement with this notion, further tests examining the functional capacity of γδ T cells from \( Id3^{-/-} \) mice revealed that they are hyper-responsive to PMA and ionomycin, producing enhanced amounts of IFNγ in response to these mitogenic stimuli (353). Furthermore, in a separate study, the increased percentage of γδ T cells in \( Id3^{-/-} \) mice is found to be due to the expansion of a \( V\gamma1.1^{+} V\delta6.3^{+} \) T cell subset, many of which expressed high levels of the innate NKT cell transcription factor, PLZF (promyelocytic leukemia zinc finger) (380). Also seemingly in opposition to the signal strength model, knock-in mutations in the TCR signaling cascade molecule SLP-76, which affects the Itk calcium flux pathway via Vav1, or the ERK/MAPK phosphorylation pathway via Nck, both lead to increased percentages of γδ T cells in the thymus. Again, this is the result of an increase in frequency and absolute numbers of \( V\gamma1.1^{+} V\delta6.3^{+} \) γδ T cells, the majority of which express PLZF, display the functional characteristics of an NKT cell, and have been characterized as self-reactive innate lymphocytes (381, 382). Thus, in the presence of Id3, these self-reactive \( V\gamma1.1^{+} \) γδ T cells may
induce TCR signals that are too strong to allow for survival, leading to their negative selection. Conversely, loss of Id3 weakens the signal produced by the Vγ1.1+ T cells, allowing for their survival and subsequent expansion. In accordance with this view, KN6 Tg γδTCR T cells in a negatively selecting background can survive and mature to a CD24lo phenotype in the absence of Id3 (367). Thus, Id3 is necessary for the development of non-auto-reactive T cells whose TCR is of the appropriate strength to permit positive selection and survive negative selection, while its expression is detrimental to auto-reactive γδ T cells whose TCR signal is too strong in the presence of Id3 to overcome negative selection and develop to maturity.

22 Notch signaling and αβ T cell development

Notch signaling is essential to enforce the T cell fate in hematopoietic progenitors entering the thymus, and to ensure the generation and survival of early DN thymocytes. At the β-selection checkpoint, Notch signals mediate a host of cellular processes that drive the transition of DN3 cells to the DP stage (132, 135, 136). Notch signals are required for the expression of the pre-TCR (136), and our laboratory has shown that Rag2−/− DN3 cells bearing a functional TCRβ must receive concurrent Notch signaling for differentiation across the β-selection checkpoint (140, 284). Notch signaling is required for more than cellular differentiation, and mediates metabolism and survival by regulating the activation of the PI3K/Akt pathway (284). Having established the critical link between Notch signaling and the PI3K pathway, the aim then became to elucidate the precise mechanism for this interaction.

In the thymus, known upstream inducers of this pathway include IL-7R (CD127) (332) and CXCR4 (99). However, whether these signaling pathways or their molecular intermediates are the targets of Notch, and the means by which Notch regulates activation of the PI3K pathway remained unclear. In Chapter II, I establish that loss of PI3K signals in the absence of Notch is not due to receptor down-regulation, suggesting the receptors inducing PI3K pathway activation are expressed, but Notch regulates the responsiveness of this pathway to external stimuli. Key to understanding this link is to appreciate that Notch mediates its effects in the nucleus, by forming a transcriptional complex that regulates expression of downstream target genes. It is in this way that Notch must affect the PI3K pathway activity through receptor proximal signals. In Chapter II, we find a role for Notch-dependent transcriptional activation of HES1 in mediating the interaction between Notch and the PI3K pathway, by down-regulating expression of PI3K.
pathway inhibitor, PTEN. The ability of HES1 to repress Pten promoter activity downstream of Notch signaling is in agreement with a previous study looking at the relationship of these factors in T-ALL (168). However, unlike a previous report suggesting that enforced expression HES1 in E14 fetal thymocytes promotes proliferation in these cells (197), I found that HES1 retroviral over-expression in DN3 cells had a deleterious effect, leading to cellular atrophy and apoptosis. This toxic affect associated with high levels of HES1 expression has been documented in other cell types, and is likely due to the strong transcriptional repressor activity of HES1 (182), which becomes dysregulated with a retroviral expression system.

In line with the study by Wendorff et al. (198), I found that the role of HES1 downstream of Notch extends beyond the β-selection checkpoint. Specifically, interfering with HES1 expression or function leads to decreased T cell proliferation as well as decreased commitment of hematopoietic progenitors to the T cell lineage. In apparent opposition to this study, however, is my finding that HES1 is absolutely required at the β-selection checkpoint. While Wendorff et al. employ HES1^{fl-Lck-Cre} mice and observe that HES1 is dispensable through and beyond β-selection, it is likely that the timing of their genetic manipulation, as well as the timing of their analysis, prevent them from observing the effect of HES1-deletion at this checkpoint. Importantly, in my own studies, I took advantage of a narrow window of opportunity when we can accurately observe the effects of decreased HES1 function in DN3 cells. After this, the few cells that manage to escape the incomplete blockage imposed by inhibiting HES1 function are able to differentiate to the DP stage and induce an extensive wave of proliferation associated with this developmental progression, making the effect less apparent. Once these cells develop to become DN4 and DP cells, which typically do not express HES1, they are no longer affected by the absence of HES1, and perhaps might even fare better in its absence.

Our finding that loss of PTEN is able to compensate for the survival and differentiation defects associated with Notch signaling withdrawal parallels previous work which reveals a similar capacity for Pten-deletion to compensate for other PI3K-inductive signaling pathways, such as pre-TCR and IL-7R in T cell development and at the β-selection checkpoint. However, while loss of PTEN is able to rescue both differentiation and cellularity defects associated with the loss of pre-TCR and IL7-R signaling, it can only rescue survival and differentiation defects associated with the absence of Notch signals, suggesting that a separate set of genes and pathways regulate proliferation downstream of Notch signaling. My results show that Notch
induction of c-Myc expression drives the proliferation of β-selected cells. Previous studies addressing the role of c-Myc in T cell development are in agreement with my findings that c-Myc is a mediator of cellular expansion (339, 340).

23 Notch signaling and γδ T cell development

Our lab and others have found that the requirement for Notch signaling is different for αβ- and γδ-lineage precursors (284, 297, 331, 365). Specifically, we have shown that γδ precursors differentiate independently of Notch signals after expression of the γδ TCR, while αβ-lineage precursors remain Notch dependent during pre-TCR selection (284). Considering that development along the γδ T cell lineage does not require concurrent Notch signaling at the selection checkpoint, the γδ TCR itself must provide the signals normally attributed to Notch function: cues which mediate survival, differentiation, and proliferation. The existing data points to a collaboration between pre-TCR and Notch signals to down-regulate the transcription factor E2A at the DN3 selection checkpoint. Both signaling cascades induce the ERK/MAPK pathway, as well as Id3, to dampen E2A activity (344, 345, 348). Considering the evidence for Notch-independent development of γδ T cells in the presence of strong γδ TCR signals, it has been proposed that the requirement for Notch signaling at this selection checkpoint depends on the magnitude by which the TCR signals can downregulate E2A expression (367). In this model, strong TCR signals leading to a high induction of Id3 can successfully suppress E protein activity to a level that allows for passage through selection, independently of Notch signaling. In contrast, weak signals such as those produced by the pre-TCR are unable to independently down-regulate E2A to levels that allow for successful traversal of the β-selection checkpoint, and thus require concurrent Notch signaling to achieve this successfully. In support of this model, Chapter III finds that over-expression of Id3 by retroviral transduction in Rag2+/Id3−/ DN3 cells was sufficient to mimic the strong signals required to promote γδ-lineage development, in the absence of both TCR expression and Notch signaling (367). Importantly, mutant forms of Id3 that do not interact with E2A (362) could not induce this TCR- and Notch-independent γδ T cell differentiation, indicating the requirement for Id3-mediated inhibition of E2A function.

Although weak γδ TCR signaling has been proposed to mimic pre-TCR signaling by giving rise to αβ-lineage DP cells that require concurrent Notch signals, the quantitative requirement for Notch is not equivalent for the two receptors. In a study using Rag1−/ γδ TCR-Tg mice (331),
which generate both αβ- and γδ-lineage cells, γδTCR-expressing precursor T cells are observed to require a greater input from Notch signaling to enter the αβ-lineage than pre-TCR-expressing cells, based on dampening of Notch signaling in a γ-secretase inhibitor dose-dependent manner. These observations suggest that the pre-TCR synergizes more efficiently with Notch signals than the γδTCR, perhaps due to its unique cell-autonomous signaling capacity, and that this characteristic of the pre-TCR may have an additional role in influencing lineage fate.

24 T-ALLs: Aberrant T cell development

Inherent to the nature of any developmental process is the need for periods of rest and cell cycle inhibition, punctuated by intervals of rapid cell division and proliferative bursts. For this reason, the functional interactions between molecular pathways that participate in T cell development often require tight regulation in order to prevent runaway metabolism and growth. In the event of their dysregulation, these interactions usually manifest themselves as key inducers and mediators of the T-ALL transformation process. This is particularly true of molecular pathways that activate pro-survival or anti-apoptotic factors, as well as inducers of proliferation and cell division.

In Chapter II, I showed that interplay between Notch, HES1 and PTEN is required for T cell development across the β-selection checkpoint. In Chapter III, I addressed the importance of MAPK pathway activation downstream of the pre-TCR and γδTCR in mediating survival, proliferation, survival, and in the case of γδ-T cells, Notch independence. Notch signaling is tightly regulated temporally, such that after selection at the DN3 stage, Notch receptor expression is rapidly downregulated. Notch signaling decreases following β-selection, leaving post-selected DN3b cells with low levels of Notch1 expression compared to pre-selected DN3a cells (76). This was shown to be a consequence of transcriptional auto-regulation by Notch1, as well as pre-TCR induction of Id3, which leads to a loss of E2A dependent Notch1 transcription (336, 366). This regulation of Notch activity, along with the auto-regulatory abilities of HES1, where high levels of HES1 protein can be inhibitory to HES1 transcription (183), serve important roles in limiting the heightened metabolic active phase of DN3 cells at their selection checkpoint. Despite this, Notch signaling dysregulation is one of the most common initiating factors of T-ALL development. Perhaps unsurprisingly then, especially considering the metabolic activity induced with Notch and PI3K signaling, the interactions between Notch,
HES1 and PTEN have also been observed in T-ALL (168). Several studies of T-ALL have also implicated c-Myc as a direct downstream target of Notch signaling, and as a critical component in transformation and the cell growth process (170, 338). Furthermore, the complex relationship between Id3 and Notch1 may be necessary to ensure downregulation of Notch post-selection, but the mitogenic signals propagated by the MAPK pathway, which lead to Id3-mediated inhibition of E2A activity, must be also restricted. Several studies have shown that loss of E2A activity promotes the transformation of T cell progenitors (65, 383). Therefore, while powerful cellular metabolic pathways need to be activated in DN3 cells to ensure successful selection to the next stage of development, cessation of these signals is equally as important to prevent uncontrolled growth, leading to malignancy. Together, this underscores the importance of regulating developmental pathways during normal T cell development.

25 Future Directions

25.1 The many mechanisms regulating PI3K pathway activity

Combining my work in Chapter II, which demonstrates the importance of Notch signals in promoting the PI3K pathway in DN3 cells differentiating along the αβ-lineage, and the work in Chapter III, which suggests that γδ TCR signals can overcome the requirement for Notch signals via strong induction of Id3, it is possible that strong induction of the ERK/MAPK pathway, and subsequently high Id3 levels, in γδ T cells promotes PI3K pathway activity in a way which mimics the Notch-mediated inhibition of PTEN expression. Several studies on tumours suggest this link is possible, as multiple methods have been observed to dysregulate the PI3K/PTEN relationship. Specifically, in vivo data of tumours show that mutations in Ras and PTEN are mutually exclusive, suggesting an interaction between these proteins such that loss of PTEN and activation of Ras mediate the same function (384). Pten<sup>−/−</sup>Lck-Cre mice also suffer from increased ERK phosphorylation (115), while PI3K has been found to interact with Ras via its catalytic subunit, p110 (385), while Egr-1 has been found to upregulate expression of PTEN messenger RNA and protein in irradiated cells (386). Future attempts to definitively characterize the interplay between the ERK/MAPK pathway, or its downstream mediator Id3, and PI3K will help shed more light on the relationship between these pathways in the DN3 cell undergoing selection.
25.2 The molecular and signal strength requirements of developing IL-17-producing γδ T cells

In light of increasing evidence that γδ T cells are an important source of IL-17, especially during an innate immune reaction (387, 388), efforts have been made to characterize these cells and map the course of their development in the thymus and periphery. While studies on the development of IL-17-producing γδ T cells have focused on their bifurcation from IFNγ-producing γδ T cells rather than from the αβ-lineage, accumulating data has suggested a unique ability of IL-17 γδ T cells to traverse thymic selection in the absence of ligand engagement, and yet remain γδ-lineage cells. This notion directly opposes the hypothesis that strength of TCR signal stems from its ligand-engagement requirements, such that weak signals arise from the ligand-independent nature of a receptor such as the pre-TCR, while strong signals are induced from γδTCR-ligand engagement.

In consideration of this recent finding, an obvious question raised is, how does the γδTCR induce a quantitatively larger signal than the pre-TCR, if it may also signal ligand-independently? The signal strength model remains valid, in the sense that antigen-naive γδ T cells that adopt the IL-17 pathway are still more potent inducers of the ERK/MAPK pathway compared to αβ-lineage T cells. However, the proposal of a ligand-mediated mechanistic basis for the generation of stronger signal seems to be invalidated, at least in the case of IL-17-producing γδ T cells. Are IL-17 cells an exception to the rule? It does not seem that way, as all Vδ, Vγ fusion pairs were found to self-dimerize, with the exception of DETC Vδ1-EPOR, Vγ5 fusion pairs (363). However, evidence does exist to indicate that IL-17 γδ T cells are unique, especially in their ability to recognize danger signals and secrete cytokine in the absence of TCR engagement. As innate effectors of host defense, IL-17 γδ T cells have been shown to employ innate receptors such as Toll-like receptors and Dectin-1 (389), or directly respond to cytokines IL-1β and IL-23 (390), to rapidly secrete IL-17 in response to danger, rather than receive stimulation via its TCR. Further research will need to be conducted in this new area of γδ T cell development before comprehensive conclusions can be reached.

If it is correct that ligand engagement does not play a necessary role for the induction of a signal strength differential between pre-TCR and γδTCR, then further examination into the mechanistic basis behind weak and strong signaling is required. Furthermore, if IFNγ- and IL-17-producing
γδ-lineage T cells both generate strong signals as measured by ERK1/2 activation, then the identity of the signaling pathway that dictates the IL-17 versus IFNγ differential lineage outcome remains to be deciphered. The identification of naturally occurring IL-17-producing γδ T cells in the thymus may help to further our understanding in this area (148). Naturally occurring Th17 cells (nTh17) have recently been shown to have differential signaling requirements than their conventional peripheral counterparts, such that reduced TCR signaling results in increased numbers of nTh17 cells while at the same time leads to defective conventional Th17 cell differentiation (149). The role of TCR signal strength in promoting and/or supporting IL-17-producing γδ T cells may similarly depend on whether one is talking about the naturally occurring thymic subset or its peripheral counterpart. Interestingly, intrathymic development of the naturally occurring IL-17-producing γδ T cells seems to require HES1, such that their numbers and ability to produce IL-17, but not IFNγ are severely decreased in the thymi of Hes1-deficient fetal mice (314). Although much has been learned since the discovery of the γ and δ TCRs and the initial characterization of the development of γδ-lineage cells, it is clear that we still have much more to learn about how the many distinguishing features of γδ T cells are generated.

26 Conclusions

This thesis examines the molecular pathways and effectors required for DN3 lineage fate decisions. The ability of a DN3 cell to differentiate along the αβ- or γδ-lineage is determined by the balance of a multitude of environmental cues. Accumulating evidence in the study of αβ versus γδ T cell fate points to a decisive role for the TCR in dictating lineage outcomes, specifically through the strength by which it signals. It is becoming increasingly clear that the ERK/MAPK pathway is central to mediating TCR signaling strength differentials, whereby strong signals lead to higher induction of this pathway. Significantly, over-expression of downstream ERK target genes, Egr and Id3, in developing thymocytes is necessary and sufficient to mimic γδTCR strong signals and mediate differentiation to the γδ-lineage, in the absence of Notch signaling and TCR expression. Considering this, I proposed a model, whereby strong TCR signals, which induce high levels of Id3, are able to suppress E protein activity beyond the threshold required for passage across selection, independently of Notch signaling,
while weak pre-TCR signals require concurrent Notch signals to sufficiently downregulate E2A to allow for differentiation beyond the β-selection checkpoint.

Inhibition of E2A activity, however, is not the only role of Notch signaling in pre-T cells traversing the β-selection checkpoint. I also identify key signaling intermediates that mediate T cell differentiation, proliferation, survival and cellular metabolism downstream of Notch. Direct Notch target, HES1, forms a critical link between Notch signals and activation of the PI3K/Akt pathway, by repressing expression of the PI3K pathway inhibitor, PTEN. This interaction is largely responsible for coordinating differentiation, survival and metabolism of pre-T cells at the β-selection checkpoint. Proliferation of β-selected cells that reach the DP stage of T cell development, however, is controlled by another Notch target gene, c-Myc.

Taken together, the results from this thesis provide a detailed examination of the molecular mechanisms that are instrumental in determining lineage fate, survival, and proliferation of differentiating thymocytes. While it is clear that the Notch, TCR, PI3K, and MAPK signaling pathways provides unique signaling outcomes critical to the successful differentiation of DN3 cells along the αβ or γδ T cell lineage, the interplay between each of these pathways is equally as important (Fig. 42). Crosstalk between these pathways ensures that DN3 cells reach or exceed certain molecular thresholds required to progress to the next stage of development, while at the same time enforces critical checkpoints and regulatory mechanisms that prevent aberrant growth, proliferation, and leukemic transformation.
Figure 42. A comprehensive model of the interactions between the Notch, TCR, PI3K, and MAPK signaling pathways involved in DN3 thymocyte differentiation along the αβ-lineage or γδ-lineage. Pre-TCR AND γδTCR induce the MAPK pathway, leading to Id3 expression and suppression of E proteins. Notch signaling also leads to inhibition of E2A via Id3 expression, activation of c-Myc and HES1, and subsequently, inhibition of PTEN. PI3K pathway signaling is activated downstream of chemokine and cytokine receptors CXCR4 and IL7-R, respectively, following engagement of the receptors with their corresponding ligands. While the MAPK pathway and PTEN have been shown to interact in cancers, the role for this interaction in T cell development remains to be determined.
References


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