Notch1 and IL-7 Receptor Interplay Maintains Proliferation of Human Thymic Progenitors while Suppressing Non-T Cell Fates

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Notch signaling is critical for T cell development of multipotent hemopoietic progenitors. Yet, how Notch regulates T cell fate specification during early thymopoiesis remains unclear. In this study, we have identified an early subset of CD34highc-kit+/flt3−/IL-7Rα− cells in the human postnatal thymus, which includes primitive progenitors with combined lymphomyeloid potential. To assess the impact of Notch signaling in early T cell development, we expressed constitutively active Notch1 in such thymic lymphomyeloid precursors (TLMPs), or triggered their endogenous Notch pathway in the OP9-Delta-like1 stroma coculture. Our results show that proliferation vs differentiation is a critical decision influenced by Notch at the TLMP stage. We found that Notch signaling plays a prominent role in inhibiting non-T cell differentiation (i.e., macrophages, dendritic cells, and NK cells) of TLMPs, while sustaining the proliferation of undifferentiated thymocytes with T cell potential in response to unique IL-7 signals. However, Notch activation is not sufficient for inducing T-lineage progression of proliferating progenitors. Rather, stroma-derived signals while sustaining the proliferation of undifferentiated thymocytes with T cell potential in response to unique IL-7 signals. Furthermore, Notch1 leads to a loss of T cell production and to the development of B cells in the thymus. Conversely, overexpression of a constitutively active form of Notch1 inhibits B lymphopoiesis and results in the appearance of developing T cells in the bone marrow (BM). Thus, Notch1 signaling appears to instruct T cell commitment at the expense of B cell differentiation in thymus-seeding progenitors with T and B potential (9–10). However, both human and mouse thymus-settling precursors retain other non-T lineage potentials, including NK cell, dendritic cells (DC), and even macrophage potential (11–18). Consequently, the question that arises is how is Notch-induced T cell fate specification of such multipotent precursors regulated during early thymopoiesis.

In both humans and mice, the most immature T cell precursors are included in the CD4+ CD8− double-negative (DN) thymocyte subset. Human DN thymocytes contain a minor population of CD34highCD33+ precursors, which retain lymphoid and myeloid lineage potential and express the highest levels of CD34, supporting the notion that this population represents the earliest T cell precursor (14–15). Such CD34highCD33+ thymocytes develop along lymphoid (T/NK) or myeloid (macrophages/DCs) pathways through separate intermediate progenitors (19), suggesting that they map to a critical precursor stage upstream of the lymphoid/myeloid bifurcation within the thymus. In fact, lymphoid commitment and loss of myeloid potential occurs in downstream CD34+CD3−CD1a− progenitors (termed pro-T cells), which are conventional T/NK bipotential intermediates, and NK cell potential is lost afterward in more mature CD34+CD3−CD1a+ (pre-T) cells, with CD1a expression correlating with T-lineage specification (18). However, TCRβ gene rearrangement, a hallmark of T-lineage commitment, is delayed in vivo until the next stage of CD4+ immature single-positive thymocytes, which undergo β selection, differentiate into conventional CD4+CD8+ double-positive (DP) thymocytes, and lose the potential to generate any progeny cell types but T cells (18, 20, 21). Whether both loss of non-T cell potentials and commitment to the T cell lineage occurs through separate or linked mechanisms dependent on Notch1 signaling remains contentious and needs to be directly approached.

Besides endogenous transcription factors, cytokines are important exogenous factors capable of regulating hemopoietic development. Lineage-specific action of cytokines mostly on their survival and proliferation effects on specific intermediate progenitors, and seems to be regulated at the level of receptor expression.
In fact, down-regulation of cytokine receptors that drive myeloid development, such as the GM-CSFR, and up-regulation of the IL-7R represent a critical step in murine lymphoid commitment (22). Also, lymphoid and myeloid intermediates in the human thymus express, respectively, the IL-7R or the GM-CSFR (19). Previous results in mice showed that the IL-7/IL-7R pathway plays a conserved nonredundant role in early thymopoesis by supporting the survival and proliferation of DN precursors (Ref. 23 and reviewed in Ref. 24). However, whereas thymocyte expansion before T cell commitment is driven by IL-7R signals, IL-7 is not required for differentiation beyond the DN2 pre-T cell stage (25).

Accordingly, IL-7R levels are tightly regulated during murine T cell development, such that expression of IL-7Rα declines after the DN2 stage, and IL-7-R signaling must be terminated before transition to the DP stage (25, 26). Besides this prominent role of IL-7 in T cell development, IL-7R signaling is dispensable for NK cell development in both humans and mice, and even for B cell development in humans (23, 25, 27).

Although much is known about the importance of Notch1 and IL-7-R signaling in T cell development, the necessary steps that precede T cell commitment very early in intrathymic differentiation remain elusive. In this study, we have identified a subset of CD34(low)CD33(c−kit)flt3(IL-7Rα+) primitive precursors in the human postnatal thymus, and have approached the interplay between Notch1 and IL-7R at this early developmental stage. Our results suggest that Notch1 and IL-7-R pathways cooperate to serve an essential aspect of T cell development, i.e., amplification of the pool of intrathymic progenitors before T cell development.

Materials and Methods

Cell isolation and flow cytometry

Human postnatal thymocytes were isolated from thymus fragments removed during corrective cardiac surgery of patients aged 1 mo to 4 years, after informed consent was provided. Thymocyte suspensions were depleted of Lin-positive cells by MACS (AutoMACS; Miltenyi Biotec). Thymic lymphoid/myeloid precursors (TLMPs) (CD34(low)CD33(CD1a+), pro-T (CD34(low)CD33(CD1a+), and pre-T cells (CD34(low)CD33(CD1a+)) were AutoMACS sorted from the Lin− fraction or sorted in a FACSVantage SE sortor (BD Biosciences) as described (19). Intracellular Notch1 domain-specific (ICN1+) Lin− and GFP+ NK cells generated from TLMPs under multilineage-supportive cytokines (BD Biosciences) were sorted based on GFP, CD13, and CD56 expression. Sorted populations were >99% pure upon reanalysis.

The following directly labeled mAbs were used: CD7-TC, CD8α-PE, CD45RA-PE, CD15-PE, and TCRβ-TC from Caltag Laboratories; CD1a-allophycocyanin, CD3ε-PE or -allophycocyanin, CD14-FITC or -PE, CD19-PE, CD24-PE, or -FITC, CD44-PE, CD45-PE, -CD13-PE-Cy5, -CD11a/Cd11b-PE, -CD11c-PE, -CD161-PE from BD Biosciences; and CDA1-RD1, CD4-PE-Cy5, CD13-PE-Cy5, CD33-PE-Cy5, CD34-PE-Cy5, GM-CSFRα-PE, IL-2R-PE, IL-7Rα-PE, and TCRβ-PE-Cy5 from Beckman Coulter. PE- or allophycocyanin-conjugated goat anti-mouse F(ab′)2, IgG1 were from Southern Biotechnology Associates and BD Biosciences, respectively. Unlabeled mAbs used were CD2 from BD Biosciences, IL-2R receptors as well as low-to-intermediate levels of IL-7Rα (Fig. 1A). Increased IL-7Rα and decreased c-kit and flt3 expression was found on downstream CD34(CD33(}

Retroviral constructs and cell transduction

The retroviral vectors encoding the entire ICN1 domain and GFP from a bicistronic transcript (MigR1-ICN1), or GFP alone (MigR1-GFP), were provided by Dr. J. C. Aster (Department of Pathology, Brigham and Women’s Hospital, Boston, MA) (28). Previous studies validated the use of GFP as a surrogate marker for ICN1 expression and confirmed successful ICN1 expression by Western blotting (8, 29). Full-length cDNA encoding human IL-7Rα was amplified by PCR from human thymocytes and subcloned into the MigR1-GFP plasmid. Specific expression of IL-7Rα on GFP+ cells was confirmed by intracellular staining in transiently transfected COS cells. Retroviral infections were performed as described (29).

Cell cultures

DC and monocytes were generated from TLMPs in RPMI 1640 cultures (BioWhittaker) supplemented with 10% FCS (Invitrogen Life Technologies) and the following recombiant human (rh) cytokines from the National Institute of Biological Standards and Controls (NIBSC): 100 IU/ml IL-7, 60 IU/ml IL-1α, 50 IU/ml IL-6, 100 IU/ml stem cell factor (SCF), and 100 IU/ml GM-CSF (referred to as myeloid/DC-supportive cultures). Addition of 50 IU/ml IL-2 (Hoffman-La Roche) or 50 IU/ml IL-15 (NIBSC) to those cultures (multilineage-supportive cultures) allowed for the simultaneous generation of NK cells.

CFU assays were performed by plating 10¹ to 3 x 10⁴ TLMPs/ml in Methocult GF (StemCell Technologies) containing 30% FCS and supplemented with 100 ng/ml SCF, 10 ng/ml IL-3, and 10 ng/ml GM-CSF (NIBSC). Colonies were counted by day 14. GM-CFU identification was based on typical morphology and flow cytometry of individual colonies.

The OP9 murine BM stroma cell line expressing either GFP (OP9) or the Notch ligand Delta-like 1 (OP9-DL1) was provided by Dr. J. C. Zühna-Pfückler (Department of Immunology, Sunnybrook Research Institute, University of Toronto, Toronto, Ontario, Canada). OP9 cocultures were performed as originally described (30) in the presence of either multilineage-supportive cytokines (see above) or 100 IU/ml rhIL-7 (NIBSC; specific activity: 10¹⁰ IU/mg) plus 50 IU/ml rhFLIP-L (NIBSC; specific activity: 10¹⁰ IU/mg). When indicated, lower amounts (20 or 5 IU/ml) of rhIL-7 were used.

Hybrid human/mouse fetal thymic organ cultures (hu/mo FTCO) were performed as described (29).

Semiquantitative RT-PCR and TCRβ gene rearrangements

Total RNA isolated using TRIzol (Invitrogen Life Technologies) was reverse transcribed into cDNA using oligo-d(T) primer and Expand Reverse Transcriptase (Roche). PCRs, performed using the same serially diluted cDNA samples shown for β-actin, were subjected to 1.5–2.5 agarose electrophoresis and visualized with ethidium bromide. The gene-specific primers, product lengths, and amplification conditions used are provided in Table I.

The V(D)J and DJ TCRβ gene rearrangement analysis was performed by PCR and Southern blotting as previously described (20).

Results

Characterization of c-kit−flt3+IL-7Rα+ progenitors with lymphomyeloid potential in the human postnatal thymus

CD34(CD44(CD1a−Lin−) immature human thymocytes include up to 35% of CD33+ cells, which express the highest CD34 surface levels (CD34(MBH)) (Fig. 1A). Such CD34(CD33)> subset (0.030 ± 0.018% of total thymocytes) includes primitive progenitors with the capacity to generate NK cells, myeloid, and plasma-cytoid DCs, and even monocyte/macrophages, in addition to T cells, suggesting that they comprise the earliest progenitors seeding the human postnatal thymus (18, 19). The myeloid potential of CD34(CD33)+ subset (Fig. 1B) showing that clonogenic colonies with a typical GM morphology were formed at frequencies lower than those of HSC (13), but higher than expected for an intrathymic precursor (Table II). Phenotypic analysis of individual colonies confirmed the generation of CD14+ macrophages, CD15+ granulocytes, and CD14(CD1a−myeloid DCs (data not shown). As a whole, available data showing that CD34(CD33)+ thymocyte precursors display a robust T cell developmental capacity (29), include NK/DC clonogenic precursors (15), and can generate GM lineage cells (Fig. 1B), provide evidence that thymus-seeding cells retain lymphoid and myeloid potential. Therefore, they will be hereafter referred to as TLMPs.

More extensive phenotypic analyses showed that virtually all TLMPs, isolated by means of CD34 and CD33 expression, expressed the c-kit− and flt3 receptors as well as low-to-intermediate levels of IL-7Rα (Fig. 1A). Increased IL-7Rα and decreased c-kit and flt3 expression was found on downstream CD34(CD33)−...
pro-T and CD34^+ CD1a^+ pre-T cells (Fig. 1A), whereas no expression of myeloid-lineage cytokine receptors such as GM-CSF was detected on TLMPs, pro-T, or pre-T cells (Table III). Therefore, human TLMPs display a c-Kit^+ flt3^+ IL-7Rox^+ phenotype (Fig. 1A), equivalent to that recently described for lymphomyeloid stem cells in the BM (31) and thymus-settling cells in mice (9).

**Activation of Notch1 signaling inhibits myeloid differentiation of human TLMPs**

To investigate the role of Notch1 in the regulation of the earliest cell fate decisions in the human thymus, TLMPs were retrovirally transduced with a bicistronic vector encoding a constitutively active form of Notch1 and GFP as a reporter (ICN1^+), or GFP alone (GFP^+), as a control. Both GFP- and ICN1-transduced TLMPs were then assayed for their myeloid potential in vitro under myeloid/DC-supportive differentiation conditions (IL-7, SCF, IL-1α, IL-6, GM-CSF) shown previously to sustain the simultaneous generation of CD14^+ CD13^+ monocyte/macrophages and CD14^+ CD13^+ myeloid DCs which express CD1a (19). As shown in Fig. 2A, a marked reduction in both frequencies and numbers of DCs was found in ICN1-transduced progenitors. Instead, Notch signaling supported myeloid differentiation of TLMPs into myeloid-lineage cells. Inhibition of myeloid differentiation was not due to a decreased cell survival of ICN1-transduced progenitors. Therefore, activation of the Notch pathway impairs myeloid/DC

### Table I. Oligonucleotide primers and amplification conditions used for RT-PCR gene expression analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>5’ Primer</th>
<th>3’ Primer</th>
<th>PCR Cycles (no.)</th>
<th>Size (bp)</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>GACAAGGCGCTGGGAGGGAGCTT</td>
<td>CTTGCCCTCCCTCTCTCTCCCA</td>
<td>20</td>
<td>356</td>
<td>55</td>
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<tr>
<td>CEBPa</td>
<td>GGCGGCTGCGCTGGGAGGGAGCTT</td>
<td>CTTGCCCTCCCTCTCTCTCCCA</td>
<td>20</td>
<td>356</td>
<td>55</td>
</tr>
<tr>
<td>Deltex1</td>
<td>GACGCTGCGCTGGGAGGGAGCTT</td>
<td>CTTGCCCTCCCTCTCTCTCCCA</td>
<td>20</td>
<td>356</td>
<td>55</td>
</tr>
<tr>
<td>Ets1</td>
<td>GACGCTGCGCTGGGAGGGAGCTT</td>
<td>CTTGCCCTCCCTCTCTCTCCCA</td>
<td>20</td>
<td>356</td>
<td>55</td>
</tr>
<tr>
<td>GABPa</td>
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<td>CTTGCCCTCCCTCTCTCTCCCA</td>
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<td>356</td>
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<tr>
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<td>CTTGCCCTCCCTCTCTCTCCCA</td>
<td>20</td>
<td>356</td>
<td>55</td>
</tr>
<tr>
<td>GATA3</td>
<td>GACGCTGCGCTGGGAGGGAGCTT</td>
<td>CTTGCCCTCCCTCTCTCTCCCA</td>
<td>20</td>
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<td>55</td>
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<tr>
<td>HEB</td>
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<td>CTTGCCCTCCCTCTCTCTCCCA</td>
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<td>Hes1</td>
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<td>CTTGCCCTCCCTCTCTCTCCCA</td>
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<td>PU.1</td>
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<td>20</td>
<td>356</td>
<td>55</td>
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<tr>
<td>RAG1</td>
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<td>CTTGCCCTCCCTCTCTCTCCCA</td>
<td>20</td>
<td>356</td>
<td>55</td>
</tr>
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</table>

### Table II. Myeloid differentiation potential of early human thymic precursors

<table>
<thead>
<tr>
<th>Progenitor No.</th>
<th>CFU-GM^a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
</tr>
<tr>
<td>10^5</td>
<td>12</td>
</tr>
<tr>
<td>3 × 10^5</td>
<td>24</td>
</tr>
<tr>
<td>10^6</td>
<td>85</td>
</tr>
<tr>
<td>3 × 10^6</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

^a The indicated numbers of human CD34^+ CD33^+ CD1a^- thymic progenitors were plated in methylcellulose cultures supplemented with SCF, GM-CSF, and IL-3. At day 14, the number of GM colonies was determined by counting. Data from three independent experiments are shown.
Sustained Notch1 activation impairs NK cell differentiation at sequential intrathymic precursor stages

To assess the impact of Notch1 signaling on NK cell fate, ICN1-transduced TLMPs were next analyzed for their differentiation potential in myeloid/DC-supportive cultures supplemented with IL-2 (or IL-15), which are permissive for multilineage (myeloid/DC/NK) differentiation (15). GFP-transduced TLMP controls gave rise within 8 days to an early wave of CD13⁺CD7⁻CD56⁻NK cells, including DCs (15, 19), followed by a second wave of CD13⁺CD7⁻CD56⁺NK cells, which became the predominant population by days 15–18 of culture (Fig. 3A). In contrast, sustained Notch1 signaling abrogated myeloid/DC differentiation, and also resulted in a marked reduction of frequencies and numbers of NK cells (8.4 ± 0.8- and 8.7 ± 1.9-fold, respectively, in six independent experiments) (Fig. 3, A and B). Notably, ICN1⁺ thymocytes blocked to myeloid/DC/NK non-T cell fates proliferated in vitro with kinetics parallel to those of GFP⁺ controls (Fig. 3A). Inhibition of NK cell differentiation induced by Notch1 was not specific of thymic precursors at the TLMP stage. Rather, Notch1 signaling also resulted in a marked reduction of frequencies and numbers (25.6 ± 4.2- and 23.4 ± 4.3-fold, respectively) of NK cells derived from downstream CD34⁺CD44⁺CD33⁻T/NK bi-potential progenitors at the pre-T cell stage, while supporting their expansion in vitro (Fig. 3, C and D). Therefore, Notch1 signaling impairs early thymocyte progenitors at successive developmental stages from adopting an NK cell fate, but supports their proliferation in vitro.

Sustained Notch1 signaling is capable of inhibiting non-T cell differentiation, but is not sufficient for inducing progression along the T cell lineage

Flow cytometry analyses were then performed to phenotypically characterize ICN1⁺ thymocytes proliferating under multilineage-differentiation conditions. Results summarized in Table III showed that ICN1⁺ thymocytes displayed a CD2⁺CD7⁻CD45RA⁻lymphoid-associated phenotype, kept expression of c-kit and flt3, but up-regulated surface IL-7Rα and cytoplasmic CD3ε expression levels, as compared with TLMPs. Also, they turned off CD33 and CD34 molecules expressed on primary TLMPs. Notably, no lineage markers (including erythroid, myeloid, NK, B, and T cells) were expressed throughout culture on ICN1⁺Lin⁻ thymocytes. Neither did they express the typical CD1a T-lineage marker in-
up-regulated in vivo at the TLMP to pro-T cell transition (20), was turned on in ICN1 \(\text{Lin}^-\) thymocytes, whereas the myeloid-related transcription factor \(C/EBP\alpha\), which is down-regulated in pro-T cells, was decreased in ICN1 \(\text{Lin}^-\) thymocytes as well. Cells overexpressing active Notch1 also showed a marked reduction of \(GATA2\), which is repressed in vivo in pro-T and pre-T cells, while \(PU.1\) expression remained unaffected. Notably, transcription factors that influence T cell commitment and early T cell differentiation such as \(HEB\), \(GABP\alpha\), and \(GATA3\), as well as the T-lineage gene \(pT\alpha\), were expressed in vivo in primary TLMPs and were maintained in vitro in ICN1-transduced precursors, while none of these genes but \(GATA3\) was expressed in NK cells derived from TLMP GFP\(^+\) controls (Fig. 4A). Finally, Notch downstream targets \(Hes1\) and \(Deltex1\) were expressed in TLMPs (Fig. 4A), suggesting that Notch signaling is active in vivo in some progenitors included within this early precursor subset. Taken together, our phenotypic and genetic studies suggest that ICN1 \(\text{Lin}^-\) proliferating thymocytes are blocked at the pro-T cell stage.

Notch signaling is required for proper TCR \(\alpha/\beta\) gene rearrangement (33), which marks irreversible commitment to the \(\alpha/\beta\) T cell lineage. Analyses on the rearrangement status of the TCR \(\beta\) locus revealed that ICN1 \(\text{Lin}^-\) undifferentiated thymocytes displayed a TCR\(\beta\) germline configuration, as they lacked not only V(D)J\(\beta\), but also DJ\(\beta\) rearrangements (Fig. 4B). Neither did they express TCR\(\gamma\) or TCR\(\delta\) gene products (not shown) indicative of T-lineage commitment. Therefore, we concluded that sustained Notch1

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**FIGURE 3.** Sustained Notch1 signaling prevents NK cell differentiation from human thymocyte progenitors at successive developmental stages. Generation of NK cells from TLMPs (A and B) or pro-T cells (C and D) overexpressing either activated Notch1 (ICN1) or GFP (GFP), under multilineage-supportive conditions. A, Absolute numbers of total cells and percentages and absolute numbers of NK cells (CD56\(^-\)CD7\(^+\)) derived from ICN1- (■) or GFP-transduced (▲) TLMPs or (○) pro-T cells, calculated on electronically gated GFP\(^+\) cells at the indicated days of culture. B, Representative profiles of CD56 and CD7 expression on GFP\(^+\)-gated progeny of ICN1-transduced or GFP-transduced TLMPs cultured for 23 days or (○) pro-T cells cultured for 19 days. Numbers represent percentages in each quadrant.

**FIGURE 4.** Gene expression, TCR\(\beta\) gene rearrangement status, and pro-T cell potential of human thymic precursors and their ICN1- or GFP-transduced progenies. A, RT-PCR was performed on normalized samples of cDNA (see \(\beta\)-actin) either from the TLMP, pro-T, and pro-T-sorted CD34\(^+\) thymocyte subsets described in Fig. 1, or from progenies of ICN1-transduced (ICN1\(\text{Lin}^-\)) and GFP-transduced (GFP\(\text{CD56}^-\)) TLMPs cultured for 15 days under multilineage-supportive cytokines. Two-fold serial dilutions of each cDNA were used. B, Southern blot analysis of DJ\(\beta\)2-JJ\(\beta\)2 rearrangements in GFP-sorted ICN1\(\text{Lin}^-\) or CD56\(^+\) NK cell progenies derived from ICN1- or GFP-transduced TLMPs, respectively, after 15 days in multilineage-supportive cultures. Total thymocytes and K562 myeloid cells served as positive and negative controls, respectively. C, Flow cytometry analysis of CD4 vs CD8 expression on electronically gated GFP\(^+\) progenies derived by day 28 in FTOC either from sorted ICN1\(\text{Lin}^-\) thymocytes expanded in vitro under multilineage-supportive conditions, or from ICN1- (ICN1) or GFP-transduced (GFP) freshly isolated TLMPs.
signaling is necessary and sufficient for inhibiting differentiation of thymocyte precursors into non-T (myeloid, DC, and NK) cell fates, but is not sufficient for inducing TCR gene rearrangement and progression along the T cell lineage. However, ICN1+/Lin- undifferentiated thymocytes could develop into conventional DP thymocytes in a hu/mo FTOC system, although less efficiently than freshly isolated TLMPs (Fig. 4C), thus providing functional evidence that they display T cell differentiation potential.

Constitutive Notch1 activation is permissive for expansion of thymocyte precursors, but does not substitute for survival and proliferation signals provided by IL-7

We found that maintenance of IL-7Rα surface expression was a key feature of ICN1+ thymocytes proliferating in vitro (Table III and Fig. 5A). In contrast, control TLMPs differentiating into NK cells and myeloid cells lost IL-7Rα and simultaneously up-regulated either IL-2Rβ, a hallmark of NK cell commitment (34), or GM-CSFRα, a myeloid-lineage marker (22), respectively, but these cytokine receptors were never coexpressed in ICN1+ thymocytes (Fig. 5A and Table III). To address whether maintenance and up-regulation of IL-7Rα expression is a by-product of progression toward the T cell lineage or is directly induced by Notch, we assessed the impact of Notch1 signaling on IL-7Rα expression at the clonal level in the T cell line Jurkat. As shown in Fig. 5B, surface expression of IL-7Rα was markedly up-regulated (13-fold) in ICN1-transduced Jurkat cells, and IL-7Rα up-regulation (5-fold increase) was observed as well in IL-7Rα-transduced Jurkat cells, included as a control. Collectively, these results support the possibility that activation of the Notch pathway is involved in the regulation of IL-7Rα surface expression in developing human thymocytes.

IL-7 is known to serve a nonredundant survival and proliferative function in early T cell development (26, 27). Therefore, we investigated whether the IL-7/IL-7R pathway is functionally involved in proliferation of ICN1+ undifferentiated thymocytes. As shown in Fig. 5C, ICN1+Lin- thymocytes proliferated exponentially with a 1.5-day doubling time in multicytokine progenitors up to day 20, and cellular recovery declined thereafter. However, ICN1+Lin- cell numbers were markedly reduced in cultures deprived of IL-7 by day 9. In fact, ICN1+Lin- thymocytes kept viable for 5–6 days upon IL-7-withdrawal, but cell viability dropped severely afterward. Therefore, survival and proliferation of ICN1+Lin- precursor thymocytes was supported by nonredundant IL-7 signals. In contrast, proliferation of NK cells derived from GFP-transduced TLMPs was IL-2 dependent, but independent of IL-7 (Fig. 5C). These results provide evidence that Notch1 signaling does not substitute for survival and proliferation signals provided by IL-7R.

To investigate the impact of IL-7R signaling on the developmental fate of human TLMPs, we next analyzed the differentiation potential of TLMPs retrovirally transduced with IL-7Rα (Fig. 6A). In contrast to ICN1+TLMPs, IL-7Rα-transduced TLMPs differentiated toward myeloid DCs and NK cells with kinetics and efficiencies similar to GFP-transduced controls (Fig. 6B). Developing non-T cells up-regulated the expected cytokine receptor (GM-CSFRα and IL-2Rβ, for myeloid and NK cells, respectively) and coexpressed IL-7Rα (Fig. 6C), but proliferated independently of IL-7 (Fig. 6D). In contrast, ectopic IL-7Rα expression was functional in T-lineage cells, as it improved cellular recoveries of both αβ and γδ T cells in FTOC supplemented or not with exogenous IL-7 (data not shown). Taken together, our results indicate that the IL-7R pathway does not influence the lineage fate of TLMPs, but provides unique survival and proliferation signals to early thymocyte precursors that have lost the potential to generate non-T cells in response to Notch1 signaling.

Notch and OP9 stroma-derived signals are concurrently required for inducing progression along the T cell lineage of proliferating thymic progenitors blocked to non-T cell fates

To next approach the impact of endogenous Notch signaling in early human thymocyte development, we used the reported
CD44 and down-regulated CD34 expression (CD34<sup>int</sup>) days. TLMPs retrovirally transduced with the human IL-7Rα, which have lost surface IL-7Rα, progressed to DP thymocytes (see below; Fig. 8). In this regard, it has been shown that IL-7 concentrations are critical for efficient T cell development of murine postnatal thymocytes developing throughout successive T cell maturation stages, but is also permissive for the maintenance of T cell precursors.

Additional experiments performed under conventional cytokine conditions for OP9-DL1 cocultures (i.e., IL-7 plus flt-3L) revealed that progression along the T cell lineage proceeded with efficiencies equivalent to those observed in multicytokine-supplemented OP9-DL1 cocultures (Fig. 8C and data not shown). However, we consistently found that T cell development was more efficient both in absolute and relative terms in the FTOC system (Fig. 4C). In this regard, it has been shown that IL-7 concentrations are critical for efficient T cell development of murine postnatal precursors, which is greatly improved in the OP9-DL1 coculture by lowering IL-7 amounts (35, 36). In contrast, we found that lowering IL-7 in our conventional OP9-DL1 cocultures from 100 to 20 or 5 IU/ml did not result in enhanced T cell production. Neither did we observe improved T cell recoveries when OP9-DL1 cocultures were treated with lower IL-7 amounts (35, 36). In contrast, we found that pro-T and pre-T cells coexisted with constant numbers of CD34<sup>high</sup>CD44<sup>high</sup> undifferentiated TLMPs in OP9-DL1 cultures during the first week of culture, whereas neither TLMPs, nor T-lineage cells survived in the OP9 coculture (Fig. 8B). Therefore, Notch signaling not only supports the proliferative expansion of human thymocytes developing throughout successive T cell maturation stages, but is also permissive for the maintenance of T cell precursors.

with up-regulated IL-7Rα were efficiently generated (7.4 ± 0.6-fold increased numbers) (Fig. 8A). Strikingly, the OP9-DL1 coculture supported the generation of undifferentiated pro-T cells for about 1 wk (14.4 ± 1.4-fold from days 3–7). Thereafter, pro-T cells differentiated into CD1a<sup>+</sup> pre-T cells, which were also 11.6 ± 0.8-fold expanded during the next 1-wk culture period, and then gave rise to DP thymocytes (Fig. 8B). More importantly, we found that pro-T and pre-T cells coexisted with constant numbers of CD34<sup>high</sup>CD44<sup>high</sup> undifferentiated TLMPs in OP9-DL1 cultures during the first week of culture, whereas neither TLMPs, nor T-lineage cells survived in the OP9 coculture (Fig. 8B). Therefore, Notch signaling not only supports the proliferative expansion of human thymocytes developing throughout successive T cell maturation stages, but is also permissive for the maintenance of T cell precursors.
Collectively, our results indicate that both constitutive and ligand-induced Notch signaling suppress the generation and/or expansion of non-T lineage precursors from TLMPs, and thus impair the development of myeloid and NK cell lineages, while simultaneously supporting the maintenance and expansion of progenitors with T cell potential. However, progression of proliferating progenitors along the T cell pathway is only supported by ligand-induced Notch signaling in the OP9-DL1 coculture. To address whether physiological Notch/DL1-induced proliferation of T cell precursors. However, Notch signaling is not sufficient to promote terminal T cell differentiation of primitive intrathymic progenitors, while maintaining the proliferation of T cell precursors. However, Notch signaling is not sufficient to promote terminal T cell differentiation, but requires additional stromal-derived signals. Notably, Notch1-induced proliferation was shown dependent on IL-7R signaling, but ectopic IL-7R expression could not replace Notch1 for the maintenance and expansion of progenitors blocked to non-T cell fates. Thus, we conclude that the concerted activation of both Notch1 and IL-7R pathways may play during this particular window that covers the initial steps of T cell development. By combining ligand-independent and DL1-like1-induced endogenous Notch activation, we show that the most prominent function of Notch1 signaling in very early T cell development is to block non-T cell differentiation of primitive intrathymic progenitors, while maintaining the proliferation of T cell precursors. However, Notch signaling is not sufficient to promote terminal T cell differentiation, but requires additional stromal-derived signals. Notably, Notch1-induced proliferation was shown dependent on IL-7R signaling, but ectopic IL-7R expression could not replace Notch1 for the maintenance and expansion of progenitors blocked to non-T cell fates. Thus, we conclude that the concerted activation of both Notch1 and IL-7R pathways may serve an essential function of amplification of the intrathymic pool of T cell progenitors, before inducing their terminal T cell differentiation in response to instructive signals provided by the thymic microenvironment.

Previous studies have shown that the human thymus contains primitive progenitors with combined lymphoid and myeloid potential (14, 18, 19), suggesting that, as recently shown in mice (9, 

**Discussion**

Hematopoietic precursors commit and differentiate to the T cell lineage in response to instructive Notch1 signals delivered intrathymically (5, 6, 9, 10). An intriguing aspect of T cell development is that commitment occurs relatively late after thymus entry, in progenitors that have already spent about 2 wk within the thymus and have undergone a 10^3-fold expansion, mostly driven by IL-7R signals (22, 24). Here, we have approached the role that Notch1 signaling may play during this particular window that covers the initial steps of T cell development. By combining ligand-independent and DL1-like1-induced endogenous Notch activation, we show that the most prominent function of Notch1 signaling in very early T cell development is to block non-T cell differentiation of primitive intrathymic progenitors, while maintaining the proliferation of T cell precursors. However, Notch signaling is not sufficient to promote terminal T cell differentiation, but requires additional stromal-derived signals. Notably, Notch1-induced proliferation was shown dependent on IL-7R signaling, but ectopic IL-7R expression could not replace Notch1 for the maintenance and expansion of progenitors blocked to non-T cell fates. Thus, we conclude that the concerted activation of both Notch1 and IL-7R pathways may serve an essential function of amplification of the intrathymic pool of T cell progenitors, before inducing their terminal T cell differentiation in response to instructive signals provided by the thymic microenvironment.

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**FIGURE 8.** Notch and OP9 stroma-derived signals support the survival and expansion of early thymic progenitors and are concurrently required for inducing proliferative thymic progenitors to progress along the T cell lineage. A, Correlated expression of CD34 vs CD44 on ex vivo-isolated TLMPs (left dot plot) and on the progenies derived from TLMPs by day 7 in OP9 (middle dot plot) and OP9-DL1 cocultures (right dot plot) supplemented with multicytokines. Gate a, TLMPs (CD34^high^CD44^high^); gate b, pro-T cells (CD34^int^CD44^low^); gate c, myeloid progenitors (CD34^int^CD44^high^). Histograms of IL-7R expression in progenies derived in OP9 and OP9-DL1 cocultures are shown on the right. Background was obtained with isotype-matched irrelevant controls (shaded histograms). B, Absolute numbers of total cells, TLMPs, myeloid progenitors, pro-T and pre-T (CD7^+^CD13^+^) cells derived from TLMPs in multicytokine-supplemented OP9 and OP9-DL1 cocultures at the indicated times. Cell numbers are referred to 10^5 input progenitors. The dot plot histogram on the right shows the expression of CD4 vs CD8 on cells recovered from OP9-DL1 cocultures by day 23. C, Absolute numbers of total cells derived from TLMPs in OP9-DL1 cocultures supplemented with the indicated amounts of rhIL-7 plus 50 IU/ml rhflt3-L either for the whole culture period (closed symbols), or for 12 days and then changed to lower rhIL-7 amounts (open symbols). Cell numbers are referred to 10^5 input progenitors. D, Absolute numbers of pre-T cells generated from ICN1-transduced (ICN1) or GFP-transduced (GFP) TLMPs in OP9-control stroma supplemented with 100 IU/ml rhIL-7 plus 50 IU/ml rhflt3-L. Pre-T cell yields were calculated per 10^5 input progenitors. The correlated expression of CD1a and IL-7Rα in cell progenies recovered from OP9 cocultures by day 10 is shown in dot plots on the right.
10, 11, 36), recent thymus immigrants in humans include multipotent progenitors distinct from HSCs. TLMPs, with T, NK, DC, and GM potential, are identified here within a subset of Lin—CD34+ thymocytes that express c-kit, flt3, and IL-7 receptors. Although formal proof that such TLMPs include B cell progenitors is still lacking, this possibility appears very likely in light of a recent report showing that CD34+CD1a- human immature thymocytes do in fact include progenitors with B lymphoid potential (13). Immature thymocytes expressing c-kit and flt3 have also been identified within the early T cell precursor population in mice (9). Moreover, c-kit+flt3+IL-7Rα+ cells have recently been characterized in murine BM as a novel subset of multipotent lymphoid-primed BM stem cells devoid of megakaryocyte/erythroidcyte potential (31). These are lymphoid-primed IL-7R+ progenitors with short-term reconstitution potential, which seem to define the first lineage commitment/restriction step of a novel route for adult HSC development (31, 37). It is thus possible that such short-term HSCs are the immediate precursors of multipotent TLMPs. Although formal proof that multilineage hemopoietic potential can be attributed to a single TLMP progenitor cell still deserves appropriate clonal approaches, the stem cell-like nature of TLMPs is also supported by their gene expression profiles, because they still lackmultilineage gene expression, a feature that precedes commitment in the hemopoietic system (2, 38). Alternatively, the observed expression of T-lineage-associated genes (i.e., GATA-3, HEB, and pTec) in TLMPs would suggest that the T cell program was already initiated at this early stage, a possibility that is further supported by the finding of Notch target gene expression in TLMPs. High levels of Hes1 transcription are also found in murine early T cell precursors, and remain essentially constant up to DN3 stage; however, Deltex1 expression is up-regulated at the next developmental stage in mice (9, 10). Supporting the immature feature of human TLMPs, recent data by Rothenberg and coworkers (39) suggest that induction of Hes1 transcription and T-lineage gene expression can be temporally uncoupled from T-lineage specification in mice. Our finding that Notch1-induced T cell development of human TLMPs occurs at the expense of alternative non-T lineage choices, including myeloid, NK cell, and DC lineages concurs with previous results in mice (10, 16) and extends recent data in humans using Notch inactivation approaches (40). In addition, the use of the OP9-DL1 culture has provided a new opportunity to show that impaired differentiation of non-T cell lineages involves active suppression by Notch. In fact, DL1-mediated Notch signaling reduced markedly the generation of intermediate myeloid progenitors from TLMPs, but if generated, endogenous Notch activation was also capable of blocking their differentiation into macrophages and DCs. A similar suppression mechanism seems to act on downstream T/NK pro-T cells to inhibit NK cell differentiation. It is thus possible that Notch1 signaling also acts at or immediately upstream of the TLMP stage to block intrathymic B cell differentiation (13), as suggested in mice (9, 10). In this scenario, gradual loss of non-T differentiation options induced by Notch could be explained in quantitative terms, as recently proposed (40, 41). Thus, increasing thresholds of Notch signaling may be required within the thymic microenvironment to sequentially suppress B, myeloid/DC, and NK cell lineage choices. Our results based on constitutive Notch1 signaling in TLMPs indicate that Notch-dependent suppression of non-T cell differentiation was not linked to progression along the T cell program beyond the pro-T cell stage, although as shown for human HSCs (42), nonmanipulated TLMPs undergo T-lineage differentiation in the OP9-DL1 culture. Although it could be argued that these discrepant outcomes rely on the strong gain of function transduction approach used, we found that Lin−ICN1+ cells proliferating under multilineage-supportive conditions were capable of progressing along the T cell pathway in a FTOC, and ICN1-transduced TLMPs also developed into T cells in OP9-control cocultures, or in FTOC. It is thus possible that Notch1 signaling is sufficient for initiating T-lineage specification (i.e., down-regulation of myeloid- and progenitor-associated genes, and RAG1 up-regulation), early after thymus seeding, but additional inductive signals provided by specific thymic microenvironments, and mimicked by the OP9 stroma, are required to promote irreversible T-lineage commitment and terminal T cell differentiation. Functional proof that ICN1−Lin− progenitor cells represent T-committed progenitors devoid of non-T cell lineage potential must still await the development of an inducible ICN expression system. An important aspect of our studies is the observation that inhibition of non-T cell differentiation by constitutive Notch signaling paralleled a powerful expansion of ICN1− progenitors blocked to non-T cell fates. In addition, endogenous Notch signaling provided by the OP9-DL1 stroma supported the maintenance of constant numbers of Lin−CD34+ primitive precursors for the first week of culture, although a marked proliferation of progenitors developing throughout the earliest T cell developmental stages (i.e., pro-T and pre-T cells) was concurrently induced. These results point to a critical survival/proliferative role of Notch in early T cell development, and suggest that the regulatory function that Notch signaling plays in self-renewal of HSCs and lymphoid and myeloid precursors (43–46) can now be extended to the intrathymic pool of T cell precursors. It is thus possible that Notch signaling leads to a short-term maintenance of TLMPs blocked for non-T cell fates, while also supporting the expansion of downstream pro-T and pre-T cells, likely through asymmetric divisions. This conclusion is further supported by previous in vivo and in vitro studies showing that inhibition of Notch signaling blocks proliferation of DN mouse thymocytes and human thymic progenitors (40, 47). As occurs in vivo, survival and expansion induced by Notch in vitro was dependent on IL-7R signaling. In contrast, suppression of non-T cell differentiation was not replaced by ectopic IL-7R expression, but required an intact Notch signal. Although these results do not imply a direct association between Notch and IL-7R, we found that TLMPs and also Jurkat T cells up-regulate surface IL-7Rα in response to Notch1 signaling. Moreover, higher IL-7Rα levels can be found in DP ICN1+ thymocytes than in their GFP+ counterparts (data not shown). These data, together with the identification of a putative CBF1/Su(H)/LAG1 binding site in the promoter of the human IL-7Rα gene (data not shown) suggest that IL-7Rα may be a direct target of Notch in T-lineage cells. Because IL-7R signals are critical in early thymopoiesis, but dispensable following T cell commitment, and must be terminated by the DN3 stage (26), it is possible that Notch1 signaling regulates the dynamic regulation of IL-7R expression throughout in vivo thymopoiesis (25). In summary, we propose that repression of non-T cell differentiation together with delayed T cell differentiation provides a mechanistic basis for how modulation of Notch1 signaling controls IL-7R-induced expansion of the precursor pool of thymocytes throughout development of the T cell lineage.

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