NOTCH1 AND IL-7 RECEPTOR INTERPLAY MAINTAINS PROLIFERATION OF HUMAN THYMIC PROGENITORS WHILE SUPPRESSING NON-T CELL FATES

Running title: Notch1 and IL-7R in expansion of intrathymic progenitors

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Summary

Notch signaling is critical for T-cell development of multipotent hematopoietic progenitors. Yet, how Notch regulates T-cell fate specification during early thymopoiesis remains unclear. Here, we have identified an early subset of $CD34^{hi}$ c-kit^{+} $flt3^{+}$ IL-7Rα^{+} cells in the human postnatal thymus, which includes primitive progenitors with combined lympho-myeloid potential. To assess the impact of Notch signaling in early T-cell development, we expressed constitutively active Notch1 in such thymic lympho-myeloid precursors (TLMPs), or triggered their endogenous Notch pathway in the OP9-Delta-like1 stroma coculture. Our results show that proliferation versus 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 natural killer 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 are concurrently required. Moreover, while ectopic IL-7R expression cannot replace Notch for the maintenance and expansion of undifferentiated thymocytes, Notch signals sustain IL-7R expression in proliferating thymocytes and induce IL-7R upregulation in a T-cell line. Thus, IL-7R and Notch pathways cooperate to synchronize cell proliferation and suppression of non-T lineage choices in primitive intrathymic progenitors, which will be allowed to progress along the T-cell pathway only upon interaction with an inductive stromal microenvironment. These data provide insight into a mechanism of Notch-regulated amplification of the intrathymic pool of early human T-cell progenitors.

Introduction

All mature blood cell types in the adult derive ultimately from a bone marrow HSC with self-renewal capacity, which restricts progressively its developmental potential through a series of intermediate progenitor stages. Transcription factors and cytokines are key regulators of lineage specification of multipotent HSC (1-3). Among transcriptional regulators, Notch1, a member of the evolutionarily conserved family of Notch transmembrane receptors (4), has recently emerged as a key instructive regulator of T-cell commitment and differentiation within the thymus (5, 6). Conditional ablation of Notch1 leads to a loss of T cell production and to the development of B cells in the thymus (7). Conversely, over-expression of a constitutively active form of Notch1 inhibits B lymphopoiesis and results in the appearance of developing T cells in the BM (8). 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, 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 $CD34^{hi}$ $CD33^{+}$ 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 $CD34^{hi}$ $CD33^{+}$ 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 $CD34^+$ $CD33^-$ $CD1a^-$ progenitors (termed pro-T cells), which are conventional T/NK bipotential intermediates, and NK cell potential is lost afterward in more mature $CD34^+$ $CD33^-$ $CD1a^+$ (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 (ISP) 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 occur 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 hematopoietic development. Lineage-specific action of cytokines relies mostly on their survival and proliferation effects on specific intermediate progenitors, and seems to be regulated at the level of receptor expression. In fact, downregulation of cytokine receptors that drive myeloid development, such as the GM-CSFR, and upregulation 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 thymopoiesis by supporting the survival and proliferation of DN precursors (23 and reviewed in 24). However, whereas thymocyte expansion prior to 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-7R 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 and DC 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-7R 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 $CD34^{hi}$ $CD33^{+}$ c-kit^{+} $flt3^{+}$ IL-$7R\alpha^{+}$ 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-7R pathways cooperate to serve an essential aspect of T-cell development; *i.e.* amplification of the pool of intrathymic progenitors prior to 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 Biotech). TLMPs ($CD34^{hi}$ $CD33^{+}$ $CD1a^{-}$), pro-T ($CD34^{+}$ $CD33^{-}$ $CD1a^{-}$), and pre-T cells ($CD34^{+}$ $CD33^{-}$ $CD1a^{+}$) were AutoMACS sorted from the Lin^{-} fraction or sorted in a FACSVantage SE sorter (BD Biosciences) as described (19). $ICN1^{+}$ Lin^{-} and GFP^{+} NK cells generated from TLMPs under multilineage-supportive cytokines (see below), 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, CD56-PE, and TCRγδ-TC from Caltag; CD1a-APC, CD3ε-PE or -APC, CD14-FITC or -PE, CD19-PE, CD34-FTIC or -PE, CD44-FITC, c-kit-APC, flt3-PE, and CD161-PE from BD Biosciences; and CD1a-RD1, CD4-PE-Cy5, CD13-PE-Cy5, CD33-PE-Cy5, CD34-PE-Cy5, CD56-PE-Cy5, GM-CSFRα-PE, IL-2Rβ-PE, IL-7Rα-PE, and TCRαβ-PE-Cy5 from Beckman Coulter. PE-, or APC-conjugated goat anti-mouse $F(ab_2)$' IgG were from Southern Biotechnology and BD Biosciences, respectively. Unlabelled mAbs used were CD2 from BD Biosciences, IL-2Rβ from Nichirei, IL-7Rα from Beckman Coulter, and glycophorin from the ATCC. Four- and three-color flow cytometry was performed using an EPICS XL (Beckman Coulter) or a FACSCalibur (BD Biosciences) cytometer, respectively. Data were collected on viable cells. Propidium iodide staining was used to exclude dead cells. Isotype-matched irrelevant antibodies (Caltag) were used to define background fluorescence.

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 (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 (BioWhittaker) cultures supplemented with 10% FCS (Invitrogen) and the following recombinant human (rh) cytokines from the National Institute of Biological Standards and Controls (NIBSC): 100 IU/ml of IL-7, 60 IU/ml IL-1α, 50 IU/ml IL-6, 100 IU/ml 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 10^3-$3x10^4$ TLMPs per ml in Methocult GF (StemCell Technologies) containing 30% FCS and supplemented with 100 ng/ml SCF, 10ng /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-like1 (OP9-DL1) was kindly provided by Dr. J.C. Zúñiga-Pflücker. OP9

cocultures were performed as originally described (30) in the presence of either multilineage-supportive cytokines (see above) or 100 IU/ml of rhIL-7 (NIBSC; specific activity: 10^8 IU/mg) plus 50 IU/ml rhFlt3-L (NIBSC; specific activity: 10^6 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 FTOC) were performed as described (29).

Semiquantitative RT-PCR and TCRβ gene rearrangements.

Total RNA isolated using Trizol (Invitrogen) was reverse transcribed into cDNA using oligo-d(T) primer and Expand Reverse Transcriptase (Roche). PCR reactions, performed using the same serially diluted cDNA samples shown for β-actin, were subjected to 1.5-2% agarose electrophoresis and visualized with ethidium bromide. The gene-specific primers, product lengths, and amplification conditions used are provided in Table 1.

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 lympho-myeloid 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^{hi}) (Fig.1A) Such CD34^{hi} CD33$^+$ subset (0.030±0.018% of total thymocytes) includes primitive progenitors with the capacity to generate NK cells, myeloid and plasmacytoid 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^{hi} CD33$^+$ thymocyte precursors was validated in conventional methylcellulose colony-forming assays (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 2). Phenotypic analysis of individual colonies confirmed the generation of CD14$^+$ macrophages, CD15$^+$ granulocytes and CD14$^-$ CD13$^+$ CD1a$^+$ myeloid DCs (data not shown). As a whole, available data showing that CD34^{hi} CD33$^+$ thymic 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 thymic lympho-myeloid precursors or 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-CSFRα could be detected on TLMPs, pro-T or pre-T cells (Table 3). Therefore, human TLMPs display a c-kit$^+$ flt3$^+$ IL-7Rα$^+$ phenotype (Fig. 1A), equivalent to that recently described for lympho-myeloid 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 Figure 2A, a marked reduction in both frequencies and numbers of DCs was found in ICN1$^+$ cultures as compared with GFP$^+$ **controls (mean ± SEM; 10.0 ± 3.9-fold and 6.2 ± 0.2-fold reduction, respectively, in 3 independent experiments).** The impaired generation of DCs correlated with **a 10.6 ± 1.6-fold decrease** of macrophages (Fig. 2B), indicating that Notch1 signaling inhibited the differentiation of TLMPs into myeloid-lineage cells. Inhibition of myeloid differentiation was not due to a decreased cell survival of ICN1-transduced progenitors. Instead, Notch signaling supported the expansion *in vitro* of thymocytes blocked to myeloid fates up to day 16-18, but then the culture declined steadily (Fig. 2A). Therefore,

activation of the Notch pathway impairs myeloid/DC differentiation of TLMPs, while supporting the expansion *in vitro* of thymocyte precursors blocked to myeloid fates.

Sustained Notch1 activation impairs NK cell differentiation at sequential intrathymic precursor stages.

To assess the impact of Notch-1 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 $CD13^+$ $CD7^-$ myeloid cells, including DCs (15, 19), followed by a second wave of $CD13^-$ $CD7^+$ $CD56^+$ NK cells, which became the predominant population by day 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 $\pm$ 0.8- and 8.7 $\pm$ 1.9-fold, respectively, in 6 independent experiments)** (Fig. 3A, 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 $\pm$ 4.2- and 23.4 $\pm$ 4.3-fold, respectively)** of NK cells derived from downstream $CD34^+$ $CD44^+$ $CD33^-$ T/NK bipotential progenitors at the pro-T cell stage, while supporting their expansion *in vitro* (Fig. 3C, 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 Notch 1 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 3 showed that $ICN1^+$ thymocytes displayed a $CD2^+$ $CD7^+$ $CD45RA^+$ lymphoid-associated phenotype, kept expression of c-kit and flt3, but upregulated 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 induced at the pre-T cell stage, or the CD4 molecule, which marks the onset of TCRβ gene rearrangements in human T-cell development (18). As a whole, the phenotype $ICN1^+$ Lin^- cells matches that of lymphoid-committed intrathymic precursors at the pro-T cell stage. Notably, the same phenotype was displayed by pro-T cells transduced with active Notch1 (data not shown).

To approach the lineage affiliation of $ICN1^+$ Lin^- proliferating thymocytes at the molecular level, we next focused on expression analysis of lineage-associated regulatory genes (32). As shown in Figure 4A, RAG-1, a hallmark of lymphoid commitment which is upregulated *in vivo* at the TLMP to pro-T cell transition (20), was turned on in $ICN1^+$ Lin^- thymocytes, whereas the myeloid-related transcription factor C/EBPα , which is downregulated in pro-T cells, was decreased in $ICN1^+$ 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, GABPα, and GATA3, as well as the T-lineage gene pTα, 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 $ICN1^+$ Lin^- proliferating thymocytes are blocked at the pro-T cell stage.

Notch signaling is required for proper TCRβ gene rearrangement (33), which marks irreversible commitment to the αβ T cell-lineage. Analyses on the rearrangement status of the TCRβ locus revealed that $ICN1^+$ Lin^- undifferentiated thymocytes displayed a TCRβ germ-line configuration, as they lacked not only V(D)Jβ, but also DJβ rearrangements (Fig. 4B). Neither did they express TCRγ or TCRδ gene products (not shown) indicative of T-lineage commitment. Therefore, we concluded that sustained Notch1 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), this 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 3 and Fig. 5A). In contrast, control TLMPs differentiating into NK cells and myeloid cells lost IL-7Rα and simultaneously upregulated 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 3). To address whether maintenance and upregulation 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 signalling on IL-7Rα expression at the clonal level in the T-cell line Jurkat. As shown in Figure 5B, surface expression of IL-7Rα was markedly upregulated (13-fold) in ICN1-transduced Jurkat cells, and IL-7Rα upregulation (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-7R 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 was functionally involved in proliferation of $ICN1^+$ undifferentiated thymocytes. As shown in Figure 5C, $ICN1^+$ Lin^- thymocytes proliferated exponentially with a 1.5-day doubling time in multicytokine cultures 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 afterwards. 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 upregulated 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 exogeneous IL-7 (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 OP9-DL1 ligand-dependent Notch activation assay

(30). In the absence of Notch ligand, OP9 stromal cells allowed TLMPs to differentiate simultaneously into myeloid DCs, monocytes and NK cells under multilineage-supportive cytokine conditions (Fig. 7). Myeloid cells reached maximal proportions by day 8 (Fig. 7A), but decreased progressively thereafter and disappeared by day 12, when NK cells became the major cell population (Fig. 7B). In contrast, TLMPs cocultured on OP9-DL1 stroma supplemented with multicytokines failed to differentiate along non-T cell pathways **(10.7 $\pm$ 1.7- and 10.7 $\pm$ 2.1-fold reduction in myeloid and NK cell production, respectively)**, but gave rise to $CD7^{+}$ $CD13^{-}$ lymphoid cells, which upregulated the CD1a T-lineage-associated marker (Fig. 7A) and progressed to DP thymocytes (see below; Fig. 8B). Kinetic analyses were then performed to assess the impact of ligand-induced Notch signaling on the generation of specific intermediate progenitors for T or non-T intrathymic differentiation pathways (19). We found that OP9 cocultures supported the generation of recognizable Lin^{-} myeloid progenitors with upregulated CD44 and downregulated CD34 expression ($CD34^{int}$ $CD44^{hi}$), which have lost surface IL-7Rα (Fig. 8A). Such myeloid precursors increased progressively in OP9 cultures up to day 7, while $CD34^{hi}$ $CD44^{hi}$ TLMPs decreased concurrently (Fig. B). In marked contrast, generation of myeloid progenitors was very inefficient in OP-9-DL1 cocultures **(3.7 $\pm$ 0.7-fold reduction in absolute numbers)**, whereas Lin^{-} $CD34^{int}$ $CD44^{lo}$ pro-T cells with upregulated IL-7Rα were efficiently generated **(7.4 $\pm$ 0.6-fold increased numbers)** (Fig.8A). Strikingly, the OP-9-DL1 coculture supported the expansion of undifferentiated pro-T cells for about one week **(14.4 $\pm$ 1.4-fold from day 3 to day 7).** Thereafter, pro-T cells differentiated into $CD1a^{+}$ pre-T cells, which were also **11.6 $\pm$ 0.8-fold** expanded during the next 1-week culture period, and then they gave rise to DP thymocytes (Fig. 8B). More importantly, we found that pro-T and pre-T cells

coexisted with constant numbers of $CD34^{hi}$ $CD44^{hi}$ 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.**

Further experiments performed under conventional cytokine conditions for OP9-DL1 cocultures (i.e. IL-7 plus Flt-3L) (30) 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 IU/ml to 20 or 5 IU/ml did not result in enhanced T-cell production. Neither, did we observe improved T-cell recoveries when OP-DL1 cocultures initiated and maintained for the first 12 days with 100 IU/ml rhIL-7 were subcultured with lower IL-7 amounts. Rather, both total cell numbers and T-cell production dropped severely in OP9-DL1 cultures following IL-7-lowering (Fig. 8C and data not shown). Survival of TLMPs and proliferation of pro-T and pre-T cells dropped as well (data not shown)

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 impairs 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/Delta signaling is the rate-limiting event for inducing T-cell differentiation in the OP9-DL1 coculture, TLMPs overexpressing constitutive active Notch1 were analyzed for their T-lineage potential upon coculture onto OP9-control stroma. As shown in Figure 8D, $ICN1^{+}$ thymocytes, but not GFP-transduced TLMPs, progressed along the T-cell pathway and gave rise to $CD1a^{+}$ IL-$7R\alpha^{+}$ pre-T cells when cocultured with OP9 cells. These results suggest that stroma-derived inductive signals are required besides Notch signaling for supporting early thymic precursors blocked to non-T cell fates to progress along the T-cell pathway

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 two wks 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 Delta-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, prior to 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 an myeloid potential (14, 18, 19), suggesting that, as recently shown in mice (9, 10, 11, 36), recent thymus immigrants in man include multipotent progenitors distinct from HSCs. Thymic lympho-myeloid progenitors, with T, NK, DC and GM potential (referred to as TLMPs), 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 ckit and flt3 have also been identified within the early T-cell precursor (ETP) population in mice (9). Moreover, $c\text{-}kit^+$ $flt3^+$ IL-7Rα^+ cells have recently been characterized in murine BM as a novel subset of multipotent lympho-myeloid BM stem cells devoid of megakaryocyte/erythrocyte (MkE) 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 hematopoietic 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, since they still lack RAG1 transcription, but display multilineage gene expression, a feature that precedes commitment in the hematopoietic system (2, 38). Alternatively, the observed expression of T-lineage-associated genes (*i.e.* GATA-3, HEB and pTα) 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 ETPs, and remain essentially constant up to DN3 stage; however, Deltex1 expression is upregulated at the next developmental stage in mice (9, 10). Supporting the immature feature of human TLMPs, recent data by Rothenberg and coworkers suggest that induction of Hes1 transcription and T-lineage gene expression can be temporally uncoupled from T-lineage specification in mice (39).

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 undergone T-lineage differentiation in the OP9-DL1 culture. **While 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*. downregulation of myeloid- and progenitor-associated genes, and RAG1 upregulation), 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^{-} $CD34^{hi}$ primitive precursors for the first wk 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. While these results do not imply a direct association between Notch and IL-7R, we found that TLMPs and also Jurkat T cells upregulate 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 CSL binding site in the promoter of the human IL-7Rα gene (not shown) suggest that IL-7Rα may be a direct target of Notch in T-lineage cells. Since 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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Footnotes

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Abbreviations. BM: bone marrow. DC: dendritic cell. DL1: delta-like1. DN: double negative. HSC: hematopoietic stem cell. ICN1: intracellular Notch1 domain. Lin: lineage. SCF: stem cell factor. TLMP: thymic lympho-myeloid progenitor. DN, double negative; DP double positive; ISP: immature single positive; MkE, megakaryocyte/ erythrocyte.

Figure legends

Figure 1. Phenotype and GM potential of primitive human CD34$^+$ intrathymic progenitors. (A) Flow cytometry analysis of CD34$^+$ thymocytes based on expression of CD1a and CD34. Electronic gates of CD34^{hi} CD1a$^-$ (a) and CD34$^+$ CD1a$^+$ (b) subsets ere performed on magnetically sorted CD34$^+$ Lin$^-$ thymocytes. Representative histograms for CD33, IL-7Rα, c-kit and flt3 expression in the TLMP (CD34^{hi} CD1a$^-$ CD33$^+$), pro-T (CD34$^+$ CD1a$^-$ CD33$^-$), and pre-T (CD34$^+$ CD1a$^+$ CD33$^-$) CD34$^+$ subsets are shown. Shaded histograms represent background fluorescence determined with isotype-matched irrelevant mAbs. MFI: mean fluorescence intensity. (B) Bright-field images of typical GM colonies generated *in vitro* from CD34^{hi} CD1a$^-$ CD33$^+$ TLMPs by day 14 in methylcellulose culture. Original magnification: X100.

Figure 2. Sustained Notch1 signaling impairs myeloid/DC differentiation of human intrathymic progenitors. Generation of DCs and monocytes/macrophages from TLMPs retrovirally transduced with constitutively active Notch1 and GFP (ICN1), or GFP alone (GFP) under myeloid/DC-supportive conditions. (A) Absolute numbers of total cells and percentages and absolute numbers of DCs (CD13$^+$ CD1a$^+$) derived from ICN1- (□) or mock-transduced (●) TLMPs calculated on electronically gated GFP$^+$ cells at the indicated culture times. (B) Representative profiles of CD13 and CD1a expression on GFP$^+$-gated progeny of ICN1-transduced or GFP-transduced TLMPs cultured for 9 days. Background fluorescence was determined with isotype-matched irrelevant mAbs.

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 mock-transduced (●) TLMPs or (C) 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 mock-transduced TLMPs cultured for 23 days or (D) pro-T cells cultured for 19 days. Numbers represent percentages in each quadrant.

Figure 4. Gene expression, TCRβ 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 β-actin) either from the TLMP, pro-T and pre-T sorted $CD34^+$ thymocyte subsets described in Figure 1, or from progenies of ICN1-transduced ($ICN1^+$ Lin^-) and GFP-transduced (GFP^+ $CD56^+$) TLMPs cultured for 15 days under multilineage-supportive cytokines. Twofold serial dilutions of each cDNA were used. (B) Southern-blot analysis of Dβ2-Jβ2 rearrangements in GFP-sorted $ICN1^+$ 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 *versus* CD8 expression on electronically-gated GFP^+ progenies derived by day 28 in FTOC either from sorted $ICN1^+$ Lin^- thymocytes expanded *in vitro* under multilineage-supportive conditions, or from ICN1- (ICN1) or GFP-transduced (GFP) freshly-isolated TLMPs.

Figure 5. Notch1 signaling is essential for maintaining IL-7R surface expression on progenies of TLMPs, but does not substitute for survival and proliferation signals provided by IL-7. (A) Flow cytometry analysis of IL-7Rα and IL-2Rβ expression in GFP^+-gated progenies of ICN1-transduced (dashed) and mock-transduced (line) TLMPs cultured under multilineage-supportive conditions for the indicated days. Background fluorescence (shaded) was determined using isotype-matched irrelevant mAbs. (B) Flow cytometry analysis of IL-7Rα expression in GFP^+-gated GFP-transduced (GFP), IL7Rα-transduced (IL-7Rα), and ICN1-transduced (ICN1) Jurkat T cells. (C) Absolute numbers of $ICN1^+$ Lin^- (upper panel) and GFP^+ NK cell progenies (lower panel) derived from ICN1-transduced and GFP-transduced TLMPs, respectively, either in multicytokine-supporting cultures (●) or in multicytokine-supporting cultures deprived of either IL-7 (▲) or IL-2 (□) from day 9.

Figure 6. Enforced IL-7Rα expression does not affect the lineage fate of TLMPs. TLMPs retrovirally transduced with the human IL-7Rα subunit and GFP, or GFP alone, were cultured under multilineage-supportive conditions. (A) IL-7Rα expression on GFP^+-gated progeny of IL-7Rα-transduced (dashed) and GFP-transduced (line) TLMPs cultured for 12 days. Background fluorescence (shaded) was determined using isotype-matched irrelevant mAbs. (B) Percentages of DC and NK cell progenies of GFP-transduced (filled bars) or IL-7Rα-transduced (open bars) TLMPs cultured for the indicated days. (C) Correlated expression of GM-CSFRα or IL-2Rβ and IL-7Rα on GFP^+-gated progenies of IL-7Rα-transduced TLMPs cultured for 8 and 12 days, respectively. (D) Absolute numbers of total cells recovered from GFP-transduced and IL-7Rα-transduced TLMPs cultured under multilineage-supportive conditions either with or without IL-7 for the indicated days.

Figure 7. Ligand-dependent endogenous Notch activation inhibits myeloid/DC and NK cell differentiation of human intrathymic progenitors.

Generation of (A) DCs and monocytes and (B) NK cells from human TLMPs cocultured on either OP9-control or OP9-DL1 stromal cells, under multilineage-supportive conditions. Flow cytometry analysis of (A) CD13 and CD1a expression and (B) CD56 and CD7 expression on progenies of TLMPs cultured for 8 days or 12 days, respectively (left histograms). Numbers represent percentages in each quadrant. Absolute numbers of DCs ($CD13^+$ $CD1a^+$), monocytes ($CD13^+$ $CD1a^-$), and NK cells ($CD56^+$ $CD7^+$) generated in the same OP9-control (filled bars) and OP9-DL1 (open bars) cultures are shown on the right. Cell numbers are referred to 10^5 input progenitors.

Figure 8. Notch and OP9 stroma-derived signals support the survival and expansion of early thymic precursors and are concurrently required for inducing proliferative thymic progenitors to progress along the T-cell lineage.

(A) Correlated expression of CD34 *versus* 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 ($CD34^{hi}CD44^{hi}$); gate b: proT cells ($CD34^{int}CD44^{lo}$); gate c: myeloid progenitors ($CD34^{int}CD44^{hi}$). Histograms of IL-7Rα 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, proT and preT ($CD7^+$ $CD13^-$ $CD1a^+$) 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 *versus* 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 100IU/ml rhIL-7 plus 50 IU/ml rhFlt3-L. PreT 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.

Table 1. Oligonucleotide primers and amplification conditions used for RT-PCR gene expression analysis

Gene	5' primer	3' primer	PCR cycles (No.)	Size (bp)	Annealing temperature (ºC)
β-actin	GCATGGAGTCCTGTGGCATCCACG	GGTGTAACGCAACTAAGTCATAG	20	356	55
CEBPα	GACACGCTGCGGGGCATCT	CTGCTCCCCTTCCTTCTCTCA	30	494	55
Deltex1	AGGGATGACCGGGATACTGC	GGCCTTTCTCGTTGTTGGGT	35	632	55
Ets1	CGATCTCAAGCCGACTCTCA	GAGGGTATAGCGGGATTCTGG	35	453	65
GABPα	TCAGCATGACCGATATAGACC	GCTGCACTGTATCCAATAAG	30	582	55
GATA2	TGTTGTGCAAATTGTCAGACG	CATAGGTGCCATGTGTCCAGC	35	278	60
GATA3	GAAGGCATCCAGACCCGAAAC	ACCCATGGCGGTGACCATGC	30	254	60
HEB	ACCACTCCATGACTCTGCAGC	AGGAGTGTGTGAGGCAGCAAC	30	442	55
Hes1	GCCAGTGTCAACACGACACCGG	TCACCTCGTTCATGCACTCG	30	310	55
pTα	CCCATCTCTCCCTGCCTTCTG	GGAGCAGGTCAAACAGCAGC	30	213	55
PU.1	TGGAAGGGTTTCCCCTCGTC	TGCTGTCCTTCATGTCGCCG	35	546	60
RAG1	CAGCGTTTTGCTGAGCTCCT	CTAGGAGAAGCCCTCAATGC	30	455	55

Table 2. Myeloid differentiation potential of early human thymic precursors

Progenitor No.	CFU-GM [a]		
	Exp. 1	Exp. 2	Exp. 3
10^3	12	4	7
$3x10^3$	24	nd	nd
10^4	85	50	67
$3x10^4$	>100	>100	nd

[a] The indicated numbers of human $CD34^+$ $CD33^{lo}$ $CD1a^-$ 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.

Table 3. Comparative phenotype of human $CD34^+$ TLMP, pro-T and pre-T thymic progenitors and the progenies derived from GFP-transduced or ICN1-transduced TLMPs cultured under multilineage-supportive conditions

	TLMP [a]	pro-T [a]	pre-T [a]	$ICN1^+$ Lin^- [b]	GFP^+ DC [c]	GFP^+ NK [c]
CD1a	– [d]	–	+	–	hi	–
CD2	+	+	+	+	+/–	+/–
cytCD3 [e]	+/–	+	+	+	–	+
CD4	–	–	–	–	+	–
CD7	+	+	+	+	lo	+
CD33	lo	–	–	–	+	+/–
CD34	hi	+	lo	–	–	–
CD45RA	+	+	+	+	lo	+
c-kit	+	lo	+/–	+	–	+
flt3	+	lo	+/–	+	–	+
GM-CSFR	–	–	–	–	+	–
IL-2Rβ	–	–	–	–	–	+
IL-7Rα	lo	+	+	+	–	–

Flow cytometry was performed on the indicated cell populations.
[a] $CD34^+$ $CD33^{lo}$ $CD1a^-$ (TLMPs); $CD34^+$ $CD33^-$ $CD1a^-$ (pro-T cells); $CD34^+$ $CD33^-$ $CD1a^+$ (pre-T cells).
[b] Lineage-negative (Lin^-; $CD3^-$, $CD4^-$, $CD13^-$, $CD19^-$, $CD56^-$, Glycophorin A^-) progeny of ICN1-transduced ($ICN1^+$) TLMPs cultured under multilineage supportive conditions during 15 days.
[c] DC and NK cell progenies of GFP-transduced (GFP^+) TLMPs cultured under multilineage supportive conditions for 8 and 15 days, respectively.
[d] –, negative; +, positive; lo, low expression; hi, high expression; +/–, heterogeneous expression.
[e] Cytoplasmic CD3ε.

Figures

Figure 1

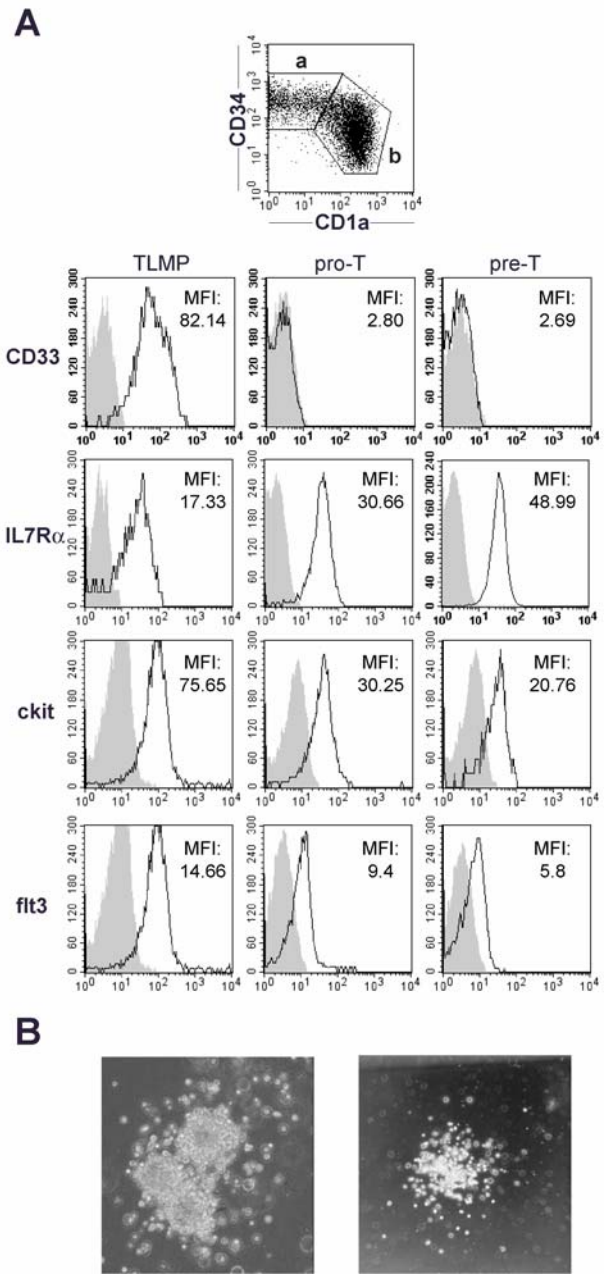


Figure 2

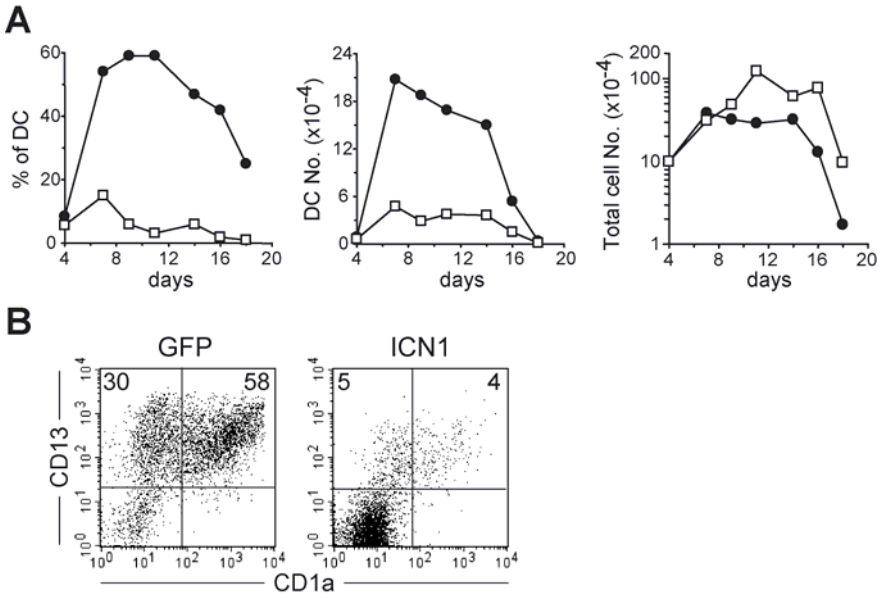

A
% of DC
DC No. (x10^-4)
Total cell No. (x10^-4)
days
B
GFP
ICN1
30
58
5
4
CD13
CD1a

Figure 3

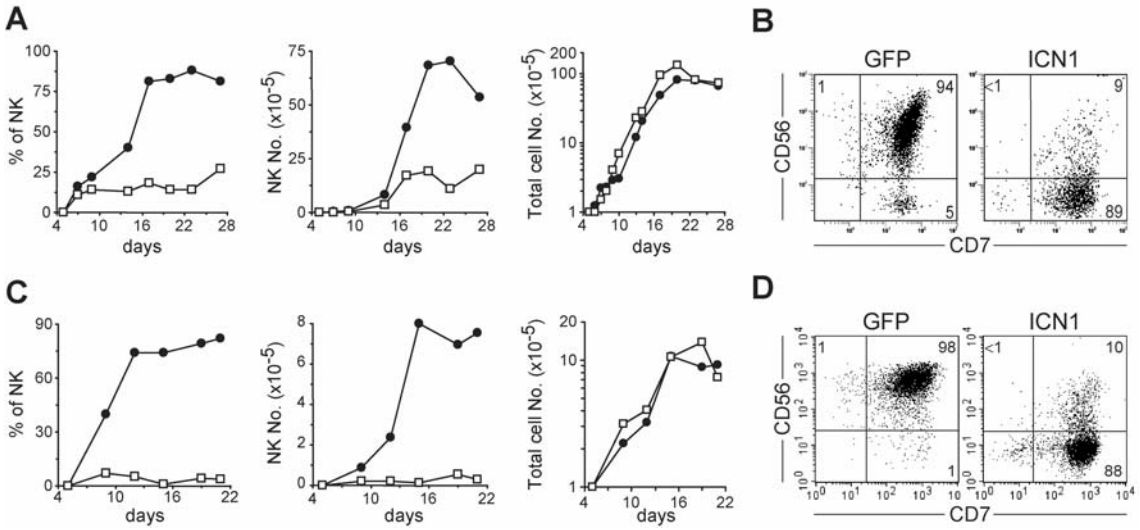


Figure 4

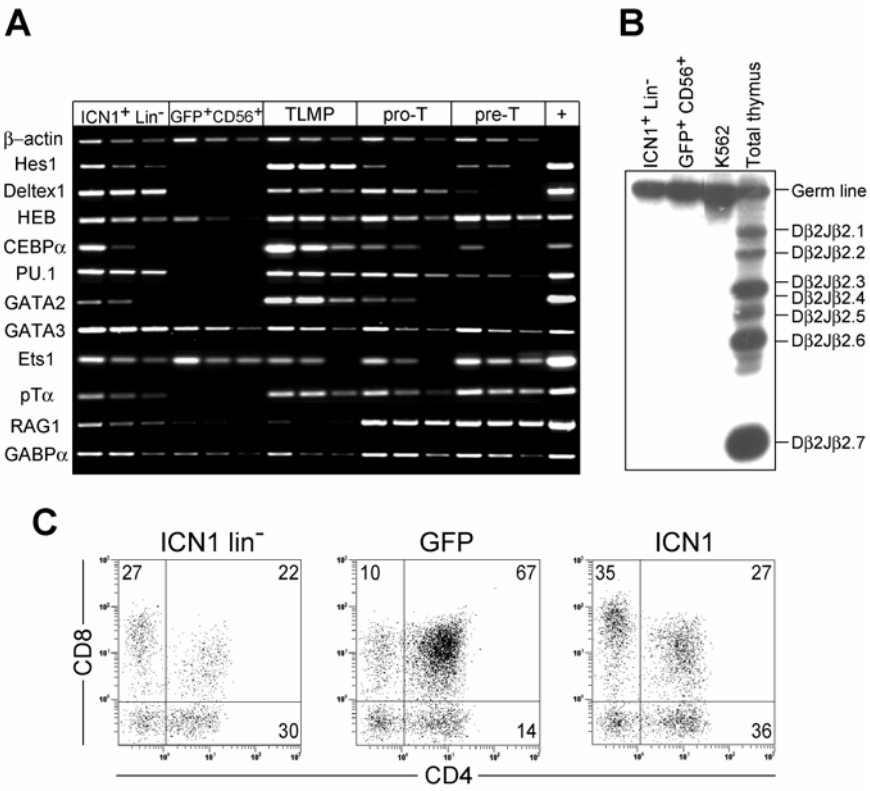

A
ICN1+ Lin-
GFP+CD56+
TLMP
pro-T
pre-T
+
β–actin
Hes1
Deltex1
HEB
CEBPα
PU.1
GATA2
GATA3
Ets1
pTα
RAG1
GABPα
B
ICN1+ Lin-
GFP+ CD56+
K562
Total thymus
Germ line
Dβ2Jβ2.1
Dβ2Jβ2.2
Dβ2Jβ2.3
Dβ2Jβ2.4
Dβ2Jβ2.5
Dβ2Jβ2.6
Dβ2Jβ2.7
C
ICN1 lin-
GFP
ICN1
CD8
CD4
27
22
30
10
67
14
35
27
36

Figure 5

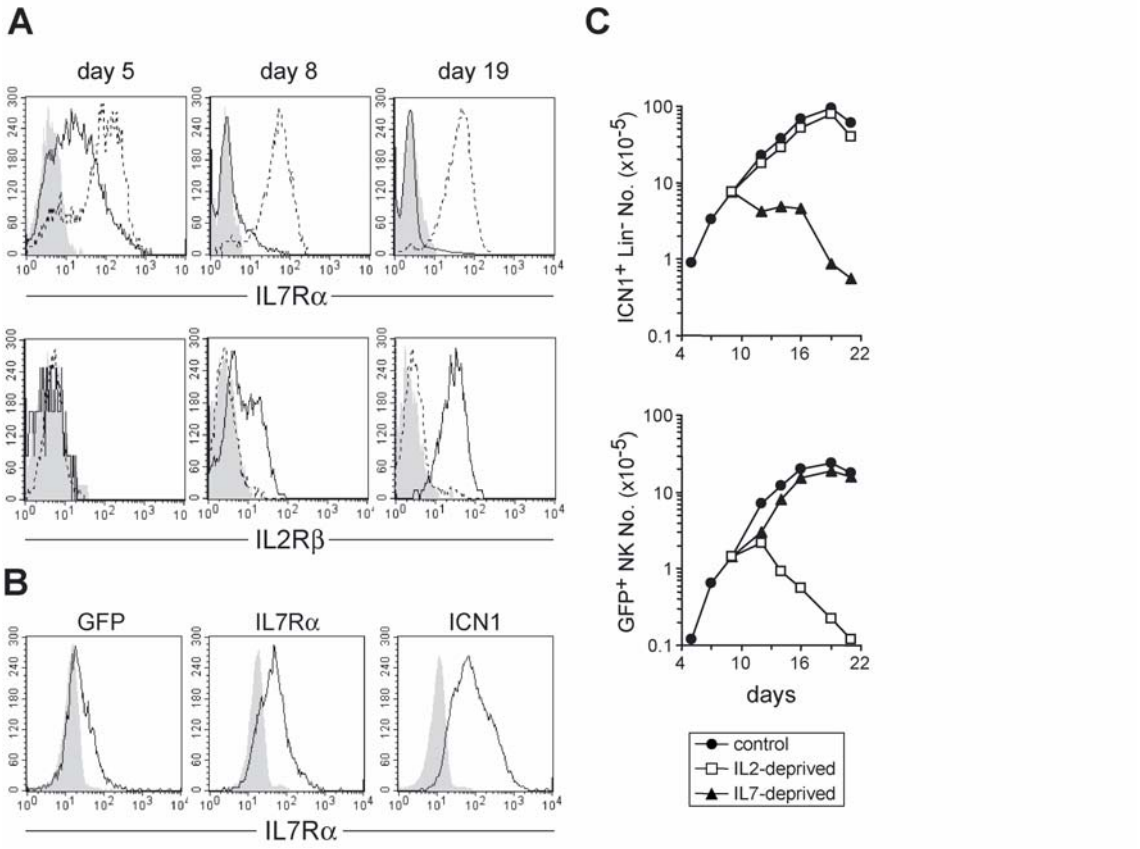

A
day 5
day 8
day 19
IL7Rα
IL2Rβ
B
GFP
IL7Rα
ICN1
IL7Rα
C
ICN1+ Lin- No. (x10-5)
GFP+ NK No. (x10-5)
days
control
IL2-deprived
IL7-deprived

Figure 6

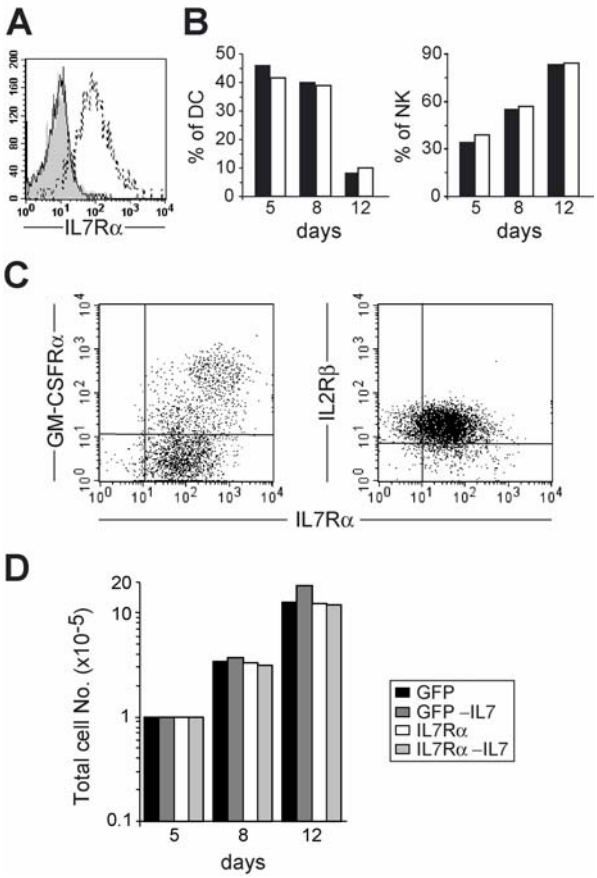

A
IL7Rα
B
% of DC
days
% of NK
days
C
GM-CSFRα
IL2Rβ
IL7Rα
D
Total cell No. (x10-5)
days
GFP
GFP –IL7
IL7Rα
IL7Rα –IL7

Figure 7

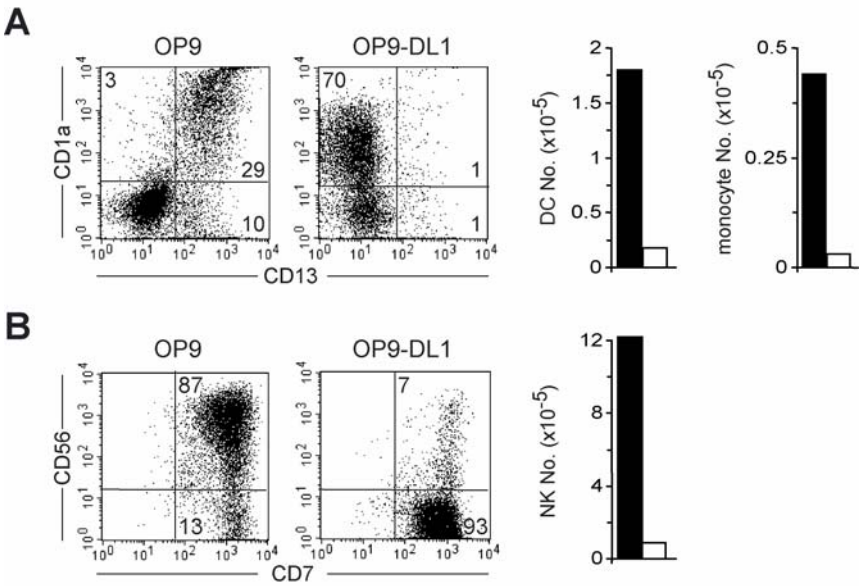
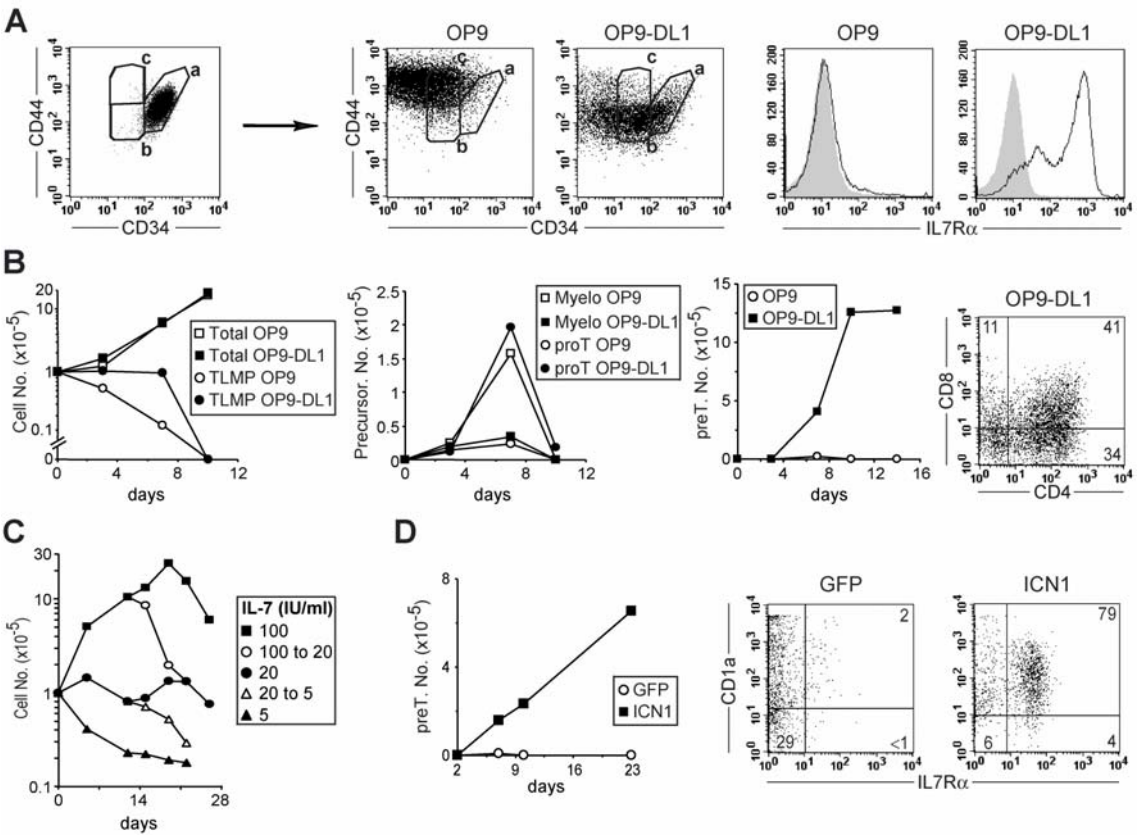

A
OP9
OP9-DL1
CD1a
CD13
3
29
10
70
1
1
DC No. (x10-5)
monocyte No. (x10-5)
B
OP9
OP9-DL1
CD56
CD7
87
13
7
93
NK No. (x10-5)

Figure 8


A
CD44
CD34
c
a
b
OP9
OP9-DL1
IL7Rα
B
Cell No. (x10-5)
Total OP9
Total OP9-DL1
TLMP OP9
TLMP OP9-DL1
days
Precursor. No. (x10-5)
Myelo OP9
Myelo OP9-DL1
proT OP9
proT OP9-DL1
preT. No. (x10-5)
OP9
OP9-DL1
CD8
CD4
11
41
34
C
IL-7 (IU/ml)
100
100 to 20
20
20 to 5
5
D
GFP
ICN1
CD1a
2
29
<1
79
6
4