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Inhibitors of DNA Binding Proteins Restrict T Cell Potential by Repressing Notch1 Expression in Flt3-Negative Common Lymphoid Progenitors

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Lineage commitment is regulated during hematopoiesis, with stepwise loss of differentiation potential ultimately resulting in lineage commitment. In this study we describe a novel population of B/NK bipotent precursors among common lymphoid progenitors in the fetal liver and the bone marrow. The absence of T cell precursor potential, both in vivo and in vitro, is due to low Notch1 expression and secondary to inhibition of E2A activity by members of the inhibitor of DNA binding (Id) protein family. Our results demonstrate a new, Id protein-dependent, molecular mechanism of Notch1 repression, operative in both fetal and adult common lymphoid progenitors, where T cell potential is selectively inhibited without affecting either the B or NK programs. This study identifies Id proteins as negative regulators of T cell specification, before B and NK commitment, and provides important insights into the transcriptional networks orchestrating hematopoiesis. The Journal of Immunology, 2012, 189: 000–000.

The hematopoietic system continuously generates mature blood cells belonging to multiple lineages. The integrity of this process depends on self-renewing multipotent hematopoietic stem cells (HSCs) that are able to sustain blood cell production throughout life. HSCs are found among a population of hematopoietic precursors coexpressing high levels of Sca-1 and c-Kit, but lacking lineage-associated markers (LSK) (1–3). Among LSK cells, expression of Flt3 (also known as Flk2) identifies multipotent progenitors (MPPs) that have lost self-renewing capacity (4). Those MPPs with the highest expression of Flt3, called lymphoid-primed MPPs (LMPPs), have little ability to generate megakaryocytes or erythrocytes, both in vivo and in vitro, but are efficient myeloid and lymphoid progenitors (5). LMPPs further differentiate to express the α-chain of the IL-7 receptor, becoming common lymphoid progenitors (CLPs) that have minimal myeloid potential but are able to give rise to T, B, and NK cells (6). It was recently shown that expression of Flt3 can be used to separate the originally defined CLP population into two major subsets: the Flt3-expressing CLPs (CLPFLt3+), which have a robust T, B, and NK cell differentiation potential, and the Flt3-negative fraction (CLPFLt3−), which is thought to include only progenitors restricted to the B lymphocyte lineage (7).

In mammals, HSCs reside and differentiate in two major primary lymphoid organs: the fetal liver (FL), responsible for most of the hematopoiesis occurring in embryonic life, and the bone marrow (BM), where blood-borne cells are produced postnatally. Whereas our understanding of hematopoiesis has relied to a large extent in the identification and purification of the various progenitor populations from adult BM, the fetal counterparts of hematopoietic precursors are much less well studied. The observation that the FL counterpart of the adult CLPs can give rise to macrophages and lymphoid tissue inducer (LTI) cells (8) led to the proposition that fetal and adult hematopoiesis follow different rules (9).

Differentiation and commitment of multipotent progenitors depend on the activation of signaling pathways and on the expression of transcription factors that induce lineage-specific genes and repress alternative cell fates. Some of these pathways and factors regulate cell fate by inducing specific genetic programs that ultimately result in lineage commitment. For example, signaling by Notch1 regulates T cell differentiation. Inactivation of Notch1 results in a block of T cell development and a striking accumulation of B cells in the thymus, at least some of which arise from T cell progenitors (10–15). Proper T cell differentiation requires tight control of Notch1 expression and activity, both inside and outside the thymus, but the regulatory mechanisms controlling its expression in lymphoid progenitors are still not fully understood. Early B cell factor (Ebf1), in contrast, plays a crucial role in the development and specification of B lineage cells. Mice deficient in this transcription factor do not express many B cell-associated genes, such as CD79a, CD79b, IgM, and V-pre-B, and the cells do not...
Lymphoid progenitors, cells, fresh isolation of adult (>8 wk old)-
derived lymphoid progenitor cells, freshly isolated BM cells were incu-
bated with biotinylated Abs to CD3, CD4, CD8, B220, CD11c, NK1.1, CD11b, Gr-1, and Ter119. In both cases lineage (Lin+) cells were removed with a MACS cell separation system (Miltenyi Biotec) according to the manufacturer’s recommendations. After depletion, Lin+ cells were stained with anti-Sca-1-FITC, anti-Flt3-PE, anti–IL-7Rα-PE-Cy7, anti–c-Kit-allophycocyanin-Cy7, anti-Ly6D-allophycocyanin, and biotinylated anti-
CD19. For the detection of biotinylated Abs, streptavidin-Pacific Blue was 
used. For the analysis of reconstituted mice, BM and spleen cells were isolated and the following Abs were used for flow cytometry: anti-CD45.1, anti-CD45.2, anti-CD11b, anti-Gr-1, anti-CD19, anti-CD11c, anti-B220, anti-IgM, anti-NK1.1, anti-CD3, anti-IgD, anti-CD8, and anti-plasmycoid dendritic cell Ag (PDCA-1). For the detection of biotinylated 
Abs, streptavidin-allophycocyanin-Cy7 and streptavidin-Pacific Blue were 
used. Abs were purchased from either BD Biosciences or eBioscience. 
Dead cells were excluded with propidium iodide. Stained cells were 
analyzed on a FACSCanto (Becton Dickinson) with FlowJo software (Tree 
Star) or sorted with a MoFlo (Beckman Coulter) or a FACSAria (Becton 
Dickinson). Intracellular staining was performed as previously done (25) 
with purified hE47 mAb (BD Biosciences), and background staining was 
assessed with an isotype control of irrelevant specificity (BD Biosciences). 
CLPs (Lin– IL-7Rα– c-Kit–Sca-1– Flt3– Ly6D–) were first sorted in the 
FL on day 15.5 after conception, and then fixed and stained. E47 and 
isotype control staining were revealed with an allophycocyanin-coupled 
anti-mouse IgG1 Ab (BD Biosciences).

Limiting dilution assays

For analysis of lymphoid/myeloid potential, single cell-sorted progenitors 
were cocultured with OP-9 (B/NK and myeloid cell potential) or OP-DL4 
(T cell potential) on 96-well plates in Opti-MEM with 10% FCS, penicillin 
(50 U/ml), streptomycin (50 μg/ml), and 2-ME (50 μM). The culture 
medium was supplemented with saturating amounts of c-Kit ligand, Flt3 
ligand, and IL-7 (38). For clonal analysis of NK/B cell potential, purified 
human IL-2 (provided by J.P. Di Santo, Institut Pasteur, Paris, France) 
was also added to the cultures. After 11–13 d, cells were harvested, stained for 
CD19, CD3, CD4, CD8, NK1.1, and CD11b, and analyzed by FACs. 
The growth of B cell colonies was scored by staining with CD19 and IgM on 
days 7–8.

In vivo reconstitutions

BM or FL HSCs, CLPLin−, and CLPLin+ were purified by cell sorting 
and injected i.v. into irradiated (600 rads) Rag2−/− mice. Four to 5 wk later, 
recipients were analyzed for the presence of donor type cells in the BM 
and spleen by flow cytometry.

Quantitative RT-PCR analysis

RNA was extracted using an RNeasy Micro kit (Qiagen) according to the 
maker’s instructions. Total cDNA was prepared using random pri-
mers and SuperScript II reverse transcriptase (Invitrogen) according to the 
manufacturer’s instructions. Quantitative PCR reactions were 
done in triplicate with TaqMan Universal Master mix (Applied Biosystems), 
and detection was done using an ABI Prism 7000 sequence detection system 
(Applied Biosystems) and normalized to the amount of hypoxanthine 
phosphoribosyltransferase (HPRT). For quantification of expression, 
the following TaqMan gene expression assays were used (all from Applied 
Biosystems): HPRT (Mm 00446968_m1), E47 (Mm 01288946_m1), 
E47 (Mm 00435501_m1), Notch1 (Mm 00435245_m1), Id2 (Mm 
00711781_m1), and Id3 (Mm 00492575_m1). Reactions were incubated at 
95°C for 10 min and then run thought 45 cycles of 95°C for 15s and 60°C 
for 1 min.

Retroviral transduction

CLPs were sorted in culture medium with IL-7 and spinoculated for 2 h at 
2500 rpm in the presence of 5 μg/ml polybrene with MigR1, MigR1-ICN1, 
MigR1-E47 retroviral supernatants as previously 
described (39). In the case of MigR1-ICN1 infection, cells were cultured 
with OP9-DL4 stroma in the presence of c-Kit ligand, Flt3 ligand, and 
IL-7. At day 11, cells were harvested and stained for CD4, CD8, and CD3. 
For MigR1-E47 transduction, cells were cultured for 36 h in medium with 
all three cytokines. Subsequently, GFP+ cells were sorted and the expression of Notch1 was measured by RT-PCR. We 
estimated the efficiency of GFP expression by analyzing GFP positive 
and GFP negative cells. GFP expression was detected by flow cytometry in 
25, 20, and 10% of the cells transduced with MigR1, MigR1-ICN1, and MigR1- 
E47, respectively.
Results

Identification of a Lin^− Sca-1^+ c-Kit^+ IL-7Rα^+ population in the FL

We analyzed lymphocyte progenitor populations in the FL at day 15.5 after conception. By flow cytometry we found a Lin^− Sca-1^low c-Kit^low IL-7Rα^+ population that phenotypically resembles bone marrow CLPs. Within this population, expression of Flt3 at the cell surface distinguished two subsets: CLP^{Flt3^+} (75–85%) and CLP^{Flt3^−} (15–25%) (Fig. 1A). Within the Lin^− Sca-1^low c-Kit^low IL-7Rα^− population, high levels of expression of Flt3 identified the LMPP population (5). The same fractions are also found in similar proportions in the BM (data not shown). Further characterization of these precursor populations revealed that fetal CLP^{Flt3^+} expressed higher amounts of CD34 than did their BM counterparts, and they also expressed low amounts of CD4 and CD11b. Additionally, fetal CLPs, both Flt3^+ and Flt3^−, expressed low levels of B220 (Supplemental Fig. 1B). In contrast, adult CLPs expressed low to undetectable levels of these surface markers (Fig. 1B, Supplemental Fig. 1B).

Expression of IL7Rα by FL CLPs and lack of T cell potential in CLP^{Flt3^−}

To evaluate the differentiation capacity of these two subsets of fetal progenitors, we performed in vitro assays at the single cell level. Individual cells were cultured with OP-9 or OP9-DL4 stroma under conditions that efficiently support lymphoid and myeloid lineage development (38). FL-derived progenitors had a higher cloning efficiency and generated larger clones compared with those from the adult (data not shown). No FL IL-7Rα^+ precursors were able to produce myeloid colonies, and CLP^{Flt3^−} cells efficiently generated all lymphoid subsets in this in vitro assay (Fig. 2A). Addition of M-CSF to the OP9 cultures did not result in the appearance of myeloid cells from fetal CLPs (data not shown). Strikingly, although 78% of individual CLP^{Flt3^−} cells efficiently generated simultaneously B and NK cell progeny, they differentiated into T cells at a very low frequency (Fig. 2A), similar to what was seen with BM CLP^{Flt3^−} (Supplemental Fig. 1D).

Although in vitro clonal assays efficiently reproduce the extension of lineage potential at the single cell level (38), they may not always reproduce the physiologic conditions cells encounter in vivo. Therefore, we determined the ability of fetal CLP^{Flt3^+} and CLP^{Flt3^−} cells to reconstitute sublethally irradiated (600 rads) alymphoid recipients. Four weeks after transfer into Ragg^-/-^- recipients, both FL-derived CLP^{Flt3^+} and CLP^{Flt3^−} generated lymphoid cells, but no myeloid cells, in the spleen of recipient mice and, in keeping with their progeny in vitro, CLP^{Flt3^−} cells were completely devoid of T cell potential in vivo, but readily gave rise to B and NK cells (Fig. 2B). It is noteworthy that a few (<1%) donor-derived CD11b^+Gr-1^+ cells could be detected in the recipients’ BM, from either CLP^{Flt3^+} or CLP^{Flt3^−} input populations, but all these cells coexpressed CD11c and PDCA-1 (data not shown), markers characteristic of plasmacytoid dendritic cells, suggesting...
that both FL CLP subsets differentiated into plasmacytoid dendritic cells, as is known for BM CLPs (40).

BM-derived CLP $\text{Flt3}^+$ cells were also devoid of T cell potential in vivo, as already observed (7), but they generated B cells and NK cells in recipient mice (data not shown). Analysis of their differentiation potential in vitro revealed that $\sim15\%$ of BM-derived CLP $\text{Flt3}^+$ cells could be identified as bipotent B/NK precursors, unable to give rise to T cells when cultured with OP9-DL4 stromal cells (Supplemental Fig. 1D). Taken together, these results indicate that expression of IL-7Ra marks lymphoid commitment, with loss of myeloid potential, during either BM or FL hematopoiesis. Furthermore, the absence of Flt3 expression identifies a B/NK cell-restricted progenitor that lacked T cell potential.

Loss of T cell potential in CLP $\text{Flt3}^-$ is due to low Notch1 expression

To understand the molecular mechanisms involved in the loss of T cell potential observed in CLP $\text{Flt3}^-$, we studied the expression pattern of Notch1, which is known to regulate T lymphocyte differentiation. We found that, coincident with their loss of T cell potential, CLP $\text{Flt3}^-$ showed lower expression of Notch1 compared with CLP $\text{Flt3}^+$, which readily generated T cells (Fig. 3A). Similar results were observed in BM-derived CLPs (Supplemental Fig. 1C).

We transduced CLP $\text{Flt3}^-$ with a GFP-expressing retroviral vector encoding ICN1, a constitutively active form of Notch1, and plated them on stromal cells in the presence of c-Kit ligand, Flt3 ligand, and IL-7. After 11–13 d culture, T cell development was assessed in the GFP+ progeny by analyzing the expression of CD4 and CD8. Retroviral expression of ICN1 in CLP $\text{Flt3}^-$ led to the acquisition of T cell potential by 27% of the cells in the culture (Fig. 3B, Supplemental Fig. 2A), indicating the acquisition of T cell potential. These data indicated that T cell potential in CLP $\text{Flt3}^-$ can be rescued by supplementing the cells with a Notch1-derived signal.

Notch1 expression in CLPs is regulated by E2A

In pluripotent hematopoietic progenitors Notch1 expression is known to be regulated by members of the E family of proteins (23, 25, 26). We observed that this is also true in CLPs because Notch1 expression was much reduced in CLP $\text{Flt3}^-$ of E2A-deficient mice (Fig. 4A). In the FL of E2A$^{+/−}$ embryos there is a less marked reduction in the number of CLPs (Supplemental Fig. 2B) than what is observed in the BM of adult mice (25). The phenotypic profiles of FL CLPs of E2A$^{+/−}$, E2A$^{−/−}$, and E2A$^{+/−}$ day 15 embryos is shown in Supplemental Fig. 2C. Transduction of CLP $\text{Flt3}^+$ with a retroviral vector encoding E47 induced a 4-fold upregulation in Notch1 transcription in these cells (Fig. 4B). CLP $\text{Flt3}^-$ cells downregulate significantly their expression of Notch1.

FIGURE 2. Differentiation potential of fetal LMPP, CLP $\text{Flt3}^+$, and CLP $\text{Flt3}^−$ subsets. (A) Single FL-derived cells from the indicated populations were sorted into 96 wells and cultured in the presence of OP9-DL4 (in conditions that support T cell differentiation) or OP9 (in conditions that support B cell, NK cell, and myeloid differentiation) stromal cells. After 11–13 d colonies were analyzed by flow cytometry for the presence of T cell progeny (left panel) or myeloid, B, and NK cells (right panel). Results are represented as the percentage of wells scoring positive for CD11b, CD19, and NK1.1. The figure shows the results obtained in one experiment representative of two (96 wells of each population in each). These results have been reproduced multiple times within different experimental layouts. (B) In vivo reconstitution ability of FL HSCs, CLP $\text{Flt3}^+$, and CLP $\text{Flt3}^−$. Sublethally irradiated Rag$^{gc−/−}$ mice were transplanted with allotype marked HSCs, CLP $\text{Flt3}^+$, or CLP $\text{Flt3}^−$ (as indicated; 800–1500 cells/recipient mouse) isolated from day 15 FL. Top panels, Expression of CD3 and CD19 among donor-derived splenocytes; bottom panels, expression of NK1.1 among donor-derived CD3$^+$ CD19$^+$ splenocytes. Plots show one representative reconstituted mouse for each group analyzed 4–5 wk after transplantation. Numbers in boxes indicate the percentage (±SD) of cells in each gate, calculated from four recipients of HSCs, five recipients of CLP $\text{Flt3}^+$ cells, and nine recipients of CLP $\text{Flt3}^-$ cells.
during the 24 h in culture with the empty virus, but also in these cells, expression of E47 induced a 2-fold upregulation of mRNA for Notch1 (Fig. 4B). These results indicate that E2A proteins are necessary for expression of Notch1 also at the CLP Flt3+ stage. Additionally, the results also demonstrated that increased activity of E2A is sufficient to upregulate Notch1 expression in CLP Flt3− cells.

To investigate whether CLP Flt3− cells represent a later developmental stage than CLP Flt3+ cells, we purified these populations and determined the kinetics of their generation of B lymphocytes in culture. Sorted CLP Flt3+ generated B lymphocytes with a delay of ~2 d, when compared with CLP Flt3−, and by day 7 of culture >30% of the CLP Flt3−-derived CD19+ cells expressed IgM, whereas only 5% of the CLP Flt3−-derived CD19+ cells were IgM+ (Supplemental Fig. 3A). It is thus likely that during lymphopoiesis CLP Flt3− cells are hierarchically positioned after CLP Flt3+ cells. To show directly that CLP Flt3+ cells give rise to CLP Flt3− cells, we cultured them on OP9 stromal cells and observed that, after 48 h culture, a percentage of CLP Flt3+ cells lose expression of Flt3 and acquire characteristics of CLP Flt3− cells, namely lower expression of Notch1 (Fig. 5). We also observed that, under these conditions, the CLP Flt3− cells arising in culture express upregulated levels of Id2 (Fig. 5), a protein that controls the activity of members of the family of E proteins (19, 41). Under the same culture conditions, purified CLP Flt3− cells did not re-express Flt3 on their surface (Supplemental Fig. 3B).

**FIGURE 4.** Notch1 expression is regulated by E47 in fetal CLPs. (A) Relative expression of Notch1 in CLP Flt3+ and CLP Flt3− purified from E47+/+ and E47−/− mice. One representative experiment of two is shown. Error bars represent the means ± SEM of triplicates. Samples were normalized for the expression of Hprt. (B) CLP Flt3+ and CLP Flt3− cells were transduced with GFP-expressing MigR1 or MigR1-E47 retroviral vectors. After 24 h culture in medium supplemented with growth factors, GFP+ cells were sorted and the relative expression of Notch1 was quantitated. For comparison, the levels of Notch1 expressed by ex vivo-isolated CLPs are shown. This result is representative of three experiments.
We found that FL CLP<sup>Flt3<sup>−</sup></sup> cells express Id<sub>2</sub> and Id<sub>3</sub> at higher levels than do CLP<sup>Flt3<sup>+</sup></sup> cells (Fig. 6B). Again, CLP<sup>Flt3<sup>−</sup></sup> showed lower expression of Notch1 compared with CLP<sup>Flt3<sup>+</sup></sup>. Furthermore, and consistent with lower E2A activity, CLP<sup>Flt3<sup>−</sup></sup> cells also expressed lower levels of Ebf1 and Pax5 than did CLP<sup>Flt3<sup>+</sup></sup> cells (Fig. 6B). No difference in the levels of mRNA for E2A could be detected in these two populations (Fig. 6B), but detection of the protein by intracellular staining (Fig. 6C) showed a small reduction of E2A in CLPFlt3<sup>−</sup> (mean fluorescence intensity of 2959) compared with CLP Flt3+ (mean fluorescence intensity of 3797).

Similar results for the expression of Id2, Id3, Notch1, Ebf1, Pax5, and E2A were obtained with CLPs isolated from the BM (Supplemental Fig. 1A, 1C), where no Ror<sup>gt+</sup> LTi cells can be found (42, 43).

Id proteins repress Notch1 expression in CLP<sup>Flt3<sup>−</sup></sup>

Because the E2A inhibitors Id2 and Id3 were upregulated in CLP<sup>Flt3<sup>−</sup></sup>, we analyzed Id-deficient embryos to assess the role of these factors in Notch1 downregulation. Analysis of mRNA expression revealed an increase of Notch1 expression in CLP<sup>Flt3<sup>−</sup></sup> of Id2<sup>−</sup>−/− mice, when compared with wild-type (Fig. 7A). It has been suggested that Id3 compensates for Id2 function, as Id2<sup>−</sup>−/− mice, although displaying a severe defect in NK development, have normal numbers of NK progenitor cells and increased expression of Id3 (47). We could not analyze Id2<sup>−</sup>−Id3<sup>−</sup>− mice because the double mutant is embryonic lethal (data not shown). However, by intercrossing Id2<sup>−</sup>− and Id3<sup>−</sup>− mice we obtained Id2<sup>−</sup>−Id3<sup>−</sup>− day 15 embryos and assessed the effect of the loss of these three Id alleles on the CLP compartment and on Notch1 expression. In the FL of Id2<sup>−</sup>−Id3<sup>−</sup>− animals CLPs display a normal phenotype.
Discussion

Lymphoid commitment is thought to follow different pathways in the BM and FL microenvironments (9) because the fetal counterpart of BM CLPs was found to give rise also to macrophages in vitro (8). However, we now showed that expression of IL-7Rα in the FL marks a population of CLPs that lacks myeloid potential, similar to the BM. The E15.5 CLPs described in this study do not give rise to detectable myeloid progeny in vivo, similarly to the ones described by Mebius et al. (8) on embryonic day 14.5. It is possible that the small burst size of fetal CLPs would render their macrophage potential undetectable in our in vitro assay.

Adult CLPs are known to be heterogeneous with respect to expression of the receptor tyrosine kinase Flt3 (7, 45). Expression of this receptor identifies lymphoid progenitors with full T, B, and NK cell potential, whereas the population of CLPFlt3− had no T cell potential and was thought to contain only committed B cell progenitors that are unable to generate NK and DC cells after transfer into C57BL/6 recipient mice (7). We found, however, that >80% of FL CLPFlt3− cells generated both B and NK progeny in vitro. Furthermore, adoptive transfer of CLPFlt3− into Rag−/− recipients led to the development of B and NK cells, demonstrating that these cells were not irreversibly committed to the B cell lineage. NK cell progeny from CLPFlt3− may be competed away in sublethally irradiated C57BL/6 recipients, because such mice, contrary to the Rag−/− animals we used, can produce NK cells from endogenous precursors.

Our results support developmental models with a fundamental segregation between the lymphoid and myeloid lineages. The lymphoid branch is identified in early progenitors by the expression of the IL-7Rα already during fetal hematopoiesis. This is consistent with fate mapping studies in adult animals showing that, in contrast to lymphocytes, the vast majority of myeloid cells develop from IL-7Rα progenitors (48).

Our work also revealed that lymphopoiesis proceeds along similar paths in fetal and adult environments. During lymphopoiesis, both fetal and adult CLPs can lose the potential to give rise to T cells before they lose NK potential. Although in the most common hierarchical models of hematopoiesis NK cells appear more closely related to the T lineages (49–54), our findings point to the existence of an intermediate stage of lymphocyte development unable to generate T cells, but with combined NK and B cell potential. This intermediate stage could be identified in both fetal and adult hematopoietic environments.

The loss of T cell potential in CLPFlt3− is due to downregulation of Notch1. Enforced expression of the E2A gene product E47 restored Notch1 expression in CLPFlt3−, as did impaired expression of the dominant-negative regulators of E2A activity Id2 and Id3. Taken together, these results indicate that Id proteins (specifically Id2 and Id3) inhibit Notch1 by counteracting E protein activity in CLPFlt3−. Because double Id2/Id3 knockout animals are not viable, we could only study T lineage potential in mutant CLPFlt3− using cells still carrying one active allele. Notably, even in this case a sizable fraction of the cells had T lineage potential. The recovery of T cell potential in the Id mutant cells is only partial, perhaps because residual Id protein activity can still impair Notch1 expression in a fraction of the cells, although no reduction is observed at the population level.

Importantly, CLPFlt3− from either Id2−/− Id3−/− or Id2−/− Id3−/− embryos generated T cells, suggesting that these two proteins compensated for each other in their function. Accordingly, and similarly to what is seen in NK precursors (47), Id3 mRNA expression was higher in CLPs from Id2−/− mice (data not shown). In the thymus, Id proteins favor NK cell development while repressing T cell potential (28–30). We now show that Id-mediated inhibition of Notch1, and consequent suppression of T cell potential, occurs also extrathymically in the FL and BM. Our findings implicate Id proteins in lineage restriction through the inhibition of E2A activity, already at the CLP stage of development.
The B cell specification factor Ebf1 is also induced by E2A (17, 22), and consistent with reduced E2A activity in CLP<sup>P<sub>B</sub></sup>-cells, we observed lower expression of Ebf1 in those cells. Engagement of CLP<sup>P<sub>B</sub></sup>- into the B lineage requires sustained IL-7 signaling, which is necessary to maintain Ebf1 and Pax5 expression (39, 46, 55, 56). After development of pro-B cells, Id protein expression is inhibited by Ebf1 (21, 57) enabling full activity of E2A, whose continued expression is indispensable later in B cell development (19, 58). At the pro-B cell stage, however, Pax5 reaches its maximal expression (18) and directly represses Notch1 (59). CLP<sup>P<sub>B</sub></sup>- cells are distinct from the recently described NK-committed precursors (60), which have very low B cell potential and high levels of IL-7Rα on their surface. Because CLP<sup>P<sub>B</sub></sup>- cells are B/NK bipotent, they are likely to be upstream of the pre-pro-NK cells, giving rise to them under conditions where the IL-7 signal is not strong enough to drive B cell differentiation. In the NK cells, giving rise to them under conditions where the IL-7 signal is not strong enough to drive B cell differentiation, the NK pathway (47).

In conclusion, this study demonstrated that Id protein expression in CLPs regulates the functional activity of E2A, thus playing a central role in the gene regulatory network (19, 61) that controls the NK pathway (47).

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Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Figure 1. (A) Bone marrow CLP^{Flt3+} and CLP^{Flt3−} populations. Adult BM was depleted of Lin^{+} cells and Lin^{−}CD19^{+}IL-7Rα^{+} cells (left panel) were analysed for the expression of c-Kit and Sca-1 (middle panel) as well as Flt3 and Ly6D (right panel). Numbers in the plots indicate the percentage of cells in each quadrant. Lineage cocktail includes CD3, CD4, CD8, B220, CD11c, NK1.1, CD11b, Gr-1 and Ter119. (B) B220 expression by FL (left panel) or BM (right panel) CLP^{Flt3+} and CLP^{Flt3−} cells. For comparison, the expression of B220 by CD19^{+} IL7Rα^{+} cells (a population highly enriched in pro-B cells) is also shown (......). (C) Expression of lymphoid genes among BM CLP. Quantitative PCR analysis of Id2, Id3, Notch1, Ebf-1 and Pax-5 mRNA in BM CLP^{Flt3+} and CLP^{Flt3−}. Error bars represent the mean ± SEM of triplicates. Samples were normalised for the expression of Hprt1. a.u. = arbitrary units. This result is representative of two experiments. (D) Differentiation potential of adult BM derived CLP^{Flt3+} and CLP^{Flt3−} subsets. Single BM derived cells from the indicated populations were sorted into 96 wells and cultured in the presence of OP9 (in conditions that support B cell, NK cell and myeloid differentiation) or OP9-DL4 (in conditions that support T cell differentiation) stromal cells. The figure shows the percentage of wells with (left graph): myeloid (CD11b^{+}); (middle graph): T (Thy1^{+}); or (right graph): B (CD19^{+} - light grey), B and NK (CD19^{+} and NK1.1^{+} - dark grey) and NK only (NK1.1^{+} - black) progeny after 11 - 13 days of culture. This result is representative of multiple experiments. ND: not detected.

Supplementary Figure 2. (A) T cell potential in CLP^{Flt3−} before or after transduction with GFP expressing MigR1 or MigR1-ICN1 retroviral vectors. 24 hours after transduction, single GFP^{+} cells were sorted into wells pre-plated with OP9-DL4 stromal cells, in conditions that support T cell differentiation. After 11-13 days colonies were analyzed for the expression of CD4 and CD8 by flow cytometry. Statistical analysis was performed with Fischer's exact test. (B) Number of CLP in the FL of E47^{+/+} or E47^{−/−} embryos at day 15.5 of gestation. Two individual embryos of each genotype, issued from two independent pairings are shown. (C) Identification of fetal liver, CLP^{Flt3+} and CLP^{Flt3−} populations in E2A^{+/+}, E2A^{+/-} and E2A^{−/−} embryos, as indicated. FL from day 15.5 was depleted of Lin^{+} cells and expression of c-Kit and Sca-1 (middle panels) as well as Flt3 (right panels) was analysed in IL-7Rα^{+}Lin^{−} fractions. Numbers in the plots indicate the percentage of cells in each gate. Lineage cocktail includes CD3, Gr-1, CD11c, NK1.1 and Ter119.
**Supplementary Figure 3.** (A) Kinetics of B cell generation *in vitro*. B cell (CD19+ IgM+) generation from single LMPP, CLP\textsuperscript{Flt3+} and CLP\textsuperscript{Flt3−} was evaluated at different time points after coculture with OP-9 cells, in conditions that support B cell differentiation. (B) Lack of generation of CLP\textsuperscript{Flt3+} from CLP\textsuperscript{Flt3−} cells. Sorted day 15 FL CLP\textsuperscript{Flt3−} were cultured for 48 hours on OP9 stromal cells, supplemented with limiting amounts of IL-7 and Flt3L. 48 hours after culture, Sca-1\textsuperscript{low}c-Kit\textsuperscript{low}IL-7Rα+CD19−α4β7− cells were analysed for Flt3 expression. Left panel shows the reanalysis of sorted CLP\textsuperscript{Flt3−} before culture (t0). Right panel shows the profile of Flt3 expression after culture (t + 48h). (C) Expression of IL7Rα in precursor populations. Black histogram: LSK (Lin−Sca-1\textsuperscript{+}c-Kit\textsuperscript{+}IL7Rα− cells); white histogram CLP (Lin−Sca-1\textsuperscript{low}c-Kit\textsuperscript{low}IL7Rα+RORC− cells); gray histogram: Lti (Lin−Sca-1\textsuperscript{low}c-Kit\textsuperscript{low}IL7Rα+RORC− cells).

**Supplementary Figure 4.** (A) Identification of FL CLP\textsuperscript{Flt3+} and CLP\textsuperscript{Flt3−} populations in Id2+/+Id3+/+ and Id2−/Id3−/− embryos, as indicated. FL from day 15.5 was depleted of Lin+ cells and expression of c-Kit and Sca-1 (middle panels) as well as Flt3 (right panels) was analyzed in IL-7Rα+Lin− fractions. Numbers in the plots indicate the percentage of cells in each gate. Lineage cocktail includes CD3, Gr-1, CD11c, NK1.1 and Ter119. (B) Number of CLP in the FL of Id2+/+Id3+/+ and Id2−/Id3−/− embryos at day 15.5 of gestation. Three individual embryos of each genotype, issued from two independent pairings are shown. (C) Enhanced T cell potential in CLP\textsuperscript{Flt3−} from Id2+/−Id3−/− embryos. Single fetal liver CLP\textsuperscript{Flt3−} cells from the indicated genotypes were sorted into wells preplated with OP9-DL4 stromal cells, in conditions that support T cell differentiation (for each condition 48 wells were analyzed in this experiment). After 11-13 days colonies were analyzed for the presence of T cells by flow cytometry. Statistical analysis was performed with Fischer's exact test. n.s.: not statistically significant.
Supplementary Figure 1

A. Flow cytometry analysis of bone marrow CLP for ITPRα, Lin + CD19, cKit, Sca-1, and Ly6D expression.

B. Histograms showing the expression of B220 in fetal liver and bone marrow CLP with isotype controls and Flt3+ and Flt3- subpopulations.

C. Expression levels of Id2, Id3, and Notch1 in FL and BM CLP subpopulations.

D. Clonogenic potential of FL and BM CLP subpopulations for myeloid, NK, and T cell lineages.
Supplementary Figure 2

Panel A: Graph showing clonogenic T cell potential (%) with bars for ex vivo, MigR1, and MigR1-ICN1. The p-value is 0.0017.

Panel B: Scatter plot showing number of cells per FL (x 10^3) for E47+/+ and E47-/-.

Panel C: Flow cytometry analysis showing Lin+CD19, IL7Rα, Sca-1, c-Kit, and Flt3 for E2A+/+, E2A+/-, and E2A-/-.
Supplementary Figure 3

A

Kinetic of IgM+ cells generation

Days

% of IgM+ cells in the CD19+ population

CLP Flt3-
CLP Flt3+
LMPP

B

Flt3
PI

t₀

t +48h

C

% of maximum

IL7Rα

LSK
CLP
LTi
### A

- **Lin + CD19**
- **IL7Rα**
- **Sca-1**
- **c-Kit**
- **Flt3**

- **Id2 +/+**
  - 3.58%
  - 69.5%
  - 83.8%
  - 9.68%

- **Id3 +/+**
  - 4.31%
  - 69.4%
  - 84.8%
  - 7.87%

### B

- **Number of cells / FL (x 10^3)**

### C

- **Clonogenic T cell Potential (%)**

- **Id2 +/+**
- **Id3 +/+**
- **Id2 -/-**
- **Id3 +/-**