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

Ana Pereira de Sousa,*† Claire Berthault,*†‡ Alessandra Granato,*†§ Sheila Dias,*‡ Cyrille Ramond,*† Claire Berthault,*† Alessandra Granato,*†§ Sheila Dias,*‡<br>Barbara L. Kee,*§# Ana Cunha,*† and Paulo Vieira*†

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 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 (CLP$^{Flt3^+}$), which have a robust T, B, and NK cell differentiation potential, and the Flt3-negative fraction (CLP$^{Flt3^-}$), 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, IgI1, and V-pre-B, and the cells do not...
proteins that reduces E protein activity and consequently lymphoid differentiation that displays B and NK cell, but not T cell, T lineage potential, revealing a novel intermediate stage in lym.

of Flt3 expression in CLPs is associated with the specific loss of resemblance the BM CLPs, and in both hematopoietic organs the loss in vitro and in vivo. Phenotypically and functionally FL CLPs progenitors in the FL that are able to generate T, B, and NK cells does not prevent B lineage differentiation under the appropriate conditions. Thus, this work uncovers a novel role for Id proteins in the regulation of the NK versus T cell fate (28–30).

In this study we identified a population of lymphoid-restricted progenitors in the FL that are able to generate T, B, and NK cells, but are devoid of myeloid differentiation potential, both in vitro and in vivo. Phenotypically and functionally FL CLPs resemble the BM CLPs, and in both hematopoietic organs the loss of Flt3 expression in CLPs is associated with the specific loss of T lineage potential, revealing a novel intermediate stage in lymphoid differentiation that displays B and NK cell, but not T cell, potential. We further demonstrate that the mechanism underlying this lineage restriction is a stage-specific upregulation of Id2/3 proteins that reduces E protein activity and consequently Notch1 expression. Importantly, the elevated expression of Id proteins in CLPId2/3− cells does not prevent B lineage differentiation under the appropriate conditions. Thus, this work uncovers a novel role for Id proteins in the regulation of E2A activity at the CLP stage.

Materials and Methods

Mice

C57BL/6 control mice (Ly5.1 and Ly5.2) were purchased from Charles River Laboratories, Rag2−/− (31), Il7−/− (32), Flt3 ligand−/− (33), and Rorcγt-GFP mice (34) were bred under specific pathogen-free conditions at the Pasteur Institute. All animal experiments were done in accordance with the guidelines of the Pasteur Institute, which were approved by the French Ministry of Agriculture. Id2−/− (35), Id3−/− (36), and E47−/− (37) mice were housed at the University of Chicago Animal Resource Center, and experiments were performed in accordance with the appropriate conditions of the National Institutes of Health with protocols approved by the Institutional Animal Care and Use Committees of the University of Chicago and the Pasteur Institute.

Cell purification

FL cells were obtained from embryos at day 15.5 gestation (with the day of appearance of vaginal plug being taken as day 0.5). Spleens were isolated from adult mice (>8 wk old). Single-cell suspensions were obtained by disruption of FL or spleen with a nylon mesh in culture medium that was HBSS with 1% FCS. BM cells were recovered by flushing the femurs with 2 ml HBSS using a 27.5-gauge needle.

Flow cytometry and sorting

For the purification of fetal-derived lymphoid progenitors cells, freshly isolated day 15.5 FL cells were incubated with biotinylated Abs to CD3, CD11c, NK1.1, Gr-1, and Ter119. For isolation of adult (>8 wk old) derived lymphoid progenitor cells, freshly isolated BM cells were incubated 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 Biotech) 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-Ly5.6-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-plasminogen deficient 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 hi-E47 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− γ− Ly5.6+) 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, CLPId2/3−, and CLPId2/3− 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 manufacturer’s instructions. Total cDNA was prepared using random primers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Quantitative PCR reactions were performed 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), EBF1 (Mm 01288946_m1), Pax5 (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 produced in Plat-E as 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 of c-Kit ligand, Flt3 ligand, and IL-7. 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 expression by flow cytometry in 25, 20, and 10% of the cells transduced with MigR1, MigR1-ICN1, and MigR1-E47, respectively.

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Results
Identification of a Lin\(^{-}\)Sca-1\(^{+}\)c-Kit\(^{+}\)IL-7Ra\(^{+}\) 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-7Ra\(^{+}\) 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-7Ra\(^{+}\) 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 IL7Ra 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-7Ra\(^{+}\) 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) allogeneic recipients. Four weeks after transfer into Rag\(2/2\) mice, 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 FLt3\(^2\) 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 \(\sim 15\%\) of BM-derived CLP FLt3\(^2\) 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-7R\(\alpha\) 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 FLt3\(^2\) is due to low Notch1 expression

To understand the molecular mechanisms involved in the loss of T cell potential observed in CLP FLt3\(^2\), 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 FLt3\(^2\) showed lower expression of Notch1 compared with CLP FLt3\(^+\), which readily generated T cells (Fig. 3A). Similar results were observed in BM-derived CLPs (Supplemental Fig. 1C).

We transduced CLP 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 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 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 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 FLt3\(^+\) with a retroviral vector encoding E47 induced a 4-fold upregulation in Notch1 transcription in these cells (Fig. 4B). CLP FLt3\(^+\) cells downregulate significantly their expression of Notch1.

**FIGURE 2.** Differentiation potential of fetal LMPP, CLP FLt3\(^+\), and CLP FLt3\(^2\) 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 FLt3\(^+\), and CLP FLt3\(^2\). Sublethally irradiated Rag\(^{gc−/−}\) mice were transplanted with allotype marked HSCs, CLP FLt3\(^+\), or CLP FLt3\(^2\) (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 FLt3\(^+\) cells, and nine recipients of CLP FLt3\(^2\) 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.

CLPFlt3+ cells are more advanced in lymphopoiesis than are CLPFlt3− cells

To investigate whether CLPFlt3− cells represent a later developmental stage than CLPFlt3+ cells, we purified these populations and determined the kinetics of their generation of B lymphocytes in culture. Sorted CLPFlt3+ generated B lymphocytes with a delay of ~2 d, when compared with CLPFlt3−, and by day 7 of culture >30% of the CLPFlt3−-derived CD19+ cells expressed IgM, whereas only 5% of the CLPFlt3+-derived CD19+ cells were IgM+ (Supplemental Fig. 3A). It is thus likely that during lymphopoiesis CLPFlt3− cells are hierarchically positioned after CLPFlt3+ cells. To show directly that CLPFlt3+ cells give rise to CLPFlt3− cells, we cultured them on OP9 stromal cells and observed that, after 48 h culture, a percentage of CLPFlt3+ cells lose expression of Flt3 and acquire characteristics of CLPFlt3− cells, namely lower expression of Notch1 (Fig. 5). We also observed that, under these conditions, the CLPFlt3− 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 CLPFlt3− cells did not re-express Flt3 on their surface (Supplemental Fig. 3B).

CLPFlt3+ cells express elevated levels of Id2 and Id3

We then went on to study the expression in CLPs of Id2 and Id3, two well-known modulators of E protein activity in lymphoid precursors (41). For that purpose it was important to exclude LTi cells because of the high amounts of Id2 they express (35), although they represent only a small fraction of the cells in our CLP gate owing to the distinctly higher levels of IL-7Rα on their surface (see Supplemental Fig. 3C).

LTi cells are found in FL, but not in BM (42, 43), and they can be identified by the expression of retinoic acid orphan receptor (ROR)yt (42–44). We therefore isolated Rorc(yt)-GFPTG mice (34). Additionally, we purified CLPs that were Ly6D+ to restrict our analysis to precursors not yet engaged in the first steps of B lineage specification (45, 46) (Fig. 6A).
We found that FL CLP^{Flt3+} cells express Id2 and Id3 at higher levels than do CLP^{Flt3+} cells (Fig. 6B). Again, CLP^{Flt3+} showed lower expression of Notch1 compared with CLP^{Flt3+}. Furthermore, and consistent with lower E2A activity, CLP^{Flt3+} cells also expressed lower levels of Ebf1 and Pax5 than did CLP^{Flt3+} 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 CLP^{Flt3+} compared with CLP^{Flt3+} (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^gt+ LTi cells can be found (42, 43).

Id proteins repress Notch1 expression in CLP^{Flt3−}

Because the E2A inhibitors Id2 and Id3 were upregulated in CLP^{Flt3+}, 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^{Flt3−} of Id2^{−/−} mice, when compared with wild-type (Fig. 7A). It has been suggested that Id3 compensates for Id2 function, as Id2^{−/−} 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^{−/−}Id3^{−/−} mice because the double mutant is embryonic lethal (data not shown). However, by intercrossing Id2^{−/−} and Id3^{−/−} mice we obtained Id2^{−/−}Id3^{−/−} 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^{−/−}Id3^{−/−} animals CLPs display a normal phenotype...
revealed no reduction in the expression of Hprt.

6 represent the means ± SEM of triplicates. Samples were normalized for the expression of Hprt, a.u., arbitrary units. (B) Single FL CLPFlt3+/− and CLPFlt3−/− cells from the indicated genotypes isolated from day 15.5 FL. One representative experiment of three is shown. Error bars represent the means ± SEM of triplicates. Samples were normalized for the expression of Hprt, a.u., arbitrary units. (B) Single FL CLPFlt3+/− and CLPFlt3−/− cells from the indicated genotypes were sorted into wells pre-plated with OP9-DL4 stromal cells, in conditions that support T cell differentiation. (A, B) Relative expression of Notch1 in purified CLPFlt3+/− and CLPFlt3−/− of the indicated genotypes isolated from day 15.5 FL. One representative experiment of three is shown. Error bars represent the means ± SEM of triplicates. Samples were normalized for the expression of Hprt, a.u., arbitrary units.

FIGURE 7. Id2/Id3 proteins inhibit Notch1 expression and T cell differentiation potential in CLPFlt3−/−. (A) Relative expression of Notch1 in purified CLPFlt3+/− and CLPFlt3−/− of the indicated genotypes isolated from day 15.5 FL. One representative experiment of three is shown. Error bars represent the means ± SEM of triplicates. Samples were normalized for the expression of Hprt, a.u., arbitrary units. (B) Single FL CLPFlt3+/− and CLPFlt3−/− cells from the indicated genotypes were sorted into wells pre-plated with OP9-DL4 stromal cells, in conditions that support T cell differentiation. After 11–13 d colonies were analyzed for the presence of T cells by flow cytometry. Shown is one experiment representative of two (in each case 48 wells were analyzed). Statistical analysis was performed with a Fischer exact test.

(Supplemental Fig. 4A) and are present in numbers comparable to what is observed in control animals (Supplemental Fig. 4B).

Remarkably, analysis of CLPFlt3−/− from Id2−/− Id3−/− embryos revealed no reduction in Notch1 expression, compared with CLPFlt3+ (Fig. 7A), and a significant, albeit partial, recovery in T precursor activity (Fig. 7B).

This result indicates that Id proteins, specifically Id2 and Id3, repress Notch1 expression and T cell potential in CLPFlt3−/−. It is of note that CLPFlt3−/− isolated from the FL of embryos of the reciprocal cross, Id2+/− Id3−/−, also have a significantly increased T cell potential when compared with Id2 or Id3 heterozygote mutants (Supplemental Fig. 4C).

We conclude, therefore, that Id2 and Id3 can compensate for each other functionally and repression of Notch1 expression depends on the total amount of Id proteins available in the cell. At early stages of hematopoiesis, Id proteins play a role in repressing Notch1 expression and in this way prevent engagement of the T cell developmental pathway.

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 Ragyc−/− 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 Ragyc−/− 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 cell lineage (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 extrathympically 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^{IgM} cells, we observed lower expression of Ebf1 in those cells. Engagement of CLP^{IgM} 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).

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References


