From progenitor cell to immune cell: cytokines and transcription factors in human hematopoietic development

Dontje, W.

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Synergy between IL-15 and Id2 promotes thymic progenitor cells to develop into NK cells
Abstract

IL-15 and the Inhibitor of DNA binding (Id)2, which negatively regulates basic-helix-loop-helix (bHLH) transcription factors, have been shown to play key roles in NK cell development in mice. Consistent with this, exogenous IL-15 stimulated human thymic T/NK cell precursors to develop into NK cells at the expense of T cells both in fetal thymic organ culture (FTOC) and in co-culture with OP9-DL1 cells, which provide a T cell permissive environment through engagement of Notch receptors. Overexpression of Id2 in thymic progenitor cells also stimulated NK cell development and blocked T cell development. This is possibly due to inhibition of the transcriptional activity of the E-protein HEB, which is the main E-protein that enhanced T cell development. Notably, Id2 increased a pool of lineage CD1a^−CD5^+ progenitor cells, which in synergy with IL-15 differentiated into NK cells. Taken together, our findings point to a dualistic function of Id2 in controlling T/NK cell lineage decisions: while T cell development is impaired by Id2, most likely by sequestering HEB, NK cell development is promoted by increasing a pool of CD1a^−CD5^+ NK cell progenitors, which requires the presence of IL-15 to differentiate into mature NK cells.
Introduction

T cells and natural killer (NK) cells are closely related cell types and share many cell surface antigens. Additionally, there are many functional similarities in particular between NK cells, TCRγδ+ T cells and CD8+ TCRαβ+ T cells. The existence of NKT cells, a specialized T cell with features of both T and NK cells, further underscores the close relationship between these two cell lineages. It is therefore not surprising that T and NK cells originate from a common precursor. Bi-potential T/NK precursor cells have been found in both the human (1) and murine (2, 3) thymus. Yet the thymus produces predominantly T cells and relatively very few NK cells, suggesting that the thymus microenvironment favors the development of T cells over NK cells. Although early cellular stages in T cell development have been defined reasonably well, the early stages of thymic NK cell development and the mechanisms regulating these steps remain poorly understood. Freud et al. (4) described the characterization of the different stages of NK cell differentiation in human secondary lymphoid organs, while Sanchez et al identified several stages of NK cell committed and uncommitted precursors in the thymus (5). In the human thymus CD34+CD1a+ cells mark an uncommitted stage in development, which can be subdivided in an early CD5− and a later CD5+ stage (6, 7). Both the CD34+CD1a+CD5− and CD34+CD1a+CD5+ precursor cells have T cell as well as NK cell potential. Upregulation of the cell surface marker CD1a on CD34+ progenitor cells is strongly, but not absolutely, associated with commitment to the T cell lineage (8) as the CD1a+CD5+ subset still has little NK cell but no DC or pDC precursor activity (8, 9).

It has been well established that E proteins, members of the basic helix-loop-helix (bHLH) family of transcription factors, play crucial roles in both T and B cell development. bHLH factors are characterized by two conserved domains, a HLH domain mediating homo- and/or heterodimerization and a basic domain allowing DNA binding of the protein dimers. Four E proteins including E12, E47, HEB, and E2-2 have been identified (10). Mice deficient for the E2A splice variants E12 and E47 lack B cells and exhibit disturbed T cell development (11). In addition, T cell development is compromised in mice lacking E2-2 or HEB (12, 13). The transcriptional activities of E proteins are controlled by inhibitor of DNA binding (Id) proteins. Id factors also contain a helix-loop-helix domain, allowing dimerization with E proteins. However, Id proteins lack the basic DNA-binding domain, and as a consequence complexes of E and Id proteins are transcriptionally inactive. In line with impaired T cell development in E protein knock out mice we have reported that the forced expression of Id3 in human progenitor cells inhibits their development into T cells and at the same time promotes NK cell development (7, 14). These findings are consistent with the observation that the number of NK cell precursors (NKP) is severely reduced in mice deficient for the related Id protein Id2 (15, 16). However, a more extensive analysis revealed that the number of NK precursors was not affected by Id2 deletion, but that development of these precursors into mature NK cells was blocked (17). No gross defects on lymphoid development were reported in Id3 deficient mice (18). Therefore, it is conceivable that Id2 is the physiological factor for NK cell development, not only in mice but also in humans. However the contribution of Id2 on human NK cell development has not been examined.

The cytokine Interleukin (IL)-15 plays an important role in NK cell development. Severe Combined Immune Deficient (SCID) patients suffering from a defect in IL-15 signaling have impaired NK cell development (19). Similarly, mice deficient for IL-15 (20) or components of the IL-15R complex (21) have strongly reduced numbers of NK cells. In addition, murine progenitor cells
cultured in FTOC with high concentrations of IL-15 are potently blocked in their TCRαβ⁺ T cell development and instead differentiate into NK cells (22). Recently it was shown that the generation of NKPs does not depend on IL-15 signaling as common gamma chain (γc) deficient mice have normal numbers of NKPs (23). However this leaves unresolved whether IL-15 affects NK cell development at the progenitor stage by inducing differentiation or by inducing survival and/or expansion at the mature NK cell stage.

Here we provide evidence that Id2 is as potent as Id3 in blocking human T cell development and stimulating NK cell development in FTOC or in a co-culture system employing OP9-DL1 cells. Since HEB is the dominant bHLH factor that enhanced human T cell development, it is reasonable to assume that Id2 mediated its effect by antagonizing HEB activity. Interestingly, thymic progenitor cells ectopically expressing Id2 gave rise to increased numbers of CD1a⁺CD5⁺ early progenitor cells, that only in synergy with IL-15 differentiated into mature NK cells. The Id2-induced progenitor cell expansion could be counteracted by co-expression of HEB. Together our findings indicate that the balance between Id2 and HEB regulates the T/NK cell bifurcation: high levels of HEB promote T and inhibit NK cell development, while high Id2 levels block T cell development and prepare a pool of IL-15 responsive NK cell precursor cells.
Materials and methods

Monoclonal antibodies and cytokines. Monoclonal antibodies to CD1a, CD3, CD4, CD5, CD8, CD56, conjugated to PE, PerCP, PeCy7, APC or APCCy7 were purchased from Becton Dickinson (BD, San Jose, CA). CD1a-PE was obtained from Beckman Coulter (Fullerton, CA). CD56-APC was from Beckman Coulter (BC, Marseille, France). The human cytokines interleukin-15 (IL-15) and stem cell factor (SCF) were obtained from R&D Systems (Abingdon, United Kingdom). IL-7 and Flt3L were a kind gift from Dr. J. Cornelissen (Erasmus University, Rotterdam, Netherlands).

Cell lines, constructs and retrovirus production. The naïve OP9 murine stromal cell line was kindly provided by Dr. T. Nakano (Osaka University, Osaka, Japan). The OP9 DeltaLike1/neo (OP9 DL1) cell line was previously established (24). Human cDNA sequences for Id2, Id3, E2-2 (Nagasawa, EJL in press), HEB (obtained from Dr. D. Littman (25)), E12, and E47 (both obtained from Dr. C. Murre (UCSD, San Diego, CA) were ligated into LZRS ires GFP or LZRS ires YFP (24) retroviral vectors. The empty constructs were used in control transductions. Retroviral supernatants were obtained from transfected Phoenix-GALV packaging cells (26).

Isolation of CD34⁺ cells from postnatal thymus. The use of postnatal thymus tissue was approved by the Medical Ethical Committee of the Academic Medical Center. Thymocytes were obtained from surgical specimens removed from children up to 3 years of age undergoing open heart surgery, with informed consent from patients in accordance with the Declaration of Helsinki. The tissue was disrupted by mechanical means and pressed through a stainless steel mesh to obtain a single cell suspension, which was left overnight at 4°C. The following day thymocytes were isolated from a Ficoll-Hypaque density gradient (Lymphoprep; Nycomed Pharma, Oslo, Norway). Subsequently, CD34⁺ cells were enriched by immunomagnetic cell sorting, using a CD34 cell separation kit (Miltenyi Biotec). The CD34⁺ thymocytes were stained with antibodies against CD34, CD1a, CD56 and BDCA2. CD34⁺CD1a⁺CD56⁺BDCA2⁺ cells, further referred to as CD34⁺CD1a⁺, were sorted to purity on a FACSARia (BD), purity of the sorted cells in all experiments was >99%.

Retroviral transduction and differentiation assays. For transduction experiments CD34⁺CD1a⁺ postnatal thymocytes were cultured overnight in Yssel’s medium (27) with 5% NHS, 20 ng/ml SCF and 10 ng/ml IL-7. The following day cells were incubated for 6 to 7 hours with virus supernatant in retronectin coated plates (30 µg/ml; Takara Biomedicals, Otsu, Shiga, Japan). The development of T and NK cells was assessed by co-culturing the mixture of transduced and non-transduced CD34⁺CD1a⁺ progenitor cells with OP9 cells in MEMα medium (Gibco) with 20% FCS (Hyclone), 5 ng/ml IL-7 and 5 ng/ml Flt3L. Flow cytometric analyses were performed on an LSRII FACS analyzer (BD).

STAT5 phosphorylation. CD1a⁺CD5⁻, CD1a⁺CD5⁺ and CD1a⁺CD5⁺ thymic subsets were sorted from the CD34⁺ MACSed postnatal thymocytes. To exclude contaminating T and NK cells the populations were sorted negative for CD3 and CD56. The flowthrough of the CD34⁺ MACs was enriched for CD56 positive cells by a second round of MACs selection using the CD56 separation kit (Miltenyi Biotec) and subsequently sorted for NK cells on basis of CD56⁻CD3⁺. Sorted cells were starved for 1 hour at
37°C and subsequently stimulated with 20 ng/ml IL-15. After methanol fixation cells were analyzed for the presence of phosphorylated STAT5 using a STAT5 (Y694) Alexa 647-conjugated antibody (BD).

Reverse transcriptase PCR. Real time polymerase chain reaction (RT-PCR) using human specific primers was performed on an iCycler PCR (Bio-Rad, Hercules, CA). Primers were: β-actin (FW) 5’-ATGGAGTTGAGGTAGTTTCG; β-actin (REV) 5’-CAAGAGATGGCCACGGCTTTCAG; Id2 (FW) 5’-CGGATATCAGCATCCTGTCC;  Id2  (REV)  5’-TCATGAACACCGCTTATTCAG;  Id3  (FW)  5’-CTTCCCATCCAGACAGCCId3;  (REV)  5’-CTGCGTTTCTGGAGGTGTCA;  E12  (FW)  5’-ACAGCGAGAAGCCCCAGA;  E12  (REV)  5’-CTGGTTTGAGGATTTGTTCC; E47 (FW) 5’-GTCGGACAAAGCGCGAGAC;  E47  (REV)  5’-ACAGGCTGCTTTGGGATTC;  HEB  (FW)  5’-CCGTGGCAGTCATCTTAGT;  HEB  (REV)  5’-GCCGATACGCGCAAACCTT;  E2-2  (FW)  5’-ATGGGAGAGAATCAAACTTA;  E2-2  (REV)  5’-CCTCCATGGCACTCTGTGA.
Results

**IL-15 stimulates NK cell and inhibits T cell development**

It is well documented that IL-15 stimulates NK cell development from uncommitted precursor cells *in vitro* (28-30). Moreover, IL-15 and IL-15R-deficient mice have strongly diminished numbers of NK cells compared to wild type mice (20, 21, 23). IL-15 is also able to block T cell development (22), suggesting that IL-15 can alter the developmental potential of bi-potential T/NK cell precursors. Indeed the data shown in Fig. 1A confirm that IL-15 can promote NK cell development and inhibit T cell development in fetal thymic organ culture (FTOC) from CD34+CD1a+ thymic progenitor cells in a dose dependent manner. In this experiment the percentage of CD56+CD3+ NK cells increased from 1.4% in the absence of IL-15 to 94% in the presence of 32 ng/ml IL-15. The percentage of CD3+ T cells decreased from 78% to 0.1%. Consistent with this the percentage of CD4+CD8+ double positive T cells was reduced from 88% to 1%. The cell recoveries per lobe were comparable, with exception of the FTOC supplemented with 32 ng/ml IL-15, which yielded twice the number of cells compared to 0, 2, or 8 ng/ml IL-15 (data not shown). Thus the total numbers of T cells decreased and that of NK cells increased with the concentration of IL-15, indicating that IL-15 promotes NK cell development and at the same time inhibits T cell development in FTOC.

Similar results were obtained when thymic progenitor cells were cultured on OP9 stromal cells expressing the Notch ligand DeltaLike1 (OP9-DL1), which have been shown to support T cell development *in vitro* in the presence of IL-7 and FLT3L (24). Increasing the dose of IL-15 to 10 ng/ml blocked T cell development, as a 20-fold reduction in absolute CD3+ T cell numbers was observed compared to no IL-15 added (Fig. 1B). Concurrently, 10-fold reduced numbers of CD4+CD8+ double positive T cells were recovered in the presence of the highest dose of IL-15 tested (10 ng/ml) when compared to the condition in which no IL-15 was added. In contrast, the absolute numbers of CD3+ CD56+ NK cells increased up to 100-fold in the presence of 10 ng/ml IL-15 compared to cultures without IL-15 (Fig. 1B). These data add further support to the notion that IL-15 stimulates NK cell development at the expense of T cell development.

**Id2 inhibits T cell development and stimulates NK cell development similar to Id3**

In addition to IL-15 also the balance of Id and E proteins plays an important role in the T/NK lineage decision. Consistent with a role for Id proteins in this lineage decision, Id2+/− mice have impaired NK cell development (16). Previously we observed that Id3 promotes human NK cell development in FTOC (14). However, since no gross defects in lymphoid development were reported in Id3 deficient mice (18), it is likely to assume that Id2 is the physiological factor involved in NK cell development. To obtain further insight in the role of Id2 as the physiological factor involved in human NK cell development, we determined mRNA expression levels of Id2 and Id3 in freshly isolated human thymic NK cells. Using real-time reverse transcription (RT)-PCR we observed that mature thymic NK cells expressed Id2 at 35-fold higher levels and Id3 at 3-fold higher levels compared to expression levels in thymic CD34+CD1a+ progenitor cells (Fig. 2A). This supports the notion that high Id levels, in particular Id2, may be favorable for human NK cell development.

To formally determine whether Id2 inhibits human T cell development in a manner similar to Id3 we overexpressed Id2 by retroviral gene transfer in CD34+CD1a+ thymic precursor cells and cultured the
cells in FTOC (Fig. 2B). Ectopic expression of Id2 in CD34⁺CD1a⁻ thymic precursors inhibited their ability to develop into T cells, as reduced percentages of CD3⁺ and CD4⁺CD8⁻ T cells were observed (Fig. 2B). On the other hand, NK cell development was enhanced by overexpression of Id2. Since Id2 can affect apoptosis of certain cell types independent of its ability to dimerize with E proteins (31), it was important to exclude that Id2 specifically induced apoptosis in T cells. We therefore tested a mutant form of Id2 (Δ-Id2) that is unable to dimerize with E proteins, but is able to protect against apoptosis (31) and demonstrated that (Δ-Id2) was unable to affect development in a FTOC, confirming the notion that Id2 inhibits T cell development by sequestering essential E proteins (Fig. 2B).

**Figure 1. IL-15 stimulates NK cell and inhibits T cell development.** (A) Sorted human CD34⁺CD1a⁻ thymocytes were cultured in an FTOC for 3 weeks in the presence of various concentrations of IL-15. Single cell suspensions were prepared and analyzed by flow cytometry. Numbers in each dot plot represents the percentage of cells in each quadrant. (B) Sorted human CD34⁺CD1a⁻ thymocytes were cultured in an OP9-DL1 co-culture for 3 weeks in the presence of various concentrations of IL-15. Cells were analyzed by flow cytometry. Shown is the fold expansion of CD3⁺CD56⁺ T cells, CD3⁺CD56⁻ NK cells, CD4⁺CD8⁻ DN cells and CD4⁺CD8⁺ T cells.
To confirm our results obtained in FTOC we co-cultured Id2 or Id3 transduced thymic progenitor cells with OP9-DL1 cells in the presence of IL-7 and FLT3L. Both Id2 and Id3 inhibited T cell development, as we observed reduced percentages and absolute numbers of CD4⁺CD8⁻ and CD3⁺ T cells after culture (Fig. 2C-F). In contrast, more NK cells developed from Id2 or Id3 transduced thymic progenitor cells when co-cultured with OP9-DL1 cells both in percentage and absolute numbers (Fig. 2E-F, and data not shown). It is noteworthy, however, that the numbers of NK cells did not steadily increase over time neither from control or Id2 transduced progenitor cells. Together these data confirm the results previously obtained in FTOC (14) and support the notion that Id2 and Id3 affect human T and NK cell development in a comparable manner.

HEB, but not E12, E47, or E2-2 promotes T cell development

Id-proteins are the natural antagonists of E proteins. Therefore, it is reasonable to assume that elevating the levels of E protein expression may counteract the Id2-induced NK progenitor cell development. Consistent with a major role of Id proteins in allowing NK cell development, we observed that NK cells expressed lower levels of E protein transcripts including E2-2, E12, E47, and HEB, as compared to the CD34⁺CD1a⁻ T/NK progenitor cells (Fig. 3A and data not shown). First we evaluated the contribution of the different E proteins to human thymic development. Knock-out studies in mice have implicated a role for all four E proteins in T cell development (11-13). Therefore we transduced HEB, E47 or E12, in CD34⁺ progenitor cells and cocultured these under T cell permissive conditions using OP9-DL1 cells. None of the E proteins affected the relative proportions of the different CD4/CD8 T cell subsets (Fig. 3B). However, calculating the absolute cell numbers revealed that HEB stimulated T cell development as indicated by an increase in the pool of CD4⁺CD8⁻ T cells (Fig. 3C). In contrast, ectopic expression of E47 or E12 slightly inhibited T cell development as compared to the GFP control transduced population. This not only suggests that HEB is the predominant E protein that contributes to T cell development, but in addition implies that HEB activity may be antagonized by Id2/3 in our experiments described above (Fig. 2).

Id2 and IL-15 synergize in NK cell development

We noticed that the number of NK cells did not steadily increase over time when control or Id2 transduced progenitors were cultured on OP9-DL1 cells (Fig. 2F). It is possible that NK cells do not survive or that development of progenitor cells is impaired in this in vitro system. Since we and others established that IL-15 plays an important role in NK cell development (Fig. 1 and (23)), we aimed to assess the effect of IL-15 on Id2 transduced thymic progenitor cells. We transduced thymic CD34⁺CD1a⁻ progenitor cells with a control-IRE5-GFP or Id2-IRE5-GFP construct and cultured the cells with or without IL-15 on OP9-DL1 cells. Since we wanted to minimize the effect of IL-15 in stimulating the proliferation of mature NK cells, we added a suboptimal concentration of IL-15 (0.5 ng/ml) in these experiments, which was 10-fold less than the lowest concentration used in the experiments described in Fig. 1B. As expected, we observed that IL-15 enhanced NK cell development both from control and Id2 transduced progenitor cells (Fig. 4). Notably, the increase in NK cell numbers was most pronounced when Id2 transduced progenitor cells were cultured with IL-15, as compared to either Id2 alone or IL-15 only (Fig. 4B). Similar results were obtained both after 2 (data not shown) and 3 weeks (Fig. 4) of culture. Taken together, these results indicate that Id2 and IL-15 act synergistically in stimulating NK cell development.
Figure 2. Id2 and Id3 block T cell and stimulate NK cell development. (A) RT-PCR analysis for Id2 and Id3 mRNA expression levels on freshly isolated postnatal thymic CD34^+CD1a^- progenitors and CD56^-CD3^- NK cells. The values for the CD34^-CD1a^- sample were set to 1 and are indicated by the dashed line. The error bars represent standard deviations of triplicate samples. One representative experiment of 2 is shown. (B) Id2, Δ-Id2, or GFP control virus transduced CD34^+CD1a^- thymic progenitor cells were cultured in FTOC. After 3 weeks the cultures were analyzed for expression of CD4/CD8 and CD3/CD56. Plots shown are gated on GFP^+ cells. (C, D) Id2, Id3, or YFP control virus transduced CD34^+CD1a^- thymic progenitor cells were co-cultured with OP9-DL1 cells in the presence of IL-7 and Flt3L. Plots shown are gated on YFP^+ cells. (C) Expression of CD4/CD8 was determined after 14 days of culture by flow cytometry. (D) Fold expansion in absolute cell numbers of the CD4/CD8 (gated CD56^- to exclude NK cells) in the transduced population was calculated on basis of total numbers of cells harvested from the cultures after 14 days, percentages of transduced cells, and percentages of each population corrected for the number of input cells. One representative experiment of 2 is shown. (E, F) Thymic CD34^-CD1a^- progenitors were transduced with control YFP or Id2 virus and co-cultured on OP9-DL1 cells for 3 weeks. Plots shown are gated on YFP^+ cells. (E) Expression of CD3/CD56 was determined after 21 days of culture by flow cytometry. Fold expansion in absolute cell numbers of CD3^-CD56^- and CD3^-CD56^+ cells in the transduced population was calculated on basis of total numbers of cells harvested from the cultures after 7, 14 and 21 days, percentages of transduced cells, and percentages of each population corrected for the number of input cells. One representative experiment of 2 is shown.
**Figure 3.** HEB, but not E12, E47, or E2-2 promotes T cell development. (A) RT-PCR analysis for E protein expression levels on freshly isolated postnatal thymic CD34⁺CD1a⁻ progenitors and CD56⁻CD3⁻ NK cells. The values for the CD34⁺CD1a⁻ sample was set to 1 and is indicated by the dashed lines. The error bars represent SDs of triplicate samples. One representative experiment of 2 is shown. (B) CD34⁺CD1a⁻ thymic progenitor cells were transduced with HEB, E47, E12, or control virus and co-cultured on OP9-DL1 cells with IL-7 and Flt3L. After 3 weeks the cultures were analyzed for the presence of cells expressing CD4 and CD8. (C) Fold expansion of CD4/CD8 expressing cells 3 weeks after culture. One representative experiment of 2 is shown.

**Figure 4.** IL-15 induces Id2 transduced thymic progenitors to develop into NK cells, which is inhibited by Notch signaling. Id2 or YFP-control transduced CD34⁺CD1a⁻ progenitors were cocultured with OP9-DL1 cells in the presence or absence of minimal amounts (0.5 ng/ml) of IL-15 for 3 weeks. Cells were analyzed by flow cytometry. (A) Percentage of CD3⁺ T cells and CD56⁺ NK cells. (B) Fold expansion of CD3-CD56⁺ NK cells.
Figure 5. Id2 controls expansion of an IL-15 responsive CD1a-CD5+ population. (A) Freshly isolated early thymic subsets were analyzed for the presence of phosphorylated STAT5 (pSTAT5) by flow cytometry after stimulation with IL-15. As positive control for pSTAT5 staining CD56+CD3 thymic NK cells were stimulated with IL-15 and unstimulated NK cells stained for pSTAT5 served as negative control. Open histograms represent stimulated cells, filled histograms represent unstimulated cells. One representative experiment of 3 is shown. (B) CD34+CD1a+ cells were Id2 or control transduced and co-cultured OP9-DL1 cells cells. The cultures were analyzed for the presence of thymic progenitor cells based on expression of CD1a and CD5 by flow cytometry. Cells are gated CD56+CD4+CD8+. (C) Fold expansion of the CD1/CD5 subsets (gated CD56+CD4+CD8+) in the transduced populations. One representative experiment of 3 is shown.

Id2 controls expansion of an IL-15 responsive CD1a-CD5+ progenitor pool

Our observations described above show that low concentrations of IL-15 induced a robust increase in NK cell numbers from Id2-transduced progenitor cells, raising the possibility that Id2 is involved in the expansion of IL-15 responsive progenitor cells. To address this, we first tested whether freshly isolated, non-T-cell committed CD34+ thymic subsets, including CD1a-CD5+ and CD1a-CD5+ cells responded to IL-15. It is known that primary human T and NK cells respond to IL-15 by phosphorylation of STAT5 (32). Therefore, the freshly isolated thymic subsets were incubated with IL-15 and analyzed for the levels of phosphorylated STAT5 (pSTAT5) by flow cytometry (Fig. 5A). In comparison with mature thymic CD56-CD3- NK cells the CD1a-CD5- cells responded only weakly to IL-15, since low pSTAT5 levels were detected, whereas the CD1a-CD5+ subset was highly susceptible to IL-15 stimulation as high levels of pSTAT5 were detected (Fig. 5A). Subsequent analysis of the CD1/CD5 progenitor subsets lacking expression of CD56/CD4/CD8 arising from Id2-transduced CD34+ progenitor cells in co-culture with OP9-DL1 cells revealed that Id2 induced expansion of CD1a-CD5+ cells (Fig. 5B and 5C). Addition of IL-15 to Id2 transduced progenitor cells generated higher absolute numbers of CD1a-CD5+ cells as compared to cultures without IL-15 (Fig. 5C). This was most notable when the cultures were analyzed after 21 days. Although IL-15 stimulated STAT5 phosphorylation in

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freshly isolated CD1a⁺CD5⁺ cells (Fig. 5A) such cells were not present in cultures of Id2 transduced cells (Fig. 5B and 5C). Together these observations support the notion that Id2 allows CD1a CD5⁺ but not CD1a⁻CD5⁺ progenitor cells to proliferate in the presence of IL-15.

**HEB inhibits Id2 induced expansion of NK cell progenitors**

Our study clearly shows that Id2 affected the T/NK lineage decision on one hand by stimulating the expansion of IL-15 responsive NK cell progenitors and on the other hand by inhibiting T cell development. Vice versa HEB stimulated T cell development, while HEB was expressed at relatively low levels in thymic NK cells. This makes it tempting to speculate that the balance between HEB and Id2 determines the outcome of the T/NK lineage decision. To test this hypothesis we double-transduced CD34⁺CD1a⁻ progenitors with HEB and Id2 or control constructs. In confirmation of our earlier observations (Fig. 2C and 2D), as compared to the control transduced cells the single Id2-transduced progenitor cells gave rise to an increase in the number of CD4⁻CD8⁻ DN cells (Fig. 6A) of which the vast majority were CD1a CD5⁺ progenitor cells (Fig. 6B). Notably, forced HEB expression was able to reduce the number of Id2-expanded CD4⁺CD8⁻CD1a⁻CD5⁺ cells to background levels (Fig. 6A and B). This may indicate that HEB inactivation by Id2 is required for proper induction of the NK cell lineage program. Furthermore, we observed that the Id2-induced block in T cell development could not be rescued by HEB. This may suggest that in the T-cell lineage Id2 not only inhibits the transcriptional activity of HEB, but more likely impairs T cell development by repressing the function of multiple E proteins.

![Figure 6](image)

**Figure 6. HEB inhibits Id2 induced expansion of NK cell progenitors.** CD34⁺CD1a⁻ thymic progenitor cells were double transduced with Id2 and HEB or control constructs. The mixture of transduced and non-transduced cell was co-cultured on OP9 DL1 cells with IL-7 and FLT3L. At 14 days the cultures were analyzed for the presence of cells expressing CD4 and CD8 (gated CD56 negative) and CD1a and CD5 (gated negative for CD56, CD4 and CD8). Shown is the fold expansion of the different subsets in the transduced populations. One representative experiment of 2 is shown.
Discussion

Here we show that the inhibitor of DNA-binding protein Id2 has a dualistic function in regulating human thymic T and NK cell differentiation. On one hand Id2 impaired T cell development, which is consistent with previous observations using Id3 transductions in human progenitors (14), and Id2 overexpression in mice both in vivo (33) and in vitro (34). On the other hand, Id2 increased the number of CD1a^+CD5^+ early progenitor cells. In agreement with these findings, NK cell development in Id2^+/− mice is impaired, which may be due to an intrinsic defect in NK cell progenitors (15, 16). In the present study we observed that Id2 only stimulates full NK cell differentiation in the presence of IL-15, indicating that expression of Id2 by itself is not sufficient. Interestingly, expansion of the NK cell progenitor pool could be counteracted by co-transduction of HEB, which, as shown here, is the main E-protein driving T cell development.

Previously we reported that in fetal thymic organ culture (FTOC) Id3 blocks human T-cell development, while strongly stimulating that of NK cells (14). Here we used the OP9 bone marrow stromal cell line expressing the Notch ligand DL1 to analyze T cell development in vitro (24, 48). Both Id2 and Id3 inhibited T cell development in this system to a similar extent. However, Id2 transduced cells co-cultured with OP9-DL1 cells only induced a mild increase in NK cell numbers relative to control transduced cells. This contradicts the results of our previous study (14) that showed a dramatic increase of numbers of mature NK cells generated from Id3-transduced CD34^+ progenitor cells in an FTOC. It has been shown that thymic stromal cells express IL-15 transcripts. A crucial role for IL-15 signaling in both human and murine NK cell development has been well established and was confirmed in this study (19, 20, 21, 36). Our data indicate that Id2 and IL-15 work synergistically in promoting NK cell development consistent with the interpretation that the effect of Id3 on NK cell development in an FTOC is the consequence of a combined action of Id3 and IL-15 produced by the thymic environment. We also observed that Id2 promoted the expansion of an early CD1a^+CD5^+ progenitor pool, which is in line with other reports showing that Id2 impairs differentiation and has a role in promoting cell proliferation (35). The in vitro generated CD1a CD5^+ progenitor cells, like their CD34^+CD1a CD5^+ ex vivo isolated thymic counterparts, are responsive to IL-15. We observed that IL-15 induced the expansion of CD1a^+CD5^+ Id2^+ NK precursor cells as well as the transition of these NK-cell precursors into mature NK cells. Attempts to isolate NK cell progenitors in humans on the basis of IL-15 responsiveness have been proven difficult as this approach reveals both NKPs and mature NK cells (36, 37). IL-15 deficient mice have normal numbers of NK cell progenitors but have severely reduced numbers of mature NK cells (23). This, however, left unresolved the question whether IL-15 is a factor required for differentiation of NK cells or whether it is merely involved in the proliferation/survival of mature NK cells. The picture emerging from our findings is that Id2 by sequestering HEB and possibly other E proteins as well, promotes development of NK cell precursors at the expense of T cell precursors, and that IL-15 is required to complete differentiation towards mature NK cells and also stimulates proliferation of mature NK cells.

Engagement of IL-15 to its receptor, consisting of the common IL-2/15-Rβ and γc subunits in addition to a unique IL-15Rα chain, results in activation of several pathways, including the JAK/STAT pathway (45). Thymic T/NK progenitors when isolated ex vivo rapidly phosphorylated STAT5 in response to IL-15 as was measured by flow cytometry. We observed that CD34^+CD1a^+CD5^+ thymocytes, which are considered to be T cell committed (1), also responded to IL-15 by phosphorylation of STAT5. This was surprising, since we show here and others have shown that IL-15
negatively regulates T cell development (22). It is possible that the contribution of the Notch signaling pathway may explain this discrepancy. Notch signaling is implicated in the emergence of T/NK precursor cells (46) and stimulation of T cell development in humans (24, 47, 48). In our studies we observed that much higher numbers of NK cells developed on parental OP9 cells as compared to OP9-DL1 cells (unpublished observation). This suggests that DL1 induced Notch signaling is able to block development of NK cells, at least in the absence of IL-15. NK cells do develop when exogenous IL-15 is added either in FTOC or in OP9-DL1 cultures as shown here and by others (22, 41). Thus, we propose that although CD34+CD1a+CD5+ thymocytes are able to respond to IL-15, when triggered by Notch in the absence of IL-15 these cells are prevented to develop into NK cells, and instead develop into T cells.

Taken together, we propose a two-step model for the T/NK cell lineage diversification in which Id2 is required to expand a progenitor pool which will only after engagement by IL-15 efficiently differentiate into NK cells. This imposes a novel role for IL-15 in differentiation of NK cell progenitors in addition to its known role in survival and expansion of mature NK cells.

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References


