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Publication date

2018

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Nagasawa, M. (2018). *Development of human plasmacytoid dendritic cells and innate lymphoid cells*.

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Chapter 3

Synergy between IL-15 and Id2 promotes the expansion of human NK progenitor cells, which can be counteracted by the E protein HEB required to drive T cell development

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ABSTRACT

The cytokine IL-15 and the inhibitor of DNA binding (Id)2, which negatively regulates the activity of basic helix-loop-helix transcription factors, have been shown to play key roles in NK cell development. Consistent with this, exogenous IL-15 added to human thymic progenitor cells stimulated their development into NK cells at the expense of T cells both in fetal thymic organ culture and in coculture with stromal cells expressing the Notch ligand Delta-like 1. Overexpression of Id2 in thymic progenitor cells stimulated NK cell development and blocked T cell development. This, in part, is attributed to inhibition of the transcriptional activity of the E protein HEB, which we show in this study is the only E protein that enhanced T cell development. Notably, Id2 increased a pool of lineage CD1a⁻CD5⁺ progenitor cells that in synergy with IL-15 furthered expansion and differentiation into NK cells. Taken together, our findings point to a dualistic function of Id2 in controlling T/NK cell lineage decisions; T cell development is impaired by Id2, most likely by sequestering HEB, whereas NK cell development is promoted by increasing a pool of CD1a⁻CD5⁺ NK cell progenitors, which together with IL-15 differentiate into mature NK cells.

INTRODUCTION

T cells and NK cells are closely related cell types and share many cell surface Ags. In addition, there are many functional similarities in particular between NK cells, TCR- $\gamma\delta^+$ T cells and CD8⁺TCR- $\alpha\beta$ 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. Bipotential T/NK precursor cells have been found in the thymus of both humans¹ and mice^{2,3}. 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, whereas Sanchez et al.⁵ identified several stages of committed and uncommitted NK cell precursors in the thymus. 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 conventional or plasmacytoid dendritic cell (pDC) precursor activity^{8,9}. It has been well established that E proteins, which are members of the basic helix-loop-helix (bHLH) family of transcription factors, play crucial roles in development of T cells, B cells, and pDCs^{7,10}. Members of the bHLH family 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 identified¹¹. Mice deficient for the E2A splice variants E12 and E47 lack B cells and exhibit disturbed T cell development¹². In addition, T cell development is compromised in mice lacking E2-2 or HEB^{13,14}. The transcriptional activities of E proteins are controlled by inhibitor of DNA binding (Id) proteins. Id factors also contain a HLH 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^{6,15}. These findings are consistent with the observation that the number of NK cell precursors (NKPs) is severely reduced in mice deficient for the Id3-related protein Id2^{16,17}. 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 blocked¹⁸. No gross defects on lymphoid development were reported in *Id3*-deficient mice¹⁹. Therefore, it is conceivable that Id2 is the physiological factor for NK cell development, not only in mice but also in humans. The role of Id2 on human NK cell development, however, has not been examined. The cytokine IL-15 has been shown to play an important role in NK cell development. SCID patients who have a defect in IL-15 signaling have impaired NK cell development²⁰. Consistent with this, human NK cell development in an in vivo humanized mouse

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model is strongly augmented when recombinant IL-15/IL-15Ra–Fc complexes were injected²¹. Similarly, mouse NK cell development depends on IL-15 as mice deficient for IL-15²² or components of the IL-15R complex²³ have strongly reduced numbers of NK cells. In addition, murine progenitor cells cultured in fetal thymic organ culture (FTOC) with high concentrations of IL-15 are potentially blocked in their TCR- $\alpha\beta^+$ T cell development and instead differentiate into NK cells²⁴. Recently, it was shown that the generation of NKPs does not depend on IL-15 signaling as common γ chain (γc)-deficient mice have normal numbers of NKPs²⁵. However, this leaves unresolved whether IL-15 affects NK cell development at the progenitor stage by inducing differentiation or by stimulating survival and/or expansion at the mature NK cell stage. In this study, 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 coculture system using OP9 stromal cells expressing the Notch ligand Delta-like 1 (OP9-DL1). Thymic progenitor cells ectopically expressing Id2 gave rise to increased numbers of CD1a⁺CD5⁺ early progenitor cells, that with IL-15 further expanded and differentiated into mature NK cells. The Id2-induced progenitor cell expansion could be counteracted by coexpression of HEB. This, together with the finding that HEB is the only bHLH factor that stimulated T cell development, suggests that the balance between Id2 and HEB regulates the T/NK cell bifurcation: high levels of HEB promote T and inhibit NK cell development, whereas high Id2 levels block T cell development and prepare a pool of IL-15 responsive NK cell progenitor cells.

MATERIALS AND METHODS

MAbs and cytokines

MAbs to CD1a, CD3, CD4, CD5, CD7, CD8, CD16, CD56, CD94, CD122, CD127, NKG2D, NKP30, NKP46, conjugated to PE, PerCP, PeCy7, APC, or APCCy7 were purchased from Becton Dickinson (BD Biosciences, San Jose, CA) or Biolegend (San Diego, CA). CD1a-PE and CD56-APC were obtained from Beckman Coulter (Marseille, France). The human cytokines IL-15 and stem cell factor were obtained from R&D Systems (Abingdon, U.K.). IL-7 and FLT3L were a kind gift from Dr. J. Cornelissen (Erasmus University, Rotterdam, The Netherlands).

Cell lines, constructs, and retrovirus production

The naive OP9 murine stromal cell line was kindly provided by Dr. T. Nakano (Osaka University, Osaka, Japan). The OP9-DL1/neo cell line was previously established²⁶. Human cDNA sequences for Id2, Id3, E2-2 (10), Id2 DHLH²⁷, HEB (obtained from Dr. D. Littman, New York University School of Medicine, New York, NY)²⁸, E12, and E47 (both obtained from Dr. C. Murre, University of California at San Diego, La Jolla, CA) were ligated into the retroviral vector LZRS-internal ribosome entry site (IRES)-GFP or LZRS-IRES-yellow fluorescent protein (YFP)²⁶. The empty constructs were used in control transductions. Retroviral supernatants were obtained from transfected Phoenix-GALV packaging cells²⁹.

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 y 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 next 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, Bergisch Gladbach, Germany). The CD34⁺ thymocytes were stained with Abs against CD34, CD1a, CD56, and BDCA2. CD34⁺CD1a⁻CD56⁻BDCA2⁻ cells, further referred to as CD34⁺CD1a⁻, were sorted to purity on a FACS Aria (BD Biosciences), 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 medium³⁰ with 5% hormonal human serum, 20 ng/ml stem cell factor, and 10 ng/ml IL-7. The following day cells were incubated for 6–7 h with virus supernatant in retronectin-coated plates (30 mg/ml; Takara Biomedicals, Shiga, Japan). The development of T and NK cells was assessed by coculturing the mixture of transduced and nontransduced CD34⁺CD1a⁻ progenitor cells in the hybrid murine/human FTOC as has been described previously³¹. IL-15 was added at the onset of the culture, the medium containing IL-15 was refreshed every week. Alternatively the mixture of transduced and nontransduced CD34⁺CD1a⁻ progenitor cells were cocultured with OP9-DL1 cells in MEM a medium (Life Technologies, Carlsbad, CA) with 20% FCS (Hyclone Laboratories, Logan, UT), 5 ng/ml IL-7, and 5 ng/ml FLT3L. Flow cytometric analyses were performed on an LSRII FACS analyzer (BD Biosciences); electronic gating was performed using FlowJo (Tree Star, Ashland, OR). Numbers in each dot plot represent the percentages of cells in the quadrants. The fold expansion in absolute cell numbers was calculated using Microsoft Excel 2007 (Microsoft, Redmond, WA) on basis of total numbers of cells harvested from the cultures, percentages of transduced cells, and percentages of each population corrected for the number of input cells. One representative experiment of two is shown.

STAT5 phosphorylation

The CD1a⁻CD5⁻, CD1a⁻CD5⁺, and CD1a⁺CD5⁺ thymic subsets were sorted from the CD34⁺ MACS-enriched postnatal thymocytes. To exclude contaminating T and NK cells, the populations were sorted negative for CD3 and CD56. The flow through 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 the basis of CD56⁺CD3⁻. Sorted cells were starved for 1 h 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 Ab (BD Biosciences).

RT-PCR

Real-time PCR using human specific primers was performed on an iCycler PCR (Bio-Rad, Hercules, CA). Primers were as follows: b-actin (FW) 5'- ATGGAGTTGAAGGTAGTTTCG-3'; b-actin

(REV) 5'-CAAGAGATGGCCACGGCTGCTTCAGC-3'; Id2 (FW) 5'-CGGATATCAGCATCCTGTCC-3'; Id2 (REV) 5'-TCATGAACACCGCTTATTCAG-3'; Id3 (FW) 5'-CTTCCCATCCAGACAGCC-3'; Id3 (REV) 5'-CTGCGTTTCTGGAGGTGTCA-3'; E12 (FW) 5' -ACAGCGAGAAGCCCCAGA-3' ; E12 (REV) 5' -CTGCTTTGGGATTACAGTTC-3' ; E47 (FW) 5' -GTCGGACAAAGCGCAGAC-3' ; E47 (REV) 5' -ACAGGCTGCTTTGGGATTC-3' ; HEB (FW) 5' -CCGTGGCAGTCATCCTTAGT-3' ; HEB (REV) 5' -GCCGATACGGCAGAAACTT-3' ; E2-2 (FW) 5' -ATGGGAGAGAATCAAACCTTA- 3' ; E2-2 (REV) 5' -CCTCCATGGCACTACTGTGA-3' ; CD161 (FW) 5' - TCTTCTCGGGATGTCTGTC-3' ; CD161 (REV) 5' -CCTGCTCTGTTGAATGTCCA-3'.

RESULTS

IL-15 stimulates NK cell and inhibits T cell development

A role for IL-15 in mouse and human NK cell development is well documented^{4,21-23,25,32,33}. In mice, IL-15 blocks T cell development²⁴, suggesting that IL-15 can alter the developmental potential of bipotential T/NK cell precursors. In this study, we investigated the role of IL-15 in the T/NK cell diversification in human. The data in Fig. 1A show that IL-15 can promote human NK cell development and inhibit T cell development in a hybrid human/mouse 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 and 94% in the presence of 32 ng/ml IL-15. The percentages of CD56⁻CD3⁺ T cells in these culture conditions decreased from 78% (no IL-15) to 0.1% (with 32 ng/ml IL-15). Consistent with this, the percentages of CD4⁺CD8⁺ double positive (DP) T cells were reduced from 87% to 1%. The total cell recoveries per lobe were comparable, with the exception of the FTOC supplemented with 32 ng/ml IL-15, which yielded twice the number of cells compared with 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 mounting concentrations 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 OP9-DL1, which have been shown to support T cell development in vitro in the presence of IL-7 and FLT3L²⁶. Increasing the dose of IL-15 to 10 ng/ml blocked T cell development, as a 20-fold reduction in absolute CD56⁻CD3⁺ T cell numbers was observed compared with the condition in which no IL-15 was added (Fig. 1B). Concurrently, 10-fold reduced numbers of CD4⁺CD8⁺ DP T cells were recovered in the presence of the highest dose of IL-15 tested (10 ng/ml) when compared with 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 with 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, it is known that 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 development¹⁷. In addition, we previously observed that Id3 promotes human NK

cell development in FTOC ¹⁵. However, because no gross defects in lymphoid development were reported in *Id3*-deficient mice ¹⁹, it is more 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 central 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 RT-PCR we observed that mature thymic NK cells expressed Id2 at 35-fold higher levels and Id3 at 3-fold higher levels compared with 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.

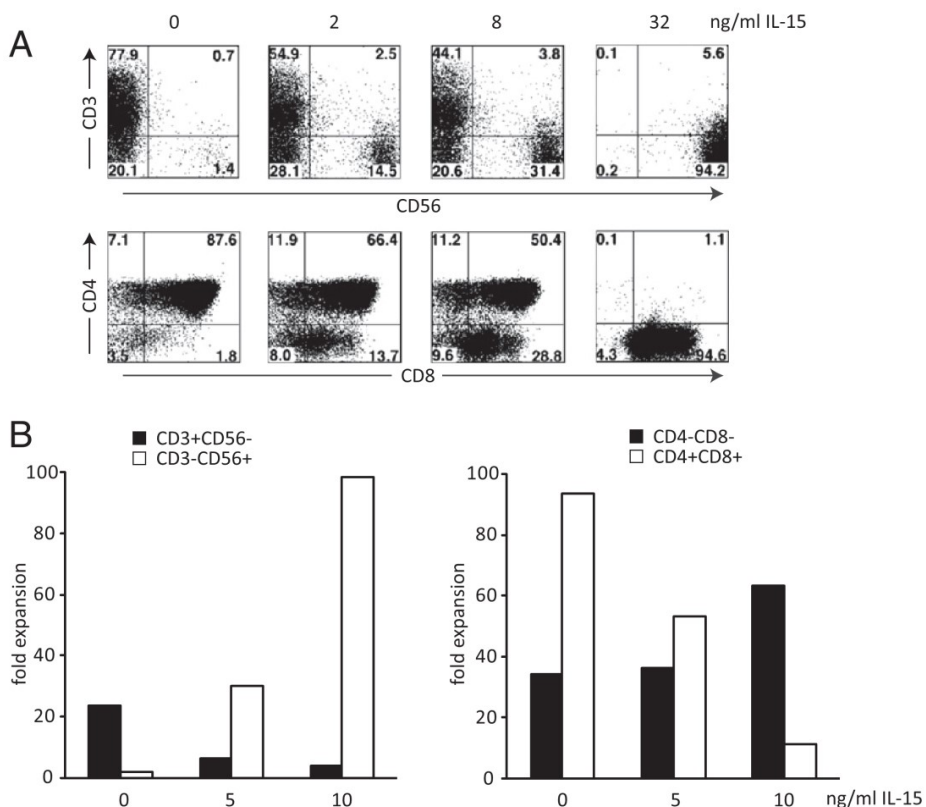


FIGURE 1. IL-15 stimulates development of NK cells and inhibits development of T cell.

(A) Sorted human CD34⁺CD1a⁻ thymocytes were cultured in a FTOC in the absence or presence of the indicated amounts of IL-15. After 3 wk, single-cell suspensions were prepared and analyzed by flow cytometry. Numbers in each dot plot represent the percentages of cells in each quadrant. This experiment has been repeated twice with similar results. (B) Sorted human CD34⁺CD1a⁻ thymocytes were cocultured with OP9-DL1 stromal cells in the absence or presence of the indicated amounts of IL-15. After 3 wk, cells were counted and 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⁺ DP T cells calculated as described in the Materials and Methods. One representative experiment of two is shown.

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To 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 CD4⁺CD8⁺ DP (near 6- fold) and CD56⁻CD3⁺ T cells (near 3-fold) were observed compared with the control transduced culture (Fig. 2B). In contrast, NK cell development was enhanced by overexpression of Id2, as the percentage of CD56⁺CD3⁻ NK cells increased by 45-fold compared with the control culture (Fig. 2B). Because Id2 can affect apoptosis of certain cell types independent of its ability to dimerize with E proteins²⁷, it was important to exclude that Id2 specifically induced apoptosis in T cells. We therefore tested a mutant form of Id2 (DId2) that is unable to dimerize with E proteins, but is able to induce apoptosis²⁷. Notably, DId2 did not affect lymphoid development in a FTOC (Fig. 2B) as compared with the control transduced culture reinforcing the notion that Id2 inhibits T cell development by sequestering essential E proteins. To rule out that Id2 transduced progenitor cells indirectly induced the development of NK cells, we analyzed the phenotype of the untransduced cells in the same culture. The phenotype of these untransduced cells resembled the phenotype of cells obtained in the GFP control transduced culture providing evidence that Id2 affects differentiation of the T and NK cell lineages in a cell-intrinsic manner (Supplemental Fig. 1).

To further confirm our results obtained in FTOC, we cocultured Id2, Id3, or control 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⁺ DP (3- to 4-fold in percentage, near 10-fold in absolute numbers, Fig. 2C, 2D) and CD56⁻CD3⁺ T cells (5- to 7-fold in percentage, near 10-fold in absolute numbers, Fig. 2E, 2F) after culture compared with control transduced cells. Development of CD56⁺CD3⁻ NK cells from Id2 and Id3 transduced thymic progenitor cells after coculture with OP9-DL1 cells, however, was hardly affected, neither in percentages nor in absolute numbers (Figs. 2E, 2F, 3). Collectively, these data are in agreement with the results previously obtained in FTOC¹⁵, and support the notion that Id2 and Id3 affect human T cell development in a comparable manner.

Id2 and IL-15 synergize in NK cell development

It was unexpected that NK cell development from Id2- or Id3- transduced progenitor cells on OP9-DL1 cells was not increased, and not when analyzing the cultures over time (Fig. 2E, 2F; data not shown). It is possible that NK cells do not survive or that development of progenitor cells is impaired in this in vitro system. Because we and others established that IL-15 plays an important role in NK cell development (Fig. 1)²¹⁻²⁴, we assessed the effect of IL-15 on Id2 transduced thymic progenitor cells. We transduced thymic CD34⁺CD1a⁻ progenitor cells with a control- IRES-GFP or Id2-IRES-GFP construct and cultured the cells with or without IL-15 on OP9-DL1 cells. Because 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; data not shown) in these experiments, which was 10-fold less than the lowest concentration used in the experiments described in Fig. 1B. Even this

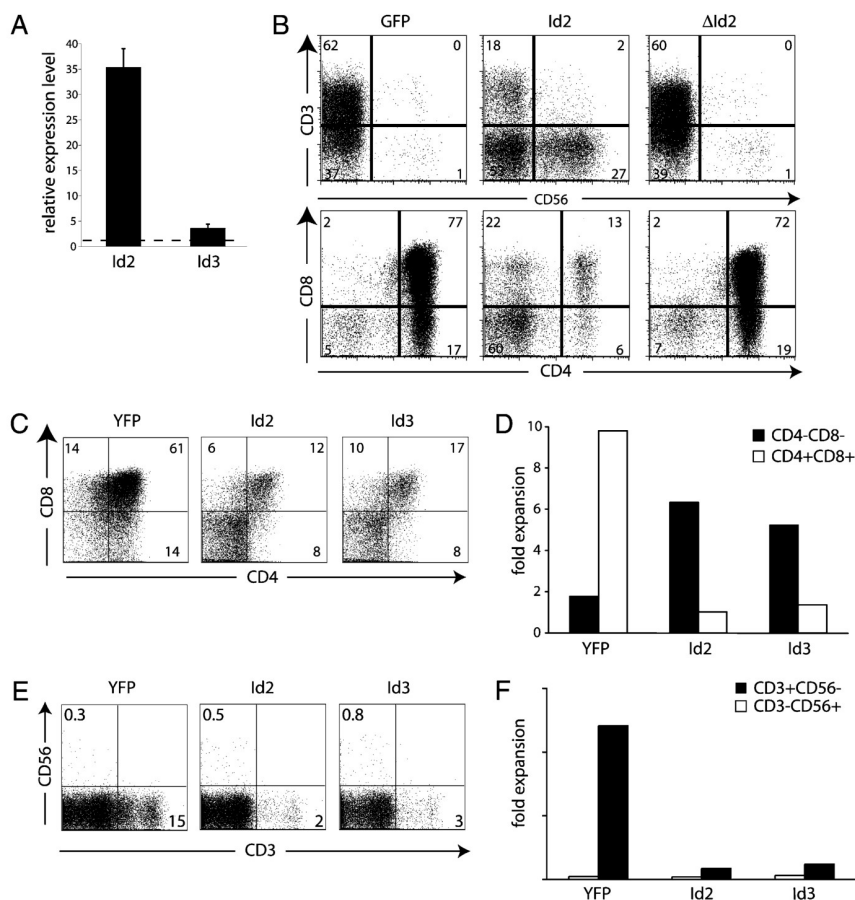


FIGURE 2. Id2 and Id3 block T cell development and stimulate NK cell development.

(A) Quantitative RT-PCR analysis for Id2 and Id3 mRNA expression levels in freshly isolated postnatal thymic CD34⁺CD1a⁻ progenitors and CD56⁺CD3⁻ thymic NK cells. The value for the CD34⁺CD1a⁻ sample was set to 1 and is indicated by the dashed line. The error bars represent SDs of triplicate samples. One representative experiment of two is shown. (B) CD34⁺CD1a⁻ thymic progenitor cells were transduced with retroviral vectors expressing Id2-IRES-GFP, ΔId2-IRES-GFP, or control IRES-GFP and cultured in FTOC. After 3 wk, the cultures were analyzed for expression of CD4/CD8 and CD3/CD56. Plots shown are electronically gated on GFP⁺ cells. (C–F) CD34⁺CD1a⁻ thymic progenitor cells were transduced with Id2-IRES-YFP, Id3-IRES-YFP, or control IRES-YFP viruses and cocultured with OP9-DL1 cells in the presence of IL-7 and FLT3L. After 14 d, cultures were analyzed by flow cytometry. One representative experiment of two is shown. (C) Dot plots showing the expression of CD4 and CD8 (gated CD56⁻ to exclude NK cells) on YFP⁺ cells. (D) Fold expansion in absolute cell numbers of the CD4⁺/CD8⁺ (gated CD56⁻ to exclude NK cells) in the YFP⁺ cells. (E) Dot plots showing the expression of CD3 and CD56 on YFP⁺ cells. (F) The fold expansion in absolute cell numbers of CD3⁺CD56⁻ T cells and CD3⁻CD56⁺ NK cells in the YFP⁺ population was calculated as described in the Materials and Methods.

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trace amount of IL-15 enhanced NK cell development from control transduced progenitor cells both in proportion and absolute numbers (Fig. 3A, 3B, Supplemental Table 1; 4/4 donors). As expected, CD3⁺ T cell development was blocked in the presence of IL-15 (Fig. 3A, Supplemental Table 1; 3/4 donors). More importantly, the increase in NK cell numbers was most pronounced when IL-15 was added to Id2 transduced progenitor cells as compared with either Id2 alone or IL-15 only (Fig. 3, Supplemental Table 1; 4/4 donors). Taken together, these results indicate that Id2 and IL-15 act synergistically in stimulating NK cell development.

All mouse thymic NK cells expressed CD127 (IL-7R α), and were independent of Id2 for their development^{18,34}. These data are seemingly in contrast with the role of Id2 that we observed in this study for development of thymic progenitor cells into human NK cells. In contrast to the mouse, however, we observed that the majority of human NK cells in the postnatal thymus are negative for CD127 expression (Supplemental Fig. 2). Similarly, the Id2-transduced NK cells lacked CD127 expression, whereas T cells generated in parallel control cultures did express CD127 (Supplemental Fig. 2). This suggests that different pathways for thymic NK cell development exist in human and mouse.

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

The observation that low concentrations of IL-15 induced a robust increase in NK cell numbers from Id2-transduced progenitor cells (Fig. 3) raises 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 progenitor subsets, including CD1a⁻CD5⁻ and CD1a⁻CD5⁺ cells, responded

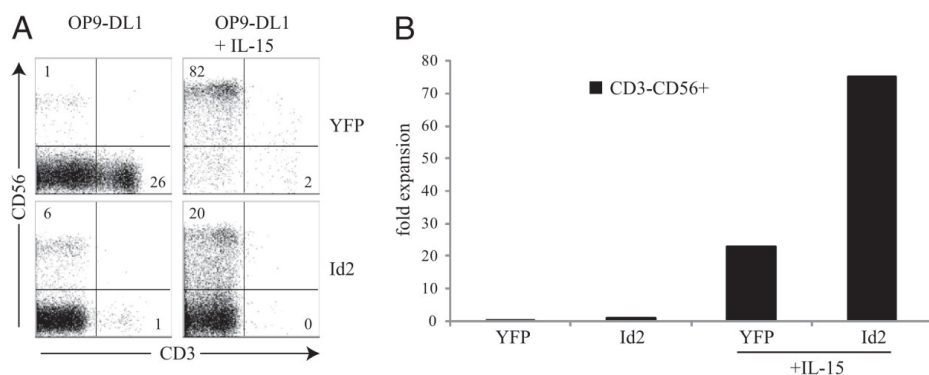


FIGURE 3. IL-15 induces Id2-transduced thymic progenitors to develop into NK cells.

Id2-IRES-YFP or control-IRES-YFP transduced CD34⁺CD1a⁻ progenitors were cocultured with OP9-DL1 cells supplemented with IL-7 and FLT3L in the absence or presence of minimal amounts of IL-15 (0.5 ng/ml). After 3 wk, the cultures were analyzed by flow cytometry for expression of CD3 or CD56. (A) Percentages of CD3⁺CD56⁻ T cells and CD56⁺CD3⁻ NK cells gated on YFP⁺ cells. (B) Fold expansion of CD56⁺CD3⁻ NK cells, either control or Id2 transduced, was calculated as described in the Materials and Methods. One representative experiment of four is shown.

to IL-15. It is known that primary human T and NK cells in response to IL-15 phosphorylate STAT5³⁵. Therefore, freshly isolated CD34⁺CD1a⁻CD5⁻ and CD34⁺CD1a⁻CD5⁺ thymic subsets were stimulated with IL-15 *in vitro* and analyzed for the levels of phosphorylated STAT5 (pSTAT5) by flow cytometry (Fig. 4A). In comparison with mature thymic CD56⁺CD3⁻ NK cells (pSTAT5; mean fluorescence intensity [MFI 452]), the CD34⁺CD1a⁻CD5⁻ cells responded weakly to IL-15, because relatively low pSTAT5 levels (MFI 144) were detected (Fig. 4A). In contrast, the CD34⁺CD1a⁻CD5⁺ subset was as susceptible to IL-15 stimulation (pSTAT5; MFI 459) as the mature thymic NK cells. As this finding might suggest that (part of) the CD34⁺CD1a⁻CD5⁺ cells are already committed to the NK cell lineage, flow cytometric analysis and quantitative PCR were performed to analyze the phenotype of the CD34⁺CD1a⁻CD5⁺ and CD34⁺CD1a⁺CD5⁺ subsets in more depth. Expression of the NK cell specific or associated markers, NKp46, NKp30, NKG2D, CD94, CD16, or CD161, was undetectable on CD34⁺ progenitor cells, but prominently expressed on thymic NK cells (Fig. 4B, Supplemental Fig. 3). As expected, CD7 was expressed on all CD34⁺ subsets and mature NK cells (Supplemental Fig. 3A). Together, these results indicate that (subsets of) CD34⁺ thymic progenitor cells are not yet NK cell committed.

Then, we wanted to gain insight in the CD1/CD5 progenitor subsets, which lack expression of CD56/CD4/CD8, that were generated from Id2-transduced CD34⁺ progenitor cells in coculture with OP9-DL1 cells. This revealed that Id2 preferentially induced the development of CD1a⁻CD5⁺ cells both in percentage and absolute cell numbers (Fig. 4C, 4D, Supplemental Table II; 3/3 donors). The addition of IL-15 to Id2-transduced progenitor cells generated even higher (35-fold) absolute numbers of CD1a⁻CD5⁺ cells as compared with cultures without IL-15 after 21 d (Fig. 4D, Supplemental Table II; 3/3 donors). Although we observed that IL-15 also stimulated STAT5 phosphorylation in freshly isolated CD34⁺CD1a⁺CD5⁺ cells (MFI 469, Fig. 4A), cells with a similar phenotype were underrepresented in cultures of Id2 transduced cells (Fig. 4C, 4D, Supplemental Table II; 2/3 donors). Together these observations support the notion that Id2 allows expansion of CD1a⁻CD5⁺ progenitor cells, but not CD1a⁺CD5⁺ progenitor cells, particularly when cultured in the presence of IL-15.

T cell development is stimulated by HEB, but not E12, E47, or E2-2

Id proteins are the natural antagonists of E proteins³⁶. Consistent with the blocking effect of Id2 and Id3 on T cell development and the stimulatory effect on NK cell development, the following can be hypothesized: either the levels of E protein expression are lower in NK cells compared with T cells or the E protein levels are constant, but their function is impaired in NK cells by elevated levels Id protein. In addition, elevating the levels of E protein expression in thymic progenitor cells might have a positive effect on T cell development, but not on NK cell development. To address these issues, we first analyzed E protein expression in freshly isolated thymic NK cells and their progenitor cells. In line with our hypothesis, we observed that NK cells expressed lower levels of all E protein transcripts, E12, E47, HEB, and E2-2, as compared with the levels in CD34⁺CD1a⁻ T/NK progenitor cells (Fig. 5A). Further, 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

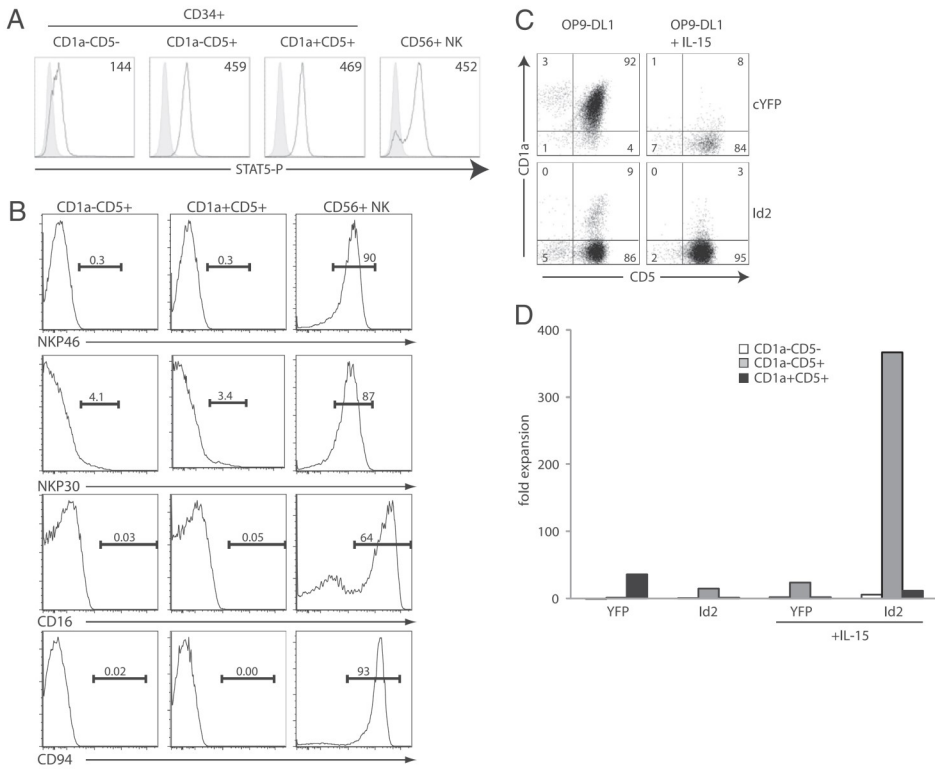


FIGURE 4. Id2 controls expansion of an IL-15 responsive CD1a⁻CD5⁺ population.

(A) Freshly isolated early thymic subsets were analyzed by flow cytometry for their ability to pSTAT5 after stimulation with IL-15. As positive control for pSTAT5 staining, CD56⁺CD3⁻ thymic NK cells were stimulated with IL-15 and as negative control, unstimulated NK cells were analyzed. Open histograms represent pSTAT5 stained cells after IL-15 stimulation, filled histograms represent medium cultured NK cells stained for pSTAT5. Numbers represent the MFI of the pSTAT5 stained cells. Unstimulated NK cells had a MFI of 42. One representative experiment of three is shown. (B) CD34-MACS and CD56-MACS enriched postnatal thymocytes were stained for indicated NK cells markers. (C) CD34⁺CD1a⁻ thymic progenitor cells were transduced with Id2-IRES-YFP or control-IRES-YFP viruses and cocultured on OP9-DL1 cells with IL-7 and FLT3L in the absence or presence of low amounts of IL-15 (0.5 ng/ml). The cultures were analyzed for the presence of cells expressing CD1a and/or CD5 by flow cytometry at 21 d of culture. Dot plots shown are electronically gated on YFP⁺CD56⁻CD4⁻CD8⁻ cells. Numbers in each dot plot represent the percentages of cells in the quadrants. (D) Fold expansion of CD1/CD5 expressing subsets after gating on YFP⁺CD56⁻CD4⁻CD8⁻ cells were calculated as described in the Materials and Methods. One representative experiment of three is shown.

Id2 and IL-15 synergize in human NK cell development

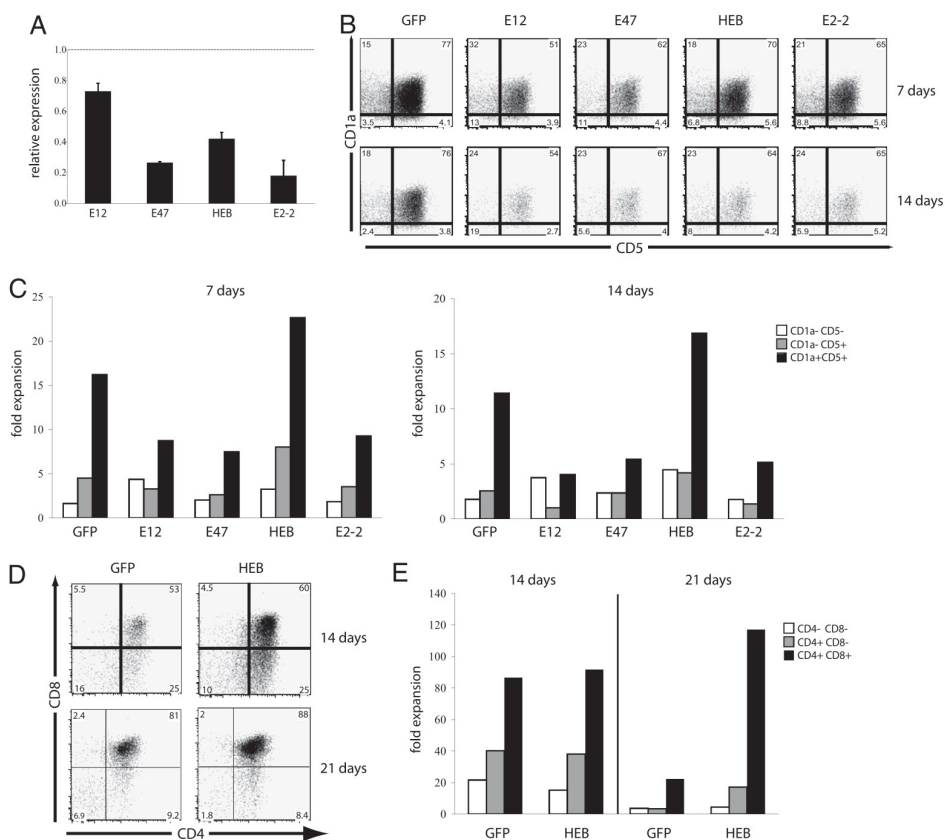


FIGURE 5. HEB, but not E12, E47, or E2-2, promotes T cell development.

(A) Quantitative RT-PCR analysis for E protein mRNA expression levels in freshly isolated postnatal thymic CD56⁺CD3⁻ NK cells compared with CD34⁺CD1a⁻ thymic progenitor cells. The values for the CD34⁺CD1a⁻ sample were set to 1 and are indicated by the dashed lines. The error bars represent SDs of triplicate samples. One representative experiment of two is shown. (B–E) CD34⁺CD1a⁻ thymic progenitor cells were transduced with HEB–IRES–GFP, E47–IRES–GFP, E12–IRES–GFP, E2-2–IRES–GFP, or control–IRES–GFP viruses and cocultured on OP9–DL1 cells with IL-7 and FLT3L. (B,C) At indicated time points, cultures were analyzed for the presence of CD1a/CD5 positive cells (gated on GFP⁺CD56⁻CD4⁻CD8⁻ cells). Fold expansion of CD1/CD5 expressing subsets after gating on YFP⁺CD56⁻CD4⁻CD8⁻ cells was calculated as described in the Materials and Methods. One representative experiment of three is shown. (D, E) Analysis of HEB/GFP-transduced cells for the expression of CD4/CD8 (gated on GFP⁺CD56⁻). Fold expansion of CD4/CD8 expressing subsets after gating on YFP⁺CD56⁻ cells was calculated as described in the Materials and Methods. One representative experiment of three is shown.

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cell development¹²⁻¹⁴. Therefore, we transduced HEB, E47, E12, or E2-2 in CD34⁺ progenitor cells and cocultured these under T cell permissive conditions using OP9-DL1 cells. None of the E proteins dramatically affected the relative proportions of the early T cell subsets based on expression of CD1a and CD5 as compared with control transduced cells (Fig. 5B). However, calculating the absolute cell numbers revealed that HEB modestly stimulated T cell development as indicated by an increase in the pool of CD1a⁺CD5⁺ T cell committed cells when compared with control transduced progenitor cells (Fig. 5C). In contrast, ectopic expression of E47, E12, or E2-2 slightly inhibited the number of developing T cells as compared with the GFP control transduced population. Analyzing later stages of T cell development revealed that HEB stimulated the generation of CD4⁺CD8⁺ DP T cells particularly in absolute cell numbers after 21 d of culture (Fig. 5D, 5E). Similarly, when HEB transduced thymic progenitor cells were allowed to differentiate in FTOC, T cell development was stimulated compared with control transduced cells (Supplemental Fig. 4). Cells transduced with a mutant form of HEB (DHEB, which lacks the DNA binding domain and as a consequence is deficient in transcription activation) resulted in similar percentages of T cell subsets compared with control FTOCs (Supplemental Fig. 4). This not only suggests that HEB is likely the most critical E protein that contributes to T cell development, but in addition implies that HEB may be the E protein antagonized by Id2/3 overexpression in our experiments described in our earlier report¹⁵ (Fig. 2).

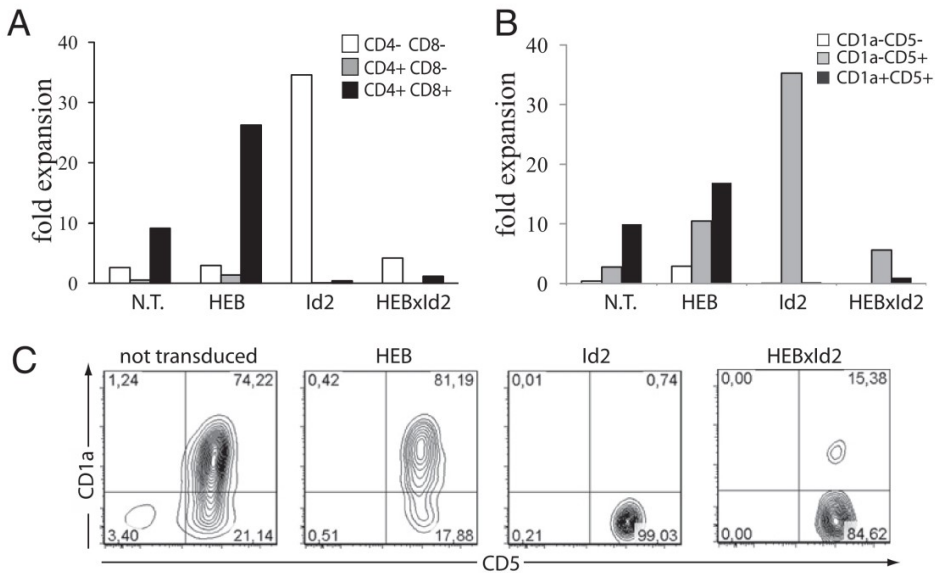


FIGURE 6. HEB inhibits Id2-induced expansion of NK cell progenitors.

CD34⁺CD1a⁻ thymic progenitor cells were double transduced with Id2-IRES-YFP and HEB-IRES-GFP. The cells were cocultured on OP9-DL1 cells with IL-7 and FLT3L. After 14 d, the cultures were analyzed for the presence of cells expressing (A) CD4 and CD8 (gated on CD56⁻ cells) and (B) CD1a and CD5 (gated on CD56⁻CD4⁻CD8⁻ cells). C, The fold expansion of the CD1a/CD5 subsets in the CD56⁻CD4⁻CD8⁻ transduced populations was calculated as described in the Materials and Methods. One representative experiment of four is shown.

HEB inhibits Id2-induced expansion of NK cell progenitors

Our study 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, we observed that HEB stimulated T cell development, whereas 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 cotransduced CD34⁺CD1a⁻ progenitors with HEB and Id2 or the appropriate control constructs. In line with our earlier observations (Fig. 2C, 2D), the single Id2-transduced progenitor cells gave rise to an increase in the number of CD4⁻CD8⁻ double negative cells (Fig. 6A, Supplemental Table III; 4/4 donors) of which the vast majority were CD1a⁻CD5⁺ progenitor cells (Fig. 6B, 6C, Supplemental Table IV; 4/4 donors) as compared with the control transduced cells. In addition, HEB alone stimulated T cell development (Fig. 6, Supplemental Tables III and IV; 4/4 donors and 3/4 donors, respectively), which confirms the results obtained in Fig. 5. Notably, forcing both HEB and Id2 expression reduced the number of Id2-expanded CD1a⁻CD5⁺ (CD4⁻CD8⁻) cells to background levels (Fig. 6B, 6C, Supplemental Tables III and IV; 4/4 donors and 4/4 donors, respectively). This suggests that the transcriptional inactivation of HEB through dimerization with Id2 is required for proper induction of the NK cell lineage program. HEB when overexpressed in thymic progenitor cells also inhibited the development of NK cells in FTOC supplemented with IL-15, suggesting that HEB prevents the IL-15 driven transition of progenitor cells into mature NK cell (Supplemental Fig. 4). Furthermore, we observed that the Id2-induced block in T cell development could not be rescued by HEB overexpression (Fig. 6), which makes it likely to assume that Id2 affects T cell development not only by inhibiting the transcriptional activity of HEB, but possibly also by repressing the function of other E proteins.

DISCUSSION

In this study, 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 progenitors¹⁵, and Id2 overexpression in mice both *in vivo*³⁷ and *in vitro*³⁸. On the other hand, Id, particularly in synergy with IL-15, increased the number of CD1a⁻CD5⁺ early human progenitor cells as well as mature NK cells. In agreement with these findings, NK cell development in mice deficient for Id2, IL-15, or IL-15 signaling components is impaired^{16,17,25}. Interestingly, the Id2-induced expansion of the NK progenitor cell pool could be counteracted by cotransduction of the bHLH factor HEB. This reflects our findings that expression of HEB, but also other E proteins, is lower in mature NK cells compared with T cell progenitor cells. Finally, we show in this study for the first time by direct comparison that HEB, in contrast to E12, E47, or E2-2, has a positive effect on the development of early human T cells.

Our results indicated that Id2 promoted the expansion of an early CD1a⁻CD5⁺ NK cell progenitor pool in the thymus. This is in line with other reports showing that Id2 has a role in promoting

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cell proliferation through direct regulation of the cell cycle³⁹⁻⁴¹. Because Id factors are known to sequester E proteins, we speculate that the Id2-induced expansion of CD1a⁻CD5⁺ progenitor cells is likely the result of inhibiting E protein activity. Contrary to what would be expected from our data shown in this report, thymic NK cell development was not inhibited in *Id2*-deficient mice¹⁸. Likely this may be explained by differences between mouse and human thymic NK cells. In mice all thymic NK cells expressed the IL-7R α chain/CD127^{18,34}, whereas, in contrast, only a minor fraction of NK cells in the human thymus expresses CD127. In line with this, we show in this study that the *in vitro* generated NK cells derived from Id2-transduced thymic progenitor cells and cultured in the presence of IL-15 did not express CD127. This could suggest that thymic NK cells that lack CD127 rely on Id2 for development and that the CD127-positive NK cells are a separate lineage, which develop independent of Id2. Although *Id2* deficiency in mice did not abrogate thymic NK cell development, it did impair the development of mature bone marrow NK cells, which is rescued by simultaneous deletion of the E2A locus¹⁸. In contrast, the numbers of mature NK cells in the blood or spleen were not restored in the *Id2/E2a* double-deficient mice¹⁸. Therefore, it was proposed that not E2A (i.e., E12 or E47), but alternative E proteins were suppressed by Id2 to allow NK cell development in the thymus. Based on our observation that HEB when overexpressed together with Id2 was able to counteract the expansion of human CD1a⁻CD5⁺ progenitor cells, it raises the possibility that downmodulation of HEB activity, but not E2A activity, by Id2 is required to permit NK cell development. Analysis of *Id2/Heb* double-deficient mice will be instrumental to confirm these findings in the mouse. Previously, we reported that Id3, when overexpressed in human progenitor cells and cultured in FTOC blocked T cell development, whereas strongly stimulating that of NK cells¹⁵. In this study, we show that Id2 similarly affected T and NK cell development in FTOC. Using OP9-DL1 feeder cells to analyze T cell development *in vitro*^{26,42}, we directly compared Id2 and Id3 and showed that both inhibited T cell development to a similar extent. These findings, together with the observation that Id2 is expressed at higher levels in thymic NK cells compared with Id3, and in addition that *Id3*-deficient mice did not have obvious defects in lymphoid development¹⁹, add weight to the notion that Id2 may be more physiologically relevant as an NK cell promoting factor. Interestingly, however, Id2-transduced cells cocultured with OP9-DL1 cells hardly affected the NK cell numbers relative to control transduced cells. At first, these findings seemed to contradict the results found when Id2- or Id3-transduced progenitor cells were cultured in FTOC¹⁵ (Fig. 2). An explanation to reconcile this discrepancy is the lack of IL-15 in the OP9 stromal cell cocultures. This is underscored by the finding that the addition of exogenous IL-15 to these cultures significantly induced the appearance of mature NK cells. Consistent with this, a crucial role for IL-15 in both human and murine NK cell development has been well documented^{20-23,43}. The exact stage in human NK cell development in which IL-15 exerts its effect has remained largely unclear. Attempts to isolate NKPs in humans on the basis of IL-15 responsiveness have been proven difficult as this approach reveals both NKPs and mature NK cells^{43,44}. Studies in mice genetically deficient for the expression of IL-15 revealed that IL-15 was required for the generation of mature NK cells, but not for the development of NKP cells²⁵. In agreement, we and others showed that IL-15 was able to stimulate mature human NK cell development (this study and²¹). In contrast, however, we observed that

IL-15 in synergy with Id2 was capable of promoting the expansion of CD1a⁻CD5⁺ NKP cells. These in vitro-generated cells are phenotypically similar to CD34⁺CD1a⁻CD5⁺ progenitor cells found in the thymus, and that, when isolated ex vivo, are responsive to IL-15. This, together with the observation that thymic stromal cells express IL-15 transcripts²⁴, underscores the notion that IL-15 may be involved in expanding human NK cells also in vivo. Collectively, our findings impose a novel role for IL-15 in human NK cell differentiation in addition to its known role in survival and expansion of mature NK cells. It is well accepted that engagement of IL-15 to its receptor, consisting of the common IL-2/15Rb and α subunits in addition to a unique IL-15R α chain, results in activation of several pathways, including the JAK/STAT pathway⁴⁵. It was unexpected, however, to observe that not only the thymic CD34⁺CD1a⁻CD5⁺ progenitor cells, but also CD34⁺CD1a⁺CD5⁺ thymocytes phosphorylated STAT5 after IL-15 stimulation. The CD34⁺CD1a⁺CD5⁺ thymocytes are considered to be T cell committed and have largely lost the capacity to differentiate into NK cells as they have initiated rearrangement of TCR-b genes^{1,6}. In the mouse, it was shown that low doses of IL-15 stimulated T cell development, particularly the TCR-g δ lineage²⁴. Also in humans, we observed that IL-15 at relatively low concentrations (,10 ng/ml) favored TCR- $\gamma\delta$ ⁺ T cell development over TCR- $\alpha\beta$ ⁺ T cell development in FTOC, although absolute T cell numbers were reduced (Y. Yasuda and H. Spits, unpublished observation). These data suggest that CD34⁺CD1a⁺CD5⁺ thymocytes, when exposed to IL-15 in the thymic microenvironment, might differentiate into TCR- $\gamma\delta$ ⁺ T cells. This would also imply that epithelial cells in the deep cortical region of the thymus, where the CD34⁺CD1a⁺CD5⁺ thymocytes reside, express IL-15. It will be of interest to investigate whether all cortical epithelial cells express IL-15 or that specific niches exist where TCR- $\gamma\delta$ lineage commitment is enforced.

Taken together, we propose a two-step model for NK cell lineage development: 1) Id2 expands the NKP pool and functionally impairs E proteins by sequestration, which results in a block in T cell development, and 2) further expansion of the NKP progenitor pool and subsequent differentiation into mature NK cells is driven by IL-15. In contrast, for T cell development to be released it is essential that Id2 levels are reduced, which then allows transcriptional activity of E proteins, in particular HEB.

ACKNOWLEDGMENTS

We thank Berend Hooijbrink for help with FACS sorting and maintenance of the FACS facility; Dr. M. Hazekamp and staff at the Leiden University Medical Center and the Amsterdam Medical Center for providing postnatal thymus tissue; and the staff of the animal facility of the Dutch Cancer Institute for excellent care of the animals.

DISCLOSURES

The authors have no financial conflicts of interest.

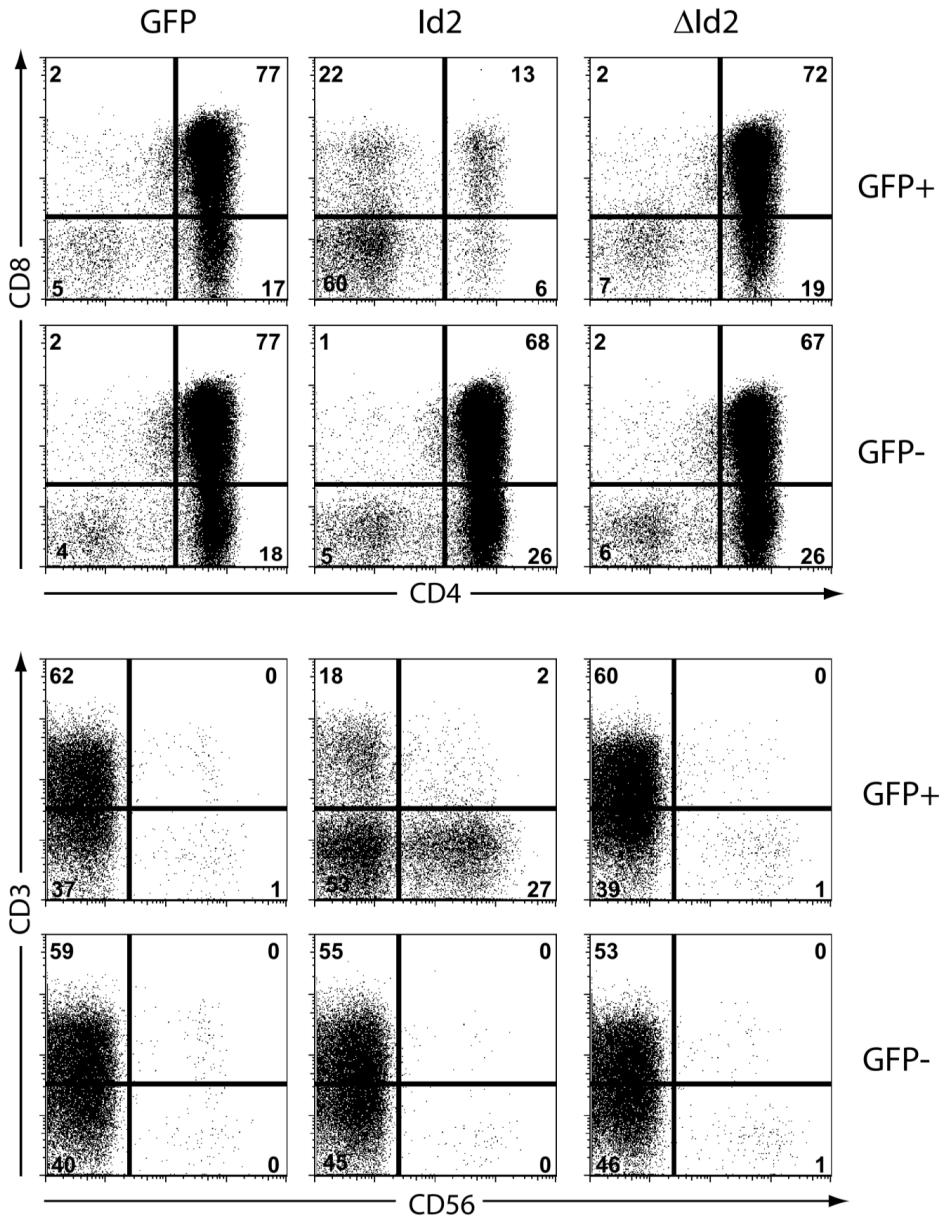
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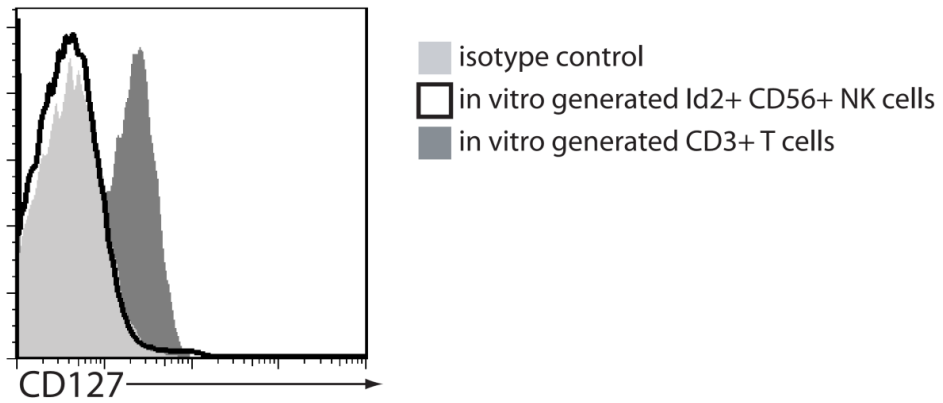
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Supplemental figure 1. Id2 blocks T cell development and stimulate NK cell development in a cell intrinsic manner.

CD34⁺CD1a⁻ thymic progenitor cells were transduced with retroviral vectors expressing Id2-IRES-GFP, ΔId2-IRES-GFP, or control IRES-GFP and cultured in FTOC. After 3 weeks the cultures were analyzed for expression of CD4/CD8 and CD3/CD56. Plots shown are electronically gated on transduced (GFP⁺) and non-transduced (GFP⁻) cells.

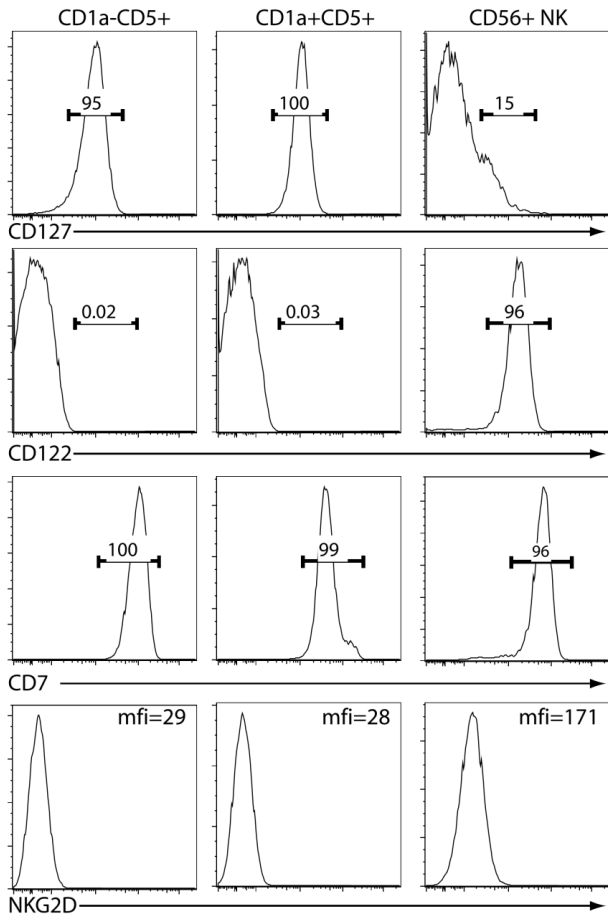


Supplemental figure 2. In vitro generated NK cells do not express CD127.

Id2-IRES-YFP or control-IRES-YFP transduced CD34⁺CD1a⁻ progenitors were co-cultured with OP9-DL1 cells supplemented with IL-7 and FLT3L in the absence or presence of 0.5 ng/ml of IL-15. YFP⁺CD3⁺ T and YFP⁺CD56⁺ NK cells were analysed for expression of CD127 at 3 weeks of culture.

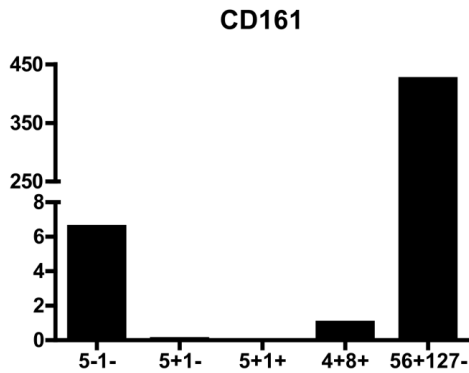
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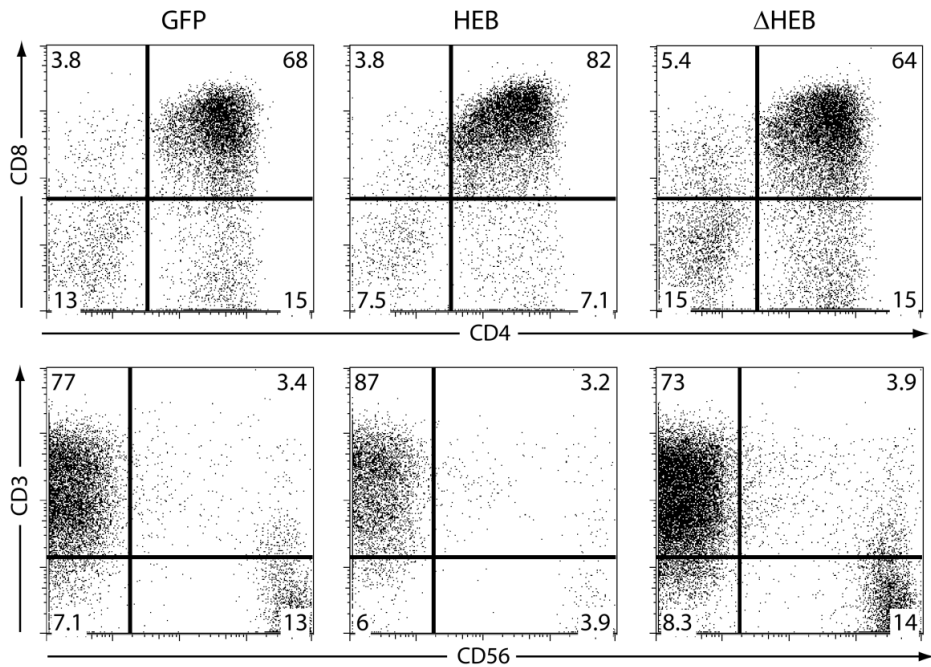
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Supplemental figure 3. Freshly isolated CD1a⁺CD5⁺ thymic progenitor cells do not express NK cell markers.

CD34-MACS and CD56-MACS enriched postnatal thymocytes were stained for indicated cell surface markers.



Supplemental figure 4. HEB inhibits IL-15 induced NK cell development.

CD34⁺CD1a⁻ thymic progenitor cells were transduced with retroviral vectors expressing HEB-IRES-GFP, Δ HEB-IRES-GFP, or control IRES-GFP and cultured in FTOC in the presence of 10 ng/ml IL-15. After 3 weeks the cultures were analyzed for expression of CD4/CD8 and CD3/CD56. Plots shown are electronically gated on GFP⁺ cells.

Supplemental table 1. IL-15 induces Id2 transduced progenitors to develop into NK cells

		2 weeks				3 weeks			
		without IL-15		with IL-15		without IL-15		with IL-15	
Exp		CD3+ CD56- T	CD3- CD56+ NK	CD3+ CD56- T	CD3- CD56+ NK	CD3+ CD56- T	CD3- CD56+ NK	CD3+ CD56- T	CD3- CD56+ NK
	1	GFP	3.2	0.7	3.2	8.3	n.d.		n.d.
Id2		0.4	10.0	1.0	189.0	n.d.		n.d.	
2	GFP	2.3	0.7	0.6	10.0	10.0	0.5	0.6	23.0
	Id2	0.2	2.1	0.6	30.0	0.2	1.0	0.5	75.0
3	GFP	7.0	0.0	2.0	12.0			1.3	22.0
	Id2	0.0	0.0	2.0	73.0			1.6	110.0
4	GFP	n.d.		n.d.		5.1	0.4	9.6	10.0
	Id2	n.d.		n.d.		1.1	0.7	2.3	33.0

The fold expression in absolute cell numbers in the GFP+ population (gated CD56-) were calculated as described in the Materials and methods section.

n.d. = not determined

Supplemental table 2. Id2 expands an IL-15 responsive CD1a⁻CD5⁺ progenitor subset

		2 weeks						3 weeks					
		without IL-15			with IL-15			without IL-15			with IL-15		
Exp		CD1a- CD5-	CD1a- CD5+	CD1a+ CD5+	CD1a- CD5-	CD1a- CD5+	CD1a+ CD5+	CD1a- CD5-	CD1a- CD5+	CD1a+ CD5+	CD1a- CD5-	CD1a- CD5+	CD1a+ CD5+
	1	GFP	4.6	15	106	9	11	94	0.4	1.5	19	n.d.	
Id2		6.4	200	17	93	664	36	0.4	10	0.9	n.d.		
2	GFP	2.2	7.1	0.3	2.7	10	1	7.9	39	1.4	2.3	19	1.4
	Id2	2.5	51	2	7.9	110	2.9	4.3	126	6.4	17	256	3.9
3	GFP	1.2	3.1	6.7	3.8	7.1	2.8	1.7	7	15	7	18	1.2
	Id2	0.3	8.2	0.2	16	84	1.6	0.2	11	0.4	32	163	6.7

The fold expression in absolute cell numbers in the GFP+ population (gated CD56-CD4-CD8-) were calculated as described in the Materials and methods section.

n.d. = not determined

Chapter 3

Supplemental table 3. HEB does not rescue Id2 induced block on T cell development

Exp.		2 weeks			3 weeks		
		CD4-CD8-	CD4+CD8-	CD4+CD8+	CD4-CD8-	CD4+CD8-	CD4+CD8+
1	not	2.6	0.5	9.1	0.8	0.1	1.4
	HEB/GFP	3	1.4	26	1.5	0.2	2.4
	Id2/YFP	35	0.1	0.4	51	1	0
	HEB/GFP x Id2/YFP	4.2	0	1.2	0	0	0
2	not	1.2	0.6	2.3	1.1	0.7	5.7
	HEB/GFP	0.3	0.3	1.9	0.1	3.5	7
	Id2/YFP	12.5	0.1	0.2	1.7	0.2	0.5
	HEB/GFP x Id2/YFP	1.7	0	0.1	2.5	0.3	0.9
3	not	2.2	0.7	1.8	0.9	0.1	0.8
	HEB/GFP	1.4	0.4	2.2	0.8	0	1.3
	Id2/YFP	4.4	0.2	0.1	5.2	0	0
	HEB/GFP x Id2/YFP	1.9	0.1	0.1	0.2	0	0
4	not	0.8	0.8	0.9			
	HEB/GFP	1.4	0.9	1.2		n.d.	
	Id2/YFP	2.2	0.4	0.1			
	HEB/GFP x Id2/YFP	2.1	0.7	0.3			

The fold expression in absolute cell numbers in the different transduced populations (gated CD56-) were calculated as described in the Materials and methods section.

n.d. = not determined

Supplemental table 4. HEB inhibits Id2 induced expansion of CD1a⁻CD5⁺ subset

Exp.		2 weeks			3 weeks		
		CD1a-CD5-	CD1a-CD5+	CD1a+CD5+	CD1a-CD5-	CD1a-CD5+	CD1a+CD5+
1	not	0.0	3.0	10.0	1.0	1.0	1.0
	HEB/GFP	3.0	10.0	17.0	1.0	3.0	2.0
	Id2/YFP	0.0	35.0	0.0	0.0	52.0	0.0
	HEB/GFP x Id2/YFP	0.0	6.0	1.0	0.0	0.0	0.0
2	not	0.8	1.6	1.7	0.2	0.4	9.3
	HEB/GFP	0.6	0.8	0.9	0.6	3.0	8.8
	Id2/YFP	0.2	12.5	0.2	0.3	2.1	0.3
	HEB/GFP x Id2/YFP	0.1	1.8	0.1	0.0	0.0	0.0
3	not	0.3	1.8	3.3	1.1	0.6	0.2
	HEB/GFP	0.1	2.0	2.4	0.3	1.7	0.4
	Id2/YFP	0.0	1.5	0.1	0.1	5.3	0.0
	HEB/GFP x Id2/YFP	0.0	2.0	0.1	0.1	0.1	0.0
4	not	0.5	1.2	0.9			
	HEB/GFP	2.3	0.6	0.6		n.d.	
	Id2/YFP	0.3	2.4	0.1			
	HEB/GFP x Id2/YFP	0.7	1.1	0.4			

The fold expression in absolute cell numbers in the different transduced populations (gated CD56-CD4-CD8-) were calculated as described in the Materials and methods section.

n.d. = not determined