

## **Inhibition of T Cell and Promotion of Natural Killer Cell Development by the Dominant Negative Helix Loop Helix Factor Id3**

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### **Summary**

Bipotential T/natural killer (NK) progenitor cells are present in the human thymus. Despite their bipotential capacity, these progenitors develop predominantly to T cells in the thymus. The mechanisms controlling this developmental choice are unknown. Here we present evidence that a member(s) of the family of basic helix loop helix (bHLH) transcription factors determines lineage specification of NK/T cell progenitors. The natural dominant negative HLH factor Id3, which blocks transcriptional activity of a number of known bHLH factors, was expressed in CD34<sup>+</sup> progenitor cells by retrovirus-mediated gene transfer. Constitutive expression of Id3 completely blocks development of CD34<sup>+</sup> cells into T cells in a fetal thymic organ culture (FTOC). In contrast, development into NK cells in an FTOC is enhanced. Thus, the activity of a bHLH transcription factor is necessary for T lineage differentiation of bipotential precursors, in the absence of which a default pathway leading to NK cell development is chosen. Our results identify a molecular switch for lineage specification in early lymphoid precursors of humans.

The earliest progenitor cells in the thymus have the capacity of developing into multiple hematopoietic lineages. Upon further development within the thymus, these progenitor cells progressively lose their multipotentiality (1). There is considerable evidence that the human (2) and the mouse thymuses (3, 4) contain bipotential T/NK progenitors. However, given the paucity of mature NK cells in the thymus, the thymic environment strongly favors development of these bipotential T/NK progenitor cells into T cells. The molecular mechanisms controlling T/NK lineage specification in the thymus are not yet elucidated, but it seems fair to assume that this developmental choice is under transcriptional control.

Several transcription factors that orchestrate lymphoid development have now been identified in the mouse. Ikaros is a key factor that affects development of all lymphoid (T, NK, and B) cells (5). Several other factors appear to be critical for development of specific lymphoid lineages like GATA-3 (6) and TCF-1 (7) for T cells and Pax5 (8), EBF (9), Sox4 (10), and the products of the E2A gene (11, 12) for B cells. The E2A proteins E12 and E47 belong to the family of basic helix loop helix (bHLH) transcription factors, which are involved in differentiation of many cell types

(13). E12 and E47 probably regulate B cell development by controlling expression of immunoglobulin, RAG, and a number of B cell-specific genes including mb-1,  $\lambda$ 5, CD19, and Pax5 (11, 12, 14). Transcriptional activity of bHLH factors is controlled by the inhibitor of DNA binding (Id) proteins. This family of HLH factors comprises four members, Id1, 2, 3, and 4, which are highly homologous in their HLH domains and have distinct tissue distributions (15, 16). Id factors can heterodimerize with bHLH factors, but lack a basic DNA binding domain, and therefore they block transcriptional activity by bHLH factors. Constitutive overexpression of Id1 under control of a B cell-specific mb-1 promoter leads to inhibition of B cell development (17), similar to the one observed in E2A-deficient animals (11, 12).

Recent evidence has suggested a role for bHLH factors in development of T cells as well. E2A-deficient mice were initially reported to have no gross abnormalities in T cell development, but the size of the thymus is smaller than that of wild-type animals (12). Mice deficient for another bHLH factor, HEB, displayed a partial block in T cell development at an early stage of development (18). Whether HEB and E2A deficiencies affect NK cell development is not known.

In this paper we have investigated the role of bHLH factors in human T and NK cell development. We have made use of retrovirus-mediated gene transfer to enforce expression of one of the Id proteins, Id3, in CD34<sup>+</sup> progenitors using a bicistronic vector harboring Id3 and the marker gene green fluorescent protein (GFP). Id3 can interact with a wide range of bHLH factors and blocks transcriptional activity of E12, E47, and HEB (19). We found that enforced expression of Id3 strongly inhibits development of CD34<sup>+</sup> progenitors to CD3<sup>+</sup> T cells in an in vitro fetal thymic organ culture (FTOC), but promotes development of NK cells.

## Materials and Methods

**Construction of the Vectors and Transduction of Target Cells.** Previously we have successfully used bicistronic vectors with a gene of interest linked to a downstream internal ribosomal entry site (IRES) and a marker gene that allow independent translation of the products of both genes in the transduced target cells (20). The IRES-GFP sequence was ligated into the LZRS vector (21), and a polylinker was placed downstream of the gag and upstream of the IRES sequences. The Id3 coding sequence was cloned from the pCDNAId3 (gift of Dr. C. Murre, University of California at San Diego, San Diego, CA) plasmid by PCR using oligonucleotide primers with appropriate linkers. The product was ligated between the XhoI and SnaBI site of the polylinker from our plasmid LZRS-linker-IRES-GFP, to obtain the retroviral vector LZRS-Id3-IRES-GFP. (A modified version of GFP [enhanced GFP] was used in this study and was obtained from Clontech, Palo Alto, CA.) Helper-free recombinant retrovirus was produced after transfection into a 293T-based amphotropic retroviral packaging cell line, Phoenix (21). Purified CD34<sup>+</sup> cells were cultured for 24 h in the presence of 10 ng/ml human IL-7 and 10 ng/ml stem cell factor (SCF) (both from R&D, Abingdon, UK). The cells were then transduced by overnight incubation with virus supernatant in the presence of 10  $\mu$ g/ml Dotap (Boehringer Mannheim GmbH, Mannheim, Germany).

**Isolation of CD34<sup>+</sup> Cells from Fetal Liver and Postnatal Thymus.** Fetal liver was obtained from elective therapeutic abortions. Gestational age was determined by crown-rump length and ranged from 14 to 17 wk. The use of this tissue was approved by the medical ethical committee of the Netherlands Cancer Institute and was contingent upon informed consent. Human fetal liver cells were isolated by gentle disruption of the tissue by mechanical means, followed by density gradient centrifugation over Ficoll-Hypaque (Lymphoprep; Nycomed Pharma, Oslo, Norway). The CD34<sup>+</sup> cells were isolated from these samples by immunomagnetic cell sorting, using a CD34 separation kit (varioMACS; Miltenyi Biotec, Bergisch Gladbach, Germany) and were further purified by sorting with a FACStar plus<sup>®</sup> (Becton Dickinson, San José, CA). Postnatal thymus samples were from children undergoing open-heart surgery. CD34<sup>+</sup> cells, enriched by immunomagnetic cell sorting, were labeled with anti-CD34 (HPCA-2; Becton Dickinson) and anti-CD1a (Coulter, Hialeah, FL) antibodies and the CD34<sup>+</sup>CD1a<sup>-</sup> cells were purified by FACS<sup>®</sup> sorting. The purity of the populations used in this study was >99%.

**Fetal Thymic Organ Cultures.** In vitro development of human T and NK cells from CD34<sup>+</sup> fetal liver cells or thymocytes was studied using the hybrid human/mouse FTOC using fetal thymuses from embryos of RAG-1-deficient mice as described (22). TCR- $\gamma/\delta$ <sup>+</sup> and NK cells were expanded in Ysells medium (23)

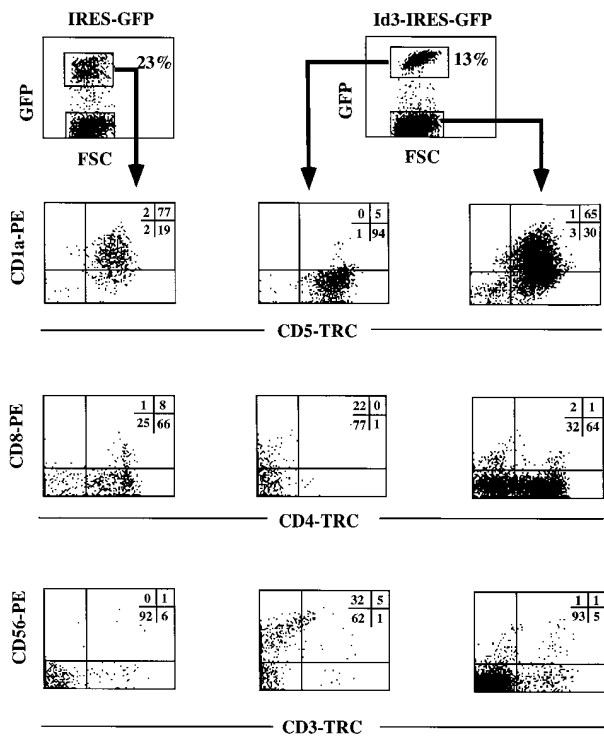
from the FTOC cultures using a feeder cell mixture as described previously (24).

**NK Cell Assays.** 10,000 CD34<sup>+</sup> sorted cells were cultured in Ysells medium in the presence of 10 ng/ml SCF, 10 ng/ml IL-7, and 100 U/ml IL-2 (Eurocetus, Amsterdam, Netherlands). After 1 wk, cells were harvested, counted, and analyzed by FACS<sup>®</sup>, using anti-CD3 and anti-CD56 (provided by Dr. J.H. Phillips, DNAX, Palo Alto, CA). The cytotoxic activity of NK cells was determined with a standard <sup>51</sup>Cr-release assay using cells of NK-sensitive (K562) and insensitive (the EBV-transformed B cell line EBV225) cell lines as target cells.

**Analysis of D-J $\beta$  Rearrangements.** D-J $\beta$  rearrangements were determined by PCR as described previously (25). Primers and probes used for TCR- $\beta$  rearrangement were: 5'-TG GTGGTC-TCTCCAGGCTCT-3' (D $\beta$ 1.1), 5'-CCAGCTGTCCAGC-CTTGACTT-3' (J $\beta$ 1.3-1.4) and 5'-CAAAGCTGTAACAT-TGTGGGGAC-3' (D $\beta$ <sub>pan</sub> probe). To control for the amount of DNA in the PCR, genomic amplification of the RAG-2 gene was performed. Primers and probe used were: 5'-TGTGAATTGCA-CAGTCTTGCCAGG-3' (RAG-2 sense), 5'-GGGTTTGT-TGAGCTCAGTTGAATAG-3' (RAG-2 antisense), and 5'-CAAGATATGGTTTGGGAAGCAACATGGGAAA-3' (RAG-2 probe).

## Results and Discussion

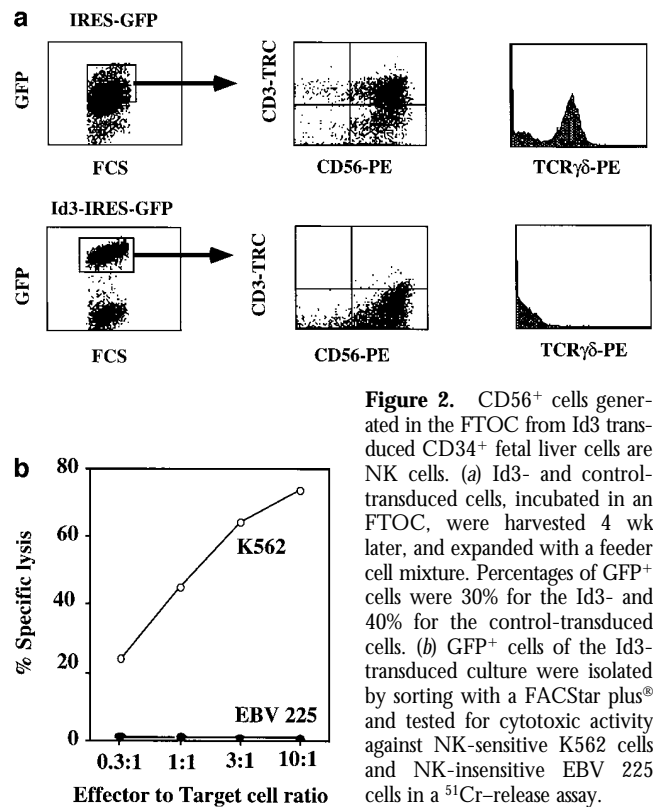
**Enforced Expression of Id3 Inhibits Development of CD1a<sup>-</sup>CD34<sup>+</sup> Thymocytes, but Promotes NK Development in an FTOC.** To study the role of bHLH factors in T cell development, we overexpressed the dominant negative HLH protein Id3 in T cell progenitors and monitored the fate of the transduced cells. Id3 has a very broad tissue distribution and CD34<sup>+</sup> fetal liver and thymic progenitor cells express Id3 messenger RNA as determined by reverse transcription PCR (results not shown). Purified CD34<sup>+</sup> fetal liver cells were cultured with a combination of SCF and IL-7 for 24 h. The cells were then transduced by overnight coculture with supernatants of packaging cells producing recombinant viruses harboring either Id3-IRES-GFP or IRES-GFP, and cultured in an FTOC for 4 wk (22). The flow cytometric analysis shown in Fig. 1 demonstrates that the GFP marker was transferred to 23% of the progeny of the IRES-GFP and 13% of the Id3-IRES-GFP-transduced progenitor cells. The patterns of CD3, CD4, and CD5 stainings of the GFP<sup>+</sup> cells from the IRES-GFP-transduced cells are identical to that of the nontransduced GFP<sup>-</sup> cells (Fig. 1). The great majority of the GFP<sup>+</sup> cells express CD1a, and very few CD56<sup>+</sup> cells were present in these samples. By contrast, cells harvested from the FTOC with Id3-IRES-GFP-transduced fetal liver cells did not express CD1a, and 32% of these cells were positive for the NK cell marker CD56. Almost no CD3<sup>+</sup> cells were observed (Fig. 1). Not only the proportions of CD56<sup>+</sup> cells were different, but also the absolute numbers since >30-fold more GFP<sup>+</sup>CD56<sup>+</sup> cells were found in the FTOC, populated with Id3-transduced CD34<sup>+</sup> cells. These data indicate that CD34<sup>+</sup> fetal liver cells, overexpressing Id3, develop preferentially into CD3<sup>-</sup>CD56<sup>+</sup> cells in the FTOC. It was important to ensure that these GFP<sup>+</sup>CD3<sup>-</sup>CD56<sup>+</sup> cells represent functional NK cells. To obtain enough cells for testing their cy-



**Figure 1.** Overexpression of Id3 inhibits generation of T cells and promotes development of NK cells from CD34<sup>+</sup> fetal liver cells. CD34<sup>+</sup> cells were isolated, transduced with viruses harboring IRES-GFP or Id3-IRES-GFP, and incubated with mouse thymic lobes at 10<sup>4</sup> cells/lobe for 4 wk. Cell suspensions were stained with the indicated TriColor (TRC) and PE-labeled antibodies and analyzed on a FACScan<sup>®</sup>. Cell recoveries of both samples were 3 × 10<sup>4</sup> cells/lobe. Percentages of transduced cells 2 d after transduction were 25% in both control- and Id3-transduced samples.

tolytic activity, we cultured the cells harvested from the FTOC with a feeder mixture consisting of irradiated PBMCs, the EBV cell line JY, PHA and IL-2. Under these conditions we can expand NK cells and TCR- $\gamma/\delta$ , but not TCR- $\alpha/\beta$  cells from an FTOC (24); the failure to obtain TCR- $\alpha/\beta$  cells is because the mouse thymic environment is unable to induce functional maturation in these cells (24). Fig. 2 demonstrates that the GFP<sup>+</sup> cells expanded from the control FTOC contained GFP<sup>+</sup> TCR  $\gamma/\delta$ <sup>+</sup> cells and a few CD56<sup>+</sup>CD3<sup>-</sup> cells. By contrast, GFP<sup>+</sup> cells expanded from the FTOC with the Id3<sup>+</sup> progenitor cells were CD56<sup>+</sup> and did not express CD3, underscoring the strong inhibitory effect of Id3 on generation of TCR- $\gamma/\delta$ <sup>+</sup> cells. The GFP<sup>+</sup>CD3<sup>-</sup>CD56<sup>+</sup> cells were sorted from the Id3-IRES-GFP cultures and were shown to be highly cytotoxic for the NK sensitive target cell K562, but not for an NK-resistant cell line (Fig. 2 b). These results confirm that the CD56<sup>+</sup>CD3<sup>-</sup>GFP<sup>+</sup> cells observed in these FTOC are functional NK cells.

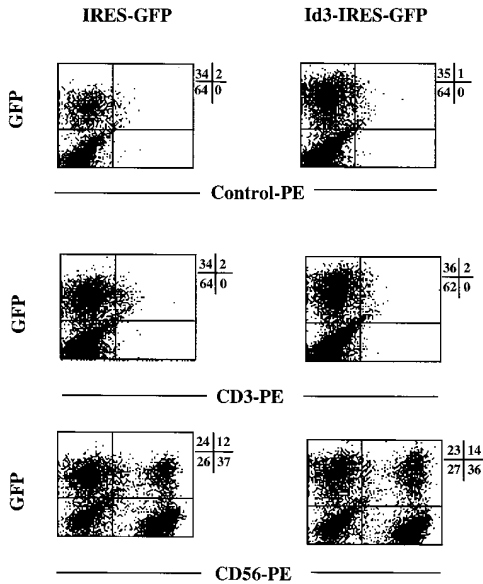
The observation that the proportion of CD56<sup>+</sup> cells in the Id3-GFP<sup>+</sup> progeny is much higher than in the control GFP progeny and in the Id3-GFP<sup>-</sup> cells in the FTOC is consistent with the notion that Id3 overexpression switches the cell fate of the bipotential thymic T/NK progenitors. An alternative possibility is that overexpression of Id3 con-



**Figure 2.** CD56<sup>+</sup> cells generated in the FTOC from Id3 transduced CD34<sup>+</sup> fetal liver cells are NK cells. (a) Id3- and control-transduced cells, incubated in an FTOC, were harvested 4 wk later, and expanded with a feeder cell mixture. Percentages of GFP<sup>+</sup> cells were 30% for the Id3- and 40% for the control-transduced cells. (b) GFP<sup>+</sup> cells of the Id3-transduced culture were isolated by sorting with a FACStar plus<sup>®</sup> and tested for cytotoxic activity against NK-sensitive K562 cells and NK-insensitive EBV 225 cells in a <sup>51</sup>Cr-release assay.

fers a growth or survival advantage to developing NK cells resulting in an overrepresentation of these cells in the FTOC. To investigate this, we studied the effect of Id3 transduction on development of CD34<sup>+</sup> fetal liver cells into NK cells in a mixture of SCF, IL-7, and IL-2 (2). All cells generated from transduced CD34<sup>+</sup> fetal liver cells and cultured with the cytokines for 2 wk were CD3<sup>-</sup> (not shown). In both Id3-IRES-GFP and the IRES-GFP cultures, we observed the same proportions of GFP<sup>+</sup> cells (35%) and an identical distribution of CD56 in the IRES-GFP<sup>+</sup> and the Id3-IRES-GFP<sup>+</sup> cells (Fig. 3). Thus, constitutive expression of Id3 in the CD34<sup>+</sup> fetal liver cells does not significantly enhance the percentage of NK cells that develop under the influence of SCF, IL-7, and IL-2. This result makes it unlikely that the increase of NK cell numbers observed in the FTOC with Id3<sup>+</sup> progenitor cells is caused by improved survival or proliferation by Id3 overexpression.

TCR- $\beta$  rearrangements are initiated in the CD1a<sup>+</sup>CD3<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup>CD34<sup>+</sup> cells (25, 26). Moreover, CD1a<sup>+</sup> cells are unable to develop into NK cells (2). This suggests that upregulation of CD1a is correlated with T cell commitment. If this is correct and if overexpression of Id3 inhibits T cell commitment, we should expect that CD1a<sup>-</sup>CD34<sup>+</sup> cells transduced with Id3 fail to develop into T cells in an FTOC. Purified CD1a<sup>-</sup>CD34<sup>+</sup> cells were cultured with SCF and IL-7, transduced, and incubated in an FTOC for 3 wk. Fig. 4 demonstrates that the control-transduced cells developed normally. By contrast, the proportion of CD3<sup>+</sup> cells that developed from Id3-transduced CD1a<sup>-</sup>

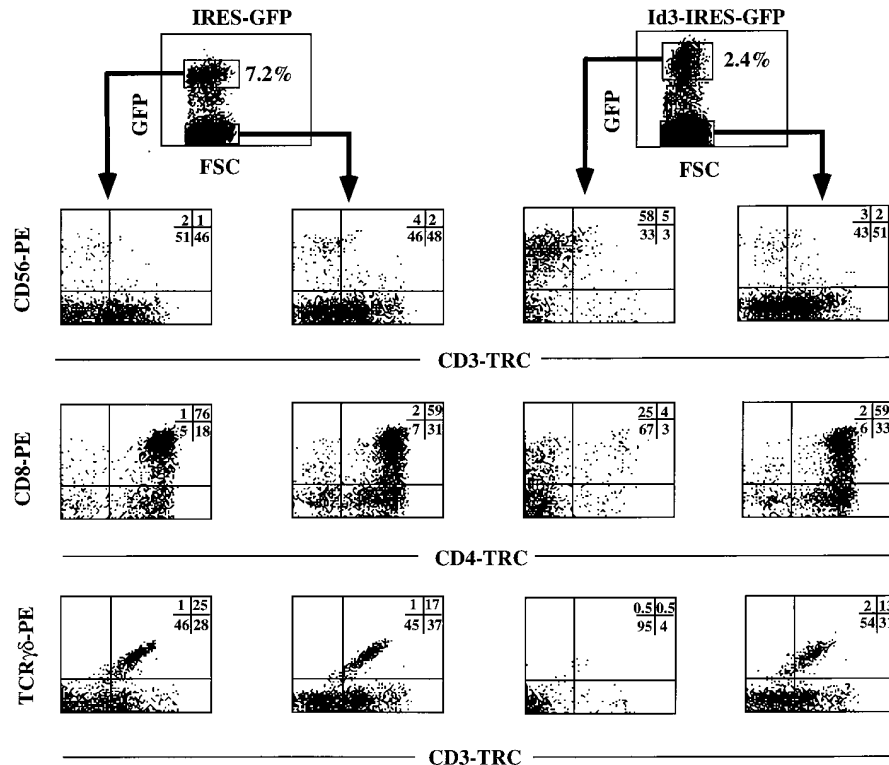


**Figure 3.** Overexpression of Id3 does not affect NK development and expansion of CD34<sup>+</sup> fetal liver cells incubated in IL-2, IL-7, and SCF. CD34<sup>+</sup> fetal liver cells were isolated, transduced, and incubated with the cytokines for 14 d.

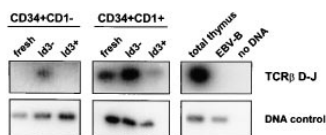
CD34<sup>+</sup> cells is strongly reduced, whereas the proportion of NK cells is dramatically increased. The absolute numbers of GFP<sup>+</sup> NK cells in the Id3-transduced samples was 10-fold higher than in the GFP<sup>+</sup> cells in the control-transduced samples. The inhibition of generation of CD3<sup>+</sup> by Id3 is

underscored by the almost complete absence of CD4<sup>+</sup> and CD4<sup>+</sup>CD8<sup>+</sup> in the cells expressing Id3-GFP. The CD8<sup>+</sup> cells observed in these cultures are CD3<sup>-</sup>CD56<sup>+</sup> cells. The Id3-IRES-GFP<sup>+</sup>CD3<sup>-</sup>CD56<sup>+</sup> could be expanded with a feeder cell mixture and displayed cytolytic activity against K562 cells (results not shown).

**Enforced Expression of Id3 Inhibits Induction of D-J $\beta$  Rearrangement by IL-7 and SCF.** T cells are defined by TCR gene rearrangements; in NK cells, these genes are in the germ line configuration (1). The fact that Id3 inhibits generation of CD3<sup>+</sup> cells and stimulates that of NK cells, raises the possibility that bHLH factors are required for processes that result in TCR rearrangements. To test this, we cultured transduced CD1a<sup>-</sup>CD34<sup>+</sup> and CD1a<sup>+</sup>CD34<sup>+</sup> cells for 7 d in IL-7 and SCF, sorted GFP<sup>+</sup> and GFP<sup>-</sup> cells, and analyzed for the presence of D-J $\beta$  rearrangements with a sensitive PCR technique (25). Fig. 5 shows that Id3 completely blocked IL-7 and SCF-mediated induction of D-J $\beta$  rearrangement in CD1a<sup>-</sup>CD34<sup>+</sup> cells. One trivial explanation for this observation is that SCF and IL-7 induce growth of a very small population of contaminating CD1a<sup>+</sup>CD34<sup>+</sup> cells that have already undergone D-J $\beta$  rearrangements; overexpression of Id3 could inhibit the growth of these contaminating cells. This is unlikely because D-J $\beta$  rearrangements were still detectable in Id3-transduced CD1a<sup>+</sup>CD34<sup>+</sup> cells recovered after 7 d of culture (Fig. 5). The ratios of the intensities of the bands hybridized with the D-J $\beta$  and genomic DNA control (RAG-2) probes in the starting CD1a<sup>+</sup>CD34<sup>+</sup> (lane 4) and in the Id3-CD1a<sup>+</sup>CD34<sup>+</sup> (lane 6) populations were the same. This ratio is increased in the untransduced CD1a<sup>+</sup>CD34<sup>+</sup> cells. These data indicate that



**Figure 4.** Enforced expression of Id3 in CD1a<sup>-</sup>CD34<sup>+</sup> thymocytes blocks T cell development and promotes NK cell development in an FTOC. CD1a<sup>-</sup>CD34<sup>+</sup> thymocytes were isolated from a thymic fragment of a 6-mo-old child, transduced and incubated in a FTOC for 4 wk at 10<sup>4</sup> cells/lobe. Cell recoveries were 5 × 10<sup>4</sup> and 6 × 10<sup>4</sup> cells/lobe for the Id3- and the control-transduced cells, respectively. Percentages of GFP<sup>+</sup> cells 2 d after transduction were 9% in both samples. Percentages of GFP<sup>+</sup> cells, harvested from the FTOC, were 7.2% in the control and 2.4% in the Id3-transduced samples.



**Figure 5.** Enforced expression of Id3 in CD34<sup>+</sup>CD1<sup>-</sup> thymocytes inhibits induction of D-J $\beta$  rearrangements after incubation in IL-7 and SCF. CD1a<sup>-</sup> and CD1a<sup>+</sup>, CD34<sup>+</sup> cells were

isolated, transduced, and incubated for 5 d in IL-7 plus SCF. After the incubation, GFP<sup>+</sup> and GFP<sup>-</sup> cells were isolated from both samples using a FACStar<sup>®</sup>. Based on GFP expression, the purity of the sorted populations was >99%. DNA was prepared after the sorting and analyzed for the presence of D-J $\beta$  rearrangements by PCR. The intensities of the hybridized band were determined with a phosphorimager (Fujix Bas 2000; Raytest Benelux BV, Straubenherdt, FRG). The ratios of D-J/genomic control were: lane 1, 0; lane 2, 0.0025; lane 3, 0; lane 4, 0.035; lane 5, 0.062; and lane 6, 0.028.

Id3 inhibits the IL-7 and SCF-mediated increase DJ $\beta$  rearrangements in CD1a<sup>+</sup>CD34<sup>+</sup> cells. It appears, therefore, that Id3 overexpression inhibits induction of D-J $\beta$  rearrangement. It remains to be determined whether the rearrangement process itself is blocked or whether an earlier step is inhibited. Our results raise the possibility that the SCF receptor (ckit) and/or the IL-7R communicate with bHLH factors as suggested previously (14).

*The Role of bHLH Factors and Id Proteins in T Cell Development.* The results presented here indicate that bHLH proteins are involved in T cell commitment. The critical role of these factors in T cell commitment is comparable to that of E2A proteins in commitment of B cells (14) and emphasizes the similarities of the mechanisms of early B and T cell development (27). Several bHLH factors, including E2A, HEB, and E2-2, can be inhibited in their action by overex-

pression of Id3. Mice with a targeted E2A null mutation that have no B cells were initially reported to have no apparent gross abnormalities in distribution of double negative (DN), double positive (DP), and mature thymocytes; however, the thymi of these mice are relatively small (11, 12). A more recent analysis of the effect of E2A deficiency on T cell development has revealed that the proportion of DP cells in the thymus of E2A<sup>-/-</sup> mice is lower than in wild-type or heterozygous E2A<sup>-/+</sup> mice. Within the DN population, an accumulation of CD44<sup>+</sup>CD25<sup>-</sup> cells and relatively few CD44<sup>low</sup>CD25<sup>+</sup> cells was observed (28). Thus it appears that E2A deficiency results in a partial inhibition of the CD25<sup>-</sup> to CD25<sup>+</sup> transition. Most interestingly, this transition is equivalent to the CD1a<sup>-</sup> to CD1a<sup>+</sup> transition in humans (Blom, B., P.C.M. Res, and H. Spits, manuscript in preparation) that is blocked by Id3. HEB may be another target for Id3. Zhuang et al. observed accumulation of DN and less DP thymocytes in the thymus of HEB<sup>-/-</sup> mice (18). Expression of the CD3-TCR complex on the thymocytes and spleen cells of the HEB-deficient mice was not analyzed, leaving the developmental stage that is blocked unresolved. It is possible that HEB, E2A, and perhaps other bHLH factors collaborate in inducing T cell development, and that a complete inhibition ensues when these factors are simultaneously inhibited by overexpression of Id3. Thus, T cell development may be dictated by a combined dosage of several bHLH factors. It is in this respect noteworthy that B cell development appears to be regulated by the combined dosage of E2A, HEB, and E2-2 proteins (18). Future studies should provide more exact information about which bHLH factors are critical for T cell development and which genes are controlled by these factors.

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