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Disruption of αβ but not of γδ T cell development by overexpression of the helix–loop–helix protein Id3 in committed T cell progenitors

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Enforced expression of Id3, which has the capacity to inhibit many basic helix–loop–helix (bHLH) transcription factors, in human CD34$^+$ hematopoietic progenitor cells that have not undergone T cell receptor (TCR) gene rearrangements inhibits development of the transduced cells into TCRαβ and γδ cells in a fetal thymic organ culture (FTOC). Here we document that overexpression of Id3, in progenitors that have initiated TCR gene rearrangements (pre-T cells), inhibits development into TCRαβ but not into TCRγδ T cells. Furthermore, Id3 impedes expression of recombination activating genes and downregulates pre-Tα mRNA. These observations suggest possible mechanisms by which Id3 overexpression can differentially affect development of pre-T cells into TCRαβ and γδ cells. We also observed that cell surface CD4$^-$CD8$^-$CD3$^-$ cells with rearranged TCR genes developed from Id3-transduced but not from control-transduced pre-T cells in an FTOC. These cells had properties of both natural killer (NK) and pre-T cells. These findings suggest that bHLH factors are required to control T cell development after the T/NK developmental checkpoint.

Keywords: basic helix–loop–helix protein/NK cell/T cells/thymus/transcription

Introduction

During the period of T cell differentiation in the thymus, there is a continuous influx of blood-borne hematopoietic progenitors (Scollay *et al.*, 1986; Donskoy and Goldschneider, 1992). While the exact identity of the thymic immigrants has yet to be determined, studies on the most primitive hematopoietic progenitor cells in both human and mouse thymus strongly suggest that these cells are not committed to the T cell lineage (Spits *et al.*, 1995; Shortman and Wu, 1996; Blom *et al.*, 1998). The earliest hematopoietic progenitor cells in the human thymus express high levels of CD34, lack CD1a (Figure 1) and have the capacity to develop into natural killer (NK) cells and dendritic cells (Sanchez *et al.*, 1994; Marquez *et al.*, 1995; Res *et al.*, 1996). CD1a$^-$CD34$^+$ cells have their γ genes in the germline configuration but contain immature (D–D and D–J) T cell receptor δ (TCRδ) gene rearrangements (Blom *et al.*, 1999). Upregulation of CD1a on CD34$^+$ cells is correlated with induction of T cell commitment as mature TCRδ rearrangements are present and TCRγ and TCRβ rearrangements commence at the CD1a$^+$CD34$^+$ stage (Ramiro *et al.*, 1996; Blom *et al.*, 1997, 1999). These cells develop into CD3$^-$CD4$^+$CD8$^-$, denoted CD4 immature single positive (ISP), cells (Galy *et al.*, 1993; Kraft *et al.*, 1993). CD4 ISP cells are fully committed to the T cell lineage as they are unable to differentiate into cells other than T cells. The great majority if not all of the CD4 ISP cells contain mature TCRδ rearrangements, and ~35% of the TCRγ alleles are rearranged in these cells. However, the majority of the TCRβ alleles (85%) of the CD4 ISP cells are still in the germlike configuration (Blom *et al.*, 1999). The hierarchy in TCR gene rearrangements found by Blom *et al.* is consistent with a recent analysis of in- and out-of-frame TCRβ and δ rearrangements, indicating that in most humans the TCRδ locus rearranges before β (Margolis *et al.*, 1997). However, the latter study also suggested that in some individuals the TCRδ locus rearranges concurrently with TCRβ. The sequence of various developmental cellular stages and the hierarchy of TCR gene rearrangements during T cell development in man and mice are similar (Shortman and Wu, 1996; Capone *et al.*, 1998; Spits *et al.*, 1998).

When developing into T cells, the progenitor cells should pass a number of clearly defined checkpoints (Godfrey and Zlotnik, 1993; Shortman and Wu, 1996; Blom *et al.*, 1998). The first one is the T/NK checkpoint when progenitor cells commit to the T cell lineage. This should occur at or around the transition of CD1a$^-$CD34$^+$ into CD1a$^+$CD34$^+$ cells. A second checkpoint is when the pre-T cell chooses to become either a TCRαβ or a TCRγδ cell; the cellular stage where this happens in the human thymus has yet to be determined. The mechanisms of the T/NK and TCRαβ/γδ lineage choices are as yet poorly understood. Recent studies in mouse and human systems have provided evidence that basic helix–loop–helix (bHLH) transcription factors are involved in induction of commitment to the T cell lineage at the T/NK checkpoint (Bain *et al.*, 1997a; Heemskerk *et al.*, 1997). bHLH factors are well characterized and have been shown to be important regulators of lineage determination and cell type specification in many organisms (Murre *et al.*, 1994). These proteins are characterized by two highly conserved bipartite domains. An HLH domain, mainly consisting of hydrophobic residues, permits hetero- or homodimerization of these proteins. A motif of basic residues mediates binding of these complexes to a con-

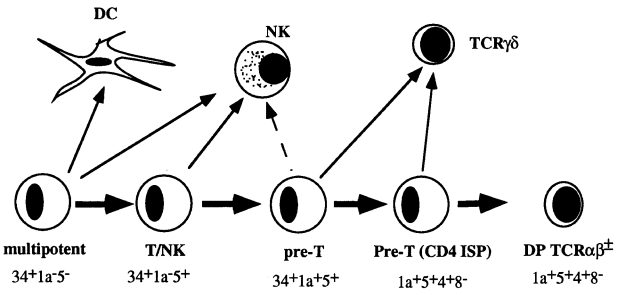

Fig. 1. A model of early developmental stages of T cell development in the human thymus.

sensus E-box (CANNTG) sequence present in promoters or enhancers of target genes (Church *et al.*, 1985; Ephrussi *et al.*, 1985). Two members of the bHLH family, E12 and E47, encoded by the E2A gene (Murre *et al.*, 1989) are essential for B cell development but are also involved in T cell development (Bain *et al.*, 1994, 1997a,b; Zhuang *et al.*, 1994). The thymuses of E2A-deficient mice have a low cellularity with a size of ~20% compared with their wild-type littermates. T cell differentiation is partially blocked at an early stage of development. Mice deficient for another bHLH factor, HEB, exhibit, besides a profound B cell defect, a partial T cell developmental block (Zhuang *et al.*, 1996). Concordant with these observations in the mouse, we have documented that inhibition of bHLH factors in human T cell precursors blocks development of these cells into T cells in an *in vitro* fetal thymic organ culture (FTOC) (Heemskerk *et al.*, 1997). In that study, we took advantage of the finding that transcriptional activity of bHLH factors is regulated by a structurally related group of proteins, generally referred to as inhibitors of DNA binding or Id proteins. This subfamily consists of four members (Id1–Id4) that have varying tissue distributions (Christy *et al.*, 1991; Sun *et al.*, 1991; Riechmann *et al.*, 1994). Id proteins possess the protein dimerization HLH domain, resulting in an ability to heterodimerize with the bHLH proteins, but as they lack the basic motif, they cannot bind DNA. Id proteins block the transcriptional activity of the bHLH proteins by sequestering these factors (Benezra *et al.*, 1990). We found that exogenous expression of one of the Id proteins, Id3, following retrovirus-mediated gene transfer in human $CD34^+$ fetal liver and thymocyte populations, inhibits T cell development in an FTOC exactly at the T/NK branch point (Heemskerk *et al.*, 1997). Most interestingly, NK cell development was promoted by enforced expression of Id3, suggesting that sequestration of bHLH factors results in a shift in cell fate from T to NK lineage cells. As Id3 interacts with E2A and HEB proteins, preventing DNA binding and transcriptional activation by these proteins (Loveys *et al.*, 1996), our findings have established an important role of bHLH factors in T cell development.

The transcriptional control mechanisms that dictate the αβ/γδ lineage decision have not yet been elucidated. However, some of the factors that contribute to the divergence of αβ/γδ lineage have been resolved using the mouse system. The occurrence of productive rearrangements at the γ and δ loci (Kang *et al.*, 1995; Livak *et al.*, 1995) is an important factor, but also external signals provided through Notch1 (Washburn *et al.*, 1997) and the pre-TCR (Aifantis *et al.*, 1998) have been implicated.

Here we provide evidence that bHLH factors, besides their role in the T/NK lineage determination, differentially affect TCRαβ/γδ lineage development in an FTOC beyond the T/NK checkpoint. We took advantage of a retroviral system that allows introduction of genes into cells at different stages of development to express Id3 in $CD1a^+CD34^+$ and CD4 ISP pre-T cells. It was observed that development of the transduced cells into TCRαβ cells was strongly blocked. In contrast, development of these cells to TCRγδ T cells was not inhibited. Strikingly, Id3-transduced but not control-transduced CD4 ISP cells have the capacity to develop into $CD3^-$ cells that lost CD1a and CD4 expression but retained rearranged TCR genes. These observations indicate that continued activities of bHLH factors are required to control development of TCRαβ cells beyond the T/NK checkpoint.

Results

Effect of constitutive Id3 expression on development of $CD1a^+CD34^+$ thymocytes in an FTOC

We observed previously that overexpression of Id3 in $CD1a^-CD34^+$ cells results in inhibition of differentiation of these cells into $CD1a^+$ cells and stimulation of NK development (Heemskerk *et al.*, 1997). Moreover, Id3 prevented initiation of TCRβ gene rearrangements in $CD1a^-CD34^+$ thymocytes cultured in interleukin (IL)-7. Our results indicated that sequestering of bHLH factors by Id3 inhibits transition of $CD1a^-$ into $CD1a^+$ progenitor cells. As the NK precursor cell activity is strongly reduced in $CD1a^+CD34^+$ thymocytes (Spits *et al.*, 1998) and TCRγ and β gene rearrangements are initiated upon upregulation of CD1a (Blom *et al.*, 1999), it was of interest to determine whether bHLH factors are necessary after acquisition of CD1a. We sorted $CD1a^+CD34^+$ thymocytes and transduced them with Id3–internal ribosome entry site (IRES)–green fluorescent protein (GFP). The cells were cultured in an FTOC for 3 weeks. In a representative experiment, 1.6×10^6 cells were recovered, of which 3% expressed high levels of the GFP marker, from five lobes which initially were seeded with 40 000 cells/lobe (8-fold expansion). The phenotypic analysis of these cells is shown in Figure 2. The proportions of double positive (DP) cells which developed from the Id3-transduced $CD1a^+CD34^+$ thymocytes were dramatically lower than observed for untransduced cells (Figure 2; 3 versus 88%). Additionally, significant fewer $TCR\alpha\beta^+$ T cells were present in the Id3-transduced cell sample (3 versus 64%). However, similar percentages of $TCR\gamma\delta^+$ T cells were recovered from the transduced and untransduced populations (1.1 versus 0.7%). Thus, the ratio TCRαβ to TCRγδ was dramatically affected by Id3 overexpression (3 versus 90). Surprisingly, the majority of $Id3^-GFP^+CD1a^+CD34^+$ cells had lost CD1a after FTOC, although all cells expressed CD5. In contrast, virtually all untransduced cells remained positive for CD1a and CD5. Though control-transduced $CD1a^+CD34^+$ thymocytes were unable to develop into NK cells, $CD3^-CD56^+$ NK cells did develop from the Id3-transduced cells (7.6% of the Id3-transduced cells versus 0% of the untransduced cells). Taken together, these data indicate that Id3 overexpression in $CD1a^+CD34^+$ cells resulted in a strong

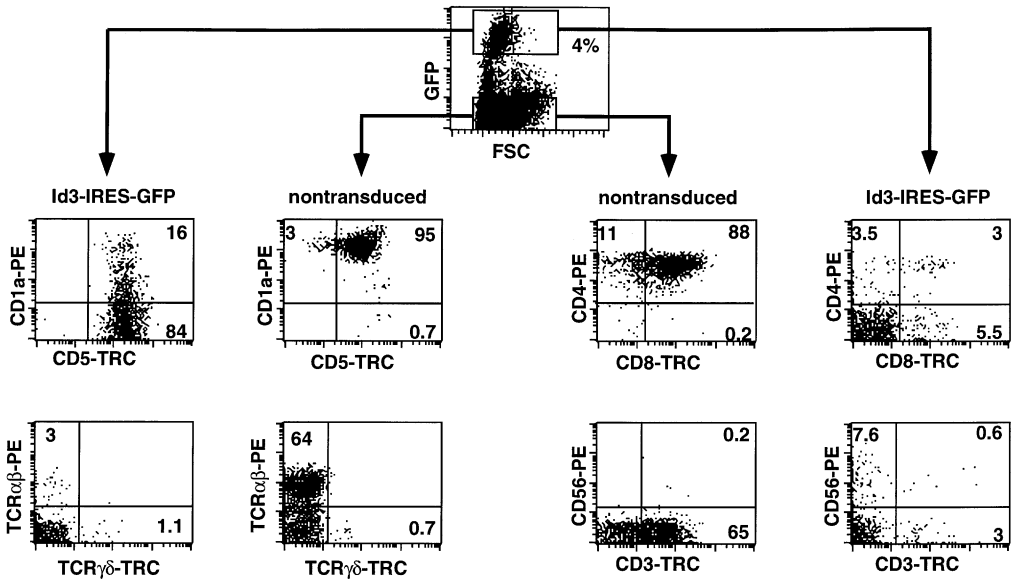

Fig. 2. Three-color flow cytometric analysis of cells recovered from an FTOC seeded with Id3-transduced $CD1a^+CD34^+$ thymocytes. Sorted $CD1a^+CD34^+$ thymocytes (>99% pure upon reanalysis) were transduced with the retrovirus containing the Id3–IRES–GFP construct and cultured in an FTOC for 3 weeks. Single cell suspensions were prepared and analyzed by flow cytometry for the expression of the indicated surface antibodies. Expression of the surface antigens is shown after electronic gating on GFP^+ or GFP^- cells. Numbers in the dot plots represent percentages of dots in each quadrant.

inhibition of αβ but not of γδ T cell development. Moreover, incubation of Id3-transduced cells in an FTOC resulted in downregulation of CD1a and led to development of $CD56^+$ cells.

Overexpression of Id3 in CD4 ISP thymocytes blocks development of TCRαβ$^+$ but not TCRγδ$^+$ T cells in an FTOC

The results presented in Figure 2 indicate that TCRαβ development from $CD1a^+CD34^+$ cells is strongly inhibited while development of TCRγδ cells is much less affected. The stage that follows the $CD1a^+CD34^+$ stage expresses CD4 but lacks CD8 (Figure 1). Ramiro *et al*. (1996) have documented that TCRβ gene rearrangements are present in the CD4 ISP. Southern blot analysis of TCRβ rearrangements in $CD34^+$ and CD4 ISP populations using Jβ1- and Jβ2-specific probes have confirmed the data of Ramiro and collaborators, but also indicated that 85% of the TCRβ alleles are still in the germline configuration (Blom *et al*., 1999). We have found, moreover, that almost all (>95%) CD4 ISP cells have rearranged TCRδ loci and that 35% of the alleles have TCRγ rearrangements (Blom *et al*., 1999). Consistent with the fact that all CD4 ISP cells have one or more TCR genes in rearranged form, they are completely unable to develop into NK cells, indicating that they are fully committed to the T cell lineage. To investigate whether Id3 influences T cell development after complete commitment to the T cell lineage, CD4 ISP cells were sorted from CD8-depleted thymocytes, transduced with viruses harboring either the control IRES–GFP or Id3–IRES–GFP DNAs and cultured for 12 days in an FTOC. Flow cytometric analysis of one of four representative experiments shown in Figure 3 revealed that 12% of the cells recovered from the FTOC were GFP^+, while 5% of the cells were $Id3^-GFP^+$. The control GFP-transduced CD4 ISP thymocytes developed into $CD1a^+CD5^+$ cells, of which the majority were $CD4^+CD8^+$ DP thymocytes (>80%) (Figure 3). Both $\alpha\beta^+$ T cells (27%) and $\gamma\delta^+$ T cells were present (0.7%). As expected, no $CD56^+$ NK cells were generated. The results of the analysis of the Id3–GFP-transduced CD4 ISP thymocytes after FTOC were strikingly different. Less than 3% of these cells expressed a TCRαβ, while 2% expressed a TCRγδ. In terms of percentages, there was even an increase of TCRγδ cells in the Id3-transduced samples. However, as shown below, the absolute numbers of TCRγδ cells in both samples are not significantly different. Thus, while Id3 overexpression strongly inhibited differentiation of CD4 ISP cells into TCRαβ cells, development of these cells into TCRγδ cells was not blocked. Another dramatic effect was the development of relatively large numbers of $CD1a^-CD3^-CD4^-CD5^+CD8^-$ cells from Id3-transduced CD4 ISP cells as 60% of these cells lacked expression of CD1a, although all cells expressed CD5 (Figure 3). Moreover, 76% of the Id3-transduced cells lacked CD4 and CD8 (Figure 3). The observation that some cells (2%) were observed that express CD56 and not CD3 may suggest that Id3 overexpression in CD4 ISP permits some of these cells to enter the NK cell lineage.

The effect of Id3 overexpression on TCRγδ development depends on the developmental stage of the transduced progenitor cells

The results shown in Figures 2 and 3 contrast with those in our previous study which demonstrated that Id overexpression in uncommitted $CD34^+$ progenitor cells inhibited their development into both TCRαβ and γδ T cells in an FTOC. This is illustrated more clearly in Table I which summarizes the results of eight experiments; two with $CD34^+$ fetal liver cells, three with $CD1a^-CD34^+$ thymic precursors and three with committed CD4 ISP cells. Table I shows that the proportions of IRES–GFP^+ and Id3–IRES–GFP^+ cells before the FTOC are similar.

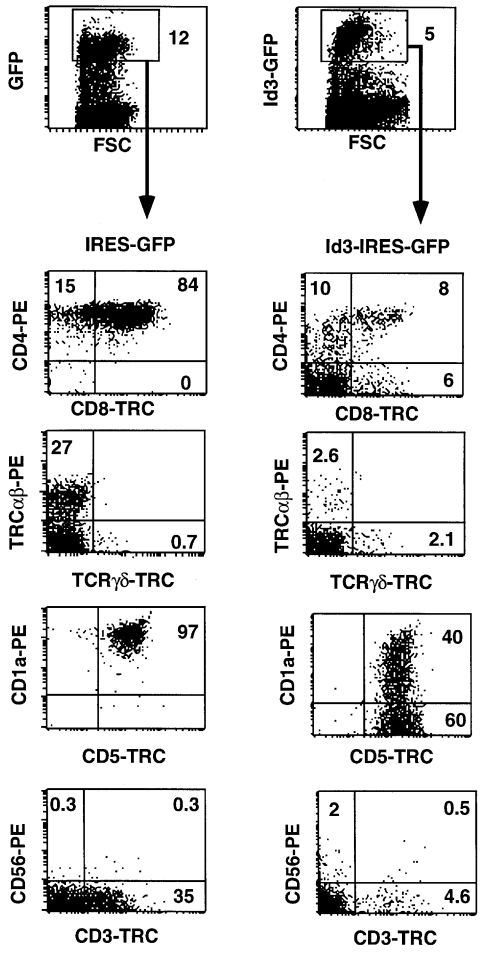

Fig. 3. Three-color flow cytometric analysis of IRES–GFP- and Id3–IRES–GFP-transduced CD4 ISP thymocytes cultured in an FTOC. CD4 ISP thymocytes were sorted (>99% pure upon reanalysis) and transduced with the retrovirus containing the control IRES–GFP or the retrovirus expressing Id3–IRES–GFP and cultured in FTOC for 12 days. Single cell suspensions were prepared and stained with PE- and TRC-conjugated antibodies against different surface antigens. Flow cytometry was performed and cells were analyzed for the indicated surface markers after gating on the control GFP^+ cells or the Id3–IRES–GFP^+ cells. Numbers in the dot plots represent percentages of dots in each quadrant.

Thus the input numbers of Id3- and control-transduced progenitor cells were comparable. In contrast, the percentages of GFP^+ cells in Id3-transduced cells after the FTOC were lower than that in the control-transduced cells, which is most likely due to the fact that proliferation induced by β selection does not occur in the Id3-transduced cells (vide infra). To allow a meaningful assessment of the effect of Id3 on T cell development, we therefore calculated the absolute number of $GFP^+TCR\alpha\beta$ and $GFP^+TCR\gamma\delta^+$ cells harvested from each experiment. Since the same number of lobes could not always be harvested, and therefore the total cell recoveries of each sample were different, we expressed the number as the amount of GFP^+ cells per 10^6 total (GFP^+ and GFP^-) cells. Control GFP-transduced cells were not included in two experiments (experiment 3 with $CD1a^-CD34^+$ thymocytes and experiment 1 with CD4 ISP cells). In these experiments, we calculated the number of untransduced TCRαβ and γδ cells that developed from the same input number as that of the Id3-transduced cells. The data presented in Table I clearly indicate that enforced expression of Id3 in $CD34^+$ fetal liver (two experiments) and $CD1a^-CD34^+$ cells (three experiments) strongly blocked development of both TCRαβ and γδ cells. Id3 expression also inhibited TCRαβ development of CD4 ISP cells in all three experiments. However, the absolute numbers of TCRγδ cells recovered from the Id3- and control-transduced CD4 ISP cells were the same. These findings indicate that after the T/NK checkpoint, bHLH factors continue to be required for TCRαβ but not for TCRγδ development.

Enforced expression of Id3 in $CD1a^-CD34^+$ cells prevents the appearance of cells with TCRδ rearrangements and with upregulated RAG1 and RAG2 mRNA and downregulates pTα mRNA

In our previous study, we demonstrated that enforced Id3 expression prevents the appearance of TCRβ rearrangements in $CD1a^-CD34^+$ cells cultured in IL-7 (Heemskerk *et al*., 1997). To determine whether Id specifically affects TCRβ rearrangements or whether Id3 interferes with TCR gene rearrangements at other loci as well, we examined the effect of Id3 on TCRδ Vδ1–Jδ1 rearrangements in $CD1a^-CD34^+$ cells cultured in IL-7 and stem cell factor (SCF) using a PCR technique. $CD1a^-CD34^+$ cells were sorted, transduced with Id3 or with the control vector and cultured for 6 days in IL-7. Since the transduced gene is expressed 2 days after the transduction procedure, Id3 is expressed for the last 4 days of the culture period. The GFP^+ cells were sorted from both cultures, and the DNA was isolated and subjected to a PCR. Figure 4 shows a band with a very low intensity following Southern blot of the PCR product from DNA of freshly isolated $CD1a^-CD34^+$ cells. This may either reflect a low level of Vδ1–Jδ1 rearrangements in this population or be due to a few contaminating $CD1a^+CD34^+$ cells in the cell preparation. The number of the Vδ1–Jδ1 rearrangements strongly increased when control-transduced cells were cultured in IL-7 and SCF (Figure 4, lane 2) and was of the same level as those detected in $CD1a^+CD34^+$ cells isolated in the same sort as $CD1a^-CD34^+$ cells (Figure 4, lane 4). Enforced expression of Id3 completely inhibited the increase of Vδ1–Jδ1 rearrangements by IL-7 and SCF (Figure 4, lane 3).

The observation that enforced expression of Id3 inhibited induction of both TCRδ and TCRβ gene rearrangements by IL-7 and SCF prompted us to investigate the effect of Id3 on expression of RAG1 and RAG2. $CD1a^-CD34^+$ and $CD1a^+CD34^+$ cells were sorted, transduced with Id3 or with the control vector and cultured for 6 days in IL-7. The GFP^+ cells were sorted from both cultures, and the RNA was isolated and reverse transcribed. The cDNA samples were subjected to PCR using primers specific for HPRT, RAG1 and RAG2. Figure 5 shows that cells overexpressing Id3 in both $CD1a^-CD34^+$ (lanes 6 and 7; the two lanes show the bands of undiluted and one-fofth diluted samples, respectively) and $CD1a^+CD34^+$ (lanes 12 and 13) have reduced levels of RAG1 and RAG2 compared with the control vector-transduced $CD1a^-CD34^+$ cells (lanes 4 and 5) and $CD1a^+CD34^+$ cells (lanes 10 and 11). In the same experiment, we also analyzed pTα mRNA levels, because it has been demonstrated that pTα, a component of the pre-TCR, is

Table I. Comparison of the effects of Id3 overexpression on the numbers of TCRαβ and γδ cells that developed from uncommitted and committed T cell progenitors

Cell population	Exp.	% Transduced cells				No. of GFP^+ cells with TCRαβ or γδ per 10^6 cells			
		Id3–IRES–GFP		IRES–GFP		Id3		Control	
		Before[a]	After[a]	Before[a]	After[a]	αβ	γδ	αβ	γδ
$CD34^+$	1	52	13	49	23	0	0	23 000	16 100
fetal liver	2	35	2	31	18	0	200	12 600	18 000
$CD1a^-CD34^+$	3	12	2	10	6	180	600	10 200	4800
thymus	4	10	1	14	14	200	0	37 800	2800
	5	36	2.6	ND		780	360	108 000	8640
$CD1a^+CD4^+CD^-$	6	8	3	ND		900	1200	24 000	800
thymus	7	3	1	4	4	700	240	17 600	240
	8	13	5	13	12	1500	1000	32 400	840

After each FTOC, we determined the total number of recovered cells, the percentage of GFP^+ cells and the phenotype. Based on these data, we calculated the total numbers of GFP^+ αβ and γδ cells. To allow for a comparison of all experiments, we expressed the numbers of recovered αβ and γδ cells per 10^6 total (GFP^+ and GFP^-) cells. In experiments 5 and 6, we calculated the numbers of untransduced cells that developed from the same input number as Id3-transduced cells.

[a]'Before' and 'after' refer to the percentage of GFP^+ cells before and after incubation of the transduced cells in the FTOC.

crucial for optimal development of TCRαβ cells, but is not required for development of $TCR\gamma\delta^+$ cells (Fehling *et al*., 1995). It is demonstrated in Figure 5 that pTα is strongly downregulated by enforced expression of Id3, both in $CD1a^-CD34^+$ (third panel, lanes 6 and 7) and in $CD1a^+CD34^+$ cells (third panel, lanes 12 and 13) as the intensities of the pTα bands in the Id3-transduced cells are even lower than in the freshly isolated $CD1a^-$ (lanes 2 and 3) and $CD1a^+CD34^+$ cells (lanes 8 and 9). We have also determined the intensities of the PCR bands using video densitometry employing the Eagle Eye still video system and Eagle Sight software. From the measured intensities, we calculated the ratios RAG1:HPRT, RAG2:HPRT and pTα:HPRT. The calculated RAG1:HPRT ratio for freshly isolated $CD1a^-CD34^+$ was 0.04; following incubation of the control, the ratio increased to 1.33, while the ratio in the Id3-transduced sample was 0.07. For the $CD1a^+CD34^+$ samples, the RAG1:HPRT ratios were 0.76, 2.1 and 0.5, respectively. A similar pattern was observed for RAG2. Although these measurements should be interpreted with caution, they suggest that overexpression of Id3 prevented upregulation of Id3 rather than downregulated expression of RAG1 and RAG2. The pattern of pTα:HPRT ratios was different. The ratio in the freshly isolated $CD1a^-CD34^+$ cells was 0.8; following culture, the control gave a ratio of 1.0 while in the Id3-transduced sample the pTα:HPRT ratio was 0.03. The pTα:HPRT ratios determined in the $CD1a^+CD34^+$ samples were 1.4, 1.9 and 0.02, respectively. These data suggest that Id3 overexpression results in downregulation of pTα mRNA.

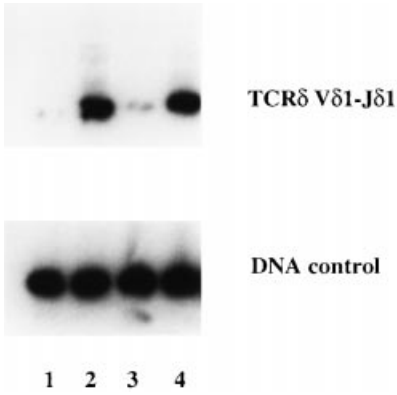

Fig. 4. TCRVδ1–Jδ1 rearrangements in freshly isolated $CD1a^-CD34^+$ and $CD1a^+CD34^+$ cells and $Id3^-$ and control-transduced $CD1a^-CD34^+$cells, cultured in IL-7 and SCF. $CD1a^-CD34^+$ and $CD1a^+CD34^+$cells were isolated and the $CD1a^-CD34^+$ cells were transduced with GFP or with Id3–GFP as described in Materials and methods. The transduced cells were cultured in a combination of IL-7 and SCF for 6 days. After this culture period, GFP^+ cells were sorted from both the Id3- and control-transduced samples (>98% pure upon reanalysis). DNA was prepared and analyzed for the presence of Vδ1–Jδ1 rearrangements by PCR. Lane 1, freshly isolated $CD1a^-CD34^+$ cells; lane 2, cultured control GFP-transduced $CD1a^-CD34^+$ cells; lane 3, cultured Id3-transduced $CD1a^-CD34^+$ cells; lane 4, freshly isolated $CD1a^+CD34^+$ cells. Amplification of the *RAG2* gene was done to control for the amount of DNA.

$CD1a^-CD4^-CD3^-$ cells recovered from Id3–IRES–GFP^+ CD4 ISP thymocytes cultured in FTOC can be expanded with IL-2 and show characteristics of both pre-T cells and NK cells

The $CD1a^-CD3^-CD4^-$ cells (Figure 3) recovered from the FTOC seeded with Id3–IRES–GFP-transduced CD4 ISP cells also expressed CD2 and CD7 (results not shown). The recovery of these cells was unexpected as no $CD3^-CD4^-$ cells were detected in the FTOC with untransduced or control-transduced CD4 ISP cells. These $CD1a^-CD2^+CD3^-CD4^-CD7^+$ cells are reminiscent of CD3 pre-NK cells (Sanchez *et al*., 1993). As pre-NK cells respond to stimulation with phytohemagglutinin (PHA) and IL-2 (Sanchez *et al*., 1993), we cultured the cells derived from the FTOC with a feeder mixture of irradiated peripheral blood mononuclear cells (PBMC), the Epstein–Barr virus (EBV) cell line JY, PHA and IL-2. The cells recovered from an FTOC seeded with Id3–IRES–GFP-transduced CD4 ISP cells were expandable and in one typical experiment we observed that 66% of the Id3–GFP^+ cells lacked CD3; the majority of these $CD3^-$ cells expressed the NK marker CD56 (Figure 6). In addition, TCRγδ cells were present in these cultures, a portion of which expressed CD56. The Id3–$GFP^+CD56^+CD3^-$ cells were sorted and

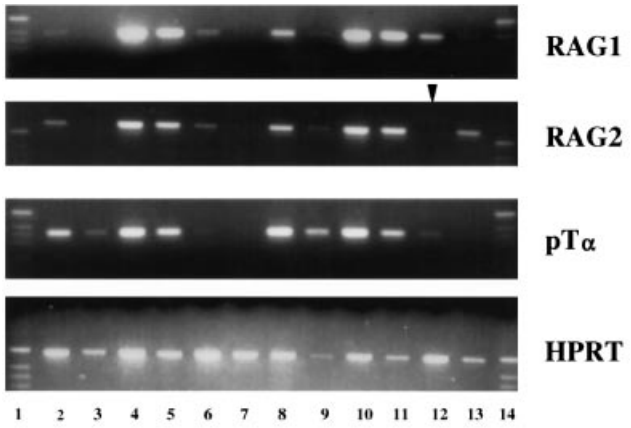

Fig. 5. RT–PCR analysis of RAG1, RAG2, pTα and HPRT expression in Id3- and control-transduced CD1a$^-$CD34$^+$ and CD1a$^+$CD34$^+$ cells, cultured in IL-7 and SCF. CD1a$^-$CD34$^+$ cells were isolated and transduced as described in the Materials and methods. The cells were cultured in a combination of IL-7 and SCF for 6 days. The GFP$^+$ cells were sorted from both cultures, and the RNA was isolated and reverse transcribed. Two cDNA samples, one undiluted (first lane of each series) and another one-fifth diluted (second lane of each series), were subjected to PCR using primers specific for HPRT, RAG1, RAG2 and pTα. Lane 1, markers; lanes 2 and 3, freshly isolated CD1a$^-$CD34$^+$ cells; lanes 4 and 5, cultured IRES–GFP-transduced CD1a$^-$CD34$^+$ cells; lanes 6 and 7, cultured Id3–IRES–GFP-transduced cells; lanes 8 and 9, freshly isolated CD1a$^+$CD34$^+$ cells; lanes 10 and 11, cultured IRES–GFP-transduced CD1a$^+$CD34$^+$ cells; lanes 12 and 13, cultured Id3–IRES–GFP-transduced CD1a$^+$CD34$^+$ cells; lane 14, markers. Lane 12 in the second (RAG2) panel, marked by an arrow, did not contain material, but since the one-fifth dilution (lane 13) shows a clear signal, RAG expression of this sample can be compared with that of lane 9 (freshly isolated CD1a$^+$CD34$^+$ cells).

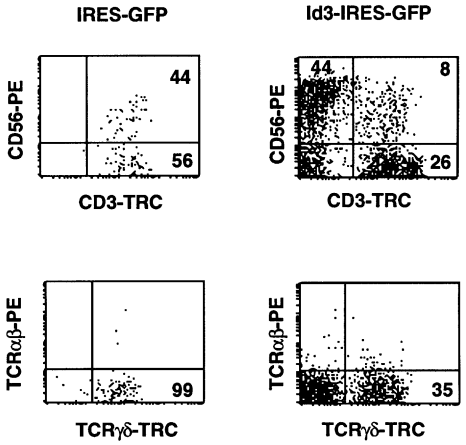

Fig. 6. CD1a$^-$CD3$^-$ cells harvested from an FTOC seeded with Id3–IRES–GFP-transduced CD4 ISP cells can be expanded in IL-2-containing medium. Id3-transduced CD4 ISP cells were cultured for 14 days in an FTOC. The cells were harvested and the GFP-positive cells were sorted using a FACStar plus and stimulated with a feeder cell mixture containing PHA and IL-2. Following 14 days of culture, the cells were stained with the indicated PE- or TRC-conjugated antibodies and analyzed on a FACScan.

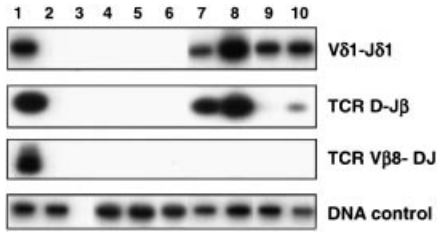

Fig. 7. TCR rearrangement analysis on CD3$^-$CD56$^+$ cells obtained from Id3–IRES–GFP-transduced cells cultured in an FTOC. Id3-transduced FL CD34$^+$ and Id3-transduced CD1a$^-$CD34$^+$, CD1a$^+$CD34$^+$ and CD4 ISP thymocytes were cultured in an FTOC, harvested and expanded for 2 weeks with feeders. Expanded cultures were sorted (>99.9% pure upon reanalysis) for Id3–GFP$^+$CD56$^+$CD3$^-$ NK cells and Id3–GFP$^+$CD56$^+$CD3$^+$ γδ$^+$ T cells. NK cells were also obtained from cytokine (IL-2, IL-7 and SCF)-induced CD34$^+$CD38$^+$ fetal liver cells and CD1a$^-$CD34$^+$ thymocytes. DNA was isolated from these cells and analyzed by PCR for TCR Vδ1–Jδ1 (first panel), TCR D–Jβ (second panel) or TCR Vβ8–DJ (third panel) rearrangements using the primer combinations described in the Materials and methods. Lanes 1–10 contain PCR products amplified from DNA that was isolated from total thymocytes (lane 1), EBV-B (lane 2), no DNA (lane 3), FL CD34$^+$CD38$^+$ plus cytokines (lane 4), fetal liver Id3$^+$CD56$^+$CD3$^-$ from the FTOC (lane 5), CD1a$^-$CD34$^+$ thymocytes plus cytokines (lane 6), CD1a$^+$CD34$^+$ Id3$^+$CD56$^+$CD3$^-$ from the FTOC (lane 7), CD1a$^+$CD34$^+$ Id3$^+$CD56$^+$CD3$^+$ from the FTOC (lane 8), CD4 ISP Id3$^+$CD56$^+$CD3$^-$ from the FTOC (lane 9) and CD4 ISP Id3$^+$CD56$^+$CD3$^+$ from the FTOC (lane 10).

these cells were shown to have cytotoxic activity against the NK-sensitive target cell line K562, but not against an NK-resistant EBV-B cell line (results not shown). In contrast, all GFP$^+$ cells expanded from the FTOC with control GFP-transduced CD4$^+$ ISP cells were TCRγδ cells. This was expected because TCRγδ but not TCRαβ harvested from the FTOC can be cultured in IL-2 (Res *et al*., 1997). More importantly, no CD3$^-$ cells were present in these cultures (Figure 6). These results indicate that overexpression of Id3 in CD4 ISP cells selectively leads to development of CD3$^-$ cells that have the capacity to respond to IL-2.

The CD3$^-$CD56$^+$ cells obtained from Id3-transduced CD4 ISP thymic precursors were derived from cells that had undergone TCR gene rearrangements. This raises the possibility that at least a proportion of the CD1a$^-$CD3$^-$ CD4$^-$CD56$^+$ cells, recovered from the FTOC and expanded in IL-2-containing medium, had rearranged TCR genes. We therefore examined whether TCRδ and TCRβ rearrangements were present in Id3–GFP$^+$CD56$^+$CD3$^-$ cells recovered from an FTOC seeded with CD4 ISP Id3-transduced thymocytes. As a positive control, we analyzed TCRδ and TCRβ rearrangements in Id3–GFP$^+$CD56$^+$ CD3$^+$TCRγδ$^+$ T cells derived from the same FTOCs. After expansion of the cells with feeders, they were sorted in a highly stringent way to completely remove mature cells (>99.9% pure) and TCR rearrangements were analyzed by PCR. As a negative control, DNA isolated from an EBV-transformed B cell line was used. As expected, the Id3–GFP$^+$CD56$^+$CD3$^+$TCRγδ$^+$ derived from CD1a$^+$CD34$^+$ and CD4 ISP cells had rearranged Vδ1–Jδ1 and DJβ gene segments (Figure 7, lanes 8 and 10, respectively) while an EBV-B cell line (lane 2) did not reveal either of the two rearranged TCR gene products. Surprisingly, analysis of DNA isolated from the Id3–GFP$^+$CD56$^+$CD3$^-$ cells, derived from FTOC-incubated Id3-transduced CD1a$^+$ CD34$^+$ and CD4 ISP thymocytes, both revealed Vδ1–Jδ1 and DJβ rearrangements (Figure 7, lanes 7 and 9, respectively). TCRβ DJ rearrangements in the Id3–GFP$^+$CD56$^+$CD3$^-$ cells derived from this sample of CD4 ISP thymocytes were very weak but became more clearly visible after a longer exposure time (data not shown). It cannot be excluded completely that the TCR gene rearrangements depicted in lanes 7 and 9 are derived from

contaminating $CD3^+$ cells but, given the stringency of our sort and the intensities of the bands in the $CD3^-$ samples compared with the $CD3^+$ samples (particularly when one compares lanes 9 and 10), we feel confident that the signals are not derived from the few contaminating ($<0.1\%$) $CD3^+$ cells. Moreover, although no TCRδ germline bands were detected in Southern blot analysis of CD4 ISP, not all CD4 ISP cells have Vδ1 to Jδ1 gene rearrangements (Blom *et al*., 1999), making it even less unlikely that the clear Vδ1–Jδ1 rearrangements detected by PCR analysis of the $Id3^+$ $CD3^-CD56^+$ cells were due to contaminating $CD3^+$ cells. Complete Vβ8–DJ rearrangements were not detectable in any subpopulation tested, except in total thymocytes (Figure 7, third panel), consistent with the fact that only a small minority of the starting CD4 ISP cells have TCRβ gene rearrangements (Blom *et al*., 1999). It is of note that $CD3^-CD56^+$ NK cells obtained from cytokine-induced $CD34^+$ fetal liver cells (lane 4) or $CD1a^-CD34^+$ thymocytes (lane 6) did not have rearrangements at either the TCRδ or TCRβ locus.

To exclude the possibility that Id3 induces rearrangements in NK cells, we analyzed NK cells obtained from an FTOC which was seeded with Id3-transduced $CD34^+$ fetal liver cells. We have shown previously that $CD34^+$ FL cells do not have rearranged TCR gene segments (Jaleco *et al*., 1997). Moreover, we observed that incubation of Id3-transduced $CD34^+$ FL cells gives rise to many NK cells in an FTOC (Heemskerk *et al*., 1997). These Id3–GFP^+CD56^+ NK cells were isolated from cells expanded in a feeder cell mixture, and Figure 7 shows that they had no rearranged TCR genes (lane 5). Also, rearrangements of the Vδ2 gene segment, which are associated with fetal T cell development (Krangel *et al*., 1990), were undetectable (data not shown).

Discussion

Recently, we have demonstrated that enforced expression of Id3 in human $CD34^+$ fetal liver cells results in a complete inhibition of TCRαβ and TCRγδ cell development and a developmental arrest of cells at the $CD1a^-CD5^+$ stage (Heemskerk *et al*., 1997). Interestingly, these $CD1a^-CD5^+$ cells selectively differentiate into $CD3^-CD56^+$ cells that display NK cell activity (Heemskerk *et al*., 1997). These findings indicate that bHLH factors are involved in the control of commitment to the T cell lineage at the T/NK branch point. We now demonstrate that overexpression of Id3 in cells downstream of this branchpoint strongly inhibits development of TCRαβ T cells, while that of TCRγδ cells is not inhibited. This is in contrast to the strong inhibition of TCRγδ cell development following Id3 expression in $CD34^+$ fetal liver cells or in $CD1a^-CD34^+$ thymocytes that represent stages before the T/NK split (Table I), and indicate that bHLH factors continue to be involved in TCRαβ development beyond the T/NK branchpoint.

One possible explanation for our observations is that Id3 overexpression interferes with TCR gene rearrangements. This may be one mechanism through which Id3 inhibits development of uncommitted progenitors into αβ and γδ T cells. Data from our previous study indicate that Id3 overexpression in $CD1a^-CD34^+$ thymocytes cultured in IL-7 prevented the appearance of cells with TCRβ DJ gene rearrangements (Heemskerk *et al*., 1997). Here we demonstrate that Id3 overexpression also prevents the appearance of cells with TCRδ rearrangements in $CD1a^-CD34^+$ cells cultured in IL-7. We also observed inhibition of upregulation of RAG1 and RAG2 in cells overexpressing Id3. It is possible that TCR gene rearrangements are directly affected by Id3 overexpression. Since TCRα rearrangements commence at a stage downstream of the CD4 ISP stage, in the DP population (Ramiro *et al*., 1996; Verschuren *et al*., 1998), continued recombinatorial activity is required for development of CD4 ISP cells into TCRαβ cells. One can, therefore, hypothesize that Id3 overexpression in CD4 ISP cells blocks TCRαβ cell development by inhibiting completion of TCRβ and initiation of TCRα gene rearrangements. The failure of Id3 to inhibit development of CD4 ISP cells may be attributed to the fact that these cells contain complete TCRδ and γ gene rearrangements (Blom *et al*., 1999). However, our data do not preclude that Id3 overexpression in uncommitted T cell progenitors results in a developmental arrest at the stage that initiates TCR gene rearrangements and does not affect recombinations directly.

The observed strong inhibition of pTα expression by Id3 overexpression suggests another, perhaps more likely, manner in which Id3 may differentially affect TCRαβ and γδ development from committed pre-T cells. Studies with pTα-deficient mice indicate that this gene is required for optimal development of TCRαβ but not of TCRγδ cells (Fehling *et al*., 1995). In this respect, it is important to note that the CD4 ISP stage is upstream of the β selection checkpoint (Ramiro *et al*., 1996; Blom *et al*., 1999). It is therefore tempting to hypothesize that enforced expression of Id3 in CD4 ISP cells leads to downregulation of pTα and thereby inhibits the proliferation that accompanies β selection. This may contribute to or perhaps be completely responsible for the observed inhibition of TCRαβ development. It is unclear whether bHLH factors directly affect pTα gene transcription. The promotor region of pTα has yet to be characterized. Interestingly, the bHLH protein E47 has been shown directly to control expression of the surrogate light chain genes, the equivalents of pTα in B cell progenitors (Sigvardsson *et al*., 1997).

The hypothesis that Id3 impedes T cell development either by affecting TCR rearrangements or pTα expression or both does not explain the observation that Id3 overexpression in $CD1a^+CD34^+$ and CD4 ISP cells results in the appearance of $CD1a^-CD3^-CD4^-CD5^+$ cells in an FTOC. These cells were never observed in FTOCs with untransduced or control GFP-transduced $CD1a^+CD34^+$ or CD4 ISP cells. We have considered the possibility that once T cell development is aborted, downregulation of CD1a and CD4 and acquisition of some NK markers occurs by default in our FTOC system. This is unlikely since enforced expression of constitutively active $p56^{lck}$ in CD4 ISP cells strongly inhibited T cell development, but did not result in the appearance of $CD1a^-CD3^-CD4^-CD5^+$ cells in an FTOC (Blom *et al*., 1999). These data indicate that $CD1a^-CD3^-CD4^-CD5^+$ cells appear as a consequence of Id3 overexpression in CD4 ISP cells. These cells could be expanded following culture in a feeder cell mixture containing IL-2. A large proportion of the $CD3^-$ cells in these cultures expressed NK markers such as CD56 (Figure 6) and CD94 (results not shown)

and displayed cytotoxic activity. Consistent with the fact that CD4 ISP cells have >95% and 15% of the TCRδ and β alleles, respectively, in rearranged form (Blom *et al*., 1999), the $CD1a^-CD3^-CD4^-CD56^+$ cells derived from CD4 ISP cells contain TCRδ and a low level of TCRβ gene rearrangements. Thus these cells have NK characteristics but they retained properties of pre-T cells. Since the expanded cells have TCR rearrangements but fail to express a TCR on the cell membrane, we should assume that the observed TCR rearrangements were unproductive. Our data suggest, therefore, that bHLH factors in $CD3^-$ pre-T cells prevent the loss of CD1a and CD4 and acquisition of the capacity to respond to IL-2 before completion of TCR rearrangements. The loss of CD1a, CD4 and CD8 and the acquisition of NK markers on cells that develop from Id3-transduced CD4 ISP cells strongly suggest that Id3 overexpression may result in a change of cell fate of some cells from the T into the NK cell lineage. Experiments to test this interesting possibility are under way.

The identity of the bHLH factors that are critical for T cell development has yet to be determined precisely. Using an RT–PCR, we have found that early T cell progenitors in the human thymus express E12, E47, HEB as well as Id1 and Id3 (results not shown). The E2A and HEB proteins have been implicated in T cell development. E2A deficiency results in a partial block in T cell differentiation at the transition of $CD44^+CD25^-$ cells into $CD44^{lo}CD25^+$ cells (Bain *et al*., 1997a). The $CD25^-$ to $CD25^+$ transition is roughly equivalent to the $CD1a^-$ to $CD1a^+$ transition in humans (Blom *et al*., 1998, Spits *et al*., 1998). Mice deficient for another bHLH protein, HEB, have perturbed T cell development with a relative accumulation of double negative and $CD3^-CD4^-CD8^+$ cells in the thymus (Zhuang *et al*., 1996). Since no further analysis of thymocytes from HEB-deficient mice has been reported (Zhuang *et al*., 1996), the exact T cell developmental stage that is affected in these mice remains to be determined. Thus, it is likely that sequestration of E2A proteins by Id3 is, at least in part, responsible for the block in T cell differentiation observed following overexpression of Id3. It seems likely, however, that other bHLH factors are implicated as well.

The data presented here provide evidence that control of T cell commitment beyond the T/NK checkpoint and completion of development of committed pre-T cells into TCRαβ cells require the continued activities of bHLH factors. The power of the retroviral system is also clearly demonstrated here. Not only does this technology offer a way to genetically modify human T cell development, it also has advantages on its own. The most important one is that genes can be introduced before or after defined checkpoints of development of individual lineages. Taking advantage of this feature, one can analyze functions of genes in a way that cannot yet be achieved easily using knock-out or transgenic technologies. Application of this technology to the mouse system and combining this with knock-out technologies will greatly extend the ability to analyze the mechanisms of lymphoid development.

Materials and methods

Isolation of subpopulations from thymus

Normal human thymocytes were obtained from thymus fragments removed during corrective cardiac surgery of patients aged 1 month to 2 years. Thymic lobes were minced gently in RPMI-1640 (Gibco-BRL Life Technologies Ltd, Paisley, UK) containing 2% (v/v) fetal calf serum (FCS; BioWhittaker, Verviers, Belgium) and antibiotics (penicillin 500 IU/ml, streptomycin 100 mg/ml, Boehringer Mannheim Biochemicals, Germany). Cells were isolated by Ficoll density gradient centrifugation (Lymphoprep, 1.077 g/ml, Nycomed Pharma, Oslo, Norway). Thymocytes recovered from the Ficoll interface were enriched for $CD34^+$ cells using a kit for separation of $CD34^+$ cells and a miniMACS (Miltenyi Biotec Inc., Sunnyvale, CA), according to the manufacturer's instructions. Cells were sorted after staining with monoclonal antibodies (mAbs) against molecular epitopes different from those recognized by the mAbs used during the miniMACS procedures. For cell sorting of $CD1a^-CD34^+$ and $CD1a^+CD34^+$ subpopulations, cells were incubated with 1 μl/10^6 cells of CD34–fluorescein isothiocyanate (FITC) (HPCA-2, Becton Dickinson & Co., San Jose, CA) and 0.5 μl/10^6 cells of CD1a–phecoerythrin (PE) (T6-RD1, Coulter Clone, Hialeah, FL) at 4°C for 30 min. To enrich for CD4 ISP cells, total thymocytes were depleted twice with CD27 mAb [9F4, gift from Dr R.van Lier, Central Laboratory of Blood transfusion (CLB), Amsterdam], CD69 mAb (L78, provided by Dr J.H.Phillips, DNAX, Palo Alto, CA) and magnetic beads (Dynabeads, Dynal, Oslo, Norway). The depleted thymocytes were stained with CD4–PE (Leu3a, Becton Dickinson), CD3–FITC (Leu4a), and CD8α–tricolor (TRC; 3B5, Caltag Laboratories, South San Francisco, CA) and sorted on the basis of $CD4^+CD3^-CD8^-$ expression. Cells were isolated by fluorescence activated cell sorting on a FACStar Plus (Becton Dickinson) equipped with an argon laser emitting at 488 nm. During the sorting procedure, cells were kept at 4°C. All fractions used in the subsequent experiments were reanalyzed after sorting and contained >99% of the cells in the selected sort gates.

Retroviral constructs and transduction

A LZRS retroviral vector was constructed comprising the Id3 cDNA (generous gift of Dr C.Murre, UCSD, San Diego, CA) and the enhanced GFP (obtained from Clontech, Palo Alto, CA) as a marker gene. The cDNAs were separated by the sequence of the IRES in order to allow individual translation of the bicistronic mRNA containing both Id3 and GFP (Id3–IRES–GFP) without generating a fusion protein (Davies and Kaufman, 1992; Pestova *et al*., 1996; Sachs *et al*., 1997). As a control, we made a retroviral vector containing the IRES–GFP sequence only (IRES–GFP). Helper virus-free recombinant retroviruses (titre 10^6/ml, as determined by transduction of mouse 3T3 fibroblast cells) were produced after transfection of the retroviral constructs into the 293T-based ΦNX-A amphotropic packaging cell line (Kinsella and Nolan, 1996) (provided by Dr G.Nolan, Stanford University, CA) and selection on puromycin. Progenitor cells were purified from thymus and cultured overnight in the presence of 10 ng/ml IL-7 (R & D systems, Abingdon, UK) and 10 ng/ml SCF (R & D) followed by incubation for 7–8 h or overnight with virus supernatant in plates coated with 30 μg/ml recombinant human fibronectin fragment CH-296 (RetroNectin™; Takara, Otsu, Japan) (Kimizuka *et al*., 1991; Hanenberg *et al*., 1996, 1997).

Hybrid human–mouse fetal thymic organ cultures

The *in vitro* development of human T cells from $CD1a^+CD34^+$ and CD4 ISP progenitor cells was studied using the hybrid human–mouse FTOC (Res *et al*., 1996). Fetal thymuses were obtained from embryos of RAG1-deficient mice on day 15–16 of gestation. The lobes were treated with 1.35 mM deoxyguanosine (Sigma, St Louis, MO) for 5 days before incubation with human progenitor cells in a hanging drop culture for 2 days followed by incubation on an air–liquid interface. Culture medium consisted of Yssel's medium (Yssel *et al*., 1984) supplemented with 2% normal human serum and 5% FCS. To analyze differentiation of human cells, the thymuses were dispersed into single cell suspensions and stained with mAbs specific for human cell surface antigens.

Expansion cultures

Cells recovered from the FTOC were expanded at a cell density of 3×10^5/ml in Yssel's medium containing a mixture of irradiated PBMC (10^6/ml; 3000 rad), the EBV cell line JY (10^5/ml; 5000 rad), PHA (100 ng/ml: Murex Diagnostics Ltd, Dartford, UK) and IL-2 (20 IU/ml; Chiron, Amsterdam, The Netherlands). Under these conditions, we can expand NK cells and $\gamma\delta^+$ T cells, but not $\alpha\beta^+$ T cells from an FTOC (Res *et al*., 1997).

Flow cytometric analysis

GFP-transduced human cells recovered from the hybrid mouse FTOC cultures were analyzed by three-color flow cytometric analysis on a FACScan (Becton Dickinson) after incubation for 30 min with the

following antibodies conjugated with the fluorochromes PE or TRC: CD3 (Leu-4), CD4 (Leu-3a), CD8 (Leu-2), CD2 (Leu-5b), CD7 (Leu-9), CD5 (Leu-1), CD45RA (Leu-18) (all from Becton Dickinson), anti-TCRαβ and anti-TCRγδ (Immunotech, Marseille, France), CD56 (NCAM 16.2, Becton Dickinson) and CD1a (T6-RD1, Coulter Clone). Data were analyzed with the Cell Quest program (Becton Dickinson).

RT–PCR assays

RNA was isolated from flow cytometric-sorted human postnatal thymocyte subpopulations using TRIzol reagent (Gibco-BRL) according to the manufacturer's instructions and reverse transcribed using a poly$(dT)_{15}$ oligonucleotide and 400 U of M-MuLV reverse transcriptase (Gibco-BRL) at 37°C for 1 h. Semi-quantitative RT–PCR was performed essentially as described previously (Blom *et al*., 1997). PCR assays were done in 30 µl reaction volumes using the appropriate diluted amounts of cDNA template, 2 mM $MgCl_2$, 0.25 mM each dNTP, 10 pmol of each primer and 0.8 U of *Taq* polymerase (Gibco-BRL) in 1× buffer (10 mM Tris–HCl pH 8.5, 50 mM KCl). Reaction conditions were as follows: 5 min denaturing step at 95°C followed by 31 cycles of 1 min at 95°C, 1 min at 55°C and 1 min at 72°C. PCR products were separated on 1% agarose gels, stained with ethidium bromide and analyzed by video densitometry using the Eagle Eye still video system and Eagle Sight software (Stratagene, La Jolla, CA). The RAG1, RAG2, pTα and HPRT primers that were used are: HPRT sense, 5′-TATGGACAGGACTGAACGTCTTGC-3′; HPRT antisense, 5′-GACACAAACATGATTCAAATCCCTGA-3′; RAG1 sense, 5′-GAACACACTTTGCCTTCTCTTTGG-3′; RAG1 antisense, 5′-CGCTTTGCCTCTTGCTTTCTCGTT-3′; RAG2 sense, 5′-TGTGAATTGCACAGTCTTGCCAGG-3′; RAG2 antisense, 5′-GGGTTTGTTGAGCTCAGTTGAATAG-3′; pTα sense, 5′-GTCCAGCCCTACCCACAGGTGT-3′; and pTα antisense, 5′-CGGGAATTCGACGTCCCTGGCTGTAGAAGCCTCTC-3′.

TCRδ gene rearrangement analysis by PCR

TCR gene rearrangements were analyzed by using DNA of ~10^4 cells in a genomic DNA PCR assay. Isolation of DNA and PCR of TCR gene rearrangements was done as described (Breit *et al*., 1991, 1993). TCRδ PCR was carried out using forward Vδ1 (5′-TGAGGAAAGCAGTCACCCTGAAC-3′) or Vδ2 (5′-ACCAAACAGTGCCTGTGTCAATAGG-3′) and Jδ1 reverse (5′-ACCTCTTCCCAGGAGTCCTCC-3′) primers. TCRβ D–J PCR was carried out using Dβ1.1 forward (5′-TGGTGGTCTCTCCCAGGCTCT-3′) and Jβ1.3–1.4 reverse (5′-CCAGCTGTCCAGCCTTGACTT-3′) primers. A 10 µl aliquot of the PCR products was run on a 2% agarose gel followed by DNA transfer to a nylon filter (Hybond N^+, Amersham, UK). Filters were pre-hybridized at 55°C for at least 1 h (6× SSC, 0.5% SDS, 5× Denhardt's, 100 mg herring sperm DNA per litre) and hybridized overnight with a ^{32}P-end-labeled oligoprobe specifically recognizing Vδ1–Jδ1 (5′-TGAGGAAAGCAGTCACCCTGAAC-3′) or TCRβ Dβpan (5′-CAAAGCTGTAACATTGTGGGGAC-3′) PCR products internal to the PCR primers. To control for the amount of DNA used in the PCR, we amplified the *RAG2* gene by using *RAG2* forward (5′-TGTGAATTGCACAGTCTTGCCAGG-3′) and *RAG2* reverse (5′-GGGTTTGTTGAGCTCAGTTGAATAG-3′) primers, which we detected with a RAG2 oligoprobe (5′-CAAGATATGGTTTGGAAGCAACATGGGAAA-3′). Filters were washed with an excess amount of 2× SSC; 0.1% SDS at 50°C for 30 min and exposed to an autoradiographic film.

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References

Aifantis,I., Azogui,O., Feinberg,J., Saint Ruf,C., Buer,J. and von Boehmer,H. (1998) On the role of the pre-T cell receptor in αβ versus γδ T lineage commitment. *Immunity*, **9**, 649–655.

Bain,G. *et al*. (1994) E2A proteins are required for proper B cell development and initiation of Ig gene rearrangements. *Cell*, **79**, 885–892.

Bain,G. *et al*. (1997a) E2A deficiency leads to abnormalities in αβ and γδ T cell development, perturbations in V(D)J rearrangement and to the rapid development of T-cell lymphomas. *Mol. Cell. Biol.*, **17**, 4782–4791.

Bain,G., Robanus Maandag,E., Te Riele,H., Feeney,A.J., Sheehy,A., Schlissel,M., Shinton,S.A., Hardy,R.R. and Murre,C. (1997b) Both E12 and E47 allow commitment to the B cell lineage. *Immunity*, **6**, 145–154.

Benezra,R., Davis,R.L., Lockshon,D., Turner,D.L. and Weintraub,H. (1990) The protein Id: a negative regulator of helix–loop–helix DNA binding proteins. *Cell*, **61**, 49–59.

Blom,B., Res,P., Noteboom,E., Weijer,K. and Spits,H. (1997) Prethymic $CD34^+$ progenitors capable of developing into T cells are not committed to the T cell lineage. *J. Immunol.*, **158**, 3571–3577.

Blom,B., Res,P.C.M. and Spits,H. (1998) T cell precursors in mice and man. *Crit. Rev. Immunol.*, **18**, 371–388.

Blom,B., Verschuren,M.C.M., Heemskerk,M.H.M., Bakker,A.Q., van Gastel-Mol,E.J, Wolvers-Tettero,I.L.M., van Dongen,J.J.M. and Spits,H. (1999) TCR gene rearrangements and expression of the pre-T cell receptor complex during T cell differentiation. *Blood*, in press.

Breit,T.M., Wolvers Tettero,I.L., Hahlen,K., Van Wering,E.J. and van Dongen,J.J. (1991) Extensive junctional diversity of γδ T-cell receptors expressed by T-cell acute lymphoblastic leukemias: implications for the detection of minimal residual disease. *Leukemia*, **5**, 1076–1086.

Breit,T.M., Wolvers-Tettero,I.L., Beishuizen,A., Verhoeven,M.A., Van Wering,E.R. and van Dongen,J.J. (1993) Southern blot patterns, frequencies, and junctional diversity of T-cell receptor-δ gene rearrangements in acute lymphoblastic leukemia. *Blood*, **82**, 3063–3074.

Capone,M., Hockett,R.J. and Zlotnik,A. (1998) Kinetics of T cell receptor β, γ, and δ rearrangements during adult thymic development: T cell receptor rearrangements are present in $CD44^+CD25^+$ pro-T thymocytes. *Proc. Natl Acad. Sci. USA*, **95**, 12522–12527.

Christy,B.A., Sanders,L.K., Lau,L.F., Copeland,N.G., Jenkins,N.A. and Nathans,D. (1991) An Id-related helix–loop–helix protein encoded by a growth factor-inducible gene. *Proc. Natl Acad. Sci. USA*, **88**, 1815–1819.

Church,G.M., Ephrussi,A., Gilbert,W. and Tonegawa,S. (1985) Cell-type-specific contacts to immunoglobulin enhancers in nuclei. *Nature*, **313**, 798–801.

Davies,M.V. and Kaufman,R.J. (1992) The sequence context of the initiation codon in the encephalomyocarditis virus leader modulates efficiency of internal translation initiation. *J. Virol.*, **66**, 1924–1932.

Donskoy,E. and Goldschneider,I. (1992) Thymocytopoiesis is maintained by blood-borne precursors throughout postnatal life. A study in parabiotic mice. *J. Immunol.*, **148**, 1604–1612.

Ephrussi,A., Church,G.M., Tonegawa,S. and Gilbert,W. (1985) B lineage-specific interactions of an immunoglobulin enhancer with cellular factors *in vivo*. *Science*, **227**, 134–140.

Fehling,H.J., Krotkova,A., Saint Ruf,C. and Von Boehmer,H. (1995) Crucial role of the pre-T-cell receptor α gene in development of αβ but not γδ T cells. *Nature*, **375**, 795–798.

Galy,A., Verma,S., Barcena,A. and Spits,H. (1993) Precursors of $CD3^+CD4^+CD8^+$ cells in the human thymus are defined by expression of CD34. Delineation of early events in human thymic development. *J. Exp. Med.*, **178**, 391–401.

Godfrey,D.I. and Zlotnik,A. (1993) Control points in early T-cell development. *Immunol. Today*, **14**, 547–553.

Hanenberg,H., Xiao,X.L., Dilloo,D., Hashino,K., Kato,I. and Williams, D.A. (1996) Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. *Nature Med.*, **2**, 876–882.

Hanenberg,H., Hashino,K., Konishi,H., Hock,R.A., Kato,I. and Williams, D.A. (1997) Optimization of fibronectin-assisted retroviral gene transfer into human $CD34^+$ hematopoietic cells. *Human Gene Ther.*, **8**, 2193–2206.

Heemskerk,M.H.M., Blom,B., Nolan,G., Stegmann,A.P.A., Bakker,A.Q., Weijer,K., Res,P.C.M. and Spits,H. (1997) Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix–loop–helix factor Id3. *J. Exp. Med.*, **186**, 1597–1602.

Jaleco,A.C., Blom,B., Res,P., Weijer,K., Lanier,L.L., Phillips,J.H. and Spits,H. (1997) Fetal liver contains committed NK progenitors, but is not a site for development of $CD34^+$ cells into T cells. *J. Immunol.*, **159**, 694–702.

Kang,J., Baker,J. and Raulet,D.H. (1995) Evidence that productive rearrangements of TCR γ genes influence the commitment of progenitor cells to differentiate into αβ or γδ T cells. *Eur. J. Immunol.*, **25**, 2706–2709.

Kimizuka,F., Taguchi,Y., Ohdate,Y., Kawase,Y., Shimojo,T., Hashino,K., Kato,I., Sekiguchi,K. and Titani,K. (1991) Production and characterization of functional domains of human fibronectin expressed in *Escherichia coli*. *J. Biochem. (Tokyo)*, **110**, 284–291.

Kinsella,T.M. and Nolan,G.P. (1996) Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. *Human Gene Ther.*, **7**, 1405–1413.

Kraft,D.L., Weissman,I.L. and Waller,E.K. (1993) Differentiation of $CD3^-4^-8^-$ human fetal thymocytes *in vivo*: characterization of a $CD3^-4^+8^-$ intermediate. *J. Exp. Med.*, **178**, 265–277.

Krangel,M.S., Yssel,H., Brocklehurst,C. and Spits,H. (1990) A distinct wave of human T cell receptor γ/δ lymphocytes in the early fetal thymus: evidence for controlled gene rearrangement and cytokine production. *J. Exp. Med.*, **172**, 847–859.

Livak,F., Petrie,H.T., Crispe,I.N. and Schatz,D.G. (1995) In-frame TCR δ gene rearrangements play a critical role in the αβ/γδ T cell lineage decision. *Immunity*, **2**, 617–627

Loveys,D.A., Streiff,M.B. and Kato,G.J. (1996) E2A basic-helix–loop–helix transcription factors are negatively regulated by serum growth factors and by the Id3 protein. *Nucleic Acids Res.*, **24**, 2813–2820.

Margolis,D., Yassai,M., Hletko,A., McOlash,L. and Gorski,J. (1997) Concurrent or sequential delta and beta TCR gene rearrangement during thymocyte development: individual thymi follow distinct pathways. *J. Immunol.*, **159**, 529–533

Marquez,C., Trigueros,C., Fernandez,E. and Toribio,M.L. (1995) The development of T and non-T cell lineages from $CD34^+$ human thymic precursors can be traced by the differential expression of CD44. *J. Exp. Med.*, **181**, 475–483.

Murre,C., McCaw,P.S. and Baltimore,D. (1989) A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. *Cell*, **56**, 777–783.

Murre,C. *et al.* (1994) Structure and function of helix–loop–helix proteins. *Biochim. Biophys. Acta*, **1218**, 129–135.

Pestova,T.V., Hellen,C.U. and Shatsky,I.N. (1996) Canonical eukaryotic initiation factors determine initiation of translation by internal ribosomal entry. *Mol. Cell. Biol.*, **16**, 6859–6869.

Ramiro,A.R., Trigueros,C., Marquez,C., San,M.J. and Toribio,M.L. (1996) Regulation of pre-T cell receptor (pTα-TCRβ) gene expression during human thymic development. *J. Exp. Med.*, **184**, 519–530.

Res,P., Martınez Caceres,E., Jaleco,A.C., Noteboom,E., Weijer,K. and Spits,H. (1996) $CD34^+CD38^{dim}$ cells in the human thymus can differentiate into T, natural killer and dendritic cells but are distinct from stem cells. *Blood*, **87**, 5196–5206

Res,P., Blom,B., Hori,T., Weijer,K. and Spits,H. (1997) Downregulation of CD1 marks acquisition of functional maturation of human thymocytes and defines a control point in late stages of human T cell development. *J. Exp. Med.*, **185**, 141–151.

Riechmann,V., Van Cruchten,I. and Sablitzky,F. (1994) The expression pattern of Id4, a novel dominant negative helix–loop–helix protein, is distinct from Id1, Id2 and Id3. *Nucleic Acids Res.*, **22**, 749–755.

Sachs,A.B., Sarnow,P. and Hentze,M.W. (1997) Starting at the beginning, middle, and end: translation initiation in eukaryotes. *Cell*, **89**, 831–838.

Sanchez,M.J., Spits,H., Lanier,L.L. and Philips,J.H. (1993) Human natural killer cell committed thymocytes and their relationship to the T cell lineage. *J. Exp. Med.*, **178**, 1857–1866.

Sanchez,M.-J., Muench,M.O., Roncarolo,M.G., Lanier,L. and Phillips,J.H. (1994) Identification of a common T/NK cell progenitor in human fetal thymus. *J. Exp. Med.*, **180**, 569–576.

Scollay,R., Smith,J. and Stauffer,V. (1986) Dynamics of early T cells: prothymocyte migration and proliferation in the adult mouse thymus. *Immunol. Rev.*, **91**, 129–157.

Shortman,K. and Wu,L. (1996) Early T lymphocyte progenitors. *Annu. Rev. Immunol.*, **14**, 29–47.

Sigvardsson,M., O'Riordan,M. and Grosschedl,R. (1997) EBF and E47 collaborate to induce expression of the endogenous immunoglobulin surrogate light chain genes. *Immunity*, **7**, 25–36.

Spits,H., Lanier,L. and Phillips,J.H. (1995) Development of human T and natural killer cells. *Blood*, **85**, 2654–2670.

Spits,H., Blom,B., Jaleco,A.C., Weijer,K., Verschuren,M.C.M, van Dongen,J.J., Heemskerk,M.H.M and Res,P.C.M (1998) Early stages in the development of human T, natural killer and thymic dendritic cells. *Immunol. Rev.*, **165**, 75–86.

Sun,X.H., Copeland,N.G., Jenkins,N.A. and Baltimore,D. (1991) Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix–loop–helix proteins. *Mol. Cell. Biol.*, **11**, 5603–5611.

Verschuren,M.C.M., Blom,B., Spits,H. and van Dongen,J.J.M. (1998) PjA-Bp expression and T cell receptor δ deletion during human T cell development. *Int. Immunol.*, **10**, 1873–1880.

Washburn,T., Schweighoffer,E., Gridley,T., Chang,D., Fowlkes,B.J., Cado,D. and Robey,E. (1997) Notch activity influences the αβ versus γδ T cell lineage decision. *Cell*, **88**, 833–843.

Yssel,H., De Vries,J.E., Koken,M., Van Blitterswijk,W. and Spits,H. (1984) Serum-free medium for the generation and the propagation of functional human cytotoxic and helper T cell clones. *J. Immunol. Methods*, **72**, 219–227.

Zhuang,Y., Soriano,P. and Weintraub,H. (1994) The helix–loop–helix gene E2A is required for B cell formation. *Cell*, **79**, 875–884.

Zhuang,Y., Cheng,P. and Weintraub,H. (1996) B lymphocyte development is regulated by the combined dosage of three basic-helix–loop–helix genes, E2A, E2-2 and HEB. *Mol. Cell. Biol.*, **16**, 2898–2905.

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