Lymphoid development; a dynamic interplay of timing and dosing
van Lent, A.U.G.

Citation for published version (APA):
van Lent, A. U. G. (2009). Lymphoid development; a dynamic interplay of timing and dosing

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
The Id2 induced block in T cell generation is overcome by TCRαβ overexpression

Anja U. van Lent, Bianca Blom, Maho Nagasawa, Rachida Siamari, Hergen Spits and Nicolas Legrand
Abstract

The thymus is permissive for development of progenitor cells into multiple lineages, including T cells, NK cells and Dendritic Cells. Commitment into a single lineage is orchestrated by the balanced expression of various transcription factors. In this study we antagonized E protein transcriptional activity by overexpression of Inhibitor of DNA binding (Id)2 to assess the requirement of E proteins in T cell development. Ectopic expression of Id2 blocked T cell development from early progenitor cells in T cell permissive conditions (co-culture with OP9-DL1 stromal cells). We showed that this block could be rescued by TCRαβ gene transfer, but not by transfer of the TCRβ-chain alone, generating non-conventional CD4−CD8− double negative mature T cells which were responsive to TCR activation. The Id2 x TCRαβ transduced progenitor cells did not pass the anticipated CD4+CD8+ DP stage, which could be partially restored by simultaneous expression of the pre-TCR-α chain. However, the development of mature CD8+ mature T cells could not be rescued by pre-TCR-α overexpression. Our data thus reveal essential requirements for E-proteins in TCRαβ T cell development apart from the processes related to the assembly of functional pre-TCR / TCR genes. Finally, we show that sustained Notch induced signals are required to seal the commitment of TCRαβ/CD3 expressing cells into the TCRαβ T cell lineage, which otherwise develop into cells phenotypically resembling NK cells or NKT cells.
Introduction

T cells develop in the thymus from early thymic progenitors (ETP), which are the most immature intrathymic progenitors, that are gradually restricted in their multi-lineage potential. In addition to T cell potential, ETP display myeloid, dendritic cell (DC), natural killer (NK) and very limited B cell potential (1). The most immature thymic subset in the human thymus expresses CD34 and CD10 but lack CD1a and CD24 (2). Inside the thymus, ETP interact with thymic stromal cells and receive extracellular signals, including Notch ligand-mediated signals, that critically regulate their commitment to the T cell lineage and early thymopoiesis. Ligation of the Notch-1 receptor by its ligands of the Delta-like family results in cleavage of the receptor and transport of the intra cellular part of Notch-1 to the nucleus leading to activation of CSL, which specifically initiates and maintains the T cell differentiation program (3). Other transcriptional factors have been shown to be essential to allow the proper continuation of the T cell commitment process, including GATA-3, TCF family factors, “E proteins”, and the Ets family factor PU.1 (4). These factors ensure not only survival of the cells in the early phases of T cell differentiation and generation of a functional T cell receptor (TCR) complex, but in addition contribute to the block in differentiation to other lineage (5-7).

The upregulation of CD7 on the CD34+CD1a- uncommitted T cell precursors marks the loss of DC and myeloid potential (8). These precursors still have the capacity to become NK cells or T cells. Ensuing upregulation of CD1a is considered to be a hallmark of human T cell lineage commitment, as CD34+CD1a+ cells are unable to develop into non-T cell fates (9-11) and have initiated TCR loci rearrangement. This recombination process occurs in a strict order under the control of a molecular machinery containing the essential recombination activating gene (RAG)-1 and RAG-2 proteins. The rearrangement of the TCRδ gene segments occurs prior to the TCRγ and TCRβ rearrangements, and the TCRα locus is last in this hierarchically-regulated process (12-14). ETP have been reported to already contain immature (D-D and D-J) TCRδ gene rearrangements, whereas the γ locus is still in germline configuration. Following CD1a upregulation, the differentiating cells exhibit mature TCRδ rearrangements and initiate TCRγ and TCRβ rearrangements. Their direct CD4 immature single positive (CD4ISP) progeny contains a significant proportion of TCRγ rearranged genes and the first rearranged V-DJ TCRβ alleles (12). Productive in-frame rearrangement of the TCR γ and δ genes will result in the expression of a TCR-γδ complex and direct differentiation along the γδ T cell lineage. In contrast, a functional TCR-β chain will pair with
the surrogate pre-TCR-α (pTα) chain in a pre-TCR complex to induce β selection, β allelic exclusion and further differentiation along the αβ T cell lineage pathway (15). The developmental outcomes of these two processes are rather distinct. Most γδ T cells never express the CD4 or CD8αβ co-receptors and they show little proliferation capacity. In contrast, pre-TCR-mediated β selection results in rapid differentiation into CD4+CD8+ double-positive (DP) cells and extensive proliferation (15). Signaling through each type of TCR at this stage of development appears to promote a particular fate at the expense of the other lineage potential (16, 17).

Apart from the nature of the rearranged TCR genes, other mechanisms have been implicated in the αβ versus γδ T lineage choice as well, including the TCR signal strength, cytokines such as IL-7, various transcription factors and Notch signaling (18). It is known that the balance between Id and E-proteins plays an important role in the T/NK lineage decision and in αβ T cell development (19). E proteins are a class of transcription factors, which consist of a helix-loop-helix (HLH) dimerization motif and a basic DNA binding domain that targets conserved E-box motifs. Members of the E-protein family include E2-2, HEB, and the E12 and E47 splice variant products of the E2A gene. E proteins have been reported to regulate the expression of surrogate and antigen receptor genes and promote TCR rearrangements (reviewed in (20)). For example, HEB and E2A are reported to initiate pTα and RAG1/2 gene expression (21, 22) and suppress progression to the DP stage in absence of an adequate pre-TCR complex (23) and accordingly HEB and E2A-deficient mice demonstrate perturbed T cell development.

Transcriptional activity of E proteins is regulated by the structurally related inhibitor of DNA (Id) proteins. This family of HLH factors can heterodimerize with the E proteins, but lacks a basic DNA binding domain. As such, they sequester the E proteins and block their transcriptional activity (24). Enforced expression of Id3 in human CD34+CD1α- thymocytes was shown to strongly suppress both TCRαβ and TCRγδ T cell development, presumably due to TCR rearrangement inhibition, and promote NK cell generation in fetal thymic organ cultures (FTOC) (25). Our group has observed that Id2 is as potent as Id3 in blocking human T cell development in vitro ((26) and Schotte et al., manuscript submitted). Additionally, the ectopically expressed Id2 in human thymic progenitor cells was shown to promote the accumulation of CD5+CD1α+ cells, which phenotypically resemble NK/T progenitor cells. These progenitor cells could be further expanded in the presence of IL-15 and differentiated into mature NK cells (Schotte et al., manuscript submitted). These results
suggest a role for Id2 in NK cell development and are in line with the observation that mice deficient for Id2 demonstrate disturbed NK cell development (27).

Furthermore, ectopic expression of Id3 in CD4ISP pre-T cells led to a specific block in αβ, but not γδ, T cell lineage development (28). Since only the TCRαβ loci at the CD4ISP stage are sufficiently rearranged to allow TCR formation, the observed outcome could be due to either interference of Id3 with TCRαβ rearrangements or a developmental arrest in TCRαβ differentiation at the stage that these rearrangements are initiated. Id3 overexpression may inhibit T cell development by decreasing the expression of the RAG and pTα genes (28). In that case it is predicted that TCRαβ T cell development can be restored by introduction of productively rearranged TCRα and TCRβ genes and/or of the pTα gene.

We have previously shown that TCRαβ gene transfer into human hematopoietic progenitors, with subsequent culture on OP9 stromal cells expressing the Notch ligand Delta-like1 (DL1), led to enhanced T cell lineage commitment and TCR surface expression, giving rise to phenotypically and functionally mature, antigen specific, CD8+ T cells (29). In this study, we indirectly addressed the role of E proteins in TCRαβ T cell development, by analyzing whether the Id2-induced block in T cell development could be rescued by the enforced expression of a complete and functional TCRαβ complex.

Materials and methods

Cell lines, constructs and retroviral production
The OP9-DL1 cell lines were previously described (30). Briefly, murine bone marrow stromal OP9 cells (31) were transduced with the LZRS IRES-neo retroviral vector engineered to express human Delta-like1 (DL1). Cells were maintained in MEMα (Invitrogen, Carlsbad CA, USA) supplemented with 20% FCS (Hyclone, Logan UT, USA). We used the following HLA-A2 restricted TCRs (32-34): HA-2 specific (HA-2.5; AV15 BV18); hCMV-pp65 (495-503) specific (AV18 BV13); MART-1 (26-35) specific (AV25 BV12). The cDNA sequences encoding the TCRα or TCRβ chain of each TCR were inserted separately, or combined via a T2A linker (35), into the multiple cloning site of LZRS vector upstream of an internal ribosomal entry site and a marker gene (either enhanced green (GFP), yellow (YFP) fluorescent protein or the truncated form of the nerve growth factor receptor ΔNGFR (36)). The retroviral constructs LZRS-Id2-IRES-GFP and LZRS-Id2-IRES-YFP were described previously (26, 37). Control vectors were empty LZRS IRES-GFP,
LZRS IRES-YFP and LZRS IRES-ANGFR. Retroviral supernatants were produced as described (25) using the 293T-based Phoenix packaging cell line (38).

Isolation of human T cell progenitors
Thymocytes were obtained from post-natal thymus (PNT) tissue of surgical specimens removed from children up to 3 years of age undergoing open heart surgery, with informed consent from patient’s parents and approval by the medical ethical committee of the Academic Medical Center of the University of Amsterdam. The thymic tissue was mechanically disrupted and pressed through a stainless steel mesh to obtain a single-cell suspension, which was left overnight at 4°C. The thymocytes were isolated the next day from a Ficoll-Paque™ Plus (GE-Healthcare, Chalfont St Giles, UK) density gradient. CD34+ cells were enriched by immunomagnetic cell sorting, using direct CD34 human progenitor cell isolation kit (varioMACS, Miltenyi Biotec, Bergisch Gladbach, Germany). The CD34+ thymocytes were stained with mAb against CD34, CD1a, CD3, CD56, and BDCA2. The CD34+CD1a-CD56-CD3-BDCA2- (further referred to as CD34+CD1a-) population was sorted using a FACS Aria (BD Bioscience, Franklin Lakes NJ, USA), to purity always ≥ 99%. Progenitors from both HLA-A2+ and HLA-A2- donors were used and behaved similarly.

Retroviral transduction
The CD34+CD1a- PNT progenitors were cultured overnight in IMDM (Invitrogen, Carlsbad CA, USA) supplemented with Yssel’s medium (39), 5% normal human serum (NHS), 20ng/mL human stem cell factor (huSCF; PeproTech, Rocky Hill NJ, USA), and 20ng/mL human interleukin-7 (kindly given by Prof. J.J. Cornelissen, Erasmus University Rotterdam). The following day, cells were incubated for 6 to 8 hours with virus supernatant in retronectin-coated plates (30 µg/mL; Takara Biomedicals, Otsu, Shiga, Japan). Transductions with control, TCR chain and Id2 expressing vectors were always performed before the start of OP9 co-cultures.

Co-cultures of human progenitor and OP9 cells
The in vitro development of human T cells was assessed by co-culturing 5-30x10⁴ CD34+CD1a- progenitor cells with 5x10⁴ OP9 or OP9-DL1 cells in MEMα (Invitrogen, Carlsbad CA, USA) with 20% FCS (HyClone, Logan UT, USA), 5ng/mL huIL-7 (PeproTech, Rocky Hill NJ, USA), and 5ng/mL human Flt-3 ligand (gift from Prof. J.J. Cornelissen, Erasmus University Rotterdam) (30). The co-cultures were supplemented every 2 to 3 days with fresh medium, and progenitor cells were transferred to fresh stromal cells every 4 to 5
days of culture.

**Flow cytometry analysis for cell surface markers**
Cell suspensions were labeled with FITC, PE, PerCP-Cy5.5, PE-Cy7, APC, APC-Cy7 or Alexa-700 coupled anti-human mAb targeting the following cell surface markers: CD3 (SK7), CD4 (SK3), CD5 (53-7.3), CD8 (SK1), CD45 (2D1), CD56 (NCAM16.2) from BD Bioscience (Franklin Lakes NJ, USA), TCRαβ (GL3) from BD Pharmingen (San Diego, CA, USA), and CD1a (T6-RD1), CD16 (3G8), TCR pan α/β (IP26A), TCR-BV12 (VER2.32.1), -BV13.1 (IMMU222) and -BV18 (BA62.6) from Beckman Coulter (Fullerton CA, USA). Dead cells were excluded based on DAPI incorporation. All washings and reagent dilutions were done with PBS containing 2% FCS and 0.02% sodium azide (NaN3). All acquisitions were performed with a LSR-II (BD Bioscience) flow cytometer interfaced to FACS-Diva software system, and the data were analyzed with the FlowJo software (Tree Star).

**Feeder mix cultures for T and NK cell expansion**
The in vitro expansion of mature T and NK lymphocytes was performed by culturing for 10 days 1-5x10^5/mL T cells with a feeder mix consisting of 2x10^6/mL irradiated (40 Gy) allogeneic PBMC from two different donors and 2x10^5/mL irradiated (80 Gy) JY Epstein-Barr virus (EBV)-transformed B-cells, as described elsewhere (40). The culture medium consisted of IMDM supplemented with Yssel's medium (39), 1% NHS, 20ng/mL phytohemagglutinin (PHA; Gibco, Grand Island, NY) and 20U/mL recombinant human IL-2 (rhIL-2; Roche, Nutley, NJ). When required, T cells were stimulated again after 10 days.

**Statistical analyses**
Data were subjected to two-tail paired or unpaired Student’s t-test analysis where indicated in the figure legends. The obtained p values were considered significant when p<0.05.

**Results**

**Overexpression of both rearranged TCRα and TCRβ chains bypasses the Id2 induced block in T cell development.**
The balance of Id and E-proteins plays an important role in the T/NK lineage decision. Previously, we reported that transduction of Id3 in CD4 ISP thymocytes resulted in impaired TCRαβ T cell development in FTOC (28). Only a small minority of CD4 ISP have completed TCRβ rearrangements and none of these cells have initiated TCRα rearrange-
ments. Since Id overexpression reduces TCRβ rearrangements (26) Id2 may inhibit differentiation of CD4ISP cells by inhibiting rearrangements of the TCRβ and/or TCRα loci. This hypothesis predicts that ectopic expression of TCR genes rescues the Id2 induced block in T cell development.

We transduced CD34+CD1a- post-natal thymic progenitor cells with a TCRβ chain (BV13) and Id2 and cultured the cells on stromal OP9 cells expressing the Notch ligand Delta-like1 (DL1). This culture system has been shown to efficiently support T cell development from both non-transduced and transduced human CD34+CD1a- postnatal thymocytes (29). After 14 days of OP9-DL1 co-culture, almost 10% of cells in the TCRβ transduced culture expressed the introduced TCRβ chain (BV13) and a full TCRαβ/CD3 complex (Fig. 1) suggesting that in these developing T cells the introduced TCRβ chain had been able to pair with endogenously rearranged TCRα chains. In contrast, TCRβ x Id2 transduced thymic progenitor cells did not express TCRαβ/CD3 on their cell surface. These results may suggest that Id2 impaired T cell development by inhibiting endogenous TCRα rearrangements. Alternatively, Id2 may restrain cell surface expression of the TCRαβ/CD3 complex. To address these issues, we introduced both TCRα and TCRβ chains in thymic progenitor cells either with or without simultaneous overexpression of Id2. After co-culture on OP9-DL1 cells we observed that at least 40% of cells expressed the introduced TCRβ chain (BV13) and the TCRαβ/CD3 complex on their cell surface. Notably, similar or higher percentages of T cells were generated when both Id2 and TCRαβ were overexpressed (Fig. 1). This indicates that Id2 does not prevent cell surface expression of the TCRαβ complex. Moreover, introduction of the rearranged TCRα and TCRβ chains can bypass the Id2 induced block in T cell development. Taken together, when enforcing Id2 expression in thymic progenitor cells, it is conceivable that impaired TCR rearrangement is causal to the observed block in T cell development.

Overexpression of a TCR increases the proportion of mature T cells generated, whereas Id2 increases the cell number.

To gain more insight in the kinetics and number of mature T cells that developed from TCRαβ x Id2 transduced progenitor cells in co-culture with OP9-DL1 cells, T cell differentiation was followed over time by flow cytometric analysis of surface markers. We compared cultures derived from thymic progenitor cells that were transduced with control vectors (Ctrl), Id2 alone (Id2), the full TCRαβ alone (TCRαβ), or both TCRαβ and Id2 (TCRαβ x Id2). The GFP, YFP and / or NGFR markers linked to the genes allowed us to track differential
outcome of cell populations. As expected, entry of progenitors into the T cell lineage was reduced in the presence of Id2 (with a small proportion of CD1a+ cells) and no CD3+ cells were generated (Fig. 2A). In contrast, the presence of the TCRαβ imposed a pronounced T cell-skewed phenotype, with a large proportion of CD3+CD1a- and CD1a+ cells (Fig. 2B). In the presence of both Id2 and TCRαβ phenotypically mature CD3+CD1a- cells were generated at higher frequency (Fig. 2A) and with significantly increased cell numbers (Fig. 2B) compared to when TCRαβ alone was expressed. Collectively, the Id2 induced block in T cell development was overcome by concomitant overexpression of TCRαβ, moreover ectopic expression of Id2 significantly increased the absolute numbers of mature T cells generated when combined with TCRαβ overexpression.

Enforced expression of both Id2 and TCRαβ yields mature TCRαβ+ T cells, which exhibit a non-conventional phenotype.

To further characterize the nature of the rescued Id2'TCRαβ+ T cells we performed an extended phenotypic analysis of the cells generated at the end of the OP9-DL1 co-culture. The population transduced with only the TCRαβ, which we previously demonstrated to be phenotypically and functionally mature TCRαβ+ T cells (29), was compared to the cells
Figure 2. T cell development in OP9-DL1 co-culture with TCR x Id2 double transduced PNT progenitors. Human CD34+CD1a+ lymphoid progenitors were purified from post-natal thymocytes and retrovirally transduced with empty control vectors or vectors containing the genes Id2 and/or a TCR and co-cultured with stromal OP9-DL1 cells and IL-7 and FLT3L (5 ng/mL each). Electronic gating on either single or double marker positive populations allowed comparison between cells containing only marker genes (Ctrl), Id2 or TCR alone, or a combination of a TCR and Id2 (TCR x Id2). (A) The fractions of human cells at different stages in T cell development (expressing CD1a and/or CD3) were followed over time and are shown for each transduced population. (B) The mature T cell numbers generated after 3 weeks in ratio to control transduced cells which were set to 1 are shown. The values are corrected for initial transduction efficiency. The results show one representative experiment out of 3. * = p<0.05
with enforced TCRαβ and Id2 expression. Evaluation of their CD4 versus CD8 profile revealed a notable difference: the majority of mature T cells generated with only the TCRαβ were CD8 single positive (SP) T cells (80%), whereas TCRαβ x Id2 mature T cells were almost exclusively CD4 CD8 double negative (DN) T cells (Fig. 3A). The large majority of CD3+ cells from both SP and DN T cell populations expressed the TCRβ chain of the introduced TCR-BV18 (Fig. 3B). We only detected TCRαβ and not TCRγδ on the surface of CD3+ cells, (Fig. 3C). As Id2 has previously been implicated in human NK cell development (Schotte et al, manuscript submitted) we also analyzed the expression of CD16 and CD56 (Fig. 3D). These cell surface markers are associated with the NK cell lineage, although CD56 is also expressed on subsets of activated T cells. We did not observe expression of CD16 in either TCRαβ or TCRαβ x Id2 transduced cultures, but CD56 was expressed on a proportion (23%) of TCR x Id2 transduced cells (Fig. 3D). The NKT cell invariant TCR-VA24 chain was not expressed on CD3+CD56+ cells (data not shown), ruling out the possibility that these cells resemble conventional NKT cells. Collectively, the majority of TCRαβ x Id2 transduced thymic progenitor cells developed into DN CD3+ T cells.

Figure 3. Phenotype of mature T cells generated in OP9-DL co-culture system from TCR x Id2 double transduced human PNT progenitors. Flow cytometric analysis of mature T cells generated in the OP9-DL co-cultures with TCR (HA2-NGFR) alone or dual TCR (HA2-NGFR) x Id2-GFP overexpression in PNTs. (A) The CD4 vs CD8 profile is on gated NGFR`GFPl (TCR only) or NGFR`GFP+ (TCR x Id2) CD3`CD1a` cells. (B) The β-chain of the HA2 TCR can be stained with an antibody against its constant BV18 domain. The staining is shown for total NGFR`GFPl (TCR) or NGFR`GFP+ (TCR x Id2) gated populations. (C) and (D) show staining against pan TCRαβ or TCRγδ complexes or CD56 and CD16 respectively, on NGFR`GFPl or NGFR`GFP+ gated subsets. Data shown is representative of four experiments.
TCR x Id2 dual transduced thymic progenitor cells develop into functionally mature T cells in OP9-DL1 co-culture.

T cells are defined by the surface expression of a functional CD3-TCR complex. To assess the ability of the TCRαβ DN T cells to respond to TCR-mediated signals, we polyclonally stimulated the cells harvested from the OP9-DL1 cultures with PHA, recombinant IL-2, and irradiated feeder cells consisting of human PBMC and EBV-transformed B cells (JY), as previously documented (40). We observed that all phenotypical mature T cells generated in OP9-DL1 co-cultures, irrespective of the expression of the transduced genes, had proliferated similarly under these conditions and expanded on average 25 times after 10 days of stimulation (Fig. 4A). As a result, it could clearly be noticed that all TCR transduced populations within the bulk culture, which initially contained the largest proportion of CD3+CD1a- cells at the start of the feeder-mix culture (Fig. 2A), were highly enriched after feeder-mix stimulation (Fig. 4B). It is notable that the fold increase in percentage of Id2 x TCR T cells is much higher, i.e. 6-fold, compared to the increase in either TCRαβ only transduced T cells, which was around 2-fold. Control transduced T cells maintained their frequencies. Preliminary experiments revealed that feeder mix cultures supplemented with the CMV TCR cognate antigen instead of PHA, resulted in an even more pronounced expansion of exclusively the CMV TCR (x Id2) transduced T cells, demonstrating antigen-specific functionality. Overall, these data indicate that mature T cells can be generated from TCRαβ x Id2 transduced thymic progenitor cells.

Developing T cells from dual TCRαβ x Id2 transduced thymic progenitor cells do not pass through the CD4+CD8+ double positive stage.

It has been well established that conventional TCRαβ T cell development requires developing thymocytes to pass through a stage in which the pre-TCR, a multimeric complex of the pre-TCRα (pTα) chain, CD3 components and a TCRβ-chain, is expressed at the cell surface. The pre-TCR contributes to the generation of CD4+CD8+ double positive (DP) thymocytes, and is shown to be crucial for the development of TCRαβ T cells, but not for TCRγδ T cells (reviewed in (41)). To determine the ability of TCRαβ x Id2 thymic progenitor cells to develop into CD4+CD8+ DP cells, we analyzed the expression of CD4 and CD8 over time in OP9-DL1 co-cultures after transducing thymic progenitor cells with the TCRα- and TCRβ-chains, with or without simultaneous Id2 overexpression. As expected, we observed that the Id2 only transduced progenitor cells consistently generated reduced percentages of CD4+CD8+ DP cells at any time-point as compared to the non-transduced (NT) progenitor cells (Fig. 5A). After 14 days the number of DP cells generated in Id2
ID2 AND TCR OVEREXPRESSION IN HUMAN THYMIC PROGENITORS

Figure 4. Dual TCR x Id2 transduced T cells produced in OP9-DL1 cocultures are functional. Bulk cultures containing control, TCR-YFP and TCR-YFP x Id2-NGFR transduced PNT were cultured for 3 weeks on OP9-DL1 and subsequently transferred to stimulation assays with irradiated feeder cells, PHA and IL-2. After 10 days the expansion rate of phenotypically mature (CD3+CD1a-) cells was calculated by comparing the number of mature cells before and after feeder mix stimulation, for control transduced (closed squares), TCR (open circles), and TCR x Id2 double transduced (closed triangles) populations (A). (B) Proportions of the different transduced populations within one culture are depicted at the start (left) and the end (right) of the feedermix stimulation. Dotplots show one experiment representative of seven.

transduced cultures was significantly reduced (6-fold) as compared to non-transduced cultures (Fig. 5B). In TCRαβ x Id2 transduced cultures this reduction was even higher (20-fold, p<0.001) as compared to control transduced cultures. The decrease in the number of DP cells generated from TCRαβ x Id2 versus TCRαβ only transduced thymic progenitor cells was not significant, but a trend could be observed (p-value = 0.14; Fig. 5B). Consistent with our previous results (29), the introduction of only a TCRβ chain inclined to stimulate the generation of high numbers of DP cells compared to non-transduced cells (p=0.06; Fig. 5B), reminiscent of pre-TCR signaling and the β-selection process. Again, concomitant overexpression of Id2 had a significant inhibitory effect on the accumulation of DP cells, both in terms of frequency (Fig. 5A) and cell number (Fig. 5B).

Our results raised the question whether diminished DP cell generation from Id2 transduced progenitor cells might be caused by reduced pre-TCR mediated signaling. It has been
reported that pTα gene expression is regulated by E2A and HEB during immature murine T cell development (42, 43) and we have observed a reduced level of the pTα transcript in cultured Id3 transduced thymic progenitor cells (28). In accordance, we observed that enforced expression of Id2 resulted in reduced levels of pTα transcripts in developing thymocytes as well (data not shown). Therefore, we assessed whether increasing the level of pTα gene expression might rescue the development of DP cells and mature CD8+ SP T cells from TCRαβ x Id2 transduced thymic progenitor cells. In line with our previous findings, we observed a high proportion of DP cells in the control and TCRαβ transduced cultures, which was strongly reduced when overexpressing Id2 simultaneously (Fig. 5C). When in addition to TCRαβ and Id2 also pTα was ectopically expressed in thymic progenitor cells, the generation of DP cells was partially rescued (Fig. 5C). However, in contrast to populations transduced with TCR, pTα or TCR x pTα in the absence of Id2 (data not shown), those that co-expressed Id2 failed to develop into CD8+ SP mature T cells. Taken together, our data indicate that Id2 inhibits the generation of DP immature T cells and CD8+ TCRαβ+ mature T cells. This is at least in part due to reduced pTα expression, although other gene expression pathways induced by E-protein activity appear to be involved as well.

Ectopic expression of Id2 results in increased expansion of lineage CD5+CD1a− progenitor cells

Overexpression of Id2 in thymic progenitor cells cultured on OP9-DL1 cells leads to expansion of CD5+CD1a− (CD3−CD4−CD8−) precursor cells (Schotte et al, manuscript submitted). To assess the effect of simultaneous TCRαβ and Id2 overexpression on the CD5+CD1a+ progenitor pool we determined the frequencies of (CD3−CD4−CD8− gated) CD5+CD1a+ progenitor cells and CD5+CD1a+ progenitors of TCRαβ or control transduced thymic progenitor cells, with or without Id2, after 9 days of OP9-DL1 co-culture. Consistent with our earlier findings, we noted a significant increase (4 fold) in the proportion of CD5+CD1a+ cells in the Id2 transduced cultures as compared to the control transduced culture (Fig. 6A). Similar percentages of CD5+CD1a+ cells were detected in Id2 only and TCRαβ x Id2 cultures, suggesting that the expansion of this CD5+CD1a+ pool is independent of TCRαβ expression (Fig. 6A). Proportions of committed CD5+CD1a+ cells were not significantly affected by enforced TCR expression (Fig. 6B).

To further examine the effect of Id2 on T cell lineage commitment, we evaluated the expression of CD1a on all cells present in the co-culture, i.e. without restrictive gating on cells that lack expression of CD3, CD4 or CD8. The fraction of CD1a+ cells was analyzed over time and
assessed for control or TCRαβ transduced thymic progenitor cells with and without simultaneous overexpression of Id2. CD1a upregulation was significantly reduced when Id2 was co-transduced, both in control and TCRαβ transduced populations (Fig. 6C). Overall, CD1a expression is not rescued by introduction of a TCRαβ in Id2 transduced progenitor cells.
Double transduced TCRαβ x Id2 thymic progenitor cells retain T/NK development potential, although sustained Notch signaling skews towards the T cell lineage

The lack of upregulation of the CD1a marker in TCR x Id2 transduced developing T cells may suggest that E protein activity is required for CD1a gene expression and/or T cell commitment. We observed that the TCRαβ x Id2 transduced thymic progenitor cells acquired the mature T cell phenotype (CD3⁺CD1a⁻) within a period as short as 5 days of co-culture on OP9-DL1 stromal cells. This phenotype of the T cells remained unaltered.

Figure 6. Overexpression of Id2 expands the frequency of CD5⁺CD1a⁻ NK/T bipotential progenitor cells from PNT in OP9-DL culture, irrespective of concomitant overexpression of a TCRαβ. Human CD34⁺CD1a⁻ post-natal thymocytes were sorted and retrovirally transduced with a control vector or a TCR (CMV, MART or HA2) with or without Id2, and co-cultured with stromal OP9-DL cells. (A) The frequencies of generated CD5⁺CD1a⁻ cells (A) and CD5⁺CD1a⁺ (B) (both gated on CD3⁺CD4⁺CD8⁻) in the various populations are shown after 9 days of culture. (C) The proportion of total CD1a expressing cells, without prior subset gating, was followed over time in the various populations. Graphs shows data of three pooled experiments.
in the continual OP9-DL1 culture over time. Previous studies have indicated that T cell commitment of early progenitors required sustained Notch signaling (44, 45). It is well documented that human thymic NK cells express CD3 proteins in their cytoplasm (46). We wondered whether the (TCRαβ x Id2) CD3⁺CD1a⁻ T cells recovered after various timelengths of OP9-DL1 co-culture were committed to the T cell lineage, or represent in fact pre-NK cells or NK cells that express a TCR. If that is the case we would expect that Notch signaling, which promotes T at the expense of NK cell development, would reduce the number of TCR bearing cells with NK cell markers like CD56. We therefore transferred the transduced cells to feeder mix culture conditions, which are permissive for the growth of both NK cells and T cells (47), at various time points after a pre-culture on OP9-DL1 stromal cells (Fig. 7A).

Control transduced thymic progenitor cells which were directly seeded into feeder mix cultures, i.e. without any pre-culturing on OP9-DL1, all developed into CD56⁺CD3⁻ NK cells (Fig. 7B - top left panel). This is in accordance with previous reports showing that human immature postnatal thymocytes give rise to phenotypically and functionally mature NK cells after 1-2 weeks of culture in presence of irradiated feeder cells and IL-2 (48). Increasing the time of pre-culturing on Notch-triggering OP9-DL1 stromal cells decreased the capacity of the cells to develop into CD56⁺CD3⁻ NK cells. Only when thymic progenitor cells had received Notch induced signals for more than 2 weeks, CD3⁺ T cells could be recovered from the feeder mix cultures (Fig. 7B - top row). After 20 days of OP9-DL1 co-culture, a population of phenotypically mature T cells (7% of total cells) had developed from thymic progenitor cells (Fig. 7A - top right panel). Once the bulk culture was transferred to the feeder mix culture, the large majority of the expanding cells consisted of CD3⁺CD56⁻ T cells, no CD56⁺CD3⁻ NK cell fraction was detected (Fig. 7B - top right panel). Transduction of thymic progenitor cells with only Id2 gave exclusive rise to CD56⁺CD3⁻ NK cells, on the condition of short periods of OP9-DL1 pre-culture (Fig. 7A/B - 2nd top row). The total number of CD56⁺CD3⁻ NK cells generated after feeder mix culture was strongly enhanced in the presence of Id2 as compared to control-transduced cells, although these numbers rapidly decreased as the length of OP9-DL1 pre-culturing was extended (Fig. 7C - NB note scale differences after different time lengths) consistent with the notion that Notch signaling reduces the NK cell progenitor potential.

TCRαβ-transduced thymic progenitor cells generated the largest proportions of CD3⁺CD56⁻ T cells in feeder mix cultures, even without OP9-DL1 pre-culture period (Fig. 7B – 3th
row). In the latter case, the generated CD3⁺CD56⁺ T cells expressed the introduced TCRβ-chain on their surface, but displayed minimal proliferation capacity. The number of T cells generated in feeder mix cultures increased with incremented time of OP9-DL1 pre-culture (Fig. 7C). Concomitant expression of Id2 in TCR-transduced thymic progenitor cells enhanced the numbers of all subsets (CD3⁺ T cells, CD3⁺CD56⁻ cells and CD3⁻CD56⁺ NK cells) formed in feeder mix cultures, as compared to control-, TCR- and Id2-transduced progenitors (Fig. 7C). Increasing the duration of Notch signaling in OP9-DL1 pre-cultures enhanced CD3⁺CD56⁺ T cell accumulation and hindered CD3⁻CD56⁺ NK cell generation, both in terms of frequency (Fig. 7B - bottom row) and absolute cell numbers (Fig. 7C).

Collectively, our results indicate that the additional enforced expression of a TCR leads to increased T cell generation in feeder mix cultures, starting from control- or Id2-transduced thymic progenitor cells pre-cultured on OP9-DL1 stromal cells. However, the expression of a complete TCRαβ is not sufficient to fully compensate for absent or limited Notch triggering in the proper generation of TCRαβ/CD3⁺ T cells. Furthermore, enforced expression of Id2 leads to increased numbers of CD56⁺CD3⁻ NK cells and CD56⁻CD3⁺ cells, but the skewing by Id2 towards the NK cell lineage is counter-balanced by increased duration of Notch-triggering in pre-culture with OP9-DL1 cells. The frequency data of generated CD3⁺CD56⁺ T versus CD3⁻CD56⁺ NK cells of the various settings are summarized in Fig. 7D. We conclude therefore that the CD3⁺CD1a⁻ cells that are generated from Id2 and TCRαβ co-transduced cells belong to the T cell lineage. Those T cells develop, however, without an intermittent expression of CD1a.

Discussion

Various studies have clearly demonstrated that E-protein activity is essential for proper human T cell development (23). Nevertheless, the entirety of processes, which are specifically dependent on E protein activity and critical for T cell development, remains unclear. The dependence on bHLH factors for TCR gene rearrangements has been recognized, but it remains to be determined to what extent other molecular processes are non-redundant and cannot be compensated for. In this study, we determined whether human T cell development in the absence of E protein activity could proceed normally, when a complete TCR was provided for by ectopic gene expression. We demonstrated that the overexpression of the Id2 gene blocked T cell development, which could be rescued by enforced expression of a full TCRαβ complex.
ID2 AND TCR OVEREXPRESSION IN HUMAN THYMIC PROGENITORS

A

OP9-DL1 co-culture

0 days 5 days 10 days 20 days

Ctrl

sorted (lin-)
CD3-CD56-

Id-2

sorted (lin-)
CD3-CD56-

TCR

sorted (lin-)
CD3-CD56-

TCR x Id-2

sorted (lin-)
CD3-CD56-

OP9-DL1 preculture:

0 days 5 days 10 days 20 days

B

Feeder mix

Ctrl

Id-2

TCR

TCR x Id-2
Figure 7. Analysis of CD3 versus CD56 profile of transduced PNT developing in OP9-DL1 culture for various durations with contiguous feeder cell stimulation. Id2, TCR and TCR x Id2 transduced PNT were cultured for 0, 5, 10 or 20 days on OP9-DL cells after which they were transferred as bulk cultures to feeder mix conditions containing irradiated feeder cells, PHA and IL-2. Cells were stained and analysed for CD3 versus CD56 expression, just before transfer (A) and 10 days after transfer to feeder mix cultures (B). Arrows clarify the cellular input of the specific feeder mix cultures. Dotplots are shown per transduced population from left to right. (C) Absolute cell numbers per subset obtained in feeder mix culture after 0 (left), 10 (middle) and 20 (right) days are depicted. The cell numbers are corrected for transduction efficiency and calculated from 1x10^5 marker(s) specific-positive cells transferred to the feeder mix culture. The 0 and 5 day pre-cultures were performed once; 10 day pre-cultures 5 times; 20 day pre-cultures 4 times. Representative dotplots are shown. (D) This is a schematic overview of the proportions of CD3+CD56− versus CD3+CD56+ cell populations generated in the feeder mix cultures in the presence or absence of Id2 and/or TCR overexpression with variable durations of OP9-DL pre-culture (= Notch (days)) to summarize the data in the dotplots of (B).
It was previously reported that Id3-transduced CD4ISP cells exhibit a block in the development of TCRαβ+ T cells, although it is still an unsolved issue whether Id3 directly affects TCR gene rearrangements or whether Id3 merely results in a developmental arrest at the stages which initiate TCR gene rearrangements (28). More recent studies have demonstrated that E-proteins specifically bind to promoters linked to the process of TCR gene rearrangements (49). We demonstrated here that Id2 x TCRβ cells were unable to express a complete TCRαβ complex and to develop into mature T cells, in contrast to cells which had received a full TCRαβ (Fig. 1). This observation suggests that TCRα rearrangements are blocked by enforced Id2 expression. Consistent with this hypothesis, expression of TCRα and β genes overcame the inhibition of development of CD3⁺ T cells by Id2. However, development of CD4⁺CD8⁺ DP cells was not rescued and the majority of TCRαβ x Id2 transduced progenitor cells differentiated into CD4⁻CD8⁻ DN CD3⁺ T cells.

The acquisition of this mature T cell phenotype without transiting through the CD4⁺CD8⁺ DP immature T cell stage suggests commitment to the γδ T cell lineage. It is possible that enforced expression of Id2, like Id3, reduced the levels of pTα expression, preventing development into DP cells and allowing the TCRαβ transduced precursors to develop into the γδ T cell lineage. Indeed, we observed that overexpression of the pTα gene, resulted in rescue of the generation of CD4⁺CD8⁺ DP cells. However, this rescue was only partial and we never observed the development of mature CD8⁺ SP αβ lineage T cells. Our results indicate that ectopic expression of Id2, in addition to inhibiting TCR gene rearrangement and reducing pTα expression, also affects development of the TCRαβ lineage via another mechanism, independent of its effect on TCR generation and pTα expression alone.

This mechanism may be sequestration of HEB, which plays an important role in the processes required for TCRαβ T cell development. In general, among all three E-protein gene knockouts, disruption of HEB induces the most severe defect in T-cell development (50-52). More specifically, previous studies have shown that HEB is required for mouse T cell development in at least three distinct developmental stages: CD4⁺CD8⁻ DN cells, ISP cells and the transition from DP to SP mature T cells (53). Mice carrying a dominant negative allele of HEB, capable of forming non-functional heterodimers with E2A protein, display a stronger and earlier block in T cell development than HEB knockout mice. This developmental defect seemed specific to the αβ T-cell lineage, since γδ T cells were still detected, even though with a slightly reduced number (53). In the context of our study, it is
notable that enforced expression of HEB was the only E-protein able to counteract the Id2 induced expansion of progenitor cells, however was unable to rescue T cell development (Schotte et al, manuscript submitted). To test this hypothesis, the ability of HEB to rescue the block in αβ T cell differentiation in TCR x Id2 transduced progenitors needs to be investigated.

Analysis of the molecular mechanisms that control TCRαβ and TCRγδ T lineage differentiation in the mouse confirm that αβ T cell development is different and probably more complex than γδ T cell development. Besides a shared group of transcription factors in both T cell lineages, progression along the αβ lineage requires a collection of unique transcription factors, as well as epigenetic and miRNA-mediated regulation (18), to enable pre-TCR expression and allelic exclusion amongst other processes. Several regulatory genes of the γδ lineage (such as the runt-domain containing factor Runx3, high-mobility group transcription factor Sox13, Notch target gene HES1, E protein inhibitor Id2 (59)) can be detected as early as the DN2 and DN3 stage (59-61), suggesting that the expression in γδ T cells is contained since the precursor phase, while differentiating αβ lineage cells suppress these genes as they develop to DP cells. It was therefore postulated that the γδ lineage may represent a default T cell differentiation pathway (18). Id2 transduced PNT differentiate neither to NK nor T cells after prolonged OP9-DL1 culture, except when a TCR is simultaneously expressed. The TCR can divert the cells into the T cell lineage. Overall, it is reasonable to speculate that the mature CD4-CD8- T cells generated from TCRαβ x Id2-transduced progenitors are the product of blocked TCRαβ T cell development by Id2 and blocked NK differentiation by Notch signaling, imposing the TCRγδ lineage decision as a default pathway in the presence of an ectopically expressed TCR.

Although we favor the idea that at least a part of the CD3+ cells that develop in the cultures from TCRαβ x Id2 transduced progenitors are γδ T cells, other CD3+ T cells may be generated as well. In the OP9-DL1 co-culture, TCR x Id2 transduced progenitors differentiate also to CD56+ TCR+ cells. These cells might represent NKT cells, but alternatively they may belong to the NK cell lineage since their proportion in the cultures is strongly reduced by prolonged Notch-1 signaling. The finding that the temporal and quantitative dosing of E protein and Notch signaling results in gradual switches in NK versus γδ-like T cell development underscores the close relationship between these cell lineages. Functional and phenotypic similarities between NK cells and γδ T cells have frequently been described. Mouse γδ T cell populations were shown to exhibit variable degrees of NK programming, which was
reflected in the expression of NK receptors such as NKp46, CD16, NKG2D and NK1.1 and reduced levels of TCR expression (62). The majority of the CD3+CD56+ cells generated in our cultures expressed additional NK markers as well, such as CD94 and NKP30, but not CD16 (data not shown).

Our data are consistent with the idea that developing T cells maintain a long period of developmental plasticity, in which T lineage commitment remains reversible (3). The final developmental choice depends on precise temporal and quantitative dosing of originally Notch-activated regulatory genes. In this differentiation program, alternative lineage fates need to be excluded and T cell specific genes need to get specifically expressed (reviewed in (3)). In human thymic lymphoid development, CD34+CD1a- pro-T cells have the potential to develop to T cells, but also NK cells and DC. Only after up-regulation of CD1a are these alternative differentiation potentials lost. In the case of TCRαβ x Id2-transduced CD34+CD1a- pro-T cells placed in OP9-DL1 culture, we observed no up-regulation of CD1a except for a small fraction of developing cells (Fig. 6C). Since commitment to the T cell lineage is not blocked by the combination of Id2 and TCRαβ one should conclude that E proteins are directly regulating CD1a.

Id2 overexpression has additional effects besides affecting TCRαβ, TCRγδ and NK cell development, because the T cell numbers generated from thymic progenitors with enforced expression of TCRαβ x Id2 were significantly increased as compared to non-transduced and TCRαβ-only transduced thymic progenitors. The presence of the Id2 gene appeared to deliver a proliferative or survival advantage, which is in accordance with previous reports demonstrating the function of E2A and HEB in blocking thymocyte proliferation prior to proper pre-TCR expression (54), and the role of Id2 in both cellular proliferation and lymphocyte survival (55, 56).

**Acknowledgments**

We thank Mirjam Heemskerk for helpful comments and discussions. This work is supported by grants from the Landsteiner Foundation for Blood Transfusion Research (LSBR, grant 2003-1365) and the Dutch Cancer Society (KWF; grant NKI 2006-3530). We thank Berend Hooibrink for cell sorting and maintenance of the FACS facility. Dr. M. Hazekamp and staff at the Leiden University Medical Center and the Amsterdam Medical Center are gratefully thanked for providing postnatal thymus tissue.
References


sus T-cell lineage decision through control of GATA-3 and Spi-B. Blood 107:2446-2452.
ID2 AND TCR OVEREXPRESSION IN HUMAN THYMIC PROGENITORS


61. David-Fung, E. S., M. A. Yui, M. Morales, H. Wang, T. Taghon, R. A. Diamond, and E. V.