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$\alpha\beta$ versus $\gamma\delta$ fate choice: counting the T-cell lineages at the branch point

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Summary

Both $\alpha\beta$ and $\gamma\delta$ T cells develop in the thymus from a common progenitor. Historically distinguished by their T-cell receptor (TCR), these lineages are now defined on the basis of distinct molecular programs. Intriguingly, in many transgenic and knockout systems these programs are mismatched with the TCR type, leading to the development of $\gamma\delta$ lineage cells driven by $\alpha\beta$ TCR and *vice versa*. These puzzling observations were recently explained by the demonstration that TCR signal strength, rather than TCR type *per se*, instructs lineage fate, with stronger TCR signal favoring $\gamma\delta$ and weaker signal favoring $\alpha\beta$ lineage fates. These studies also highlighted the ERK (extracellular signal regulated kinase)-Egr (early growth response)-Id3 (inhibitor of differentiation 3) axis as a potential molecular switch downstream of TCR that determines lineage choice. Indeed, removal of *Id3* was sufficient to redirect TCR $\gamma\delta$ transgenic cells to the $\alpha\beta$ lineage, even in the presence of strong TCR signal. However, in TCR non-transgenic *Id3* knockout mice the overall number of $\gamma\delta$ lineage cells was increased due to an outgrowth of a V γ 1V δ 6.3 subset, suggesting that not all $\gamma\delta$ T cells depend on this molecular switch for lineage commitment. Thus, the $\gamma\delta$ lineage may in fact be a collection of two or more lineages not sharing a common molecular program and thus equipollent to the $\alpha\beta$ lineage. TCR signaling is not the only factor that is required for development of $\alpha\beta$ and $\gamma\delta$ lineage cells; other pathways, such as signaling from Notch and CXCR4 receptors, cooperate with the TCR in this process.

Keywords

$\alpha\beta$ T cells; $\gamma\delta$ T cells; lineage choice; T-cell receptor; Id3; PLZF

Introduction

T-cell sublineages can be divided into two large classes: $\alpha\beta$ and $\gamma\delta$ T cells. It is believed that the choice between $\alpha\beta$ and $\gamma\delta$ fates is the first lineage decision made by progenitors after they commit to the T-cell lineage. Historically $\alpha\beta$ and $\gamma\delta$ lineages were defined by the T-cell receptor (TCR) type. CD4[−]CD8[−] [double negative (DN)] thymocytes rearrange three out of four TCR loci: *Tcrb*, *Tcrg*, and *Tcrd*. The cells at this stage are arrested in proliferation and require TCR expression to re-enter cell cycle. If a cell succeeds in an in-frame *Tcrb* rearrangement, it expresses TCR β in a complex with the germline-encoded pre-TCR α (pT α) chain. Expression of this complex – pre-TCR – leads to a burst of proliferation, upregulation of the CD4 and CD8 coreceptors, silencing of *Tcrg* and initiation of *Tcra* rearrangement

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(which results in the excision of the *Tcrd* locus). If *Tcra* is productively rearranged, CD4⁺CD8⁺ [double positive (DP)] thymocytes express TCRαβ at the cell surface and can further differentiate, for instance towards CD4⁺ ('helper') or CD8⁺ ('killer') lineages. Progression through the DP stage is believed to be a hallmark of αβ lineage commitment.

Progenitors that productively rearrange *Tcrg* and *Tcrd* loci express the γδTCR at the cell surface. These cells likewise undergo a burst of proliferation, but in wildtype (wt) mice the majority of them avoid progression through the DP stage and egress to the periphery with a CD4⁺CD8⁺ (or, more rarely, with CD4⁺CD8⁺ or CD4⁺CD8⁺) phenotype. As a common molecular program provides a firmer basis for lineage definition than the expression of a single receptor (TCR), the αβ and γδ lineages are currently defined on the basis of progression through the DP stage (αβ) or lack thereof (γδ lineage). As the rescue of the quiescent thymocytes depends on pre-TCR or γδTCR expression, these processes are termed β- and γδ- selection, respectively.

Over time it became clear that the correlation between TCR type and lineage fate was not always perfect (Table 1). Premature TCRαβ expression in DN thymocytes, which takes place in the majority of TCR-transgenic strains, leads to the appearance of TCRαβ⁺ cells that resemble γδ T cells in their surface phenotype and ability for rapid effector responses (1), that do not rearrange endogenous *Tcra* loci (2), and that do not progress through the DP stage, as revealed by fate mapping experiments (3). DP cells, however, are also present in TCRαβ transgenic animals even in the absence of pTα (4), suggesting that the αβTCR can drive both αβ and γδ lineage differentiation. Early TCRαβ expression is not merely a feature of TCR transgenic systems as it can also happen in wt mice due to rare premature *Tcra* rearrangements driven by the *Tcrd* enhancer (5).

In contrast, most TCRγδ transgenic mice studied so far generate some DP cells even when bred to a *Rag* (*recombination-activating gene*)-deficient background, albeit the frequency of DP cells is variable (6,7). Furthermore, in TCR non-transgenic mice incapable of expression of either a pre-TCR or an αβTCR, such as TCRβ^{-/-} (8,9) and pTα^{-/-}TCRα^{-/-} (4), DP cells are likewise detected. There is evidence for the presence of a small population of TCRγδ-driven DP cells in wt mice (8). Thus although the pre-TCR drives exclusively differentiation of αβ lineage cells, both TCRαβ and TCRγδ are compatible with either fate. Interestingly, the pre-TCR seems to be a relatively novel evolutionary acquisition as pTα homologues, readily detected in all sequenced mammalian genomes, cannot be found in other vertebrates (10). Thus the 'non-canonical' relationship between TCR type and lineage fate observed in pTα-deficient animals and other knockout/transgenic systems may represent vestiges of once mainstream developmental pathways.

TCR signal strength as the lineage determining factor

If TCR type does not determine lineage fate but there is an obvious correlation between lineage choice and TCR composition in wt mice, what is the fate-determining factor? Two elegant studies suggested that TCR signal strength, rather than TCR type *per se*, determines the lineage choice (7,12). Both studies took advantage of the fact that the γδTCR is compatible with both γδ and αβ lineage fates. On the one hand, the αβ (DP) compartment in γδTCR transgenic mice was enhanced when TCR signal strength was attenuated by *CD3ζ* hemizygosity (12) or *lck* deficiency (7). On the other hand, increased signal strength in TCR transgenic mice on CD5^{+/-} or CD5^{-/-} backgrounds led to a decrease in DP cell numbers. In the KN6 transgenic system, where transgenic TCR recognizes β2-microglobulin (β2m)-dependent major histocompatibility complex (MHC) class Ib molecules T10 and T22 (13,14), *β2m* deficiency leads to a dramatic increase in DP cell numbers at the expense of

DN TCR $\gamma\delta^+$ cells with a mature phenotype (7). Thus, stronger TCR signal favors $\gamma\delta$ and weaker signal favors $\alpha\beta$ lineage development.

These studies firmly established the role of TCR signal strength in $\alpha\beta$ versus $\gamma\delta$ lineage choice. However, some data were compatible with lineage choice prior to TCR expression (so called pre-commitment) (15,16). CD25⁺CD44⁺ immature thymocytes that do not yet express TCR can be subdivided on the basis of IL-7R α expression. Although IL-7R α^{hi} and IL-7R α^{low} cells were able to generate both lineages and in both cases $\gamma\delta$ lineage cells were in the minority, IL-7R α^{hi} cells generated more $\gamma\delta$ lineage cells than the IL-7R α^{low} fraction (15). This result is compatible with the concept of pre-commitment; however, alternative explanations are possible. For instance, increased *Tcrd* rearrangement observed in the IL-7R α^{hi} fraction (15) can explain the increased ability to generate $\gamma\delta$ lineage cells.

To reconcile the pre-commitment model with the obvious role of TCR signal strength in the development of $\alpha\beta$ and $\gamma\delta$ lineages, it was suggested that the TCR signal may 'confirm' the lineage choice made by a TCR-independent mechanism (17,18): if the TCR signal strength matches the chosen lineage, then the cell continues to differentiate along the selected fate, whereas in case of a mismatch the development may be blocked and the cell eliminated. Indeed, it is easy to imagine that a strong TCR signal – for instance in the KN6 transgenic system in the presence of TCR ligand – deletes $\alpha\beta$ pre-committed T cells, and these cells are rescued in the absence of the ligand thus restoring the DP compartment.

As it is impossible to distinguish between instructive and confirmatory roles of TCR in lineage choice *in vivo* or in bulk cultures, we followed the fate of single TCR $\gamma\delta^+$ progenitors (19) in the OP9-DL1 coculture system (20) that can support the development of both $\alpha\beta$ and $\gamma\delta$ lineages (21). A substantial fraction of single immature TCR $\gamma\delta^+$ thymocytes gave rise to DP cells when cultured on OP9-DL1 cells. However, when the progeny of the same TCR $\gamma\delta$ -expressing cell which gave rise to DP cells received a strong TCR signal, they irreversibly acquired a mature $\gamma\delta$ lineage phenotype (19). Thus $\gamma\delta$ versus $\alpha\beta$ fate choice can be made after TCR expression, an observation that supports an instructive role of TCR signaling in lineage commitment.

Strong TCR signal: is the ligand required?

As at least in case of KN6 TCR transgenic cells a ligand is required to prevent diversion of the cells to the $\alpha\beta$ lineage (7), it is tempting to speculate that the same is true for other $\gamma\delta$ lineage cells. However, information on endogenous $\gamma\delta$ TCR ligands and their role in development is extremely scarce. In fact KN6 ligands, MHC class Ib molecules T10 and T22, are the only endogenous molecules that were formally shown to interact with a murine $\gamma\delta$ TCR (13,14,22). For human $\gamma\delta$ T cells, recognition of CD1c (23) and the stress-induced MHC-like molecule MICA (24,25) by their TCRs was demonstrated; however, the role of these molecules in $\gamma\delta$ lineage commitment could not be easily addressed. Many other endogenous molecules including but not restricted to isopentenyl pyrophosphate (26,27), cardiolipin (28), surface-expressed F1-ATPase (29), the non-classical MHC molecule Qa-1(30), an insulin-derived peptide (31), and the Skint1 receptor (32,33) were suggested as possible $\gamma\delta$ TCR ligands; however, formal evidence is still lacking. The role of these putative ligands in $\gamma\delta$ T cell development, with the exception of Skint1, has not been addressed.

Skint1 was discovered by mapping the gene responsible for a defect in the differentiation of skin dendritic epidermal V γ 5V δ 1 T cells (DETCs) [here and below V γ nomenclature, after Helig and Tonegawa (34)] in a substrain of FVB mice (32,33). DETCs represent the first wave of fetal $\gamma\delta$ T cells. They develop in the thymus around days 14-16 of embryonic development and home to the epidermis of the skin. In FVB/Tac mice, V γ 5V δ 1 cells were present in fetal thymi but did not acquire a mature phenotype and failed to populate the skin

(their niche was taken over by other $\gamma\delta$ T cells). The developmental block was rescued at the level of surface phenotype by crosslinking the TCR with an antibody, suggesting that the phenotype may be caused by the absence of the V γ 5V δ 1 TCR ligand expression (32). The defect was later mapped to the *Skint1* gene, encoding a transmembrane protein that is expressed by thymic stroma and keratinocytes (33), supporting the idea that it may encode a TCR ligand. However, formal evidence for a Skint1 – V γ 5V δ 1 TCR interaction is still missing. It was also not tested whether some V γ 5V δ 1 cells were redirected to the $\alpha\beta$ lineage in the absence of Skint1.

Some evidence supports the hypothesis that $\gamma\delta$ TCRs can generate ligand-independent signals as does the pre-TCR (35,36). Indeed, although in the KN6 system, TCR $\gamma\delta$ expressing cells were redirected to the $\alpha\beta$ lineage when T10/T22 expression was abolished by β 2m deficiency, in TCR non-transgenic mice comparable numbers of $\gamma\delta$ T cells bound T22 tetramers in wt and β 2m^{-/-} mice (37). However, it was noted that the only element of $\gamma\delta$ TCR that is required for binding of T10/T22 is D δ 2 (38), and interaction affinity between TCR and T10/T22 varies widely (39). Thus, it is possible that tetramer-binding cells in β 2m^{-/-} mice have TCRs different from those found in wt mice, are selected on ligands other than T10/T22, and merely cross-react with the T22 tetramers (40).

It is not clear whether the strong TCR signal required for $\gamma\delta$ lineage commitment is always generated by an interaction with a cognate ligand or whether it can also result from ligand-independent signaling. Identification of endogenous $\gamma\delta$ TCR ligands or formal proof of ligand-independent signaling is required to address this question.

The problem of a ‘useful’ $\gamma\delta$ TCR repertoire

$\gamma\delta$ TCRs are potentially the most diverse antigen receptors, in part due to the possibility to utilize more than one D segment in the TCR δ CDR3 loop (41-43). Moreover, at least in the case of T10/T22-restricted $\gamma\delta$ TCRs, the TCR δ CDR3 loop is sufficient to recognize the ligand (22,38,39), a feature not shared by studied $\alpha\beta$ TCRs and reminiscent of antigen recognition by antibodies. This observation raises a question of how a ‘useful’ $\gamma\delta$ TCR repertoire is selected. Indeed, if a strong TCR signal is sufficient for $\gamma\delta$ lineage differentiation, $\gamma\delta$ lineage cells may theoretically be selected on any extracellular molecule present in the thymus.

In $\alpha\beta$ T-cell development, MHC restriction of the TCR repertoire is imposed at least in part by CD4 and CD8 coreceptors that sequester the pool of available lck (44). Absence of both coreceptors leads to the availability of ‘free’ lck and rescues some $\alpha\beta$ T-cell development in MHC-deficient animals due to positive selection of non-MHC-restricted $\alpha\beta$ T cells (44). This selection in the absence of coreceptors resembles $\gamma\delta$ T-cell development, as $\gamma\delta$ T cells do not progress through the CD4⁺CD8⁺ stage.

V γ 5V δ 1 and V γ 6V δ 1 TCRs of $\gamma\delta$ T cells developing in fetal thymi have restricted diversity due to the lack of expression of terminal deoxynucleotidyl transferase (TdT) (required for the generation of junctional diversity) and to the restricted use of V γ gene segments in embryonic thymocytes. However, this explanation is not valid for $\gamma\delta$ T cells generated later in ontogeny. Thus, whether and how positive selection in the thymus leads to the generation of a ‘useful’ TCR $\gamma\delta$ repertoire is not clear at present.

The ERK-Egr-Id3 axis in $\alpha\beta$ versus $\gamma\delta$ lineage choice

The fact that TCR signal strength determines lineage fate implies the presence of a molecular switch capable of transforming an analog TCR signal strength into a binary lineage choice. The original work on the role of TCR signal strength in lineage commitment

(7,12) hinted that the ERK (extracellular signal regulated kinase)-Egr (early growth response)-Id3 (inhibitor of differentiation 3) axis may play such a role: ERK was more phosphorylated in TCR $\gamma\delta$ - than in pre-TCR-expressing thymocytes (12) and in KN6 thymocytes in the presence of the ligand (7). Correspondingly, the Egr transcription factors and their target, inhibitor of E protein function Id3, were induced in KN6 cells more strongly when the ligand was present (7).

Enforced expression of Egr1 disrupted the development of $\alpha\beta$ lineage cells from the $\beta 2m^{-/-}$ KN6 fetal thymic organ cultures (7). Likewise, it interfered with the development of $\alpha\beta$ lineage cells from wt but not $Id3^{-/-}$ progenitors – with a compensatory increase in TCR $\gamma\delta^{+}$ cells (7). As the Egr family consists of several members with overlapping functions, the authors further focused on the role of Id3 in $\alpha\beta$ versus $\gamma\delta$ lineage commitment. To this end, they bred KN6 TCR transgenic mice to the $Id3^{-/-}$ background. In the absence of Id3, large numbers of $\alpha\beta$ lineage (DP) cells were generated even when the $\gamma\delta$ TCR ligand was expressed (45), suggesting that $Id3$ upregulation was required for $\gamma\delta$ lineage commitment. Moreover, retroviral overexpression of $Id3$ in the absence of TCR (in Rag-deficient thymocytes) was sufficient to confer to them the ability to secrete IFN γ (45). In TCR non-transgenic mice, $Id3$ deficiency led to a dramatic decrease in V γ 5 T cells in the skin and in V γ 4 T cells in the spleen. It was previously reported that the enforced expression of Id3 disrupts $\alpha\beta$ but not $\gamma\delta$ T-cell development (46).

Id proteins negatively regulate the function of the E protein family transcription factors. They can heterodimerize with E proteins but lack a DNA-binding domain and thus function in a dominant-negative fashion (47). Inhibition of E protein functions is also believed to be required for $\alpha\beta$ lineage development, and E47 deficient $Rag^{-/-}$ thymocytes bypass the β -selection checkpoint (48,49). Thus, inhibition of E protein activity seems to be required for the differentiation of both lineages. It was suggested that the level of this inhibition may define the lineage fate, with stronger induction of Id3 by stronger TCR signal resulting in $\gamma\delta$ lineage differentiation (45).

How can an analog signal from TCR result in binary lineage choice? Wiest and colleagues (40) suggest that the duration of the signal rather signal intensity *per se* may play a role. It was previously reported that in Swiss 3T3 fibroblasts, transient ERK activation led to upregulation of the immediate early gene *c-Fos* but fails to stabilize c-Fos protein by phosphorylation, whereas when signaling was sustained and active ERK was still present when the c-Fos protein was produced, it stabilized c-Fos by phosphorylation (50). It is conceivable that ERK activation is sustained in presence of the ligand in the KN6 system and this in turn leads to stabilization of the immediate early gene *Egr1* (40). Indeed, the level of Egr1 upregulation in the presence of the ligand was about threefold on the level of mRNA but was almost 25-fold on the protein level (40), providing a potential mechanism for amplification of gradual differences in the signal into a near on/off switch.

The striking observation that the ERK-Egr-Id3 axis is involved in $\gamma\delta$ lineage commitment in the KN6 system, and that in the absence of $Id3$ the cells are redirected to the $\alpha\beta$ lineage, suggests that this is the molecular switch that determines $\alpha\beta$ versus $\gamma\delta$ lineage choice.

Id3-independent $\gamma\delta$ T cells

While Id3 expression was required for $\gamma\delta$ lineage development in the KN6 system and for the development of V γ 5 and V γ 4 cells in wt animals (45), the overall numbers of $\gamma\delta$ T cells in TCR non-transgenic Id3-deficient mice were increased due to an outgrowth of a V γ 1V δ 6.3 population (45,51,52). These cells had previously been shown to have an unusual surface phenotype (Thy1^{low}, some NK1.1⁺ and CD4⁺) and are unique among $\gamma\delta$ T cells in

their ability to produce IL-4 together with IFN γ (53). As these features are characteristic of NKT cells, these V γ 1V δ 6.3/V δ 6.4 $\gamma\delta$ T cells are often referred to as $\gamma\delta$ NKT cells (53,54).

Recently others (55,56) and we (57) demonstrated that these cells express the transcription factor PLZF (promyelocytic leukemia zinc finger), previously shown to be required and sufficient for the acquisition of innate-like properties by NKT cells (58,59). Like NKT cells, V γ 1V δ 6.3 cells from PLZF-deficient mice are incapable of rapid cytokine responses, suggesting that this transcription factor plays similar roles in NKT and V γ 1V δ 6.3 functional maturation.

Id3 independence of the PLZF-positive subset may in part be explained by the fact that these cells – similarly to $\alpha\beta$ NKT cells (60) – express a high level of Id2, another Id family member (T.K., H.v.B., unpublished observations). *Id2* was previously reported to be a direct PLZF target in myeloid cells (61). However, in *Rag1*^{-/-} V γ 1V δ 6.4 TCR transgenic mice, which normally generate large numbers of PLZF⁺ $\gamma\delta$ T cells (57), PLZF deficiency did not alter the balance between $\alpha\beta$ (DP) and $\gamma\delta$ lineage cells, suggesting that PLZF and its regulation of *Id2* were dispensable for $\gamma\delta$ lineage commitment of V γ 1V δ 6.4 cells (T.K., H.v.B., manuscript in preparation). As neither PLZF nor Id3 are required for $\gamma\delta$ lineage commitment of V γ 1V δ 6.3/V δ 6.4 cells, molecular mediators of this process are yet to be identified.

The fact that the role of TCR signal strength was initially established for the Id3-dependent $\gamma\delta$ lineage (7) raises the question of whether strong TCR signal is also required for the commitment of Id3-independent $\gamma\delta$ T cells. As both in $\gamma\delta$ (V γ 1V δ 6.3/V δ 6.4 cells) and in $\alpha\beta$ (NKT cells) compartments PLZF expression is associated with a very restricted combination of TCR chains and as both cell types are believed to undergo agonist selection (62), we decided to test if PLZF can be induced by strong TCR signal. We were able to induce PLZF in polyclonal immature TCR $\gamma\delta$ ⁺ thymocytes in cell culture by antibody-mediated TCR crosslinking in the presence (but not in the absence) of OP9 stroma (57), suggesting that PLZF may indeed be induced *in vivo* by agonist selection. Moreover, the conditions of these cultures were identical to those used in our experiments with lineage diversion of immature TCR $\gamma\delta$ ⁺ thymocytes (see above) (19), suggesting that PLZF⁺ $\gamma\delta$ T cells also require strong TCR signal for lineage commitment.

Although PLZF was readily induced by TCR crosslinking in culture, it was not upregulated *in vivo* by the presence of the TCR ligand in HY and KN6 TCR transgenic mice (T.K., H.v.B., unpublished observations) suggesting that additional stimuli were required. NKT cells are selected on other thymocytes (63) and require costimulatory signal from SLAM (signaling lymphocytic activating molecule) receptors that in turn require the adapter molecule SAP (SLAM-associated protein) (64). In the absence of SAP, NKT cell development is blocked (65). As V γ 1V δ 6.3 cell numbers are also reduced in SAP-deficient mice, albeit not as dramatically as numbers of NKT cells (55,57), it is conceivable that PLZF⁺ $\gamma\delta$ T cells are likewise selected on other thymocytes and that some costimulatory interaction in the course of this selection is required for PLZF induction. This interaction, however, is unlikely to be mediated by SLAM receptors, as PLZF is induced in SAP-deficient V γ 1V δ 6.3 cells, though not to the levels observed in wt cells (55).

Intriguingly, V γ 1V δ 6.3 T cells are increased in numbers not only in *Id3* knockouts but also in mice deficient for the tyrosine kinase Itk (56,66), the transcription factor KLF2 (67), as well as in mice with a mutated adapter molecule SLP-76 (SH2 domain-containing lymphocyte phosphoprotein of 76 kDa)(55). At least for KLF2 (67) and Itk (66) deficient cells, the mechanism of the increase in the V γ 1V δ 6.3 compartment was cell autonomous, as inferred from experiments with mixed bone marrow chimeras. Itk and SLP-76 are important

for TCR signaling, and Id3 is induced by TCR signaling. These facts were used as an argument against PLZF induction *in vivo* by a strong TCR signal (55). However, if TCR signals have nothing to do with PLZF upregulation, it is difficult to explain why its expression correlates with two TCR types, the V α 14 TCR of invariant NKT cells and the V γ 1V δ 6.3/V δ 6.4 TCR, and why PLZF expression can be induced by anti-TCR antibodies. One possibility is that some branches of TCR signaling (i.e. those that require Itk and tyrosines at positions 112, 128, and 145 in SLP-76) indeed inhibit PLZF induction (or expansion of PLZF⁺ cells), whereas others promote it. An alternative explanation, suggested by Lauritsen and colleagues (45), is that some of the V γ 1V δ 6.3 cells may be deleted by a very strong TCR signal and are rescued when TCR signaling is somewhat attenuated. Indeed, the junctional composition of TCR chains in Id3^{-/-} (52) V γ 1V δ 6 cells when compared to wt cells (53,68) seems to be overlapping but distinct. This finding may suggest that some cells that are normally deleted escape elimination in Id3 knockouts.

Does a single $\gamma\delta$ lineage exist?

The notion that some $\gamma\delta$ T cells strictly require Id3 for lineage commitment and are diverted to the $\alpha\beta$ lineage in its absence (45) while others do not require Id3 and even increase in number in Id3 knockouts (45,51,52,55) raises a question of whether such an entity as the $\gamma\delta$ lineage exists. Indeed, there is an important difference in how we currently define $\alpha\beta$ and $\gamma\delta$ lineages.

$\alpha\beta$ lineage cells are defined on the basis of a well-characterized molecular program, associated with the progression through the DP stage, some parts of which, including upregulation of CD4 and CD8 coreceptors and transcription factor ROR γ t (69), are documented to take place in all cells of the $\alpha\beta$ lineage. DP cells can be viewed as common uncommitted progenitors of all $\alpha\beta$ sublineages (although some suggestive evidence for pre-commitment to the CD8 $\alpha\alpha$ IEL lineage does exist) (70).

The $\gamma\delta$ lineage is defined on the basis of acquisition of functional maturity without progression through the DP stage. A common molecular program, characteristic of all developing $\gamma\delta$ lineage cells, has not been identified so far. The only pan- $\gamma\delta$ T-cell marker identified to date is the $\gamma\delta$ TCR itself. Although several molecules including sox13 (16) and ICER (71) were suggested for this role, some $\gamma\delta$ subsets do not express these molecules (19, and authors' unpublished observations). However, markers for $\gamma\delta$ sublineages can be readily identified (72,73).

This is probably not surprising, as most $\alpha\beta$ lineage cells in wt mice are driven by ligand-independent pre-TCR signaling, and the $\alpha\beta$ TCR is assembled only at the DP stage. Thus all TCR (and antigen presenting cell)-specific signals are received by the cell only after the common $\alpha\beta$ lineage program has brought the cell to the DP stage. On the contrary, as there is no pre-TCR analogue in $\gamma\delta$ T-cell development, a cell is likely to receive signals specific for a particular $\gamma\delta$ TCR once it is expressed on the cell surface. This may lead to an execution of 'sublineage-specific' programs right at the transition through the TCR controlled checkpoint. Thus, it is conceivable that multiple equipollent $\gamma\delta$ lineages diverge from each other and from the $\alpha\beta$ lineage at the first TCR-controlled checkpoint.

Different requirements for Notch signaling in $\alpha\beta$ and $\gamma\delta$ lineage development

In addition to TCR signaling, other pathways also regulate the transition through the β -/ $\gamma\delta$ -selection checkpoint. Notch signaling, important throughout T-cell development, was initially thought to determine the $\alpha\beta$ versus $\gamma\delta$ lineage fate (74,75). However, later studies

demonstrated that rather than instructing lineage choice, Notch signaling was absolutely required for $\alpha\beta$ lineage development, whereas $\gamma\delta$ lineage cells were relatively Notch-independent (6,21,76). It was shown that *Id3* expression by $\gamma\delta$ lineage cells was necessary and sufficient for this relative Notch independence (45). Interestingly, downregulation of E47 was blocked in pre-TCR- but not in $\gamma\delta$ TCR-expressing cells in the absence of Notch signaling (45), and it was suggested that weak pre-TCR signals required Notch signaling to counteract E protein activity, whereas strong signals from the $\gamma\delta$ TCR did not (45).

Although Notch1 expression as well as expression of its targets quickly diminishes after β -selection (76), several studies – both in culture (6,21,77) and *in vivo* (78) – demonstrated that Notch signaling is absolutely crucial at and after the β -selection checkpoint. The exact role of Notch at this stage of T-cell development, however, remains poorly understood. Ciofani *et al.* (79) demonstrated that Notch signaling was required for the survival of pre- β -selected cells, as *Rag*-deficient DN3 thymocytes quickly disappeared from cultures in the absence of Notch ligands but persisted in cultures where Notch ligands were present. As pre- β -selection DN3 cells do not proliferate, this result clearly indicates that Notch is required for the survival of DN3 cells. To address the role of Notch signaling beyond cell survival, it is crucial to compensate the survival defect. In one such attempt, Ciofani *et al.* (77) utilized constitutively active protein kinase C overexpression that increased cell yield from DN3 cultures in the absence of Notch ligands but did not lead to DP progression. However, neither cell proliferation nor the ability of surviving cells to differentiate to DP in secondary cultures were addressed in these experiments, making it difficult to conclude that Notch was crucial for differentiation *per se*. To address these issues, we performed a similar experiment with *vav-bcl2* transgenic (80) DN3a thymocytes. The *bcl2* transgene efficiently rescued the survival of thymocytes in the absence of Notch signaling (Fig. 1A). Although the cells cultured on OP9-GFP (without Notch ligand) showed diminished proliferation (Fig. 1C), some of them did progress to the DP stage (Fig. 1B). This may be the consequence of a sufficient level of Notch signaling having been received *in vivo*. To overcome this problem, we further sorted undifferentiated ($CD4^-CD8^-$) undivided cells from these cultures and subcultured them for another four days in the presence or absence of Notch ligands. Cells cultured for the first four days on OP9-GFP readily proliferated and progressed to the DP stage after transfer to OP9-DL1. The same cells cultured on OP9-GFP showed no signs of proliferation or differentiation (Fig. 1B,C). Thus, we conclude that Notch signaling is directly required for proliferation and differentiation towards the $\alpha\beta$ lineage.

Several direct Notch targets relevant to T-cell development were identified over last years. This includes *pTa* (81), *c-Myc* (82), and *IL-7Ra* (83). *IL-7Ra* signaling inhibits progression to the DP stage (84). Indeed, in OP9-DL1 cocultures, the presence of IL-7 delays progression to the DP stage, and addition of antibodies blocking the IL-7 receptor reverses this effect but does not alter differentiation of cells cultured in the absence of exogenous IL-7 (T.K., H.v.B., unpublished observations). IL-7 signaling is therefore not required for β -selection. We tested whether ectopic expression of *pTa* and *c-Myc* in presence of the *vav-bcl2* transgene was sufficient to drive DP progression in the absence of Notch ligands. To this end, we crossed *vav-bcl2* transgenic mice to mice that express *pTa* ectopically under the *lck* promoter and infected thymocytes from these animals with a retrovirus encoding *c-Myc*. DN3a thymocytes ectopically expressing *bcl2*, *pTa*, and *c-Myc* differentiated normally on OP9-DL1 monolayers but were still incapable of proliferation and progression to the DP stage in the absence of Notch ligands (Fig. 2). Thus our knowledge of Notch targets, relevant in $\alpha\beta$ lineage differentiation is incomplete and further studies are required to identify the missing parts of this pathway.

The role of CXCR4 signaling in $\alpha\beta$ lineage development

Both pre-TCR and Notch signaling are required for $\alpha\beta$ lineage development, but are not sufficient to drive the cell beyond the β -selection checkpoint, as DP cells do not develop in a feeder-free system in presence of recombinant Notch ligands (85,86). The chemokine receptor CXCR4 was recently shown to be required for transition through the β -selection checkpoint (87,88). Somatic *CXCR4* deficiency has a multi-organ embryonic lethal phenotype (89), and the thymic phenotype of the first floxed *CXCR4* deficiency (90) was perplexing: a complete block at the DN1 stage prior to the expression of *lck-Cre*. Recently, however, CXCR4 was conclusively identified as a non-redundant actor in β -selection, serving not only as a chemoattractant but also as a developmental cue. The two effects could only be uncoupled in the reductionist single-environment OP9-DL1 culture system.

An independently generated floxed CXCR4 allele showed only a partial block at β -selection when bred to *lck-Cre* (87), with pre-selection thymocytes appearing lost in the medulla, failing to home to the subcapsular zone and undergoing increased apoptosis. CXCR4-deleted early thymocytes were also impaired in DP progression *in vitro*, indicating that in addition to directional signals, CXCR4 signaling promotes some combination of survival, proliferation, and differentiation during β -selection. A complementary approach revealed that downstream of CXCR4, the phosphoinositide 3-kinase (PI3K) subunit isoforms p110 γ and p110 δ were both required for optimal DP progression (88). In a key experiment, the combination of recombinant CXCL12, IL-7, Flt3L and Notch ligand DLL4 for the first time allowed for limited progression of DN3 cells to DP in a stromal cell-free system. This key step in the molecular dissection of cell surface receptors in β -selection is, however, not the last. Other G-protein-coupled receptors may be involved, as treatment of OP9-DL1 cultures with pertussis toxin blocks DP progression completely (T.K. and H.v.B., unpublished data).

β -selection: pushing the cell off the ledge?

One aspect of the β -selection checkpoint is the seeming ease with which it can be bypassed. It is not surprising that activation of TCR signaling even in the absence of TCR, e.g. by expression of constitutively active *lck* (91), leads to DP progression. It is also not surprising that the deletion of the phosphatase PTEN (phosphatase and tensin homolog), which counteracts PI3K (92), or the ectopic co-expression of the costimulatory molecule CD28 and its ligand B7-2 (93), which can activate PI3K, bypasses the β -selection checkpoint, as PI3K activity is involved in pre-TCR and CXCR4 signaling (88). PI3K phosphorylates PIP2 (phosphatidylinositol-4,5-bisphosphate) to form PIP3 (phosphatidylinositol-3,4,5-trisphosphate), which in turn is required for positioning of the Akt kinase at the plasma membrane. Expression of constitutively active Akt, which does not require PIP3 for membrane localization, also leads to β -selection bypass (79). Inhibition of E protein activity, which is in part mediated by Id3, is also a part of β -selection process (47). The deletion of E47, that also leads to checkpoint bypass (48), can likewise mimic an aspect of physiological pre-TCR signaling. Similarly, β -selection was bypassed in mice deficient for the transcription factor Ikaros (94). It is less obvious why the overexpression of intracellular Notch1 rescinds the necessity for TCR signaling (95). However, here one can argue that as pre-TCR and Notch signaling cooperate in driving the cell past the checkpoint, their targets may overlap, and thus a supraphysiological Notch signal may activate the expression (or repression) of targets that normally require cooperation between the two pathways. It is more difficult to explain why activation of the Wnt signaling pathway by expression of constitutively active β -catenin also drives DP progression in the absence of pre-TCR (96). At the time these experiments were performed, it was thought that canonical Wnt signaling, which requires the stabilization of β -catenin, was crucial for normal T-cell development (97). However, later it was shown that *β -catenin* deletion led to a mild thymic phenotype

(98) or no phenotype at all (99) even in the absence of its homolog γ -catenin (100,101), suggesting that Wnt signaling, or at least its canonical branch, is not vital for T-cell development. β -selection is also bypassed under other conditions, including the NOD genetic background [DP cells are present in NOD.*Rag*^{-/-} mice (102)] and in mice deficient for the serine/threonine kinase Pim-1 (103); however, it is unclear which signaling pathways are responsible here. Even the expression of the SV40 large T antigen (which counteracts p53 and Rb and thus suppresses apoptosis and activates cell proliferation) in *Rag*-deficient thymocytes led to the development of CD4⁺CD8⁺ lymphoma (the phenotype of non-malignant thymocytes was not addressed in this study) (104). Consistent with these observations, mice deficient in both *Rag2* and *p53* have a significant DP population in the thymus (105), albeit the mechanism for that is unclear. Similarly, *p53* deficiency rescues the partial block in β -selection caused by *CD3 γ* deficiency (106), although this effect is visible in 6-week-old mice but not in 10-day-old mice, consistent with a slow accumulation of initially rare DP cells due to improved survival.

It is possible that these signaling pathways – even those which are dispensable for normal T-cell development – all have a common set of downstream targets that are specifically required in DN3 to DP progression. However, it is also conceivable that rather than activating an $\alpha\beta$ T-cell-specific transcription program directly, they merely drive a change in some basic cell state (e.g. in cell cycle or metabolism), which is sufficient to induce DP progression. In such a scenario, a DN3 cell would be already poised to become a DP cell, and a change in a cell state (e.g. cell cycle entry) would trigger the progression.

In OP9-DL1 cultures, the differentiation of DN3a cells correlates well with proliferation, as only those cells that have divided several times begin to upregulate CD4 and CD8 (Fig. 3). Cell proliferation *per se* can be required, for instance, for the dilution of a factor that blocks differentiation (such as E47) and/or be required for chromatin remodeling.

Concluding remarks

The molecular mechanisms governing the first TCR-controlled checkpoint, the choice between $\alpha\beta$ and $\gamma\delta$ lineages, are starting to come into focus. The analog TCR signal is digitized by the Erk-Egr-Id3 axis, with strong signal instructing the $\gamma\delta$ and weak signal instructing the $\alpha\beta$ fate. These findings, however, add an unexpected twist to the model, as some $\gamma\delta$ T cells are independent of Id3 and express PLZF, which drives a unique NKT-like phenotype. Thus, under the ill-defined $\gamma\delta$ umbrella, upon close inspection, may lie a cluster of full-fledged developmental lineages with discrete molecular programs, producing peripheral $\gamma\delta$ T cells with distinct functions.

The lack of known ligands for most $\gamma\delta$ TCRs remains the main obstacle in the definition of distinct $\gamma\delta$ lineages. The absence on most $\gamma\delta$ T cells of known lck-interacting coreceptors, which in the $\alpha\beta$ lineage mediate class I or class II restriction by sequestering the limiting lck, suggests that $\gamma\delta$ TCRs, like BCRs, may recognize many geometric shapes on an antigen; this hypothesis is supported by the first crystal structure of a $\gamma\delta$ TCR in complex with its ligand. The presence in some $\gamma\delta$ T cells of unknown lck-sequestering coreceptors cannot be excluded; these could recognize any ligand and drive the restriction of some $\gamma\delta$ T cells, not necessarily to an MHC-like molecule.

While $\gamma\delta$ thymocytes are relatively independent of Notch signaling, $\alpha\beta$ lineage cells die rapidly in its absence. By compensating the survival defect using transgenic *bcl2*, we show here that Notch signaling is absolutely required not only for the survival but also for the proliferation and differentiation of developing $\alpha\beta$ thymocytes. Recent work identifies another receptor-ligand pair, CXCR4 and CXCL12, as contributors to β -selection via the PI3K cascade, although it remains to be determined which combination of survival,

proliferation, and differentiation this signal promotes. Importantly, this work presents a reconstitution of the thymic environment permissive for β -selection in a stroma-free culture using recombinant ligands.

While the biochemical reconstitution of β -selection had proven difficult until now, a wide variety of both relevant and irrelevant genetic abnormalities, such as the deletion of *E47* or *Ikaros*, allow cells to bypass this checkpoint. These findings lead us to the hypothesis that the DN3 cell is already poised on the brink of DP-ness, requiring a generic stimulus—such as entry into the cell cycle, which is downstream of many pathways known to bypass β -selection—to realize its fate.

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Fig. 1. Notch signaling is required for differentiation and proliferation of $\alpha\beta$ T-cells

(A). A *vav-bcl2* transgene compensates for the survival defect in the absence of Notch signaling. 10^5 *vav-bcl2* transgenic (blue) or wt (red) DN3a cells were plated on OP9-DL1 cells in the presence of 1 μ M of γ -secretase inhibitor to block Notch signaling. On days 4 and 8, cells were harvested, and live cells were counted. (B, C). *vav-bcl2* transgenic DN3a cells were plated on OP9-DL1 or OP9-GFP monolayer as indicated. On day 4, cells were analyzed for CD4/CD8 expression (B) and CFSE dilution (C). Undivided CD4⁺CD8⁺ cells were sorted, plated on OP9-DL1 or OP9-GFP monolayer as indicated, and analyzed again four days later.

Fig. 2. Ectopic expression of *c-Myc*, *bcl2* and *pTa* is insufficient to compensate for the absence of Notch signaling

DN thymocytes from *lck-pTa/vav-bcl2* double transgenic mice were spin-infected with c-Myc encoding retrovirus or empty vector on OP9-DL1 monolayer. DN3a thymocytes were sorted 2 days after infection, labeled with CellTrace Violet and plated on OP9-GFP (top row) or OP9-DL1 (bottom row). Proliferation of GFP+ and GFP- cells (A) and CD4/CD8 expression on GFP+ cells (B) was assessed after 4 days of culture.

Fig. 3. Link between proliferation and differentiation

Wt DN3a thymocytes were sorted, labeled with CFSE and cultured on OP9-DL1 monolayers. After 4 days cells were harvested, stained for CD4 and CD8 α and analyzed by FACS. CD4 and CD8 α fluorescence was summed electronically. Arrows indicate cells that went through 3 or 4 divisions.

Table 1Relationship between $\alpha\beta/\gamma\delta$ lineages and TCR expression

	“Canonical”	“Non-canonical”	
$\gamma\delta$ lineage	TCR $\gamma\delta$	prematurely expressed TCR $\alpha\beta$	in $\alpha\beta$ TCR Tg mice (1) (2) (3) possibly - a minor population in wt mice (5)
$\alpha\beta$ lineage	pre-TCR $\rightarrow$ TCR $\alpha\beta$	TCR $\alpha\beta$, TCR $\gamma\delta$	in $pT\alpha^{-/-}$ mice (11) in $Rag^{-/-}$ TCR $\gamma\delta$ Tg mice (6,7) a minor population in wt mice (8) in species without a pre-TCR ?