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Temporal Predisposition to αβ and γδ T Cell Fates in the Thymus

Pablo Pereira, Laurent Boucontet, and Ana Cumano

How T cell progenitors engage into the γδ or αβ T cell lineages is a matter of intense debate. In this study, we analyzed the differentiation potential of single thymocytes from wild-type and TCRγδ-transgenic mice at two sequential early developmental stages. Double-negative (DN) 3 progenitors from both wild-type and transgenic mice retain the capacity to engage into both pathways, indicating that full commitment is only completed after this stage. More importantly, DN2 and DN3 progenitors from TCRγδ transgenic mice have strong biases for opposite fates, indicating that developmentally regulated changes, other than the production of a functional TCR, altered their likelihood to become a γδ or an αβ T cell. Thus, unlike the differentiation in other hematopoietic lineages, T cell progenitors did not restrict, but rather switch their differentiation potential as they developed. The Journal of Immunology, 2012, 188: 1600–1608.

T cell development in the thymus is determined by cis-acting genetically defined developmental programs and trans-acting interactions with stromal cells, other thymocytes, and soluble factors (1, 2). αβ T cells and γδ T cells, defined by the chains they use to form their clonotypic receptors, develop in the thymus from a common progenitor. These progenitors are found among double-negative (DN) CD4—CD8—thymocytes. Based on the expression of CD25, CD44, and CD117, progenitor thymocytes can be organized according to the following maturation sequence: CD25^+CD44^+CD117^- (DN1) → CD25^-CD44^-CD117^- (DN2 or pre-T cells) → CD25^-CD44^+CD117^- (DN3 or pre-T cells) → CD25^-CD44^-CD117^- (DN4) (3). Completed TCRγ and TCRδ rearrangements are already detectable at the DN2 stage and terminate at the DN3 stage (4, 5). At this point, Vβ to DJβ rearrangements begin (6). Progression beyond the DN3 stage requires surface expression of either TCRγ or a pre-TCR (7, 8), composed of a TCRβ-chain paired with an invariant pre–TCRα-chain (9). Formation of a productive TCRγδ allows the development of γδ T cells that remain DN (8, 10, 11). Development of γδ-lineage cells is accompanied by increased transcription of TCRδ and, presumably, TCRγ genes (12). However, TCRγ is not strictly required for the differentiation of γδ-lineage cells, as evidenced by the fact that early expression of a TCRβ (mostly seen in TCRαβ-transgenic [Tg] mice) produces TCRβ^+ DN cells with characteristics of γδ T cells (13–16). By contrast, αβ-lineage cells subsequently differentiate to the DN4 and CD4^-CD8^- double-positive (DP) stages. Concomitant with DP cell differentiation is the silencing of TCRγ genes and the rearrangement of TCRδ genes, which results in the excision of the TCRδ locus, thus precluding TCRγδ expression in αβ-lineage cells (17, 18). In normal animals, most DP cells carry productive TCRβ-chains, suggesting that they originated from cells that expressed a pre-TCR at the DN3 developmental checkpoint [these cells are usually referred to as “β-selected” cells and the checkpoint is referred to as “β-selection” (7)]. Notably, TCRγδ can also mediate β-selection–like differentiation to the DP stage. This is most evident in animals unable to express a functional pre-TCR, in TCRγδ Tg mice in vivo (8, 10, 19–23), and in cultures in which TCRγδ-expressing progenitor cells are allowed to develop in vitro (24–26). Like β-selected DP cells, γδ-selected DP cells silence TCRγ gene expression and undergo TCRα rearrangements, indicating that they are bona fide αβ-lineage cells (21). The absence of productive TCRβ rearrangements in γδ-selected DP cells, together with their silencing of TCRγ genes, preclude them from expressing TCR/CD3 complexes at that stage, resulting in an abortive differentiation. Lack of intracytoplasmic TCRβ expression (TCRβic) and surface CD3 expression can be used to distinguish them from β-selected DP cells. Thus, high levels of TCRγδ and simultaneous expression of CD4 and CD8 define the earliest populations that unequivocally represent commitment to the γδ and αβ lineages, respectively (27, 28).

Models of αβ and γδ lineage commitment vary in the extent to which TCRγδ and the pre-TCR are proposed to instruct commitment (instructive models) or reinforce a previous commitment event (selective models) (1). Selective models propose that lineage commitment is stochastic and results in a binary choice prior to TCR expression, whereby daughter cells commit to one or the other lineage. Instructive models, by contrast, propose that lineage commitment follows TCR expression. The strict instructive model cannot be easily reconciled with observations that TCRγδ can drive the generation of αβ-lineage–committed DP cells or that early expression of a Tg TCRαβ produces TCRαβ^+ DN cells with characteristics of γδ T cells, described above. It was recently proposed that the strength of the signal, rather than the TCR isoform, determines lineage selection, with strong TCR signals promoting a γδ-like fate and weaker signals promoting αβ T cell differentiation through DP intermediates (29). Although they are usually considered to favor the instructive model, experiments supporting the signal strength model (30, 31) are compatible with
selective models, as well (32–34). More importantly, variations in signal capacity of TCRs may interfere with commitment, as well as with selective events that may be different in developing αβ and γδ lineage cells (33, 34). More recent experiments showed that a fraction of TCRγδ+ DN3-like cells, which can give rise to αβ-lineage cells in vitro, could be diverted to the γδ T cell lineage when a strong TCR signal was mimicked by TCR cross-linking (25), thus providing further support for this model. However, concerns with this interpretation have been put forward (34). It seems difficult to reconcile these two models (33–37), suggesting that they are incomplete.

A key question in the development of αβ and γδ T cells and germaine to lineage commitment is the definition of the exact point at which the two lineages diverge [i.e., the point(s) of commitment]. Selective and instructive models predict that commitment occurs before or after TCR expression, respectively. Using the OP9-DL1 coculture system (38), it was proposed that commitment initiates at the DN2 stage and is completed at the DN3 stage. This was based on the finding that single DN3 cells give rise to either TCRαβ+ or TCRγδ+ cells, but rarely both (24). However, the absence of CD4 and CD8 expression analysis precludes definitive conclusions with regard to αβ/γδ-lineage commitment, particularly because TCRγδ expression in progenitor cells is known to result in the production of both γδ and DP cells (24). Furthermore, these experiments do not allow the discrimination of true lineage commitment from αβ T cell fate consequent to the inability of a progenitor cell to form a TCRγδ as the result of unsuccessful rearrangement at their TCRγδ/TCRβ loci. Such discrimination can only be made by the analysis of progenitor cells capable of expressing TCRγδ. In this study, we analyzed, at the clonal level, the potential of progenitors cells isolated from wild-type (WT) and Tg-gδ mice to differentiate into αβ- and γδ-lineage cells in vitro. Our results identified a successive change in thymocyte content that alters the likelihood of a cell becoming a γδ or αβ T cell, emphasized by DN2 and DN3 progenitors with an identical TCRγδ displaying divergent fate outcomes when assessed at the clonal level. They also indicated the existence of mechanisms operating in vivo that preclude expression of a TCRγδ in many progenitor cells, thus favoring and allowing αβ-lineage development in WT and Tg-gδ animals, respectively.

Materials and Methods

Mice

C57BL/6 (B6) mice were obtained from Iffa-Credo (L’Abresle, France), B6 CD45.1, B6 mice Tg for rearranged Vγ1Vδ4Cγ4 and Y66D2J6b1 chains (Tg-gδ) (39), pTα-deficient mice, and TCRβ enhancer (Eβ)-deficient mice (23) were maintained in our animal facilities. All animal procedures were in compliance with the guidelines of the committee on animal experience (23) and were approved by the French Ministry for strategy and definition of sorting gates, we unexpectedly observed that no progenitor generated progeny containing exclusively γδ T cells (Fig. 2B). About half of the DN2 cells gave rise to both αβ and γδ T cells, whereas the remainder generated exclusively DP cells. Single DN3 cells gave rise to progeny containing only DP cells (75%) or DP cells and γδ T cells.
Thus, DN2 and DN3 cells produce similar progeny, with the only difference being an increase in the frequency of cells giving rise exclusively to αβ-lineage cells as development progresses. This could be due, at least in part, to the accumulation of nonfunctional TCRγ and/or TCRδ rearrangements in DN3 cells that renders them and their progeny incapable of expressing a TCRγδ.

Similar analyses performed with progenitors from Tg-γδ mice also produced unexpected results. First, ~70% of DN2 cells from Tg-γδ mice gave rise to exclusively γδ cells, whereas none generated exclusively αβ-lineage cells (Fig. 2B, 2C). The lack of αβ-only progeny from Tg-γδ DN2 cells, as well as that of γδ-only progeny from WT DN2 cells, was not a result of using the OP9-DL1 culture system, because their absence was also observed in clonal analyses performed in fetal thymus organ cultures (FTOC; Fig. 2D). Furthermore, many DP cells originating from Tg-γδ DN2 cells were not β selected, as suggested by their lack of TCRβic chains (Fig. 2E). Perhaps more surprising was the finding that none of the DN3 cells from Tg-γδ mice produced progenies containing exclusively γδ-lineage cells, in clear contrast with their DN2 counterparts (Fig. 2B). DN3 cells from Tg-γδ mice gave rise to progeny resembling those produced from WT DN3 cells (Fig. 2C), albeit with a higher frequency of clones giving rise to cells of both lineages (Fig. 2B), indicating that the apparent αβ-lineage commitment of WT DN3 cells is due, at least in part, to the inability of these cells to express a TCRγδ.

Collectively, these results are not readily compatible with pre-commitment models that consider the αβ/γδ lineage decision as

![Figure 1](http://www.jimmunol.org/)
intracellular TCR analyses shown in different experiments. As previously reported (24), WT DN2 and DN3 cells analyzed the development of gd TCR3, probably due to their more immature state and lower extent of gd differentiation into DP cells compared with DN3 cells. WT DN2 cells differentiated into DP cells with similar efficiency and kinetics as did WT DN2 cells, indicating that the potential to develop into gd T cells is not limited to the DN3 stage and into gd lineage cells at the DN2 stage and into gd lineage cells at the DN3 stage.

Developmental progression is accompanied by decreased gd-lineage developmental potential

To better understand the differences between WT and Tg-gd progenitors, we cultured small numbers (100 cells) of adult WT and Tg-gd mice on OP9-DL1 monolayers and analyzed the development of gd and DP cells at different time points. As previously reported (24), WT DN2 and DN3 cells differentiated efficiently into DP cells and gd T Cells. WT DN2 cells displayed delayed kinetics and a slightly greater efficiency of differentiation into gd lineage cells than did WT DN3 cells (Fig. 3), probably due to their more immature state and lower extent of TCRγ and TCRδ rearrangements. DN3 cells from Tg-gd mice differentiated into DP cells with similar efficiency and kinetics as did WT DN3 cells, as well as into gd T Cells with a 5-fold higher efficiency and with accelerated kinetics compared with DN3 cells from WT mice (Fig. 3, bottom right panels). By contrast, DN2 cells from Tg-gd mice produced ~10-fold less DP cells and ~5-fold more gd T cells than did WT DN2 or DN3 cells (Fig. 3, top right panels). Furthermore, most DP cells originating from Tg-gd DN2 cells never exhibited high levels of CD3 at the cell surface (compare the fraction of cells expressing high levels of CD3 among TCRγ-gd-negative cells in DN2 cultures from WT and Tg-gd mice; Fig. 3), suggesting that they were gd selected rather than β selected. Because of the silencing of TCRγ genes and their absence of functional TCRβ-chains, gd-selected DP cells do not express TCR/CD3 complexes. Of note, DN2 cells from Tg-gd mice differentiated into DP cells with faster kinetics than did DN2 cells from WT mice, a likely consequence of the gd selection mediated by TCRγ in some of their progeny.

To identify a more physiological setting in which only signals mediated through polyclonal gdTCRs that rely on endogenous rearrangements for their expression could drive T cell differentiation, we analyzed the developmental potential of DN2 and DN3 cells from Eβ-knockout (KO) mice in similar assays (Supplemental Fig. 2A). Like DN2 cells from Tg-gd mice, Eβ-KO DN2 cells produced progeny consisting of roughly equal numbers of gd T cells and TCRβδ-negative DP cells. Interestingly, gd T cells developed with similar kinetics in cultures from Eβ-KO and WT DN2 cells, indicating that gdTCRs are expressed with similar kinetics and efficiency in both strains. Also similar to Tg-gd DN3 cells and different from their DN2 counterparts, DN3 cells from Eβ-KO mice produced ~20-fold more DP cells than gd T cells, although their progression beyond the DN3 stage was mediated...
through γδTCRs. Of note, differentiation of DN3 cells was more inefficient in Eβ-KO mice than in WT or Tg-γδ mice, and 10-fold more DN3 cells were required to obtain progenies of similar size, indicating that expression of a γδTCR in DN3 cells is rather inefficient.

When we performed similar kinetic studies including DN1 and two transitional populations (DN2–3 [lin $^{CD117^+CD44^+CD25^+}$] and DN3–4 [lin $^{CD117^+CD44^+CD25^{int}}$]) and plotted the ratio of the maximum number of γδ- to αβ-lineage DP cells generated from those cultures, two important points become apparent (Fig. 4). First, this ratio was more or less constant until the DN2–3 transitional stage, and it decreased linearly thereafter, identifying a window from the late DN2 stage to the DN3 stage during which the differential production of γδ- or αβ-lineage cells takes place. The decreased ratio in DN3 cells compared with that of earlier progenitors was due to decreased production of γδ T cells rather than to increased production of DP cells (Fig. 3), indicating that developmental progression is accompanied by decreased γδ-lineage cell production. Second, a similar pattern with parallel slopes was observed in progenitor cells from WT, Tg-γδ, and Eβ-KO mice (Fig. 4, Supplemental Fig. 2B), indicating that the decrease in γδ-lineage developmental potential, along with T cell differentiation, is independent of the likelihood of a progenitor cell to productively rearrange TCRγ- and TCRδ-chains that coexpress at the cell surface, as well as of the expression of a functional pre-TCR; therefore, this decrease is likely to be developmentally regulated. These results are most compatible with the notion of a developmental switch occurring before the DN3 stage, which alters the likelihood of progenitor cells developing as γδ- or αβ-lineage cells. However, differential proliferation and/or survival of γδ T cells originating from DN2 or DN3 cells in culture could also explain, at least in part, these results.

**Differentiation of DP and γδ cells from DN2 or DN3 cells occurs with opposite kinetics**

The γδ bias of DN2 cells, compared with the αβ bias of DN3 cells, in Tg-γδ mice predicts a difference in the kinetics at which cells of the two lineages will be generated from DN2 and DN3 cells in WT mice in vitro. To test this prediction, we cloned DN2 and DN3 cells in OP9-DL1 coculture system and analyzed the generation of γδ T cells and DP cells at different time points in independent clones. Fig. 5 shows the results for progeny of 10 DN2 cells (Fig. 5A) and 4 DN3 cells (Fig. 5B) in which cells from both lineages were detected. CD4 versus CD8 and CD3 versus TCRδ profiles of progeny from four representative DN2 cells and four DN3 cells at different time points are shown in Supplemental Fig. 3. The appearance of γδ T cells in cultures originating from single DN2 cells preceded that of DP cells by ~2 d in every clone analyzed. Conversely, in cultures originating from DN3 cells, DP cells preceded γδ cells by ~2 d. The late development of γδ cells from DN3 cells was independent of whether differentiation beyond the DN3 stage occurred through β or γδ selection, as evidenced by the high or low levels, respectively, of CD3 expressed at the cell surface of the developing DP cells (Fig. 5C). However, the nature of the selecting receptor influenced the total number of γδ-lineage cells that developed in these cultures (Fig. 5B, 5C). These results demonstrated intrinsic differences between DN2 and DN3 progenitors with regard to their ability to differentiate into γδ- or αβ-lineage cells in WT mice. They also revealed the
In vitro DN2 cells from adult Tg-\(g_\alpha\)- mice.

Figure 5. Different predisposition of DN2 and DN3 cells to generate \(g_\delta\)- and \(g_\alpha\)-lineage cells in vitro. DN2 cells (one cell/well) and DN3 cells (five cells/well) from WT mice were cultured in OP9-DL1 monolayers in the presence of IL-7. On the indicated days, half of the cultures were harvested and analyzed by flow cytometry for the presence of TCR

DN3–DN4 transitional cells expressed detectable levels of surface TCR

Timing and levels of expression of TCR

Figure 6. Surface expression of TCR-\(g_\delta\) in progenitors from WT and Tg-\(g_\delta\) mice. DN thymocytes from WT and Tg-\(g_\delta\) mice were stained with FITC-labeled anti-CD3, PE-labeled anti-\(\alpha\), allophycocyanin-labeled anti CD117, PE-Cy7-labeled anti-CD25, allophycocyanin-Cy7-labeled anti-CD4, and a mixture of lineage-specific biotin-labeled Abs specific for CD4, CD8, CD19, CD11b, CD11c, TCR\(\beta\), and NK1.1, followed by streptavidin-Pacific blue. A, CD25 and CD117 profiles of lineage-negative thymocytes from WT (left panel) and Tg-\(g_\delta\) (middle panel). The definition of DN2-like to DN4-like cells is depicted in the right panel. B, Surface TCR-\(g_\delta\) expression in the indicated DN-like populations, as defined above, from WT (filled graphs) and Tg-\(g_\delta\) (dashed lines).

Most progenitor cells never express TCR-\(g_\delta\), even in Tg-\(g_\delta\) mice

In vitro DN2 cells from adult Tg-\(g_\delta\) mice differentiate primarily into \(g_\delta\)-lineage cells. The same cells in vivo produce normal numbers of \(g_\alpha\)-lineage cells, presumably through DN3 intermediates. A partial solution to this paradox came from the analysis of surface expression of TCR-\(g_\delta\) in different subsets of progenitor cells. Timing and levels of expression of TCR-\(g_\delta\) were similar in WT and Tg-\(g_\delta\) mice, being detectable for the first time at the DN3 stage (Fig. 6). Furthermore, only a small fraction of DN3 and DN3–DN4 transitional cells expressed detectable levels of surface TCR-\(g_\delta\), even in Tg-\(g_\delta\) mice (1% and 1.9% of DN3 cells and 5.5% and 19.3% of DN3–DN4 cells in WT and Tg-\(g_\delta\) mice, respectively; Fig. 6B). These results indicated the existence of mecha-

nisms operating in vivo and precluding expression of TCR-\(g_\delta\) in the majority of progenitor cells. Of note, TCR-\(\alpha\) cells that fell into the DN4-like gate represented the majority of TCR-\(g_\delta\) thymocytes, which express no or low levels of c-kit, CD25, and CD44.

The same TCR-\(g_\delta\) induces the differentiation of \(g_\alpha\)- and \(g_\delta\)-lineage cells in vivo

The above results help to explain the apparently normal developmental of \(g_\alpha\)-lineage cells in Tg-\(g_\delta\) mice (Fig. 1). In these animals, lack of expression of the transgenes in many progenitor cells allows normal TCR\(\beta\) rearrangements and, consequently, normal differentiation of \(g_\alpha\)-lineage cells (Fig. 7A). To investigate whether lineage-potential switch during development observed in vitro operates in vivo, we crossed Tg-\(g_\delta\) mice with mice deficient in pre-TCR assembling (pTr-KO and E\(\beta\)-KO mice, which lack pTr-chains or TCR-\(\beta\)-chains, respectively). We reasoned that, if this were the case, stochastic expression of the transgenes at different developmental points should result in the development of both \(g_\delta\) T cells and \(g_\alpha\)-lineage DP cells mediated by a unique TCR. As shown in Fig. 7B, compared with their non-Tg littersmates, both pTr-KO Tg-\(g_\delta\) mice and E\(\beta\)-KO Tg-\(g_\delta\) mice contained 6- and 40-fold more \(g_\delta\) and DP thymocytes, respectively. Thus, as previously shown for other TCR-\(g_\delta\) Tg lines (21), our Tg-\(g_\delta\) TCR induced the differentiation of both \(g_\alpha\)- and \(g_\delta\)-lineage cells in vivo.

Environmental independence of the size of the \(g_\delta\) thymocyte population

The lack of TCR-\(g_\delta\) expression in many progenitor cells observed above could be part of the normal developmental program in the adult thymus that favors the generation of \(g_\alpha\) cells over \(g_\delta\) cells. Alternatively, it may result from dysregulation imposed by the TCR transgenes, which notably increases the number of progenitors capable of expressing a functionally identical TCR-\(g_\delta\) that may compete for factors or niches that, although not limiting in WT animals, become limiting in Tg-\(g_\delta\) mice. To distinguish these possibilities, we generated radiation bone marrow chimera that...
FIGURE 7. The same TCRγδ induces the differentiation of αβ- and γδ-lineage cells in vivo. A, Intracellular TCRγδ staining of DN3 and DP cells from WT and Tg-γδ mice. Numbers represent mean ± SD of positive cells in six individual mice/group analyzed at 6–8 wk of age. B, Numbers of DP cells (left panel) and γδ thymocytes (right panel) in WT, Eβ-KO, and pTα-KO mice expressing TCRγδ transgenes (Tg-γδ) or not (LM). Data are mean ± SD of three to six mice/group analyzed individually at 3–4 wk of age.

were reconstituted with mixtures of WT and Tg-γδ bone marrow cells at different ratios. We reasoned that if niches or factors limit the development of γδ thymocytes in Tg animals, we should find a similar number of Tg-γδ T cells in all of the chimeras, irrespective of the proportion of WT and Tg progenitors. As shown in Fig. 8, there was a linear correlation between the number of WT and Tg-γδ thymocytes developing in the chimeras compared with the number of WT and Tg progenitors injected. This result excluded any possible effect of environmental factors in limiting the number of γδ thymocytes in Tg-γδ mice. Rather, it indicated that the number of γδ thymocytes present in Tg-γδ mice reflects the maximum number of γδ-lineage cells that could develop when all progenitors carry functionally rearranged TCRγ- and TCRδ-chains.

**Discussion**

The data presented in this article revealed three important observations that are germane to αβ/γδ lineage commitment. First, although we were unable to identify a progenitor cell from WT mice that would develop exclusively as γδ cells in either the OP9-DL1 system or in FTOC, such a progenitor was evident in the DN2 population of Tg-γδ mice. This suggested that commitment to the γδ-lineage occurs only after expression of TCRγδ, contradicting selective models of αβ/γδ lineage choice, which state that a binary choice occurs before rearrangement of the TCR genes. The finding that there are no γδ-committed DN2 cells in WT mice, in contrast to their Tg counterparts, is not unexpected. The stochastic nature of the rearrangement process, together with the fact that not all combinations of TCRγ and TCRδ are expressed at the cell surface (11, 42), results in many daughter cells being unable to express TCRγδ. However, a major implication of this finding is that progenitor cells that fail to express a selectable TCRγδ still retain the potential to differentiate into αβ-lineage cells.

A second important observation is that, during the DN2 to DN3 transition, there are intrinsic changes in the progenitors that alter their likelihood to differentiate into γδ- or αβ-lineage cells. This is best illustrated by our experiments showing that DN2 and DN3 cells with identical TCRs have different T cell fate outcomes when assessed at the clonal level (Fig. 2); however, it is also evident in progenitors from WT animals (Fig. 4) and in those from Eβ-KO mice that are unable to form a pre-TCR and that, therefore, can only signal through TCRγδ (Supplemental Fig. 2). It could be argued that this loss of γδ-lineage potential during development does not reflect intrinsic changes in developing cells but rather a successful depletion of γδ-committed cells from a heterogeneous population of γδ- and αβ-committed cells that commit stochastically during the DN2 to DN3 transition. However, such a mechanism cannot explain the high frequency of DN2 cells from Tg-γδ mice that differentiate exclusively into γδ-lineage cells (and the absence of DN2 cells that differentiate exclusively into αβ-lineage cells in the same mouse), without postulating that αβ-lineage commitment is accompanied by the repression of TCRγδ expression. But this postulate is incompatible with the development of γδ-selected DP cells in vivo and in vitro. Importantly, the predisposition to differentiate into αβ-lineage cells is maintained for some time after expression of TCRγδ, as evidenced by the fact that about one fifth of the TCRγδ+ DN3 cells retain the potential for both αβ and γδ T cell differentiation (25).

The third important observation is that there is an absence of surface expression of TCRγδ in the majority of cells transitioning from DN2 to DN4 stages, even when they carry functionally rearranged TCRγ and TCRδ chains known to pair at the cell surface. This is unlikely to be a Tg artifact, because the number of γδ T cells that develop in the thymus is a direct function of the likelihood that a progenitor will assemble and express a selectable TCRγδ. However, mRNA expression of the Tg Vγ1 chain in progenitor cells coincides with the time when the TCRγ locus opens for rearrangement in WT mice (39), and the ontogenic time at which protein expression of the Tg TCR is detectable (Fig. 6) parallels that of endogenous TCRγδ, indicating a seemingly normal expression of the Tg chains in Tg-γδ mice. Similar mechanisms
likely to operate in WT animals. Thus, only 0.5–1% of WT DN3 cells express detectable levels of surface TCRγδ (12, 25), whereas 15–20% of the same DN3 population expresses intracellular TCRβ-chains and, consequently, the pre-TCR (44, 45), frequencies also reported in preselected DN3 cells (12, 44). Although TCRγδ requires two successful rearrangement events, as opposed to one for the pre-TCR, the expected frequency of pre-TCR–expressing cells should not exceed that of TCRγδ–expressing cells by $>2$-fold (11). Thus, mechanisms precluding surface expression of TCRγδ are required to explain the 10–40-fold higher frequency of pre-TCR–expressing cells over TCRγδ–expressing cells in the WT DN3 population. The molecular nature of these mechanisms is unknown, but they may be related to the relatively low TCRγ transcription previously found in DN2 and DN3 cells from Tg-γδ mice (39). These mechanisms are likely unrelated to the change in lineage potential discussed above. Their major consequence is that αβ-lineage differentiation is favored over γδ-lineage differentiation in adult mice.

We propose a revised model for T cell-lineage determination that integrates these results and observations documented in the literature. Our results indicated that DN2 cells are predisposed to differentiate into γδ cells and, as they advance through development to the DN3 stage, progressively lose γδ-lineage potential while acquiring αβ-lineage potential. During the DN2 to DN3 transition, expression of TCRγδ results in γδ cell differentiation, as originally proposed by Pardoll et al. (46) and Allison and Lanier (47). Likely, the differentiation of γδ cells at this stage is fast, such that putative intermediate populations are difficult to detect in steady-state analyses ex vivo. The γδ cells developing through this pathway initially express CD117 and represent $\sim$5% of the total γδ thymocytes in WT animals and $\sim$50% in Tg-γδ mice at any given time point (39). Therefore, c-kit expression may be a marker of newly generated γδ cells that develop from DN2–DN3 transitional cells. Because of the potential loss of c-kit expression, the contribution of this pathway to the total γδ compartment is difficult to determine. However, the finding that 50–70% of the γδ thymocytes contain incomplete DJβ (44, 48) rearrangements strongly suggests that more than half of the γδ thymocytes differentiated before the onset of V-DJβ rearrangements at the DN3 stage.

By contrast, DN3 cells are predisposed toward αβ-lineage differentiation. Many such cells will be unable to form a functional TCRγδ and will depend on β selection for further differentiation, thus matching their predisposition to the expression of the correct receptor. However, a small fraction of them express TCRγδ and enter the αβ pathway through TCRγδ selection. In the normal thymus, these cells may be outcompeted by β-selected cells and disappear before the DP stage (49), but they constitute the DP pool in TCRβ-deficient mice or in Tg-γδ mice in a RAG-deficient background (8, 10, 19–23). As shown in this study and previously (25), a small fraction of their progeny will differentiate into γδ cells, indicating that lineage commitment only occurs after TCR expression. One possible scenario is that during their differentiation from DN3 to DP, a fraction of the cells stochastically switch their genetic program and attempt to develop as γδ-lineage cells. Cells that succeed in expressing TCRγδ continue differentiating along this pathway, whereas cells that fail to do so die. Consistent with this interpretation is the recurrent (7, 48, 50, 51), although not absolute (44), finding that a large fraction of TCRβ-chains present in γδ cells from adult mice are functional, suggesting that their precursors were initially selected through the pre-TCR. Moreover, the presence of TCR-negative apoptotic DN4 cells that have been β selected (52) is predicted by this interpretation. Finally, the late development of γδ cells from single DN3 progenitors in vitro and the fact that most DN3 from Tg-γδ mice are not yet lineage fixed are best explained within this scenario. Thus, although consistent with stochastic models of αβ/γδ-lineage commitment, these results place the commitment point after expression of the TCR. Alternatively, one may consider that the strength of signal resulting from expression of TCRγδ in γδ-selected DN3 cells moves cells away from their initial αβ-lineage pathway and commits them to the γδ lineage. As discussed elsewhere (34), the signal-commitment model cannot accommodate the fact that the same TCRγδ can drive the generation of DP and TCRγδ cells without invoking heterogeneity in the signal received by progenitor cells expressing identical TCRRs at the same developmental stage and that can only be stochastic in nature.

In conclusion, our results indicated two points of commitment during T cell differentiation. The first one is developmentally regulated and sequential, rather than binary, allowing a fraction of progenitor cells to develop as γδ–lineage cells during a developmental window. After that, the αβ lineage of development is favored but not fixed, and commitment to the γδ lineage is still possible. The model of αβ/γδ-lineage commitment presented in this article provides a rationale for the fact that, in normal animals, DN2 cells are endowed with a set of genes that is important for proper γδ T cell function but that is no longer expressed at the DN3 and DN4 stages (16, 53). It can also accommodate heterogeneity in the DN2 population that has been interpreted as support for stochastic models of lineage development (54), because decreased levels of expression of IL-7R mostly correlate with the DN2 to DN3 transition. It also accommodates the existence of γδ cells expressing functional TCRβ-chains (45) due to late development of γδ cells after β selection (52). Finally, it proposes heterogeneity in the development of γδ T cells that might be linked to the development of different functional subsets (55).

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Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure 1: γδ thymocytes from Tg-γδ mice respond normally to TCR mediated signals

(A) DN thymocytes from WT and Tg-γδ mice cultured for 4 hours in the presence of PMA and Ionophoro and Brefeldin A were stained with Abs specific for CD3, TCRδ and TNFα. Data shows intracellular TNFα staining of electronically gated γδ thymocytes from WT (left) and Tg-γδ (right) mice. (B) Mixed DN thymocytes from CD45.2 WT and CD45.1 Tg-γδ mice were labeled with CFSE and cultured for 3 days in plates coated with anti TCRδ Abs in the presence of IL-2. After 4 hours of incubation in uncoated wells, cells were stained with Abs specific for CD3, TCRδ and CD45.1. Data are shown as CFSE fluorescence in electronically gated γδ thymocytes of WT and Tg-γδ origin as indicated.
Supplemental Figure 2: Lineage potential switch along T cell development is independent of TCR isotype

DN2 (100 cells/well) or DN3 (100 cells/well WT and Tg-γδ; 1000 cells/well EβKO) were cultured in OP9-DL1 monolayers in the presence of IL-7. (A) Absolute number of γδTCR+ (γδ-lineage cells; right panels) and DP (αβ-lineage cells; left panels) per well found at the indicated days in cultures initiated with DN2 (top panels) or DN3 (bottom panels) cells. (B) Ratio of the maximum number of γδTCR+ and DP cells obtained in these cultures
Supplemental Figure 3. Different predisposition of DN2 and DN3 cells to generate γδ- and αβ-lineage cells in vitro.

DN2 (1 cell/well) and DN3 (5 cells/well) from WT mice were cultured in OP9-DL1 monolayers in the presence of IL-7. At the indicated days, half of the cultures were harvested and analyzed by flow cytometry for the presence of TCRγδ (γδ-lineage cells) and CD4 and CD8 (αβ-lineage cells).

Shown are CD4/CD8 (left) and CD3/TCRδ (right) profiles of progeny from four representative DN2 (top) or DN3 (bottom) cells at the indicated days.