Temporal Predisposition to $\alpha\beta$ and $\gamma\delta$ T Cell Fates in the Thymus

Pablo Pereira, Laurent Boucontet and Ana Cumano

*J Immunol* published online 11 January 2012
http://www.jimmunol.org/content/early/2012/01/11/jimmunol.1102531
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: 000–000.
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 TCRαβ as the result of unsuccessful rearrangement at their TCRγ/TCRδ 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-γδ 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-γδ 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 Vγ1Vδ4Cγδ and V66D02/81 chains (Tg-γδ) (39), pTα-deficient mice, and TCRβ enhancer (βE)-deficient mice (23) were maintained in our animal facilities. All animal procedures were in compliance with the guidelines of the committee on animal experimentation of the Institut Pasteur and were approved by the French Ministry of Agriculture.

Radiation chimeras

Irradiated (1200 rad) B6 mice were injected with a total of 2 × 10⁶ T cell-depleted bone marrow cells from CD45.1 Tg-γδ mice and CD45.2 WT mice at different ratios. T cell depletion was performed by one-step killing at 37°C for 45 min with anti-Thy1 (1H1), anti-CD4 (RL 174), and anti-CD8 (H0-2.2) mAbs and rabbit complement (Cedarlane Laboratories, Hornby, ON, Canada), followed by centrifugation over a density gradient using Lympholyte M (Cedarlane Laboratories).

Abs, staining, and cell sorting

Fluorochrome-labeled anti-CD25 (PC61), anti-CD44 (IM7), anti-CD117 (BD8), anti-CD3ε (145-2C11), anti-CD6 (GL3), anti-TCRB (H57-597), anti-NC1.1 (PK136), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD11b (M1/70), anti-CD11c (N418), anti-Gr-1 (RB6-8C5), anti-CD19 (1D3), anti-CD45.1 (A20), and anti-CD45.2 (1D4) mAbs and streptavidin were purchased from BD Bioscience, eBioscience, or BioLegend (San Diego, CA).

Cell surface labeling was performed, as described (39). Cells were analyzed in a FACS Calibur, FACS Canto I, or FACS Canto II (Becton Dickinson, Franklin Lakes, NJ) and analyzed with either CELLQuest pro software (BD Bioscience) or FlowJo software. For sorting, thymocytes were stained with FITC-labeled anti-CD44, PE-labeled anti-CD25, allophycocyanin-labeled anti-CD117, and a mixture of biotin-labeled Abs specific for CD3, CD4, CD8, CD19, CD11b, CD11c, NK1.1, TCRβ, and TCRδ, followed by PE-Cy7-labeled streptavidin and sorted with a MoFlo cell sorter (Cytomxrix, Fort Collins, CO). Unless otherwise specified, these biotin-labeled Abs were used to define the lin− cells in our experiments.

Cell cultures

OP9-DL1 cells were produced in the laboratory and maintained in OptimEM media (Life Technologies) supplemented with 2-ME, antibiotics (Life Technologies), and 10% FCS. Cocultures of progenitor cells were performed in the same culture media with a monolayer of 2000 OP9-DL1 cells/well and the indicated numbers of progenitor cells. Media were supplemented with mouse rIL-7. In the kinetic experiments, 500–1000 OP9-DL1 cells were added at some point after removing half of the cells for analysis.

Results

Normal development of αβ-lineage cells in adult Tg-γδ mice

We described several aspects of the development of T cells in Vγ1Vδ6 Tg-γδ mice (39, 40). The rearranged chains were isolated as large cosmids, thus ensuring physiological expression of the transgenes (39). Compared with WT mice, Tg-γδ mice displayed comparable numbers of αβ-lineage DP and single-positive thymocytes and an ∼5-fold increase in the number of γδ thymocytes (Fig. 1A, 1B). Numbers of early T cell progenitors also compared well between WT and Tg-γδ mice (Fig. 1C–E), indicating that the transgenes resulted in no loss of progenitors, which was then compensated for during development. Cells bearing the Tg TCR appear to be bona fide γδ-lineage cells, as evidenced by their ability to respond to TCR ligation in a manner similar to WT γδ T cells (Supplemental Fig. 1), as well as by a number of phenotypic and molecular criteria that have been used in the past to differentiate αβ- and γδ-lineage cells (39).

Clonal analysis of T cell progenitors from WT and Tg-γδ mice

To analyze the developmental potential of progenitor cells, we used the OP9-DL1 culture system, which allows the differentiation of single progenitor cells to both αβ and γδ cell lineages. Unlike an earlier report (24), we determined αβ and γδ T cell lineage potential by the presence, within the progeny, of DP and TCRγδ+ cells, respectively.

Limiting-dilution analyses of DN2 and DN3 cells isolated from WT and Tg-γδ mice are shown in Fig. 2A. About one in two DN2 cells and one in nine DN3 cells could develop to a sizable clone, regardless of whether they originated from normal or Tg-γδ mice. These frequencies compared well with previously published frequencies in WT mice (24, 41). The different response of DN2 and DN3 cells likely reflects the fact that DN2 cells proliferate in response to IL-7 before differentiating, whereas DN3 cells require a functional pre-TCR/TCR to differentiate further (38, 41). However, the presence of functionally rearranged TCRγδ and δ genes results neither in an advantage nor in a disadvantage of precursor frequencies at these developmental stages, suggesting that many DN3 cells do not express the Tg TCR (see later discussion).

When the progenies of single DN2 and DN3 cells from WT mice were analyzed (see Fig. 1B 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 DP 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 and γδ T cells (25%).
T cells (25%) (Fig. 2B, 2C). 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 TCRβc 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.** Development of αβ- and γδ-lineage cells in Tg-γδ mice. A, Flow cytometric analyses of WT and Tg-γδ thymocytes for CD4 and CD8 (top panels) or CD3 and TCRγδ (bottom panels). Numbers indicate frequencies of cells in the quadrants (top panels) or the frequency of γδ thymocytes (bottom panels). B, Total number of αβ-lineage DP and single-positive cells and γδ-lineage cells in the thymus of WT and Tg-γδ mice. Each symbol represents an individual mouse. DN thymocytes from WT (C) and Tg-γδ mice (D) were stained with FITC-labeled anti-CD44, PE-labeled anti-CD25, allophycocyanin-labeled anti CD117, and a mixture of lineage-specific biotin-labeled Abs specific for CD3, CD4, CD8, CD19, CD11b, CD11c, TCRβ, TCRγδ, and NK1.1, followed by streptavidin–PE–Cy7. Left panels, CD44 and CD117 profiles of lin− thymocytes showing the gate used to define CD117 bright cells. Right panels, CD25 and CD44 profiles of lin− CD117 bright cells (top panels) or lin− cells (bottom panels) and the gates used to define the DN1 to DN4 populations. Numbers indicate frequencies of cells in the indicated gate. E, Total number of the indicated progenitor cells in the thymus of WT and Tg-γδ mice. Each symbol represents an individual mouse.
showed marked bias for developing into gd cells varies with the developmental stage of the progenitor cell. Differentiated efficiently into DP cells and points. As previously reported (24), WT DN2 and DN3 cells to better understand the differences between WT and Tg-\(\gamma\delta\)-lineage developmental potential, we cultured small numbers (100 cells) of adult DN2 and DN3 cells from WT and Tg-\(\gamma\delta\)-lineage progenitors and to provide quantification of their developmental progression is accompanied by decreased \(\gamma\delta\)-lineage developmental potential. To identify a more physiological setting in which only signals mediated through polyclonal \(\gamma\delta\)-TCRs that rely on endogenous rearrangements for their expression could drive T cell differentiation, we analyzed the developmental potential of DN2 and DN3 cells from E\(\beta\)-knockout (KO) mice in similar assays (Supplemental Fig. 2A). Like DN2 cells from Tg-\(\gamma\delta\) mice, E\(\beta\)-KO DN2 cells produced progeny consisting of roughly equal numbers of \(\gamma\delta\)-negative cells in DN2 cultures from WT and Tg-\(\gamma\delta\) mice; Fig. 3), suggesting that they were \(\gamma\delta\)-selected rather than \(\beta\) selected. Because of the silencing of TCR\(\gamma\) genes and their absence of functional TCR\(\beta\)-chains, \(\gamma\delta\)-selected DP cells do not express TCR/CD3 complexes. Of note, DN2 cells from Tg-\(\gamma\delta\) mice differentiated into DP cells with faster kinetics than did DN2 cells from WT mice, a likely consequence of the \(\gamma\delta\) selection mediated by TCR\(\gamma\delta\) in some of their progeny. To identify a more physiological setting in which only signals mediated through polyclonal \(\gamma\delta\)-TCRs that rely on endogenous rearrangements for their expression could drive T cell differentiation, we analyzed the developmental potential of DN2 and DN3 cells from E\(\beta\)-knockout (KO) mice in similar assays (Supplemental Fig. 2A). Like DN2 cells from Tg-\(\gamma\delta\) mice, E\(\beta\)-KO DN2 cells produced progeny consisting of roughly equal numbers of \(\gamma\delta\) T cells and TCR\(\beta\)-negative DP cells. Interestingly, \(\gamma\delta\) T cells developed with similar kinetics in cultures from E\(\beta\)-KO and WT DN2 cells, indicating that \(\gamma\delta\)TCRs are expressed with similar kinetics and efficiency in both strains. Also similar to Tg-\(\gamma\delta\) DN3 cells and different from their DN2 counterparts, DN3 cells from E\(\beta\)-KO mice produced ∼20-fold more DP cells than \(\gamma\delta\) 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 CD117thCD44mCD25⁺] and DN3–4 [lin CD117⁺CD44⁺CD25m]) 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 increased production of DP cells (Fig. 3), indicating that developmental progression is accompanied by decreased γδ T 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 TCRγ- 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

---

**FIGURE 3.** Development of γδ- and αβ-lineage cells from WT and Tg-γδ T cell progenitors in OP9-DL1 cultures. One hundred DN2 cells (lin CD117⁺CD44⁺CD25⁺) or DN3 cells (lin CD117⁺CD44⁺CD25⁺) isolated from adult WT and Tg-γδ mice were cultured with OP9-DL1 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 TCRγδ (γδ-lineage cells) and CD4 and CD8 (αβ-lineage cells), as indicated. Right panels. Absolute cell numbers of γδ cells and DP cells originating in these cultures from DN2 (top panels) or DN3 (bottom panels) progenitor cells. One representative experiment of four is shown.

**FIGURE 4.** Loss of γδ-lineage potential as T cell progenitors advance in development. One hundred DN1 (lin CD117⁺CD44⁺CD25⁺), DN2 (lin CD117⁺CD44⁺CD25⁺), DN2–3 (lin CD117⁺CD44⁺CD25⁺), DN3 (lin CD117⁺CD44⁺CD25⁺), and DN3–4 transitional cells (lin CD117⁺CD44⁺CD25⁺) isolated from WT and Tg-γδ mice were cultured and analyzed on different days, as in Fig. 3. Data represent the ratio of the maximum number of γδ-lineage cells (γδTCR⁺ cells) and αβ-lineage cells (DP cells) obtained in these cultures.
plasticity of DN3 cells, which, although initially prone to develop as αβ-lineage cells, produce progeny that can revert this phenotype and develop as γδ cells. A similar plasticity of progenitors after TCRγδ expression was shown recently (25).

Most progenitor cells never express TCRγδ, even in Tg-γδ mice

In vitro DN2 cells from adult Tg-γδ mice differentiate primarily into γδ-lineage cells. The same cells in vivo produce normal numbers of αβ-lineage cells, presumably through DN3 intermediates. A partial solution to this paradox came from the analysis of surface expression of TCRγδ in different subsets of progenitor cells. Timing and levels of expression of TCRγδ were similar in WT and Tg-γδ 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 TCRγδ, even in Tg-γδ mice (1% and 1.9% of DN3 cells and 5.5% and 19.3% of DN3–DN4 cells in WT and Tg-γδ mice, respectively; Fig. 6B). These results indicated the existence of mechanisms operating in vivo and precluding expression of TCRγδ in the majority of progenitor cells. Of note, TCRδ+ cells that fell into the DN4-like gate represented the majority of TCRγδ thymocytes, which express no or low levels of c-kit, CD25, and CD44.

The same TCRγδ induces the differentiation of αβ- and γδ-lineage cells in vivo

The above results help to explain the apparently normal development of αβ-lineage cells in Tg-γδ mice (Fig. 1). In these animals, lack of expression of the transgenes in many progenitor cells allows normal TCRβ rearrangements and, consequently, normal differentiation of αβ-lineage cells (Fig. 7A). To investigate whether lineage-potential switch during development observed in vitro operates in vivo, we crossed Tg-γδ mice with mice deficient in pre-TCR assembling (pTα-KO and Eβ-KO mice, which lack pTα-chains or TCRβ-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 γδ T cells and αβ-lineage DP cells mediated by a unique TCR. As shown in Fig. 7B, compared with their non-Tg littermates, both pTα-KO Tg-γδ mice and Eβ-KO Tg-γδ mice contained 6- and 40-fold more γδ and DP thymocytes, respectively. Thus, as previously shown for other TCRγδ Tg lines (21), our Tg-γδ TCR induced the differentiation of both αβ- and γδ-lineage cells in vivo.

Environmental independence of the size of the γδ thymocyte population

The lack of TCRγδ expression in many progenitor cells observed above could be part of the normal developmental program in the adult thymus that favors the generation of αβ cells over γδ 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 TCRγδ that may compete for factors or niches that, although not limiting in WT animals, become limiting in Tg-γδ mice. To distinguish these possibilities, we generated radiation bone marrow chimeras that...
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 thymocytes in either the OP9-DL1 system or in FTOC, such a progenitor was evident in the mice that would develop exclusively as αβ-lineage cells. 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 selectible 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 at the cell surface, both in the chimeras shown in Fig. 8, as well as in non-Tg animals carrying only one or two functional alleles of Cδ (43), thus excluding a possible role for environmental factors in limiting the number of γδ thymocytes in Tg-γδ mice. Furthermore, 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 TCRγ, indicating a seemingly normal expression of the Tg chains in Tg-γδ mice. Similar mechanisms are

FIGURE 7. The same TCRγδ induces the differentiation of αβ- and γδ-lineage cells in vivo. A, Intracellular TCRβ 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.

FIGURE 8. The size of the γδ thymocyte population in WT and Tg-γδ mice is independent of extrinsic factors. Irradiated B6 mice were reconstituted with 2 × 10^6 T cell-depleted bone marrow cells from CD45.2 WT mice and CD45.1 Tg-γδ mice, at the indicated ratios. Depicted are numbers of γδ thymocytes of WT or Tg-γδ origin analyzed 10 wk after reconstitution. Each symbol represents the mean of three mice analyzed individually.
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 TCRγ 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 ~5% of the total γδ thymocytes in WT animals and ~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 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 TCRs 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).

Acknowledgments
We thank Bruno Silva-Santos, Dan Pennington, and Adrian Hayday for discussion and critical reading of previous versions of the manuscript.

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 γδ TCR+ (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 γδ TCR+ 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.