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The Developmental Fate of T Cells Is Critically Influenced by TCRγδ Expression

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Summary

Differentiation of γδ and αβ T cells from a common precursor cell depends on productive rearrangement and expression of TCRγδ or TCRβ genes, but whether it is an instructive or a stochastic mechanism that is responsible for this process is unclear. We report that expression of the productively rearranged TCRγ transgene competitively inhibits αβ thymocyte development under conditions where TCRβ gene rearrangement is limiting. The status of TCRδ gene rearrangements in the remaining αβ-lineage cells indicates that the effect is mediated by the intact γδ receptor. Paradoxically, in TCRβ$^{-/-}$ mice, γδ receptor expression can also drive differentiation of some αβ-lineage cells. To resolve this paradox, we provide evidence for a minor population of γδ-dependent αβ-lineage cells in normal mice. The results indicate that the T cell lineage commitment process is either error-prone or stochastic.

Introduction

αβ and γδ T cells arise intrathymically from a common lymphocyte progenitor. Development of αβ T cells normally involves an initial phase in which a CD4$^-$CD8$^-$ (double-negative [DN]) progenitor cell expresses the pre–T cell receptor (pre-TCR) complex, consisting of a TCRβ chain associated with a nonpolymorphic surrogate α chain, pTα (Fehling et al., 1995). These cells undergo substantial proliferation and develop into CD4$^+$ CD8$^+$ (double-positive [DP]) cells that subsequently express TCRα genes and are subject to thymic selection processes. γδ T cells appear earlier in fetal thymic development than αβ T cells. Compared to differentiation of αβ T cells, γδ T cell differentiation involves considerably less cell proliferation and is not dependent on pTα expression.

The mechanisms that underlie the central γδ/αβ cell fate decision in T cell development are not fully understood (reviewed by Shortman and Wu, 1996; Kang and Raulet, 1997). Successful differentiation of both types of T cell requires productive rearrangement of the relevant TCR chains (in-frame without termination codons at their V(D)J junction). An early proposal that lineage determination involves selective rearrangement of only the appropriate TCR genes in precommitted progenitor cells has been refuted, since rearrangements of the "inappropriate" receptor genes, such as γ and δ genes in αβ T cells (Hayday et al., 1985; Garman et al., 1986; Dudley et al., 1995; Kang et al., 1995; Livak et al., 1995; Wilson et al., 1996) and β genes in γδ T cells (Dudley et al., 1994; Dudley et al., 1995; Burtrum et al., 1996; Mertsching and Ceredig, 1996; Vicari et al., 1996), are allowed and indeed frequently observed. The available evidence suggests, however, that successful expression of TCRγ and TCRδ genes in progenitor cells influences αβ versus γδ T cell development.

Despite several early reports that expression of TCRγ and TCRδ transgenes in all developing T cells failed to prevent αβ T cell development (Bonneville et al., 1989; Dent et al., 1990; Ishida et al., 1990; Sim et al., 1995), recent results indicate that TCRγδ transgene expression often causes a substantial reduction in αβ T cell development. The effect was more pronounced when TCRγδ transgenic cells developed as a minor population in competition with normal stem cells (Bonneville et al., 1989; Dent et al., 1990; Washburn et al., 1997). Most significant are recent studies in normal mice that demonstrate that although γ and δ genes are frequently rearranged in αβ-lineage cells, most of the rearrangements are nonproductive and therefore cannot encode a functional TCR chain. Eighty percent or more of TCRγ and δ gene rearrangements in αβ-lineage cells were shown to be nonproductive, a frequency greater than the 66% that could be expected of a random process (Dudley et al., 1995; Kang et al., 1995; Livak et al., 1995). Taken together, the results suggest that expression of γ and/or δ TCR chains impedes cells from differentiating along the αβ lineage. However, it has not been established whether the lineage choice is determined by γδ receptors per se or by putative pre-TCR complexes containing γ or δ chain. Furthermore, the issue remains clouded by the observation that some αβ-lineage cells still arise in TCRγδ transgenic mice, an event that has not been adequately explained (Dent et al., 1990; Kersh et al., 1995; Sim et al., 1995).

Two general models have been considered to account for γδ versus αβ T cell lineage commitment. "Instructive" models propose that precursor cells are bipotent prior to TCR expression. Immature DN thymocytes that successfully rearrange γ and δ genes and produce a γδ TCR will mature along the γδ lineage, usually retaining the DN phenotype. In contrast, cells that successfully rearrange TCRβ genes and consequently express a pre-TCR will differentiate into DP thymocytes and develop along the αβ lineage. In one version of this model, complete (V-J or V(D)J) γ and δ gene rearrangements occur before the final step in β rearrangement (V to D-J), and only cells that fail to become γδ cells can go on to attempt to become αβ T cells (Allison and Lanier, 1987; Pardoll et al., 1987). Another version suggests a competitive scenario in which complete γ, δ, and β rearrangements occur more or less concurrently, so that cells that happen first to make productive γ and δ rearrangements will become γδ cells, whereas initial successful rearrangement at the β locus stimulates development along the αβ lineage (Livak et al., 1995).

The alternative models, referred to here as "stochastic" models, propose that commitment is independent of, and probably precedes, receptor gene rearrangement (Winoto and Baltimore, 1989; Ishida et al., 1990).

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Rearrangements of the TCRβ, γ, and/or δ genes occur in each committed cell, but only cells that make productive rearrangements of the TCR genes that match the predetermined cell fate are allowed to contribute to the mature lineages. Both the stochastic and instructive models assume that cells that fail to express any TCR cannot mature or persist.

To assess some of the predictions of the models of T cell development, we have examined the effect of a rearranged TCRγ transgene on αβ versus γδ development. By expressing only one chain of the γδ TCR as a transgene, the effects of γδ expression on the development of the two T cell lineages can be examined in a thymic environment that is not grossly altered in its cellular composition. The extent to which γδ TCR^+ progenitor cells contribute to the αβ lineage could be assessed by determining the effects of γ transgene expression on the abundance of in-frame TCRδ rearrangements in αβ-lineage cells. The data demonstrate that γδ expression serves to prevent most progenitor cells from populating the αβ lineage, but paradoxically can induce small numbers of progenitor cells to differentiate into DP cells. These results demonstrate that the lineage-determining mechanism is to some extent imperfect. These findings put important constraints on both major models proposed to account for the T cell lineage determination.

Results

The Effects of the γ Transgene on αβ T Cell Development Are Dependent on the Efficiency of Endogenous TCRβ Gene Rearrangements

Expression of a functionally rearranged TCRγ transgene (Vγ2-Jγ1Cγ1 from the G8 γδ T cell clone [Dent et al., 1990]) resulted in a greater than 2-fold increase in the number of DN γδ TCR^+ thymocytes when compared to nontransgenic littermates (Table 1). An average of 90% of these cells expressed the Vγ2 chain, encoded by the transgene (data not shown), whereas only 40% of nontransgenic DN γδ TCR^+ cells were $V\gamma2^+$. Increased numbers of γδ cells were observed in all four transgenic lines analyzed, even those with only one or two transgene copies (data not shown).

Although the transgene affected the prevalence of γδ T cells in otherwise normal mice, it had no consistent effect on the number of thymocytes (Figure 1A), DP cells (Table 1), or mature αβ thymocytes (data not shown), regardless of transgene copy number. However, the transgene clearly affected the number of αβ-lineage cells when the TCRβ gene rearrangement was limiting. As one means of limiting β gene rearrangement, we crossed the TCRγ transgenic mice to $TCR\beta^{-/-}$ mice (Mombaerts et al., 1992) to generate TCRγ transgenic $TCR\beta^{+/-}$ mice. Compared to nontransgenic $TCR\beta^{+/-}$ littermates, adult $TCR\beta^{+/-}$ transgenic mice exhibited a clear reduction in the total number of thymocytes, by an average of 2-fold, largely because of a reduction in the number of DP thymocytes (Figure 1A and Table 1). Mature thymocyte numbers were also reduced significantly, whereas the number of DN thymocytes was unaffected by the transgene (data not shown). As in $TCR\beta^{+/+}$

Table 1. Thymocyte Subsets in Normal and γ Transgenic Mice Harboring Wild-type or Mutant TCRβ Alleles

TCRβ and γTg Genotypes		TN subsets					DP Cells	γδ Cells
		$CD25^-CD44^+$	$CD25^+CD44^+$	$CD25^+CD44^-$	$CD25^{int}CD44^-$	$CD25^-CD44^-$		
$\beta^{+/+}$	Tg^-	8.3 ± 2.8	4.9 ± 1.4	21.1 ± 6	4.1 ± 1.3	16.2 ± 6.2	1981.0 ± 638	7.0 ± 2.1
	Cells (n = 5)							
	% in S phase	5.9% ± 0.1%	28.3% ± 0.6%	18.9% ± 0.4%	50.4% ± 3.5%	51.0% ± 1.7%	10.7% ± 0.1%	9.8% ± 0.4%
	Tg^+	5.6 ± 1.7	4.9 ± 1.6	23.0 ± 6.8	4.3 ± 1.5	18.9 ± 6.7	2043.0 ± 429	15.6 ± 3.2
	Cells (n = 4)							
	% in S phase	17.0% ± 2.0%	38.8% ± 2.3%	23.2% ± 1.7%	53.0% ± 3.5%	52.4% ± 2.5%	11.3% ± 0.4%	10.1% ± 0.6%
$\beta^{+/-}$	Tg^-	6.0 ± 1.0	2.0 ± 0.2	27.5 ± 3.6	12.0 ± 2.3	26.5 ± 3.6	1485.0 ± 136	9.0 ± 1
	Cells (n = 12)							
	% in S phase	11.9% ± 1.2%	23.9% ± 3.5%	13.0% ± 1.9%	45.5% ± 4.6%	47.2% ± 2.7%	10.4% ± 0.2%	10.8% ± 1.0%
	Tg^+	4.4 ± 0.6	1.4 ± 0.2	14.9 ± 2.0	10.0 ± 1.0	28.2 ± 2.4	821.0 ± 78	17.0 ± 2
	Cells (n = 22)							
	% in S phase	13.8% ± 2.2%	29.1% ± 2.2%	21.3% ± 2.8%	51.1% ± 3.7%	39.5% ± 2.0%	10.7% ± 0.5%	10.8% ± 1.1%
$\beta^{-/-}$	Tg^-	1.5 ± 0.2	1.9 ± 0.3	43.3 ± 3.7	5.4 ± 0.7	7.6 ± 0.7	23.0 ± 3	17.0 ± 1
	Cells (n = 27)							
	% in S phase	12.6% ± 1.3%	35.5% ± 2.9%	12.5% ± 1.3%	27.8% ± 1.6%	20.1% ± 1.2%	13.7% ± 1.6%	16.4% ± 1.1%
	Tg_+	1.5 ± 0.2	1.8 ± 0.4	21.2 ± 3.7	6.1 ± 1.0	17.4 ± 2.4	213.0 ± 37	21.0 ± 4
	Cells (n = 17)							
	% in S phase	16.2% ± 0.7%	33.9% ± 1.7%	19.7% ± 1.1%	37.4% ± 1.3%	28.6% ± 4.5%	12.0% ± 0.8%	15.0% ± 1.0%

Values represent average numbers of cells ($\times 10^5$) per thymus or percentages of cells in the subset in S phase, as indicated, ± SEM.

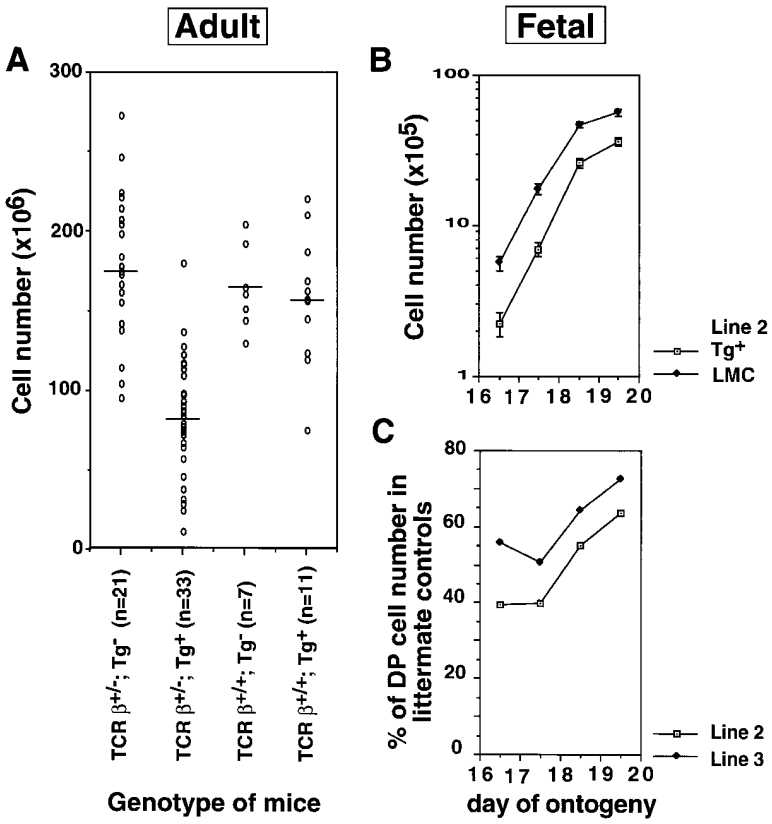

Figure 1. Inhibition of αβ Thymocyte Development in TCRγ Transgenic Mice

(A) Decreased thymocyte numbers in TCRβ$^{+/-}$ transgenic mice. Circles represent values from individual mice; bars indicate average cell numbers in each group (TCRβ$^{+/-}$, Tg$^-$ = 177.3 × 10^6, Tg$^+$ = 83.7 × 10^6; TCRβ$^{+/+}$, Tg$^-$ = 163.6 × 10^6, Tg$^+$ = 156.6 × 10^6).

(B) Absolute DP thymocyte cell numbers are reduced in transgenic fetuses. Comparison of littermates from one to four litters at the indicated times is shown for transgenic line 2 (13 copies). Values represent mean and standard error of the means. LMC, nontransgenic littermate control.

(C) Reduction in transgenic DP thymocyte cell numbers during fetal ontogeny is shown as a ratio of DP thymocyte numbers in transgenic fetuses to littermate nontransgenic control mice for two transgenic lines (line 3, 3 copies) at the indicated times.

mice, transgene expression in TCRβ$^{+/-}$ mice resulted in a 2-fold increase in TCRγδ$^+$ thymocytes (Table 1).

As a second approach to limit β gene rearrangement, we examined fetal, as opposed to adult, TCRβ$^{+/+}$ mice. In TCRβ$^{+/+}$ mice at embryonic days 16–17, near the time DP cell development normally commences, γ transgene expression resulted in a 2-fold reduction in the number of DP cells (Figure 1B). A similar, but less pronounced, effect was also seen in another transgenic founder line with a lower transgene copy number (Figure 1C). The reduction in DP thymocytes in transgenic mice gradually diminished thereafter and was negligible by 3 days after birth (data not shown). Collectively, the results show that the TCRγ transgene causes a reduction in the number of DP cells, but only when the extent of TCRβ chain generation is limiting.

Cells with Functional γ and δ TCR Chains Are Selectively Excluded from the DP Population

Reduced DP cell development in γ transgenic mice is likely to reflect selective exclusion of cells that express the γδ TCR from this developmental compartment. Previous reports demonstrated that only 20% or fewer of rearranged Vγ and Vδ genes are rearranged in-frame in αβ-lineage cells—significantly fewer than the 33% expected if selection against γ or δ chain expression had not occured in these cells (Dudley et al., 1995; Kang et al., 1995; Livak et al., 1995). To explain why selection against in-frame rearrangements was incomplete, it was proposed that the selection was exerted against expression of the γδ TCR, rather than against expression of γ or δ chains alone. Hence, an appreciable fraction of cells that had rearranged γ or δ productively, but not both, would escape selection. If this explanation is correct, expression of the TCRγ transgene in all cells should cause a profound decrease in the occurrence of endogenous in-frame TCRδ gene rearrangements in αβ-lineage cells.

The polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) assay, which displays amplified junctional regions of rearranged TCR genes (Dudley et al., 1994), was used to estimate the frequency of in-frame δ gene rearrangements in sorted DP thymocytes from γ transgenic or nontransgenic littermates. In-frame rearrangements are represented by bands at 3 bp increments. In TCRβ$^{+/-}$ mice, TCRγ transgene expression strongly depressed the frequency of in-frame Vδ4 and Vδ5 rearrangements in sorted DP thymocytes from TCRβ$^{+/-}$ mice, from approximately 20% in nontransgenic mice to 5%–9% for Vδ4, and to 7%–10% for Vδ5 (Figure 2). The results suggest that the 2-fold reduction in the number of DP cells in the γ transgenic mice is caused in large part by a stringent exclusion from the DP population of cells that generates in-frame δ rearrangements. A similar effect of the transgene was also observed in TCRβ$^{+/+}$ mice (see below), despite the failure of the transgene to reduce DP cell numbers. These results suggest that exclusion of TCRγδ$^+$ cells from the DP compartment also occurs in transgenic TCRβ$^{+/+}$ mice, but that this effect is obscured by compensatory mechanisms. These data provide direct evidence that cells coexpressing productively rearranged

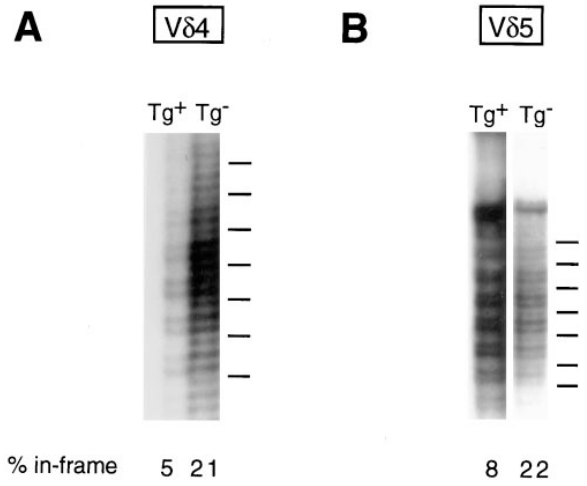

Figure 2. Selective Depletion of Cells with In-Frame δ Rearrangements from the DP Population in TCRγ Transgenic Mice

The frequencies of in-frame (A) TCR Vδ4 and (B) TCR Vδ5 gene rearrangements are reduced in transgenic TCRβ$^{+/-}$ mice. Bars on the side of the autoradiograph indicate in-frame bands as determined by DNA sequencing ladders run in parallel on the same gels; numbers on the bottom are percentages of in-frame bands as determined by densitometric analysis using a Phosphorimager. One representative experiment of three is shown. (Average in-frame gene rearrangement frequencies from three independent experiments: Tg$^+$ Vδ4 = 6.7%, Vδ5 = 8.7%; Tg$^-$ Vδ4 = 21.7%, Vδ5 = 23.3%). Frequencies of in-frame TCRδ gene rearrangements in transgenic mice are significantly different from the values from nontransgenic littermates: $p < 0.005$, Student's t test.

γ and δ genes are largely excluded from the DP population.

Thymocytes in the Transgenic TCRβ$^{+/-}$ Mice Exhibit a Normal Rate of Cell Turnover

The proliferative status of DP cells in transgenic or nontransgenic mice was examined in a series of bromodeoxyuridine (BrdU) labeling experiments (Lucas et al., 1993; Penit et al., 1995). The turnover rate of the TCRβ$^{+/-}$ DP population (i.e., the time required to label the population after initiating continuous exposure to BrdU) was identical in transgenic and nontransgenic littermates (Figure 3A). The transgene also had no effect on the fraction of TCRβ$^{+/-}$ DP cells that labeled with BrdU after a 1 hr pulse (Table 1), conditions reported to label only cells in S phase (Lucas et al., 1993). These data suggest that the proliferation and death rates of DP cells in the γ transgenic mice were not grossly altered, a finding consistent with the proposition that the transgene reduces the extent of differentiation of DN cells into DP cells. That the transgene's effects were observed only when TCRβ rearrangement was limiting is also consistent with this conclusion. Furthermore, the number of precursor CD25$^+$CD44$^-$ triple-negative (TN) (CD3$^-$CD4$^-$CD8$^-$) thymocytes was reduced in the TCRβ$^{+/-}$ transgenic mice (Table 1), in agreement with the notion that a fraction of these cells had been diverted to the γδ lineage. There was a slight increase in the percentage of these cells in S phase in the transgenic mice (Table 1), supporting the proposal that γδ expression stimulates some cell division in this population, or alternatively,

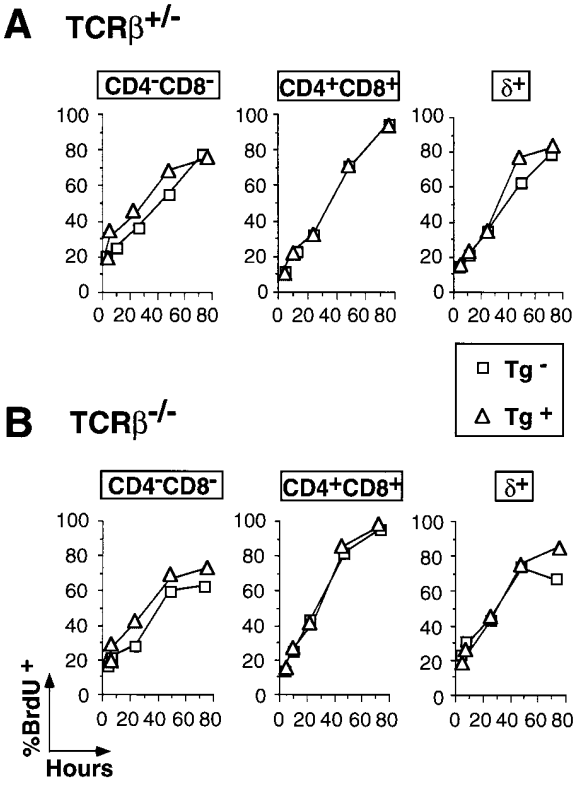

Figure 3. BrdU Incorporation Kinetics of Transgenic Thymocytes

(A) TCRβ$^{+/-}$ γ transgenic and littermate controls were labeled for various times with BrdU: 1 hr (Tg$^-$ n = 5, Tg$^+$ n = 6), 8 hr (Tg$^-$ n = 4, Tg$^+$ n = 3), 24 hr (Tg$^-$ n = 6, Tg$^+$ n = 16), 48 hr (Tg$^-$ n = 3, Tg$^+$ n = 3), and 72 hr (Tg$^-$ n = 2, Tg$^+$ n = 4) after BrdU administration. The percentage BrdU incorporation in different thymic subsets was determined by flow cytometry.

(B) TCRβ$^{-/-}$ γ transgenic and littermate controls were labeled 1 hr (Tg$^-$ n = 5, Tg$^+$ n = 3), 8 hr (Tg$^-$ n = 3, Tg$^+$ n = 3), 24 hr (Tg$^-$ n = 17, Tg$^+$ n = 11), 48 hr (Tg$^-$ n = 2, Tg$^+$ n = 3), and 72 hr (Tg$^-$ n = 4, Tg$^+$ n = 1) with BrdU and analyzed.

that the diversion of γδ lineage cells from this population enhances the representation of proliferating αβ lineage cells. The turnover rates of different TN subsets (Godfrey and Zlotnik, 1993) were similar in γ transgenic and nontransgenic mice, suggesting that the death and proliferation rates were not grossly altered by the transgene (data not shown).

Similarly, the increased number of γδ thymocytes in γ transgenic mice was accompanied neither by alterations in the kinetics of labeling of γδ thymocytes (Figure 3A) nor in the proportion of these cells in S phase (Table 1). The data are consistent with the proposition that the transgene increases the likelihood that precursor cells differentiate into γδ TCR$^+$ cells, while having an opposite effect on the differentiation of precursor cells into DP cells.

DP Thymocytes in TCRβ$^{-/-}$ Mice

Adult TCRβ$^{-/-}$ mice develop small and variable numbers of DP cells (averaging 2.3 × 10^6 DP cells per thymus; Table 1) (Mombaerts et al., 1992; Passoni et al., 1997),

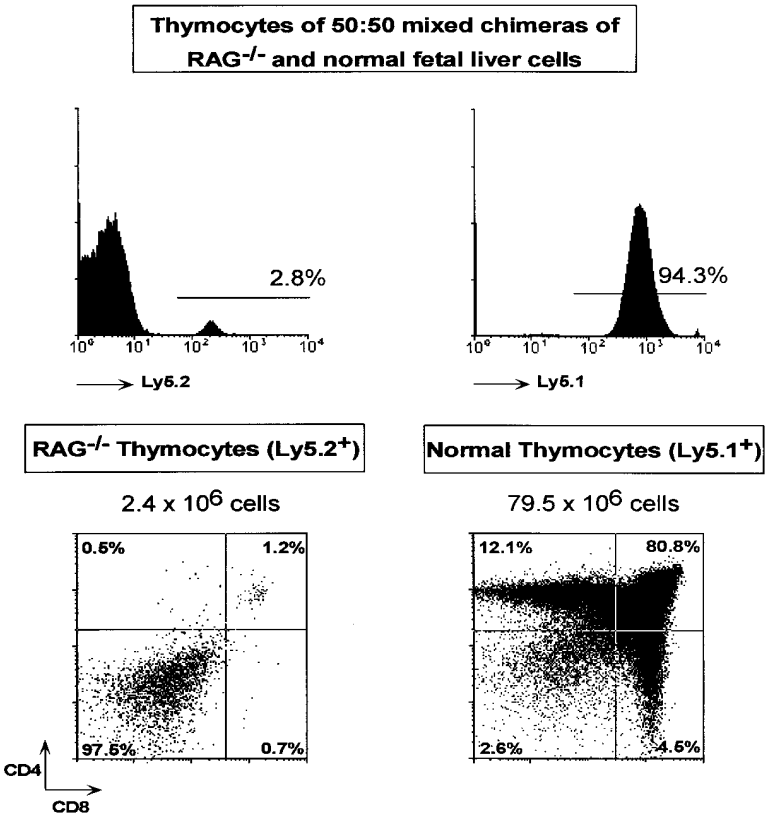

Figure 4. RAG-1$^{-/-}$ Thymocytes Do Not Generate Significant Numbers of DP Thymocytes in the Presence of Codifferentiating Normal Thymocytes

B6 Ly5.1 congenic recipients reconstituted with 50:50 mixture of fetal liver cells were stained with mAbs specific for CD4, CD8, Ly5.1 (normal B6 thymocytes), and Ly5.2 (RAG-1$^{-/-}$ thymocytes). (Top) Profiles showing the composition of developing donor thymocytes. (Bottom) Profiles representing CD4/CD8 expression patterns in Ly5.1- or Ly5.2-gated populations. RAG-1$^{-/-}$ thymocytes stained with the same combination of mAbs show 0% DP thymocytes. Control stainings using a mixed population of adult RAG-1$^{-/-}$ and B6 Ly5.1 thymocytes at various ratios show background signals (approximately 0.5% DP thymocytes detectable) in the gated RAG-1$^{-/-}$ thymocyte population. The flow cytometric profiles shown are representative results from one mouse of a group of six transplanted mice. Similar results were obtained when adult bone marrow cells were used as donor cells.

cells that are mostly absent in TCR$\beta^{-/-}$TCR$\delta^{-/-}$ double-deficient mice. In light of the evidence that TCR$\gamma\delta$ expression prevents production of DP cells in TCR$\beta^{+/-}$ and TCR$\beta^{+/+}$ mice, it is paradoxical that $\gamma\delta$ expression apparently stimulates production of DP thymocytes in TCR$\beta^{-/-}$ mice.

We examined the role of TCRγ and δ expression on the development of these enigmatic DP thymocytes in TCR$\beta^{-/-}$ mice in more detail. First, we addressed whether the effects of TCRδ expression on DP cell development are indirect, in line with previous studies showing that developing $\gamma\delta$ or $\alpha\beta$ T cells can induce neighboring SCID (severe combined immunodeficient) thymocytes to differentiate into DP cells (Shores et al., 1990; Lynch and Shevach, 1993). To the contrary, we found that in fetal liver chimeras where normal and recombination-deficient RAG-1$^{-/-}$ stem cells were mixed in equal proportion and allowed to codifferentiate, essentially no development of DP cells from the RAG-1$^{-/-}$ progenitors could be detected (Figure 4). These data indicate that the previous findings suggesting transinduction of DP cell development may depend on specific properties of the SCID mutation.

Analysis of TCRδ rearrangements in the TCR$\beta^{-/-}$ DP cells also indicated a cell-autonomous requirement for TCR expression for the development of these cells. A very high percentage of Vδ4 and Vδ5 rearrangements (80%–86%, Figure 5) were in-frame in purified DP cells from TCR$\beta^{-/-}$ mice, suggesting that δ chain expression was required for the development of these cells. Similar results were recently reported by Passoni et al. (1997), who also observed selection for in-frame rearrangements of some Vγ genes in these DP cells. This strong selection for in-frame TCRδ and γ rearrangements contrasted dramatically with the selection against in-frame δ and γ rearrangements observed in DP cells from normal mice ($\leq$20% in-frame; Figure 2) (Dudley et al., 1995; Livak et al., 1995). Taken together, these results support the notion that DP cell development normally requires expression of TCRs and that the DP cells in TCR$\beta^{-/-}$ mice are derived from TCR$\gamma\delta$-expressing progenitor cells.

Despite the requirement for TCRδ and γ expression for the production of DP cells in TCR$\beta^{-/-}$ mice, other properties of the cells suggest that they have adopted the $\alpha\beta$ lineage. First, expression of the Vγ2-Jγ1Cγ1 TCRγ gene was strongly repressed in these cells (Figure 6), despite the high levels of this rearrangement detected in their genomic DNA (data not shown). This property is shared with DP cells from normal mice (Figure 6) as well as normal peripheral $\alpha\beta$-lineage cells (Garman et al., 1986; Ishida et al., 1990). The low expression of TCRγ genes in DP cells from TCR$\beta^{-/-}$ mice is in accord with their low or absent levels of cell-surface $\gamma\delta$ TCR (Mombaerts et al., 1992 and data not shown). These observations suggest that in TCR$\beta^{-/-}$ mice, only the progenitor cells of the DP cells must express TCR$\gamma\delta$ and that γ expression is subsequently repressed. A second property shared by DP cells from normal and TCR$\beta^{-/-}$ mice is the expression of the pTα gene (Figure 6) (Saint-Ruf et al., 1994). In fact, the DP cells in TCR$\beta^{-/-}$ mice expressed several-fold higher levels of pTα than did DP cells in normal mice, perhaps reflecting a lesser proliferation of the DP cell population in TCR$\beta^{-/-}$ mice, leading to less dilution of total pTα mRNA. In contrast, TCR$\gamma\delta^{+}$

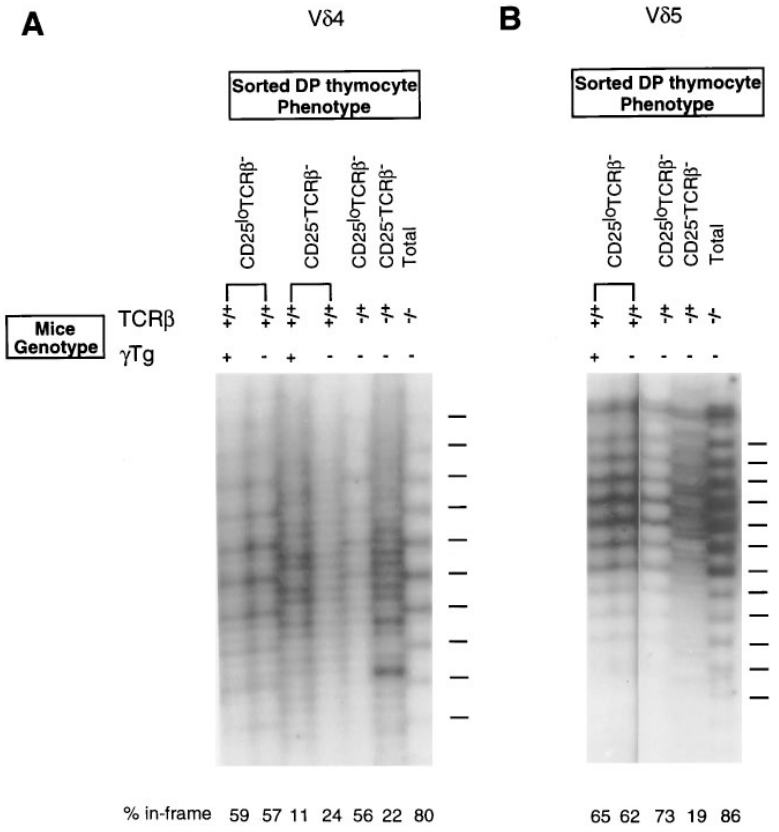

Figure 5. TCRδ Gene Rearrangement Status in CD25^{lo} and CD25^{-} DP Thymocytes

Increased frequencies of in-frame (A) TCR Vδ4 and (B) TCR Vδ5 gene rearrangements in sorted CD25^{lo}TCRαβ$^{null/lo}$ DP thymocytes from various mice. Bars on the side of the autoradiograph indicate in-frame bands as determined by DNA sequencing ladders run at the same time; numbers on the bottom are percentages of in-frame bands as determined by densitometric analysis using a Phosphorimager. Transgenic CD25^{-} DP thymocytes show the lowest frequencies of in-frame δ gene rearrangements, while sorted DP thymocytes (total) from TCRβ$^{-/-}$ have the highest frequencies of in-frame δ gene rearrangements. DNA from sorted thymocyte populations were PCR amplified, radiolabeled, and electrophoresed on a sequencing gel at the same time, and results from one such experiment are shown. Similar results were obtained in another independent cell sorting experiment.

thymocytes did not express pTα (Figure 6) (Bruno et al., 1995). Finally, previous studies have demonstrated extensive rearrangement of TCRα genes in thymocytes of TCRβ$^{-/-}$ mice, most of which are likely to have occurred in DP thymocytes (Mombaerts et al., 1992; Mertsching et al., 1997). Collectively, the gene expression pattern and cell-surface antigen expression profiles suggest that the DP thymocytes in TCRβ$^{-/-}$ mice belong to the αβ lineage, not the γδ lineage.

To further examine the role of TCRγδ expression in the production of DP cells in TCRβ$^{-/-}$ mice, we generated TCRβ$^{-/-}$ mice that also expressed the Vγ2-Jγ1Cγ1 transgene. Surprisingly, the absolute number of TCRγδ^{+} thymocytes was not increased significantly by the transgene (Table 1), perhaps because γδ cell numbers, already enhanced by TCRβ deficiency, had reached a maximum. However, the transgene increased the number of DP thymocytes substantially, by an average of 9-fold (Table 1), resulting in an average 3-fold increase in total thymus cellularity (nontransgenic [Tg^{-}] = 8.5 ±

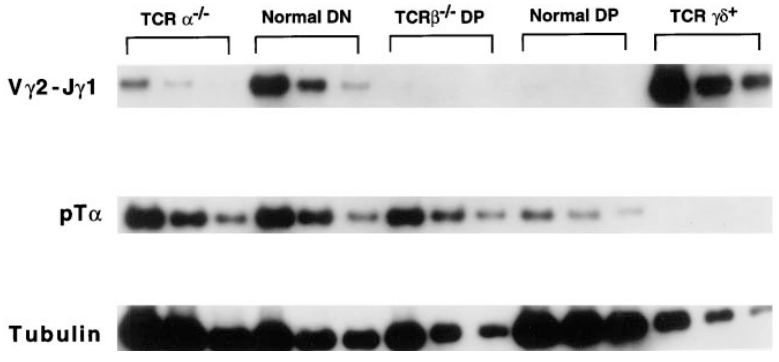

Figure 6. Gene Expression Pattern of TCRβ$^{-/-}$ DP Thymocytes Resembles That of Conventional DP Cells

TCRβ$^{-/-}$ DP thymocytes do not express the rearranged Vγ2 gene but do express the pTα gene as determined by semiquantitative radioactive reverse transcriptase PCR. These properties are shared with DP cells from normal mice and TCRα$^{-/-}$ mice; TCRα$^{-/-}$ mice produce normal DP cells that cannot undergo subsequent thymic selection. RNAs from sorted thymocyte populations (except for TCRα$^{-/-}$ thymocytes, which were unfractionated) were reverse-transcribed, and serial 3-fold dilutions of cDNAs were PCR-amplified in the presence of a radioactive nucleotide. The highest initial amount of cDNA used for control PCR of tubulin gene represents 3-fold dilution of the cDNAs of corresponding sample PCRs. Samples without the reverse transcription step did not give detectable signals (data not shown).

3.7×10^6, n = 58; transgenic [Tg^+] = $28.4 \pm 12.1 \times 10^6$, n = 31 [average ± SD]), albeit with considerable mouse-to-mouse variation. The effect of the transgene was to skew the ratio of DP cells to γδ cells in TCRβ$^{-/-}$ mice, from approximately 1:1 in nontransgenic mice to 10:1 in the transgenic mice.

The increased number of DP cells in the transgenic TCRβ$^{-/-}$ mice was accompanied by alterations in the cell number of different precursor TN subsets. The CD25$^+$CD44$^-$ subset, in which TCR gene rearrangement is normally ongoing, was decreased by 2-fold (Table 1), suggesting that a larger proportion of these cells was diverted to later developmental stages in either the γδ or the αβ lineage. Consistent with this proposition, the subsequent CD25$^-$CD44$^-$ stage contained more than twice as many cells as was observed in nontransgenic TCRβ$^{-/-}$ mice, suggesting increased differentiation of late-stage DN cells. Thus, some of the increase in the DP population in transgenic TCRβ$^{-/-}$ mice is probably due to increased differentiation of DN cells into DP cells. However, some of the increase may also be due to increased proliferation of DP cells, since this population exhibits the greatest relative increase in cellularity (Table 1). The turnover rates of DP, DN, and γδ T cells (Figure 3B) or the precursor TN subsets (data not shown) were not significantly altered by the transgene. The proportions of various thymocyte subsets in S phase were also comparable (Table 1), suggesting that the death rates of DP cells were not grossly altered. These data suggest that in TCRβ$^{-/-}$ mice, the γ transgene may enhance the extent of differentiation of DN cells into DP cells and the proliferation of DP cells.

γδ-Dependent DP Thymocytes in Normal Mice

The paradox that γδ expression is required for the production of DP cells in TCRβ$^{-/-}$ mice, but that it prevents DP cell production in TCRβ$^+$ mice, can be interpreted in two ways. One possibility is that γδ-dependent generation of DP cells occurs only in the absence of TCRβ expression. Alternatively, γδ-dependent DP cells constitute only a minor subset in normal (TCRβ$^{+/+}$ or TCRβ$^{+/-}$) mice because of the very large number of conventional DP cells in these mice.

To distinguish between these possibilities, we attempted to identify γδ-dependent DP cells in nontransgenic TCRβ$^{+/+}$ and TCRβ$^{+/-}$ mice. Indeed, we were able to identify a DP subpopulation in these mice that harbored predominantly in-frame TCRδ gene rearrangements, unlike the bulk of DP cells, which are enriched for out-of-frame δ rearrangements. This task was aided by the finding that low levels of CD25 are expressed by some DP thymocytes in TCRβ$^{-/-}$ mice, as seen in Figure 7A. Though reproducible, the stained cell population did not clearly separate from the unstained population, suggesting a continuum of low CD25 expression on these DP cells. CD25 expression is extinguished in most DP cells in TCRβ$^+$ mice, but by gating on the small CD25^{lo}TCR$^{null/lo}$ subset, we could detect a very small fraction of CD25^{lo}TCRαβ$^{null/lo}$ DP cells (Figure 7B). The most compelling evidence that these cells represent a bona fide DP subpopulation similar to the DP thymocytes found in TCRβ$^{-/-}$ mice came from PCR-RFLP analysis of DNA from these cells, which were isolated in a two-step procedure. The results revealed that purified CD25^{lo}TCRαβ$^{null/lo}$(CD3$^-$) DP thymocytes contained 57%–62% in-frame Vδ4 and Vδ5 rearrangements (Figure 5), considerably enriched compared to the expected random frequency of 33%, and even more enriched compared to CD25$^-$ or unseparated DP cells, which were selectively depleted of in-frame δ rearrangements (19%–24% in-frame; Figures 2 and 5) (Dudley et al., 1995; Livak et al., 1995). A similar outcome was observed in an independent sorting experiment (data not shown) and in fractionated DP cells from TCRβ$^{+/-}$ mice (Figure 5). These results established that many of the CD25^{lo}CD3$^-$ DP cells from normal mice, like the cells from TCRβ$^{-/-}$ mice, had been selected at some stage for expression of the TCRδ chain. In contrast, the bulk of DP cells from normal mice, which are CD25$^-$ cells, had been subjected to selection against TCRδ expression (Figure 5).

Further evidence that the CD25^{lo} DP population is γδ dependent came from analysis of the γ transgenic mice. Expression of the γ transgene in otherwise wild-type mice caused a specific 6- to 7-fold increase in the size of this DP subpopulation (Figure 7B). An even larger increase in the proportion (but not in absolute cell numbers) of this subpopulation was observed in γ transgenic TCRβ$^{+/-}$ mice (data not shown), consistent with the reduction in the total number of DP cells in these mice. In both types of mice, the CD25^{lo} DP cells were enriched for in-frame δ rearrangements (Figure 5). The increased size of this DP subset observed in γ transgenic TCRβ$^+$ mice is in accord with the increased size of the similar DP subset in γ transgenic TCRβ$^{-/-}$ mice. In contrast, the majority population of conventional DP cells in adult TCRβ$^{+/-}$ mice and in fetal TCRβ$^{+/+}$ mice was diminished by the expression of the γ transgene. Taken together, the data suggest the existence of two subsets of DP cells in normal mice: a major one depleted of in-frame δ rearrangements and a minor one enriched for in-frame δ rearrangements, which are oppositely affected by TCRγ transgene expression.

Discussion

TCRγδ Expression Hinders Development of DP Cells

The possibility that the previously reported selection against productive γ and δ rearrangements in αβ-lineage cells depends on γ or δ pairing with an unidentified pre-TCR chain is refuted by our finding that γ transgene expression in all thymocytes caused a sharp reduction in the frequency of in-frame δ rearrangements in the DP population. The results indicate that a cell is impeded from contributing to the DP population only if it expresses both γ and δ chains. This conclusion is supported by the observed reduction in the number of DP cells in adult TCRβ$^{+/-}$ mice and in fetal TCRβ$^{+/+}$ mice harboring the transgene. The data were obtained in a relatively normal physiological setting, where both γδ and αβ T cell development can occur and the function of the transgene-encoded γ chain is dependent on pairing with the endogenous δ TCR chain.

The data suggest that γδ expression reduces the

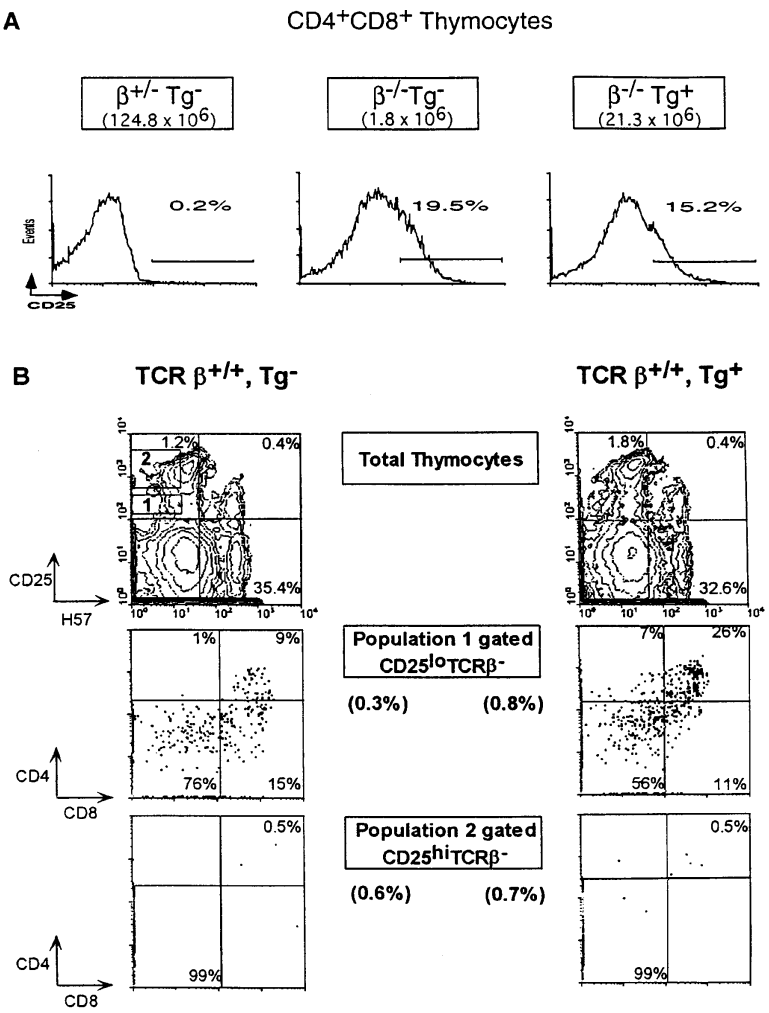

Figure 7. Detection of CD25^{lo} DP Thymocytes in Normal Mice and the Effect of the γ-Transgene on These Cells

(A) Low but significant CD25 expression on DP thymocytes of TCRβ$^{-/-}$ mice. Unfractionated thymocytes were stained with mAbs specific for CD4, CD8, and CD25. Representative CD25 expression profiles of gated CD4^{+}CD8^{+} population are shown. DP thymocyte numbers are shown in the brackets.

(B) Identification of CD25^{lo}TCRαβ$^{null/lo}$ DP thymocytes in normal mice. Unfractionated thymocytes from transgenic TCRβ$^{+/+}$ and nontransgenic littermates were stained with mAbs specific for TCRβ chain, CD25, CD4, and CD8 (top profiles) and representative results from analysis of 2 × 10^{5} cells/sample are shown. Gated population 1 represents CD25^{lo} TCRαβ$^{null/lo}$ thymocytes, and gated population 2 represents CD25^{hi}TCRαβ$^{null/lo}$ thymocytes. Many of the events are crowded on the axes in some of the plots, because of the compensation settings required for the four-color analysis. The numbers in the brackets are the percentages of total thymocytes in the corresponding gated population. Small percentages of DP thymocytes are detectable in the gated CD25^{lo}TCRαβ$^{null/lo}$ population in normal mice, which is consistently and specifically increased in the transgenic mice. This staining pattern was specific for CD25^{lo}TCRαβ$^{null/lo}$ population, since no other thymic subpopulations analyzed showed similar staining results and there were essentially no positive staining events in the CD4^{+}CD8^{+} quadrant when gated CD25^{hi}TCRαβ$^{null/lo}$ cells were analyzed.

probability that a cell will differentiate into a DP cell, rather than simply affecting the proliferation or death rates of DP cells. First, the BrdU labeling data suggest that the proliferation and death rates of DP cells were not grossly altered by the transgene, although the data are compatible with some alteration in these parameters (J. K. and D. H. R., unpublished data). Second, γ transgene expression reduced DP cell numbers in adult TCRβ$^{+/-}$ mice but not in adult TCRβ$^{+/+}$ mice, suggesting that the capacity of progenitor cells to differentiate into DP cells is determined by the likelihood of β versus γδ expression. If γδ expression simply reduced DP cell proliferation or viability, the effect should be equally evident in TCRβ$^{+/+}$ and TCRβ$^{+/-}$ mice.

Although reduced DP cell differentiation was evident in adult γ transgenic TCRβ$^{+/+}$ mice based on the reduction in in-frame TCRδ rearrangements, steady-state DP cell numbers were normal. We propose that homeostatic mechanisms to maintain normal thymus cellularity can compensate for the reduced rate of DP cell differentiation in these mice as well as in nontransgenic TCRβ$^{+/-}$ mice, but not when DP cell development is further reduced, as in γ transgenic TCRβ$^{+/-}$ mice.

That some DP cell development still occurs in γ transgenic mice is expected, since the γ transgene should affect only the cells that produce in-frame δ rearrangements. Only five of nine, or fewer, progenitor cells should harbor in-frame δ rearrangements, depending on the efficiency of δ recombination. The few DP cells harboring in-frame δ rearrangements in γ transgenic mice (Figure 2) probably comprise a few γδ-dependent DP cells, thymocytes with termination codons at their Vδ-Jδ junctions, cells in which the expressed δ chain fails to pair with the transgenic γ chain, or cells that fail to express the transgenic γ chain.

The Generation of Some DP Cells Is γδ TCR–Dependent

γδ expression clearly impedes many progenitor cells from differentiating into DP cells, but, paradoxically, γδ expression can also support the differentiation of some precursors into DP cells. The resulting DP cells in TCRβ$^{-/-}$ mice exhibited several characteristics of conventional αβ-lineage DP cells, such as pTα expression and repression of γ gene transcription. Based on the clear enrichment of in-frame δ and Vγ2 rearrangements in the population (Figure 5) (Passoni et al., 1997) and our inability to achieve significant transinduction of DP cells from RAG-1$^{-/-}$ progenitor cells in mixed chimera experiments, we conclude that the differentiation of these DP

cells requires cell-autonomous expression of γδ receptors. These γδ-dependent DP cells may be the counterpart of αβ TCR–expressing cells with properties of γδ lineage cells that have been observed in TCRαβ transgenic mice (Bruno et al., 1996).

Despite their similarity to "conventional" DP cells in most respects, a fraction of γδ-dependent DP cells in TCRβ$^{-/-}$ mice do display an unusual phenotype, in that they express low levels of CD25. One possible explanation for this observation is that the residual CD25 expression could be retained from the CD25^{+} DN stage, if the cells undergo less replication during the transitional stages than is typical for conventional DP cells (Crompton et al., 1994).

The observation that γδ-dependent DP cells also exist in normal (TCRβ$^{+/+}$ and TCRβ$^{+/-}$) mice indicates that their development is not an artifact dependent on impaired development of conventional DP cells. The CD25^{lo} DP population in normal mice contained predominantly in-frame δ rearrangements, whereas such rearrangements were selected against in conventional CD25^{-} DP cells from the same mice. Since the CD25^{lo} population represents only approximately 20% of DP cells in TCRβ$^{-/-}$ mice, it is likely that many of the γδ-dependent DP cells in normal mice went undetected in our experiments. Extrapolating, the actual number of γδ-dependent DP cells in normal mice is probably several-fold higher than 5×10^4 cells/thymus. Nevertheless, based on the relative numbers of CD25^{lo} DP cells, TCRβ$^{+/+}$ and TCRβ$^{+/-}$ mice most likely contain fewer γδ-dependent DP cells than TCRβ$^{-/-}$ mice, perhaps by a factor of 10. The reduced number of these cells in normal mice could reflect a nonspecific decrease due to the large numbers of other cells in the thymus or the action of a specific inhibitory signal that emanates from developing, conventional αβ-lineage cells (see below).

Expression of the γ transgene caused a 9-fold increase in the number of γδ-dependent DP cells in TCRβ$^{-/-}$ mice and also in normal mice as judged by the increased number of CD25^{lo} DP cells. The γδ-dependent DP cells in γ transgenic mice, like those in nontransgenic mice, contained primarily in-frame δ rearrangements. The effect of the γ transgene on the development of these unique DP cells suggests that the differentiation of these cells depends on expression of both γ and δ chains. The 9-fold increase in DP cells induced by the transgene is somewhat greater than might be predicted if it were assumed that the only effect of the transgene was to provide in-frame γ rearrangements to each progenitor cell. It is possible that some of the increase is due to greater proliferation of DP cells induced by the sustained expression of the γ transgene in these cells.

Implications of the Results for Models of γδ versus αβ T Cell Lineage Commitment

The findings that γδ expression can both impede and promote DP cell development, and that DP cell development can occur in the absence of TCRβ expression, either refute or put major constraints on the various models of αβ versus γδ cell lineage determination that have been proposed. Instructive versus stochastic models are considered separately below.

Instructive Models

Based on the supposition that DP cell development depends primarily on signaling through the TCRβ/pTα receptor complex, it has been proposed that precursor cells that produce in-frame rearrangements of the TCRβ gene are induced to differentiate into DP cells (Livak et al., 1995). However, TCRβ signaling cannot participate in the genesis of the γδ-dependent DP cells observed here and elsewhere in TCRβ$^{-/-}$ mice, which are incapable of producing functional β chains. Furthermore, it has been reported that a significant fraction of γδ cells in normal and TCRα$^{-/-}$ mice contain in-frame β rearrangements (Dudley et al., 1994, 1995; Burtrum et al., 1996; Mertsching and Ceredig, 1996), indicating that functional β expression does not lead invariably to development along the αβ lineage. These considerations suggest that γδ-dependent DP cells generally do not arise by TCRβ-dependent signaling.

The existence of γδ-dependent DP cells argues against a stringent instructive model of αβ versus γδ lineage determination. However, the data are consistent with instructive models that incorporate an imperfect, or "leaky," fate-determining mechanism (Kang et al., 1995; Kang and Raulet, 1997; Washburn et al., 1997). In such a model, a fraction of progenitor cells that express γδ receptors aberrantly adopts the αβ-lineage differentiation program. One version of this model invokes a fixed low error rate, which might occur if the signal transduction process linking receptor expression to fate determination were error prone. In this case, the fraction of cells adopting the incorrect fate might be expected to be constant. Our data indicate that the number of γδ-dependent DP cells (incorrect fate) can vary independently of the number of γδ cells (correct fate), as illustrated by a comparison of TCRβ$^{-/-}$ mice that do or do not express the γ transgene. As already discussed, however, these discrepancies in steady-state cell numbers could reflect limitations in the sizes of cell populations or altered emigration rates that obscure a constant relationship between the rate of γδ cell development and the rate of γδ-dependent DP cell development.

One can also envisage a leaky instructive model in which the rate at which cells take on the inappropriate fate depends on the thymic environment in which the cells are differentiating. It was recently demonstrated that the impaired production of DP cells in TCRβ$^{-/-}$ mice can be reversed by expression of an activated *notch* transgene in developing T cells, and that the dosage of *notch* genes in a developing thymocyte can influence its fate (Washburn et al., 1997). In cell fate decisions controlled by *notch*, interactions between equivalent neighboring cells can predispose the two cells to acquire opposite fates (Artavanis-Tsakonas et al., 1995). If some cells differentiating along the γδ lineage are neighbored by other γδ lineage cells, it is possible that some of these cells may acquire the inappropriate fate and differentiate into DP cells. This lateral induction of cell fate may occur relatively rarely in normal mice. However, in TCRβ$^{-/-}$ mice, in which differentiation of conventional αβ-lineage cells is blocked, differentiating γδ cells may neighbor each other more frequently, leading to a higher rate of γδ-dependent DP cell development, consistent with our data. Results obtained with mixed

chimeras prepared with normal and TCRγδ transgenic bone marrow cells are also consistent with this interpretation (Washburn et al., 1997).

Stochastic Models

The available data argue strongly against the original version of the stochastic model, which proposed that commitment to the αβ lineage would prevent a cell from undergoing δ gene rearrangements or from ever expressing γδ receptors (Winoto and Baltimore, 1989; Ishida et al., 1990). However, the results to date, including documentation of the existence of γδ-dependent DP cells, can be explained by a modified version of this model. The model proposes that stochastically committed cells require TCR signaling, either β/pTα or γδ mediated, to proceed to the DP stage of differentiation. Survival, proliferation, and/or further differentiation of these DP cells may require expression of TCRαβ. Hence, committed αβ-lineage cells that expressed TCRβ, but not TCRγδ, would differentiate into conventional DP cells and subsequently have the potential to differentiate into mature αβ T cells. Having expressed β first, further rearrangements of γ and δ genes might be inhibited as a result of either a specific feedback inhibition process or because the cells rapidly differentiate into DP cells. Either mechanism would account for the low frequencies of DP cells with productive rearrangements of both γ and δ genes. αβ lineage–committed cells that failed to express TCRβ but that did express TCRγδ could differentiate into γδ-dependent DP cells, but would fail to differentiate further. The small numbers of these cells in normal mice could imply that γδ-expressing αβ-lineage progenitor cells have a relatively small probability of successfully differentiating into DP cells or that they proliferate less than conventional DP cells. The increased numbers of these cells in TCRβ$^{-/-}$ mice might result from an increased likelihood that these cells will make in-frame γ and δ rearrangements, assuming that the inability to make a β chain provides more time for γ and δ rearrangements to occur. Alternatively, the paucity of conventional DP cells may relieve competition for thymic "space." The γ transgene would be expected to increase the probability that αβ-committed progenitor cells make a γδ receptor. The relatively large 9-fold effect of the γ transgene could be due to a combination of factors described above: an increase in the number of cells that successfully produce γδ receptors, and possibly increased proliferation of such cells.

Experimental Procedures

Mice

Transgenic founder mice were generated by injection of the rearranged G8 TCRγ gene (kindly provided by S. Hedrick, University of California, San Diego) into pronuclei of (C57BL/6 (B6) × CBA)F1 zygotes. Founders were then backcrossed to B6 mice for at least two generations before analysis. B6 TCRβ$^{-/-}$ mice were purchased from The Jackson Laboratories (Bar Harbor, ME). These mice were crossed to the B6-backcrossed γ transgenic mice. Transgenic TCRβ$^{+/-}$ and TCRβ$^{-/-}$ mice were obtained by intercrossing the pups.

Antibodies

Anti-Vγ2 TCR (UC3–10A6), anti-δ TCR (GL3), anti-β TCR (H57.597), anti-Ly5.1 (A20.1.7), and anti-Ly5.2 (104.2.1) monoclonal antibodies (mAbs) were purified and conjugated with fluorescein isothiocyanate (FITC) or biotin according to standard protocols. Anti-CD8α-Tricolor (53-6.7), anti-CD44-FITC (IM7.8.1), anti-CD44-biotin, and anti-CD25-phycoerythrin (PE) (PC61.5.3) mAbs and streptavidin-Tricolor were purchased from Caltag (San Francisco, CA). Anti-CD3ε-FITC was obtained from Pharmingen (San Diego, CA); rat anti-CD3 mAb from Serotech (Oxford, UK); goat anti-rat immunoglobulin G (IgG)–Red613 and anti-CD4−Red613 from Gibco (Grand Island, NY); and strepavidin-PE from Molecular Probes (Eugene, OR).

Flow Cytometry

One to three hundred thousand viable (forward and side scatter–gated) thymocytes were analyzed with an EPICS XL-MCL flow cytometer (Coulter). Composites of flow cytometric profiles were generated using the WinMDI program (John Trotter, Salk Institute, San Diego, CA). Cell sorting was performed on an ELITE cell sorter (Coulter). For four-color sorting of the CD25^{lo}TCRαβ^{-} DP population, thymocytes were depleted of TCRβ^{+} cells by using biotinylated H57 mAb and a magnetic-activated cell sorter, resulting in a three- to five-fold enrichment. The depleted cells, which still included TCRβ^{lo} cells, were stained with anti-CD4-Red613, anti-CD8-Tricolor, anti-CD25-PE, and anti-CD3ε-FITC (to eliminate residual CD3^{+} cells, including TCRγδ^{+} thymocytes) followed by four-color flow cytometric cell sorting for CD4^{+}CD8^{+}CD25^{lo}CD3^{-} thymocytes. CD25^{lo} DP and CD25^{-} DP thymocytes were sorted at the same time.

Radiation Chimeras

B6 Ly5.1 congenic mice were lethally irradiated (950 rad) in two split doses. Timed fetal liver cells (E15) or adult bone marrow cells from RAG-1$^{-/-}$ (Ly5.2) and B6 Ly5.1 mice were mixed 50:50, and 1 × 10^{7} cells were injected into recipients. Reconstituted mice were analyzed 2–3 months later.

PCR-RFLP Analysis

Analysis of TCRδ gene rearrangements was carried out essentially as described (Dudley et al., 1995), using the same PCR primers. Genomic DNA was isolated from sorted thymocyte populations, and DNA from at least 5000 cell equivalents was used for PCR using a standard 35-cycle protocol and *Pfu* polymerase (Stratagene). PCR products were then digested with restriction enzymes (HinfI for Vδ4-Jδ1 PCR products and HaeIII for Vδ5-Jδ1 PCR products), and the resulting fragments were end-labeled using T4 polynucleotide kinase (Boehringer Mannheim) and [γ-^{32}P]ATP (Amersham). The labeled products were precipitated with the aid of carrier RNA and separated on a 6% denaturing polyacrylamide sequencing gel. Band sizes were determined by a control sequencing reaction of a known DNA fragment and an end-labeled PBR322 DNA ladder that were run alongside the PCR products. Densitometric analysis was performed using a Phosphorimager (Molecular Dynamics).

BrdU Labeling

BrdU incorporation and analysis by flow cytometry was done as previously described (Tough and Sprent, 1994; Penit et al., 1995). For continuous labeling, BrdU (0.9 mg) was injected intraperitoneally into the mice at 8 hr intervals. The thymocytes were isolated at 1 (two injections 2 hr apart), 8, 24, 48, and 72 hr after BrdU administration. DN thymocytes were isolated by complement lysis of CD4 and CD8 thymocytes utilizing AD4(15) and RL172 ascites fluids. The BrdU labeling of total thymocytes was analyzed using four-color cytometry with anti-CD4-613, anti-CD8-670, and anti-TCRβ-biotin, followed by streptavidin-PE and anti BrdU-FITC (Becton Dickinson) mAbs. BrdU labeling of γδ thymocytes was analyzed using anti-δ-biotin (GL3) on purified DN thymocytes. CD25 and CD44 subpopulations of DN thymocytes were analyzed on purified DN thymocytes from which CD3^{+} cells were gated out of the analysis by utilizing a rat anti-CD3 mAb (Serotech) with secondary goat anti-rat IgG-613 (Gibco). The samples were then blocked with rat immunoglobulin (Caltag) and stained with anti-CD25-PE, anti-CD44-biotin with streptavidin 670, and anti-BrdU-FITC. The S-phase cell cycle analysis shown in Table 1 was based on the following numbers of mice: TCRβ$^{+/+}$ Tg^{-}, n = 2, Tg^{+}, n = 4; TCRβ$^{+/-}$ Tg^{-}, n = 6, Tg^{+}, n = 6; and TCRβ$^{-/-}$ Tg^{-}, n = 9, Tg^{+}, n = 6.

Reverse Transcriptase PCR

RNA was purified from various thymocyte subpopulations using Ultraspec RNA solution (Bioteck, Houston, TX). Reverse transcription of 1–2 μg of RNA was performed using oligo-dT primers and avian reverse transcriptase (both from Boehringer Mannheim). cDNA was then serially diluted 3-fold and PCR was performed for 28 cycles with the addition of 1.0 μCi of [α-^{32}P]-dCTP (Amersham) per sample in a total reaction volume of 50 μl. The starting concentration of tubulin PCR reactions was 3-fold lower than the corresponding target-gene PCRs. The following PCR primers were used: 5′ Vγ2, CTGGGAATTCAACCTGGCAGATGA, 3′ Jγ1, GGGAAGCTTACCAGA GGGAATTACTATGAG; and 5′ tubulin, CAGGCTGGTCAATGTGGCA ACCAGATCGGT, 3′ tubulin, GGCGCCCTCTGTGTAGTGGCCTTTGG CCCA. The published primer sequences (Saint-Ruf et al., 1994) were used to analyze pTα gene expression.

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