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T Cell Receptor γ Gene Regulatory Sequences Prevent the Function of a Novel TCRγ/pTα Pre–T Cell Receptor

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Summary

Expression of a TCRγ transgene in RAG-1$^{-/-}$ mice resulted in the development of a limited number of CD4$^+$CD8$^+$ (DP) thymocytes. In vivo treatments with anti-TCRγ antibody enhanced the number of DP thymocytes, demonstrating that TCRγ chains were expressed on the cell surface in the absence of δ, α, or β chains. Mutations in pTα or CD3ε genes abolished transgene-induced DP cell development, indicating that TCRγ can associate with pTα and CD3 to form a novel pre-TCR. With a transgene containing additional regulatory sequences, TCRγ expression was down-regulated in DP cells, and little DP cell development occurred. Thus, the function of the endogenous TCRγ/pTα is limited by the transcriptional down-regulation of TCRγ genes that normally accompanies DP cell development.

Introduction

Normal development of αβ and γδ T lymphocytes requires a properly coordinated generation and expression of functional TCR chains. Somatic recombination of TCRγ, δ, β, and α genes occurs early in T cell differentiation in the thymus. If a thymic precursor produces functional TCRγ and δ chains, it can develop into a γδ cell; conversely, generation of productive TCRβ gene rearrangements permits a precursor to differentiate along the αβ lineage. The immature CD4$^-$CD8$^-$CD3$^-$ (triple negative, TN) thymocyte subset that contains precursors for T cells can be ordered into developmental intermediates by the expression of CD25 and CD44 molecules in the following maturational sequence: CD25$^-$44$^+$ to CD25$^+$44$^+$ to CD25$^+$44$^-$ to CD25$^-$44$^-$ (Godfrey et al., 1993). Maturation to the CD25$^+$ stage is independent of TCR expression and requires extrinsic growth factors, the most critical being IL-7 and SCF (Rodewald et al., 1997). TCR gene rearrangements are initiated during the transition from CD25$^+$44$^+$ to CD25$^+$44$^-$ stages. The latter stage marks a major checkpoint in T cell development, wherein cells destined to develop into αβ T cells must express a functional TCRβ chain in order to efficiently proceed to the CD25$^-$CD44$^-$ stage (Mombaerts et al., 1992a; Shinkai et al., 1993; Dudley et al., 1994; Fehling et al., 1995). The successful TCRβ$^+$ cells proliferate extensively and differentiate into immature αβ lineage CD4$^+$CD8$^+$ (double positive [DP]) thymocytes.

CD25$^+$CD44$^-$ TN cells can also differentiate into CD4$^-$CD8$^-$ (double negative [DN]) γδ thymocytes if both TCRγ and TCRδ genes are rearranged productively. This process differs in important ways from αβ T cell development. First, TCRγ and δ genes undergo rearrangement more or less coordinately, whereas β genes are rearranged considerably before the α genes (Godfrey et al., 1994; Petrie et al., 1995; Wilson et al., 1996). Indeed, there is no evidence to date to suggest that the TCRγ or -δ chain functions separately with putative surrogate chains (Passoni et al., 1997; Kang et al., 1998). Second, unlike αβ lineage cells, γδ lineage thymocytes do not appear to undergo extensive proliferation during development and constitute only approximately 1% of thymocytes. The less extensive cell expansion suggests that γδ receptor expression by these cells imparts a distinct signal, in quality or quantity, compared to the signal imparted by the pre-TCR in αβ lineage cells.

The TCRβ-dependent transition of CD25$^+$44$^-$ TN thymocytes to later stages of T cell development requires association of the TCRβ chain with the surrogate α chain (pTα) and the CD3 signaling molecules. Mice with defects in expression of the functional pre-TCR complex are characterized by an arrest in development of most or all differentiating thymocytes at the CD25$^+$44$^-$ TN stage; mutations in the RAG (Mombaerts et al., 1992b; Shinkai et al., 1992) and *scid* (Rothenberg et al., 1993) genes, which are necessary for TCR gene rearrangement, the TCRβ gene (Mombaerts et al., 1992a), the pTα gene (Fehling et al., 1995), and the CD3ε gene (Malissen et al., 1995) result in a severe T cell developmental block. In RAG$^{-/-}$ mice, for example, the developmental arrest results in an approximately 100-fold reduction in thymus cellularity, which can be reversed by expression of a productively rearranged TCRβ chain transgene in the mice (Shinkai et al., 1993). These results and others (Dudley et al., 1994) have clearly established that the pre-TCR complex is necessary for normal differentiation of precursor cells into αβ lineage DP thymocytes.

The requirement for the pre-TCR in the development of DP cells can be overcome by several experimental manipulations that bypass or mimic pre-TCR signaling. Injection of MAbs against the CD3ε chain can stimulate DP cell development in RAG$^{-/-}$ (Jacobs et al., 1994; Shinkai and Alt, 1994) or pTα$^{-/-}$ mice (Fehling et al., 1997), as can the expression of activated Lck (Mombaerts et al., 1994), a signal downstream of the TCR, and activated Ras (Swat et al., 1996), a signal downstream of

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various receptor/tyrosine kinases, including Lck. Furthermore, expression of activated Notch protein in TCRβ$^{-/-}$ mice enhances the development of DP thymocytes (Washburn et al., 1997). All of these manipulations result in the production of nearly normal numbers of DP thymocytes. Other inductive signals only partially restore DP cell numbers, such as sublethal irradiation of RAG$^{-/-}$ (Zuniga-Pflucker et al., 1994; Guidos et al., 1995) and *scid* (Danska et al., 1994) mice, expression of a CD4 transgene (Norment et al., 1997), mutation of the *p53* suppressor gene (Jiang et al., 1996), or overexpression of the antiapoptotic *bcl-2* transgene (Linette et al., 1994) in RAG$^{-/-}$ mice.

TCRγδ expression also impacts αβ T cell development. αβ lineage cells, including DP cells and mature αβ T cells, exhibit a selective depletion of productive TCRγ and -δ gene rearrangements (Dudley et al., 1995; Kang et al., 1995; Livak et al., 1995). Additional transgenic studies indicate that coexpression of the γ and δ TCR chains in a progenitor cell, as a γδ TCR, usually prevents the cell from contributing to the DP cell population (Dent et al., 1990; Livak et al., 1997; Kang et al., 1998). These findings suggest that differential signaling from the γδ receptor and the TCRβ/preTα receptor result in different developmental outcomes for developing T cells, but the underlying mechanisms are not well understood. Importantly, it is unclear whether some of the differential signaling arises because only TCRβ can pair with the pTα subunit, or whether the pre-TCR function is regulated by a lineage-restricted expression of the components of the pre-TCR. Here, we have investigated this issue by generating mice that express a TCRγ chain, but not TCRβ, TCRδ, or TCRα chain, in developing T cells. The results indicate that TCRγ chain can in fact interact with pTα and support limited DP cell development. However, we demonstrate that the repression of TCRγ gene transcription that normally occurs in αβ lineage T cells (Garman et al., 1986; Ishida et al., 1990) prevents significant development of DP cells by this pathway. These results have significant implications for the developmental processes that regulate the γδ/αβ cell fate decision.

Results

TCRγ Transgene Expression in RAG-1$^{-/-}$ Mice Leads to DP Thymocyte Development

To generate mice in which TCRγ is expressed in developing T cells, but TCRβ, δ, and α are not, we generated RAG-1$^{-/-}$ mice that harbor a rearranged TCRγ transgene. The TCRγ transgene (Vγ2-Jγ1-Cγ1) was derived originally from the G8 γδ T cell that has been described in detail previously (Dent et al., 1990). We were surprised to observe that expression of the TCRγ transgene alone in RAG-1$^{-/-}$ mice led to the generation of appreciable numbers of DP thymocytes (Figure 1A), whereas DP cell development was never observed in nontransgenic RAG-1$^{-/-}$ mice. In transgenic RAG-1$^{-/-}$ mice generated with a high copy transgene founder line (13 copies), the proportion of DP thymocytes ranged from 29% to 52% of total thymocytes, whereas transgenic RAG-1$^{-/-}$ mice with a lower transgene copy number (three copies)

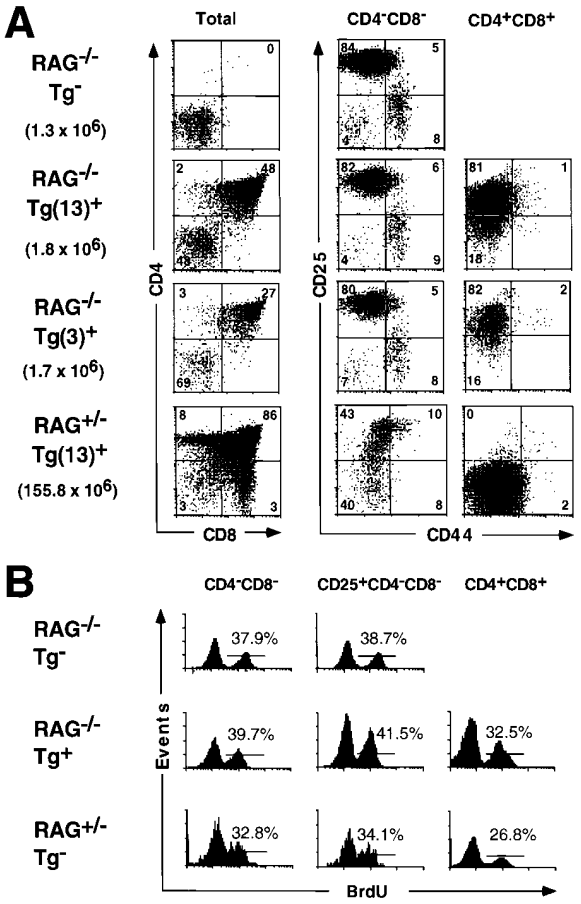

Figure 1. Generation of CD25^{int}CD4^{+}CD8^{+} Thymocytes by the TCRγ Transgene Expression in RAG$^{-/-}$ Mice

(A) Presence of DP thymocytes in TCRγ transgenic RAG-1$^{-/-}$ mice. Littermates (5 weeks old) for B6 transgenic line 2 (13 copies of the transgene) that had been backcrossed twice to B6 RAG-1$^{-/-}$ mice were stained with MAbs specific for CD4, CD8, CD25, and CD44 and analyzed by four-color flow cytometry. Representative profiles from the same analysis of another transgenic RAG$^{-/-}$ line (three copies of the transgene) are also shown. The numbers in the lower parentheses represent the total number of thymocytes from each mouse. The numbers in the quadrants represent the percentage of cells in the particular quadrant.

(B) Proportions of BrdU-labeled DP and DN thymocytes are similar among RAG$^{-/-}$, γ transgenic RAG$^{-/-}$, and normal littermate mice. BrdU was administered over a 24 hr period, and the cells were analyzed after staining with MAbs specific for CD4, CD8, CD25, and BrdU.

showed a lower proportion (range: 15%–31%) of DP thymocytes (Figure 1A). As expected, development of mature single positive T cells was blocked in the γ transgenic RAG$^{-/-}$ mice. Although TCRγ transgene expression induced significant differentiation of precursor cells to DP thymocytes in RAG-1$^{-/-}$ mice, the average total absolute thymocyte number in the transgenic RAG$^{-/-}$ mice (1.8×10^6, n = 15) was not greatly elevated from that of nontransgenic littermates (1.3×10^6, n = 10) and was much lower than the number of thymocytes in normal mice or TCRβ transgenic RAG$^{-/-}$ mice (approximately 10^8). Therefore, compared to the TCRβ chain, the transgenic TCRγ chain allows only limited development and/or proliferation of DP cells.

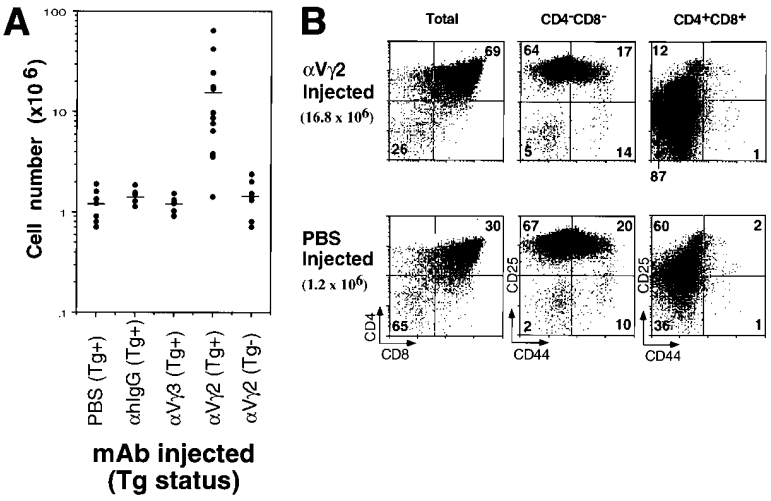

Figure 2. Increased Thymus Cellularity and DP Thymocyte Number in Mice Injected with Anti-Vγ2 Transgenic TCR MAb

(A) Total thymocyte numbers in trangenic RAG$^{-/-}$ mice injected with anti-transgenic Vγ2 TCR MAb (UC3) are compared to those injected with control MAbs (anti-Vγ3 TCR or anti-hamster IgG alone) or saline. Also shown are thymocyte cell numbers in nontransgenic RAG$^{-/-}$ littermates injected with anti-transgenic Vγ2 TCR MAb. Bars indicate average cell numbers in each group (PBS injected: 1.2×10^6; anti-hIgG injected: 1.5×10^6; anti-Vγ3 TCR MAb injected: 1.2×10^6; anti-Vγ2 TCR MAb injected: 15.9×10^6; anti-Vγ2 TCR MAb injected into nontransgenic RAG$^{-/-}$ mice: 1.4×10^6).

(B) Increased proportion of CD25$^-$ DP thymocytes in transgenic RAG$^{-/-}$ mice injected with anti-transgenic Vγ2 TCR MAb. Thymocytes isolated 5 days after the Ab or saline injection were stained with MAbs specific for CD4, CD8, CD25, and CD44. Representative profiles from four-color flow cytometric analysis are shown. The compensation settings required for the four-color analysis force many of the events to become crowded on the axes in some of the plots. The numbers in the parentheses refer to the total number of thymocytes from the mice analyzed.

Measurement of thymidine analog BrdU incorporation showed that the DP thymocytes generated by TCRγ transgene expression were labeled to a similar proportion (average ± SEM = 31.4 ± 1.5%, n = 5) as normal DP thymocytes (28.2 ± 0.6%, n = 4) during a continuous 24 hr BrdU administration (Figure 1B). The proportions of total DN cells and of the CD25$^+$ DN subsets that had incorporated BrdU were also virtually indistinguishable among the normal, transgenic RAG-1$^{-/-}$ and nontransgenic RAG-1$^{-/-}$ littermate animals. The similar labeling patterns suggest that the lower number of DP cells in γ transgenic RAG-1$^{-/-}$ mice as compared to wild-type mice may reflect a lower number of cell divisions and/or a reduced differentiation rate of these cells rather than a dramatically increased rate of cell death. Consistent with this possibility, sorted DP cells from the transgenic RAG-1$^{-/-}$ mice survived as well as normal DP cells when maintained in culture overnight (data not shown).

The DP thymocytes generated by the TCRγ transgene expression were HSA^{hi}, CD5^{lo}, fas^{null} (data not shown), in contrast to normal DP thymocytes, which are HSA^{hi}, CD5^{int}, fas^{hi}. Moreover, the DP thymocytes were predominantly CD25^{int}, clearly distinguishing them from normal CD25$^-$ DP thymocytes that arise through the signals delivered by the TCRβ/pTα complex (Figure 1A). It is possible that CD25 expression is retained on these cells from the CD25$^+$ TN stage of thymocyte differentiation, perhaps because the DP thymocytes from TCRγ transgenic RAG-1$^{-/-}$ mice undergo less cell division than normal DP thymocytes. Consistent with this possibility, there were few CD25$^-$44$^-$ TN intermediate stage thymocytes in the transgenic RAG-1$^{-/-}$ mice (Figure 1A). A similar reduction or absence of CD25$^-$44$^-$ TN thymocytes has been previously reported in DP thymocyte development from precursor cells in CD3ζ$^{-/-}$ (Crompton et al., 1994), TCRβ$^{-/-}$ (Mombaerts et al., 1992a; Godfrey et al., 1994), and pTα$^{-/-}$ (Fehling et al., 1995; Fehling et al., 1997) mice. All of these mouse strains also harbor CD25^{int} DP cells.

Transgenic TCRγ Chain Is Expressed on the Surface of RAG-1$^{-/-}$ Thymocytes

To examine how the transgenic TCRγ chain functions in RAG-1$^{-/-}$ mice, we first determined whether it was expressed on the cell surface. In flow cytometric analysis, we could not reliably detect the transgenic Vγ2 TCR on the cell surface, suggesting that the cell surface expression, if any, was very low (data not shown). Therefore, an in vivo functional assay was used to assess cell surface expression of the γ chain. Based on the earlier findings that MAbs specific for the CD3ε chain can induce the generation and expansion of DP thymocytes (Jacobs et al., 1994; Shinkai and Alt, 1994; data not shown), we determined whether MAb specific for the transgenic γ chain could induce the generation of DP cells. Injection of anti-Vγ2 MAb led to an 11-fold average increase in thymocyte number in RAG-1$^{-/-}$ γ transgenic mice 5 days after injection but had no effect in nontransgenic RAG-1$^{-/-}$ mice (Figure 2A). This increase was largely accounted for by the enlargement of DP population in the transgenic mice, in terms of increased proportion of the DP cells (55%–85% of total, Figure 2B) and an average 16-fold increase in the numbers of DP cells. Furthermore, the Ab injection resulted in increased expression of the CD5 and fas antigens on DP cells (data not shown) and down-regulation of CD25 (Figure 2B). These phenotypic alterations were not seen in mice injected with saline alone (Figures 2A and 2B) or with an isotype-matched control MAb directed against another Vγ TCR chain (Figure 2A; data not shown). These results strongly suggested that the TCRγ chain was expressed on the surface of RAG-1$^{-/-}$ thymocytes at functionally relevant levels, and that signals resulting from the cross-linking of this receptor mimic the TCRβ/pTα pre-TCR signals that permit DP cell development.

Evidence for an Alternative CD3–Associated Pre-TCR Complex Composed of TCRγ and pTα

To assess the role of the CD3 complex in mediating the effects of the transgene in RAG-1$^{-/-}$ mice, transgenic

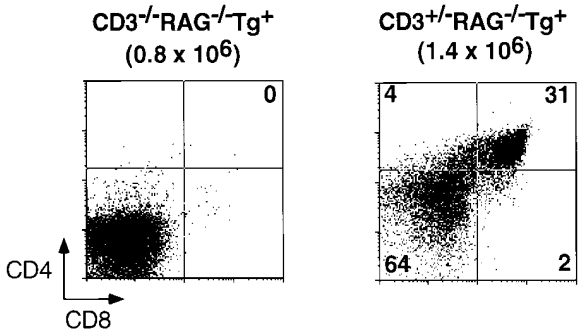

Figure 3. CD3ϵ Chain Is Necessary for TCRγ Chain–Mediated DP Thymocyte Generation

Thymocytes of TCRγ transgenic CD3ϵ$^{-/-}$RAG-1$^{-/-}$ and CD3ϵ$^{+/-}$RAG$^{-/-}$ littermate mice were stained with anti-CD4 and CD8 MAbs. Three other TCRγ transgenic CD3ϵ$^{-/-}$RAG-1$^{-/-}$ mice showed identical results.

RAG-1$^{-/-}$ mice were crossed to CD3ϵ$^{-/-}$ mice (Malissen et al., 1995) to generate TCRγ transgenic RAG-1$^{-/-}$ CD3$^{-/-}$ mice. Expression of the transgene in the double deficient mice did not stimulate development of DP thymocytes, nor did it alter the phenotype of DN thymocytes compared to those in CD3ϵ$^{-/-}$ mice (Figure 3; data not shown). Injection of MAb specific for the transgenic Vγ2 TCR chain into transgenic RAG-1$^{-/-}$ CD3$^{-/-}$ mice also had no effect (data not shown). Hence, TCRγ-mediated generation and expansion of DP thymocytes absolutely requires the CD3 signaling complex.

The observation that TCRγ signaling in RAG$^{-/-}$ mice depends on the CD3 complex suggested that γ chains may associate with a partner chain other than TCRδ to form a novel pre-TCR complex. To test the role of the pTα chain in DP thymocyte generation mediated by the TCRγ chain, TCRγ transgenic RAG$^{-/-}$ mice were crossed to pTα$^{-/-}$ mice (Fehling et al., 1995). Previous analysis has shown that RAG$^{-/-}$pTα$^{-/-}$ thymocytes are identical in phenotype to RAG$^{-/-}$ thymocytes, with all thymocytes being arrested in development at the CD25^{+} 44^{-} TN stage (Fehling et al., 1997).

No DP thymocytes developed in TCRγ transgenic pTα$^{-/-}$RAG$^{-/-}$ mice, demonstrating that DP cell development induced by the TCRγ transgene in RAG$^{-/-}$ mice was strictly dependent on expression of the pTα chain (Figure 4). The data unequivocably demonstrate the requirement of both pTα and CD3 signaling molecules for the TCRγ-induced development of DP thymocytes from precursor cells. Since the RAG$^{-/-}$ mice cannot express any other TCR chains, the results strongly suggest that TCRγ and pTα form a novel CD3−associated functional pre-TCR complex. Thus, pTα can potentially function with two distinct types of TCR chain, β and γ, that define two mutually exclusive T cell subsets. Unlike the pre-TCR containing the TCRβ chain, the pre-TCR containing the TCRγ chain induces DP cell development only inefficiently, unless the receptor is cross-linked with antireceptor Abs.

Endogenous TCRγ Chain Mediates DN to DP Transition Inefficiently

Although the transgenic TCRγ chain can form an alternate pre-TCR to allow precursor cells to differentiate into DP thymocytes in RAG$^{-/-}$ mice, the question remained whether the endogenous TCRγ chain can function in the same manner. To address this issue, we first analyzed the extent of DP thymocyte generation in TCRβ$^{-/-}$δ$^{-/-}$ mice, in which only TCRγ and α genes can potentially produce functional TCR chains. In adult, newborn (data not shown), and fetal (Figure 5A) double deficient mice, but not in RAG-1$^{-/-}$ mice, very small numbers of DP thymocytes were detected (range: 0.3% to 1.2% of total thymocytes, n=7). While previous studies of β$^{-/-}$δ$^{-/-}$ mice emphasized the absence of DP

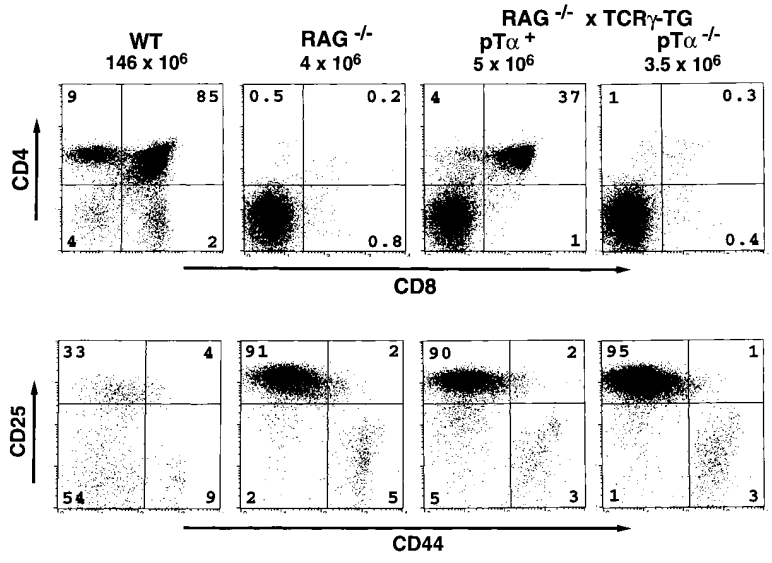

Figure 4. The Generation of DP Thymocytes in TCRγ Transgenic Mice Is Completely Dependent on pTα

(Upper) CD4/8 staining pattern of thymocytes from mice with the indicated genotype (WT, wild-type C57BL/6; RAG$^{-/-}$, RAG-1-deficient; RAG$^{-/-}$ × TCRγ-TG, RAG-1-deficient, G8 TCRγ transgenic expressing [pTα^{+}] or lacking [pTα$^{-/-}$] the pTα gene). Thymocytes were stained with Abs specific for CD4 and CD8. Before sample acquisition, propium iodide was added to efficiently exclude dead cells. The numbers on top of each profile refer to the total number of thymocytes from the particular mouse analyzed.

(Lower) CD25/44 staining pattern of TN thymocytes was examined by gating out thymocytes positive for CD3, CD4, CD8, CD19, GR-1, and MAC-1 (all FITC-conjugated). CD25-biotin/strepavidin-APC and CD44-PE staining pattern was then analyzed in FITC-negative/low cells. The data presented are representative of four independent experiments and confirm that the DP thymocyte generation in TCRγ transgenic RAG$^{-/-}$pTα^{+} mice occurs without significant down-modulation of CD25 at the TN stage.

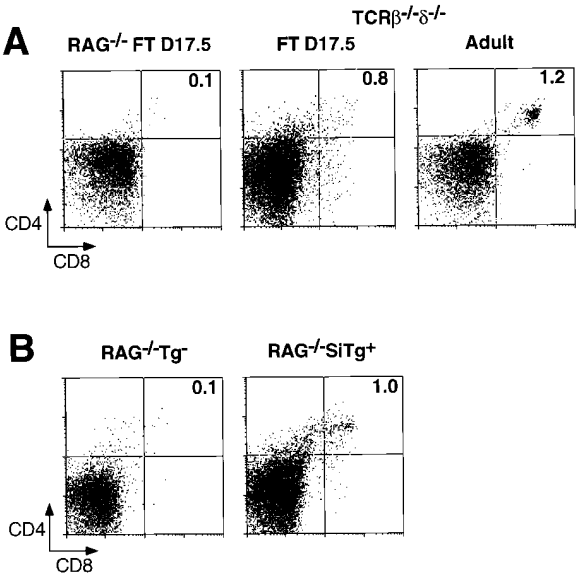

Figure 5. DP Thymocytes Are Generated Inefficiently in TCRβ$^{-/-}$δ$^{-/-}$ and in γSi Transgenic Mice

(A) Representative CD4/CD8 profiles of fetal and 5-week-old adult thymocytes of TCRβ$^{-/-}$δ$^{-/-}$ mice compared to RAG-1$^{-/-}$ mice that lack DP thymocytes.

(B) Expression of TCRγSi transgene in RAG$^{-/-}$ mice also leads to the development of very few DP thymocytes. Thymocytes from RAG-1$^{-/-}$ littermates from the mating of γSi transgenic RAG$^{+/-}$ and non-transgenic RAG$^{-/-}$ mice were stained with anti-CD4 and CD8 MAbs. Another independently derived γSi transgenic RAG$^{-/-}$ line showed identical results.

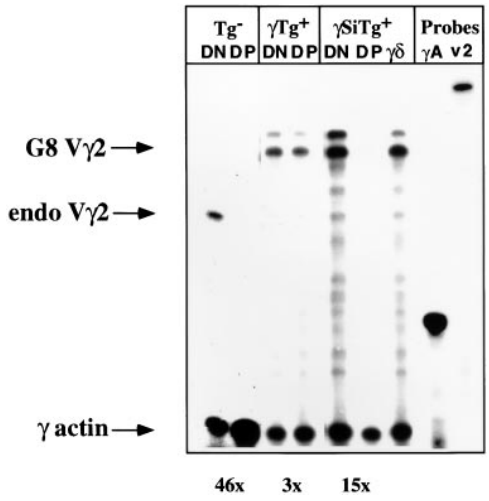

Figure 6. Expression of the TCRγSi Transgene Is Down-Modulated in αβ Lineage DP Thymocytes in Normal Mice

RNAse protection assay using G8 VDJ junction sequence-specific riboprobe shows that TCRγSi transgene expression (γSiTg^{+}), like endogenous γ gene expression, is significantly reduced in sorted DP thymocytes compared to that of sorted DN and TCRγδ^{+} thymocytes. The estimated extent of reduction in rearranged Vγ2 gene expression from DN to DP thymocytes as determined by the densitometric analysis is shown at the bottom. Levels of TCRγ gene expression in sorted DN and DP thymocytes from nontransgenic B6 mice (Tg^{-}) and TCRγ transgenic mice (γTg^{+}), where the γ transgene expression is maintained in DP thymocytes, are shown for comparison. Similar results were obtained with another TCRγSi founder line. Arrows indicate the identities of protected RNA fragments, including γ actin mRNA used here as a loading control; endo Vγ2 refers to protected endogenous rearranged Vγ2 gene with distinct VDJ junction sequences. Undigested probes (γA, γ actin; v2, G8 VDJ junction) are shown on the right.

thymocytes in these mice, small numbers of DP cells can also be seen in previously published data (Mombaerts et al., 1992a; Passoni et al., 1997). Like the DP thymocytes in TCRγ transgenic RAG-1$^{-/-}$ thymocytes, the DP thymocytes in TCRβ$^{-/-}$δ$^{-/-}$ mice were also CD25^{int} (data not shown). Hence, the endogenous TCRγ gene may be capable of inducing some DP cell generation, but it may do so much less efficiently than the TCRγ transgene.

One possible explanation for the poor functional activity of the endogenous γ gene versus the TCRγ transgene is that the endogenous TCRγ genes are transcriptionally repressed in DP cells and other resting αβ lineage cells (Garman et al., 1986; Ishida et al., 1990), whereas the transgene is constitutively expressed in these cells (Figure 6; data not shown). To assess the significance of γ gene repression in pre-TCR-mediated generation of DP cells, we employed another set of TCRγ transgenic mice in which the TCRγ transgene is regulated similarly to the endogenous TCRγ (Vγ2-Jγ1Cγ1) gene. The alternate form of the TCRγ transgene (γSi) contained an additional 3′ flanking genomic sequence (approximately 10 kb in size) downstream of the Cγ1 enhancer. Expression of the TCRγSi transgene was suppressed 12- to 15-fold in normal αβ lineage DP thymocytes when compared to the transgene-specific RNA level in DN thymocytes (Figure 6). In contrast, the transgene expression was not down-regulated in TCRγδ^{+} thymocytes. Expression of the transgene in normal mice increased the absolute numbers and proportions of γδ thymocytes to the same extent as did the ectopically expressed TCRγ transgene (Figure 7), suggesting that the TCRγSi transgene functions similarly to the original TCRγ transgene in promoting enhanced γδ cell development. The increased fraction of Vγ2^{+} cells among DN cells in the transgenic mice was reflected by a comparable increase in the level of Vγ2 transcripts in transgenic DN cells compared to normal DN cells. Therefore, it is likely that the bulk of the increased transcript levels in the transgenic mice resulted from a larger fraction of DN cells expressing rearranged Vγ2 genes, as opposed to a large increase in the level of γ transcripts in each DN cell. Consistent with this conclusion, the levels of cell surface γδ receptors in the transgenic mice were comparable to those in normal mice (Figure 7).

TCRγSi transgenic mice were crossed to RAG-1$^{-/-}$ mice to generate TCRγSi^{+}RAG-1$^{-/-}$ mice. Unlike the original TCRγ transgene, the TCRγSi transgene in RAG-1$^{-/-}$ mice promoted the development of only very small numbers of DP cells (0.4% to 1% of total thymocytes, n = 5), indistinguishable from the numbers observed in TCRβ$^{-/-}$δ$^{-/-}$ double deficient mice (Figure 5B). Similar to the DP thymocytes in TCRγ transgenic RAG$^{-/-}$ and in TCRβ$^{-/-}$δ$^{-/-}$ mice, the few DP thymocytes in TCRγSi transgenic mice were also CD25^{int} (data not shown). Identical results were obtained with an additional TCRγSi transgenic line, which also expressed the transgene at relatively high levels in DN thymocytes.

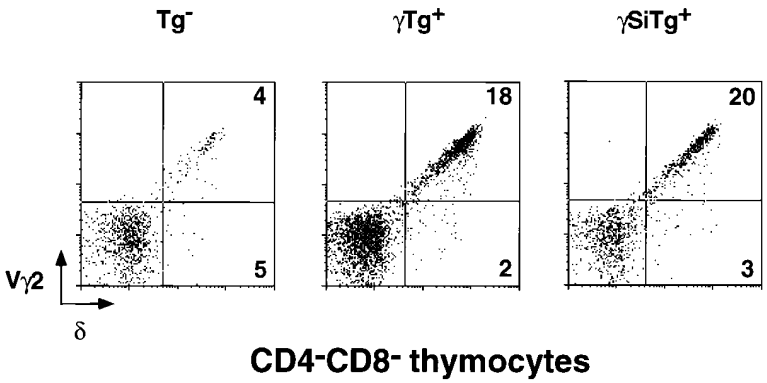

Figure 7. Both γ and γSi Transgene Expression in Normal Mice Lead to an Increased TCRγd+ Thymocyte Development

Gated DN thymocytes from TCRγ or TCRγSi transgenic mice were stained with MAbs specific for Vγ2 TCR and pan-TCRγδ. Both types of transgenic mice show a greater than 2-fold increase in the proportion of γδ thymocytes when compared to nontransgenic littermates. Most of γδ thymocytes from the transgenic mice express Vγ2 TCR.

The lower capacity of the TCRγSi transgene to induce DP cell development cannot be attributed to generally lower transgene expression in DN cells, because transgene RNA levels in DN cells of TCRγSi transgenic mice were similar to the levels in the lower copy TCRγ transgenic line in which much higher numbers of DP thymocytes were generated (Figures 1A and 6; data not shown). The data support the conclusion that the failure of the TCRγSi transgene to support significant DP cell development is due to the suppression of TCRγSi transgene expression that occurs in αβ lineage T cells.

One other possible explanation for the smaller number of DP thymocytes in TCRγSi RAG-1$^{-/-}$ transgenic mice is that the suppression of TCR expression in DP thymocytes results in the rapid disappearance of these cells. This possibility appears unlikely, however, since DP cells persisted for at least a month in other scenarios where DP cell development was induced in the absence of TCR expression, such as in RAG$^{-/-}$ mice injected with MAbs specific for CD3ε (Shinkai and Alt, 1994). Instead, the data suggest that the smaller number of DP cells in TCRγSi RAG-1$^{-/-}$ transgenic mice is most likely due to reduced differentiation of precursor cells to DP thymocytes.

Discussion

If asked why endogenous TCRγ chains cannot support development of DP thymocytes, most researchers in the field would probably suggest that it is because γ chains cannot pair with pTα chains. This possibility is refuted by our results, which provide persuasive evidence that TCRγ, pTα, and CD3 can interact to generate an alternative pre-TCR capable of stimulating the development of DP thymocytes. The observation that both the TCRγ and -β chains can function with pTα indicates an unexpected degree of promiscuity in the specificity of pTα for its partner chain. The alternative pre-TCR stimulates DP cell development substantially less well than the conventional pre-TCR. Significantly, in normal mice, the capacity of the alternative pre-TCR to stimulate DP cell development is limited by the transcriptional repression of γ genes that normally accompanies DP cell development. Therefore, although the γ chain can pair with pTα and induce differentiation towards the αβ lineage, differentiation is normally stalled at this point due to repression of γ expression. Hence, this transcriptional regulation further ensures preferential generation of sustained signals through the TCRβ/pTα instead of the TCRγ/pTα complex in developing precursor cells. Our results highlight the role of transcriptional repression of γ expression in reinforcing the lineage decision made by developing progenitor cells.

The coordinate requirement for TCRγ, pTα, and CD3ε for DP cell development in the absence of other TCR chains, coupled with the evidence that TCRγ is expressed on the cell surface in these mice, strongly suggests that the three proteins are physically associated. This conclusion is supported by the observation that anti-Vγ2 Abs, like anti-CD3ε Abs, could stimulate enhanced DP cell generation in the γ transgenic RAG-1$^{-/-}$ mice. Direct biochemical demonstration of the alternative pre-TCR is hampered by the extremely low levels of pre-TCR expression, which makes detection of even the conventional β/pTα pre-TCR in thymocytes exceedingly difficult (Groettrup et al., 1993).

The generation of DP thymocytes in γ transgenic RAG-1$^{-/-}$ mice is only approximately 1% as efficient as the generation of DP thymocytes in β transgenic RAG-1$^{-/-}$ mice. The DP cells that are generated also exhibited distinct phenotypic characteristics, typical of DP cells observed in other systems where DP cell generation is inefficient (Crompton et al., 1994; Fehling et al., 1995; Kang et al., 1998). The low efficiency of DP cell generation induced by the γ/pTα pre-TCR could be due to quantitative differences in γ/pTα function compared to the β/pTα pre-TCR. The fact that anti-Vγ2 MAb stimulation of the putative γ/pTα receptor resulted in enhanced numbers of DP cells, which exhibit a phenotype characteristic of the DP cells in normal mice, supports the notion that the γ/pTα receptor delivers weak signals that can be enhanced by receptor cross-linking. The quantitative difference could arise if there are few γ/pTα receptors per cell, as might occur if the γ chain associates poorly with pTα. Alternatively, each γ/pTα receptor could elicit weaker signals than each β/pTα receptor, due to inefficient signal transducing capacity of the receptor. The possibility that the difference is due to the absence of a ligand for the γ/pTα receptor should also be considered. However, this possibility may be less likely in light of evidence that β/pTα receptor function may not require a ligand, since neither the Vβ extracellular domain (Krimpenfort et al., 1989) nor the extracellular domain of pTα is absolutely required for efficient DP cell generation (Irving and Killeen, 1998).

Alternatively, signals transmitted by the TCRγ/pTα

pre-TCR might be qualitatively distinct from those elicited by the TCRβ/pTα complex. For example, the γ/pTα receptor may induce differentiation of DP cells, without providing a separate signal necessary for proliferation that is supplied by the conventional pre-TCR.

Considering that the extracellular domain of TCRβ is not required for efficient DP cell generation and that the β chain (like the γ chain) contains only a very small cytoplasmic domain, the possibility arises that the relevant difference(s) between the β and γ chains with respect to pre-TCR function lies in the transmembrane region. All four TCR chains, as well as pTα, contain a charged lysine residue in the transmembrane region that is thought to mediate associations with negatively charged residues in the transmembrane domains of CD3 subunits. Interestingly, the transmembrane domain of TCRβ chain contains a second charged residue, glutamic acid, that is lacking in the TCRCγ1 chain. This glutamic acid residue may form a charged pair with a corresponding second charged residue (arginine) of the pTα transmembrane domain, enhancing associations between β and pTα. Alternatively, the glutamic acid residue may mediate interactions with other signaling molecules.

While the transgenic γ chain drives the production of appreciable numbers of DP cells, endogenously encoded γ chains do so much less efficiently. This discrepancy may be attributed to the fact that only a fraction of progenitor cells in normal mice are expected to rearrange TCRγ genes productively, but this explanation cannot account for much of the difference. The results provide evidence that the repression of γ gene transcription that normally occurs as progenitor cells differentiate into DP cells is important in preventing the endogenous γ chains from promoting appreciable DP cell generation. We compared the original transgene with a longer (γSi) transgene that is identical except that it contains additional downstream regulatory sequences. Both transgenes were expressed at similar levels in DN cells, but unlike the original transgene, expression of γSi was repressed in DP cells. The endogenous γ genes are also transcriptionally repressed in DP cells as well as in other αβ lineage cells (Garman et al., 1986; Ishida et al., 1990; Wilson et al., 1996). In γSi transgenic RAG$^{-/-}$ mice only very small numbers of DP cells were generated, similar to the situation in TCRβ$^{-/-}$δ$^{-/-}$ mice. In both cases, the strongly reduced numbers of DP thymocytes probably occurs because the down-regulation of the TCRγ/pTα pre-TCR complex prevents sustained generation of signals necessary to allow significant differentiation of precursor cells into DP thymocytes. The transcriptional repression in DP cells of the γSi transgenic construct will be described in detail elsewhere.

At what point during T cell development does γ gene repression normally occur, and how do our results fit into models of the γδ/αβ T cell fate decision? In a previously proposed stochastic model of the γδ/αβ lineage decision, DN cells undergo a stochastic commitment to γδ or αβ lineage, but both types of committed cells remain capable of rearranging β, γ, and δ genes (Kang and Raulet, 1997; Livak et al., 1997; Kang et al., 1998). In one version of this model, pTα expression is restricted to αβ committed DN cells (Fehling et al., 1997; Mertsching et al., 1997). As discussed in detail elsewhere, the observed selection against productive γ and δ genes in αβ lineage cells (Dudley et al., 1995; Kang et al., 1995; Livak et al., 1995) persuasively demonstrates that both γ and δ genes can be expressed in putative αβ-restricted DN cells. Hence, it cannot be argued that γ genes are normally constitutively transcriptionally repressed in these cells (Ishida et al., 1990), thereby preventing DP cell generation via the γ/pTα pre-TCR. However, it is plausible that γ/pTα function in putative αβ-restricted DN cells is blunted by γ gene repression that occurs later, perhaps during the DN to DP transition. Thus, there may be a requirement for sustained γ/pTα signaling to mediate this differentiation event. We have made a similar proposal (Kang et al., 1998) in accounting for the capacity of the γδTCR to stimulate the generation of DP cells (Iwashima et al., 1991; Kersh et al., 1995; Livak et al., 1997). The γδTCR ranks between the β/pTα pre-TCR and the γ/pTα pre-TCR in its capacity to stimulate this transition. The superior activity of the γδ receptor compared to the γ/pTα receptor in inducing DP cell development could be attributed to its greater abundance, a longer half-life, or to its potential capacity to deliver stronger signals.

Alternatively, in the instructive model of γδ/αβ T cell lineage determination (Allison and Lanier, 1987; Dudley et al., 1995; Livak et al., 1995; Kang and Raulet, 1997; Kang et al., 1998), an uncommitted pTα-expressing DN cell is capable of rearranging β, γ, and δ genes, and the developmental outcome is dictated by differential receptor signaling. In this model, β/pTα receptors efficiently induce DP cell generation and proliferation, and γδ TCRs mainly promote differentiation of γδ cells. The TCRγ chain can associate with the pTα chain, but signals from the pre-TCR would transcriptionally silence the γ gene expression, effectively limiting the generation of DP thymocytes using this pathway. Thus, modified versions of both the instructive and stochastic models of lineage commitment can account for the activity of TCRγ chain in concert with the pTα/CD3 complex.

It is now evident that four TCR complex isoforms can induce DP cell development with varying efficiencies: the β/pTα pre-TCR (Groettrup et al., 1993; Fehling et al., 1995), the αβ TCR (Buer et al., 1997), the γδ TCR (Livak et al., 1997; Passoni et al., 1997; Kang et al., 1998), and the γ/pTα pre-TCR. In addition to the inherently lower capacities of the γδ and γ/pTα receptors to stimulate DP cell generation, transcriptional down-regulation of the γ and probably the δ genes (Wilson et al., 1996) limits DP cell generation via signals emanating from these receptors. The DP cells that do arise as a result of the expression of γ/pTα and γδ receptors presumably do not differentiate further, since these receptors may not be capable of undergoing normal positive selection events, in large part as a result of transcriptional silencing of the receptor genes. While a detailed picture is emerging of the activities of different receptor isoforms in T cell progenitors, an understanding is lacking of the basic developmental mechanisms and precisely how expression of the different receptor isoforms impact these processes. Resolution of these issues will likely require methods to follow all of the descendants of single early progenitor cells or the delineation of distinctive phenotypes for putative lineage committed early progenitor cells.

Experimental Procedures

Mice

Generation of G8 TCRγ transgenic mice (Kang et al., 1998) and pTα$^{-/-}$ mice (Fehling et al., 1995) have been described previously. G8 TCRγSi transgene contains an additional 10 kb fragment (from a BALB/c genomic clone kindly provided by Dr. L. Hood, Seattle) downstream of the Cγ1 enhancer. B6 TCRγ or TCRγSi transgenic mice were backcrossed to RAG-1$^{-/-}$ mice (kindly provided by Dr. H. Sakano, University of California, Berkeley) to generate γ transgenic RAG$^{-/-}$ mice that were then backcrossed to CD3ε$^{-/-}$ mice (Malissen et al., 1995) (kindly provided by Dr. R. Perlmutter, University of Washington, Seattle) or pTα$^{-/-}$RAG$^{-/-}$ mice to generate γ transgenic RAG$^{-/-}$CD3ε$^{-/-}$ or γ transgenic RAG$^{-/-}$pTα$^{-/-}$ mice. Mice were typed by PCR and Southern blots using published primers and probes and by flow cytometric analysis of peripheral blood lymphocytes. TCRβ$^{-/-}$δ$^{-/-}$ mice (Mombaerts et al., 1992a) were purchased from the Jackson Laboratories (Bar Harbor, ME). In some experiments, C57BL/6 (B6, from IFFA-Credo, France) mice were used as wild-type controls. For fetal thymocyte isolation, the day of the plug was designated as day 0.5. Mice were bred and maintained in specific pathogen-free facilities at the University of California, Berkeley, and at the Basel Institute for Immunology (Switzerland).

Antibodies, In Vivo Antibody Treatment, and Flow Cytometry

Anti-Vγ2 TCR (UC3-10A6), anti-Vγ3 TCR (F536), anti-δTCR (GL3), anti-CD5 (53-7.3), anti-CD95 (Jo2), and anti-HSA (J11d) 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), and anti-CD25−PE (PC61.5.3) MAbs were purchased from CALTAG (San Francisco, CA); anti-CD4−FITC (H129.19), anti-CD8α-FITC, anti-CD11b-FITC (MAC1α), anti-CD19-FITC (1D3), anti-CD25−biotin (7D4), anti-CD44−PE, and anti-CD3ε-FITC (2C11) MAbs were from Pharmingen (San Diego, CA); anti-CD4−Red613 or anti-CD4-PE were from GIBCO (Grand Island, NE); and strepavidin-PE or strepavidin-APC were from Molecular Probes (Eugene, OR). Purified, red blood cell–lysed thymocytes were stained with the relevant Abs at saturating concentrations, and side- and forward-gated 1–2 × 10^5 labeled cells were analyzed by four-color cytometry using an EPICS XL-MCL flow cytometer (Coulter) or a FACSCalibur (Becton Dickinson, Mountain View, CA.) and the CellQuest program (Becton Dickinson). Some analysis was performed with an additional step of gating out propidium iodide (PI)–positive cells to exclude dead thymocytes. Cells were sorted using an ELITE cell sorter (Coulter). Flow cytometric profiles were analyzed using the WinMDI program (John Trotter, Salk Institute, San Diego, CA).

For in vivo assay of transgenic TCR function, recipient mice were injected i.p. with 50–250 μg of purified hamster anti-Vγ2 TCR MAb followed by 150 μg of purified anti-hamster IgG (Pierce, Rockford, IL) administration 6–8 hr post primary Ab injection to cross-link the receptors. Alternatively, 50–100 μg of anti-Vγ2 Ab ascite was injected i.p. into RAG$^{-/-}$ recipient mice. Ab-treated mice were analyzed 4–7 days post injection.

RNAse Protection Assay

Riboprobe specific for the γ transgene expression was generated using T7 RNA polymerase from a linearized pKS Bluescript vector (Stratagene) construct containing the KpnI–BsrI fragment (273 bp) of G8 transgene that spans the VDJ junction. Control riboprobe specific for γ-actin mRNA was generated using SP6 RNA polymerase. RNase protection assay was carried out essentially as described (Maniatis et al., 1982) using 5 μg of total RNA purified from sorted thymocyte populations. RNA was isolated using Ultraspec RNA (Biotecx, Houston, TX) according to the manufacturer's protocol. Each sample contained both probes to normalize quantitative variations in input RNA. Densitometric analysis was performed using a Phosphorimager (Molecular Dynamics).

BrdU Labeling

BrdU incorporation and analysis by flow cytometry was performed as previously described (Tough and Sprent, 1994; Penit et al., 1995). In brief, BrdU (0.9 mg) was injected i.p. into the mice at 8 hr intervals. The thymocytes were isolated at 24 hr posttreatment and stained with anti-CD25−PE, anti-CD4-613, and anti-CD8-670 MAbs, followed by fixation, permeablization, and staining with BrdU-FITC (Becton Dickinson) MAb. Side- and forward-gated 10^5 labeled cells were analyzed by four-color cytometry using EPICS V (Coulter).

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