The Common Cytokine Receptor γ Chain and the Pre-T Cell Receptor Provide Independent but Critically Overlapping Signals in Early α/β T Cell Development

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Summary

Intracellular signals emanating from cytokine and antigen receptors are integrated during the process of intrathymic development. Still, the relative contributions of cytokine receptor signaling to pre-T cell receptor (TCR) and TCR-mediated differentiation remain undefined. Interleukin (IL)-7 interactions with its cognate receptor complex (IL-7Rα coupled to the common cytokine receptor γ chain, γ_c) play a dominant role in early thymopoiesis. However, α/β T cell development in IL-7–, IL-7Rα–, and γ_c-deficient mice is only partially compromised, suggesting that additional pathways can rescue α/β T lineage cells in these mice. We have investigated the potential interdependence of γ_c- and pre-TCR–dependent pathways during intrathymic α/β T cell differentiation. We demonstrate that γ_c-dependent cytokines do not appear to be required for normal pre-TCR function, and that the rate-limiting step in α/β T cell development in γ_c^- mice does not involve TCR-β chain rearrangements, but rather results from poor maintenance of early thymocytes. Moreover, mice double mutant for both γ_c and pre-Tα show vastly reduced thymic cellularity and a complete arrest of thymocyte differentiation at the $CD44^+CD25^+$ cell stage. These observations demonstrate that the pre-TCR provides the γ_c-independent signal which allows α/β T cell development in γ_c^- mice. Thus, a series of overlapping signals derived from cytokine and T cell receptors guide the process of α/β thymocyte development.

Key words: thymus • interleukin • lymphocyte • development • knockout

The common cytokine receptor γ chain (γ_c)[1] forms a critical functional component of the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15 (for a review, see reference 1). Naturally occurring mutations in γ_c are responsible for X-linked severe combined immunodeficiency disease (SCIDX1) in humans, characterized by a complete absence of T and NK cells, while B cells are present (for reviews, see references 2 and 3). Targeted deletion of γ_c in mice also provokes a wide variety of defects in lymphoid development, including a complete absence of NK cells, γ/δ T cells, and gut-associated lymphoid tissue (4–6). Like SCIDX1 patients, γ_c^- mice have some mature peripheral B cells, but these mice also show a remarkable degree of α/β T cell development (4–8). These data demonstrate the important role of γ_c-dependent signals in lymphopoiesis, but also suggest that fundamental differences exist between the mechanisms that permit α/β T cell development in humans and mice.

Analysis of single cytokine- or cytokine receptor–deficient mice has identified which γ_c-dependent signals are responsible for some of the observed developmental defects seen in γ_c^- mice. NK cell differentiation is critically linked to the expression of the IL-2Rβ chain (9), thereby implicating IL-2– and/or IL-15–mediated signaling pathways in the development of these cells. Since IL-2 mutant mice develop NK cells (10), this suggests that IL-15 (or another IL-2Rβ–binding ligand) is required for the differentiation of this subset (for a review, see reference 11). In contrast, the defect in γ/δ T cell development in γ_c^- mice appears strictly IL-7 dependent (12). Moreover, since IL-7 was ini-

[1] *Abbreviations used in this paper:* BrdU, bromo-deoxyuridine; DN, double negative; DP, double positive; γ_c, common cytokine receptor γ chain; RAG, recombination activating gene; SP, single positive; TN, triple negative; Tg, transgene; TRI, Tricolor.

tially identified as an important growth factor for T and B cell precursors (13, 14), this would explain the severely reduced thymic cellularity and defects in bone marrow B cell and intrathymic precursors found in IL-7 and IL-7Rα mutant mice (15–17).

The biological consequences of γ_c-dependent receptor engagement for α/β T cell development include signals which can potentially promote cell survival, proliferation, and/or differentiation. Experimentally, however, it has been difficult to conclusively define which of these processes are adversely affected in the absence of γ_c. In theory, the absence of IL-7 signaling in γ_c^- mice could potentially limit thymocyte development by affecting the survival and/or expansion of intrathymic precursors, or by reducing the efficiency of the recombination process. Evidence for the latter has been suggested by reports that the expression of functionally rearranged TCR-α/β transgenes in γ_c- or IL-7Rα–deficient mice augmented total thymocyte numbers (18, 19). However, enforced expression of the antiapoptotic factor Bcl-2 could also rescue α/β T cell development in these mice (20–22), supporting a major role for IL-7/γ_c signaling in promoting a survival program. In all of these cases, thymic reconstitution was not complete, suggesting either that IL-7/γ_c influences both survival and recombination, or that intrathymic development involves additional mechanisms which are critically dependent on this receptor complex, such as the efficiency of pre-TCR assembly or function.

In this report, we have analyzed the potential interplay between γ_c-dependent cytokine pathways and signaling through the pre-TCR for α/β T cell development.

Materials and Methods

Animals and Cell Preparation. γ_c^- mice (5), TCR-$\alpha^{-/-}$ mice (23), pT$\alpha^{-/-}$ mice (24), recombination activating gene (RAG)-$2^{-/-}$ mice (25), and their control littermates were maintained in a specific pathogen–free animal facility (CNRS/CDTA, Orleans, France). γ_c^-/pT$\alpha^{-/-}$ mice were generated by crossing $\gamma_c^{+/-}$ female mice to pT$\alpha^{-/-}$ male mice, and female offspring carrying the γ_c mutation were identified by PCR (26) and backcrossed to pT$\alpha^{-/-}$ males. γ_c^-/pT$\alpha^{-/-}$ male mice were then identified by PCR. γ_c^-/TCR-$\alpha^{-/-}$ mice were generated in a similar fashion using TCR-$\alpha^{-/-}$ male mice. All mice were on a mixed (129Ola × C57Bl/6) background and were used between 3 and 6 wk of age. Cells isolated from thymus and spleen were prepared as described previously (5).

Antibodies and Immunofluorescence Analysis. The following mAbs were used as conjugates to fluorescein (FITC), phycoerythrin (PE), Tricolor (TRI), or biotin: CD3 (145-2C11), CD4 (GK1.5), CD8β (35-5.8), TCR-α/β (H57-597), TCR-γ/δ (GL3), HSA (J11d), CD44 (Pgp-1), CD25 (PC-61), IL-7Rα (A7R34), c-kit (2B8), NK-specific (DX5), and B220 (RA3-6B2). Biotinylated mAbs were revealed with either streptavidin-TRI or streptavidin-allophycocyanin (APC; Caltag). Cell suspensions were lysed of erythrocytes and depleted of B cells using sheep anti–mouse Ig–coated magnetic beads (Dynal). Cells were stained in microtiter plates (2×10^6 cells/well in 50 μl), using combinations of directly conjugated mAbs. Simultaneous four-color cell sorting and analysis were performed on a FACSVantage® flow cytometer (Becton Dickinson). Dead cells were excluded by gating based on forward and side scatter characteristics. Sorted populations were routinely 97% pure upon reanalysis.

Cell Cycle Analysis. In vivo labeling of S phase cells with bromo-deoxyuridine (BrdU; Sigma) was performed by a single intraperitoneal BrdU injection at a dose of 50 mg/kg body wt 15 min before killing. Thymocyte subsets were sorted using a FACSVantage®, and cells incorporating BrdU were identified as described previously (27) using an FITC-coupled anti-BrdU mAb (Becton Dickinson) and a FACScan® flow cytometer.

Intracellular Staining for Bcl-2, pTα, and TCR-β Chains. Bcl-2 staining was performed as described previously (28). For intracellular staining of pTα or TCR-β chains, surface antigens were stained as above and cells were fixed with 0.1% paraformaldehyde. Cells were subsequently permeabilized in 0.1% saponin before incubation with biotinylated anti–TCR-β (29) or anti-pTα (30) mAbs and finally with streptavidin-APC before analysis on a FACSCalibur® flow cytometer.

TCR-β Rearrangements. TCR-β V(D)J rearrangements were studied as described (31). In brief, genomic DNA was amplified using a combination of two 5′ primers specific for Vβ6 and Vβ8 together with a 3′ primer hybridizing to the 3′ region of Jβ2.5. PCR products were fractionated on 2% agarose gels, transferred to nylon membranes, and probed by Southern hybridization using a ^{33}P-labeled oligonucleotide specific for the 5′ region of Jβ2.5. Five distinct PCR products ranging in size from 850 to 150 bp are expected depending on the rearrangements of the Vβ segment with either Jβ2.1, Jβ2.2, Jβ2.3, Jβ2.4, or Jβ2.5.

Results

Abnormal Development of Early Thymocyte Progenitors in γ_c^- Mice. We analyzed intrathymic development in γ_c^- mice to better define the nature of any developmental blocks resulting from the absence of γ_c. When comparing absolute numbers of $CD4^-CD8^-$ double negative (DN) cells, $CD4^+CD8^+$ double positive (DP) cells, and $CD4^+CD8^-$ and $CD4^-CD8^+$ single positive (SP) mature T cells, the DN and CD8 SP populations were more affected by the absence of γ_c (Fig. 1 A). While both DP and CD4 SP cells were reduced by 15–20-fold (similar to the overall decrease in thymic cellularity), the DN and CD8 SP subsets were reduced almost 40-fold. The DN compartment contains both early thymocyte precursors ($CD3^-$) as well as mature $CD3^+$ TCR-γ/δ and TCR-α/β cells. While γ/δ T cells do not develop in γ_c^- mice (4, 28), DN α/β T cells are present (32). To specifically evaluate γ_c^- TCR^- thymocyte progenitors, we studied CD44 and CD25 expression on $CD3^-CD4^-CD8^-$ (TN) thymocytes by four-color immunofluorescence analysis. Previous studies have demonstrated that these cells differentiate through the following stages: $CD44^+CD25^- \rightarrow CD44^+CD25^+ \rightarrow CD44^-CD25^+ \rightarrow CD44^-CD25^-$ (33; for a review, see reference 34).

Compared with controls, immature thymocytes from γ_c-deficient mice showed altered patterns of CD44 and CD25 expression (Fig. 1 B). The most immature $CD44^+CD25^-$ TN cells, which can also give rise to B cells, NK cells, and thymic dendritic cells (35; for a review, see reference 36), were found in normal frequency, but were clearly reduced in absolute numbers. More striking was the relative accu-

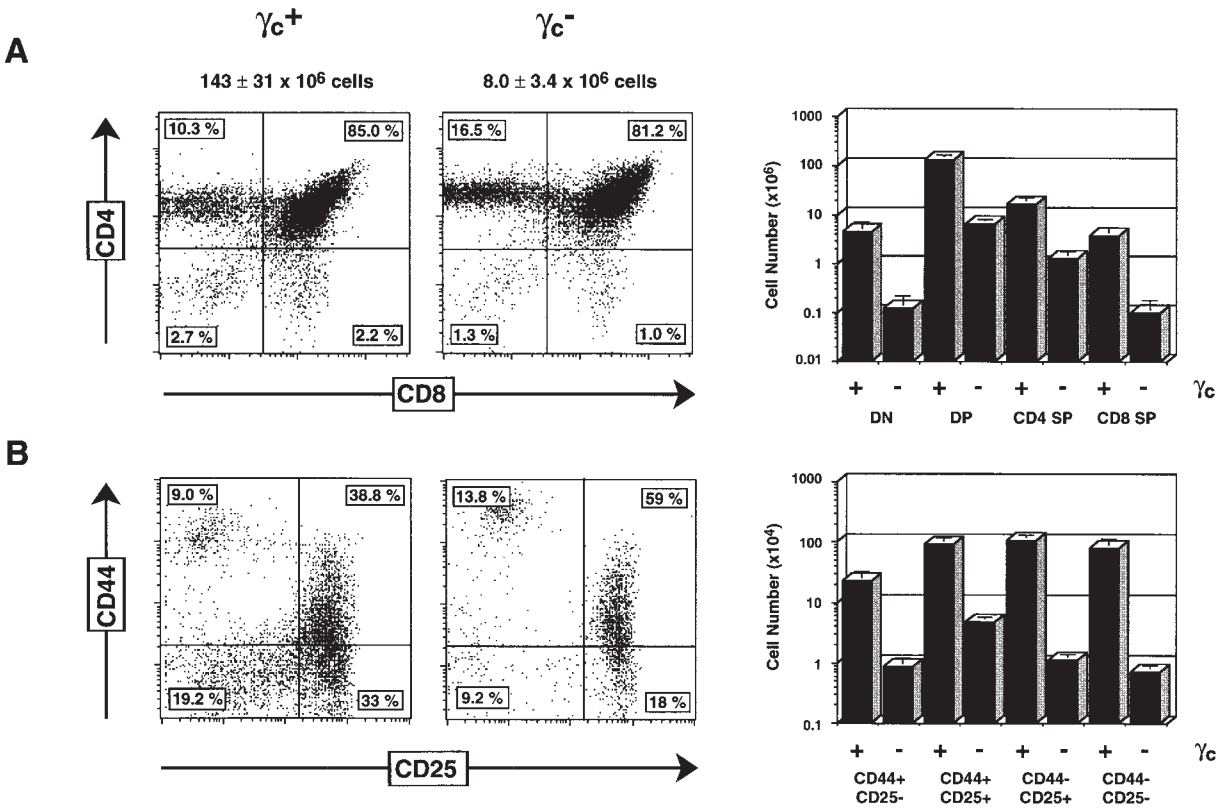

Figure 1. Early thymocyte development in control and γ_c^- mice. (A) Thymocytes from 3–4-wk-old γ_c-deficient mice and their littermate controls were stained using CD4-PE and CD8β-FITC, and absolute numbers of DN, DP, CD4 SP, and CD8 SP cells were calculated (mean ± SD from 10 mice of each genotype). (B) Thymocytes were stained with a combination of FITC-conjugated antibodies (CD3, CD4, CD8β, TCR-α/β, TCR-γ/δ, B220, DX5, and Gr-1), CD44-PE, and CD25-biotin followed by streptavidin-TRI. CD44 versus CD25 expression is shown on electronically gated $FITC^-$ TN thymocytes. Absolute numbers were calculated (mean ± SD) from six mice of each genotype.

mulation of cells at the $CD44^+CD25^+$ stage, which were almost twofold increased in frequency compared with controls (Fig. 1 B). Moreover, absence of γ_c was associated with a clear defect in the development of cells beyond the $CD44^+CD25^+$ stage. Percentages of $CD44^-CD25^+$ and $CD44^-CD25^-$ cells were reduced by a factor of 2 and were markedly reduced (>100-fold) in absolute numbers (Fig. 1 B).

Altered Cell Proliferation in γ_c^- Thymocyte Precursors. The block in thymocyte maturation seen in γ_c^- thymi could result from abnormal differentiation, reduced cell survival, and/or proliferative defects. The ability of γ_c^- thymocyte precursors to incorporate the analogue BrdU was used as a measure of intrathymic proliferation. The number of cells in S phase of the cell cycle was analyzed after a single injection of BrdU (Fig. 2 A). Only a fraction of the immature thymocytes are labeled under these conditions, and it is clear that T cell precursors in γ_c^- mice show defects in proliferation, as both $CD44^+CD25^-$ and $CD44^+CD25^+$ cells have markedly reduced BrdU incorporation relative to their γ_c^+ counterparts (the $CD44^-CD25^+$ and $CD44^-CD25^-$ subsets were not analyzed in γ_c^- thymi due to their extremely small absolute numbers; these subsets were normally labeled in γ_c^+ controls [data not shown]). DP cells from γ_c^- mice were similarly affected.

Decreased BrdU incorporation in γ_c^- thymocyte precursors could result from decreased survival of these cells. γ_c-dependent cytokines have been shown to promote lymphocyte survival by maintaining levels of antiapoptotic factors such as Bcl-2 and Bcl-X_L (37, 38). Therefore, we examined intracellular levels of Bcl-2 in immature thymocytes from γ_c^- mice and their control γ_c^+ littermates. γ_c^- precursors had markedly reduced levels of Bcl-2 compared with their γ_c^+ counterparts (Fig. 2 B). Moreover, γ_c^- cells showed elevated cell surface staining with Annexin V (Fig. 2 C), indicating commitment to the apoptotic process (39). Taken together, these results are consistent with a critical role for γ_c cytokines in the survival and expansion of thymocyte precursors, the absence of which could account in part for their reduction in absolute cell numbers.

Pre-TCR Signaling in the Absence of γ_c. Having demonstrated a major role for γ_c in the most immature T cell precursors, we next addressed the impact of the γ_c mutation on pre-TCR signaling. The activity of the pre-TCR is presumed to begin at the $CD44^-CD25^+$ cell stage when functional TCR-β chain rearrangements are achieved (24, 40). The role of γ_c-dependent cytokines in pre-TCR function has not been previously examined, although it has been suggested that cytokines (like IL-7) may play a role in the expansion of pre-T cells as they differentiate towards

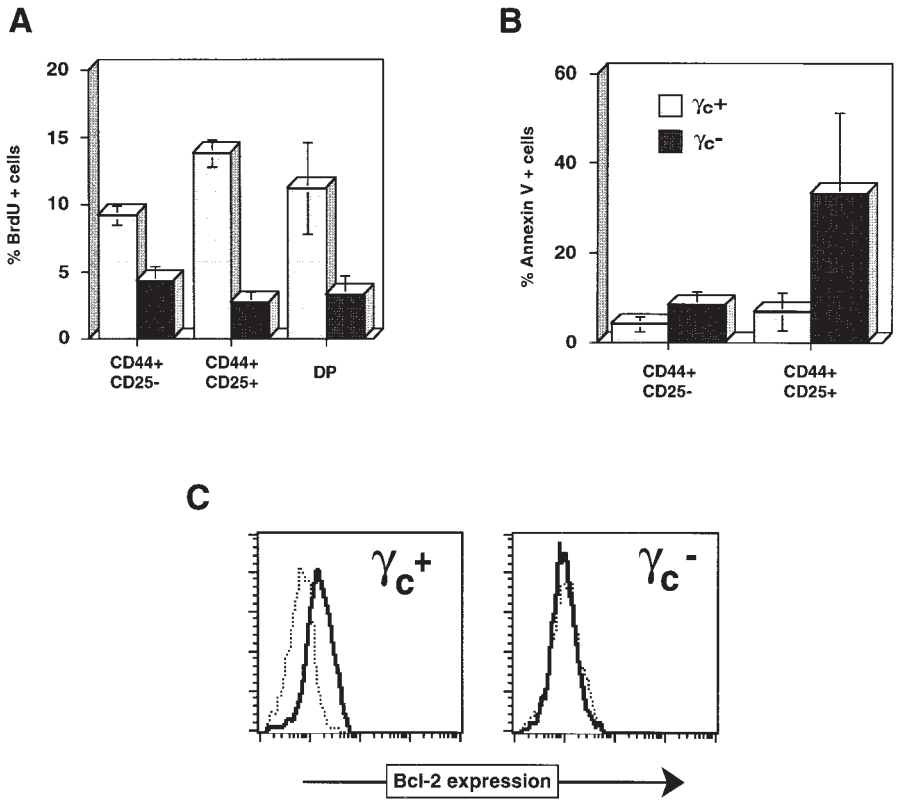

Figure 2. Altered survival and proliferation of early thymocyte precursors in the absence of γ_c. (A) Abnormal BrdU incorporation in γ_c^- thymocytes. Mice received a single pulse of BrdU before killing. Indicated thymocyte subsets were sorted, and percentages of cells incorporating BrdU were analyzed. (B) Annexin V staining of γ_c^+ or γ_c^- thymocyte subsets. Cells were stained for TN cells (see Fig. 1), CD25, and Annexin V. Propidium iodide–negative $CD25^-$ and $CD25^+$ TN cells were electronically gated, and percentages of Annexin V–positive cells were calculated (mean ± SD) from four mice of each genotype. (C) Intracellular Bcl-2 staining of DN thymocyte precursors was performed; γ_c^- thymocytes demonstrated severely reduced Bcl-2 levels (thick lines). Thin lines, isotype control staining.

the DP stage. This hypothesis gains support from the fact that $CD25^+$ intrathymic precursors express IL-7Rα (41). Moreover, the defect in intrathymic maturation in γ_c^- mice and the marked depletion in $CD44^-CD25^+$ and $CD44^-CD25^-$ cells could be the consequence of abnormal pre-TCR assembly or function.

Although the pre-TCR has its main role in the transit of early α/β T cell precursors to the DP stage (for a review, see reference 42), additional mechanisms independent of the pre-TCR have been described which can permit the generation of DP cells in vivo (43, 44). These include effects mediated by γ/δ TCRs and via α/β TCRs due to early rearrangements at the TCR-α locus. To focus on the pre-TCR–mediated pathway and to exclude these alternative pathways of DP cell generation, we crossed γ_c^- mice (which lack γ/δ T cells) with TCR-$\alpha^{-/-}$ mice (23; to eliminate TCR-α expression). Thymocyte differentiation was examined in these double mutants. Introduction of the TCR-α mutation did not further alter the pattern of differentiation of thymocytes to the DP stage compared with γ_c^- mice or further diminish their total thymic cellularity (Fig. 3 A). These results demonstrate that early TCR-α rearrangements do not play a major role in the differentiation of DP cells in γ_c^- mice.

Pre-TCR signaling results in β selection, i.e., the preferential expansion of early thymocytes expressing a single, functionally rearranged TCR-β chain (for a review, see reference 45). β selection was assessed in control, γ_c^-, and pT$\alpha^{-/-}$ thymocytes by intracellular staining of TCR-β expression (Fig. 3 B). As reported previously (44), pT$\alpha^{-/-}$ mice show clear defects in β selection as demonstrated by decreased intracellular levels of TCR-β chains in DP thymocytes compared with control mice. In contrast, γ_c^- DP thymocytes demonstrated intracellular TCR-β levels comparable to controls (Fig. 3 B, and see below).

We also considered that pre-TCR function might be affected due to reduced synthesis of TCR-β chains, thereby limiting the assembly of a pre-TCR complex. Since TCR-β V(D)J rearrangements are detected at the $CD44^-CD25^+$ cell stages (40, 46), and previous studies have suggested that IL-7/CD127/γ_c interactions may be important for RAG expression during the TCR recombination process (for a review, see reference 47), we tested whether defects in TCR-β rearrangements were responsible for the developmental block in γ_c^- thymi. γ_c^- mice were bred with mice bearing a functionally rearranged TCR Vβ8.2 transgene (Tg) (48), and thymocyte development was analyzed. As shown in Fig. 3 C, γ_c^-/TCR-β Tg^+ mice demonstrated no change in the distribution or absolute numbers of thymocytes compared with non-Tg γ_c^- littermates. The lack of discernible effect of the TCR-β Tg in γ_c^- mice was not due to an inability to express this TCR, as surface levels of Vβ8.2 were equivalent in γ_c^+ and γ_c^- total thymocytes and $CD25^+$ thymocyte precursors (data not shown). These results suggest that defects in the TCR-β chain rearrangement process are not rate limiting in the absence of γ_c, and are consistent with a role for γ_c in promoting survival and proliferation of early thymocytes.

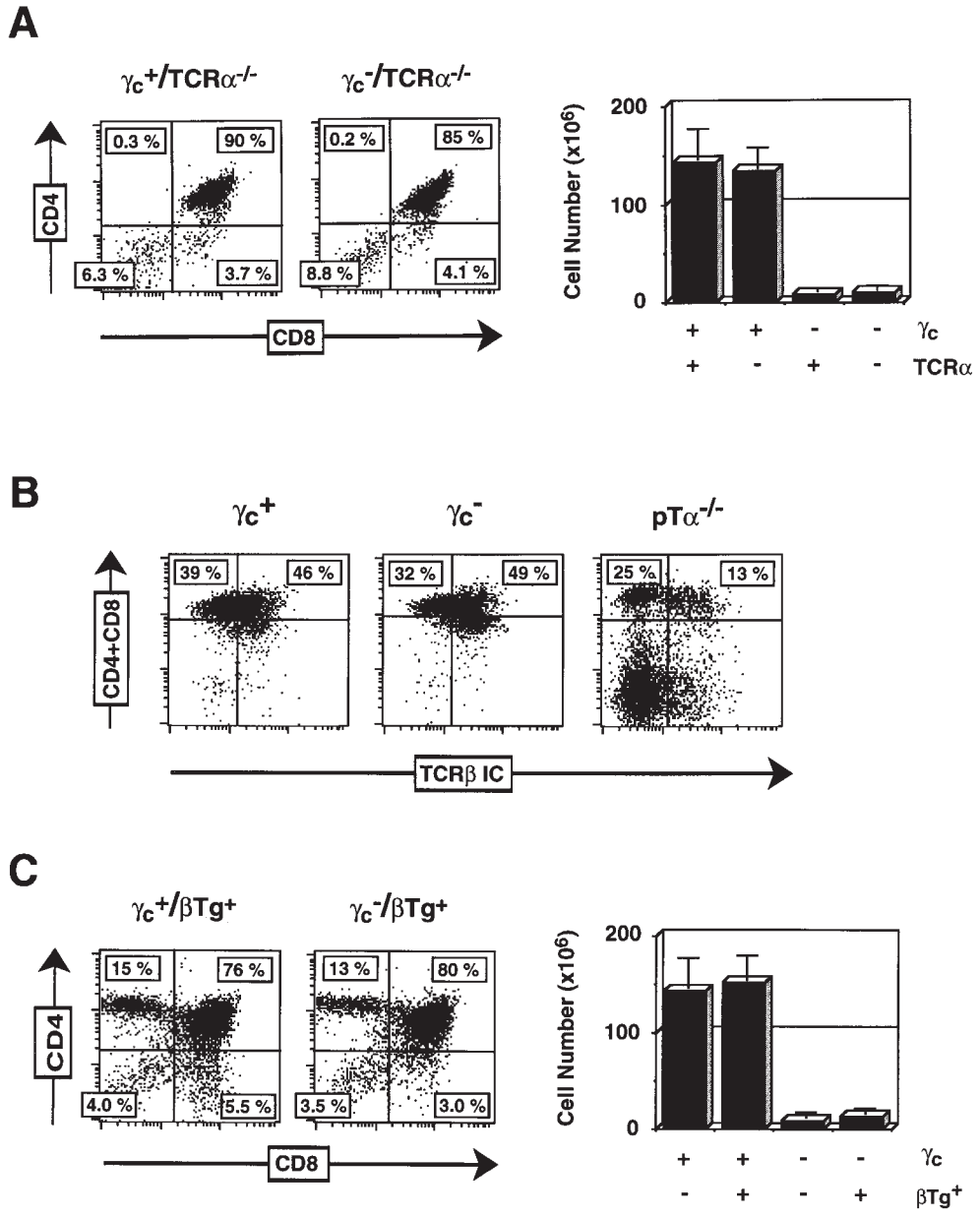

Figure 3. Pre-TCR function in the absence of the γ_c chain. (A) CD4/CD8 profiles of thymocytes from 3–4-wk-old TCR-α–deficient and TCR-α/γ_c double-deficient mice. Total thymocyte numbers were calculated (mean ± SD) from six mice of each genotype. (B) Intracellular TCR-β chain expression in thymocytes from γ_c-deficient, pTα-deficient, or wild-type mice. (C) Thymocyte CD4/CD8 profiles from mice bearing a functional TCR Vβ8.2 Tg on γ_c-deficient or wild-type backgrounds. Absolute thymocyte cell numbers (mean ± SD) were derived from four to six mice of each genotype.

γ_c and Pre-TCR Signaling Pathways Compensate for Each Other in α/β T Cell Development. To address the relative interdependence of γ_c and pre-TCR signals, we intercrossed the γ_c and pTα null strains to generate mice lacking both these molecules. Thymocyte development was analyzed in 3–4-wk-old double mutant mice ($\gamma_c^-/pT\alpha^{-/-}$) as well as single mutants for γ_c or pTα and wild-type mice.

Consistent with previous reports (5, 24), the thymi from either γ_c or pTα mutant mice demonstrated a reduction in the size (data not shown) and cellularity (20–30-fold; Fig. 4). In contrast, $\gamma_c^-/pT\alpha^{-/-}$ thymi were severely hypoplastic and showed a drastic reduction (~4,000-fold) in absolute cell numbers (Fig. 4). Thymocyte cell surface phenotype was further characterized in these mice (Fig. 4, A and B). $pT\alpha^{-/-}$ thymi showed an incomplete block in thymocyte development with an accumulation of cells at the DN stage; however, pTα-deficient thymocytes are capable of further maturation to DP and SP mature cells. In marked contrast, thymi from double mutant $\gamma_c^-/pT\alpha^{-/-}$ mice contained only immature DN cells (Fig. 4 A). Using CD44 and CD25 markers, γ_c^- thymi demonstrated the characteristic incomplete block at the $CD44^+CD25^+$ to $CD44^-CD25^+$ transition (Fig. 4 B), whereas the accumulation of cells at the $CD44^-CD25^+$ stage in pTα-deficient mice coincides with pre-TCR–mediated cellular expansion and differentiation to the DP stage (for a review, see reference 45). Strikingly, residual thymocytes from $\gamma_c^-/pT\alpha^{-/-}$ mutant mice showed a developmental block with a complete arrest of differentiation at the $CD44^+CD25^+$ stage (Fig. 4 B). To our knowledge, this is the first description of mutant mice harboring

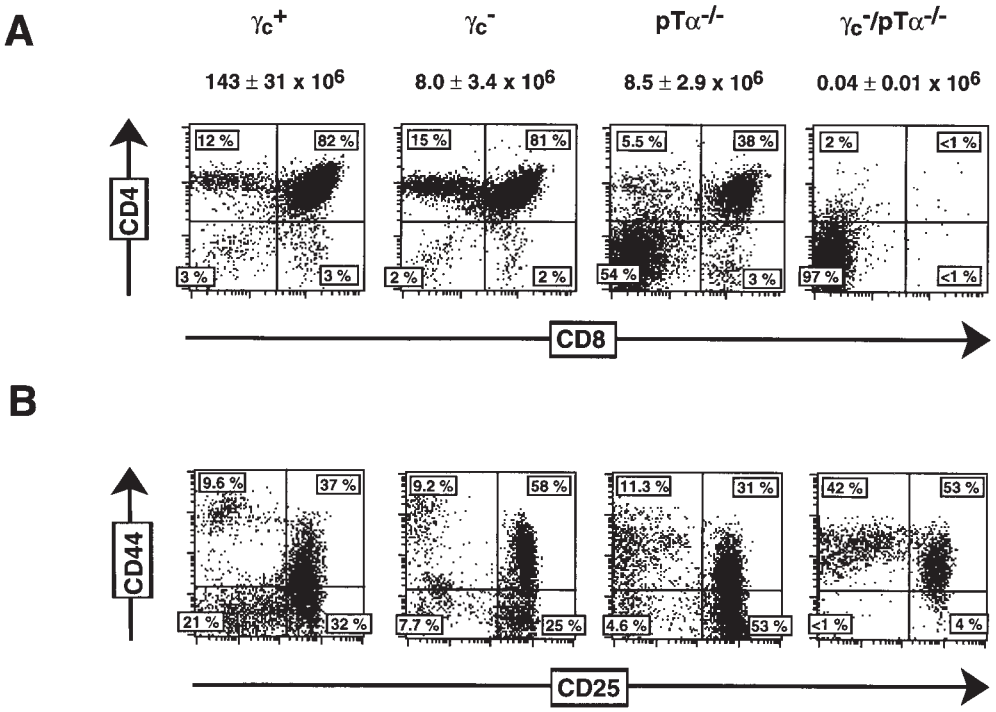

Figure 4. Intrathymic development in $\gamma_c/pT\alpha^{-/-}$ double mutant mice. (A) CD4/CD8 profiles of thymocytes from 3-wk-old mice. (B) CD44/CD25 expression on gated TN thymocytes (see Fig. 1). For these experiments, thymi from four to six $\gamma_c/pT\alpha^{-/-}$ double mutant mice were pooled. Absolute thymocyte cell numbers (mean ± SD) were calculated for the indicated mice (n = 6–10 for each genotype).

this particular intrathymic defect. In terms of absolute cell numbers, $\gamma_c^-/pT\alpha^{-/-}$ thymi contained ~4×10^4 TN precursors, all of which were $CD44^+$, and therefore similar to the number of $CD44^+$ TN precursors found in γ_c^- thymi (Fig. 1 B). However, unlike γ_c^- or $pT\alpha^{-/-}$ single mutant mice, the arrest in thymic development in $\gamma_c^-/pT\alpha^{-/-}$ double mutant thymi was complete, as no mature T cells were found intrathymically or in the peripheral lymphoid organs (data not shown, and see below). These results (a) define a critical period of intrathymic development (the $CD44^+CD25^+$ to $CD44^-CD25^+$ transition) in which signals delivered by γ_c and the pre-TCR pathways appear to overlap, and (b) suggest that pre-TCR signals are responsible for rescue of α/β T cell development in γ_c^- mice.

It would follow from these results that a pre-TCR can form at the $CD44^+CD25^+$ stage. Although several studies have reported the rearrangement status of early thymocyte subsets (40, 46, 49), no studies to date have examined pre-TCR protein expression in these cells. Using reagents specific for the TCR-β (29) and a newly developed antibody against the pTα chain (30), we characterized intracellular pre-TCR components in early thymocytes from γ_c^+ and γ_c^- mice (Fig. 5). At the $CD44^+CD25^+$ stage, thymocytes demonstrate uniform intracellular staining for pTα chains, whereas the level of pTα expression increases slightly as the cells mature to become $CD44^-CD25^+$. A small fraction of $CD44^+CD25^+$ cells (3.0 ± 1%; n = 4) also stain intracellularly for TCR-β protein; this fraction increases to ~20% as these cells downregulate CD44 expression (Fig. 5). TCR-β and pTα protein expression on a per cell basis was not qualitatively or quantitatively altered in γ_c^- thymocytes (Fig. 5). These data conclusively demonstrate that a pre-TCR can form during the $CD44^+CD25^+$ to $CD44^-CD25^+$ transition, a point at which intrathymic precursors express IL-7Rα/γ_c (41). These results suggest that γ_c and pre-TCR signals are independent and overlapping for intrathymic development.

Characterization of the $\gamma_c^-/pT\alpha^{-/-}$ Thymic Rudiment. Due to the severe reduction in thymocyte cell numbers in $\gamma_c^-/pT\alpha^{-/-}$ mice, a PCR-based strategy (31) was used to identify any TCR-β rearrangements present in these mutant thymi. DNA from control, γ_c^-, or $pT\alpha^{-/-}$ thymi contained abundant TCR-β V(D)J rearrangements, which were diverse with respect to junctional sequences present in the CDR3 region (Fig. 6; and data not shown). In contrast, rearrangements from $\gamma_c^-/pT\alpha^{-/-}$ mutant thymi were reduced in overall amounts, although samples derived from independent thymi contained multiple and different bands, indicating rearrangements to different Jβ segments (Fig. 6). Sequence analysis of these PCR products revealed unique Vβ CDR3 sequences, suggesting that the observed reduction in rearrangements was related to the paucity of absolute cell numbers and not to a restricted rearrangement potential (data not shown). Finally, TCR-β rearrangements were absent from the spleens of $\gamma_c^-/pT\alpha^{-/-}$ double mutant mice, demonstrating that the intrathymic block in α/β T cell development was complete and that no mature α/β T cells were produced that could seed the peripheral lymphoid organs (Fig. 6).

Discussion

α/β T cells are generated intrathymically through a series of developmental steps involving survival, expansion,

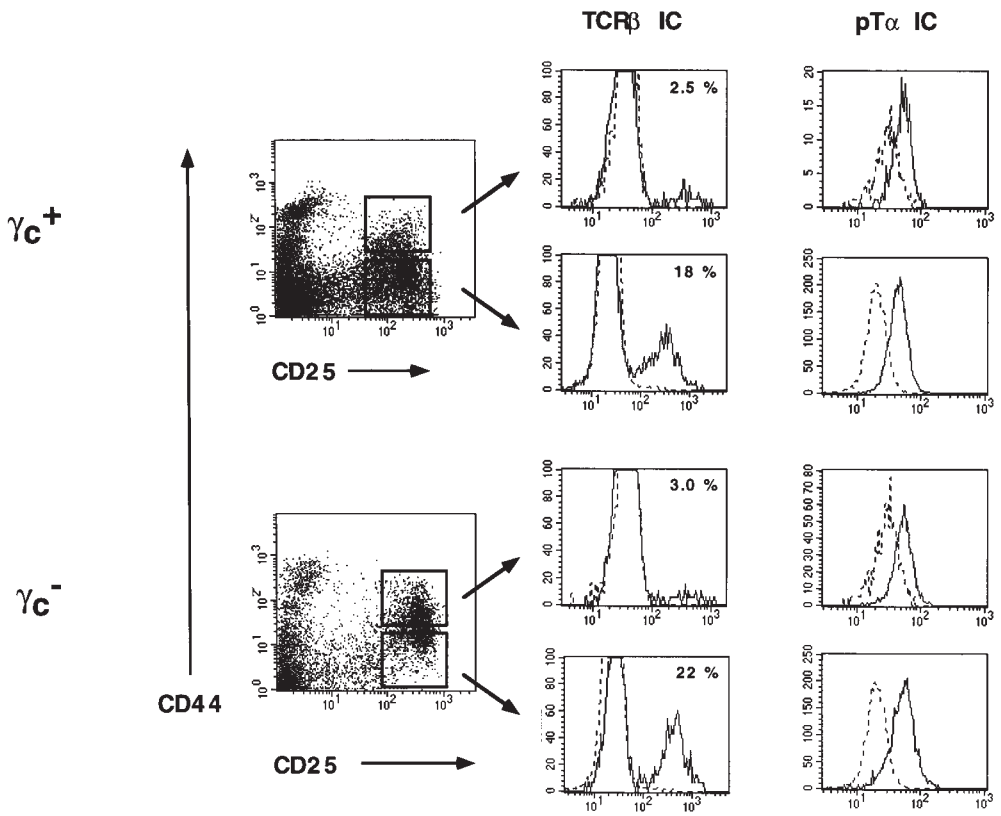

Figure 5. Intracellular expression of pTα and TCR-β chain in $CD25^+$ thymocyte precursors. Thymocytes from γ_c^+ or γ_c^- mice were surface stained for TN cells (see Fig. 1), CD44, and CD25, fixed, and permeabilized with saponin before detection of intracellular (IC) TCR-β or pTα chains. Gated $CD44^+CD25^+$ and $CD44^-CD25^+$ thymocyte subsets are boxed. Negative controls (dotted lines) are staining of thymocytes from RAG-2–deficient (for TCR-β) or pTα-deficient (for pTα) mice.

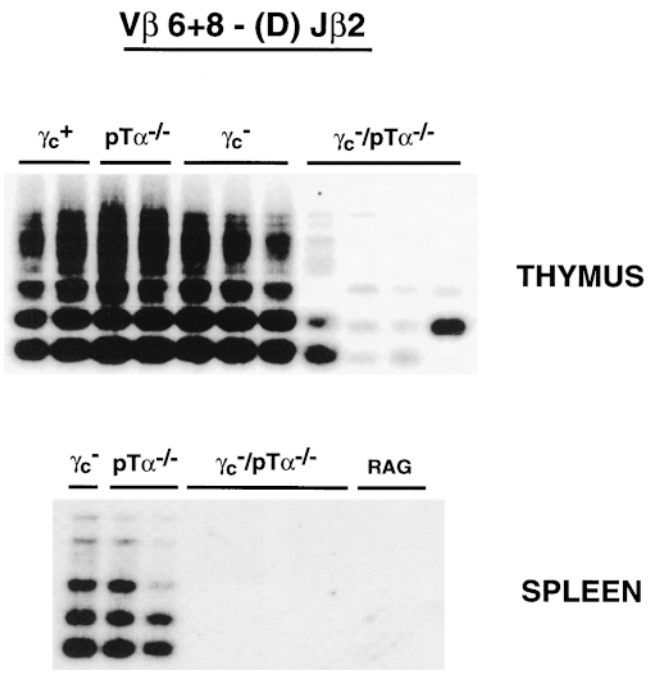

Figure 6. TCR-β rearrangements in thymus and spleen. Genomic DNA from the indicated mice were amplified by PCR using a combination of primers specific for TCR Vβ6 and Vβ8 (sense) and a primer specific for the 3′ region of TCR Jβ2.5 (antisense). Amplification products were detected by blot hybridization using a probe specific for the 5′ region of TCR Jβ2.5.

and differentiation of immature precursor cells. The γ_c chain plays a critical role in this process, primarily by relaying signals from stromal cell–derived IL-7 to developing thymocytes. The essential role of IL-7 in early thymocyte differentiation has been difficult to define because this cytokine has been postulated both to act as a trophic factor and to influence the TCR rearrangement process (for a review, see reference 47). Moreover, deficiencies in IL-7/IL-7Rα/γ_c abrogate γ/δ T cell development, whereas α/β T cell development is permissive, suggesting either a differential role for the IL-7 receptor complex in the generation of these two T cell subsets or the existence of compensatory pathways that rescue α/β T cells in the absence of IL-7/IL-7Rα/γ_c. In this report, we identify the pre-TCR as a rescue mechanism for α/β T cell development in γ_c^- mice. This result suggests that a model of intrathymic differentiation involves an overlapping series of signals derived from growth factors and TCRs that guide the maturation process (Fig. 7). This model is consistent with the permissive nature of thymocyte development in c-kit–, IL-7Rα–, γ_c-, and pTα-deficient mice (4–6, 15–17, 24, 50), and sheds light on the compensatory signaling pathways that exist to insure α/β T cell differentiation in these mutant mice.

Before the expression of a rearranged TCR chain, immature thymocytes are maintained and proliferate in response to factors provided within the thymic milieu (for a review, see reference 51). Although several cytokines have been shown to act on thymic precursors, stem cell factor

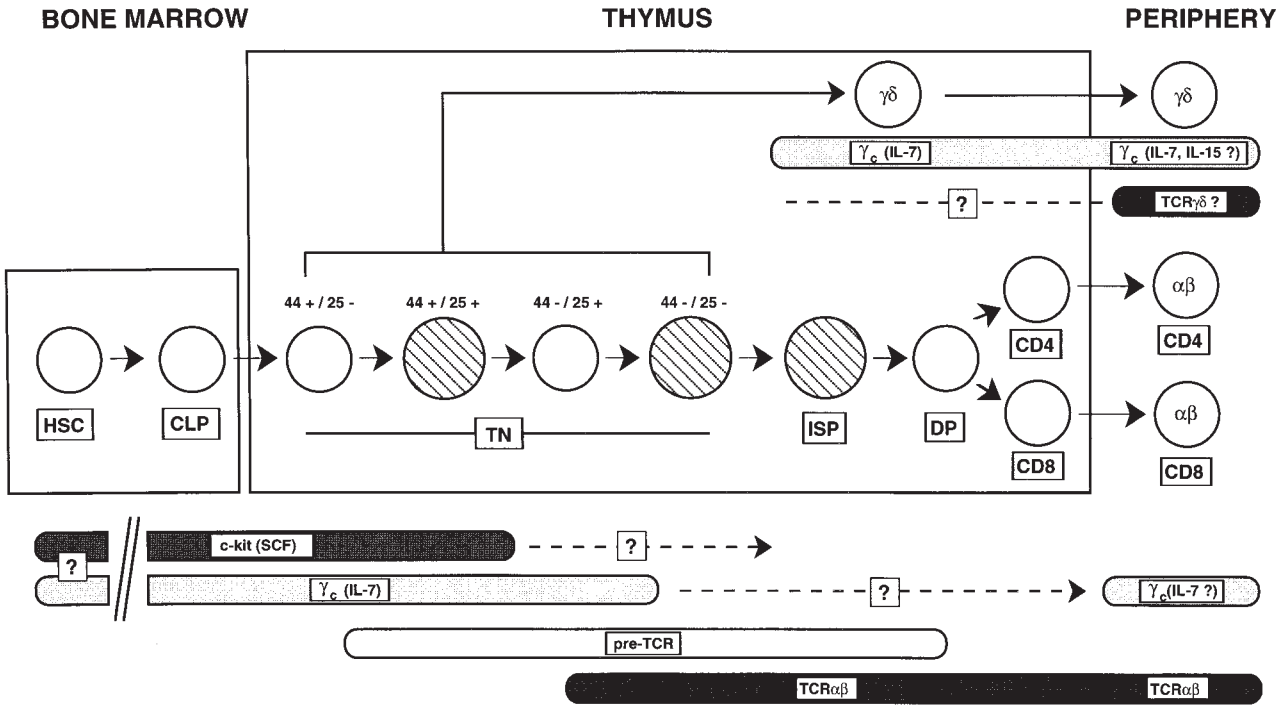

Figure 7. Cytokine and TCR signaling in intrathymic development. Hematopoietic stem cells (HSC) give rise to thymocytes via common lymphoid progenitor cells (CLP). Within the thymus, TN precursor cells can be further subdivided based on expression of CD44 and CD25. Immature SP cells (ISP) are the immediate precursors of DP thymocytes, which after tolerance induction via α/β TCR selection develop into $CD4^+$ or $CD8^+$ SP cells which exit the thymus to seed peripheral lymphoid organs. γ/δ T cells can derive from the various subsets of TN cells. Concerning the molecules required to generate and maintain α/β T cells, growth factor receptors including c-kit and the common γ chain (γ_c) in concert with the pre-TCR and TCR-α/β appear to provide overlapping signals which guide the developmental process. In the absence of either IL-7Rα, γ_c, or the pre-TCR, signals provided by adjacent pathway(s) permit a limited degree of α/β thymocyte development. In contrast, when two adjacent signals are absent (for example, in c-kit $\times$ γ_c or $\gamma_c \times$ pTα double mutants), α/β T cell development is abrogated. Moreover, α/β T cells require continual TCR triggering in the periphery. In contrast, γ/δ T cells appear strictly cytokine dependent, both intrathymically and in the periphery. Hatched circles, cycling cells.

(SCF) and IL-7 remain the two dominant factors that can promote their survival and/or expansion (14, 50, 52). The receptors for stem cell factor (c-kit) and for IL-7 (the IL-7Rα/γ_c complex) are coexpressed on early thymocytes (41, 53, 54), and proliferation of these cells is reduced in the absence of c-kit or IL-7Rα/γ_c (50, 55; and this report). The permissive nature of thymocyte development in c-kit or IL-7Rα/γ_c mutants implies redundancy in the pathways that maintain early precursors. The hypothesis that c-kit and IL-7Rα/γ_c signals could compensate for each other at this stage is strongly supported by the complete abrogation of thymocyte development (before the $CD44^+CD25^-$ cell stage) in mice deficient in both c-kit and γ_c (26). Thus, for cells up to the $CD44^+CD25^-$ stage, c-kit and γ_c act synergistically to maintain cells before TCR rearrangements. The essential nature of c-kit and γ_c signals cannot be replaced by other growth factors at this stage of development (26).

At the transition from the $CD44^+CD25^+$ stage to the $CD44^-CD25^+$ stage, rearrangements of the TCR-β chain begin (40, 46, 49). Since IL-7– and γ_c-deficient thymocyte precursors are most severely affected at this stage (55; Fig. 1 B), the failure to signal through γ_c could have an adverse effect on the TCR rearrangement process. Consistent with this hypothesis, previous studies have demonstrated that transgenic expression of a functionally rearranged TCR-α/β (against the male-specific [HY] antigen in association with $H\text{-}2D^b$) could partially restore total thymocyte numbers in γ_c- and IL-7Rα–deficient mice (18, 19). However, these experiments failed to rule out potential effects associated with TCR signaling under conditions of positive selection, since increases in thymic cellularity were only observed in $H\text{-}2D^b$ female mice. Here we show that the same TCR-β alone has no effect on thymocyte development in γ_c^- mice. This observation strongly suggests that defective TCR-β rearrangements alone cannot account for the abnormal α/β T cell development in the absence of γ_c, and supports the idea that poor survival and reduced proliferation of the $CD44^+CD25^+$ and subsequent thymocyte subsets are the major limiting factors for α/β T cell development in these mice.

The results presented here identify the pre-TCR as an independent and essential signal which acts in concert with γ_c during α/β thymopoiesis. Although in principle the failure to signal through γ_c could have an adverse effect on pre-TCR assembly or function, we find that β selection and pre-TCR–mediated expansion appear γ_c independent. However, the essential role of the pre-TCR in γ_c^- mice

is clearly illustrated by the γ_c/pTα double mutants. In these mice, thymocyte development proceeds only to the $CD44^+CD25^+$ stage (thereby delimiting the role of the c-kit pathway), and implies that further development requires either γ_c or pre-TCR signals. As indicated above, γ_c can act via IL-7 to maintain early thymocytes at this stage (20–22, 55) and might also serve to drive differentiation to the $CD44^-CD25^+$ stage where TCR rearrangements are ongoing (40, 46, 49). How, then, could the pre-TCR rescue γ_c-deficient cells at such an early stage?

To address this issue, we examined expression of TCR-β and pTα proteins in $CD44^+CD25^+$ and $CD44^-CD25^+$ thymocytes from γ_c^+ and γ_c^- mice. Our results clearly demonstrate that a pre-TCR complex can potentially form in a small subset of $CD44^+CD25^+$ cells. The expression of a pre-TCR at this stage could thereby provide a compensatory mechanism in γ_c^- mice to enable α/β T cell development. Moreover, the "window" of pre-TCR expression was similar in γ_c^+ and γ_c^- mice. In this respect, γ_c and pre-TCR signals are independent and overlapping at this stage of intrathymic development (Fig. 7).

The signaling cascades initiated from γ_c and pre-TCRs appear distinct. γ_c receptors activate the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway (for a review, see reference 56), whereas the pre-TCR uses immunoreceptor tyrosine-based activation motif (ITAM)-containing CD3 components which couple to the src family and ZAP-70/syk family tyrosine kinases (for reviews, see references 42 and 57). How the pre-TCR mediates proliferation of late thymocyte precursors is unknown, but our results indicate that this process does not require γ_c-dependent cytokines. Further work will be required to identify the mechanism by which triggering the pre-TCR engages the cell cycle.

Our observations provide insights into the difference between α/β and γ/δ T cell development in IL-7/IL-7Rα/γ_c-deficient mice (12, 17, 58). Although the pre-TCR is capable of rescuing TCR-α/β cells in γ_c^- mice, γ/δ T cells lack an equivalent mechanism and therefore must rely on other signals for their final intrathymic differentiation. We have previously shown that transgenic expression of rearranged TCR-γ or TCR-γ/δ chains failed to rescue γ/δ T cell development in γ_c^- mice, suggesting that γ_c-dependent cytokines provide the dominant signals for γ/δ T cell survival and/or proliferation both intrathymically and in the periphery (28). On the other hand, peripheral maintenance of α/β T cells requires continual TCR stimulation (for reviews, see references 59 and 60) but appears less γ_c dependent in their development.

Finally, our model suggests that differences in T cell development between human and murine γ_c deficiency might be related to species-specific differences in the function of c-kit, IL-7/IL-7Rα/γ_c, or the pre-TCR. Little is known about the specific patterns of expression of these molecules with regard to the various stages of intrathymic development in humans. Moreover, the documented differences between human and mouse pTα cytoplasmic sequences (61) could result in differential signaling properties of the pre-TCR between species. Support for this model will require further studies focusing on these pathways in human thymocyte development.

We thank D. Guy-Grand (Paris), M. Malissen, B. Malissen (Marseille), and H.-R. Rodewald (Basel) for stimulating discussions.

Supported by grants from the Institut National de la Santé et de la Recherche Médicale, the Association pour le Recherche sur le Cancer, and the Ligue Nationale Contre le Cancer. The Basel Institute for Immunology was founded and is supported by F. Hoffmann-La Roche.

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Received for publication 9 September 1998 and in revised form 17 November 1998.

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