

Essential and Partially Overlapping Role of CD3 γ and CD3 δ for Development of $\alpha\beta$ and $\gamma\delta$ T Lymphocytes

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Summary

CD3 γ and CD3 δ are two highly related components of the T cell receptor (TCR)–CD3 complex which is essential for the assembly and signal transduction of the T cell receptor on mature T cells. In gene knockout mice deficient in either CD3 δ or CD3 γ , early thymic development mediated by pre-TCR was either undisturbed or severely blocked, respectively, and small numbers of TCR- $\alpha\beta$ ⁺ T cells were detected in the periphery of both mice. $\gamma\delta$ T cell development was either normal in CD3 δ ^{-/-} mice or partially blocked in CD3 γ ^{-/-} mice. To examine the collective role of CD3 γ and CD3 δ in the assembly and function of pre-TCR and in the development of $\gamma\delta$ T cells, we generated a mouse strain with a disruption in both CD3 γ and CD3 δ genes (CD3 $\gamma\delta$ ^{-/-}). In contrast to mice deficient in either CD3 γ or CD3 δ chains, early thymic development mediated by pre-TCR is completely blocked, and TCR- $\alpha\beta$ ⁺ or TCR- $\gamma\delta$ ⁺ T cells were absent in the CD3 $\gamma\delta$ ^{-/-} mice. Taken together, these studies demonstrated that CD3 γ and CD3 δ play an essential, yet partially overlapping, role in the development of both $\alpha\beta$ and $\gamma\delta$ T cell lineages.

Key words: CD3 γ • CD3 δ • T cell receptor–CD3 complex • T cell development • knockout mouse

During thymocyte development, the genes coding for TCR- α and - β , pre-TCR- α (pT α), and the associated CD3 proteins (CD3 γ , δ , ϵ , and ζ) are expressed in a temporal order (1). The pre-TCR–CD3 complex, consisting of pT α , TCR- β , and CD3 proteins, plays a major role in early thymocyte development and in the transition from CD4⁻CD8⁻ (double negative, DN) to CD4⁺CD8⁺ (double positive, DP) cells, as targeted mutations in pT α , TCR- β , RAG, and CD3 genes all result in an arrest of T cell development at the DN CD44⁻CD25⁺ check point (2, 3). Subsequently, TCR- α replaces pT α and the resulting TCR–CD3 complex mediates signal transduction cascades leading to further T cell development (2). Compared with $\alpha\beta$ T cell development, $\gamma\delta$ T cell development is less defined (4, 5). The majority of thymic $\gamma\delta$ T cells do not express CD4 or CD8 antigens (6), and pT α and TCR- β are not involved in the development of $\gamma\delta$ T cells (7, 8). However, CD3 proteins are required for the development of this lineage (2).

Ample biochemical studies have shown that the CD3 proteins are important for assembly and efficient surface ex-

pression of TCR (9). In each TCR–CD3 complex, there are two copies of CD3 ϵ and CD3 ζ , yet only one copy of the highly homologous CD3 γ and CD3 δ (10–12). CD3 ϵ forms heterodimers with CD3 γ and CD3 δ , and can also exist as a CD3 $\epsilon\epsilon$ homodimer, whereas CD3 ζ exists as a CD3 $\zeta\zeta$ homodimer (11, 13, 14). TCRs lacking CD3 γ , δ , ϵ , or ζ can reach the cell surface, albeit 10–100-fold less efficiently than wild-type receptors, because of a certain degree of redundancy in their assembly potential (15, 16). In immature thymocytes, the CD3 proteins are expressed (17–19), before the expression of pT α and TCR- β (1). Thus, CD3 proteins can be a part of the pre-TCR–CD3 complex or part of a clonotype-independent CD3 (CIC) complex (20). In these complexes, CD3 $\gamma\epsilon$ dimers are consistently detected (20, 21), and some studies indicated the presence of a small quantity of CD3 $\delta\epsilon$ dimers (18–21). This led to the notion that CD3 γ may be preferentially required over CD3 δ in the assembly of pre-TCR complexes (22).

Recent studies on mutant mice deficient in either the CD3 γ or CD3 δ gene in part support this notion. Whereas transition from DN to DP $\alpha\beta$ thymocytes appears to be normal in CD3 δ ^{-/-} mice (23), $\alpha\beta$ T cell development in CD3 γ ^{-/-} mice is blocked at the DN CD44⁻CD25⁺ check point (24). However, the blockade in T cell development in

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CD3 γ ^{-/-} mice is incomplete, as small numbers of DP thymocytes were found and TCR- $\alpha\beta$ ⁺ T cells were detected in the periphery (24). Moreover, in either mutant a considerable number of $\gamma\delta$ T cells is present (23, 24). Therefore, it is likely that CD3 γ and CD3 δ play an essential, yet to some extent redundant, role in early development of T cells.

To examine the issue of partial overlap in function between CD3 γ and CD3 δ , a mouse strain with a disruption in both the CD3 γ and CD3 δ genes (CD3 $\gamma\delta$ ^{-/-}) would be useful. A CD3 $\gamma\delta$ ^{-/-} mouse, however, could not be generated by breeding the CD3 δ ^{-/-} and CD3 γ ^{-/-} mice, because the genes coding for CD3 γ , δ , and ϵ are located in a single gene cluster and a mere 1.4-kb intergenic sequence separates the first exons of CD3 γ and CD3 δ genes (25). Therefore, we generated CD3 $\gamma\delta$ ^{-/-} mice by deleting the promoters and exons 1 of both genes.

Materials and Methods

Generation of CD3 $\gamma\delta$ ^{-/-} Mice. The targeting construct was generated by standard methods. In brief, a genomic DNA clone containing a 15.5-kb fragment of CD3 $\gamma\delta$ genes was isolated from a 129/sv mouse genomic DNA library (provided by Dr. Manley Huang, GenPharm Int., Mountain View, CA) and subcloned into pBluescriptSK+ (Stratagene, La Jolla, CA). A 2.8-kb SalI-XhoI DNA fragment containing the PGK-TK^r gene was isolated from pPGK-TK (provided by Dr. Manley Huang), and ligated to the XhoI site of pPGK-hygro^r (hyg^r) (a gift of Dr. Richard Mortensen). A 1.9-kb XbaI-XbaI intronic fragment between exon 1 and 2 of CD3 δ was obtained by XbaI digestion of the 15.5-kb CD3 $\gamma\delta$ genomic DNA fragment. And a 3-kb intronic fragment between exon 1 and 2 of CD3 γ was obtained by first subcloning a 5-kb EcoRI-XbaI fragment into SK+ followed by a HindIII cut, so that a HindIII site from the polylinker region of the plasmid was transferred to one end of the 3-kb fragment. The 1.9-kb XbaI-XbaI fragment and the 3-kb HindIII-HindIII fragment were inserted into the 5' and 3' sites of the PGK-Hyg^r gene. In the resulting construct, a 3.1-kb DNA fragment containing the 1.4-kb intergenic DNA fragment between the CD3 γ and CD3 δ genes and exons 1 of both genes were replaced by the 2.8-kb PGK-Hyg^r cassette. 10 μ g of purified targeting molecules were electroporated into 10⁷ J-1 ES cells. ES cells were positively selected by hygromycin-B at 200 μ g/ml and negatively selected by FIAU at 0.2 μ M. 355 clones were selected and examined by Southern blots for homologous recombination using a 0.8-kb (StuI-XbaI) 5' probe located outside of the construct. Eight clones were identified as targeted clones, which were confirmed by another Southern analysis with a hyg^r probe. Four of the targeted clones were injected into the blastocysts of either C57BL/6 or BALB/C origin, and 90–100% fur color chimerism was observed in 45 founder mice. Test breeding of the chimeras indicated that all of the males ($n = 28$ from 3 embryonic stem [ES] clones) transmitted the ES cell genome. Four males were mated to C57BL/6 females to generate heterozygous mice, and homozygous CD3 $\gamma\delta$ ^{-/-} lines were obtained by sibling breeding. Identical results were obtained from homozygous CD3 $\gamma\delta$ ^{-/-} lines of different ES clones.

Flow Cytometric Analysis. Single cell suspensions of thymocytes, LN cells, spleen cells, PBL, and small intestine intraepithelial lymphocytes (iIEL) were prepared as described (26, 27). Three-color staining of the cells was performed as previously reported elsewhere (28).

RNA Analysis. Northern blot analysis was performed as described (29).

Results

Generation of CD3 $\gamma\delta$ ^{-/-} Mice. To generate mice deficient in both CD3 γ and CD3 δ gene expression, a 3.1-kb DNA fragment containing the promoters (25) and exons 1 of the CD3 γ and CD3 δ genes was replaced by a PGK-Hyg^r cassette (Fig. 1 A). The PGK-hyg^r cassette was chosen here over the PGK-neo^r cassette to prevent a possible suppressive effect of the PGK-neo^r on neighboring gene expression (30, 31). Homozygous mice carrying this mutation in the CD3 γ and δ genes were generated (Fig. 1 B). Northern blot analysis demonstrated that the expression of both CD3 γ and CD3 δ mRNA was absent in the CD3 $\gamma\delta$ ^{-/-} thymocytes (Fig. 2). Moreover, no aberrant expression of the truncated CD3 γ or δ mRNAs were ever detected in Northern blotting of thymocytes from more than 20 CD3 $\gamma\delta$ ^{-/-} mice. However, the expression of the neighboring CD3 ϵ gene and the nonlinked CD3 ζ was normal (Fig. 2), and pT α expression was detected (data not shown).

$\alpha\beta$ T Cell Development in the CD3 $\gamma\delta$ ^{-/-} Mice. Total cellularity of the thymi of CD3 $\gamma\delta$ ^{-/-} mice was 2–5% of that in wild-type or heterozygous littermates (Fig. 3 A). Flow cytometric analysis of the thymocytes showed that these cells are DN, with the majority of them being CD44⁺CD25⁺c-Kit⁻Sca-1⁺, identical to the thymocytes found in RAG^{-/-} mice (Fig. 3 B). Northern blot analyses of the thymocytes of CD3 $\gamma\delta$ ^{-/-} mice did not detect the mRNA for rearranged TCR- α and TCR- β genes, whereas only the 1.0-kb germline C β mRNA was detectable (Fig.

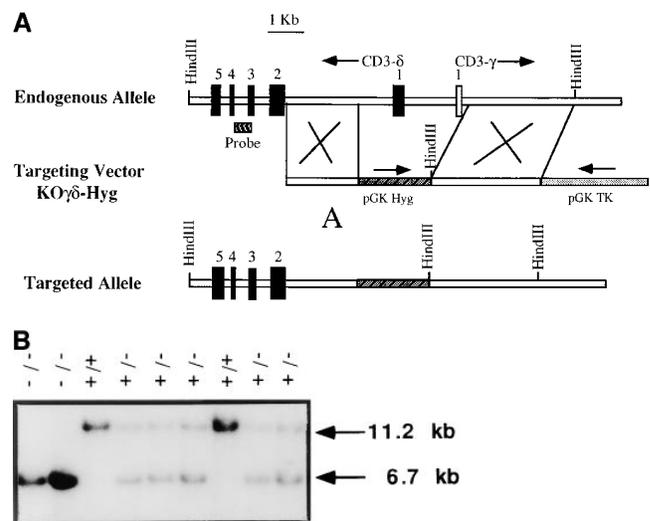


Figure 1. Disruption of CD3 $\gamma\delta$ genes. (A) Diagram of the CD3 $\gamma\delta$ targeting vector for homologous recombination. Exons 1–5 of the CD3 δ gene and exon 1 of the CD3 γ gene are numbered. Arrows indicate the transcriptional orientations of the CD3 $\gamma\delta$ genes. The 0.8-kb probe was used for screening the ES cell clones and for Southern analysis of tail DNA. (B) Southern blot analysis of tail DNA.

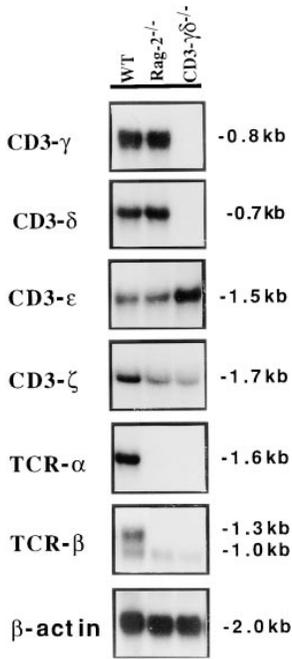


Figure 2. TCR-CD3 expression in the CD3γδ^{-/-} mice. Northern blotting of thymocytes from wt, RAG^{-/-} and CD3γδ^{-/-} mice for the expression of CD3γ, δ, ε, ζ, and TCR-α and -β. The respective probes are indicated on the left, and the sizes on the right. Two mice of each type were analyzed in this blot.

2). Consistent with these analyses, no mature αβ⁺ T cells were detected in the LN, the spleen, or the gut of the CD3γδ^{-/-} mice (Figs. 3 C and 4 C, Table 1). B cell development appeared unaffected (Table 1). Taken together, αβ T cell development in CD3γδ^{-/-} mice is blocked at the same DN CD44⁻CD25⁺ check point as RAG^{-/-} mice (32, 33).

γδ T Cell Development in CD3γδ^{-/-} Mice. Next, γδ T cell development in CD3γδ^{-/-} mice was examined. As shown in Fig. 4, A and B, γδ T cells were absent in the thymus and periphery of CD3γδ^{-/-} mice. Since γδ T cells normally account for only a very small fraction of thymocytes and peripheral T cells, we assessed γδ T cell development in the small intestine, where γδ T cells represent a major population of the iIEL in wild-type mice. In CD3γδ^{-/-} mice, γδ T cells were again nondetectable in the intestine (Fig. 4 C). However, normal number of CD8αα⁺B220⁺CD32⁺NK1.1⁻ cells, representing T cell progenitors in the gut (27) could be detected in the gut of CD3γδ^{-/-} mice (Fig. 4, C-E, Table 1, and data not shown). Therefore, these analyses indicate that deficiency in CD3γ and δ completely blocked γδ T cell development beyond the CD8αα⁺ stage.

Discussion

We report here that in the CD3γδ^{-/-} double mutant mice, intrathymic development is completely arrested at the DN CD44⁻CD25⁺ prothymocyte stage, a central check point at which pre-TCR begins to mediate further thymocyte differentiation into the DP stage. This observation indicates that the function of pre-TCR is completely abrogated in CD3γδ^{-/-} mice. In contrast, in recently reported CD3δ^{-/-} mice, thymic development is undisturbed

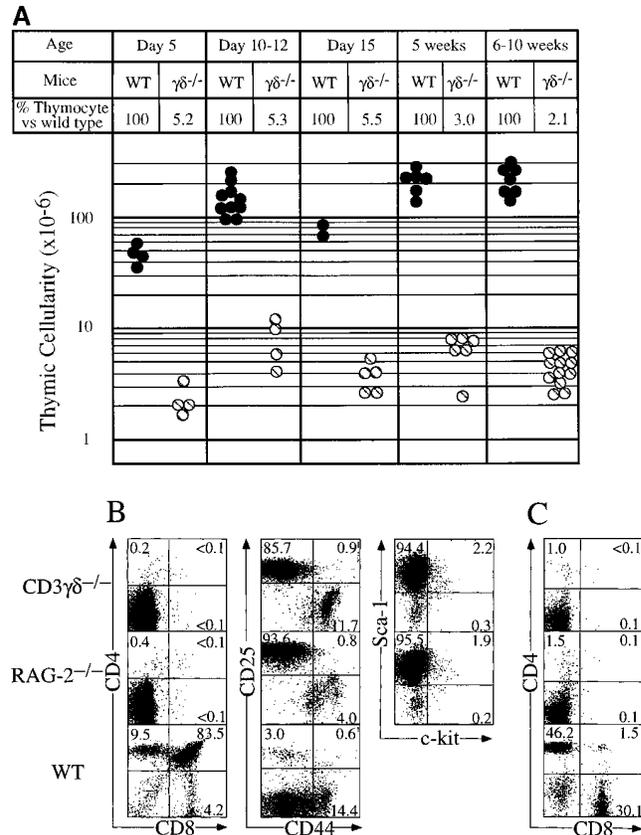


Figure 3. T cell deficiency in the CD3γδ^{-/-} mice. (A) Thymic cellularity of the mutant mice. Each symbol represents the total number of thymocytes from a mouse. The ages of the mice are indicated. For each age group, the average number of thymocytes from mutant mice was compared with that of wild-type (including CD3γδ^{+/-}) littermates or age-matched, wild-type mice (28). (B) Flow cytometric analysis of thymocytes from CD3γδ^{-/-}, RAG-2^{-/-} and wild-type mice for surface expression of CD4, CD8, CD44, CD25, Sca-1, and c-Kit. (C) Flow cytometric analysis of peripheral lymph node cells for surface expression of CD4 and CD8.

up to the DP stage (23), whereas the transition from DN to DP stages was severely but not completely blocked in CD3γ^{-/-} mice (24). The phenotypes of CD3δ^{-/-} and CD3γ^{-/-} mice are consistent with the biochemical evidence that CD3γ is preferentially required over CD3δ in prothymocytes for the assembly of the pre-TCR-CD3 complex (22). However, the present data revealed that CD3δ also participated *in vivo* in the assembly and function of the pre-TCR-CD3 complex. Moreover, small numbers of TCR-αβ⁺ T cells were detected in the periphery of CD3δ^{-/-} and CD3γ^{-/-} mice, but were absent in CD3γδ^{-/-} mice. These observations are consistent with the biological evidence that in mature T cells, the TCR-CD3 complex lacking either CD3γ or δ could sometimes be detected on the cell surface at reduced levels. However, no surface expression of the TCR-CD3 complex could be detected in cells lacking both CD3γ and δ (15, 16). Taken together, CD3γ and CD3δ collectively play an essential, yet partially overlapping, role in the assembly and function of the pre-TCR. It is most likely that in the absence of

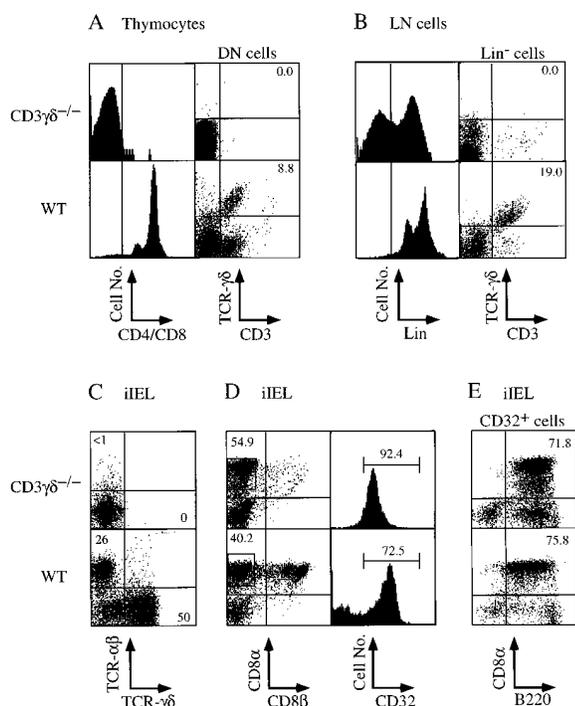


Figure 4. Flow cytometric analysis of the $\gamma\delta$ T cell compartment in $CD3\gamma\delta^{-/-}$ mice. (A) Thymocytes were stained with anti-TCR- $\gamma\delta$ -biotinylated (detected with RED-670), anti-CD3-PE, and anti-CD4/CD8-FITC. (Left) Profile of CD4/CD8 expression. (Right) Profile of TCR- $\gamma\delta$ and CD3 expression in the analytically gated DN cells. (B) Lymph node cells were similarly analyzed as in A, except that a mixture of FITC-conjugated antibodies, i.e., anti-CD4, -CD8, -TCR- $\alpha\beta$, -B220, -Mac-3, and Gr-1 (collectively termed Lin), was used. (C) Expression of TCR- $\alpha\beta$ and TCR- $\gamma\delta$ in iIEL. (D) iIEL were stained with anti-CD8 α , anti-CD8 β , and anti-CD32. (Left) Profile of CD8 α /CD8 β expression. (Right) Profile of CD32 expression in the analytically gated CD8 $\alpha\alpha^{+}$ cells. The CD8 $\alpha\alpha^{+}$ cells were all CD8 $\alpha\alpha^{+}$ and were predominantly CD32⁺. (E) iIEL were stained with anti-CD8 α , anti-B220, and anti-CD32, and the profile of CD8 α /B220 expression in the analytically gated CD32⁺ cells is shown.

CD3 γ and CD3 δ , pre-TCR cannot be expressed on the surface of prothymocytes.

In addition to the structural requirement, CD3 γ and CD3 δ may regulate pre-TCR function through the signaling capacity of the immunoreceptor tyrosine-based activation motifs (ITAMs) presented in their cytoplasmic domains (34). It is known that not every ITAM plays a distinct role in pre-TCR function. For instance, pre-TCR function is competent in mutant mice deficient in the CD3 ζ cytoplasmic domain (35). Moreover, the defect in pre-TCR function in CD3 $\gamma^{-/-}$ (24), CD3 $\zeta^{-/-}$ (36), or RAG $^{-/-}$ (19, 27, 37) mice can be overcome by anti-CD3 ϵ -mediated cross-linking. However, the same anti-CD3 ϵ treatment in vivo in CD3 $\gamma\delta^{-/-}$ mice failed to relieve the block at the DN check point (data not shown). Since the anti-CD3 ϵ antibody used in all of these studies, namely 2C11 (or 500A2), binds CD3 ϵ efficiently when either CD3 γ or CD3 δ is presented but poorly when both CD3 γ and CD3 δ are missing (38; data not shown), the lack

Table 1. T Cell and B Cell Compositions in $CD3\gamma\delta^{-/-}$ Mice

Tissue	Cell	$CD3\gamma\delta^{-/-}$	Wild type
Thymus	Cellularity (% of wt)	2.5 ± 1.1	100 ± 21
LN	TCR- $\alpha\beta^{+}$	0	73 ± 9
	B220 ⁺ CD19 ⁺	84 ± 2	19 ± 4
Spleen	TCR- $\alpha\beta^{+}$	0	35 ± 3
	B220 ⁺ CD19 ⁺	67 ± 6	47 ± 10
iIEL	TCR- $\alpha\beta^{+}$	0	41 ± 5
	TCR- $\gamma\delta^{+}$	0	50 ± 6
	CD8- α^{+}	64 ± 5	66 ± 10

A total of 9–14 mice of each type were analyzed in five independent experiments, and data are pooled and shown as mean \pm SD. Thymic cellularity was determined as shown in Fig. 3 A. Data for the peripheral lymphoid tissues and iIEL were obtained by cytometric analyses, and represent the percent of cells positive for the indicated marker in the whole lymphocyte population.

*CD8 α^{+} iIEL are CD8 $\alpha\alpha^{+}$ CD32⁺B220⁺NK1.1⁻ T cell precursors in $CD3\gamma\delta^{-/-}$ mice (Fig. 4; 27). In wild-type mice, CD8 α^{+} iIEL consist of both CD8 $\alpha\alpha^{+}$ and CD8 $\alpha\beta^{+}$ cells, and are mostly TCR- $\alpha\beta^{+}$ CD3⁺ (27).

of thymocyte differentiation upon 2C11 treatment of $CD3\gamma\delta^{-/-}$ mice might be explained by the following non-exclusive possibilities: (a) pre-TCR could not be expressed on the surface of $CD3\gamma\delta^{-/-}$ prothymocytes; (b) the inefficient binding of 2C11 to CD3 ϵ on the surface of $CD3\gamma\delta^{-/-}$ prothymocytes results in a weak signal that is below the threshold level for further thymic development; and (c) the cytoplasmic domains of CD3 γ and CD3 δ collectively play an essential role in pre-TCR function. The last possibility, nevertheless, is less likely because it has been shown that under artificial circumstances, either CD3 ϵ or CD3 ζ cytoplasmic domain alone can independently generate signals for thymocyte development to the DP stage (39). Thus, the ultimate assessment of the physiological role of the cytoplasmic domains of CD3 γ and CD3 δ awaits the generation of mutant mice in which the cytoplasmic domains of CD3 γ and CD3 δ are deleted.

An important observation of this study was that $\gamma\delta$ T cell development was completely blocked in the $CD3\gamma\delta^{-/-}$ mice. In comparison, $\gamma\delta$ T cell development was partially blocked in the $CD3\gamma^{-/-}$ mice and was undisturbed in $CD3\delta^{-/-}$ mice (23, 24). Thus, this study demonstrated that CD3 δ also plays a role in regulating the development of the $\gamma\delta$ T cell lineage, and CD3 γ and CD3 δ collectively are essential for $\gamma\delta$ T cell development. Like their regulation of $\alpha\beta$ T cell development, CD3 γ and CD3 δ may regulate $\gamma\delta$ T cell development by their structural contribution and/or signaling capacity. Nevertheless, the function of CD3 γ or CD3 δ for $\gamma\delta$ T cells may not be a duplication of their respective roles for $\alpha\beta$ T cells. For instance, although surface expression of TCR- $\alpha\beta$ is severely reduced (8–10-fold) in $CD3\delta^{-/-}$ mice, their TCR- $\gamma\delta$ expression is only mildly

(less than twofold) reduced (23). On the other hand, severe reduction of both TCR- $\alpha\beta$ and TCR- $\gamma\delta$ expression in CD3 $\gamma^{-/-}$ mice indicated a pivotal role of CD3 γ in the assembly of TCR- $\alpha\beta$ -CD3 and TCR- $\gamma\delta$ -CD3 complexes (24). Taken together, it is likely that the complete block in $\gamma\delta$ T cell development in CD3 $\gamma\delta^{-/-}$ mice was a result of the incomplete TCR- $\gamma\delta$ -CD3 complex not being expressed on cell surface in the absence of CD3 γ and CD3 δ . It remains to be investigated whether the cytoplasmic domains of CD3 γ and CD3 δ also have distinct functions in the development of $\gamma\delta$ T cells.

In conclusion, in the CD3 $\gamma\delta^{-/-}$ mice, early thymic development mediated by pre-TCR was completely blocked, and TCR- $\alpha\beta^+$ and TCR- $\gamma\delta^+$ T cells were absent. These observations are different from those made on either CD3 $\delta^{-/-}$ or CD3 $\gamma^{-/-}$ mice, in which pre-TCR function was either undisturbed or incompletely blocked, as TCR- $\alpha\beta^+$ and TCR- $\gamma\delta^+$ T cells were detected in the periphery. Taken together, these studies demonstrated that CD3 γ and CD3 δ play an essential, yet partially overlapping, role in the development of both $\alpha\beta$ and $\gamma\delta$ T cell lineages.

This work was supported by National Institutes of Health grants CA 74233 (to B. Wang) and AI35714 and R37-17651 (to C. Terhorst).

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Received for publication 26 May 1998 and in revised form 22 July 1998.

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