Essential and Partially Overlapping Role of CD3γ and CD3δ for Development of αβ and γδ T Lymphocytes

By Baoping Wang,* Ninghai Wang,* Mariolina Salio,* Arlene Sharpe,§ Deborah Allen,* Jian She,* and Cox Terhorst*

From the *Division of Immunology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215; and the ‡Department of Pathology, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115.

Summary
CD3γ and CD3δ 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 CD3δ or CD3γ, early thymic development mediated by pre-TCR was either undisturbed or severely blocked, respectively, and small numbers of TCR-αβ T cells were detected in the periphery of both mice. γδ T cell development was either normal in CD3δ-/- mice or partially blocked in CD3γ-/- mice. To examine the collective role of CD3γ and CD3δ in the assembly and function of pre-TCR and in the development of γδ T cells, we generated a mouse strain with a disruption in both CD3γ and CD3δ genes (CD3γδ-/-). In contrast to mice deficient in either CD3γ or CD3δ chains, early thymic development mediated by pre-TCR is completely blocked, and TCR-αβ+ or TCR-γδ+ T cells were absent in the CD3γδ-/- mice. Taken together, these studies demonstrated that CD3γ and CD3δ play an essential, yet partially overlapping, role in the development of both αβ and γδ T cell lineages.

Key words: CD3γ • CD3δ • T cell receptor-CD3 complex • T cell development • knockout mouse

During thymocyte development, the genes coding for TCR-α and -β, pre-TCR-α (pTα), and the associated CD3 proteins (CD3γ, δ, ε, and ζ) are expressed in a temporal order (1). The pre-TCR-CD3 complex, consisting of pTα, 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 pTα, 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 pTα and the resulting TCR-CD3 complex mediates signal transduction cascades leading to further T cell development (2). Compared with αβ T cell development, γδ T cell development is less defined (4, 5). The majority of thymic γδ T cells do not express CD4 or CD8 antigens (6), and pTα and TCR-β are not involved in the development of γδ 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 expression of TCR (9). In each TCR-CD3 complex, there are two copies of CD3ε and CD3ζ, yet only one copy of the highly homologous CD3γ and CD3δ (10–12). CD3ε forms heterodimers with CD3γ and CD3δ, and can also exist as a CD3ε homodimer, whereas CD3ζ exists as a CD3ζζ homodimer (11, 13, 14). TCRs lacking CD3γ, 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 pTα 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, CD3γε dimers are consistently detected (20, 21), and some studies indicated the presence of a small quantity of CD3δε dimers (18–21). This led to the notion that CD3γ may be preferentially required over CD3ε in the assembly of pre-TCR complexes (22).

Recent studies on mutant mice deficient in either the CD3γ or CD3δ gene in part support this notion. Whereas transition from DN to DP αβ thymocytes appears to be normal in CD3δ-/- mice (23), αβ T cell development in CD3γ-/- mice is blocked at the DN CD44-CD25+ check point (24). However, the blockade in T cell development in...
CD3γδ−/− mice is incomplete, as small numbers of DP thymocytes were found and TCR-αβ+ T cells were detected in the periphery (24). Moreover, in either mutant a considerable number of γδ T cells is present (23, 24). Therefore, it is likely that CD3γ and CD3δ 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 CD3γ and CD3δ, a mouse strain with a disruption in both the CD3γ and CD3δ genes (CD3γδ−/−) would be useful. A CD3γδ−/− mouse, however, could not be generated by breeding the CD3δ−/− and CD3γ−/− mice, because the genes coding for CD3γ, δ, and ε are located in a single gene cluster and a mere 1.4-kb intergenic sequence separates the first exons of CD3γ and CD3δ genes (25). Therefore, we generated CD3γδ−/− mice by deleting the promoters and exons 1 of both genes.

Materials and Methods

Generation of CD3γδ−/− Mice. The targeting construct was generated by standard methods. In brief, a genomic DNA clone containing a 15.5-kb fragment of CD3γδ genes was isolated from a 129/nu 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-TKr gene was isolated from pPGK-TKr (provided by Dr. Manley Huang), and ligated to the XhoI site of pPGK-hygromycine (hyg) (a gift from Dr. Richard M ortensen). A 1.9-kb XbaI-XbaI intronic fragment between exon 1 and 2 of CD3δ was obtained by XbaI digestion of the 15.5-kb CD3γδ genomic DNA fragment. And a 3-kb intronic fragment between exon 1 and 2 of CD3δ 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 gene. In the resulting construct, a 3.1-kb DNA fragment containing the 1.4-kb intergenic DNA fragment between the CD3γ and CD3δ genes and exons 1 of both genes were replaced by the 2.8-kb PGK-Hyg′ cassette. 10 μg of purified targeting molecules were electroporated into 107 J-1 ES cells. ES cells were positively selected by hygromycin-B at 200 μg/ml and negatively selected by FIAU at 0.2 μM. 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′ 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 CD3γδ−/− lines were obtained by sibling breeding. Identical results were obtained from homozygous CD3γδ−/− 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).

Results

Generation of CD3γδ−/− Mice. To generate mice deficient in both CD3γ and CD3δ gene expression, a 3.1-kb DNA fragment containing the promoters (25) and exons 1 of the CD3γ and CD3δ genes was replaced by a PGK-Hyg′ cassette (Fig. 1 A). The PGK-hyg′ cassette was chosen here over the PGK-neo′ cassette to prevent a possible suppressive effect of the PGK-neo′ on neighboring gene expression (30, 31). Homozygous mice carrying this mutation in the CD3γ and δ genes were generated (Fig. 1 B). Northern blot analysis demonstrated that the expression of both CD3γ and CD3δ mRNA was absent in the CD3γδ−/− thymocytes (Fig. 2). Moreover, no aberrant expression of the truncated CD3γ or δ mRNAs was ever detected in Northern blotting of thymocytes from more than 20 CD3γδ−/− mice. However, the expression of the neighboring CD3δ gene and the nonlinked CD3γ was normal (Fig. 2), and pTα expression was detected (data not shown).

αβ T Cell Development in the CD3γδ−/− Mice. Total cellularity of the thymi of CD3γδ−/− mice was 2–5% of that in wild-type or heterozygous litters (Fig. 3 A). Flow cytometric analysis of the thymocytes showed that these cells are DN, with the majority of them being TCR-β−/− mice (Fig. 3 B). Northern blot analyses of the thymocytes of CD3γδ−/− mice did not detect the mRNAs for rearranged TCR-α and TCR-β genes, whereas only the 1.0-kb germline Cβ mRNA was detectable (Fig. 3 B).
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 and 4, 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 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 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 CD36−/− 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 CD36 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
CD3γ and CD3δ, pre-TCR cannot be expressed on the surface of prothymocytes.

In addition to the structural requirement, CD3γ and CD3δ 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 CD3ζ cytoplasmic domain (35). Moreover, the defect in pre-TCR function in CD3γδ−/− (24), CD3ζ−/− (36), or RAG−/− (19, 27, 37) mice can be overcome by anti-CD3ε-mediated cross-linking. However, the same anti-CD3ε treatment in vivo in CD3γδ−/− mice failed to relieve the block at the DN check point (data not shown). Since the anti-CD3ε antibody used in all of these studies, namely 2C11 (or 500A2), binds CD3ε efficiently when either CD3γ or CD3ζ is present but poorly when both CD3γ and CD3ζ are missing (38; data not shown), the lack of thymocyte differentiation upon 2C11 treatment of CD3γδ−/− mice might be explained by the following non-exclusive possibilities: (a) pre-TCR could not be expressed on the surface of CD3γδ−/− prothymocytes; (b) the inefficient binding of 2C11 to CD3ε on the surface of CD3γδ−/− prothymocytes results in a weak signal that is below the threshold level for further thymic development; and (c) the cytoplasmic domains of CD3γ and CD3ζ 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 CD3ε or CD3ζ 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 CD3γ and CD3ζ collectively plays an essential role in pre-TCR function. Like their regulation of CD3ζ and CD3ζ, the cytoplasmic domain of CD3ζγδ−/− mice is severely reduced (8–10-fold) in CD3ζγδ−/− mice, their TCR-αβ+ T cells may be a duplication of their respective roles for αβ T cells. For instance, although surface expression of TCR-αβ is severely reduced (8–10-fold) in CD3ζγδ−/− mice, their TCR-γδ expression is only mildly

**Table 1.** T Cell and B Cell Compositions in CD3γδ−/− Mice

| Tissue     | Cell            | CD3γδ−/− | Wild type |
|------------|-----------------|----------|-----------|
| Thymus     | Cellularity (% of wt) | 2.5 ± 1.1 | 100 ± 21  |
| LN         | TCR-αβ+         | 0        | 73 ± 9    |
| B220+CD19⁺ |                 | 84 ± 2   | 19 ± 4    |
| Spleen     | TCR-αβ⁺         | 0        | 35 ± 3    |
| B220⁺CD19⁺ |                 | 67 ± 6   | 47 ± 10   |
| iIEL       | TCR-αβ⁺         | 0        | 41 ± 5    |
| TCR-γδ⁺    |                 | 0        | 50 ± 6    |
| CD8α⁺γδΔ⁻  |                 | 64 ± 5   | 66 ± 10   |

A total of 9–14 mice of each type were analyzed in five independent experiments, and data are pooled as shown as mean ± SD. Thymic cellularity was determined as shown in Fig. 3A. Data for the peripheral lymphoid tissues and iIEL were obtained by cytomtric analyses, and represent the percent of cells positive for the indicated marker in the whole lymphocyte population. *CD8α⁺⁺ iIEL are CD8α⁺⁺CD3⁺⁺B220⁺⁺NK1.1− T cell precursors in CD3γδ−/− mice (Fig. 4). In wild-type mice, CD8α⁺⁺ iIEL consist of both CD8α⁺⁺⁺ and CD8αβ⁺ cells, and are mostly TCR-αβ⁺⁺CD3⁺⁺ (27).
Taken together, it is likely that the complete block in γδ T cell development in CD3γδ−/− mice was a result of the incomplete TCR-γδ–CD3 complex not being expressed on cell surface in the absence of CD3γ and CD3δ. It remains to be investigated whether the cytoplasmic domains of CD3γ and CD3δ also have distinct functions in the development of γδ T cells.

In conclusion, in the CD3γδ−/− mice, early thymic development mediated by pre-TCR was completely blocked, and TCR-αβ+ and TCR-γδ+ T cells were absent. These observations are different from those made on either CD3δ−/− or CD3γ−/− mice, in which pre-TCR function was either undisturbed or incompletely blocked, as TCR-αβ+ and TCR-γδ+ T cells were detected in the periphery. Taken together, these studies demonstrated that CD3γ and CD3δ play an essential, yet partially overlapping, role in the development of both αβ and γδ T cell lineages.

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