The CD3γ chain is essential for development of both the TCRαβ and TCRγδ lineages

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CD3γ and CD3δ are the most closely related CD3 components, both of which participate in the TCRαβ–CD3 complex expressed on mature T cells. Interestingly, however, CD3δ does not appear to participate functionally in the pre-T-cell receptor (TCR) complex that is expressed on immature T cells: disruption of CD3δ gene expression has no effect on the developmental steps controlled by the pre-TCR. Here we report that in contrast with CD3δ, CD3γ is an essential component of the pre-TCR. We generated mice selectively lacking expression of CD3γ, in which expression of CD3δ, CD3ε, CD3ζ, pTα and TCRβ remained undisturbed. Thus, all components for composing a pre-TCR are available, with the exception of CD3γ. Nevertheless, T-cell development is severely inhibited in CD3γ-deficient mice. The number of cells in the thymus is reduced to <1% of that in normal mice, and the large majority of thymocytes lack CD4 and CD8 and are arrested at the CD44⁻CD25⁺ double negative (DN) stage of development. Peripheral lymphoid organs are also practically devoid of T cells, with absolute numbers of peripheral T cells reduced to only 2–5% of those in normal mice. Both TCRαβ and TCRγδ lineages fail to develop effectively in CD3γ-deficient mice, although absence of CD3γ has no effect on gene rearrangements of the TCRβ, δ and γ loci. Furthermore, absence of CD3γ results in a severe reduction in the level of TCR and CD3ε expression at the cell surface of thymocytes and peripheral T cells. The defect in the DN to double positive transition in mice lacking CD3γ can be overcome by anti-CD3ε-mediated cross-linking. CD3γ is thus essential for pre-TCR function.

Keywords: CD3γ-deficient/gene targeting/pre-TCR/T-cell development

Introduction

Based on surface expression of several key markers, intrathymic T-cell differentiation can be subdivided into several distinct developmental stages. The most immature thymocytes reside within the CD4⁻CD8⁻ double negative (DN) population, comprising only 1–3% of the total number of thymocytes. Within the DN population, the most immature subset has a c-kit⁺CD44⁺CD25⁻ phenotype and gives rise to c-kit⁺CD44⁺CD25⁺ cells. The c-kit⁺CD44⁺CD25⁻ DN subset has the TCRγ, δ and β loci in germline configuration and is not yet fully committed to the T-cell lineage (Wu *et al.*, 1991; Ardavin *et al.*, 1993; Godfrey *et al.*, 1993; Carlyle *et al.*, 1997); this population contains progenitors for NK, B, T and thymic dendritic cells. The c-kit⁺CD44⁺CD25⁺ population represents so-called pro-T-cells, in which some steps towards T-cell commitment have occurred. c-kit⁻CD44⁻CD25⁺ early and c-kit⁻CD44⁻CD25⁻ late pre-T-cells represent populations in which TCR gene rearrangements have been initiated and irreversible commitment to T-cell development has occurred (Godfrey *et al.*, 1993, 1994; Wilson *et al.*, 1994; Shortman *et al.*, 1996). Cells that have undergone productive TCRβ gene rearrangement progress to the CD4⁺CD8⁺ double positive (DP) stage via CD4 or CD8 immature single positive intermediates (reviewed in Fehling and Von Boehmer, 1997), with a concomitant burst in cell proliferation (Hoffman *et al.*, 1996). Following completion of TCRα rearrangements and production of a mature TCRαβ complex, DP thymocytes are subjected to positive and negative selection (reviewed in Kisielow and Von Boehmer, 1995; Guidos, 1996). Thymocytes surviving this selection process shut off expression of either the CD4 or CD8 co-receptors. The resulting CD4 and CD8 single positive (SP) cells represent the end product of intrathymic αβ T-cell development, and will eventually exit the thymus and migrate to the peripheral lymphoid organs (reviewed in Kisielow and Von Boehmer, 1995; Guidos, 1996).

Within the DN pre-T-cell population, the transition from the c-kit⁻CD44⁻CD25⁺ to the c-kit⁻CD44⁻CD25⁻ stage of development is the first major checkpoint that thymocytes encounter during their developmental program. Only cells that produce a functional TCRβ protein expand and proceed to the next developmental stage. This selection process is mediated by the pre-TCR (Dudley *et al.*, 1994; Levelt and Eichmann, 1995; Fehling and Von Boehmer, 1997). Signaling through the pre-TCR terminates recombination at the TCRβ locus and promotes cell-cycle entry (Hoffman *et al.*, 1996), expansion and maturation to the DP stage. Gene targeting experiments have helped to identify essential components of the pre-TCR (reviewed in Borst *et al.*, 1996; Malissen and Malissen, 1996), which is minimally composed of a heterodimer of a conventional TCRβ chain and the monomorphic pTα chain, as well as non-covalently linked CD3 subunits (Groettrup *et al.*, 1993; Jacobs *et al.*, 1994; Saint-Ruf *et al.*, 1994; Van Oers *et al.*, 1995; Berger *et al.*, 1997). TCRβ- and pTα-deficient mice (Mombaerts *et al.*, 1992a; Fehling *et al.*, 1995), as well as RAG⁻/⁻ mice (Mombaerts *et al.*, 1992b; Shinkai *et al.*, 1992) which cannot generate a TCRβ chain, exhibit an arrest early in T-cell development at the CD44⁻CD25⁺ DN stage. Introduction of a productively

rearranged TCRβ transgene into RAG$^{-/-}$ mice relieves the developmental blockade and allows progression to the DP stage, providing direct evidence for the essential role of the TCRβ chain within the pre-TCR complex (Shinkai *et al.*, 1993). Nevertheless, in contrast to the absolute developmental arrest observed in RAG$^{-/-}$ mice, some thymocytes in TCRβ$^{-/-}$ and pTα$^{-/-}$ mice do progress to the DP stage. This may be due to signaling through an aberrant pre-TCR complex, possibly reflecting the ability of other TCR chains to replace TCRβ and pTα to some extent (Buer *et al.*, 1997). In addition, as yet undefined indirect effects from the presence of other TCR-expressing cell types may contribute to development of DP cells (Shores *et al.*, 1990; Lynch and Shevach, 1993).

Some insights into how the pre-TCR may function as a molecular sensor have recently been obtained. The pTα chain does not appear to contribute to pre-TCR signaling: expression of a transgene encoding tailless pTα in pTα$^{-/-}$ mice restores thymopoiesis in these mice (Fehling *et al.*, 1997). The possible contribution of the CD3ζ chain to the pre-TCR also appears to be resolved: introduction of a signaling-deficient CD3ζ transgene into CD3ζ$^{-/-}$ mice restored thymopoiesis, indicating that CD3ζ is an essential building block of the pre-TCR, but that its signaling capacity is not absolutely required for thymocyte development (Love *et al.*, 1993; Malissen *et al.*, 1993; Ohno *et al.*, 1993; Shores *et al.*, 1994). There is, however, abundant evidence for a link between CD3 components and downstream signaling events in pre-TCR-mediated signal transduction. Treatment of RAG$^{-/-}$ or TCRβ$^{-/-}$ fetal thymic organ cultures (FTOC) or injection of RAG$^{-/-}$ or RAG$^{-/-}$×pTα$^{-/-}$ mice *in vivo* with anti-CD3ε monoclonal antibody leads to the generation of DP thymocytes (Levelt *et al.*, 1993; Jacobs *et al.*, 1994; Shinkai and Alt, 1994; Levelt *et al.*, 1995; Fehling *et al.*, 1997). Consistent with these functional data, low levels of CD3ε can be detected on the surface of pro- and pre-T-cells (Groettrup *et al.*, 1993; Jacobs *et al.*, 1994; Shinkai and Alt, 1994; Wiest *et al.*, 1994; Berger *et al.*, 1997). Whether the CD3ε subunit itself contributes to the function of the pre-TCR has yet to be resolved since CD3ε$^{-/-}$ mice unexpectedly also lacked transcription of CD3δ and showed severely decreased levels of CD3γ transcription (Malissen *et al.*, 1995). Nevertheless, the developmental block at the CD44$^-$CD25$^+$ pre-T-cell stage observed in these CD3ε-deficient mice (Malissen *et al.*, 1995) is in keeping with the notion that CD3γ, δ and/or ε components are required for pre-TCR function. The CD3δ chain, however, appears to be dispensable for pre-TCR function: thymopoiesis proceeds normally up to the DP stage in CD3δ$^{-/-}$ mice (Dave *et al.*, 1997). The lack of a defect in pre-T-cell development in CD3δ-deficient mice thus points towards the CD3ε and the CD3γ chains as the likely components that endow the pre-TCR with signaling function.

Here we examine the *in vivo* contribution of the CD3γ chain to T-cell development in CD3γ-deficient mice. Although TCRβ, pTα, CD3ε, CD3δ and CD3ζ are expressed in the thymus of these mice, a lack of CD3γ results in a phenotype identical to that of other mutant mouse strains in which essential components of the pre-TCR are missing. CD3γ thus has a crucial role in pre-T-cell development which cannot be replaced by other CD3 components.

Results

Generation of CD3γ null-mutant mice

CD3γ null-mutant mice were generated by gene targeting in embryonic stem (ES) cells using CD3γ–Δ4 as a targeting vector (Figure 1A). Two homologous recombinants were identified out of 192 colonies tested, and both were used for the generation of chimeric mice (Figure 1B). Germline transmission was obtained with both recombinants and heterozygous mice were intercrossed to produce mice homozygous for the CD3γ mutation (Figure 1C). The two independently-derived strains of CD3γ null-mutant mice displayed a similar phenotype and were alternately used in the experiments described in this study.

To examine the production of CD3γ mRNA transcripts in CD3γ$^{-/-}$ mice, total RNA extracted from wild-type (WT), heterozygous and homozygous CD3γ-mutant thymocytes, as well as RAG-1$^{-/-}$ thymocytes, was analyzed by Northern blot analysis. CD3γ$^{-/-}$ thymocytes lacked any detectable expression of CD3γ mRNA, whereas in CD3γ$^{+/+}$, CD3γ$^{+/-}$ and RAG-1$^{-/-}$ thymocytes, CD3γ transcripts of the expected size (0.8 kb) were present in abundance (Figure 2A). Since transcription of the CD3γ gene has been documented to peak at the CD25$^+$ DN stage (Wilson and MacDonald, 1995), the increased CD3γ mRNA expression level observed in RAG-1$^{-/-}$ thymocytes compared with WT thymocytes probably reflects the preponderance of CD44$^-$CD25$^+$ DN thymocytes in RAG-1$^{-/-}$ mice. RT–PCR analysis of total thymocyte RNA confirmed the complete absence of intact CD3γ mRNA (543 bp) in CD3γ$^{-/-}$ thymocytes, although some aberrant truncated transcripts were produced corresponding in size (411 bp) to CD3γ transcripts lacking exon 4 (Figure 2B).

Given the fact that the mouse CD3γ, δ and ε genes are clustered together on the genome and reside within 50 kb of each other (Saito *et al.*, 1987), and given the precedent that CD3ε$^{-/-}$ mice unexpectedly lacked transcription of CD3δ and displayed severely decreased levels of CD3γ mRNA (Malissen *et al.*, 1995), we next investigated mRNA expression levels of other CD3 components in CD3γ$^{-/-}$ mice. Northern blot analysis indicated that introduction of the pgk-neo cassette within the CD3γ gene had no discernible effect on the transcriptional activity of the closely linked CD3δ and CD3ε genes (Figure 2A). Equivalent amounts of CD3δ and CD3ε mRNA were detected in CD3γ$^{-/-}$ thymocytes compared with CD3γ$^{+/+}$, CD3γ$^{+/-}$ and RAG-1$^{-/-}$ thymocytes (Figure 2A). Furthermore, the CD3ζ gene is expressed in CD3γ$^{-/-}$ thymocytes, albeit at lower levels than in WT and heterozygous mutant thymocytes (Figure 2A), but comparable with the level observed in RAG-1$^{-/-}$ thymocytes. This is consistent with the fact that CD3ζ mRNA-production peaks at the DP thymocyte stage (Wilson and MacDonald, 1995) which is not reached effectively in CD3γ$^{-/-}$ mice (see below). pTα and full-length TCRβ and TCRα mRNA could also be detected in CD3γ$^{-/-}$ thymocytes (Figure 2A and B; data not shown). Similar to the quantity of CD3ζ transcripts, the quantity of TCRβ and TCRα transcripts is somewhat lower, reflecting impairment in development beyond the DN stage (see below).

To analyse TCR gene rearrangements, a DNA–PCR assay was performed on DNA derived from CD3γ$^{+/+}$, CD3γ$^{+/-}$, CD3γ$^{-/-}$ and RAG-1$^{-/-}$ thymocytes. Absence of

A

B

C
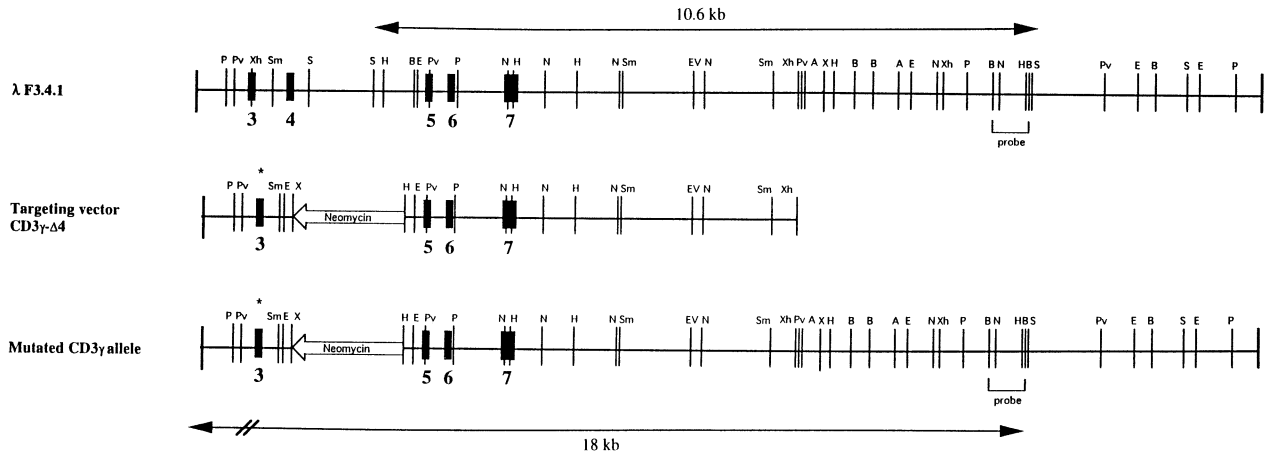
Fig. 1. Disruption of the CD3γ gene by homologous recombination. (**A**) Partial organization of the CD3γ locus (top), structure of the targeting vector CD3γ–Δ4 (middle) and structure of the disrupted CD3γ gene following homologous recombination (bottom). The asterisk indicates the location of the destroyed *Xho*I site in exon 3 resulting in a frame shift further downstream in case a truncated mRNA is produced by skipping exon 4 during splicing events. The probe used for the hybridization and the predicted fragment sizes generated by the endogenous and targeted alleles after *Sac*I digestion are depicted. Restriction enzymes: A, *Apa*I; B, *Bam*HI; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; N, *Nsi*I; P, *Pst*I; Pv, *Pvu*II; S, *Sac*I; Sa, *Sal*I; Sm, *Sma*I; X, *Xba*I; Xh, *Xho*I. (**B**) Southern blot analysis of *Sac*I-digested DNA extracted from ES cell line HM-1 and two independently derived homologous recombinant ES clones HM-1.23 and HM-1.57. (**C**) Southern blot analysis of *Sac*I-digested tail DNA derived from WT (CD3γ$^{+/+}$), heterozygous mutant (CD3γ$^{+/-}$) and homozygous mutant (CD3γ$^{-/-}$) mice.
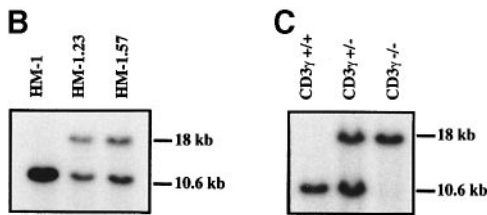
CD3γ had no discernible effect on the occurrence of TCRβ gene rearrangements, since V-DJ rearrangements were as extensive in the thymus of CD3γ$^{-/-}$ mice as in the thymus of WT and CD3γ$^{+/-}$ mice (Figure 2C). This is consistent with the Northern blot data (Figure 2A), which showed production of 1.3 kb full-length TCRβ mRNA transcripts in CD3γ$^{-/-}$ thymocytes. TCRγ and TCRδ gene rearrangements also proceeded undisturbed (Figure 2C). Taken together, these findings document that the absence of CD3γ is of no consequence to the extent of TCR gene rearrangements. Furthermore, expression of all known components of the pre-TCR complex, with the exception of the CD3γ chain, is intact in CD3γ$^{-/-}$ mice.

Thymocyte development in CD3γ$^{-/-}$ mice

The thymus of 6- to 8-week-old CD3γ$^{-/-}$ mice is dramatically reduced in size, averaging 1×10^6 cells compared with 200×10^6 cells observed in heterozygous and WT littermate controls. Although all thymocyte subsets characterized on the basis of expression of CD4 and CD8 co-receptors could be detected in CD3γ homozygous mutant mice, ~85–90% of the thymocytes were of the CD4$^-$CD8$^-$ DN phenotype, whereas in WT and heterozygous mice this population comprised only 1–3% (Figure 3A, top panel). Nevertheless, the absolute number of CD4$^-$CD8$^-$ thymocytes within CD3γ$^{-/-}$ mice is equivalent to the number of CD4$^-$CD8$^-$ thymocytes within WT and heterozygous littermate controls, suggesting an early block in development at the transition to the DP stage. More detailed analysis of the DN population present in CD3γ$^{-/-}$ mice by

monitoring for cell-surface expression of CD44 and CD25 revealed that progression beyond the CD44$^-$CD25$^+$ DN stage is severely impaired in these mice, as demonstrated by a dramatic increase in the proportion of CD44$^-$CD25$^+$ cells and an almost complete absence of the CD44$^-$CD25$^-$ subset. In contrast, this latter subset represents ~30% of the DN population in CD3γ$^{+/+}$ and CD3γ$^{+/-}$ mice (Figure 3A, bottom panel). These findings are consistent with a crucial role for the CD3γ chain in pre-TCR function. In addition, these data document that none of the CD3 components which are present in the thymus of CD3γ-deficient mice (Figure 2A) can replace CD3γ.

Some thymocytes in CD3γ$^{-/-}$ mice appear to escape the developmental block imposed by absence of CD3γ, to the extent that some 5% DP and 5–10% SP cells can still be detected in the thymus (Figure 3A, top panel). This might reflect the ability of an incomplete pre-TCR to drive development of pre-T-cells, albeit less efficiently, or of other pre-TCR-independent processes to promote pre-T-cell development (Shores *et al.*, 1990; Lynch and Shevach, 1993; Buer *et al.*, 1997). Expecting a more exacerbated phenotype, we investigated thymopoiesis in newborn CD3γ$^{-/-}$ mice by staining for CD4 and CD8 coreceptors and observed a more dramatic developmental block at the transition to the DP stage (Figure 3B, top panel). In these young mice >97% of the thymocytes resided in the CD4$^-$CD8$^-$ DN compartment. Analysis of CD44 and CD25 expression in DN cells of newborn mice revealed that these cells, like those from adult mice, are arrested at the CD44$^-$CD25$^+$ DN stage, frequently with

increased numbers of thymocytes within the CD44$^+$CD25$^-$ compartment as well (Figure 3B, bottom panel).

Surface expression of CD3ε was investigated in the thymocyte subsets that are characterized based on the differential expression of CD4 and CD8 coreceptors. CD3ε surface-expression can be observed within all thymocyte subsets in CD3γ$^{-/-}$ mice, albeit at greatly reduced levels (Figure 4A). The hierarchy of the expression levels, however, is comparable with that in the normal thymus, with SP cells expressing the highest levels, followed by CD4$^+$CD8$^+$ cells and finally CD4$^-$CD8$^-$ cells, in which

CD3ε expression is practically undetectable. The reduced expression levels are likely to reflect a role for CD3γ in (pre-)TCR complex formation and/or transport to the cell surface since mRNA expression levels for CD3ε are comparable between WT- and CD3γ-homozygous mutant mice (Figure 2A). Analysis of CD25 expression revealed that downregulation of CD25 at the DN stage is severely impaired in the absence of CD3γ. In CD3γ-deficient mice ~55–70% of the DP thymocytes still express CD25, whereas in WT littermate controls this population comprises only 1–2% (Figure 4B). It has been suggested that this failure to downregulate CD25 is due to the generation of these DP cells in the absence of β-selection (Fehling and Von Boehmer, 1997), in which differentiation to the DP stage is induced without the concomitant cell expansion (Crompton *et al.*, 1994).
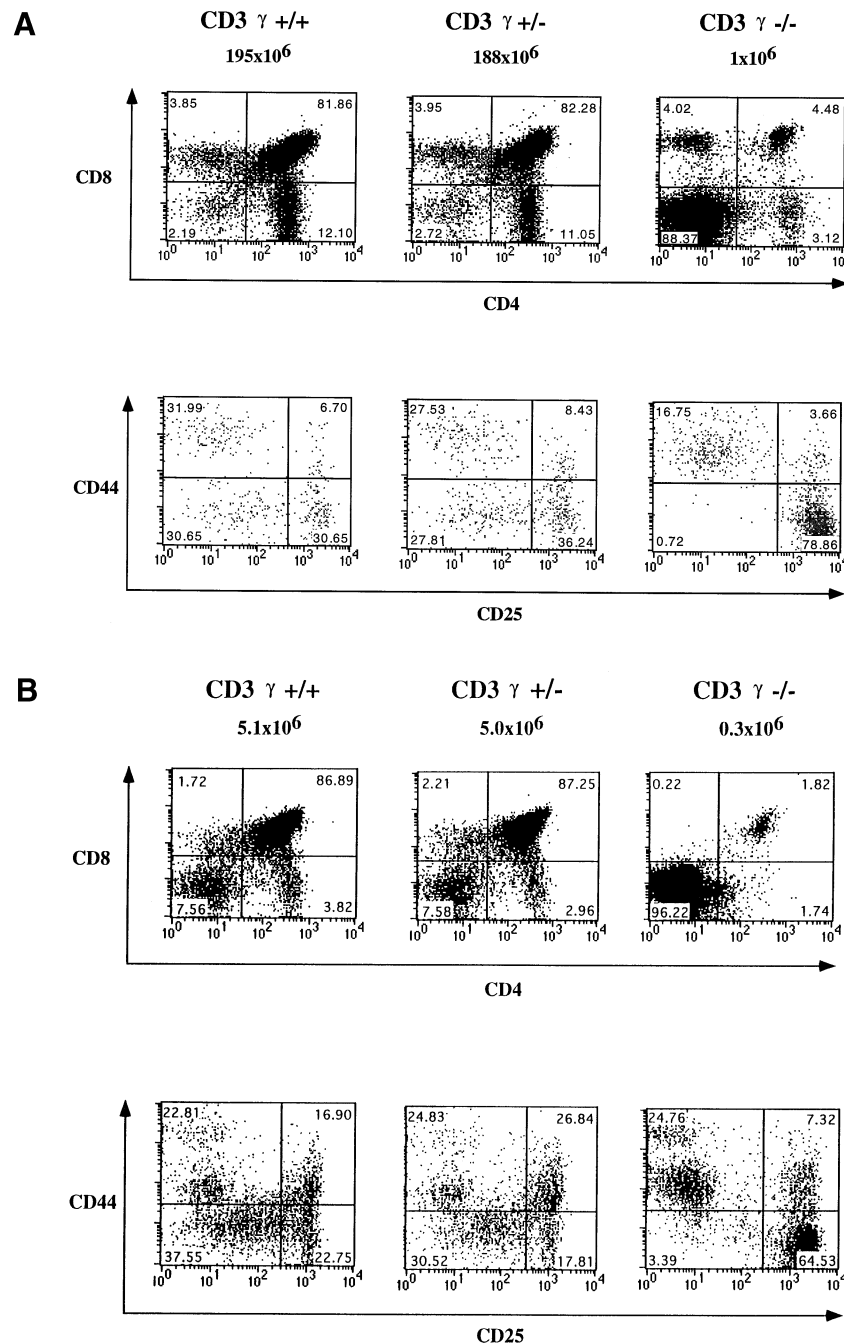
Taken together, the observations in the adult and new-born CD3γ$^{-/-}$ mice demonstrate that absence of the CD3γ chain results in a block in thymocyte development at a similar developmental stage as in RAG-1, CD3ε, TCRβ and pTα knock-out mice. These findings therefore document a crucial role for CD3γ in the pre-TCR complex. One of the ways in which CD3γ may function in the pre-TCR is by contributing to complex assembly, which is a prerequisite for transport from the ER to the cell surface. It is clear that CD3ε-expression levels on thymocytes from CD3γ$^{-/-}$ mice (Figure 4A) are severely compromised, and incomplete complex formation alone could be sufficient for reduced pre-TCR function. In a non-mutually exclusive hypothesis, the lack of unique CD3γ-dependent signaling events may further contribute to a reduction in pre-TCR function; the current production of mice lacking only the CD3γ-immunoreceptor tyrosine-based activation motif (ITAM) will resolve this issue.
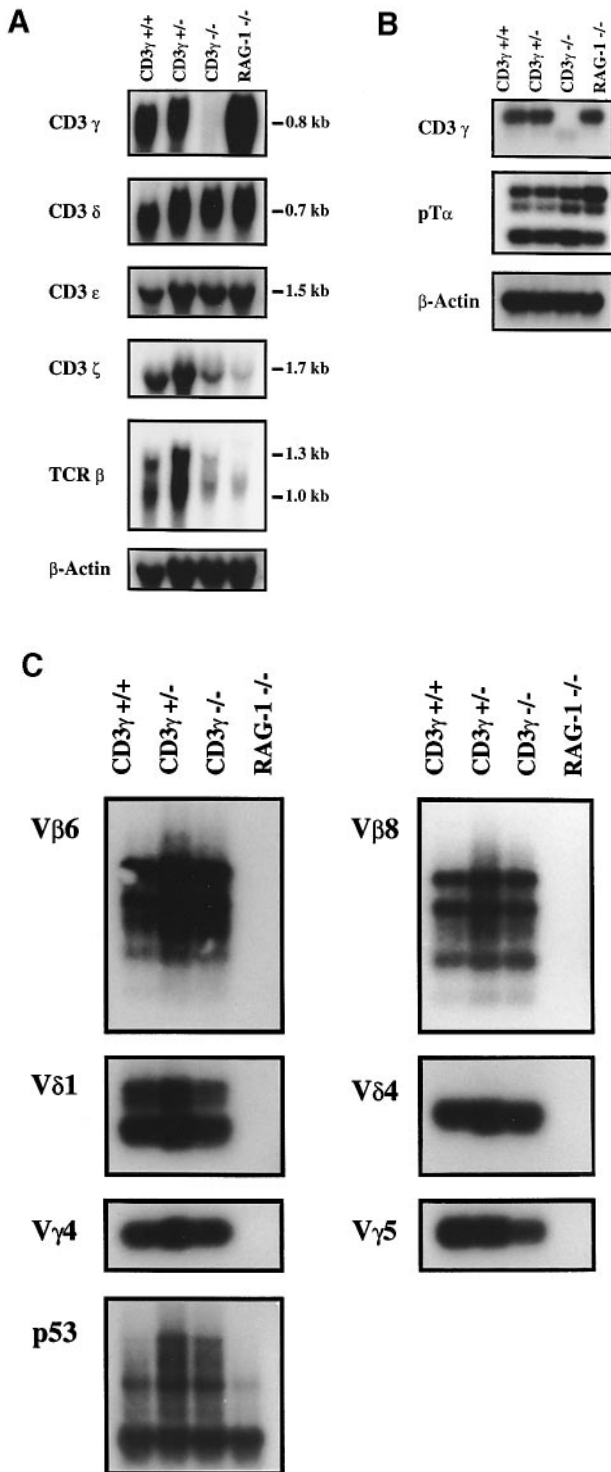
T cells in peripheral lymphoid organs of CD3γ$^{-/-}$ mice

As some SP thymocytes are generated in CD3γ$^{-/-}$ mice (Figure 3A), we addressed whether SP T cells can be detected in the peripheral T-cell repertoire. Indeed, small

Fig. 2. Expression of all components of the pre-TCR as well as TCR gene rearrangements are intact in CD3γ-deficient mice. (**A**) Northern blots were hybridized with cDNA probes corresponding to CD3γ, CD3δ, CD3ε, CD3ζ and TCRβ. To assess loading consistency, samples were hybridized with a β-actin probe. (**B**) RT–PCR products were probed by Southern blot hybridization with a cDNA probe corresponding to CD3γ (top) or an oligonucleotide specific for pTα (middle). β-actin was amplified and hybridized with a β-actin probe to demonstrate the presence of proper template in each sample (bottom). (**C**) DNA derived from CD3γ$^{+/+}$, CD3γ$^{+/-}$, CD3γ$^{-/-}$ and RAG-1$^{-/-}$ thymocytes was analyzed by DNA–PCR for the occurrence of TCRγ, δ and β gene rearrangements. TCRβ rearrangement was examined using Vβ8- and Vβ6-specific primers in combination with a Jβ2.5 primer. These primers amplify Vβ8/6–Jβ2.1 (850 bp), Vβ8/6–Jβ2.2 (650 bp), Vβ8/6–Jβ2.3 (380 bp), Vβ8/6–Jβ2.4 (240 bp) and Vβ8/6–Jβ2.5 (150 bp) fragments. Vδ1- and Vδ4-specific primers were used in combination with a Jδ1 primer to amplify rearranged TCRδ fragments of 290 bp and 170 bp, respectively. In addition, Vγ4- and Vγ5-specific primers were used in combination with a Jγ1 primer to amplify Vγ4–Jγ1 and Vγ5–Jγ1 rearranged TCRγ fragments. PCR products were probed by Southern blot hybridization with a cDNA probe corresponding to Jβ2 or a Jδ1- or Jγ1-specific oligonucleotide. As expected, thymocytes of RAG-1$^{-/-}$ mice do not reveal TCRγ, δ or β rearrangements. Genomic p53 was amplified (640 bp) and hybridized with a p53 cDNA probe to demonstrate that proper template was present in each sample.

A

CD3 γ +/+
195x10^6

CD3 γ +/-
188x10^6

CD3 γ -/-
1x10^6

B

CD3 γ +/+
5.1x10^6

CD3 γ +/-
5.0x10^6

CD3 γ -/-
0.3x10^6

Fig. 3. Thymopoiesis beyond the CD44$^-$25$^+$ DN stage is severely impaired in CD3γ$^{-/-}$ mice. Flow cytometry analysis of thymocytes from (**A**) 6- to 8-week-old or (**B**) newborn CD3γ$^{+/+}$, CD3γ$^{+/-}$ and CD3γ$^{-/-}$ mice. Total thymocytes were monitored for the expression of CD4-PE versus CD8-Biotin plus SA-Tricolor (top panels), while the expression of CD25-Biotin plus SA-Tricolor versus CD44-PE was analyzed within the CD4$^-$CD8$^-$ thymocyte subset (bottom panels). The percentage of cells within each quadrant is indicated. The absolute number of thymocytes detected in the WT, heterozygous mutant and homozygous mutant genotype is depicted above the corresponding dot display.

numbers of B220$^-$ cells that express either CD3, CD4 and/or CD8 can be detected in the spleen and lymph nodes of these mice (Figure 5A). This population contains both CD4 SP and CD8 SP T cells, in a ratio comparable with those observed in heterozygous and WT littermate controls (Figure 5B). Nevertheless, the absolute number of peripheral T cells is significantly decreased, representing only 2–5% of the number of T cells in normal mice for lymph nodes, and only 20% of the T-cell number in normal mice for the spleen. In contrast, B-cell development proceeds unaltered in CD3γ$^{-/-}$ mice. Although the relative

number of peripheral B220$^+$ cells that do not express CD3, CD4 or CD8 is increased (Figure 5A), the absolute number of mature B220$^+$IgM$^+$ B cells in the spleen and lymph nodes of CD3γ-deficient mice was unchanged (data not shown).

Since the level of TCR expression on thymocytes from CD3γ$^{-/-}$ mice is significantly reduced (Figure 4A), we determined whether TCR expression on peripheral T cells is also affected by absence of the CD3γ chain. To what extent the different CD3 components contribute to efficient surface expression of mature TCRαβ complexes has not

A

CD3 γ +/+

CD3 γ -/-

CD3ε

B

CD3 γ +/+

CD3 γ -/-

CD25
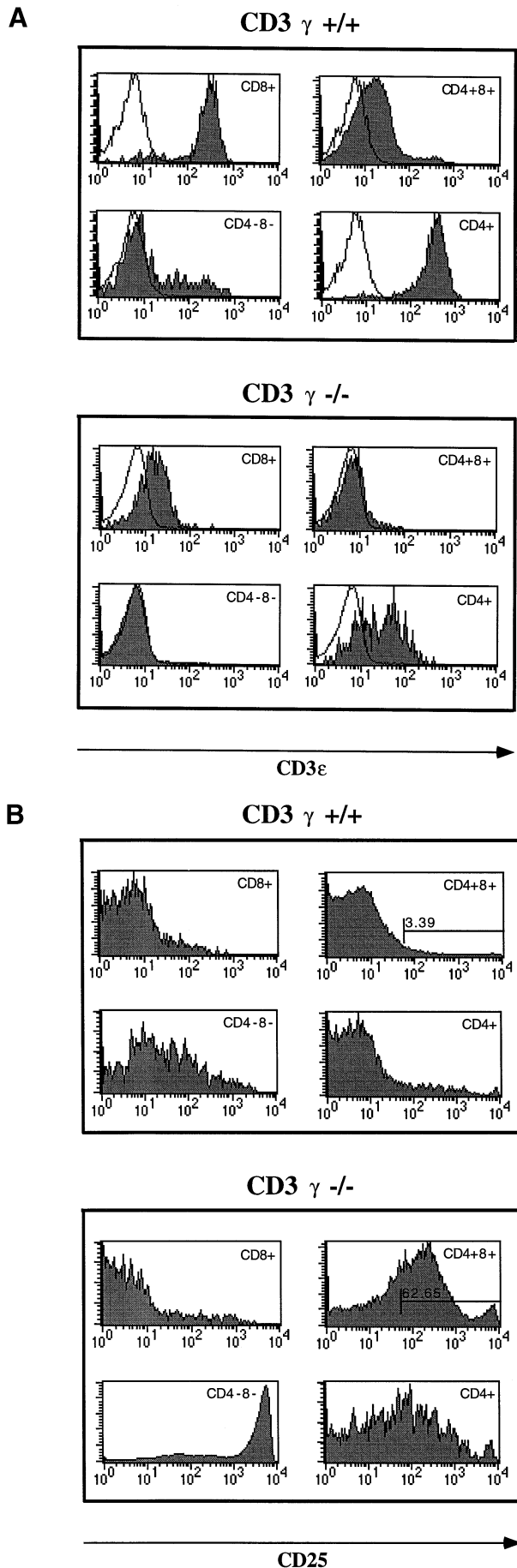
been fully resolved (Sussman *et al.*, 1988; Hall *et al.*, 1991; Kappes and Tonegawa, 1991; Buferne *et al.*, 1992; Geisler, 1992; Dave *et al.*, 1997), but a dependency on CD3γ has been reported (Buferne *et al.*, 1992; Geisler, 1992). The present findings clearly support the latter observations; in mice lacking CD3γ, CD3ε expression on peripheral T cells is severely reduced (Figure 6), reaching only 5–10% of the levels observed on WT peripheral T cells. Together with the findings on reduced CD3ε expression on the different thymocyte subsets in CD3γ-deficient mice (Figure 4A), it can be concluded that CD3γ represents an essential structural component that contributes to assembly and/or transport of the TCR–CD3 complex.

Development of γδ T cells in CD3γ$^{-/-}$ mice

The above findings suggesting a block in development at the CD44$^-$CD25$^+$ DN stage indicate a defect in αβ T-cell development. Indeed, as illustrated in Figure 7A and B through TCRβ versus CD3ε staining (top panels), αβ T-cell development is severely affected by lack of CD3γ. Next we investigated to what extent the defect in pre-T-cell development in CD3γ$^{-/-}$ mice affected development of the TCRγδ lineage as well. A severe defect in γδ T-cell development can be observed in mice lacking CD3ε (Malissen *et al.*, 1995) which suggests that γδ T cells, like αβ T cells, require signaling either through a pre-TCR-type structure or a mature γδ TCR complex to expand and/or differentiate. However, γδ T-cell development proceeds undisturbed in mice lacking TCRβ, pTα or CD3δ, and the γδ T cells of such mice express normal levels of the TCRγδ heterodimer (Mombaerts *et al.*, 1992a; Fehling *et al.*, 1995; Dave *et al.*, 1997). If γδ T cells, analogous to αβ T cells, are subject to a pre-TCR-like selection step as recently suggested (Passoni *et al.*, 1997), such a pre-TCR-type structure does not involve pTα, TCRβ or CD3δ. In addition, CD3ζ-chain-deficient mice exhibit normal γδ T-cell development (Malissen *et al.*, 1993; Ohno *et al.*, 1993), while their αβ T-cell development is compromised.

Our results on the generation of the TCRγδ lineage in CD3γ$^{-/-}$ mice support a requirement for CD3γ in the development of this lineage as well (Figure 7). First, and most importantly, although the relative number of TCRγδ positive T cells in both the thymus and lymph nodes of CD3γ$^{-/-}$ mice was comparable, or at best slightly increased, the absolute number of γδ T cells in CD3γ$^{-/-}$ mice was drastically reduced (Figures 7A and B, bottom panels). In CD3γ-deficient mice, 4×10^4 thymic γδ T cells were detected compared with $3-4 \times 10^6$ thymic γδ T cells in WT and heterozygous mice. In the lymph nodes of CD3γ-deficient mice, a 4- to 5-fold reduction in the absolute number of peripheral γδ T cells was observed when compared with WT and heterozygous mice. Although it is clear that in both the thymus and the lymph nodes of

Fig. 4. Cell-surface expression of CD3ε is drastically reduced in thymocytes from CD3γ$^{-/-}$ mice, and downregulation of CD25 at the DN stage is also severely impaired. Total thymocytes of 6- to 8-week-old WT and CD3γ homozygous mutant mice were analyzed by three-parameter flow cytometry for the expression of (**A**) CD3ε-FITC (grey) or an irrelevant mAb (white) or (**B**) CD25-Biotin plus SA-Tricolor in the several cell populations that are characterized, based on the differential expression of CD4-PE versus CD8-Biotin plus SA-Tricolor (A) or CD8-FITC (B).
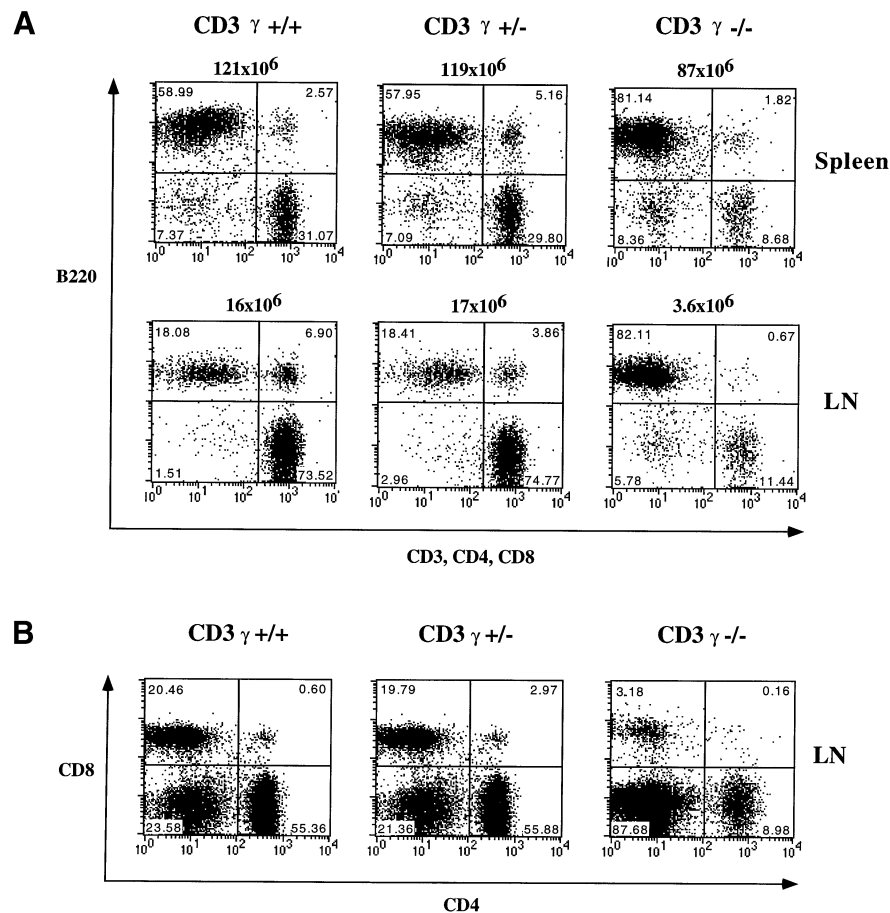
Fig. 5. Small numbers of peripheral T cells can be detected in the spleen and lymph nodes of CD3γ$^{-/-}$ mice, while B-cell development is unaffected. Flow cytometry analysis of spleen and lymph node (LN) cells of 6- to 8-week-old CD3γ$^{+/+}$, CD3γ$^{+/-}$ and CD3γ$^{-/-}$ mice. Total cell populations were monitored for the expression of (**A**) a cocktail of CD3, CD4, CD8–FITC versus B220-Biotin plus SA-Tricolor or (**B**) CD4-PE versus CD8-Biotin plus SA-Tricolor. The percentage of cells within each quadrant is indicated. The absolute number of spleen and lymph node cells detected in the WT, heterozygous mutant and homozygous mutant genotypes is depicted above the corresponding dot display.

CD3γ-deficient mice the level of surface CD3ε expression is severely reduced on all T cells (Figure 7), and some γδ T cells may therefore escape detection. CD3γ-deficient mice entirely lack the population of bright CD3ε-expressing cells in the DN thymocyte subset, which includes the γδ T-cell compartment (Figure 4A). In absolute terms, therefore, the γδ T-cell population fails to develop properly in CD3γ-deficient mice. This is dramatically different from the situation in mice deficient in pTα, TCRβ or CD3δ, in which the paucity of αβ T cells results in a relative, and frequently absolute, increase in the representation of γδ T cells (Mombaerts *et al.*, 1992a; Fehling *et al.*, 1995; Dave *et al.*, 1997), with as much as 35% of the DN compartment occupied by γδ T cells. We conclude that, although TCRγ and TCRδ rearrangements occur normally in CD3γ-deficient mice (Figure 2C) and some γδ T cells are generated, the expansion and/or further differentiation of this population is severely hampered by lack of CD3γ.

The reduced levels of TCR–CD3 expression in CD3γ-deficient mice are consistent with a structural contribution of the CD3γ chain to assembly and/or efficient transport of TCR–CD3 complexes to the cell surface. This applies to both the TCRαβ (Figures 6 and 7A and B) and TCRγδ lineages (Figure 7A and B). The defect in αβ T-cell development initiates at the pre-TCR level. The defect in

the generation of the TCRγδ lineage in CD3γ$^{-/-}$ mice is consistent with impairment in either pre-TCR-like, TCRγδ-driven expansion and/or differentiation steps during γδ T-cell development.

The defect in pre-T-cell development in CD3γ-deficient mice can be overcome by anti-CD3ε-mediated cross-linking

Thymic lobes from embryonic-day-14 CD3γ-deficient or RAG-1$^{-/-}$ mice were treated in FTOC with anti-CD3ε mAb to address whether the severe blockage in early T-cell development in CD3γ-deficient mice is a consequence of a lack of pre-TCR function. As shown previously, exposure of RAG-1$^{-/-}$ fetal thymic lobes to anti-CD3ε leads to the efficient development of DP thymocytes (Levelt *et al.*, 1993) and a concomitant cell expansion of 12- to 15-fold (Figure 8, bottom panel). Interestingly, exposure of CD3γ-deficient fetal thymic lobes to anti-CD3ε also resulted in the generation of DP thymocytes (Figure 8, top panel). These DP thymocytes comprised, like DP thymocytes in anti-CD3ε-treated RAG-1$^{-/-}$ fetal thymic lobes, ~45–55% of the total cell population (Figure 8). Furthermore, although not as pronounced as in the anti-CD3ε-treated RAG-1$^{-/-}$ fetal thymic lobes, a burst in cell proliferation of 4- to 5-fold could be observed in anti-CD3ε-treated, CD3γ-deficient fetal thymic lobes (Figure 8, top panel).

CD3 γ +/+

CD3 γ -/-

CD3ε
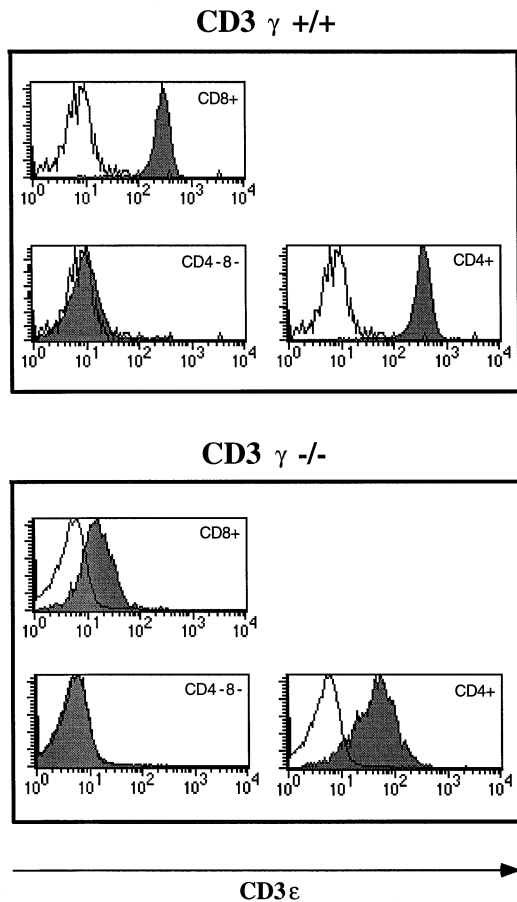
Fig. 6. Absence of CD3γ is associated with strongly reduced levels of CD3ε on peripheral T cells of CD3γ$^{-/-}$ mice. Lymph node cells of 6- to 8-week-old WT and CD3γ homozygous mutant mice were analyzed by three-parameter flow cytometry for the expression of CD3ε–FITC (grey) or an irrelevant mAb (white) in the several cell populations that are characterized based on the differential expression of CD4-PE versus CD8-Biotin plus SA-Tricolor.

These data therefore document that the defect in the DN to DP transition in mice lacking CD3γ can be overcome by anti-CD3ε-mediated cross-linking, suggesting that without CD3γ the pre-TCR is functionally incompetent. CD3γ can contribute to pre-TCR function at several levels, including provision of unique signaling features and participation in complex assembly. The finding that CD3ε expression is severely diminished in CD3γ-deficient mice (Figures 4 and 6) is consistent with the latter explanation. While future experiments will address the issue of whether CD3γ also contributes a unique signaling capability, present data show that CD3γ is essential for pre-TCR function.

Discussion

Maturation of thymocyte precursors from the CD4$^-$CD8$^-$ DN to the CD4$^+$CD8$^+$ DP stage of development requires productive rearrangement of the gene segments encoding the TCRβ chain (Dudley *et al.*, 1994; Levelt and Eichmann, 1995; Hoffman *et al.*, 1996; Fehling and Von Boehmer, 1997). Whether productive TCRβ gene rearrangements have occurred is assessed using the pre-TCR complex. The data presented in this report provide genetic evidence for the requirement of the CD3γ chain in the pre-TCR complex. Transition from the CD44$^-$CD25$^+$ to the CD44$^-$

CD25$^-$ DN stage is severely impaired in CD3γ$^{-/-}$ mice, with only very few cells progressing to the DP and SP stage. Small numbers of SP T cells reach the peripheral lymphoid organs in CD3γ$^{-/-}$ mice, but these mature T cells, like immature thymocytes, express ~10-fold lower levels of CD3ε and TCRαβ or TCRγδ compared with normal T cells. Consistent with these results, TCR expression was dramatically reduced in a CD3γ-negative variant of the human T-cell line Jurkat (Geisler, 1992). CD3γ thus clearly affects the surface-expression levels of both the signaling modules and the TCR-module of the TCR–CD3 complex, and might already contribute to T-cell development in that way. In addition, CD3γ may be necessary to achieve sufficient surface expression levels of the pre-TCR, which was recently shown to require exit from the ER/*cis*-Golgi to function properly (O'Shea *et al.*, 1997). The data are also consistent with a non-mutually exclusive hypothesis of a role for CD3γ as a unique signaling component in the pre-TCR, which cannot be replaced by the other CD3 proteins. This latter hypothesis is based on recent studies which indicated that ITAM motifs derived from different CD3 chains bind to different cytosolic mediators (Letourneur and Klausner, 1992; Osman *et al.*, 1996). CD3γ may thus contribute to pre-TCR and TCR-signaling in various ways, and additional CD3γ-dependent developmental checkpoints remain to be defined. The developmental defect at the pre-T-cell stage imposed by the absence of CD3γ can be rescued by anti-CD3ε-mediated cross-linking, indicating that the CD3γ chain is not required for functional CD3ε expression on pre-T-cells. Furthermore, these data suggest that the pre-TCR is functionally incompetent in the absence of CD3γ. This may be a consequence of incomplete complex assembly, incomplete signaling or both. While future experiments will have to resolve the way in which CD3γ contributes to pre-TCR function, the present data establish CD3γ as an essential component of the pre-TCR.

The peripheral phenotype of the CD3γ null-mutant mice is consistent with the primary immunological defects described for CD3γ-deficient patients. In these patients, a reduced T-cell compartment in the periphery has been reported (Arnaiz-Villena *et al.*, 1991, 1992), as well as impairment in several functions associated with T-cell activation (Pérez-Aciego *et al.*, 1991). Importantly, the present data provide an explanation for the immune deficiency observed in these patients: without CD3γ, T-cell development barely progresses beyond the DN stage, resulting in a severe deficiency generating a functional T-cell compartment.

Biochemical studies on the subunit composition of the pre-TCR complex have revealed that although the full array of known TCR signaling subunits (CD3γδε and ζ) is available to the pre-TCR in primary pre-T-cells, CD3δε modules are much less represented than CD3γε modules (Ley *et al.*, 1989; Jacobs *et al.*, 1994, 1996; Wiest *et al.*, 1994; Berger *et al.*, 1997). The present finding that absence of CD3γ has such a dramatic effect on pre-TCR function, while absence of CD3δ (Dave *et al.*, 1997) does not hamper thymocyte development up to the DP stage, is consistent with the notion that such CD3δε modules cannot replace the function of the CD3γε modules in the pre-TCR.

Existing information on the biogenesis of mature TCRαβ–CD3 complexes (Minami *et al.*, 1987; Alarcon
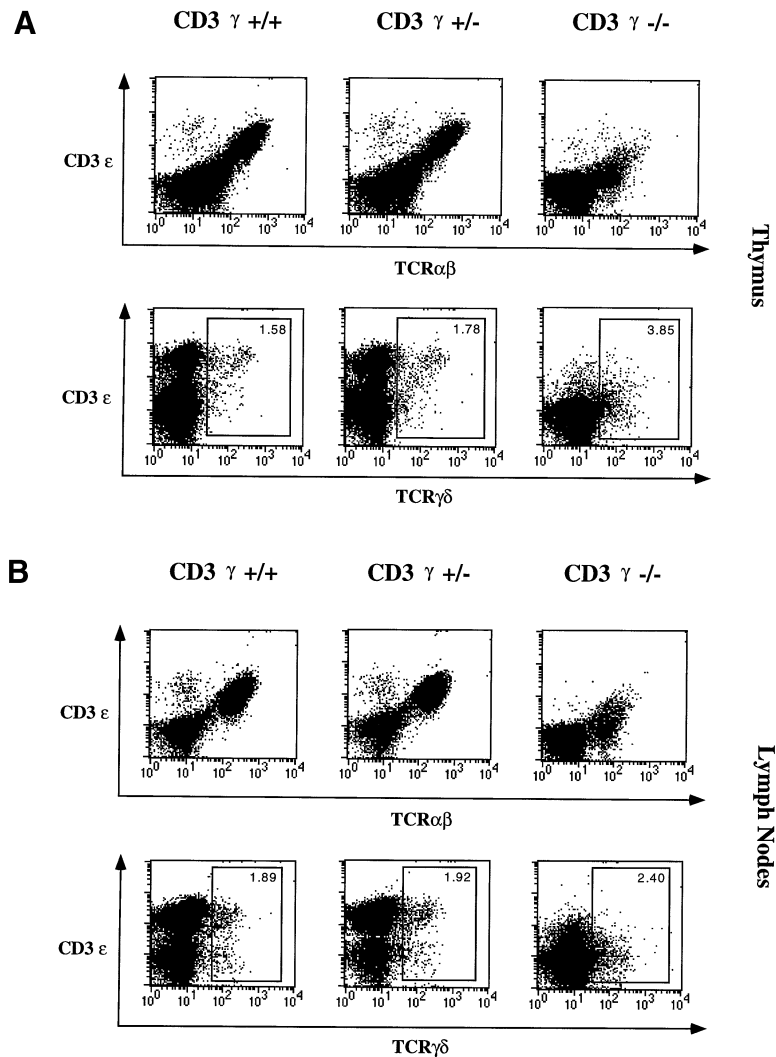
**Fig. 7.** Development of both αβ T cells and γδ T cells is affected in CD3γ-deficient mice. (**A**) Thymocytes and (**B**) lymph node cells of 6- to 8-week-old CD3γ$^{+/+}$, CD3γ$^{+/-}$ and CD3γ$^{-/-}$ mice were analyzed by flow cytometry for the expression of TCRαβ–FITC (top panels) or TCRγδ–FITC (bottom panels) versus CD3ε-PE. The percentage of cells within the depicted gates is indicated in each dot display.

et al., 1988; Bonifacino *et al.*, 1988; Klausner *et al.*, 1990; Koning *et al.*, 1990; Kearse *et al.*, 1995) also supports the observations of differential roles of the CD3 components in the pre-TCR. One of the initial steps in the assembly of a TCRαβ–CD3 complex is the formation of TCRβCD3γε and TCRαCD3δε trimeric building blocks (Alarcon *et al.*, 1988; Manolios *et al.*, 1990; Kearse *et al.*, 1995), and CD3δ may therefore primarily come into play after completion of TCRα chain formation. Upon recruitment of a CD3ζ homodimer, the complete TCRαβ–CD3 complex exits the endoplasmic reticulum and is transported to the cell membrane (Klausner *et al.*, 1990; Kearse *et al.*, 1995). Differences in the ectodomain of the CD3γ and the CD3δ subunit are thought to accommodate interactions with the TCRβ and TCRα chain, respectively (Brenner *et al.*, 1985; Manolios *et al.*, 1990; Wegener *et al.*, 1995; Dietrich *et al.*, 1996). Therefore, the phenotype exhibited by the CD3δ$^{-/-}$ mice (Dave *et al.*, 1997) is consistent with recruitment of the CD3δ subunit primarily into a mature TCRαβ–CD3 complex after TCRα rearrangement has occurred, while CD3γ is recruited into both the pre-TCR complex as well as the mature TCRαβ–CD3 complex due to its association with the TCRβ chain.

In CD3γ$^{-/-}$ mice, some cells are able to progress through the DP to the SP stage in the thymus. Remarkably, the number of SP thymocytes is as high as the number of DP cells, indicating that positive and/or negative selection steps are also severely disturbed by the lack of CD3γ. Differentiation to the DP thymocyte stage in CD3γ-deficient mice is reminiscent of observations in other mutant mouse strains in which pre-TCR function is disrupted: in both TCRβ$^{-/-}$ and pTα$^{-/-}$ mice, some DP thymocytes are generated (Mombaerts *et al.*, 1992a; Fehling *et al.*, 1995). DP thymocytes may be generated through alternate receptors not containing pTα (Buer *et al.*, 1997); the TCRαβ and TCRγδ appear to be capable of functioning as such an alternative. Alternatively, other TCR-expressing thymocytes may contribute to pre-TCR-independent pre-T-cell development through indirect mechanisms (Buer *et al.*, 1997). These hypotheses have been suggested by the observation that in TCRβ$^{-/-}$× TCRδ$^{-/-}$ or pTα$^{-/-}$×TCRδ$^{-/-}$ mice or RAG$^{-/-}$ mice, thymocyte development beyond the CD44$^-$CD25$^+$ DN stage is more rigorously blocked (Mombaerts *et al.*, 1992b; Buer *et al.*, 1997) than in TCRβ$^{-/-}$ or pTα$^{-/-}$ mice. Furthermore, adoptive transfer experiments have documented that

Hamster IgG ## 145-2C11
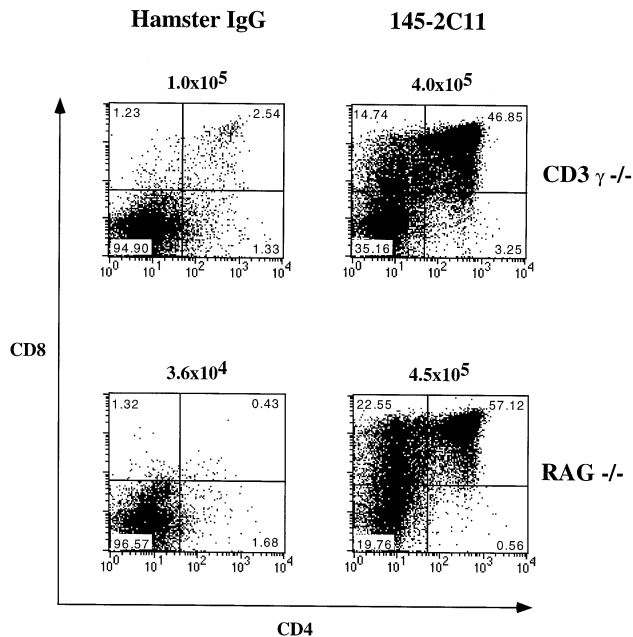
Fig. 8. The defect in pre-T-cell development in CD3γ-deficient mice can be overcome by anti-CD3ε-mediated cross-linking. Day 14 fetal thymic lobes of CD3γ-deficient (top panel) and RAG-1$^{-/-}$ (bottom panel) mice were treated with anti-CD3ε mAb (right panel) or hamster IgG (left panel) for 6 days and analyzed by flow cytometry for the expression of CD4-PE versus CD8-Biotin plus SA-Tricolor. The percentage of cells within each quadrant is indicated. The absolute number of fetal thymocytes detected per lobe is depicted above each corresponding dot display.

intravenous injection of γδ T cells or transfer of normal bone marrow cells into SCID mice induces the development of SCID-derived DP thymocytes in the absence of clonal expansion (Shores *et al.*, 1990; Lynch and Shevach, 1993). However, given the practically negligible number of γδ T cells in CD3γ-deficient mice, it is unlikely that the few DP thymocytes generated in these mice are an indirect consequence of the effects of γδ T cells. Rather, the pre-T-cells in CD3γ-deficient mice that did proceed in their development may have used the CD3δ subunit which, albeit inefficiently, may have compensated for the loss of CD3γ. Biochemical analysis will have to be performed to determine whether CD3δ is present in the pre-TCR of CD3γ$^{-/-}$ mice.

In summary, the present data document that the developmental step which is under the control of the pre-TCR complex cannot occur without the CD3γ chain. Given the strongly reduced levels of surface CD3ε in CD3γ$^{-/-}$ mice, the results provide genetic evidence for a structural role of the CD3γ chain in the (pre-)TCR. While the status of developmental checkpoints during the DP and SP stage of thymocyte maturation in CD3γ null-mutant mice remains to be investigated in more detail, it is clear that the primary defect lies at the level of the inability of the pre-TCR to function.

Materials and methods

Generation of CD3γ null-mutant mice

A mouse genomic clone encompassing the CD3γ gene was isolated from a 129 SVJ phage library (Stratagene). To construct the targeting vector CD3γ–Δ4, a 10.2 kb fragment comprising CD3γ exons 3–7 was subcloned into plasmid pBS–SKII (Stratagene). A 2.4 kb *Sma*I–*Eco*RI fragment

encompassing exon 4 (encoding the transmembrane region of the CD3γ protein) was replaced by a 1.8 kb pgk-neo cassette. Furthermore, a mutation was introduced destroying the *Xho*I site in exon 3, resulting in a frame shift further downstream, in case a truncated mRNA is produced by skipping exon 4 during splicing events. The CD3γ–Δ4 targeting vector was electroporated into the HM-1 ES cell line derived from 129 OLA mice. Clones resistant to neomycin were individually screened by Southern blot analysis for homologous recombination events, using *Sac*I-digested DNA and a probe located outside the targeting construct which recognizes a 10.6 kb WT fragment and an 18 kb recombinant fragment. Two homologous recombinants (HM-1.23 and HM-1.57) were identified out of 192 colonies tested and injected into C57BL/6 blastocysts to generate chimeric mice. Male chimeric mice were subsequently crossed to female FVB mice. Germline transmission was obtained with both clones and heterozygous mice were intercrossed to produce homozygous CD3γ$^{-/-}$ mice.

Mice

Mice were maintained under specific pathogen-free conditions in the animal colony of the Netherlands Cancer Institute and analyzed at 6- to 8-weeks-old unless indicated otherwise. RAG-1-deficient mice have been described in detail elsewhere (Mombaerts *et al.*, 1992b).

Northern blot analysis

Total RNA was prepared from thymocytes using the basic guanidine method as described in Ausubel *et al.* (1995). Twenty-five μg of total RNA was fractionated on a 1% formaldehyde-agarose gel and transferred onto Hybond N$^+$ (Amersham). Blots were hybridized with cDNA probes corresponding to CD3γ (Haser *et al.*, 1987), CD3δ (Van den Elsen *et al.*, 1985), CD3ε (Clevers *et al.*, 1988), CD3ζ (Weissman *et al.*, 1988), TCR Cβ (Caccia *et al.*, 1984) and TCR Cα. Hybridization to a β-actin probe was performed to control for RNA quality and quantity.

RT–PCR analysis

Total RNA was prepared from thymocytes using TRIzol^{TM} (Gibco-BRL) according to the manufacturer's protocol. Oligo dT primer and MMLV reverse transcriptase were used for cDNA conversion in a total volume of 20 μl. PCRs were performed in 1×PCR buffer (see below) in 50 μl total volume, and contained 3 μl (for amplification of CD3γ) or 5 μl (for amplification of pTα) cDNA template. Primers used were as follows: CD3γ forward (5′-GAGCAGAGGAAGGGTCTGGCT-3′); CD3γ reverse (5′-CTTCTTCCTCAGTTGGTTTCC-3′); pTα forward (5′-CATGCTT-CTCCACGAGTG-3′); and pTα reverse (5′-CTATGTCCAAATTCTGT-GGGTG-3′). PCRs were performed as follows: denaturing, 1 min at 94°C; annealing, 1 min at 60°C for pTα and 2 min at 65°C for CD3γ; and extension, 1.5 min at 72°C, all for a total of 35 cycles. PCR products were separated on a 1.5% agarose gel, denatured in 1.5M NaCl, 0.5M NaOH, neutralized in 1.5M NaCl, 1.0M Tris (pH 7.4) and transferred onto Hybond N$^+$ (Amersham). Blots were hybridized with a pTα-specific oligonucleotide (5′-CAGGTACTGTGGCTGAGCCTACTG-3′) or a CD3γ-specific cDNA probe (Haser *et al.*, 1987).

β-actin was amplified as a template control and hybridized with a β-actin probe. PCR primers used were: β-actin forward (5′-GTGG-GCCGCTCTAGGCACCAA-3′); and β-actin reverse (5′-CTCTTTG-ATGTCACGCACGATTTC-3′).

DNA–PCR analysis

Rearrangement of the TCRγ, δ and β loci was assayed at the genomic level as described (D'Adamio *et al.*, 1992; Itohara *et al.*, 1993). In brief, thymocytes were lysed in 200 μl of lysis mixture, containing 1×PCR buffer [670 mM Tris (pH 8.8), 67 mM MgCl$_2$, 0.1 M 2-β-mercapto-ethanol, 67 μM EDTA, 166 mM (NH$_4$)$_2$SO$_4$], 0.45% NP-40, 0.45% Triton X-100 and proteinase K (100 μg/ml). Cells were incubated at 56°C for 1 h, and proteinase K was subsequently inactivated at 96°C for 10 min. PCRs were performed in 50 μl total volume and contained 4 μl template DNA. Primers used to amplify rearranged DNA were as follows: Vγ4 (5′-TGTCCTTGCAACCCCTACCC-3′); Vγ5 (5′-TGTGC-ACTGGTACCAACTGA-3′); Jγl (5′-CAGAGGGAATTACTATGAGC-3′); Vδ1 (5′-GGGATCCTGCCTCCTTCTACTG-3′); Vδ4 (5′-CCGCTT-CTCTGTGAACTTCC-3′); Jδ1 (5′-CAGTCACTTGGGTTCCTTGTCC-3′); Vβ6 (5′-GAAGGCTATGATGCCGTCTCG-3′); Vβ8 (5′-TCCCTG-ATGGGTAGAAGGCC-3′); and Jβ2.5 (5′-TAACACGAGGAGCCG-AGTGC–3′). PCRs were performed as follows: denaturing, 1 min at 94°C; annealing, 1 min at 70°C for TCR–Vβ8, 1 min at 58°C for TCR–Vβ6 and TCRδ and 2 min at 50°C for TCRγ; extension, 1.5 min at 72°C, all for a total of 33 cycles. PCR products were separated on a 1.5% agarose gel, denatured in 1.5 M NaCl, 0.5 M NaOH, neutralized

in 1.5 M NaCl, 1.0 M Tris (pH 7.4) and transferred onto Hybond N$^+$ (Amersham). Blots were hybridized with a Jγl-specific oligonucleotide (5′-TGAATTCCTTCTGCAAATACCTTG-3′), a Jδ1-specific oligonucleotide (5′-GTTCCTTGTCCAAAGACGAGTT-3′) or a Jβ2-specific cDNA probe.

p53 was amplified as a template control and hybridized with a p53 cDNA probe. PCR primers used were: p53 forward (5′-TCACTGC-ATGGACGATCTGTTGC-3′); and p53 reverse (5′-GATGATGGTAA-GGATAGGTCGGCG-3′).

Flow cytometry

Single cell suspensions of thymus and peripheral lymphoid organs were prepared in PBA (1×PBS, 1% BSA, 0.02% NaN$_3$) aliquoted into wells of a 96-well plate (2×10^4–1×10^5 cells/well), and pelleted at 200 *g* for 2 min. Cells were resuspended in 20 µl of anti-FcγRII/III (clone 2.4G2; final concentration 1 µg/ml) to reduce nonspecific staining, and incubated for 10 min at 4°C. Staining for cell-surface antigen expression was performed at saturating mAb concentrations for 20–30 min at 4°C. Cells were washed twice in 100 µl of PBA and incubated with second-step reagent if necessary. Finally, all samples were washed twice and resuspended in 100 µl of PBA. Cells were analyzed on a Becton–Dickinson FACScan with Lysis II software. Forward- and side-scatter gating was used to exclude dead cells from the analysis.

Biotinylated, FITC- or PE-conjugated antibodies specific for murine CD3ε (clone 145–2C11), CD4 (clone RM4–5), CD8β (clone 53–5.8), CD25 (clone 7D4), CD44 (clone IM7), B220 (clone RA3–6B2), TCRαβ (clone H57–597) and TCRγδ (clone GL3) were obtained from PharMingen. R-PE anti-mouse CD4 (clone CT–CD4) was purchased from Caltag. Where appropriate, streptavidin-Tricolor (Caltag) was used as a second-step reagent.

Fetal thymic organ cultures (FTOC)

Fetal thymic lobes were prepared from CD3γ-deficient or RAG-1$^{-/-}$ mice at day 14 of gestation. They were cultured on filter discs on gelfoam in Iscove's modified Dulbecco's medium supplemented with 10 mM HEPES buffer, non-essential amino acids, 4 mM L-glutamine, penicillin, streptomycin (all from Gibco-BRL), 5×10^{-5} M 2-β-mercaptoethanol, and 20% fetal calf serum. Exposure to 10 µg/ml anti-CD3ε clone 145–2C11 (Leo *et al.*, 1987) or 10 µg/ml irrelevant hamster IgG (Jackson Immuno-Research) was started at the beginning of the culture. After 6 days, single cell suspensions were prepared and thymocytes were examined by FACS analysis for surface expression of CD4 and CD8.

Acknowledgements

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