The CD3-γδε and CD3-ζ/η Modules Are Each Essential for Allelic Exclusion at the T Cell Receptor β Locus but Are Both Dispensable for the Initiation of V to (D)J Recombination at the T Cell Receptor-β, -γ, and -δ Loci

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Summary

The pre-T cell receptor (TCR) associates with CD3-transducing subunits and triggers the selective expansion and maturation of T cell precursors expressing a TCR-β chain. Recent experiments in pre-Tα chain-deficient mice have suggested that the pre-TCR may not be required for signaling allelic exclusion at the TCR-β locus. Using CD3-ε- and CD3-ζ/η-deficient mice harboring a productively rearranged TCR-β transgene, we showed that the CD3-γδε module and the CD3-ζ/η module, and by inference the pre-TCR/CD3 complex, are each essential for the establishment of allelic exclusion at the endogenous TCR-β locus. Furthermore, using mutant mice lacking both the CD3-ε and CD3-ζ/η genes, we established that the CD3 gene products are dispensable for the onset of V to (D)J recombination (V, variable; D, diversity; J, joining) at the TCR-β, TCR-γ, and TCR-δ loci. Thus, the CD3 components are differentially involved in the sequential events that make the TCR-β locus first accessible to, and later insulated from, the action of the V(D)J recombinase.

T cells can be divided into two subsets based on the structure of their TCR. In the adult mouse, most T cells express a TCR heterodimer consisting of α and β chains, whereas a minor population expresses an alternative TCR isoform made of γ and δ chains. Each of these four TCR chains includes a clonally variable (V)1 region encoded by genes that are assembled via somatic site-specific DNA recombination reactions. These reactions, termed V(D)J rearrangements (D, diversity; J, joining), result in the random recombination of V and J gene segments in TCR-α and TCR-γ chain genes, and of V, D, and J gene segments in TCR-β and TCR-δ genes. V(D)J joining reactions may result either in productive rearrangements that maintain an open reading frame throughout the gene, or in an out-of-frame nonfunctional gene. Because T lymphocytes are diploid cells, this recombination process could, in principle, generate T cell clones expressing two productively rearranged TCR-α or TCR-γ chain combinations. In the mouse, the expression of a productively rearranged TCR-β chain transgene has been shown to prevent complete V-(D)J rearrangement of endogenous TCR-β genes (1), and this has led to the assumption that α/β T cell precursors have developed feedback inhibition mechanisms to ensure that most mature T cell clones express one, and only one, TCR-α/β chain combination. These mechanisms are referred to as allelic exclusion.

Intrathymic T cell development proceeds through discrete stages that can be defined on the basis of the configuration of TCR gene loci, and the expression of surface markers such as CD4 and CD8. Accordingly, the most immature thymocytes express neither CD4 nor CD8 and are called double negative (DN) cells. Late DN cells can mature into CD4+CD8+ (double positive, DP) cells, a small percentage of which develop further into CD4+CD8− or CD4−CD8+ (single positive, SP) cells. Based on the expression of CD25 and CD44, DN cells have been subdivided further and shown to develop according to the following maturation sequence: CD44-CD25−→CD44+CD25−→CD44+CD25−→CD44+CD25−→CD44+CD25−→CD44−/low CD25− (2). TCR-β gene rearrangements precede rearrangements at the TCR-α locus and proceed in two separate steps involving an initial D→J joining event and a subsequent V→(D)J rearrangement. TCR-β gene rearrangements start at, or at the transition
to, the CD44$^{-low}$CD25$^+$ DN stage (2, 3), whereas the first measurable TCR-$\alpha$ rearrangements occur during, or immediately after, the transition to the DP stage (4, 5). When maturing T cells fail to rearrange their TCR genes, rearrange them nonproductively, or express TCR-$\alpha/\beta$ combinations with inappropriate specificities, they are generally arrested at discrete developmental control points (see reviews in references 6 and 7). Molecular sensors have evolved to couple the transition through these control points to the attainment of certain landmark events in T cell development. For instance, one of these sensors, known as the pre-TCR, operates at the CD44$^{-low}$CD25$^+$ DN stage and couples further maturation to the prior achievement of productive TCR-$\beta$ gene rearrangements.

In the pre-TCR, TCR-$\beta$ is disulfide linked with a polypeptide encoded by a nonrearranging gene and denoted as the pT$\alpha$ chain (8). To exert its function, the pre-TCR needs to associate with both the CD3-$\gamma$ and CD3-$\zeta$ dimers (9–13), and signal via the protein tyrosine kinases lck and fyn (14–17). It has been proposed that the pre-TCR/CD3 complex triggers the selective proliferation of TCR-$\beta^+$ DN cells and concurrently drives their progression to the DP developmental stage (such transition is often denoted as TCR-$\beta$ selection). Moreover, considering that the expression of a productively rearranged TCR-$\beta$ transgenic gene inhibits most endogenous V$\beta$ to D$\beta$J$\beta$ rearrangements (see above), it has been suggested that the TCR-$\beta$ chain, and by extension the pre-TCR/CD3 complex, plays a pivotal role in the enforcement of allelic exclusion at the TCR-$\beta$ locus. Therefore, disruption of the gene coding for the pT$\alpha$ subunit should have prevented assembly of a functional pre-TCR complex and affected the establishment of allelic exclusion at the TCR-$\beta$ locus. However, in pT$\alpha^{-/-}$ thymocytes, expression of a transgene coding for a functional TCR-$\beta$ chain was found to inhibit endogenous V$\beta$ to D$\beta$J$\beta$ rearrangements to almost the same extent as in a pT$\alpha^{-/-}$ background (18, 19). Assuming that no other gene products can compensate for the loss of pT$\alpha$ (e.g., the products of prematurely expressed TCR-$\alpha$ genes; reference 20), these data are inconsistent with the suggestion that the pre-TCR/CD3 complex is involved in signaling allelic exclusion at the TCR-$\beta$ locus.

We have previously generated mice with a targeted mutation of the CD3-$\epsilon$ gene (referred to as CD3-$\epsilon^{AS}$; reference 12). This mutation abolishes the expression of intact CD3-$\epsilon$ polypeptides, dramatically reduces the transcription rate of the neighboring CD3-$\gamma$ and CD3-$\delta$ genes, and totally blocks the progression beyond the CD44$^{-low}$CD25$^+$ stage. The thymocytes found in CD3-$\epsilon^{AS}$ mice contain readily detectable levels of CD3-$\zeta$, TCR-$\beta$, and pT$\alpha$ transcripts. However, the lack of CD3-$\gamma/e$ and CD3-$\delta/e$ dimers is likely to prevent their pT$\alpha$-TCR-$\beta$ and CD3-$\zeta$ dimers from participating in the assembly of functional pre-TCR/CD3 complexes. The CD3-$\epsilon^{AS}$ mice present several experimental advantages relative to pT$\alpha$-deficient mice. First, their thymuses contain an enriched source of CD44$^{-low}$CD25$^+$ DN cells devoid of contaminating downstream $\alpha/\beta$ T cell subsets and in which TCR-$\beta$ gene rearrangements do happen normally. Second, the CD3-$\epsilon^{AS}$ mutation does prevent the development of $\gamma/\delta$ T cells and permits the analysis of early $\alpha/\beta$ T cell development in a microenvironment insulated from the adventitious effects resulting from the presence of $\gamma/\delta$ T cells (2, 20, 21). Therefore, by obviating some of the experimental limitations associated with pT$\alpha^{-/-}$ mice, the CD3-$\epsilon^{AS}$ mice constitute a particularly appropriate model to determine whether the CD3-$\gamma/e$ module, and by inference the pre-TCR, is essential for the establishment of allelic exclusion at the TCR-$\beta$ locus. Here we report on experiments showing that the CD3-$\gamma/e$ and the CD3-$\zeta/\eta$ modules of the pre-TCR play each a pivotal role in allelic exclusion at the TCR-$\beta$ locus. In contrast, analysis of CD3-$\epsilon^{AS}$ mice demonstrates that the on-set of V$\gamma$ to (D)J$\beta$ recombination at the TCR-$\beta$, TCR-$\gamma$, and TCR-$\delta$ loci can occur in the absence of CD3 subunits.

Materials and Methods

Mice. The CD3-$\epsilon^{AS}$ mice and CD3-$\zeta/\eta^{-/-}$ mice have been described (12, 22). R recombination activation gene (RAG)-1/$^{-/-}$ mice were originally obtained from E. Spanopoulou (The Rockefeller University, New York; 23). The P14 TCR-$\beta$ transgenic mice (line 128) express a TCR-$\beta$ cDNA (V$\beta$1.1-D$\beta$-J$\beta$2.4) derived from the T cell clone P14 (24). TCR-$\beta$ transgenic mice were typed for the presence of the transgene by PCR analysis of tail DNA. TCR-$\beta$ transgenic mice were crossed with CD3-$\delta$$^+$ and CD3-$\zeta/\eta$-deficient mice to obtain CD3-$\delta$$^+$ and CD3-$\zeta/\eta$-deficient TCR-$\beta$ mice. CD3-$\epsilon^{AS}$CD3-$\zeta/\eta^{-/-}$ double-deficient mice were derived from CD3-$\epsilon^{AS}$CD3-$\zeta/\eta^{-/-}$ matings. Mice were housed in a specific pathogen-free animal facility in accordance with institutional guidelines. Mice were between 4 wk and 3 mo old when analyzed.

Antibodies and Flow Cytometry. Biotinylated, FITC-, or PE-conjugated antibodies against CD3-e (2C11), CD4 (H129.19), CD8 (S3.67), CD25 (7D4), CD44 (Gp-1), and TCR V$\beta$ (F23.1) were purchased from Pharmingen (San Diego, CA). Biotinylated antibodies against M $\alpha$-1 (M1/170), B220 (RA3-6B2), and Gr-1 (RA6-8c5) were from CALTAG Labs (Tebu, Le Perray en Yvelines, France). Biotinylated antibodies were revealed with streptavidin tri-color (CALTAG Labs). Cells were stained with saturating levels of antibodies and 5–50 $\times 10^3$ events (gated on forward and side scatter) were acquired using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and analyzed with Lysis II software.

Isolation of CD25$^+$ T lymphocytes. CD25$^+$ thymocytes were sorted using a FACStar Plus©. Before sorting, thymus cell suspensions were enriched for CD4 CD8 CD3$^-$ cells by one round of complement-mediated killing with a mixture of IgM anti-CD4 (clone RL172.4), IgM anti-CD8 (clone 31M), and IgG2b anti-CD3 (clone 17A2) antibodies. Viable cells were retrieved by a density cut using Ficoll-paque (Pharmacia, Orsay, France), stained with propidium iodide and an FITC-conjugated anti-CD25 antibody, and sorted for CD25$^+$ cells.

Intraepithelial Staining. Expression of the transgenic TCR-$\beta$ chain within the CD25$^+$ thymocyte subset was assessed by intraepithelial/extracellular staining of thymocytes. Cells were first stained for CD25 as described above. After washing in PBS supplemented with 3% FCS (PBS/FCS), cells were fixed in PBS plus 4% paraformaldehyde for 20 min at room temperature, followed by two washing steps in PBS. Cells were then permeabilized in
PBS/FCS containing 0.1% saponin (Roth, Lauterbourg, France) for 10 min at room temperature. Intracellular staining with a PE-conjugated anti-VPβ (M R S.2) antibody diluted in PBS/FCS plus 0.1% saponin was performed for 20 min at room temperature and followed by three washing steps on a rocking platform using PBS/FCS plus 0.1% saponin. Finally, cells were resuspended in PBS and analyzed on a FACScan®.

RNA-PCR Amplification. RNA samples were extracted from total (CD3-ε<sup>+/−</sup>) and CD3-ε<sup>−/−</sup> TCR-β gene rearrangements) by TRIzol® (GIBCO BRL, Cergy Pontoise, France) as recommended by the manufacturer. Before conversion to cDNA, RNA samples were treated with DNAase-I and RNase free (Pharmacia). Conversion to cDNA was done on 1 µg of total RNA using the Ready-to-Go™ T-primed first strand kit (Pharmacia), 1/15 of each reaction was used for PCR amplification. The pair of Cβ2 primers used to detect transcripts incorporating the TCR Cβ exon was as described in reference 25. They are denoted as primers 1 and 2 in Fig. 3. The sequences of the other PCR primers used in these experiments were: CDR3 P14s: 5′-GTGATGCGGGGGGGGGCGGACAC-3′, and β-globin 3′UT: 5′-GGGGCATAGCCACACACGCCACCA-3′, and denoted in Fig. 3 as primers 3 and 4, respectively. The amplified products were analyzed on 1.5% agarose gel, transferred to nylon membrane (Gene Screen Plus), and hybridized using a 32P-labeled oligonucleotide probe. Hybridizing bands were quantitated using a phosphorimager (BAS 100; Fuji, R aytex France S.A.R.L., France). The oligonucleotide primers used for the analysis of TCR-β chain gene rearrangement were as described in reference 26. PCR-based analysis of TCR-α and TCR-β gene rearrangements was as previously described (27). Before the analysis of the relative levels of TCR gene rearrangements, the quality and quantity of DNA present in each sample were checked by amplifying the nonrearranging thiorox gene (M trx) using primers MTRX1: 5′-AGGGTAAAGCCTGCTATGG-3′ and MTRX2: 5′-AGTTTGTTCCTCGATCCC-3′.

**Results**

A Productively Rearranged TCR-β Transgene Is Unable to Activate the DP Stage in the Absence of CD3-ε. To determine whether the expression of a TCR-β chain...
The P14 TCR-β transgene was able to inhibit endogenous Vβ to DJββ rearrangements in the absence of CD3-e subunit, CD3-e<sup>−/−</sup> mice were crossed with transgenic mice carrying a productively rearranged VPβ<sup>+</sup> TCR-β chain derived from the P14 T cell clone (24, 28). When expressed in a wild-type background, this TCR-β transgene prevented endogenous β-locus gene rearrangements, as judged by the fact that most of the SP thymocytes developing in these mice were VPβ<sup>+</sup> (Fig. 1, compare transgenic [WT TCR-β] and nontransgenic [WT] wild-type panels). As shown in Fig. 1 A, CD3-e<sup>−/−</sup> TCR-β mice had thymuses that did not develop past the DN stage and contained absolute cell numbers similar to nontransgenic CD3-e<sup>−/−</sup> thymuses. Thus, expression of the P14 TCR-β transgene was unable to restore T cell development in CD3-e<sup>−/−</sup> mice. To specify more precisely the effect of the TCR-β transgene on early T cell development, we analyzed the CD44/CD25 profile of wild-type and CD3-e<sup>−/−</sup> DN thymocytes that developed in the absence or presence of the P14 TCR-β transgene. To this end, we gated on cells that were negative for CD3, CD4, CD8, B cell– (B220), granulocyte– (Gr-1), and macrophage– (Mac-1) specific markers (2). As shown in Fig. 2, comparison of DN cells from transgenic (WT TCR-β) and nontransgenic (WT) wild-type mice indicated that in the former there was a marked increase in the percentage of CD44<sup>−/low</sup>CD25<sup>−</sup> thymocytes at the expense of their immediate CD44<sup>−/low</sup>CD25<sup>−</sup> precursors. This finding is in line with previous data showing that TCR-β transgenic mice exhibit CD44<sup>−/−</sup>CD25<sup>−</sup> cell compartments the size of which are intermediate between those found in nontransgenic and TCR-α/β transgenic mice (19, 20). Such observations have been generally accounted for by the fact that CD44<sup>−/low</sup>CD25<sup>−</sup> cells equipped with a productively rearranged TCR-β transgene progress on average much more rapidly to the CD44<sup>−/low</sup>CD25<sup>−</sup> stage than their nontransgenic counterparts (29). Interestingly, the DN cells found in the CD3-e<sup>−/−</sup> and CD3-e<sup>−/−</sup> TCR-β mice were both arrested at the same CD44<sup>−/low</sup>CD25<sup>−</sup> stage and lacked not only the CD44<sup>−/low</sup>CD25<sup>−</sup> cells proper, but also most of the CD44<sup>−/low</sup>CD25<sup>low</sup> to − promonitors. Thus, it is likely that in the absence of CD3-γε module, pTα-TCR-β<sup>−/−</sup> heterodimers were prevented from assembling into functional pre-TCR complexes and unable to rescue the blockade in thymic development observed in CD3-e<sup>−/−</sup> mice. Alternatively, the onset of expression of the P14 TCR-β transgene during T cell development may have occurred only after the CD44<sup>−/low</sup>CD25<sup>−</sup> stage and accounted for its failure to rescue T cell development in CD3-e<sup>−/−</sup> mice.

Considering that the P14 TCR-β transgene consists of a TCR-β cDNA placed under the control of the H-2K<sup>b</sup> promotor and Ig heavy chain enhancer, its expression within the CD25<sup>+</sup> DN compartment should have depended solely on the activation of its transcription. To ascertain the presence of P14 TCR-β transcripts within the CD25<sup>+</sup> DN cell populations from CD3-e<sup>−/−</sup> TCR-β and CD3-e<sup>−/−</sup> TCR-β thymuses, we devised an RNA-PCR assay that specifically detected the P14 TCR-β transcripts (see legend of Fig. 3). As shown in Fig. 3 A, transcripts originating from the P14 TCR-β transgene were readily detectable in the CD3-e<sup>−/−</sup> TCR-β sample and in the CD25<sup>+</sup> cells sorted from CD3-e<sup>−/−</sup> TCR-β thymuses. In contrast, RNA extracted from CD3-e<sup>−/−</sup> thymocytes contained no detectable P14 TCR-β transcripts. Note that upon amplification with a pair of primers specific for the first exon of the TCR β2 gene, the CD3-e<sup>−/−</sup> RNA showed an hybridizing band corresponding to endogenous (DJ)-Cβ and V(DJ)-Cβ transcripts (12). To exclude any potential posttranslational regulation affecting the expression of the transgenic P14 TCR-β chains, CD3-e<sup>−/−</sup> TCR-β thymocytes were further analyzed by intracellular staining with an antibody (F23.1: anti-Vβ8) specific for the product of the Vβ gene segment used by the P14 transgenic TCR-β chain. As shown in Fig. 3 B, most of the CD25<sup>+</sup> cells found in CD3-e<sup>−/−</sup> TCR-β thymuses expressed the intracellular transgenic TCR-β chain. Therefore, these re-

**Figure 2.** Comparison of the triple negative thymocyte subsets from wild type (WT) mice and CD3-e<sup>−/−</sup> mutant mice in the presence or absence of P14 TCR-β transgene (TCR-β). Thymocytes were stained with anti-CD3, -CD4, -CD8, -B220, -Mac-1, and Gr-1 (all biotinylated and detected with streptavidin tricolor), anti-CD44-PE, and anti-CD25-FITC. The position of the window (R1) used to identify the DN T lineage cells is shown in the top row for each type of mouse. In the bottom row, the DN T lineage cells were analyzed for the expression of CD25 and CD44. The percentage of cells found within each quadrant is indicated.
The P14 TCR-β transgene was expressed predominantly at the CD44+/CD25+ stage, and no P14 TCR-β transgene expression was observed at the DN or DP stages. These results indicate that both the transcription and translation of the P14 TCR-β transgene were effective at the CD44+/CD25+ stage. Consistent with the latter results, introduction of the P14 TCR-β transgene in RAG-1−/− mice was found to rescue the progression to the DP stage (data not shown).

Aillic Excision of Endogenous TCR-β Transgenic Dendritic Cells. Disruption of the CD3-ζ/η gene incompletely blocks the DN to DP transition and plausibly corresponds to a leaky mutation of the pre-TCR sensor (see review in reference 31). Accordingly, CD3-ζ/η−/− mice have small thymuses that contain 2–30-fold less DP cells than wild-type littermates. These DP cells appear to have been generated via TCR-β rearrangements from nontransgenic CD3-e555 mice that lack the intron, primers for PCR amplification can be chosen to distinguish amplification products corresponding to transgenic transcription (expected size: 0.8 kb) from those resulting from adventitious DNA contamination (expected size: 1.6 kb). Accordingly, an antisense primer specific for the third untranslated region of the human β globin gene (primer 4) was used in combination with a sense primer (primer 3) straddling the sequence corresponding to the third complementarity region of the P14 TCR-β gene. RNA extracted from nontransgenic CD3-e555 mice was also included as a negative control. A second pair of primers (denoted 1 and 2) was used in parallel to detect both endogenous and transgenic transcripts containing the TCR-β gene segments. The products resulting from amplification with primer pairs 1 + 2 (TCR-β e2 exon) and 3 + 4 (TCR-β e2 Tg) were gel fractionated, blotted, and hybridized with a Cβ2-specific probe (p). The localization of specific primers is indicated by arrowheads and the transcription start site of the TCR-β gene by an arrow. Control PCR were set up in parallel using a pair of primers specific for the actin gene to control for the quantity and quality of RNA in each sample, run on agarose gel, and revealed by ethidium bromide staining (A d). The presence of the transgenic P14 TCR-β chain within the CD25+ subset was confirmed in CD3-e555 mice by intracellular staining with an antibody (F23.1) specific for the Vβ8 gene segment used by the P14 TCR-β chain. RAG-1−/− mice were also included as negative controls. Cytoplasmic staining of the CD25+ compartment from nontransgenic CD3-e555 mice revealed <1% F23.1+ cells.

AillicExcision of Endogenous TCR-β Transgene Rarrangements is Ineffective in CD3-ζ/η−/− TCR-β T Transcripts. Disruption of the CD3-ζ gene incompletely blocks the DN to DP transition and plausibly corresponds to a leaky mutation of the pre-TCR sensor (see review in reference 31). Accordingly, CD3-ζ/η−/− mice have small thymuses that contain 2–30-fold less DP cells than wild-type littermates. These DP cells appear to have been generated via TCR-β rearrangements since almost all of them express intracellular TCR-β chains, a situation that contrasts with that observed in pTα−/− mice (20) and is consistent with the complete absence of γδ T cells in CD3-ζ/η−/− mice. However, the DP cells found in CD3-ζ/η−/− thymuses can be distinguished from bona fide wild-type DP cells because they have a limited content of rearranged TCR-α gene segments (32), exhibit a reduced sensitivity to dexamethasone-induced apoptosis (15), and part of them still express CD25.
The split pattern of phenotypic changes elicited by the pre-TCR in the absence of CD3-ε/δ subunit is likely to reflect the fact that different cellular responses have different activation thresholds (e.g., the strength of stimulation required for the induction of the CD4 and CD8 genes being lower than that required for triggering efficient VαJα recombination). Along that line, it was interesting to analyze whether the CD3-ε/δ subunit of the pre-TCR was required for the establishment of allelic exclusion at the TCR-β locus. To this end, CD3-ε/δ−/− mice were crossed with the P14 TCR-β transgenic mice and the effect of the β transgene on endogenous β locus determined with the DNA-PCR assay described in the above paragraph. Note that the levels of TCR-β gene rearrangement found in CD3-ε/δ−/− thymocytes are similar to those found in wild-type littermates (12), and that the introduction of the P14 TCR-β transgene in CD3-ε/δ−/− mice did not

Figure 4. Expression of a transgenic TCR-β chain does not inhibit endogenous Vβ to Dβ-Jβ rearrangements in the absence of CD3-ε polypeptide. (A) Relative levels of TCR-β rearrangements in CD25− cells sorted from CD3-ε−/− (WT CD25−) and CD3-ε−/− TCR-β (TCR-β CD25−) thymus, and total thymocytes from CD3-ε−/− and CD3-ε−/− TCR-β mice. Identical sorting windows were set up on CD25high DN cells for both the CD3-ε−/− and CD3-ε−/− TCR-β samples. Considering that they contain >90% CD44highCD25high DN cells (see Fig. 2), the CD3-ε−/− and CD3-ε−/− TCR-β thymuses were not subjected to sorting before analysis. The extent of Dβ-Jβ and Vβ-Dβ-Jβ rearrangements were analyzed by DNA-PCR. The relative positions of the PCR primers within the TCR-β locus are depicted by arrows in the bottom diagram. Products derived from PCR reactions involving the intronic Jβ2 3′ primer with Dβ2- (top), Vβ5- (middle) or Vβ8- (bottom) specific 5′ primers were gel fractionated and detected with the intronic probe depicted at the bottom (probe). Note that the cDNA-based P14 TCR-β transgene (Vβ8.1-Dβ-Jβ2.4) is not detectable with the pair of primers used to reveal endogenous Vβ8-Jβ2 rearrangements. For each sample, dilutions of DNA template corresponding to 1×10⁵, 2×10⁴, and 3×10⁴ cell equivalent were analyzed. (B) Quantification of the results shown in A. Hybridizing bands were scanned using a phosphorimager and the relative percentages of rearrangements compared to those present in CD25− cells from CD3-ε−/− (WT) mice.

Figure 5. Expression of a transgenic TCR-β chain does not inhibit endogenous Vβ to Dβ-Jβ rearrangements in the absence of CD3-ε/δ polypeptide. The relative levels of TCR-β rearrangements found in CD3-ε/δ−/− (WT), CD3-ε/δ−/− TCR-β (TCR-β), and CD3-ε/δ−/− TCR-β thymocytes were determined as described in the legend of Fig. 4.
lead to any change in thymocyte cellularity and surface phenotype (data not shown). As shown in Fig. 5, the level of Db2 to Jβ2 rearrangement was similar in DNA extracted from wild-type (WT) TCR-β wild-type (TCRβ), and TCR-β CD3-ζ/η−/− mice. As previously documented for CD3-ε-deficient mice (see above), allelic exclusion of the endogenous TCR-β locus was severely compromised in the absence of CD3-ζ/η polypeptide (Fig. 5, compare V to DJ rearrangements in lanes TCRβ and TCRβ CD3-ζ/η−/−). Thus, these data suggest that the signals conveyed by the partial pre-TCR/CD3 complexes found in CD3-ζ/η-deficient mouse are unable to trigger TCR-β allelic exclusion.

The onset of V to DJ recombination at the TCR-β, -γ, and -δ Loci can occur in the absence of both CD3-ε and CD3-ζ/η polypeptides. The molecular mechanisms regulating the development of B cells and α/β T cells display striking similarities (see review in references 33 and 34). For instance, pre-B cells express a B cell analogue of the pre-T cell receptor called the pre-B cell receptor. The pre-B cell receptor associates with Igα/Igβ transducing subunits and triggers both the selective amplification/maturation of IgH pre-B cells and establishment of allelic exclusion at the IgH locus (34). Igβ-deficient mice show a complete block in B cell development at a stage corresponding to the CD 44−/lowCD25+ stage of T cell development (35). Interestingly, VH to DJ/Hz rearrangements were found to be severely reduced in Igβ-deficient mice, whereas DH to JH rearrangements proceeded normally. This indicated that Ig-β may play an important regulatory role in the onset of VH to DJ/Hz recombination. When bred separately, the CD3-ε−/− and CD3-ζ/η−/− mutations had no discernible effect on the occurrence and extent of Vβ to Dββ recombination (Figs. 4 and 5). Therefore, the V to DJ recombination events affecting TCR-β and IgH loci may be subjected to distinct regulatory signals. It is also possible, however, that the CD3-ε and CD3-ζ/η chains play redundant regulatory roles in the onset of Vβ to Dββ recombination. To address this question, mice lacking both proteins were derived from a F2 intercross between CD3-ε−/− and CD3-ζ/η−/− mice. As shown in Fig. 6, mice lacking both CD3-ε and CD3-ζ/η chains had thymuses the size and surface phenotype of which closely resemble those found in parental CD3-ε−/− and CD3-ζ/η−/− mice. As shown in Fig. 7, mice lacking both CD3-ε and CD3-ζ/η chains had thymuses displayed markedly different CD4/CD8 phenotypes, the latter closely resembling in size and composition those developing in CD4/CD8 double-mutant mice (Fig. 6).

![Figure 6](image-url)

Figure 6. T cell development in CD3-ε−/− and CD3-ζ/η−/− double mutant mice. Mice with eΔ5/Δ5ζ/η+/+g+/+, eΔ5/Δ5ζ/η−/+, eΔ5/Δ5ζη+/+, and eΔ5/Δ5ζη−/− genotypes were derived from an F2 intercross between CD3-ε−/− and CD3-ζ/η−/− mutant mice. Total thymocytes were analyzed by flow cytometry for the expression of CD4 versus CD8 (A) and CD25 versus CD44 (B). The percentages of cells found in each quadrant is indicated.

![Figure 7](image-url)

Figure 7. Vβ to Dββ rearrangements are not affected in CD3-εΔ5/Δ5 CD3-ζ/η−/− double mutant mice. The relative levels of TCR-β rearrangements found in eΔ5/Δ5ζ/η+/+g+/+, eΔ5/Δ5ζ/η−/+, and eΔ5/Δ5ζη+/+ in CD3-ε−/− and CD3-ζ/η−/− thymocytes were determined as described in the legend of Fig. 4.
Thus, in the absence of CD3-ζ/η chains, the CD3-ε55 mutation manifests a clear gene-dosage effect, suggesting that in a CD3-ζ/η-less context, it is the CD3-ε subunits that limit the number of pre-TCR subcomplexes available for driving the transition to the DP stage.

Considering that thymocytes that lack both CD3-ε and CD3-ζ/η genes are still capable of reaching the CD44-low/CD25+ DN stage during which Vβ to Dββ rearrangements normally happen (Fig. 6 B), we analyzed the status of their TCR-β loci using the DNA-PCR assay previously described in the legend of Fig. 4. As shown in Fig. 7, CD3-ε55/CD3-ζ/η−/− double mutant mice contained Dβ-->Jβ and Vβ-->Dββ rearrangements, the extent of which was similar to those found in the parental single mutant thymocytes. Finally, we examined the effects of the lack of both CD3-ε and CD3-ζ/η on the rearrangement of TCR-γ and -δ genes using a DNA-PCR approach (27). As shown in Fig. 8, the absence of both CD3-ε and CD3-ζ/η had little effect on the extent and timing of TCR-γ and -δ gene rearrangements.

Discussion

We showed that CD3-γδε and CD3-ζ/η modules are each essential for the establishment of allelic exclusion at the TCR-β locus. Their mandatory contribution to the activation of this negative feedback loop probably relates to the role they play in the assembly and function of the pre-TCR. In contrast, analysis of TCR-β transgenic, pTα−/− mice showed that TCR-β chains can trigger allelic exclusion without being associated with a pTα chain (18, 19). However, in the two experimental systems used to assess the role of pTα in the establishment of allelic exclusion at the TCR-β locus, significant variations were observed in the levels of inhibition of endogenous TCR-β gene rearrangements and accounted for by the presence of distinct TCR-β transgene copy numbers and/or insertion sites (18, 19). Regardless of these variations, the discrepancy that exists between the pTα- and CD3-ε-deficient mice with regard to the establishment of allelic exclusion at the TCR-β locus can be explained by the presence within the CD25+-DN cells of low constitutive levels of Vα→αα recombination that occur before signaling through the pre-TCR. In pTα−/− mice, and only in pTα−/− mice, the resulting TCR-α chains are likely to contribute to the premature assembly of TCR-α/β complexes capable of signaling maturation as well as allelic exclusion via their associated CD3 subunits (20). However, if Vα→αα rearrangements do occur in CD25+ DN cells, it is at a frequency at least 100-fold lower than that observed in DP cells (32). Thus, premature TCR-α chain expression can only account for part of the effects observed with the transgenic TCR-β chain in the absence of pTα. As suggested by Krotkova et al. (19), the capacity of the transgenic TCR-β to signal allelic exclusion independently of pTα may also relate to its capacity to be expressed in a phosphatidylinositol-linked form at the surface of CD25+ cells. (As discussed in the legend of Fig. 1, we have not been able to detect P14 TCR-β chains on the surface of CD3-ε55/CD3-ζ/η−/− TCR-β thymocytes.) Therefore, the occurrence of TCR-β allelic exclusion in the absence of pTα chain is likely to result from the combination of inappropriate expression of the transgenic TCR-β chains and premature TCR-α chain expression. Irrespective of these considerations, our data clearly exclude a model in which TCR-β chains can signal TCR-β allelic exclusion in the mere absence of any of the CD3 components thought to be part of the pre-TCR/CD3 sensor.

Our data also bear on the causal relationships between pre-TCR-induced cell proliferation and the establishment of TCR-β allelic exclusion. It has been suggested that pre-TCR-induced cell cycle progression is essential for the establishment of allelic exclusion at the TCR-β locus (36–39; see also references 40 and 41 in the case of B cell development). As outlined in Fig. 9, one or more rounds of DNA replication are speculated to enable the reprogramming of the chromatin structure of the TCR-β loci and make them inaccessible to the V(D)J recombinase. According to that model, the lack of TCR-β allelic exclusion observed in the CD3-ε55/CD3-ζ/η−/− thymuses would be fully accounted for by the fact that their TCR-β pTα heterodimers are prevented from inducing cell cycle entry. Mice carrying a mutation in the lck gene display a pronounced thymic atrophy associated with a dramatic reduction in the number of DP cells (42). In these mutant mice, TCR-β gene allelic exclusion is not severely compromised as the presence of a productively rearranged TCR-β transgene resulted in an almost complete inhibition of endogenous TCR-β gene rearrangements (43). Considering that TCR-β transgenic, CD3-ζ/η−/− thymuses display the same composition and cellularity as TCR-β transgenic, lck−/− thymuses (compare our data with those of Wallace et al., reference 43), it came as a surprise to find that there was in the former a clear dissociation between the transition to the DP stage and the establishment of TCR-β gene allelic exclusion. Thus, in the
of a pre-TCR complex (step 2). As soon as assembled, this complex triggers (step 3) the transition beyond the CD44−/low CD25− stage and activates a negative feedback loop that will close the accessibility of the second, partially rearranged, allele (allele b) to the V(D)J recombinase (continuous lines sandwiching the TCR-β allele), thereby restricting such a T cell to the expression of only a single TCR-β chain allele. The p56lck kinase (lk) constitutes one of the effectors operating downstream of the pre-TCR/CD3 complex since the overexpression of a catalytically active form of p56lck inhibits endogenous Vβ to D(β)J rearrangements while inducing coincidently the transition to the DP stage (30). As proposed previously (29), time delay along this negative feedback loop, and/or the existence of a few cells in which Vβ to D(β)J rearrangements can be attempted quasimultaneously on both β alleles, may explain the presence of rare cells with two productively rearranged TCR-β alleles (52, 53). Based on the comparison of TCR-β transgenic, p56lck−/−, and TCR-β transgenic, CD3-ζ/−/− mice (see Discussion), it is tempting to speculate that TCR-β gene allelic exclusion is brought about via two contingent pathways: one of which (step 4b), by inducing cell proliferation and DNA replication, enables the reprogrammation of the chromatin structure at the TCR-β locus, and thereby permits factor(s) induced by the second pathway (step 5) to act and render the TCR-β locus inaccessible to further V(D)J recombination. Note that the degradation of RAG-2, which results from cyclin-dependent kinase phosphorylation (loop 4a; reference 38) and occurs during the burst of divisions associated with the transition from the DN to the DP stage, appears to constitute a fail-safe mechanism not essential for the execution of TCR-β allelic exclusion (39).

absence of CD3-ζ/− subunit, TCR-β selection may have led to differentiation rather than proliferation and, consistent with the above model, resulted in the lack of TCR-β gene allelic exclusion. However, the frequency of dividing early DP cells is only slightly smaller in CD3-ζ/−/− mice than in wild-type littermates, indicating that CD3-ζ/−-less pre-TCR complexes are still capable of triggering cell cycle entry (9). Collectively, these observations suggest that burst of cell divisions induced by the pre-TCR may be enabling rather than inductive for the establishment of TCR-β gene allelic exclusion, and that the pre-TCR is likely to contribute additional signals to effect TCR-β gene allelic exclusion. According to this view and under physiological conditions, the signals emanating from both the lck- and CD3-ζ/−-less pre-TCR complexes suffice to trigger cell cycle entry and CD4/CD8 expression, whereas only those emanating from the former can reach the higher thresholds plausibly required to activate the regulatory loop required for mediating allelic exclusion (denoted as 5 in Fig. 9). However, it should be noted that upon massive and artefactual cross-linking, even the partial pre-TCR complexes expressed at the surface of CD3-ζ/−/− DN thymocytes are capable of inducing both maturation to the DP stage and TCR-β gene allelic exclusion (as suggested by the finding that most of the CD3-ζ/−/−/low CD25− DP cells that develop after injection of anti-CD3-ε antibodies do not contain intracellular TCR-β chains; reference 44). Therefore, our results are reminiscent of those obtained with the TCR complexes expressed on mature T cells (e.g., reference 45) in that they suggest that different pre-TCR-mediated responses display distinct activation thresholds.

Complexes consisting of calnexin and of CD3-ζ/ε or CD3-δ/ε pairs can be expressed at low levels at the surface of DN thymocytes (46). Upon cross-linking with anti-CD3-ε antibodies, they can induce the progression to the DP stage even in the absence of TCR-β and pTα chains (10, 11, 47). It is unlikely, however, that such CD3-calnexin complexes have a normal signaling function before pre-TCR expression as CD3-εL/A transgenic mice produce T cells that can reach the CD44−/low CD25+ stage and faithfully initiate Vβ to D(β)J rearrangements (12). Our analysis of thymocytes lacking both the CD3-ε and CD3-ζ/− subunits emphasizes that the CD3 subunits start to function only immediately before the CD44−/low CD25+ stage and CD44−/low CD25− transition (i.e., at a time when the pre-TCR is expected to operate). Based on the above results, the observation that overexpression of various CD3-ε transgenes blocks thymocyte development before the CD25+ DN stage (44), can be plausibly accounted for by the fact that when overexpressed the CD3-ε polypeptides can sequester...
ments. These results suggest that TCR-a/b gene rearrangements are probably not subjected to stepwise epigenetic controls analogous to those that affect TCR-a and TCR-a/b gene rearrangements and rely on the sequential expression of CD3-associated pre-TCR and TCR sensors. Finally, in the case of the a/b T cell lineage, it should be emphasized that the raison d’être of the pre-TCR may be that a/b T cells undergo a second step of selection known as TCR-a/b selection, and that there is a limited number of stromal cell niches capable of supporting such a selection event (49). Thereby, by triggering the selective expansion and maturation of only those T cell precursors expressing a TCR-a/b chain, the pre-TCR is likely to allow this limited number of cell niches not to be swamped with nonselectable (i.e., TCR-a/b-) DP cells, and maximize the efficacy of TCR-a/b selection.

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