

The CD3- $\gamma\delta\epsilon$ 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-T α 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- $\gamma\delta\epsilon$ and CD3- ζ/η modules, 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 alleles and therefore more than one TCR- α/β or TCR- γ/δ 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^{-/low}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→DJ rearrangement. TCR- β gene rearrangements start at, or at the transition

¹Abbreviations used in this paper: D, diversity; DN, double negative; DP, double positive; J, joining; PBS/FCS, PBS supplemented with 3% FCS; RAG, recombination activation gene; SP, single positive; V, variable.

to, the CD44^{-/low}CD25⁺ DN stage (2, 3), whereas the first measurable TCR- α 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- α/β 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- β gene rearrangements.

In the pre-TCR, TCR- β is disulfide linked with a polypeptide encoded by a nonrearranging gene and denoted as the pT α chain (8). To exert its function, the pre-TCR needs to associate with both the CD3- γ/ϵ and CD3- ζ 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- β ⁺ DN cells and concurrently drives their progression to the DP developmental stage (such transition is often denoted as TCR- β selection). Moreover, considering that the expression of a productively rearranged TCR- β transgene inhibits most endogenous V β to D β J β rearrangements (see above), it has been suggested that the TCR- β chain, and by extension the pre-TCR/CD3 complex, plays a pivotal role in the enforcement of allelic exclusion at the TCR- β locus. Therefore, disruption of the gene coding for the pT α subunit should have prevented assembly of a functional pre-TCR complex and affected the establishment of allelic exclusion at the TCR- β locus. However, in pT α ^{-/-} thymocytes, expression of a transgene coding for a functional TCR- β chain was found to inhibit endogenous V β to D β J β rearrangements to almost the same extent as in a pT α ^{+/+} background (18, 19). Assuming that no other gene products can compensate for the loss of pT α (e.g., the products of prematurely expressed TCR- α 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- β locus.

We have previously generated mice with a targeted mutation of the CD3- ϵ gene (referred to as CD3- $\epsilon^{\Delta 5}$; reference 12). This mutation abolishes the expression of intact CD3- ϵ polypeptides, dramatically reduces the transcription rate of the neighboring CD3- γ and CD3- δ genes, and totally blocks the progression beyond the CD44^{-/low}CD25⁺ stage. The thymocytes found in CD3- $\epsilon^{\Delta 5/\Delta 5}$ mice contain readily detectable levels of CD3- ζ , TCR- β , and pT α transcripts. However, the lack of CD3- γ/ϵ and CD3- δ/ϵ dimers is likely to prevent their pT α -TCR- β and CD3- $\zeta 2$ dimers from participating in the assembly of functional pre-TCR/CD3 complexes. The CD3- $\epsilon^{\Delta 5/\Delta 5}$ mice present several experimental advantages relative to pT α -deficient mice. First, their thymuses constitute an enriched source of CD44^{-/low}CD25⁺ DN cells devoid of contaminating downstream α/β T cell subsets and in which TCR- β gene

rearrangements do happen normally. Second, the CD3- $\epsilon^{\Delta 5}$ mutation does prevent the development of γ/δ T cells and permits the analysis of early α/β T cell development in a microenvironment insulated from the adventitious effects resulting from the presence of γ/δ T cells (2, 20, 21). Therefore, by obviating some of the experimental limitations associated with pT α ^{-/-} mice, the CD3- $\epsilon^{\Delta 5/\Delta 5}$ mice constitute a particularly appropriate model to determine whether the CD3- $\gamma\delta\epsilon$ module, and by inference the pre-TCR, is essential for the establishment of allelic exclusion at the TCR- β locus. Here we report on experiments showing that the CD3- $\gamma\delta\epsilon$ and the CD3- ζ/η modules of the pre-TCR play each a pivotal role in allelic exclusion at the TCR- β locus. In contrast, analysis of CD3- $\epsilon^{\Delta 5/\Delta 5}$ CD3- ζ/η ^{-/-} double mutant mice established that the onset of V to (D)J recombination at the TCR- β , TCR- γ , and TCR- δ loci can occur in the absence of CD3 subunits.

Materials and Methods

Mice. The CD3- $\epsilon^{\Delta 5/\Delta 5}$ mice and CD3- ζ/η ^{-/-} mice have been described (12, 22). Recombination activation gene (RAG)-1^{-/-} mice were originally obtained from E. Spanopoulou (The Rockefeller University, New York; 23). The P14 TCR- β transgenic mice (line 128) express a TCR- β cDNA (V β 8.1-D β -J β 2.4) derived from the T cell clone P14 (24). TCR- β transgenic mice were typed for the presence of the transgene by PCR analysis of tail DNA. TCR- β transgenic mice were crossed with CD3- $\epsilon^{\Delta 5}$ and CD3- ζ/η -deficient mice to obtain CD3- $\epsilon^{\Delta 5/\Delta 5}$ TCR- β and CD3- ζ/η ^{-/-} TCR- β mice. CD3- $\epsilon^{\Delta 5/\Delta 5}$ CD3- ζ/η ^{-/-} double-deficient mice were derived from CD3- $\epsilon^{\Delta 5/\Delta 5}$ \times CD3- ζ/η ^{-/-} 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- ϵ (2C11), CD4 (H129.19), CD8 (53-6.7), CD25 (7D4), CD44 (Pgp-1), and TCR V β 8 (F23.1) were purchased from PharMingen (San Diego, CA). Biotinylated antibodies against Mac-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 tricolor (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⁺ Thymocytes. 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^{high} cells.

Intracellular Staining. Expression of the transgenic TCR- β chain within the CD25⁺-thymocyte subset was assessed by intracellular/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-V β 8 (MR5.2) 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- $\epsilon^{\Delta 5/\Delta 5}$ and CD3- $\epsilon^{\Delta 5/\Delta 5}$ TCR- β samples) or CD25^{high} (sorted from the CD3- $\epsilon^{+/+}$ TCR- β sample) thymocytes using TRIzol[™] (GIBCO BRL, Cergy Pontoise, France) as recommended by the manufacturer. Before conversion to cDNA, RNA samples were treated with DNaseI-RNase free (Pharmacia). Conversion to cDNA was done on 1 μ g 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 C β 2 primers used to detect transcripts incorporating the TCR C β 2 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 P14 β : 5'-GTGATGCCGGGG-GGCGGAACAC-3'; and β -globin 3'UT: 5'-GGCATTAGC-CACACCAGCCACCA-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; NEN Life Science Products, LeBlanc Mesnil, France), and hybridized using a 5'-kinased oligonucleotide (C β 2A; reference 26).

Detection of Endogenous TCR Rearrangement. Total or fractionated thymocytes were solubilized in lysis buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 2 mM MgCl₂, 0.45% Nonidet P-40, 0.45% Tween 20, 60 μ g/ml proteinase K) at a concentration of 10⁷ cells/ml. After overnight incubation at 56°C, samples were heated to 95°C for 30 min to inactivate proteinase K. Dilutions of

the template genomic DNA (corresponding to 10⁵, 2 \times 10⁴, and 10⁴ cell equivalent per 10 μ l) were prepared for each sample to demonstrate that there is a linear relationship between product yield and the number of input target sequences. PCR amplifications were done in a final volume of 50 μ l and included 10 μ l of template DNA solution, 1 μ M of each primer, 200 μ M of each dNTP, 2.5 μ l of PCR buffer (166 mM [NH₄]₂SO₄, 670 mM Tris-HCl [pH 8.8], 1 mg/ml BSA), 0.2 U Taq DNA polymerase (GIBCO BRL) and MgCl₂ at a final concentration of 2.5 mM. Each cycle consisted of incubation at 94°C for 1 min, followed by annealing at 63°C for 2 min and extension for 10 min at 72°C, and was repeated 24 times. 25 μ l of the reaction was fractionated on a 1.0% agarose gel, transferred to nylon membrane (Gene-Screen Plus) and hybridized with ³²P-labeled oligonucleotide probes. Hybridizing bands were quantitated using a phosphorimager (BAS 100; Fuji, Raytest 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- γ and TCR- δ chain 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 throrax gene (Mtrx) using primers MTRX1: 5'-AGGGTAAGCTGTGCTATGG-3' and MTRX2: 5'-AGT-AGTGTTCCTCAGTCCCC-3'.

Results

A Productively Rearranged TCR- β Transgene Is Unable to Activate the Transition to the DP Stage in the Absence of CD3- ϵ . To determine whether the expression of a TCR- β chain

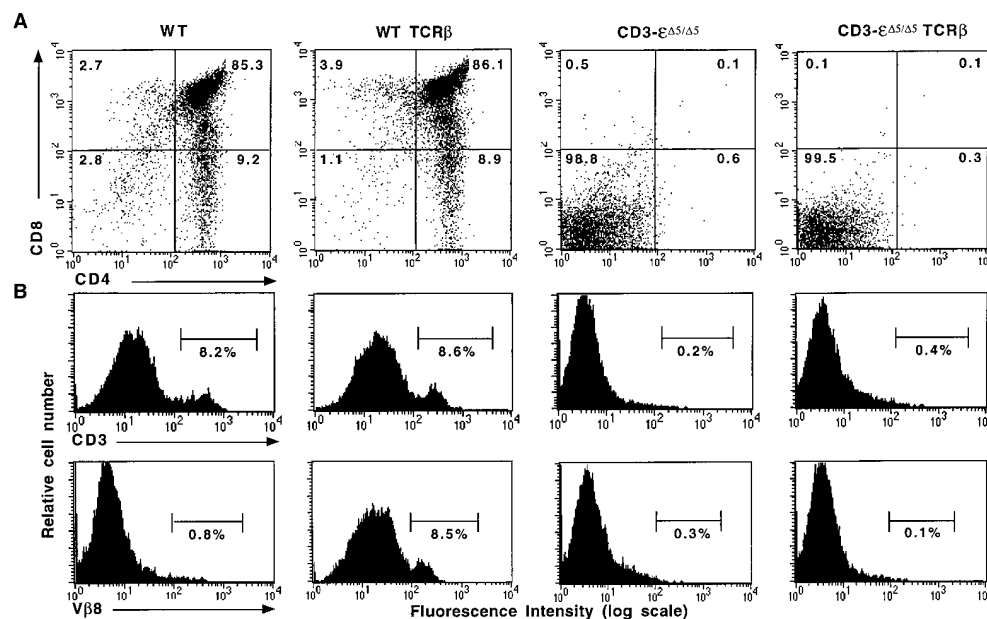


Figure 1. A transgene encoding a productively rearranged TCR- β gene does not restore T cell development in CD3- ϵ -deficient mice. Mice with CD3- $\epsilon^{+/+}$ (WT), CD3- $\epsilon^{+/+}$ TCR- β (WT TCR- β), CD3- $\epsilon^{\Delta 5/\Delta 5}$ and CD3- $\epsilon^{\Delta 5/\Delta 5}$ TCR- β genotypes were derived from a F2 intercross between the TCR- β transgenic line P14 TCR- β (TCR- β) and CD3- $\epsilon^{\Delta 5/\Delta 5}$ mutant mice. (A) Thymocytes were analyzed by flow cytometry for the expression of CD4 versus CD8. The percentage of cells found in each quadrant is indicated. (B) Thymocytes were analyzed for the expression of CD3- ϵ and V β 8. Percentage of CD3^{high} and V β 8^{high} cells are indicated. Considering that the exon coding for the epitope recognized by the 2C11 anti-CD3- ϵ antibody has been deleted in the CD3- $\epsilon^{\Delta 5}$ mutant gene, and that CD3- $\epsilon^{\Delta 5/\Delta 5}$ thy-

mocytes do not express detectable levels of TCR- β chain at their surface (12), the histograms obtained after staining CD3- $\epsilon^{\Delta 5/\Delta 5}$ thymocytes with anti-CD3- ϵ or anti-V β 8 antibodies were used as genuine negative control histograms. Note that in contrast to the situation previously observed in TCR- β transgenic SCID mice (50) and TCR- β transgenic RAG^{-/-} mice (51) where transgenic TCR- β chains are expressed as monomers without CD3- ϵ and in a phosphatidyl inositol-linked form, we have not been able to detect P14 TCR- β transgenic chains on the surface of CD3- $\epsilon^{\Delta 5/\Delta 5}$ TCR- β thymocytes after staining with the C β -specific antibody H57.597 (data not shown) and V β 8-specific antibody F23.1 (compare the CD3- $\epsilon^{\Delta 5/\Delta 5}$ and CD3- $\epsilon^{\Delta 5/\Delta 5}$ TCR- β histograms). Whether such difference resulted from the use of the P14 TCR- β transgene or is rather due to the CD3- $\epsilon^{\Delta 5/\Delta 5}$ background remains to be determined.

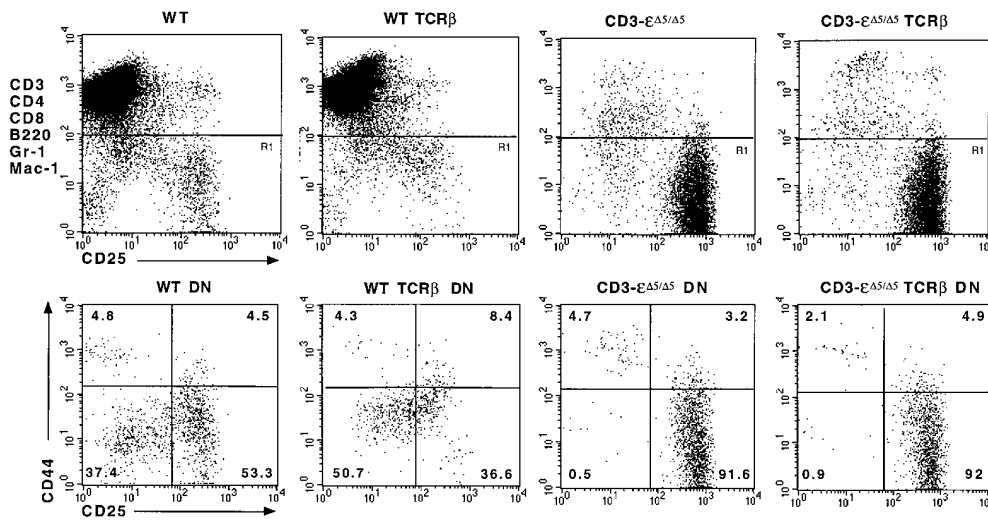


Figure 2. Comparison of the triple negative thymocyte subsets from wild type (*WT*) mice and $CD3\text{-}\epsilon^{\Delta5/\Delta5}$ mutant mice in the presence or absence of P14 TCR- β transgene (*TCR β*). Thymocytes were stained with anti-CD3, -CD4, -CD8, -B220, -Mac-1, and Gr-1 (all biotinylated and 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.

transgene was able to inhibit endogenous $V\beta$ to $DB\beta$ rearrangements in the absence of CD3- ϵ subunit, $CD3\text{-}\epsilon^{\Delta5/\Delta5}$ mice were crossed with transgenic mice carrying a productively rearranged $V\beta8^+$ 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 $V\beta8^+$ (Fig. 1, compare transgenic [*WT TCR- β*] and nontransgenic [*WT*] wild-type panels). As shown in Fig. 1 A, $CD3\text{-}\epsilon^{\Delta5/\Delta5}$ TCR- β mice had thymuses that did not develop past the DN stage and contained absolute cell numbers similar to nontransgenic $CD3\text{-}\epsilon^{\Delta5/\Delta5}$ thymuses. Thus, expression of the P14 TCR- β transgene was unable to restore T cell development in $CD3\text{-}\epsilon^{\Delta5/\Delta5}$ 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 $CD3\text{-}\epsilon^{\Delta5/\Delta5}$ 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 $CD44^{-/low}CD25^{-}$ thymocytes at the expense of their immediate $CD44^{-/low}CD25^+$ precursors. This finding is in line with previous data showing that TCR- β transgenic mice exhibit $CD44^{-/low}CD25^+$ 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 $CD44^{-/low}CD25^+$ cells equipped with a productively rearranged TCR- β transgene progress on average much more rapidly to the $CD44^{-/low}CD25^{-}$ stage than their nontransgenic counterparts (29). Interestingly, the DN cells found in the $CD3\text{-}\epsilon^{\Delta5/\Delta5}$ and $CD3\text{-}\epsilon^{\Delta5/\Delta5}$ TCR- β mice were both arrested at the same $CD44^{-/low}CD25^+$

stage and lacked not only the $CD44^{-/low}CD25^{-}$ cells proper, but also most of the $CD44^{-/low}CD25^{low}$ to - intermediates. Thus, it is likely that in the absence of CD3- $\gamma\delta\epsilon$ module, pT α -TCR- β^{P14} heterodimers were prevented from assembling into functional pre-TCR complexes and unable to rescue the blockade in thymic development observed in $CD3\text{-}\epsilon^{\Delta5/\Delta5}$ mice. Alternatively, the onset of expression of the P14 TCR- β transgene during T cell development may have occurred only after the $CD44^{-/low}CD25^+$ stage and accounted for its failure to rescue T cell development in $CD3\text{-}\epsilon^{\Delta5/\Delta5}$ mice.

Considering that the P14 TCR- β transgene consists of a TCR- β cDNA placed under the control of the H-2K^b promoter and Ig heavy chain enhancer, its expression within the $CD25^+$ DN compartment should have depended solely on the activation of its transcription. To ascertain the presence of P14 TCR- β transcripts within the $CD25^+$ DN cell populations from $CD3\text{-}\epsilon^{+/+}$ TCR- β and $CD3\text{-}\epsilon^{\Delta5/\Delta5}$ 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 $CD3\text{-}\epsilon^{\Delta5/\Delta5}$ TCR- β sample and in the $CD25^+$ cells sorted from $CD3\text{-}\epsilon^{+/+}$ TCR- β thymuses. In contrast, RNA extracted from $CD3\text{-}\epsilon^{\Delta5/\Delta5}$ 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 C β 2 gene, the $CD3\text{-}\epsilon^{\Delta5/\Delta5}$ RNA showed an hybridizing band corresponding to endogenous (D)J-C β and V(D)J-C β transcripts (12). To exclude any potential posttranslational regulation affecting the expression of the transgenic P14 TCR- β chains, $CD3\text{-}\epsilon^{\Delta5/\Delta5}$ TCR- β thymocytes were further analyzed by intracellular staining with an antibody (F23.1: anti- $V\beta$ 8) specific for the product of the $V\beta$ gene segment used by the P14 transgenic TCR- β chain. As shown in Fig. 3 B, most of the $CD25^+$ cells found in $CD3\text{-}\epsilon^{\Delta5/\Delta5}$ TCR- β thymuses expressed the intracellular transgenic TCR- β chain. Therefore, these re-

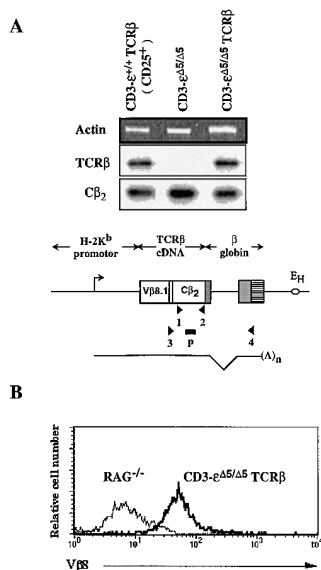


Figure 3. Assessment of TCR- β transgene expression by RNA-PCR and intracytoplasmic staining. (A) CD25⁺ cells sorted from TCR- β transgenic wild-type thymuses (CD3- ϵ ^{+/+} TCR- β) and total thymocytes from TCR- β transgenic CD3- ϵ ^{Δ 5/ Δ 5} mice (CD3- ϵ ^{Δ 5/ Δ 5} TCR- β) were analyzed for the presence of transcripts originating from the P14 TCR- β transgene using the RNA-PCR strategy depicted in the bottom diagram. The P14 TCR- β cDNA is expressed under the control of the H-2K^b promoter and IgH chain intronic enhancer (E_H). The 3' end of the P14 TCR- β cDNA is linked to a genomic fragment of the human β globin gene that provides both an intron and a polyadenylation sequence (24). Owing to the presence of this intron, primers for PCR amplification can be chosen to distinguish amplification products corresponding to transgene 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 3' 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- ϵ ^{Δ 5/ Δ 5} thymocytes 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 C β 2 exon. The products resulting from amplification with primer pairs 1 + 2 (TCR C β 2) and 3 + 4 (TCR- β Tg) were gel fractionated, blotted, and hybridized with a C β 2-specific probe (p). The location of specific primers are indicated by arrowheads and the transcription start site of the TCR- β transgene 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 (*Actin*). (B) The presence of the transgenic P14 TCR- β chain within the CD25⁺ subset present in CD3- ϵ ^{Δ 5/ Δ 5} transgenic β thymocytes was revealed by intracellular staining with an antibody (F23.1) specific for the V β 8 gene segment used by the P14 TCR β chain. RAG-1^{-/-} thymocytes were also included as negative controls. Cytoplasmic staining of the CD25⁺ compartment from nontransgenic CD3- ϵ ^{Δ 5/ Δ 5} mice revealed <1% F23.1⁺ cells.

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sults indicate that both the transcription and translation of the P14 TCR- β transgene were effective at the CD44^{-/low} CD25⁺ DN stage. Consistent with the latter results, introduction of the P14 TCR- β transgene in RAG-1-deficient mice was found to rescue the progression to the DP stage (data not shown).

Allelic Exclusion of Endogenous TCR- β Gene Rearrangements Is Absent in CD3- ϵ ^{Δ 5/ Δ 5} TCR- β Thymocytes. Having established that the P14 TCR- β transgene was expressed properly at the stage at which allelic exclusion is expected to take place, we then determined its impact on TCR- β gene allelic exclusion in the presence or absence of the CD3- ϵ mutation. This can be assessed using a DNA-PCR assay that provides an estimation of the relative levels of D β -J β and V β -D β J β rearrangements in various cell samples (30). As depicted in Fig. 4 (*bottom diagram*), primers complementary to V β or D β gene segments were used in com-

bination with a primer positioned immediately 3' to the J β 2 cluster, allowing amplification of rearranged, but not germline, V β gene segments. The resulting PCR products were visualized by hybridization with a J β 2-specific probe after electrophoresis and blot transfer. Hybridizing bands were quantitated using a phosphorimager and the relative levels of rearrangements expressed as percentages of those observed in wild-type CD25⁺ T cells (Fig. 4 B). The results shown in Fig. 4 corresponded to rearrangements of D β 2 (*top*), V β 5 (*middle*), and V β 8 (*bottom*) to each of the six J β 2 gene segments and were generated using CD25⁺ cells sorted from CD3- ϵ ^{+/+} (WT CD25⁺) and CD3- ϵ ^{+/+} TCR- β (*TCR β CD25⁺*) thymuses, and total thymocytes from CD3- ϵ ^{Δ 5/ Δ 5} and CD3- ϵ ^{Δ 5/ Δ 5} TCR- β mice. Consistent with previous data indicating that β chain gene allelic exclusion acts at a point subsequent to D β -J β joining events (1), the levels of D β 2 to J β 2 rearrangements were almost equally high in all four samples (Fig. 4, A and B, *top*). Comparison of CD3- ϵ ^{Δ 5/ Δ 5} thymocytes and wild-type CD25⁺ cells indicated that the former contained V \rightarrow DJ rearrangements that were as extensive as those found in wild-type CD25⁺ thymocytes (Fig. 4, lanes WT CD25⁺ and CD3- ϵ ^{Δ 5/ Δ 5}). As previously documented, using total thymocytes and TCR- β transgenes unrelated to the one used herein (18, 19, 30), expression of the P14 TCR- β transgene in wild-type CD25⁺ DN cells (Fig. 4, lane *TCR β CD25⁺*) resulted in a dramatic reduction of endogenous V β rearrangements (~13% of control). The latter result confirms that the P14 TCR- β transgene is expressed in a functional form at the CD25⁺ DN stage, capable of mediating allelic exclusion at the TCR- β locus and preventing expression of a second TCR- β chain on the cell surface of transgenic SP thymocytes (Fig. 1 B). In contrast, the simultaneous presence of the CD3- ϵ ^{Δ 5} mutation (lane *TCR β CD3- ϵ ^{Δ 5/ Δ 5}*, Fig. 4) prevented the effects of the transgenic TCR- β chain and permitted rearrangements of endogenous TCR V β gene segments to occur at very substantial levels (85–91% of control). Thus, these data indicate that a functional TCR- β transgene does not inhibit endogenous V β to D β J β rearrangements in the absence of CD3- ϵ subunit.

Allelic Exclusion of Endogenous TCR- β Gene Rearrangements Is Ineffective in CD3- ζ / η ^{-/-} TCR- β Thymocytes. 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 from 2–30-fold less DP cells than wild-type littermates. These DP cells appear to have been generated via TCR- β selection since almost all of them express intracellular TCR- β chains, a situation that contrasts with that observed in pT α ^{-/-} mice (20) and is consistent with the complete absence of γ / δ 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

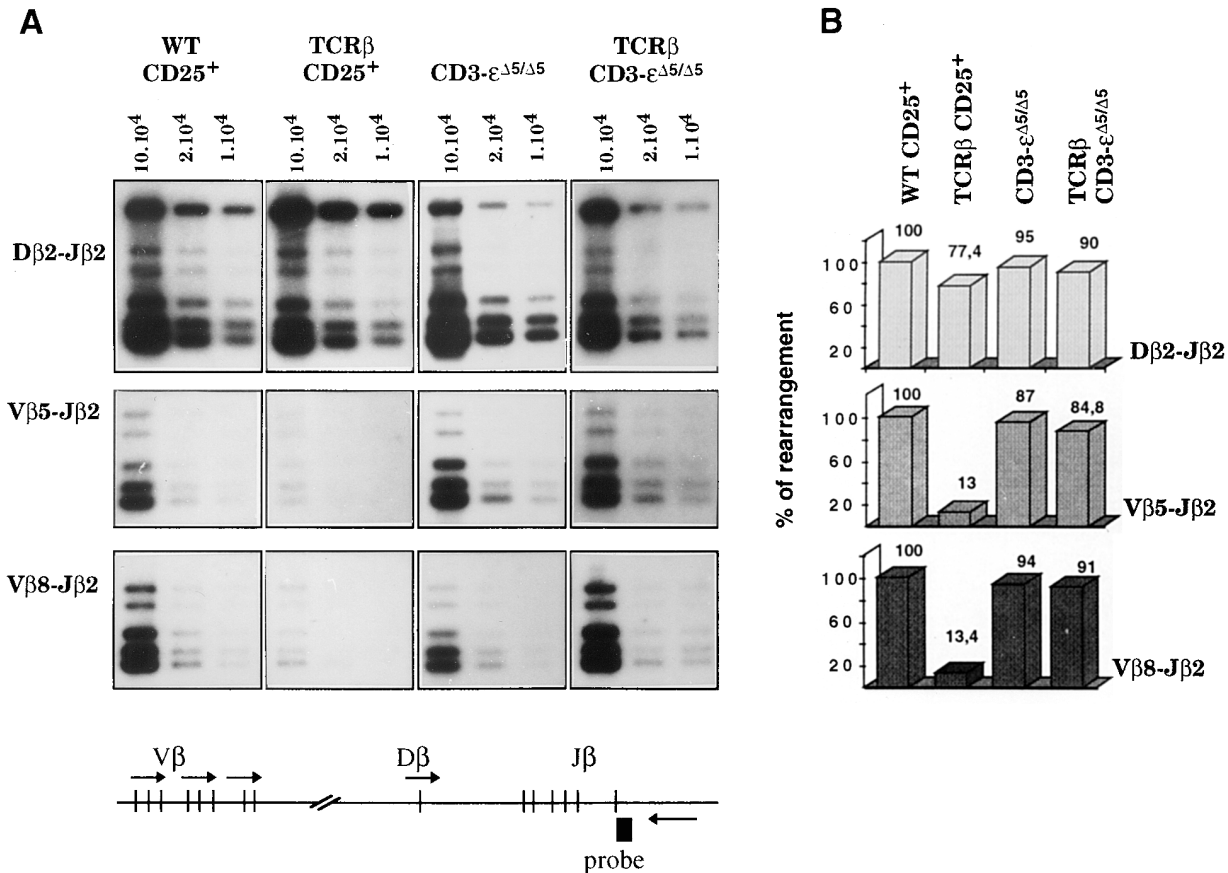


Figure 4. Expression of a transgenic TCR- β chain does not inhibit endogenous V β to D β J β rearrangements in the absence of CD3- ϵ polypeptide. (A) Relative levels of TCR- β rearrangements in CD25⁺ cells sorted from CD3- ϵ ^{+/+} (WT CD25⁺) and CD3- ϵ ^{+/+} TCR- β (TCR β CD25⁺) thymuses, and total thymocytes from CD3- ϵ ^{Δ5/Δ5} and CD3- ϵ ^{Δ5/Δ5} TCR- β mice. Identical sorting windows were set up on CD25^{high} DN cells for both the CD3- ϵ ^{+/+} and CD3- ϵ ^{+/+} TCR- β samples. Considering that they contain >90% CD44^{-/low}CD25^{high} DN cells (see Fig. 2), the CD3- ϵ ^{Δ5/Δ5} and CD3- ϵ ^{Δ5/Δ5} TCR- β thymuses were not subjected to sorting before analysis. The extent of D β -J β and V β -D β J β 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 J β 2 3' primer with D β 2- (top), V β 5- (middle) or V β 8- (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 (V β 8.1-D β 3-J β 2.4) is not detectable with the pair of primers used to reveal endogenous V β 8-J β 2 rearrangements. For each sample, dilutions of DNA template corresponding to 1×10^5 , 2×10^4 , and 1×10^4 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.

(9). The split pattern of phenotypic changes elicited by the pre-TCR in the absence of CD3- ζ/η 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 V α - \rightarrow J α recombination). Along that line, it was interesting to analyze whether the CD3- ζ/η subunit of the pre-TCR was required for the establishment of allelic exclusion at the TCR- β locus. To this end, CD3- ζ/η ^{-/-} 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- ζ/η -deficient thymocytes are similar to those found in wild-type littermates (12), and that the introduction of the P14 TCR- β transgene in CD3- ζ/η ^{-/-} mice did not

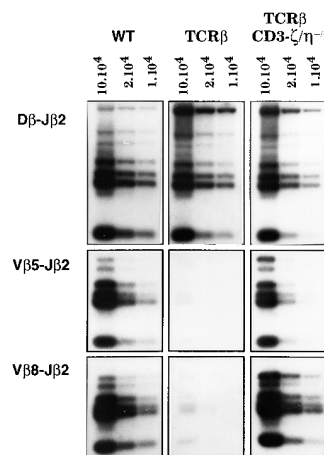


Figure 5. Expression of a transgenic TCR- β chain does not inhibit endogenous V β to D β J β rearrangements in the absence of CD3- ζ/η polypeptide. The relative levels of TCR- β rearrangements found in CD3- ζ/η ^{+/+} (WT), CD3- ζ/η ^{+/+} TCR- β (TCR β), and CD3- ζ/η ^{-/-} TCR- β thymocytes were determined as described in the legend of Fig. 4.

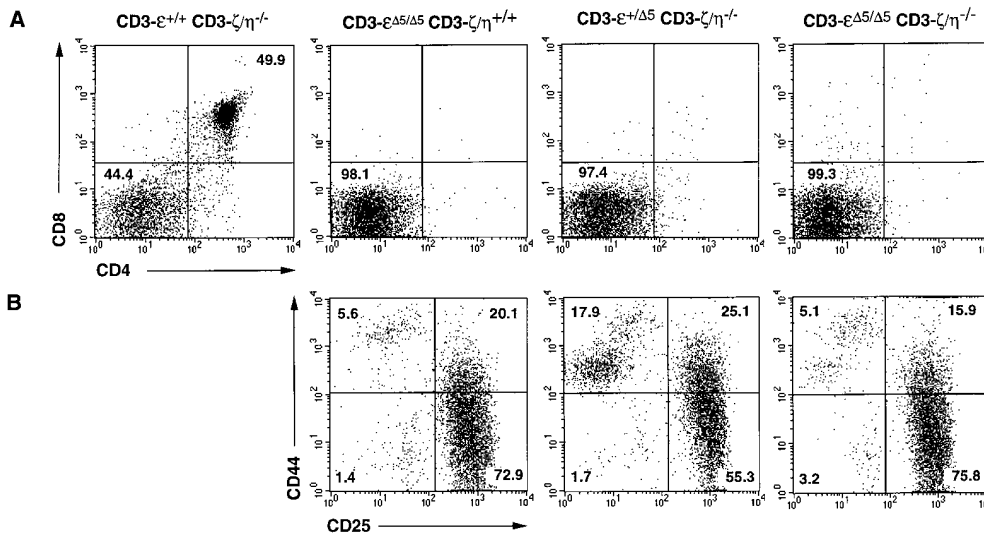


Figure 6. T cell development in $CD3-\epsilon^{\Delta5/\Delta5}CD3-\zeta/\eta^{-/-}$ double mutant mice. Mice with $\epsilon^{\Delta5/\Delta5}\zeta/\eta^{+/+}$, $\epsilon^{+/+}\zeta/\eta^{-/-}$, $\epsilon^{+/+\Delta5}\zeta/\eta^{-/-}$, and $\epsilon^{\Delta5/\Delta5}\zeta/\eta^{-/-}$ genotypes were derived from an F2 intercross between $CD3-\epsilon^{\Delta5/\Delta5}$ and $CD3-\zeta/\eta^{-/-}$ 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.

lead to any change in thymocyte cellularity and surface phenotype (data not shown). As shown in Fig. 5, the level of D β 2 to J β 2 rearrangement was similar in DNA extracted from wild-type (WT), TCR- β wild-type (*TCR β*), and TCR- β $CD3-\zeta/\eta^{-/-}$ mice. As previously documented for $CD3-\epsilon$ -deficient mice (see above), allelic exclusion of the endogenous TCR- β locus was severely compromised in the absence of $CD3-\zeta/\eta$ polypeptide (Fig. 5, compare V to DJ rearrangements in lanes *TCR β* and *TCR β $CD3-\zeta/\eta^{-/-}$*). Thus, these data suggest that the signals conveyed by the partial pre-TCR/CD3 complexes found in $CD3-\zeta/\eta$ -deficient mice 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 $CD3-\epsilon$ and $CD3-\zeta/\eta$ 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 $Ig\alpha/Ig\beta$ transducing subunits and triggers both the selective amplification/maturation of IgH^+ pre-B cells and establishment of allelic exclusion at the IgH locus (34). $Ig\beta$ -deficient mice show a complete block in B cell development at a stage corresponding to the $CD44^{-/low}CD25^+$ stage of T cell development (35). Interestingly, VH to DHJH rearrangements were found to be severely reduced in $Ig\beta$ -deficient mice, whereas DH to JH rearrangements proceeded normally. This indicated that $Ig\beta$ may play an important regulatory role in the onset of VH to DHJH recombination. When bred separately, the $CD3-\epsilon^{\Delta5}$ and $CD3-\zeta/\eta$ mutations had no discernible effect on the occurrence and extent of V β to D β J β 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 $CD3-\epsilon$ and $CD3-\zeta/\eta$ chains play redundant regulatory roles in the onset of V β to D β J β recombination. To ad-

dress this question, mice lacking both proteins were derived from a F2 intercross between $CD3-\epsilon^{\Delta5/\Delta5}$ and $CD3-\zeta/\eta^{-/-}$ mice. As shown in Fig. 6, mice lacking both $CD3-\epsilon$ and $CD3-\zeta/\eta$ chains had thymuses the size and surface phenotype of which closely resemble those found in parental $CD3-\epsilon^{\Delta5/\Delta5}$ mice (Fig. 7, compare the CD4/CD8 and CD44/CD25 profiles of panels $\epsilon^{\Delta5/\Delta5}\zeta/\eta^{+/+}$ and $\epsilon^{\Delta5/\Delta5}\zeta/\eta^{-/-}$). Interestingly, $\epsilon^{+/+}\zeta/\eta^{-/-}$ and $\epsilon^{+/+\Delta5}\zeta/\eta^{-/-}$ thymuses displayed markedly different CD4/CD8 phenotypes, the latter closely resembling in size and composition those developing in $\epsilon^{\Delta5/\Delta5}\zeta/\eta^{-/-}$ double-mutant mice (Fig. 6).

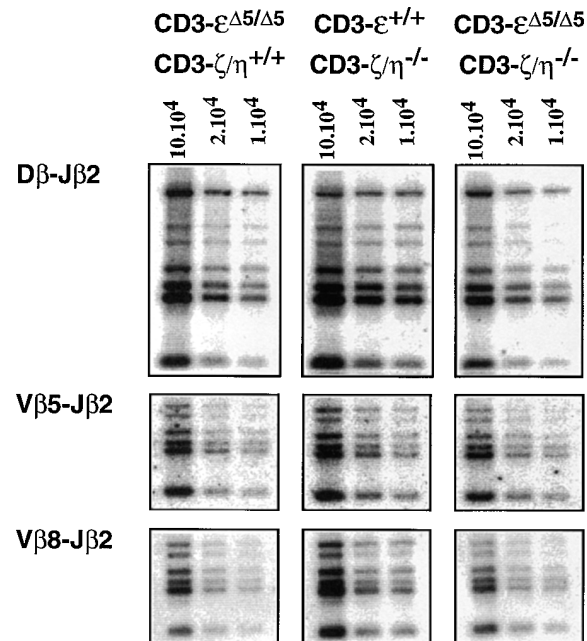


Figure 7. V β to D β J β rearrangements are not affected in $CD3-\epsilon^{\Delta5/\Delta5}CD3-\zeta/\eta^{-/-}$ double mutant mice. The relative levels of TCR- β rearrangements found in $\epsilon^{\Delta5/\Delta5}\zeta/\eta^{+/+}$, $\epsilon^{+/+}\zeta/\eta^{-/-}$, and $\epsilon^{\Delta5/\Delta5}\zeta/\eta^{-/-}$ thymocytes were determined as described in the legend of Fig. 4.

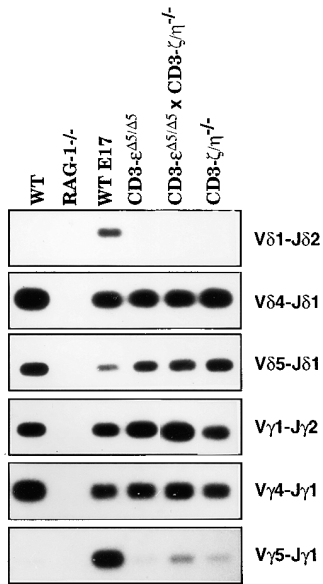


Figure 8. Relative levels of TCR- γ and TCR- δ gene rearrangements in RAG-1^{-/-}, CD3- $\epsilon^{\Delta 5/\Delta 5}$, CD3- $\zeta/\eta^{-/-}$, CD3- $\epsilon^{\Delta 5/\Delta 5}$, CD3- $\zeta/\eta^{-/-}$, and wild-type thymocytes. DNA extracted from thymocytes of fetuses at day 17 of gestation (*wt E17*) and of 4–6-wk-old wild-type (*WT*) and mutant mice was amplified with PCR primer pairs specific for the V $\delta 1$ -J $\delta 2$, V $\delta 4$ -J $\delta 1$, V $\delta 5$ -J $\delta 1$, V $\gamma 1$ -J $\gamma 2$, V $\gamma 4$ -J $\gamma 1$, and V $\gamma 5$ -J $\gamma 1$ rearrangements. PCR products were gel fractionated and the corresponding Southern blots hybridized with labeled oligonucleotide probes.

Thus, in the absence of CD3- ζ/η chains, the CD3- $\epsilon^{\Delta 5}$ 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 V β to D β J β recombination normally happens (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- $\epsilon^{\Delta 5/\Delta 5}$ CD3- $\zeta/\eta^{-/-}$ double mutant mice contained D β →J β and V β →D β J β 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- $\gamma\delta\epsilon$ 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, pT $\alpha^{-/-}$ mice showed that TCR- β chains can trigger allelic exclusion without being associated with a pT α chain (18, 19). However, in the two experimental systems used to assess the role of pT α 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 pT α - and CD3-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 V α →J α recombination that occur before signaling through the pre-TCR. In pT $\alpha^{-/-}$ mice, and only in pT $\alpha^{-/-}$ 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 V α -J α 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 pT α . As suggested by Krotkova et al. (19), the capacity of the transgenic TCR- β to signal allelic exclusion independently of pT α may also relate to its capacity to be expressed in a phosphatidyl inositol-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- $\epsilon^{\Delta 5/\Delta 5}$ TCR- β thymocytes.) Therefore, the occurrence of TCR- β allelic exclusion in the absence of pT α 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 preTCR-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- $\epsilon^{\Delta 5/\Delta 5}$ thymuses would be fully accounted for by the fact that their TCR- β pT α 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- $\zeta/\eta^{-/-}$ 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

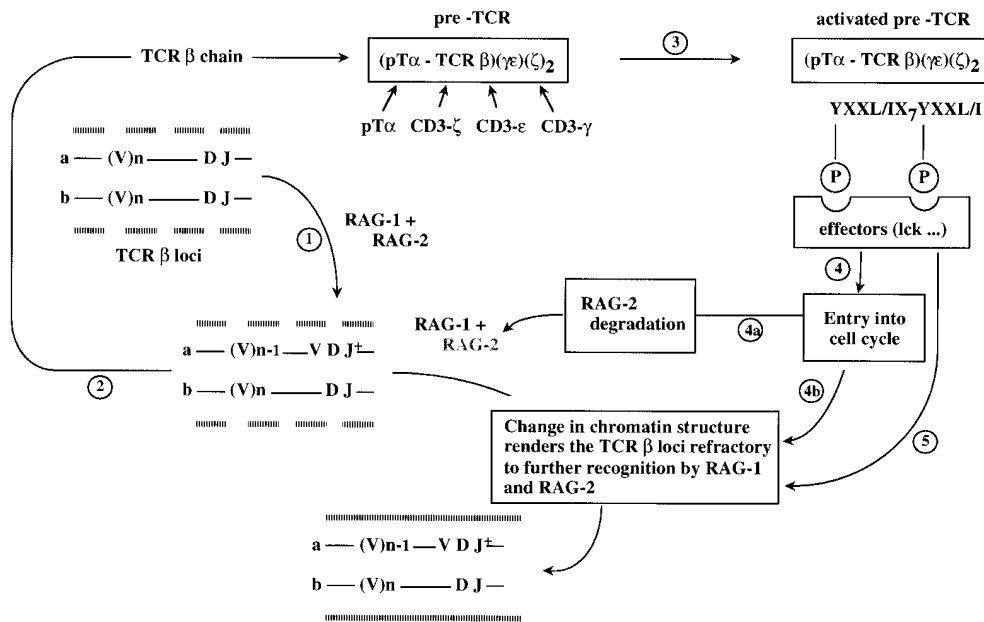


Figure 9. A model accounting for the role of the pre-TCR/CD3 complex in the establishment of allelic exclusion at the TCR-β locus. Upon entering the CD44^{-low} CD25⁺ compartment, the TCR Vβ gene segment cluster [(V)n] become accessible to the V(D)J recombinase (dashed lines sandwiching the TCR-β alleles a and b). At that stage of development, the pTα and CD3 components of the pre-TCR are already available and it is the TCR-β polypeptides that constitute the rate limiting factor in the assembly of the pre-TCR/CD3 complex. From a cohort of nine CD44^{-low} CD25⁺ triple negative thymocytes, three are expected to produce a functional Vβ gene (VDJ⁺) as a result of their first attempt of rearrangement (step 1, see reference 29). The resulting TCR-β polypeptide participates in the assembly

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 alleles), thereby restricting such a T cell to the expression of only a single TCR-β chain allele. The p56^{lck} kinase (*lck*) constitutes one of the effector operating downstream of the pre-TCR/CD3 complex since the overexpression of a catalytically active form of p56^{lck} inhibits endogenous Vβ to DβJβ rearrangements while inducing coincidentally 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 DβJβ 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, p56^{lck}^{-/-}, and TCR-β transgenic, CD3-ζ/η^{-/-} 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 reprogramming 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/η 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/η^{-/-} mice than in wild-type littermates, indicating that CD3-ζ/η-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 that view and under physiological conditions, the signals emanating from both the *lck*- and CD3-ζ/η-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 threshold 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/η^{-/-} 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/η^{-/-} 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-γ/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-ε^{Δ5/Δ5} mice produce T cells that can reach the CD44^{-low}CD25⁺ stage and faithfully initiate Vβ to DβJβ rearrangements (12). Our analysis of thymocytes lacking both the CD3-ε and CD3-ζ/η chains emphasizes that the CD3 subunits start to function only immediately before the CD44^{-low}CD25^{high} to 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

effector or adaptor molecules belonging to signaling cassettes involved in the progression to the CD25⁺ DN stage (e.g., those operated by c-kit and the common cytokine receptor γ chain; reference 48). Collectively, our findings strongly suggest that 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. In contrast, during B cell development, the Ig β transducing subunit appears to play a unique role in the initiation of VH to DHJH recombination, independent of, and prior to, its function as a component of the pre-B cell receptor (34, 35). Additionally, we have found that none of the CD3 components are required for the completion of TCR- γ and TCR- δ chain gene rearrangements. These results suggest that TCR- γ and TCR- δ gene

rearrangements are probably not subjected to stepwise epigenetic controls analogous to those that affect TCR- α and TCR- β gene rearrangements and rely on the sequential expression of CD3-associated pre-TCR and TCR sensors. Finally, in the case of the α/β T cell lineage, it should be emphasized that the *raison d'être* of the pre-TCR may be that α/β T cells undergo a second step of selection known as TCR- α/β 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- β chain, the pre-TCR is likely to allow this limited number of cell niches not to be swamped with nonselectable (i.e., TCR- β ⁻) DP cells, and maximize the efficacy of TCR- α/β selection.

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