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Restoration of early thymocyte differentiation in T-cell receptor β-chain-deficient mutant mice by transmembrane signaling through CD3ε

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ABSTRACT **Thymic repertoire selection requires the expression of the αβCD3 T-cell receptor (TCR) together with the coreceptors CD4 and CD8. The appearance of CD4 and CD8 on thymocytes is the hallmark of a complex maturation step, accompanied by downregulation of the interleukin 2 receptor (IL-2R) α chain, arrest of rearrangement (i.e., allelic exclusion) of the TCR β-chain locus, a burst of cell divisions, and reduction in cell size. This maturation step is inhibited in TCR β-chain-deficient mouse strains and may depend on surface expression of an immature TCR complex containing CD3 and TCR β chains but no TCR α chain. Here we show that the $CD4^+8^+$ double-positive (DP) stage can be induced by treatment of fetal thymic organ cultures with anti-CD3ε monoclonal antibodies in several TCR β-chain-deficient mouse strains: severe combined immunodeficient (scid) mice, mice carrying a mutation in the recombination activating gene 1 (Rag-1), or mice carrying a deletion in the TCR β-chain locus itself. These findings suggest that CD3ε is expressed on the thymocyte surface independent of and prior to the TCR β chain. The data are consistent with the notion that in wild-type mice the DP stage is induced by transmembrane signaling through an immature CD3–TCR β-chain complex, which can be bypassed by crosslinking of CD3ε alone.**

In the thymus, T-lymphoid precursor cells develop into mature T cells. The development of thymocytes is a complex process, marked by the rearrangement of the T-cell receptor (TCR) gene loci and sequential surface expression of stage-specific glycoproteins. An early stage of thymocyte development is defined by the expression of interleukin 2 receptor α chain (IL-2Rα) (1, 2). At this stage, rearrangement of the TCR β-chain gene locus takes place (3). An important control point in subsequent thymocyte differentiation is maturation to the $CD4^+8^+$ stage. This maturation step is accompanied by loss of IL-2Rα expression, a burst of cell divisions, and arrest of further rearrangement associated with allelic exclusion of the TCR β-chain locus (3–5). Following the wave of proliferations, the double-positive (DP) thymocytes become small, slowly cycling cells. During the DP stage, rearrangement of the TCR α-chain gene locus takes place (3). When functional TCR α-chain gene rearrangement has been accomplished, a complete TCR αβ-chain–CD3 complex can be expressed on the cell surface. Repertoire selection may now take place, leading to the development of self-restricted, self-tolerant $CD4^+$ or $CD8^+$ single-positive (SP) T cells (6, 7).

Surface expression of and signal transduction through a TCR–CD3 complex may also play a role prior to repertoire selection that is in maturation to the DP stage. In mice unable to produce TCR β chains, either due to a mutation in the TCR β-chain locus itself or due to a deficiency in the rearrangement machinery (scid or Rag-1 or -2 mutants), thymocyte development is blocked at the $CD4^-8^-$ double-negative (DN) stage (8–11). Recent studies have shown that, in contrast to mature T cells, immature thymocyte cell lines are able to express CD3 components on the cell surface together with the TCR β-chain polypeptide in the absence of TCR α, γ, or δ chains (12, 13) or without any TCR polypeptide chain (14, 15). Expression of an immature TCR β-chain–CD3 complex has also been detected on the cell surface of thymocytes derived from TCR β-chain transgenic Rag-2 mutant mice (16) or TCR β-chain transgenic Rag-1 mutant mice (8). These findings lead to the hypothesis that immature thymocytes receive a signal from an immature TCR β-chain–CD3 complex, confirming successful rearrangement of the TCR β-chain gene locus and instructing the thymocyte to continue its maturation process. This hypothesis is supported by the finding that thymocyte development in mice deficient for the tyrosine kinase $p56^{lck}$ (lck) (17) or overexpressing a dominant-negative form of lck (18) is also blocked at the DN stage. In mice overexpressing functional lck, maturation to the DP stage was normal, but rearrangement of the TCR β-chain locus was blocked, probably reflecting the mechanism for allelic exclusion (19). In agreement with these results, we and others (20, 21) have shown that treatment of day 14 fetal thymic organ cultures (FTOCs) with anti-CD3ε monoclonal antibodies (mAbs) prevented the subsequent appearance of TCR β chains and of full-length β-chain transcripts (which are indicative of V → DJ rearrangement; V, variable; D, diversity; J, joining) and caused a pronounced accumulation of truncated transcripts (indicative of D → J rearrangement). This effect may correspond to the arrest of rearrangement associated with allelic exclusion of the TCR β-chain locus. Moreover, the anti-CD3ε mAb treatment resulted in accelerated transition of $CD4^-8^-$ thymocytes into the DP stage (20). Therefore, we hypothesized that CD3ε is expressed on the cell surface before rearrangement of the TCR β-chain locus and that its ligation leads to induction of DP thymocytes. In this paper, we examined this hypothesis by testing DN thymocytes from several TCR β-chain-deficient mouse strains for their inducibility to DP cells by anti-CD3ε mAb.

MATERIALS AND METHODS

Mice. Fourteen day pregnant female C.B.-17 scid mice (9), Rag-$1^{+/-}$ mice (10), and TCR-$\beta^{+/-}$ mice (8) were obtained from the specific pathogen-free breeding facility at the Max-Planck-Institute für Immunbiologie in Freiburg, Germany. Rag-$1^{+/-}$ and Rag-$1^{-/-}$ fetuses were obtained from hetero-

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Abbreviations: TCR, T-cell receptor; IL-2R, interleukin 2 receptor; DP, double positive; SP, single positive; DN, double negative; FTOC, fetal thymic organ culture; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

zygous (Rag-$1^{+/-}$) females mated with homozygous (Rag-$1^{-/-}$) males. The same breeding schedule was used to obtain the TCR β-chain mutant fetuses.

mAbs. Anti-TCR β-chain antibody H57-597 (22), anti-TCR δ-chain antibody GL-3 (23), and anti-CD3ε antibody 145-2C11 (24) (all hamster IgGs) were isolated from culture supernatants by affinity chromatography over protein A columns (Pharmacia). Anti-FcRγII/III antibody 2.4G2 (25) (rat IgG) was isolated by affinity chromatography over a protein G column (Pharmacia). Purified anti-CD3ε antibody 500A2 was purchased from PharMingen. Flow cytometry was with labeled anti-Lyt-2 (53.6-7), anti-L3T4 (RM-4-5), anti-TCR β-chain (H57-597), anti-CD3ε (500A2), anti-TCR δ-chain (GL-3) (all purchased from PharMingen), and fluoresceinated anti-IL-2Rα antibody 5A2 (26), using a FACScan flow cytometer (Becton Dickinson).

FTOCs. Fetal thymic lobes were prepared from fetuses at day 14 of gestation. They were cultured for 6 days on filter discs floating on 1 ml of Iscove's modified Dulbecco's medium supplemented with 10% selected fetal calf serum, 2% glutamine, and 1% kanamycin in Costar 12-well dishes at 37°C in 7% CO_2/93% air, similar to the method of Jenkinson *et al.* (27), as described (28). The lobes were suspended for 2 hr in medium supplemented with antibodies and thereafter placed on filter discs floating on the same medium until analysis. Exposure to antibodies was started at the beginning of culture. After 6 days, single cell suspensions were made on the filter discs with a plunger. Cells were counted in a Neubauer chamber.

Flow Cytometry. Two- and three-color stainings were performed with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, and biotin-labeled antibodies. As a third color, Tricolor-conjugated streptavidin (Medac, Hamburg, F.R.G.) was used. Intracellular stainings were performed as described (20).

RESULTS AND DISCUSSION

scid Mice. FTOCs derived from scid mice were cultured for 6 days in the presence of two different anti-CD3ε mAbs (145-2C11 or 500A2) at 25 μg/ml recognizing different epitopes of the CD3ε molecule (29). Control FTOCs were treated with hamster IgG, anti-TCR β-chain mAb, anti-TCR δ-chain mAb, or anti-FcRγII/III mAb, respectively. Representative results are shown in Fig. 1. Culture of FTOCs with normal hamster IgG (Fig. 1 *A–C*) or with other control mAbs (data not shown) did not induce the transition of $CD4^-8^-$IL-$2R\alpha^+$ cells to the $CD4^+8^+$IL-$2R\alpha^-$ stage (Fig. 1 *A–C*). Treatment with anti-CD3 mAbs resulted in (*i*) production of DP cells (Fig. 1 *D* and *G*); (*ii*) an increase in the number of small cells (Fig. 1 *F* and *I*), most of which were DP cells (data not shown); (*iii*) a decrease in the proportion and absolute number of IL-$2R\alpha^+$ cells (Fig. 1 *E* and *H*); (*iv*) a 3- to 5-fold increase in absolute cell numbers per thymic lobe (Fig. 1 *A*, *D*, and *G*). The $CD4^+$ and $CD8^+$ SP thymocytes are probably cells on their way to the DP stage. The induced scid thymocytes were negative for intracellular TCR β chain (data not shown).

Rag-1 Mutant Mice. The second TCR β-chain-deficient strain analyzed was the Rag-1 mutant. In the experiment shown in Fig. 2, thymic lobes derived from four individual day 14 Rag-1 mutant fetuses and four heterozygous (normal) littermates were cultured pairwise, culturing one lobe with hamster IgG (25 μg/ml) and the other lobe with anti-CD3ε mAb (145-2C11) (25 μg/ml). After 6 days, cell suspensions were prepared from individual lobes and analyzed by flow cytometry. Rag-1 mutant fetuses (Fig. 2 *A–C*) differed from

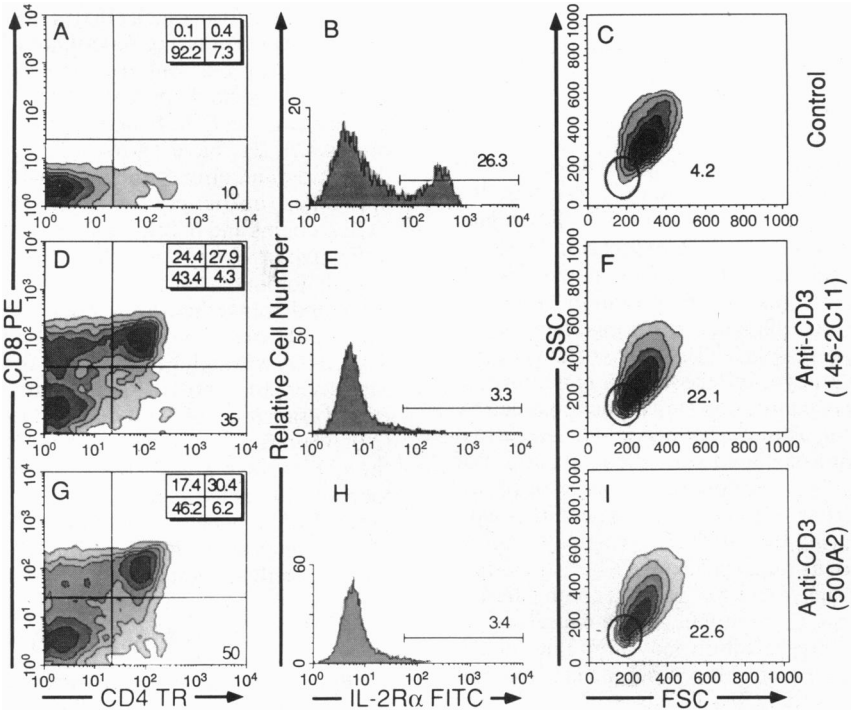

FIG. 1. Downregulation of IL-2Rα and induction of CD4 and CD8 on scid thymocytes. FTOCs derived from scid fetuses were cultured in medium containing hamster IgG (*A–C*), anti-CD3ε mAb 145-2C11 (*D–F*), or anti-CD3ε mAb 500A2 (*G–I*) and analyzed by three-color flow cytometry. Contour plots on the left (*A*, *D*, and *G*) represent fluorescence intensities of CD4 and CD8. Histograms (*B*, *E*, and *H*) represent the fluorescence intensity of IL-2Rα. Contour plots on the right (*C*, *F*, and *I*) represent gated forward-scatter (FSC) and sideward-scatter (SSC) profiles. Absolute cell numbers per lobe (×10^3) are shown in the lower right corner of *A*, *D*, and *G*. Control cultures reveal the block in thymic development at the $CD4^-8^-$ stage with between 25% and 50% IL-$2R\alpha^+$ cells and very few small cells (*A–C*). Transition into the $CD4^+8^+$IL-$2R\alpha^-$ compartment and increased absolute cell numbers were induced in the anti-CD3 mAb-treated cultures (*D–I*). Most $CD4^+8^+$ cells are found in the population of small cells (data not shown).

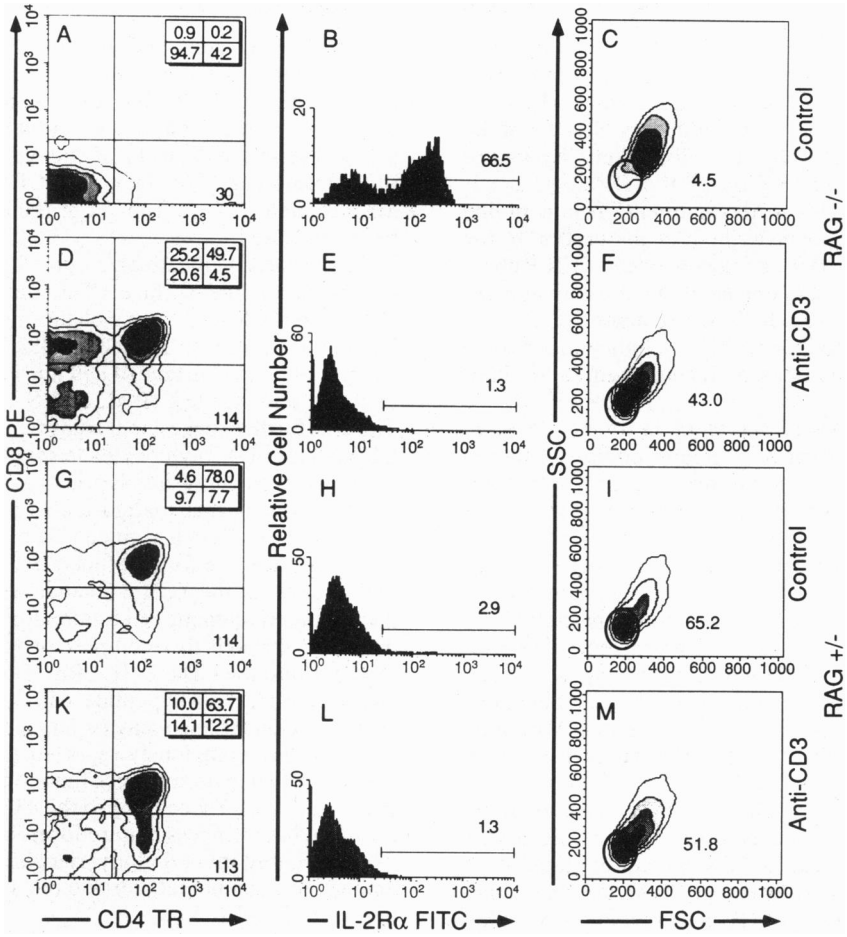

FIG. 2. Downregulation of IL-2Rα and induction of CD4 and CD8 on Rag-1 mutant thymocytes. FTOCs derived from Rag-1 mutant fetuses (*A–F*) and heterozygous littermates (*G–M*) were cultured in medium containing hamster IgG or anti-CD3ε mAb and analyzed by three-color flow cytometry. Contour plots on the left (*A*, *D*, *G*, and *K*) represent fluorescence intensities of CD4 and CD8. Histograms (*B*, *E*, *H*, and *L*) represent fluorescence intensity of IL-2Rα. Contour plots on the right (*C*, *F*, *I*, and *M*) represent gated forward-scatter (FSC) and sideward-scatter (SSC) profiles. Absolute cell numbers per lobe ($\times 10^3$) are shown in the lower right corner of *A*, *D*, *G*, and *K*. Heterozygous littermates show normal development of the $CD4^+8^+IL\text{-}2R\alpha^-$ compartment (*G–I*). This is not significantly influenced by anti-CD3 mAb treatment (*K–M*). Rag-1 mutant FTOCs show a block in development at the $CD4^-8^-IL\text{-}2R\alpha^+$ stage (*A–C*). Transition into the $CD4^+8^+IL\text{-}2R\alpha^-$ compartment and increased absolute cell numbers were induced in the anti-CD3 mAb-treated cultures (*D–F*). Most $CD4^+8^+$ cells are found in the population of small cells (data not shown).

heterozygous littermate fetuses (Fig. 2 *G–I*) by the absence of $CD4^+8^+IL\text{-}2R\alpha^-$ cells (10). Results within the group of Rag-1 mutant fetuses or within the group of littermates were very similar; results from one fetus of each group are shown. In anti-CD3ε mAb-treated FTOCs derived from Rag-1 mutant fetuses (Fig. 2 *D–F*) $CD4^+8^+IL\text{-}2R\alpha^-$ thymocytes of small size are produced in numbers similar to FTOCs from normal littermates (Fig. 2 *G–M*).

Together, these data show that scid or Rag-1 mutant thymocytes can be induced to differentiate with anti-CD3ε mAb. However, we could not detect surface expression of CD3ε by conventional flow cytometry, in agreement with previous results (9, 10). Since responsiveness to anti-CD3ε mAb should depend on the expression of this molecule by the responding cells, we analyzed CD3ε expression within non-induced and induced Rag-1 mutant thymocytes by staining for surface CD8 and intracellular CD3ε. Thymic lobes derived from individual day 14 Rag-1 mutant fetuses were cultured as described above, and individual lobes were analyzed by flow cytometry with FITC-labeled anti-CD8 mAb for surface stainings and PE-labeled anti-CD3ε mAb for intracellular stainings. Results from one Rag-1 mutant, representative of all others in the group, are shown. All induced $CD8^+$ cells expressed CD3ε intracellularly (Fig. 3*B*). In addition, the absolute cell numbers (Fig. 3) show a 2-fold expansion of the $CD8^-$ $CD3\varepsilon IC^+$ population but no expansion of the $CD3\varepsilon IC^-$ population. This suggests that respon-

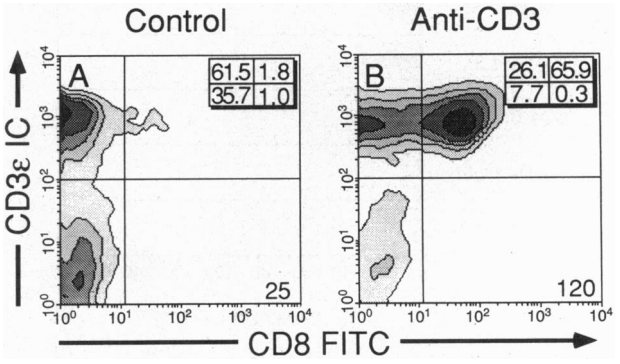

FIG. 3. Expression of intracellular CD3ε in Rag-1 mutant thymocytes. FTOCs derived from Rag-1 mutant fetuses were cultured in medium containing hamster IgG or anti-CD3ε mAb and analyzed by flow cytometry. Contour plots represent fluorescence intensities of CD8 and intracellular CD3. Absolute cell numbers per lobe ($\times 10^3$) are shown in the lower right corners. Culturing of FTOCs derived from Rag-1 mutant mice results in the development of $CD8^+$ cells and expansion of the $CD8^-CD3\varepsilon IC^+$ population. All $CD8^+$ cells express the CD3ε chain.

siveness to anti-CD3ε mAb requires expression of CD3ε, most likely exposed on the cell surface in small quantity.

Loss of IL-2Rα expression is almost complete in anti-CD3ε mAb-treated FTOCs from Rag-1 mutant mice (Fig. 2), whereas CD8 expression has occurred in only 60–70% of the CD3εIC$^+$ population (Fig. 3). It is therefore likely that loss of IL-2Rα expression and acquisition of the DP stage occur sequentially upon anti-CD3ε mAb treatment. This is in line with the sequence of events described for maturation to the DP stage in normal mice after expression of the TCR β chain (3, 4). We did not observe a decrease in the absolute number of Pgp-1^{hi} cells upon anti-CD3ε mAb treatment of FTOCs from Rag-1 mutant mice (data not shown), indicating that the treatment accelerates thymocyte development at a later stage.

TCR β-Chain Mutant Mice. The block in thymic development in TCR β-chain mutant mice is less complete than in scid and Rag-1 mutant mice (8). We investigated whether this partial block could also be restored upon anti-CD3ε mAb treatment. Thymic lobes derived from individual TCR β-chain mutant fetuses and normal littermates were cultured pairwise, one lobe in medium containing normal hamster IgG (25 μg/ml) and the other in medium containing anti-CD3ε mAb (25 μg/ml). After 6 days, cell suspensions were prepared, and a small aliquot of each pair was typed by flow cytometry using anti-TCR β-chain mAb. Corresponding cell suspensions were pooled and stained for analysis. We found that the number of DP cells in control FTOCs from TCR β-chain mutant fetuses was one-fifth to one-fourth of that found in normal littermates (Fig. 4). In *in vivo* matured thymuses, a 5-fold difference is found at birth, and the deficiency in DP thymocytes in TCR β-chain mutated mice becomes increasingly severe with age (P.M. and S.T., unpublished data). The number of DP cells in anti-CD3ε mAb-treated FTOCs was 3-fold higher than in control cultures from TCR β-chain mutant fetuses corresponding to two-thirds of that observed in cultures from normal littermates. Furthermore, downregulation of IL-2Rα in the DN population was detected. These data show that thymocytes from TCR β-chain mutant mice are inducible to mature to DP cells upon exposure to anti-CD3ε mAb.

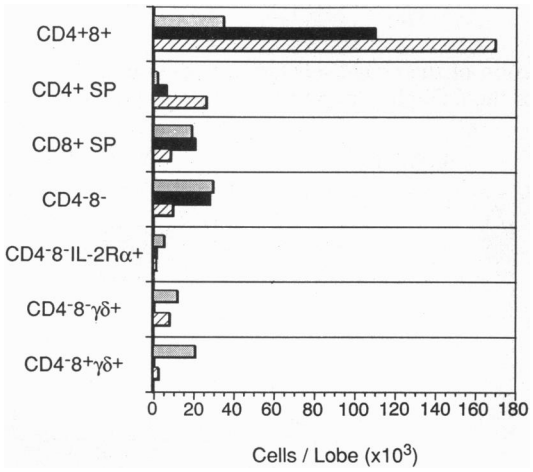

FIG. 4. Absolute cell numbers of thymic subpopulations in FTOCs derived from TCR β-chain mutant mice ($\beta^{-/-}$), cultured with anti-CD3ε mAb (solid bars) or normal hamster IgG (stippled bars) and from normal littermates cultured with normal hamster IgG (hatched bars). The main effects of the anti-CD3ε mAb treatment are increased production of DP cells, downregulation of IL-2Rα expression, and loss of TCR-δ$^+$ cells. Total cell numbers per lobe were 8.5 × 10^4 and 16.0 × 10^4 for control and anti-CD3ε mAb-treated lobes from TCR β-chain mutant fetuses, respectively, and 22.0 × 10^4 for normal control lobes.

In control FTOCs from TCR β-chain mutant fetuses 50% of the CD4$^-$8$^-$ cells and all CD4$^-$8$^+$ cells consisted of γδ cells, whereas these populations were γδ$^-$ in the anti-CD3ε mAb-treated FTOCs. No intracellular TCR δ chain was detectable in these populations (data not shown), excluding a possible internalization of the γδ receptor by anti-CD3ε mAb treatment. The lack of TCR-δ$^+$ cells in anti-CD3ε mAb-treated FTOCs from TCR β-chain mutants remains to be investigated.

Conclusions. Collectively, our data suggest that CD3ε is expressed on the surface of immature thymocytes in the absence of TCR α, β, γ, or δ chains in minute but nevertheless functional amounts. Indeed, CD3ε has been found to be expressed on the surface of cell lines derived from thymomas of Rag-1 mutant mice (P.M. and S.T., unpublished results). The data further suggest that transmembrane signaling through CD3 induces DN thymocytes to mature to the DP stage. The signaling event may involve lck, as suggested by previous results showing that maturation to the DP stage is severely inhibited in lck-deficient mice (17) or in mice overexpressing dominant-negative forms of lck (18). Our data are consistent with the role of the TCR β chain in early thymocyte development as a component of an immature CD3 complex; signals delivered through this complex drive CD4$^-$8$^-$IL-2Rα$^+$ thymocytes into the CD4$^+$8$^+$IL-2Rα$^-$ stage. The surface expression of the CD3ε polypeptide without TCR β chain in TCR β-chain-deficient mice may be an inaccuracy of nature. However, we have previously reported evidence consistent with the notion that in normal mice functional CD3ε is expressed on the thymocyte surface prior to the TCR β chain (20). It is thus possible that even less mature forms of the CD3 complex may exist, possibly involving invariant surrogate polypeptides in analogy to immunoglobulin-like receptors expressed on some immature B cells (30).

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