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The TCRβ Enhancer Is Dispensable for the Expression of Rearranged TCRβ Genes in Thymic DN2/DN3 Populations but Not at Later Stages

Christian E. Busse, Anna Krotkova,[1] and Klaus Eichmann[2]

The Eβ enhancer has been shown to be dispensable for germline transcription of nonrearranged TCRβ segments but appears to be required for TCRβ V to DJ rearrangement. Eβ dependency of the subsequent expression of VDJ-rearranged TCRβ genes in thymic subpopulations has so far not been analyzed. We generated transgenic mice, using a Vβ8.2Dβ1Jβ1.3-rearranged TCRβ bacterial artificial chromosome, which lacked Eβ, and monitored transgene expression by flow cytometry using Vβ-specific mAbs and an IRES-eGFP reporter. Transgene expression was found in double negative (DN)2 and DN3 but not at later stages of thymopoesis. There was no toxicity associated with the transgene given that apoptosis in DN3, DN4 was not increased, and the number of DN4 cells generated from DN3 cells in reaggregate thymic organ cultures was not diminished. The transgenic TCRβ gave rise to a pre-TCR, as suggested by its ability to suppress endogenous TCRβ rearrangement, to facilitate β-selection on a TCRβ-deficient background and to inhibit γδ T cell lineage development. The results suggest that the Vβ8.2 promoter is sufficient to drive expression of rearranged TCRβ VDJ genes Eβ independently in DN2/DN3 but not at later stages. ***The Journal of Immunology,*** **2005, 175: 3067–3074.**

During thymic development, T cells mature from a CD4/CD8 double negative (DN)[3] via the CD4/CD8 double positive (DP) stage to either CD4 or CD8 single positive (SP) cells. By using CD25 and CD44, the DN stage can be further subdivided into four consecutive populations, which are termed DN1–DN4 (1). V(D)J rearrangements of the TCRβ, γ, and δ loci start in DN2 and are completed in DN3 (2). To survive in the DN4 and mature to the DP stage, αβ T cells are subjected to β-selection, a process that is critically depended upon signaling by the pre-TCR and ensures that only cells with a functionally rearranged *TCRβ* gene proceed in development (3). T cell development in animals with deficiencies in pre-TCR signaling is blocked at the DN3 stage (4–8). Cross-linking CD3ε via anti-CD3ε Abs (9) can overcome the block in pre-TCR-deficient animals and produce a single wave of DP cells. Development of γδ T cells does not depend on a pre-TCR, and the γδ TCR is expressed only after the cells have reached the DN4 stage (6, 10–12).

At least two types of regulatory elements are involved in the control of transcription and accessibility for recombination of the *TCRβ* locus. Vβ promoters initiate germline transcription of the individual Vβ segments, but their role in Vβ→DβJβ rearrangements remains controversial. Experiments using transgenic (tg) mice carrying a TCRβ minilocus in which the decamer sequence of the Vβ8.3 promoter was mutated (13) suggested that the promoter is necessary for the expression but not for the rearrangement of Vβ segments. However, this study could not exclude the possibility that the promoter of the upstream Vβ5.2 segment had facilitated the accessibility to the recombination machinery. A more recent report (14) showed that the knockout of the Vβ13.1 promoter reduced the usage of the Vβ13.1 segment in Vβ→DβJβ rearrangements 5- to 10-fold but lead to complete lack of Vβ13.1$^+$ T cells in the periphery. Unexpectedly, these knockout mice showed germline transcripts of Vβ13.1, which were initiated by previously uncharacterized sequences upstream of the deleted promoter region. Taken together, both studies show that the Vβ promoter is crucial for expression of rearranged *TCRβ* genes, but the requirement for the promoter and promoter-depended germline transcription in Vβ→DβJβ rearrangements remains uncertain.

The element that has been studied best is the Eβ enhancer, a 0.5-kB segment that is located 6 kb 3′ of Cβ2 exon 4 (15, 16). Absence of Eβ results in hypermethylation and histone H3 hypoacetylation of the DβJβ1/DβJβ2 region and a lack of DβJβ1/DβJβ2 germline transcripts (17). These changes seem to be confined to the DJC region of the *TCRβ* locus because the same study could not detect alterations in epigenetic modifications or in germline transcription of the Vβ5.2 and Vβ14.1 segments. Recently, it has been shown for the germline promoter pDβ1 that the basal transcriptional machinery is still recruited in the absence of Eβ but that Eβ is required for subtle changes in the chromatin structure that seem to be required for activation of transcription (18). Eβ is capable of mediating tissue and developmental stage-specific Vβ→DβJβ rearrangements of a TCRβ minilocus in vivo (19, 20). Eβ$^{-/-}$ mice do not show Vβ→DβJβ rearrangements (21, 22) and present a phenotype similar to that of TCRβ$^{-/-}$ animals (23). Several studies have addressed the question of germline transcription of *Vβ* genes in the presence (24, 25) and absence (17, 26, 27) of Eβ. Using Rag$^{-/-}$ and anti-CD3ε-induced Rag$^{-/-}$ mice as a source of DN and DP cells, respectively, these reports showed that

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[3] Abbreviations used in this paper: DN, double negative; DP, double positive; SP, single positive; tg, transgenic; BAC, bacterial artificial chromosome; PFGE, pulsed field gel electrophoresis; WT, wild type; i.c., intracellular; RTOC, reaggregated thymic organs culture; ISP, immature SP; PI, propidium iodide.

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Vβ germline transcription is independent of Eβ, prominent in the DN population, and down-regulated to a certain extend (4- to 5-fold with the exception of Vβ14.1 (25)) upon induction of DP cells. No experiments have so far been done to characterize the role of Eβ in the expression of rearranged *TCRβ* genes in thymic subpopulations.

In the present study, we used a bacterial artificial chromosome (BAC), encoding a functionally rearranged *TCRβ* gene plus reporter (Vβ8.2Dβ1Jβ1.3Cβ1-IRES-eGFP) in the genomic context of a *TCRβ* locus lacking the Eβ enhancer. We show that expression of the TCRβ transgene is confined to the DN2/DN3 populations but nevertheless induces all developmental effects ascribed to a functional pre-TCR (β-selection, TCRβ allelic exclusion, inhibition of γδ T cell development).

Materials and Methods

BAC modification and sequencing

The construction of the initial TCRβ BAC encoding the HY-TCRβ-chain has been described elsewhere (28). Insertion of a 10.2-kb DNA fragment encoding for Dβ1Jβ1.3-Cβ1-IRES-eGFP-Dβ2-Jβ2.3 into this TCRβ BAC was done via the pSV1 shuttle vector system as described in Ref. 29. The IRES-eGFP cassette was a gift from Dr. R. Ketteler (Max-Planck-Institute of Immunobiology, Freiburg, Germany). *Pst*I restriction digest and Southern blotting were used to confirm correct integration. PFGE was performed using the CHEF Mapper XA system (Bio-Rad) with the following parameters: buffer, 0.5× Tris-borate EDTA; temperature, 14°C; gel, 0.8% NEEO agarose (Roth); electrophoresis, 6 V/cm, 120° included angle, 17:32 h running time, switch interval 0.3–15.51 s, linear ramping. TOPO Shotgun Subcloning kit (Invitrogen Life Technologies) was used to subclone the BAC. Subclones (384) were sequenced, yielding 63% coverage of the BAC. The following primers were used to detect either the wild-type (WT) or the Δ*Eβ* locus: CACTTTAAGAACCACGCCTGC (common 5′), TTA CAGTGCTTGATAGTTATTACC (WT 3′), and AGGCACATAA GAGGATGCTGG (ΔEβ 3′).

Mice and transgenesis

The BAC was linearized by *Not*I digestion before injection. PFGE was used to confirm that the DNA was not degraded. Pronuclear injections were performed in the transgenic facility of the Max-Planck-Institute. Unless otherwise mentioned, experiments were done with mice on a FVB/N background. The mice used in the TCRβ null experiment had been backcrossed to C57BL/6 for four generations (N4). Mice were genotyped by PCR, using the following primers for eGFP (BAC transgene) or TCRβ WT and null (30) allele: CGGCATCAAGGCCAACTTCAAGAG (GFP 5′), CTTGTA CAGCTCGTCCATGCCGAG (GFP 3′), AGGGACCAGGCTTTTGGTA ATAGG (Jβ1.2), GGCTTCCTTCTCCAAAA TAGAGCG (Jβ1.3), and GCCTTCTATCGCCTTCTTGACGAG (Neo 3′ end). Primers used for TCRβ WT/KO genotyping were specific for the respective alleles and did not recognize the transgene. All mice were housed in a conventional unit of the Max-Planck-Institute animal facility and were used at 5–7 wk of age.

FACS stainings

Blocking of FcRs was done by incubating single-cell suspensions with 2.4G2 hybridoma supernatant before the staining procedure. The following mAbs were used for flow cytometry (all from BD Pharmingen): anti-CD4-biotin (H129.19), anti-CD4-PerCP (RM4-5), anti-CD8-biotin (53-6.7), anti-CD25-biotin (7D4), anti-CD25-PE-Cy7 (PC61), anti-CD44-biotin (IM7), anti-CD161c-biotin (PK136), anti-TCRβ-APC (H57-597), anti-TCRδ-PE (GL3), anti-Vβ4-PE (KT4), and anti-Vβ8.1/2-PE (MR5-2). The Alexa647 (Molecular Probes) labeled anti-Vβ8.2 (F23.2) mAb was a kind gift from Dr. J. Kirberg (Max-Planck-Institute of Immunobiology). Second-step staining of biotinylated mAbs was done using streptavidin-PerCP or streptavidin-APC-Cy7 (BD Pharmingen). Apoptosis was measured with the annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen). For intracellular (i.c.) staining, cells were fixed with 1% paraformaldehyde (3 h at 4°C) and permeabilized with 0.25% saponin (20 min). After permeabilization, all subsequent steps were done in PBS/FCS (2%) containing 0.1% saponin. To rule out unspecific binding of fluorophore-conjugated mAbs to i.c. structures, controls were incubated with 5 μg of purified H57-597 or GL3 mAb during permeabilization. Cells were analyzed on a FACSCalibur or LSRII (BD Biosciences) flow cytometer and the data was evaluated using CellQuest, FACSDiVa (BD Biosciences) or FlowJo (Tree Star). For sorting of DN thymocytes, cell suspensions were first depleted of $CD4^+$ and $CD8^+$ cells by MACS (Miltenyi Biotec) and afterward sorted on a MoFlo cell sorter (DakoCytomation).

Reaggregated thymic organs cultures (RTOCs)

RTOCs were done according to Ref. 31. Briefly, fetal thymic lobes were taken from E15.5 BALB/c embryos and treated with 1.35 mM deoxyguanosine (Sigma-Aldrich) for 6 days. Lobes were then disaggregated with trypsin/EDTA (PAA). A total of 1×10^6 thymic stroma cells was mixed with thymocytes from tg mice and reaggregated on polycarbonate filters (Nuclepore 0.8 μm; Whatman), floating on IMDM + FCS (10%) + L-glutamine (4 mM) + kanamycin (100 μg/ml) + 2-ME (35 μM). RTOCs were cultured for the indicated time periods at 37°C and 7% CO_2.

VDJ rearrangement PCR

Genomic DNA from 1×10^5 sorted thymocytes was prepared by proteinase K digestion of the cells followed by isopropanol precipitation. The amount of DNA used in the experiments was adjusted according to serial dilution PCR using insulin-specific primers. The VDJ rearrangement PCRs were done using the following primers: AGCTGTCTCCTACTATC GATTTCC (3′ of Jβ2.6), GATATGCGAACAGTATCTAGGCCA (Vβ4), and TGTA CTGGTACCGACAGGATTCAG (Vβ6). The following program was used for amplification: 94°C/2 h; (94°C/30 min; 68°C/40 min; 72°C/2 h) × 40; 72°C/7 h.

RT-PCR analysis

RNA from sorted thymocytes was isolated using TRI Reagent (Sigma-Aldrich) and afterward subjected to DNase treatment (DNA-Free; Ambion). Reverse transcription with oligo(dT) primers used SuperScriptII $RNaseH^-$ (Invitrogen Life Technologies). The following primers were used to amplify the full length Vβ8.2 cDNA: ATGGGCTCCAGGCTCT TCTTCGTG (Vβ8.2 exon 1) and GATCTGGCTTCATGAATTCTT TCTTTTGAC (Cβ1 exon 4 + linker). The purification and cloning of the PCR product was done using the TOPO-XL PCR cloning kit (Invitrogen Life Technologies).

Results

A TCRβ BAC lacking the Eβ enhancer

To assess the β-selection potential of different VβDβJβ joints we created a BAC coding for the HY-TCRβ-chain in the genomic context of the *TCRβ* locus (Fig. 1*a*). An exact description of the BAC has been published elsewhere (28). By homologous recombination, the existing Vβ8.2Dβ2Jβ2.3 joint was replaced by a 10.2-kb fragment containing the following elements: the joint to be tested (Vβ8.2Dβ1Jβ1.3), Jβ1.4-Cβ1, an IRES-eGFP reporter placed directly behind the stop codon of Cβ1 exon 4, Dβ2, and Jβ2.1-Jβ2.3. PFGE of a BAC clone in which the targeting procedure had been successful suggested that a deletion had occurred because the *Not*I-linearized BAC was ~50 kb shorter than expected. Additional digests (Fig. 1*b*) confirmed this and hinted that a deletion had occurred in the 3′ region of the BAC. Shotgun sequencing of 384 clones derived from this BAC yielded 3 clones spanning a deletion, starting at 4.8 kb 3′ of the Cβ2 stop codon (1.1 kb 5′ of the Eβ *Hpa*I site) and extending 44.6 kb downstream. PCR using a pair of primers, which were located 5′ and 3′ of the deletion, yielded a product when the ΔEβ BAC was used as template but not with a BAC containing the 3′ part of the germline TCRβ locus. Conversely, a PCR in which the 3′ primer was replaced with a primer located in the very 5′ part of the deletion yielded a product with the germline but not with the ΔEβ BAC used as a template (Fig. 1*c*).

Mice carrying the complete original BAC showed normal expression of the TCRβ transgene during thymic development and in the periphery (28). Realizing that our ΔEβ BAC should enable us to monitor TCRβ tg expression driven by the Vβ8.2 promoter alone, we decided to generate mice that carried this BAC as a transgene. For pronuclei injections, we used the FVB/N mouse strain because it carries the *TCRβ*^{a} allele (32), which contains a large deletion in the Vβ region, encompassing—among other Vβs—Vβ8. Two founder animals were obtained whose offspring

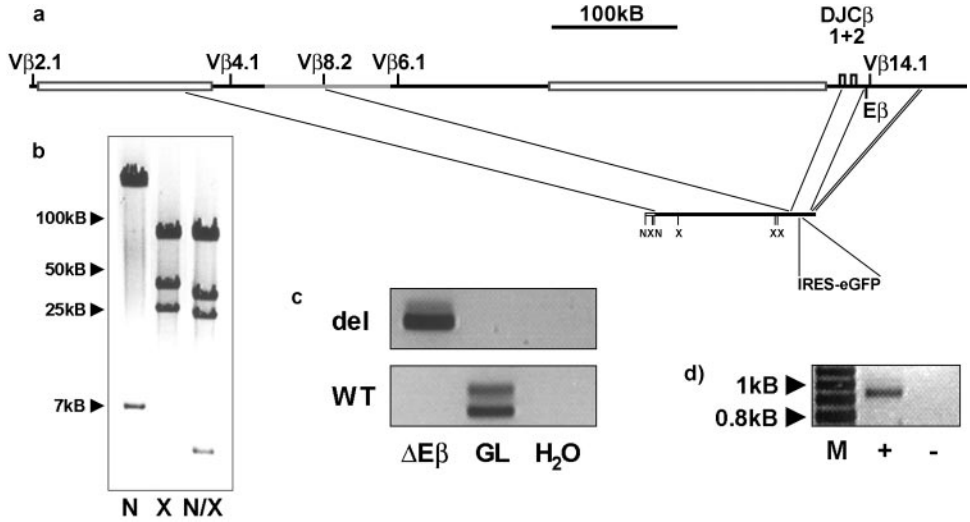

FIGURE 1. A rearranged TCRβ BAC lacking the Eβ enhancer region. *a*, Scheme of the genomic *TCR*β^b locus (*upper lane*) and the BAC (*lower lane*) used in this study. Long, sleek boxes indicate the trypsinogen repeat regions within the *TCR*β locus; small boxes above the baseline indicate the two DβJβCβ clusters. Selected Vβ elements are indicated by marks above the baseline. The gray baseline marks the genomic region that is deleted in the *TCR*β^a allele. The cleavage sites of the restriction enzymes used in *b* are indicated (X, *Xho*I; N, *Not*I). *b*, 0.5 μg of ΔEβ BAC DNA was digested with the indicated restriction enzymes and subsequently separated via PFGE (X, *Xho*I; N, *Not*I). *c*, PCR with primers either specific for the ΔEβ (del) or the unmodified (WT) *TCR*β locus was performed using either the BAC containing the 44-kB Eβ deletion (ΔEβ) or a BAC containing the 3′ part of the germline *TCR*β locus (GL) as template. *d*, DN3 thymocytes of tg mice were sorted, and total RNA was isolated. RT-PCR was performed using primers recognizing Vβ8.2 exon 1 and Cβ1 exon 4 (M, marker; +, +RT; −, −RT).

showed very similar phenotypes. Therefore, the results of only one of the lines are shown below. RT-PCR analysis of DN3 thymocytes of tg mice revealed a full-length Vβ8.2-Cβ1 transcript (Fig. 1*d*). Cloning of the PCR product and sequencing of four of these clones confirmed the specificity of the PCR and did not reveal any consistent mutations at the cDNA level (data not shown).

Thymic expression pattern of the ΔEβ BAC transgene

When compared with WT littermates, tg animals showed a ~4-fold decrease in thymic cellularity with a slight increase in the percentage of DP thymocytes (Tab 1). The absolute number of CD4 SP thymocytes was decreased ~10-fold. Although the percentage of CD8 SP thymocytes was not changed significantly, their absolute number was also ~4-fold decreased. Cells accumulated in the TCRβ^{low}CD69$^-$ stage, while the number of CD69$^+$ thymocytes was reduced substantially. The percentage of TCRβ^{high} thymocytes was significantly reduced (3-fold) in comparison to WT animals. These results are consistent with an incomplete block in the DP population somewhat before positive selection. As some of the CD8 SP cells belong to the immature SP (ISP) subset, a less pronounced reduction than that of CD4 cells is in agreement with this hypothesis.

As FVB/N mice do not possess endogenous Vβ8 genes, we could use anti-Vβ8.1/8.2 or anti-Vβ8.2 mAbs to specifically stain the TCRβ-chain encoded by the transgene. In the thymi of tg mice, we were unable to detect any cells, which expressed Vβ8.1/8.2 on the cell surface (Table I). The pre-TCR is expressed in the DN and the CD8 ISP stages of T cell development and occurs in very low abundance on the cell surface (33). Thus, because expression of the Vβ8.2 transgene in the context of the pre-TCR would have escaped the detection by surface staining, we used i.c. stainings. By this means, we detected transgene expression in the majority of DN2 cells and in some DN3 cells but not in DN4 cells and further developed thymocytes of tg mice (Fig. 2, *a* and *b*). Over 85% of the gated Vβ8.1/8.2$_{ic}^+$eGFP$^+$ population were found to be CD4$^-$CD8$^-$CD161c$^-$CD25$^+$, consistent with transgene expression predominantly in DN2/DN3 (Fig. 2*c*). TCRβ_{ic}^+Vβ8.1/8.2$_{ic}^-$ cells, i.e., cells expressing endogenous TCRβ-chains, were detected in DN3 and DN4 cells, in ~10% of DP cells, and in ~50% of SP cells (Fig. 2, *a* and *b*). Intriguingly, the vast majority of DP cells and ~50% of SP cells (predominantly CD4 SP cells; data not shown) expressed no i.c. TCRβ-chains at all (Fig. 2*a*). These results suggested that the TCRβ tg, while being expressed only in DN2/DN3, inhibited the generation of cells expressing endogenous

Table I. *Thymic subpopulations in WT, ΔEβ tg, TCR$\beta^{-/-}$, and TCR$\beta^{-/-}$ X ΔEβ tg mice*

Population	WT[a]	tg[a]	*t* test[b]	TCR$\beta^{-/-}$[c]	TCR$\beta^{-/-}$ xtg[c]	Population	WT[c]	tg[c]
Thymic cellularlarity	$7.9 \pm 3.7 \times 10^7$	$2.2 \pm 1.4 \times 10^7$	signif.	2.0×10^6	6.6×10^6			
DN	2.0 ± 0.4%	2.6 ± 1.0%	n.s.	84.2%	14.9%	TCRβ^{low}CD69$^-$	74.6%	92.9%
DP	84.4 ± 1.4%	89.5 ± 0.3%	signif.	11.2%	76.0%	TCRβ^{low}CD69$^+$	9.9%	1.8%
CD4 SP	11.0 ± 2.1%	4.4 ± 0.9%	signif.	2.8%	2.2%	TCRβ^{high}Vβ69$^+$	12.5%	2.5%
CD8 SP	2.7 ± 0.5%	3.4 ± 0.1%	n.s.	1.8%	7.0%	TCRβ^{high}CD69$^-$	3.6%	2.9%
TCRβ^{high} [d]	17.7 ± 1.4%	6.7 ± 0.6%	signif.	0.6%	2.7%	TCRβ^{high}Vβ8.2$^+$	0.2%	0.3%

[a] Numbers shown are arithmetic means ± SD, obtained from at least two independent experiments.
[b] Numbers of WT and tg animals were compared using Student's *t* test at a test level of $p = 0.01$ (signif., significant; n.s., not significant).
[c] Data from a single representative mouse.
[d] TCRβ surface expression levels in TCR$\beta^{-/-}$ mice were determined using the MR5–2 mAb.

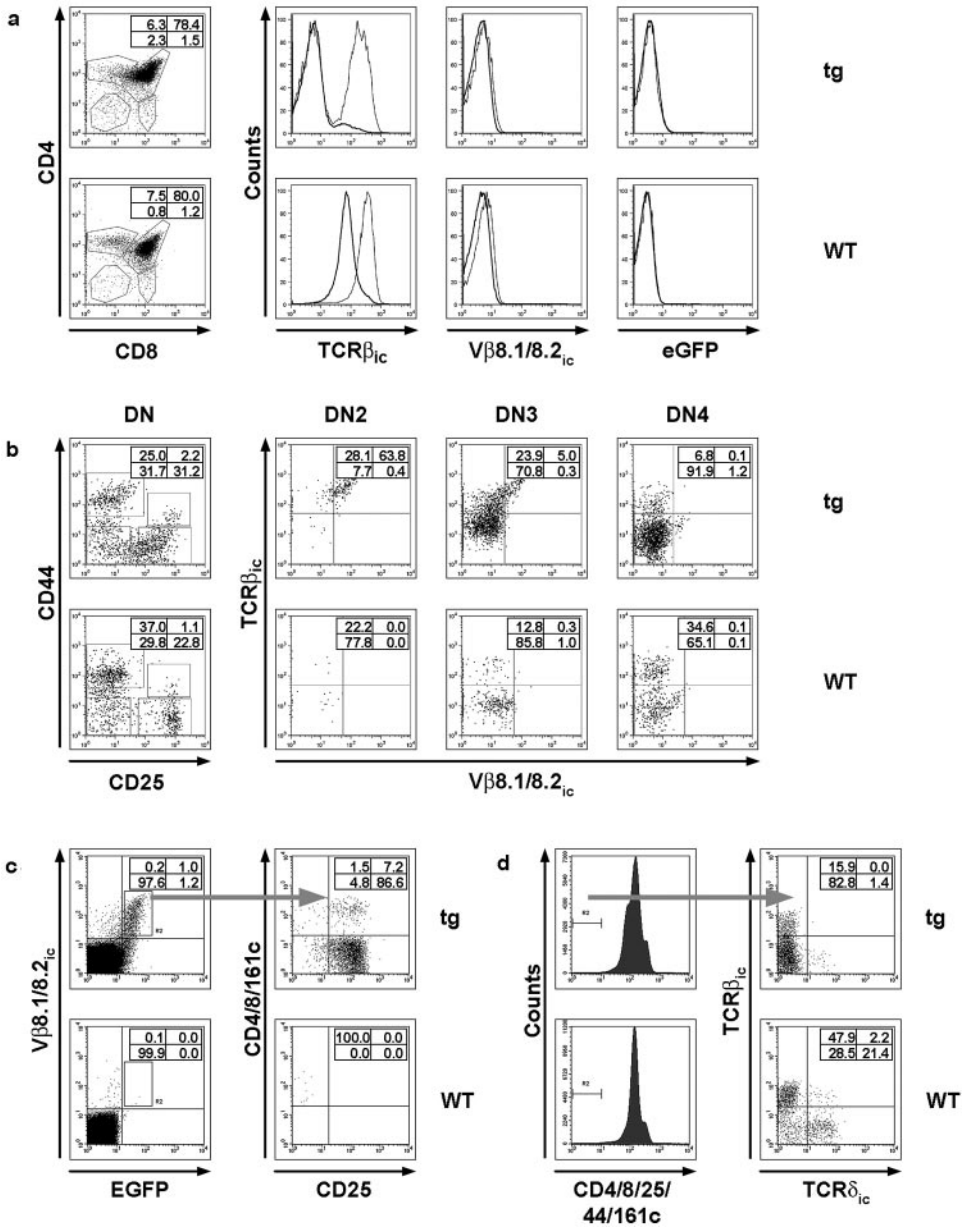

FIGURE 2. Transgene expression is restricted to the DN2/DN3 populations. Single-cell suspensions were prepared from WT or tg thymi. The cells were analysis by flow cytometry using mAbs against CD4, CD8, CD25, CD44, and CD161c (NK1.1) for surface and TCRβ, TCRδ, and Vβ8.1/8.2 for i.c. staining. *a*, Normalized histograms are depicted for TCRβ_{ic}, Vβ8.1/8.2$_{ic}$, and eGFP in DP (bold line) and combined CD4/CD8 SP cells (fine line). The TCRβ_{ic}^- SP cells in tg mice are predominantly CD4 SP cells (data not shown). *b*, TCRβ_{ic} and Vβ8.1/8.2$_{ic}$, two parameter dot plots are shown for DN2, DN3, and DN4 cells gated as shown in the *left frames*. Quadrants are set according to blocking controls using excess of unlabeled Ab. *c*, Analysis of the gated Vβ8.1/8.2$^+$eGFP$^+$ population for CD4, CD8, CD161c, and CD25. *d*, Analysis of the DN4 population for intracellular TCRβ and TCRδ. Quadrants are set according to blocking controls using excess of unlabeled Ab.

TCRβ-chains but nevertheless permitted a certain degree maturation of thymocytes that did not express TCRβ.

TCRβ transgenes have been shown to inhibit $\gamma\delta$ T cell lineage development, in part due to inhibition of endogenous TCRγ gene rearrangements (12, 34). To analyze whether this effect was also associated with a TCRβ tg whose expression is restricted to DN2/DN3, we analyzed i.c. TCRβ vs TCRδ proteins in the DN4 population of tg and WT mice (Fig. 2*d*). The percentage of TCRδ_{ic}^+ cells was severely (>15-fold) reduced in comparison to WT littermates. The results suggest that the TCRβ tg, despite its restricted expression in DN2/DN3, is capable of inhibiting $\gamma\delta$ T cell lineage development.

Down-regulation of the TCRβ transgene during the DN3 stage in tg mice

The DN2/DN3 restricted expression pattern could be due either to down-regulation of transgene expression or to death of transgene expressing cells in DN3. To distinguish between these possibilities, we sorted GFP$^+$ and GFP$^-$ DN3 cells of tg mice and placed them into reaggregate thymic organ cultures (RTOCs). Approximately 50% of the input GFP$^+$ DN3 cells were Vβ8.1/8.2$_{ic}^+$ (data not shown; see Fig. 2*c*). Analyses of cultures of GFP$^+$ cells showed that already at day 2 the percentage of Vβ8.1/8.2$_{ic}^+$ DN3 cells had dropped to 12%, indicating that >70% of the Vβ8.1/8.2$_{ic}^+$ DN3 cells had stopped transgene expression (Fig. 3). This became even more obvious at days 4 and 7, when only ~1.7 and 1%, respectively, of all DN3/DN4 cells where still Vβ8.1/8.2$_{ic}^+$. The data also show that the TCRβ tg is down-regulated before CD25, i.e., while the cells are still in DN3. In contrast to the GFP$^+$ cells, progeny of the GFP$^-$ cells expressed the transgene neither at day 2 nor at day 7. Importantly, the proportions of DN4 cells derived from GFP$^-$ and from GFP$^+$ DN3 cells were similar, suggesting that transgene expression is not toxic for the cells. The greater total cell number derived from GFP$^-$ DN3 cells appears to be due to a more efficient production of DP cells.

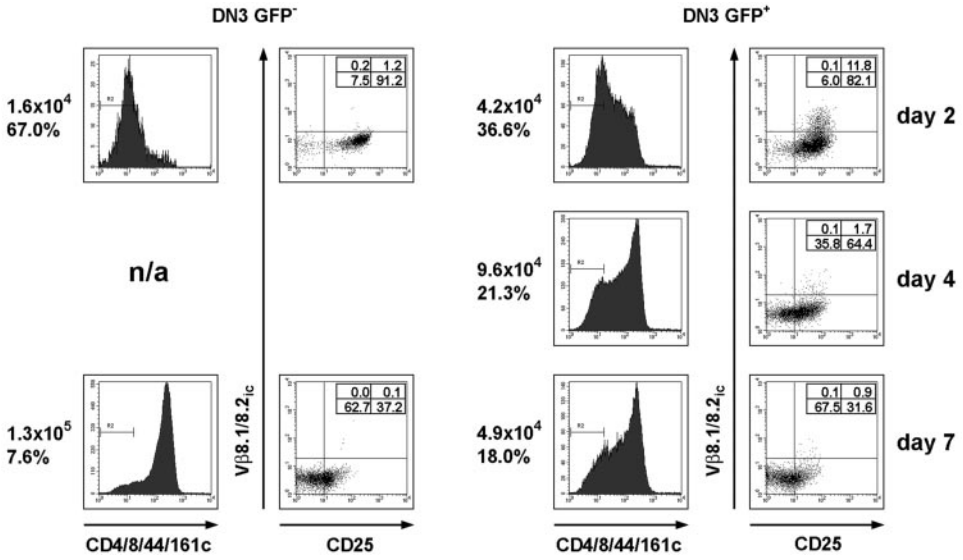

FIGURE 3. Development of tg thymocytes in vitro. eGFP$^+$ (1×10^5) or eGFP$^-$ (4×10^4) DN3 cells from tg mice were put into RTOCs. RTOCs were analyzed after the indicated time points. The numbers left of the histogram indicate the total amount of cells retrieved from the RTOC and the percentage of events within the CD4$^-$D8 $^-$D44$^-$D161c$^-$ gate.

No increased apoptosis in DN3/DN4 cells of tg mice

To further address the putative toxicity of the transgene, we analyzed the proportions of apoptotic cells in DN3 and DN4 cells of tg and WT mice. Apoptosis of thymocytes that fail to pass β-selection takes place in DN4 (11). Because we found a substantial increase of TCR$\beta_{ic}{}^-$TCR$\delta_{ic}{}^-$ cells in this population of tg mice (Fig. 2*c*), it was possible that these cells, due to down-regulation of the transgene, could have failed β-selection and become apoptotic. To address this question, we used annexin V/propidium iodide (PI) double stainings, allowing to distinguish early apoptotic (annexin V$^+$PI $^-$ from late apoptotic (annexin V$^+$PI$^+$) and necrotic (annexin V$^-$PI$^+$) cells (Fig. 4). Because we had to use FITC-conjugated annexin, the annexin signal of the viable DN3 population of tg animals includes the GFP fluorescence and is therefore brighter than that of WT DN3 cells. In DN4 cells, this effect is less pronounced, in line with the shutdown of transgene expression in this population. Nevertheless, annexin V$^+$ early apoptotic cells could be clearly distinguished from annexin V$^-$ viable cells. In agreement with our previous observations (11), only negligible numbers of apoptotic cells were seen in the DN3 subsets of both tg and WT mice, whereas the DN4 subsets of both types of mice contained significant numbers of apoptotic cells. No significant difference in the percentages of early apoptotic cells were seen in DN4, whereas the percentage of late apoptotic cells was lower in tg mice than in WT animals (20.4 vs 36.1%), a phenomenon that could be attributed to the enlarged population of β-selected DN4 cells in tg animals (see *Discussion*). The results suggest that transgene expression is not associated with an increase of apoptotic cells in the DN3/DN4 populations of tg mice.

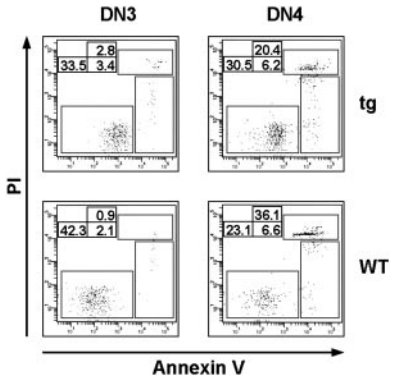

FIGURE 4. Apoptosis is not substantially altered in tg mice. Thymocytes from WT and tg mice were stained with mAbs against CD4, CD8, CD25, CD44, and CD161c. Subsequently, the cells were labeled with Annexin V/PI and analyzed by flow cytometry. Because annexin V-FITC and eGFP are detected on the same fluorescence channel, the DN3 population from tg animals is shifted toward the right.

Inhibition of endogenous TCRβ *locus rearrangements in tg mice*

TCRβ transgenes usually inhibit endogenous TCRβ rearrangements, a phenomenon reflecting *TCR*β locus allelic exclusion. We determined endogenous TCRβ rearrangements at the DNA and protein levels. For DNA analyses, Vβ8.1/8.2$_{ic}{}^+$ and Vβ8.1/8.2$_{ic}{}^-$ DN3 cells of tg mice were sorted, and TCRβ VDJ rearrangements were assayed by semiquantitative PCR using primers for Vβ4.1 and Vβ6.1, which are located 5′ and 3′, respectively, of the deletion in the *TCR*β^a allele (32) (Fig. 5*a*). Sorted DN3 cells of WT littermates and splenocytes of Rag-2$^{-/-}$ mice were used as controls. This experiment showed clearly that rearrangements of the endogenous loci were drastically, albeit not completely, suppressed in the DN3 population of tg mice. Of note, no differences were seen in the extent of inhibition of rearrangements between Vβ8.1/8.2$_{ic}{}^+$ and Vβ8.1/8.2$_{ic}{}^-$ cells, indicating that the latter subset had passed through a phase of transgene expression as well. For analyses at the protein level, we monitored expression of Vβ8.2 and Vβ4.1 in DN3 and DN4 by FACS analysis (Fig. 5*b*). Although ~10% of all TCR$\beta_{ic}{}^+$ DN3 and DN4 cells in WT mice are Vβ4.1$_{ic}{}^+$, no Vβ4.1$_{ic}{}^+$ DN3 cells and only a few Vβ4.1$_{ic}{}^+$ DN4 cells (~5% of WT) could be detected in tg animals. Nevertheless, we observed a population of TCR$\beta_{ic}{}^+$Vβ8.1/8.2$_{ic}{}^-$ cells in the DN4 subset, obviously expressing endogenous TCRβ rearrangements. This population is ~5-fold reduced compared with WT mice, which is consistent with the assumption that a small number of endogenous *TCR*β genes rearrange in DN3 and are expanded by β-selection in DN4. Taken together, these experiments show that the ΔEβ transgene is capable of mediating allelic exclusion, similar to other TCRβ transgenes (35).

β-Selection on a TCRβ-deficient background

Several of the experiments described above indicated that the DN2/DN3-restricted TCRβ tg was nevertheless capable of inducing β-selection. For example, the TCR$\beta_{ic}{}^-$TCR$\delta_{ic}{}^-$ subset of DN4, usually containing apoptotic cells that had failed β-selection

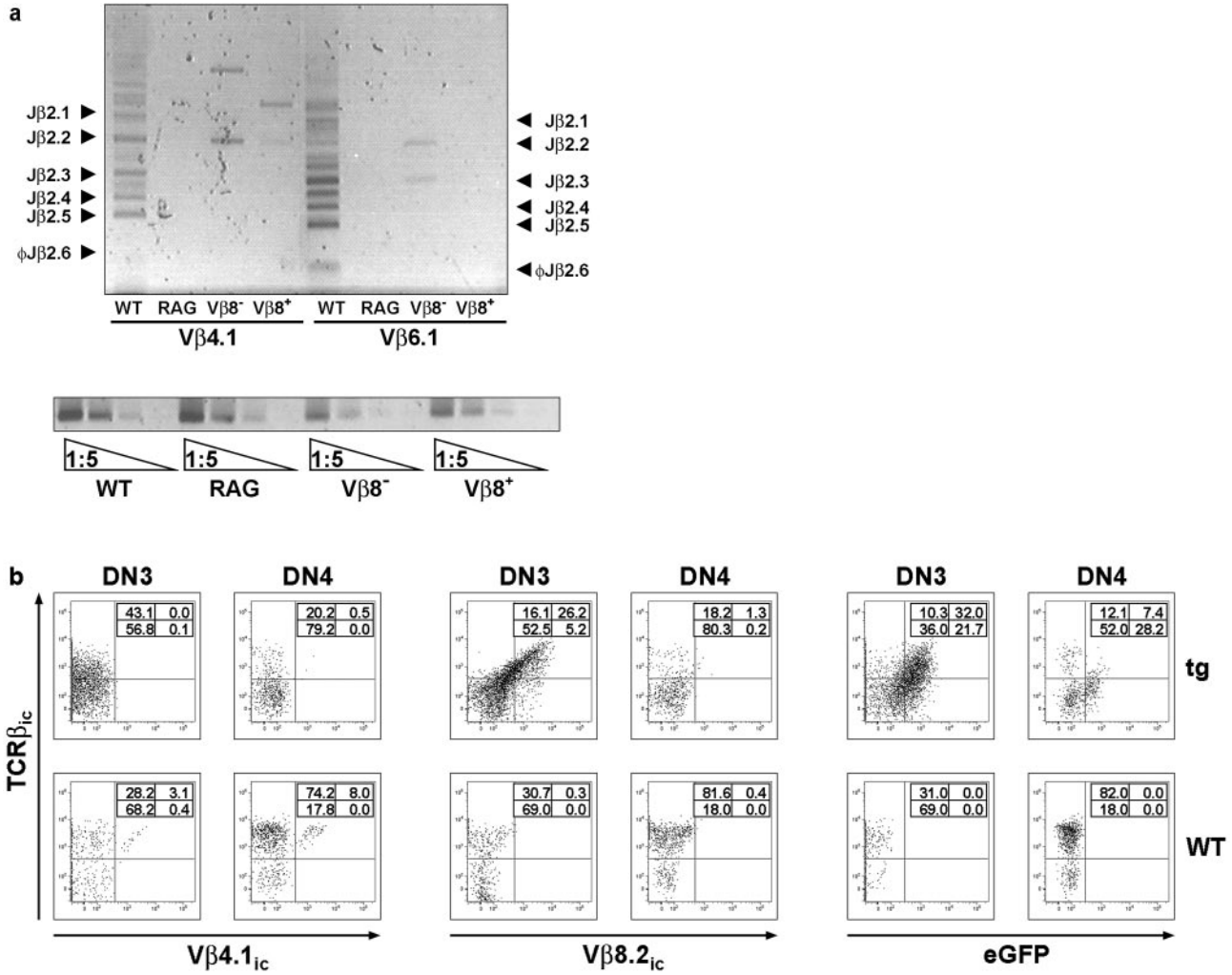

FIGURE 5. Allelic exclusion of endogenous *TCRβ* loci in tg mice. *a*, PCRs for Vβ4.1→Jβ2 and Vβ6.1→Jβ2 rearrangements were performed on genomic DNA of DN3 thymocytes of WT mice, splenocytes of Rag-2$^{-/-}$ mice, and Vβ8.1/8.2$_{ic}^{+}$ or Vβ8.1/8.2$_{ic}^{-}$ DN3 thymocytes of tg mice. The arrowheads show the expected size of rearrangements of the Vβ4/Vβ6 segment to the indicated Jβ2 segment. Usage of equivalent amounts of DNA was confirmed by serial dilution PCR using insulin-specific primers. *b*, Six-color FACS analysis of thymocytes from WT and tg mice. Cells were surface stained for CD4, CD8, CD25, CD44, and CD161c and intracellulary stained for TCRβ, Vβ8.2, and Vβ4.1.

(11), was drastically increased in tg mice without showing increased numbers of apoptotic cells (see Figs. 2*b* and 5). Moreover, as shown in Fig. 2*b*, the CD25 expression pattern in the tg mice revealed a large population with intermediate expression, which was not seen in WT mice. This population could represent DN3 cells that prematurely down-regulated CD25 as a result of β-selection by the TCRβ tg. To definitively clarify this point, we crossed the TCRβ tg mice with TCRβ knockout mice. In comparison with TCRβ$^{-/-}$tg^{-} mice, TCRβ$^{-/-}$tg^{+} animals presented an ~3-fold increase in the number of total thymocytes (Fig. 6). FACS analysis showed that this increase was mainly due to an expansion of the DP population (76 vs 11%), corresponding to a >20-fold increase in absolute numbers of DP cells. In addition, increased numbers of both types of SP cells were observed. The results suggest that the TCRβ tg was capable of partially restoring the generation of DP cells, which is severely compromised in TCRβ$^{-/-}$tg^{-} animals. Interestingly, as in all previous experiments, no significant expression of Vβ8.1/8.2 (<1% of the cells) could be detected beyond the DN3 stage (data not shown). These data suggest that the transgene in fact is able to mediate β-selection and perhaps some further maturation as well.

Discussion

In this report, we describe a ΔEβ TCRβ BAC tg mouse that shows a DN2/DN3-restricted expression pattern of the transgene. The restricted expression is likely due to the lack of Eβ, although the deletion in the BAC is more extended so that a possible contribution of other control elements is not excluded. We observed similar phenotypes in two independent tg lines, thus excluding effects caused by the integration site. The TCRβ joint encoded by the transgene was cloned from the TCRδ$_{sf}^{+}$ DN4 population. Although previous studies failed to confirm the existence of nonfunctional TCRβ-chains (Ref. 36; A. Rolink, unpublished observations), we considered the possibility that the TCRβ tg does not support αβ T cell development. However, the product of the transgene exhibited all three major hallmarks of a TCRβ-chain that can give rise to a functional pre-TCR. First, it was capable of mediating allelic exclusion of endogenous *TCRβ* loci (35) as shown on the DNA and protein levels. Second, it is capable of facilitating β-selection because its introduction onto a TCRβ$^{-/-}$ background alleviated the developmental block in the production of DP cells in these knockout mice (5). Third, it suppressed γδ T cell development (34), a phenomenon that has been shown to be at least

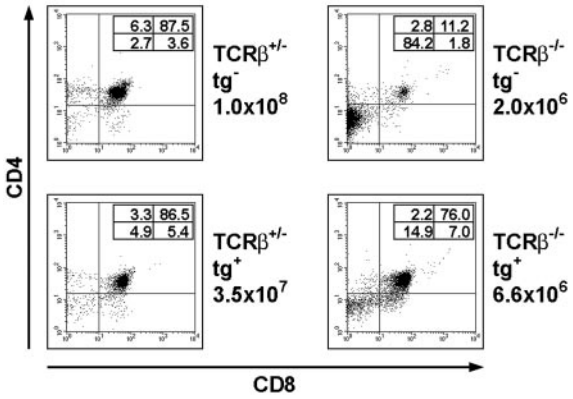

FIGURE 6. Effects of the transgene on a TCR$\beta^{-/-}$ background. The transgene was crossed onto a TCR$\beta^{-/-}$ background. Thymocyte development in different littermates was assessed by staining for CD4 and CD8 followed by FACS analysis. The number on the right side of dot plots indicates the overall thymic cellularity.

in part caused by suppression of TCRγ rearrangements due to early expression of a pre-TCR in DN3 (12).

We also considered the possibility that transgene expressing cells did not survive, either due to toxicity of one of the products of the transgene or to negative selection by a putative superantigen. Toxicity was ruled out by two types of experiments. First, annexin V/PI stainings revealed no significant increase of apoptotic cells in the tg DN3 population and a decrease rather than an increase in apoptotic cells in the tg DN4 population. Second, GFP$^+$ DN3 cells developed into Vβ8.1/8.2$_{ic}^-$ DN3 and subsequently into Vβ8.1/8.2$_{ic}^-$ DN4 cells in RTOCs, showing that transgene expressing cells do not die but rather shutdown expression of the transgene. Finally, transgene expressing cells are β-selected in vivo as they give rise to DP cells on a TCR$\beta^{-/-}$ background. Superantigen deletion had to be considered because the FVB mouse strain lacks an endogenous Vβ8.2 segment. Therefore, it was conceivable that a retrovirally derived superantigen could mediate a strong interaction between the tg Vβ8.2 chain and the endogenous H-2^q MHC molecules, resulting in deletion of the transgene-expressing cells. However, as superantigen-mediated deletions take place at the DP and not at the DN3 stage, it is unlikely that the loss of Vβ8.2$^+$ cells is caused by this mechanism. In addition, loss of transgene expression was also seen in RTOCs using BALB/c-derived thymic stroma, which expresses MHC molecules of the H-2^d haplotype and supports development of Vβ8.2$^+$ T cells.

What is the origin of the GFP$^-$ DN3 population? As transgene expression starts in DN2 and GFP$^-$DN3 cells do not give rise to Vβ8.1/8.2$_{ic}^+$ cells in RTOCs, it is likely that these cells may have previously expressed the transgene and have already down-regulated it, placing them at a later developmental stage than the GFP$^+$ DN3 cells. On the other hand, while being in a similar developmental stage as the GFP$^+$ cells, GFP$^-$ DN3 cells may never express the transgene due to mechanisms such as position effect variegation. We favor the first mechanism because of the suppression of endogenous TCRβ VDJ rearrangements, which is equally pronounced in both the Vβ8$^+$ and Vβ8$^-$ DN3 cells of tg mice. In case of the second mechanism, the GFP$^-$ cell population, which accounts for a majority of DN3 cells, should not show allelic exclusion of the endogenous *TCRβ* loci. This assumption is not at variance with the small number of DN3 cells that show endogenous TCRβ V$\rightarrow$DJ rearrangements. TCRβ transgenes never cause complete suppression of endogenous TCRβ rearrangements, presumably because tg expression in DN2 does not give rise to a pre-TCR due to lack of CD3ϵ expression in this population (10).

We envisage thymocyte development in the ΔEβ tg mice as consisting of two tiers, one driven by the TCRβ tg and the other by endogenous TCRβ-chains. The tg TCRβ is expressed in most of the DN2 cells and gives rise to the formation of a pre-TCR as soon as CD3 components are expressed in early DN3 (10), thus suppressing endogenous TCRβ and TCRγ rearrangements. Signals through the pre-TCR initiate β-selection of a large proportion of this population, driving development though the pre-TCR-dependent checkpoint into the DN4 and DP stages. Transgene expression is terminated in DN3, resulting in lack of intracellular TCRβ in most DN4 and DP cells, as well as in premature termination of proliferation and reduced numbers of DP cells. The DP stage is the end point of most TCRβ_{ic}^-TCRδ_{ic}^- cells, but a minority may even proceed to the SP stage, as indicated by the TCRβ_{ic}^- SP subset. In parallel, a few DN3 cells produce in-frame rearrangements of endogenous TCRβ loci, express these TCRβ-chains, and develop into TCRβ_{ic}^+TCRδ_{ic}^- DN4, DP, and SP cells, in line with the ~5-fold decrease in the positively selected CD69$^+$ population. Because of pre-TCR and TCR expression in this population, the TCRβ_{ic}^+ cells proliferate and undergo positive selection normally, finally giving rise to ~50% of all SP cells.

Unexpectedly, tg mice showed an ~3-fold drop in the ratio of CD4:CD8 SP cells. This phenotype is reminiscent of the one seen in WT/TCR$\alpha^{-/-}$ mixed bone marrow chimeras (37), which contain two cell populations: one that is competent of developing into the SP and one that is arrested in the DP stage. This study also reported a decreased CD4:CD8 ratio, suggesting to the investigators that the selection of CD8 SP cells in normal mice may be limited by the amount of available MHC class I molecules, whereas selection of CD4 SP cells may be limited by the generation of MHC class II-restricted TCR. This effect may also be responsible for our observations because also in ΔEβ tg mice only a minor proportion of DP cells are capable of maturing to the SP stage. In addition, the less pronounced reduction in CD8 SP cells may be due to ISP cells in this population.

Our study shows that Eβ is dispensable for transcription of a rearranged TCRβ tg in the DN2/DN3 population but not at later stages of thymopoesis. In normal mice, some *TCRβ* genes that rearrange in DN2 and early DN3 cells may indeed be expressed Eβ independently, but most rearrangements likely take place after Eβ-dependent expression commences in DN3. Previous reports (17, 26, 27) described germline Vβ transcripts in the thymi of anti-CD3ϵ-induced, Eβ-deficient Rag$^{-/-}$ mice and interpreted this observation as Eβ-independent transcription in the DP population. However, these studies did not use purified DP cells so that the results could be due to the DN cells that are still present in anti-CD3ϵ-induced Rag$^{-/-}$ thymi. Alternatively, induction of Rag$^{-/-}$ thymocytes with anti-CD3ϵ may represent too brief a stimulus that is sufficient to drive the cells over the β-selection checkpoint but insufficient to induce Eβ dependence of germline transcription, a process that might require prolonged pre-TCR signaling. As a third possibility, transcription of rearranged *TCRβ* genes may have different regulatory requirements than germline transcription. We have shown previously that transcription of rearranged *TCRβ* genes in DN3 is subject to regulation by signaling through the CD3 complex, whereas this is not the case for later developmental stages (38, 39). These results agree with the present data in suggesting that a drastic change takes place in the regulation of the expression of *TCRβ* genes during the DN3 stage.

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Disclosures

The authors have no financial conflict of interest.

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