Revision of T cell receptor α chain genes is required for normal T lymphocyte development

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To become mature $\alpha\beta$ T cells, developing thymocytes must first assemble a T cell receptor (TCR) β chain gene encoding a TCR β chain that forms a pre-TCR. These cells then need to generate a TCR α chain gene encoding a TCR α chain, which, when paired with the TCR β chain, forms a selectable $\alpha\beta$ TCR. Newly generated VJ α rearrangements that do not encode TCR α chains capable of forming selectable $\alpha\beta$ TCRs can be excised from the chromosome and replaced with new VJ α rearrangements. Such replacement occurs through the process of TCR α chain gene revision whereby a V α gene segment upstream of the VJ α rearrangement is appended to a downstream J α gene segment. A multistep, gene-targeting approach was used to generate a modified TCR α locus (TCR α^{sj}) with a limited capacity to undergo revision of TCR α chain genes. Thymocytes from mice homozygous for the TCR α^{sj} allele are defective in their ability to generate an $\alpha\beta$ TCR. Furthermore, those thymocytes that do generate an $\alpha\beta$ TCR have a diminished capacity to be positively selected, and TCR $\alpha^{sj/sj}$ mice have significantly reduced numbers of mature $\alpha\beta$ T cells. Together, these findings demonstrate that normal T cell development relies on the ability of developing thymocytes to revise their TCR α chain genes.

V(D)J recombination | gene rearrangement | T cell repertoire | $\alpha\beta$ T cell

The checkpoints imposed on developing T cells mandate that they generate T cell receptor (TCR) α and β chains that form an $\alpha\beta$ TCR capable of positive selection (1). TCR α and β chain gene assembly is precisely regulated within the context of normal thymocyte development. TCR β chain genes are assembled in CD4⁻/CD8⁻ [double negative (DN)] thymocytes (2–4). Expression of a TCR β chain, as a pre-TCR, results in signals that promote cellular expansion and developmental stage progression to the CD4⁺/CD8⁺ [double positive (DP)] stage of thymocyte development (5). Pre-TCR signals also promote TCR α chain gene assembly and prohibit further TCR β chain gene assembly through the process of allelic exclusion (5–7). Because allelic exclusion prohibits DP thymocytes from generating new TCR β chains, these cells must generate TCR α chains that form an $\alpha\beta$ TCR capable of positive selection if they are to become mature T cells.

The genes encoding the TCR α chain are intermingled with TCR δ chain genes in the TCR α/δ locus (Fig. 1) (8). TCR α variable region genes are assembled from V α and J α gene segments. There are ≈ 100 V α gene segments clustered in the 5' region of the mouse TCR α/δ locus and 61 J α gene segments, spanning ≈ 60 kb, clustered in the 3' region of the locus (Fig. 1) (8). The V α and J α gene segments are oriented such that any rearrangement occurs by deletion, excising the intervening DNA from the chromosome (8).

Thymocytes entering the DP compartment from the DN compartment express low TCR β chain levels, as the pre-TCR, and are referred to as TCR β^{low} DP thymocytes. Initial V α rearrangements in these cells are targeted to the most 5' J α gene segments through the activity of the T early α (TEA) promoter (9). A nonproductive VJ α rearrangement could be followed by a VJ α rearrangement on the other allele. Alternatively, a new VJ α rearrangement could be generated on the same allele as the nonproductive VJ α rearrangement (Fig. 1) (10, 11). This process

Fig. 1. Mouse TCR α/δ locus. The V α , J α , D δ , and J δ gene segments are shown as are the TCR α (C α) and TCR δ (C δ) constant region genes. The TEA promoter (TEAp) and TCR α enhancer (E α) are shown. Examples of initial and revision VJ α rearrangements and the circular extrachromosomal reciprocal products from these rearrangements are shown. The TCR α/δ locus is not drawn to scale.

occurs when an upstream V α gene segment rearranges to a J α gene segment downstream of the nonfunctional VJ α rearrangement (revision VJ α ; see Fig. 1). Because all V α to J α rearrangements occur by deletion, the nonproductive VJ α rearrangement will be excised from the chromosome and replaced by a new, potentially productive VJ α rearrangement (Fig. 1).

DP thymocytes that have successfully generated an $\alpha\beta$ TCR express higher TCR β chain levels and are referred to as TCR β^{int} DP thymocytes. TCR β^{int} DP thymocytes continue to undergo V α to J α rearrangements until recombinase activating gene (RAG) -1 and -2 expression is terminated by the signals of positive selection (12–18). Thus, a productive VJ α rearrangement that does not encode a TCR α chain capable of positive selection could be excised and replaced with a new VJ α (Fig. 1) (15, 19–21). Furthermore, in some instances, VJ α rearrangements encoding TCR α chains that form autoreactive $\alpha\beta$ TCRs could be similarly excised from the chromosome and replaced with a new VJ α rearrangement, possibly sparing the developing T cell from death mediated by negative selection (19, 22).

The murine TCR α/δ locus has ≈ 100 V α and 61 J α gene segments. Thus, developing T cells could have many opportunities to excise nonfunctional (nonproductive or productive but nonselectable) VJ α rearrangements from the chromosome and replace them with new, potentially functional VJ α rearrangements on the same allele. The extent to which normal T cell development relies on this process, hereafter referred to as TCR α gene revision, is not known. We have used a multistep,

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Abbreviations: TCR, T cell receptor; DN, double negative; DP, double positive; TEA, T early α ; RAG, recombinase activating gene; SP, single positive.

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Fig. 2. The $\text{TCR}\alpha^{\text{SJ}}$ allele. Shown is the generation of the pTEAp/JαKI-targeting construct and schematics of the targeted alleles as discussed in the text and *Materials and Methods*. The 5' homology arm (5'H), 3' homology arm (3'H), neomycin-resistant gene (Neo) and *loxP* sites (filled triangles), TEA promoter (TEAp), TEA exon (TEAe), and the position of probe 8 (filled rectangle) are shown. A Southern blot of EcoRV-digested genomic DNA from $\text{TCR}\alpha^{\text{SJ/SJ}}$, $\text{TCR}\alpha^{\text{SJ/+}}$, and $\text{TCR}\alpha^{+/+}$ mice by using probe 8 is also shown.

gene-targeting approach to generate a modified $\text{TCR}\alpha/\delta$ locus ($\text{TCR}\alpha^{\text{SJ}}$) with a single functional $\text{J}\alpha$ gene segment. By analyzing $\text{TCR}\alpha^{\text{SJ/SJ}}$ mice, we demonstrate that $\text{TCR}\alpha$ gene revision is required for thymocytes to efficiently move through two distinct checkpoints of normal thymocyte development. These findings have important implications for T cell development and for the regulation of $\text{TCR}\alpha$ chain gene assembly.

Materials and Methods

Generation of the pTEAp/JαKI-Targeting Construct. A 0.2-kb fragment containing the $\text{J}\alpha 56$ segment was amplified by PCR (5' primer, 5'-CGCG-GGATCC-TGGCTTCATCAGTTA-GATTTC-3'; 3' primer, 5'-CGCG-CTCGAG-AGGTGACG-GCTTATGTTTAAAA-3'). A 2.6-kb PCR fragment containing the TEA promoter, TEA exon, and $\text{J}\alpha 61\psi$ segment was also amplified by PCR (5' primer, 5'-CGCG-GTCGAC-GATCTC-CTGGGGCCCTACCT-3'; 3' primer, 5'-CGCG-GGATCC-CCTTTCGCTCTCTTGGGACA-3'). The 0.2- and 2.6-kb fragments were subcloned into pBluescript (Stratagene) to generate pTEAp/Jα (Fig. 2). pTEAp/JαKI was assembled by using the pLNTK-targeting vector, the 2.8-kb fragment from pTEAp/Jα, a 3.3-kb 5' homology arm (nucleotides 81594–84851; accession no. M64239), and a 1-kb 3' homology arm (nucleotides 84851–85800; accession no. M64239), as shown in Fig. 2 (23).

Southern and Northern Blot Analyses. Southern and Northern blot analyses were performed as described (24). Appropriate generation of the $\text{TEA5'loxP-3'TEA/J}\alpha$ allele was confirmed by Southern blot analysis of HindIII-digested genomic DNA by using a 0.6-kb Apal fragment containing the $\text{J}\alpha 6$ gene segment as a 5' probe (the 8.5-kb wild-type allele vs. the 8.9-kb targeted allele). Appropriate targeting at the 3' end was confirmed by Southern blot analysis of SpeI-digested genomic DNA (the 6.5-kb wild-type allele vs. the 3.5-kb targeted allele) by using a 0.4-kb probe generated by PCR (5' primer, 5'-CGCG-

CTCGAG-TGACTTGTGCCTGTCCCTAA-3'; 3' primer, 5'-CGCG-GGTACC-TGGCGTTGGTCTCTTTGAAG-3'). After Cre-mediated deletion, the $\text{TCR}\alpha^{\text{SJ}}$ allele was identified by Southern blot analysis of EcoRV-digested genomic DNA hybridized with probe 7, which has been described (the 14.5-kb $\text{TEA5'loxP-3'TEA/J}\alpha$ allele vs. the 7.1-kb $\text{TCR}\alpha^{\text{SJ}}$ allele) (25). The RAG-1 probe is a 1.3-kb EcoRI cDNA fragment. Probe 8 (TEA probe), the $\text{C}\alpha$ probe, and the GAPDH probe have been described (24).

Flow Cytometry. Flow cytometric analyses were performed as described (19) by using phycoerythrin-conjugated anti-CD4 and anti- $\text{TCR}\beta$, CyChrome-conjugated anti-CD4, phycoerythrin-Cy7-conjugated anti-CD4, allophycocyanin-conjugated anti-CD8, and FITC-conjugated anti-Vα2, -Vα3.2, -Vα8.3, -Vα11, and -CD8 (Pharmingen).

BrdUrd Labeling. Mice were injected i.p. with either two doses of 1.5 mg of BrdUrd (Sigma) separated by 1 h during single-pulse experiments or 1.5 mg of BrdUrd every 12 h for the indicated number of days during continuous labeling experiments. BrdUrd incorporation in thymocytes was assayed by flow cytometry by using a BrdUrd FITC-flow kit (Pharmingen).

Results

Generation of the $\text{TCR}\alpha^{\text{SJ}}$ Allele. We used a previously described (26) embryonic stem cell line that has a single *loxP* site targeted 5' of the TEA promoter (see Fig. 2; TEA5'loxP). A 2.6-kb fragment immediately downstream of this *loxP* site that contains the TEA promoter, TEA exon, and $\text{J}\alpha 61$ gene segment was appended to a 0.2-kb fragment containing the $\text{J}\alpha 56$ gene segment and its flanking recombination signal sequence to generate pTEAp/Jα (Fig. 2). The pTEAp/JαKI-targeting vector was used to target the TEAp/Jα cassette and the *loxP*-flanked neomycin resistance gene downstream of the $\text{J}\alpha 1$ gene segment on the

Fig. 3. T cell development in $\text{TCR}\alpha^{\text{SJSJ}}$ mice. (A) Total thymocyte numbers in 5-week-old $\text{TCR}\alpha^{\text{SJSJ}}$ (SJSJ ; $n = 4$) and wild-type $\text{TCR}\alpha^{+/+}$ ($+/+$; $n = 5$) mice ($P < 0.05$). (B) Flow cytometric analysis of $\text{TCR}\alpha^{\text{SJSJ}}$ and $\text{TCR}\alpha^{+/+}$ thymocytes by using anti-CD4 and anti-CD8. (C) Absolute numbers of $\text{CD4}^+\text{CD8}^+$ DP and CD4^+ SP and CD8^+ SP thymocytes in $\text{TCR}\alpha^{\text{SJSJ}}$ and $\text{TCR}\alpha^{+/+}$ mice. (D) Absolute numbers of splenic $\alpha\beta$ T cells and the number of CD4^+ and CD8^+ splenic $\alpha\beta$ T cells in $\text{TCR}\alpha^{\text{SJSJ}}$ and $\text{TCR}\alpha^{+/+}$ mice.

$\text{TEA5}'\text{loxP}$ allele generating the $\text{TEA5}'\text{loxP-3}'\text{TEA/J}\alpha$ allele (Fig. 2). Expression of the Cre recombinase in these embryonic stem cells led to a 66-kb deletion between the loxP sites 5' of the TEA promoter and 3' of the neomycin resistance gene generating the $\text{TCR}\alpha^{\text{SJ}}$ allele (Fig. 2).

The $\text{TCR}\alpha^{\text{SJ}}$ allele differs from the wild-type $\text{TCR}\alpha/\delta$ locus in that the 64-kb $\text{J}\alpha$ gene segment cluster, containing 61 $\text{J}\alpha$ gene segments, has been replaced by a 0.5-kb fragment containing two $\text{J}\alpha$ gene segments ($\text{J}\alpha 61$ and $\text{J}\alpha 56$). The regions immediately 3' and 5' of the $\text{J}\alpha$ gene segment cluster, including the TEA promoter, are unaltered on the $\text{TCR}\alpha^{\text{SJ}}$ allele. The $\text{J}\alpha 61$ gene segment was included because many of initial $\text{V}\alpha$ rearrangements occur to the $\text{J}\alpha 61$ gene segment (25). However, the $\text{J}\alpha 61$ gene segment is a pseudogene with two translation termination codons in the correct reading frame. Therefore, on the $\text{TCR}\alpha^{\text{SJ}}$ allele, only the $\text{J}\alpha 56$ gene segment can give rise to a functional $\text{VJ}\alpha$ rearrangement. It is important to note that comparison of $\text{TCR}\alpha$ cDNAs isolated from wild-type DP thymocytes and mature $\alpha\beta$ T cells revealed similar fractions that use the $\text{J}\alpha 56$ gene segment (data not shown). Thus, there is not a significant selection bias for or against $\text{TCR}\alpha$ chains encoded by genes that include the $\text{J}\alpha 56$ gene segment. Chimeric mice were generated from $\text{TCR}\alpha^{\text{SJ}/+}$ embryonic stem cells, and germ-line transmission of the $\text{TCR}\alpha^{\text{SJ}}$ allele was achieved.

$\text{TCR}\alpha^{\text{SJSJ}}$ Mice Have Increased Thymocytes but Reduced Mature $\alpha\beta$ T Cells Numbers. $\text{TCR}\alpha^{\text{SJSJ}}$ mice have increased total thymocyte numbers compared with wild-type $\text{TCR}\alpha^{+/+}$ mice (Fig. 3A). Flow cytometric analyses primarily revealed an increase in the number of DP thymocytes (Fig. 3B and C). It is notable that this increase in DP thymocyte numbers is coupled with a reduction in the number of CD4^+ and CD8^+ SP thymocytes in $\text{TCR}\alpha^{\text{SJSJ}}$

Fig. 4. $\text{V}\alpha$ gene segment use in $\text{TCR}\alpha^{\text{SJSJ}}$ mice. (A) Flow cytometric analysis of $\text{V}\alpha 2$, $\text{V}\alpha 3.2$, $\text{V}\alpha 8.3$, and $\text{V}\alpha 11$ expression by lymph node $\alpha\beta$ T cells in $\text{TCR}\alpha^{+/+}$ ($+/+$; open bar) and $\text{TCR}\alpha^{\text{SJSJ}}$ (SJSJ ; filled bar) mice. Four mice were included in each group, and the SD is indicated. (B) Use of the $\text{V}\alpha 2.2$, $\text{V}\alpha 2.8$, $\text{V}\alpha 2.5$, $\text{V}\alpha 2.3$, $\text{V}\alpha 2.1$, and $\text{V}\alpha 2.7$ family members in $\text{V}\alpha 2$ cDNAs isolated from $\text{TCR}\alpha^{+/+}$ (open bar; $n = 45$) and $\text{TCR}\alpha^{\text{SJSJ}}$ (filled bar; $n = 21$) mice. A schematic with the position of the different $\text{V}\alpha 2$ gene segment family members in the $\text{V}\alpha$ gene segment cluster is shown drawn to scale (21).

mice (Fig. 3B and C). Furthermore, $\text{TCR}\alpha^{\text{SJSJ}}$ mice also have reduced numbers of mature CD4^+ and CD8^+ splenic $\alpha\beta$ T cells (Fig. 3D). Thus, despite an increase in the number of DP thymocytes, $\text{TCR}\alpha^{\text{SJSJ}}$ mice have reduced numbers of mature $\alpha\beta$ T cells. This reduction is not caused by a significant bias in $\text{V}\alpha$ gene segment use on the $\text{TCR}\alpha^{\text{SJ}}$ allele as evidenced by flow cytometric analyses, revealing that the fraction of mature T cells using the $\text{V}\alpha 2$, $\text{V}\alpha 3.2$, $\text{V}\alpha 8.3$, and $\text{V}\alpha 11$ gene segments was similar when comparing wild-type and $\text{TCR}\alpha^{\text{SJSJ}}$ $\alpha\beta$ T cells (Fig. 4A). Furthermore, the frequency of use of the different $\text{V}\alpha 2$ gene segments scattered throughout the $\text{V}\alpha$ gene segment cluster was not significantly different when comparing $\text{TCR}\alpha$ cDNAs derived from wild-type and $\text{TCR}\alpha^{\text{SJSJ}}$ $\alpha\beta$ T cells (Fig. 4B).

DN to DP Transition Is Not Perturbed in $\text{TCR}\alpha^{\text{SJSJ}}$ Mice. The increased numbers of DP thymocytes in $\text{TCR}\alpha^{\text{SJSJ}}$ mice could be caused by an increase in the generation of these cells from DN thymocytes. Germ-line transcription and rearrangement of $\text{TCR}\alpha$ chain gene segments does not occur in DN thymocytes but rather is delayed until the DP stage of thymocyte development (Fig. 5A) (27–31). Likewise, germ-line transcription of the $\text{TCR}\alpha^{\text{SJ}}$ allele is also delayed until the DP stage of thymocyte development (Fig. 5A). This delay is confirmed by Northern blot analysis of RNA isolated from $\text{TCR}\alpha^{\text{SJSJ}}\cdot\text{RAG-2}^{-/-}$ DN thymocytes and DP thymocytes from $\text{TCR}\alpha^{\text{SJSJ}}\cdot\text{RAG-2}^{-/-}$ mice bearing a $\text{TCR}\beta$ transgene by using the TEA exon and $\text{C}\alpha$ probes (Fig. 5A). Compared with the wild-type $\text{TCR}\alpha$ allele, the germ-line $\text{TCR}\alpha$ transcripts templated by the $\text{TCR}\alpha^{\text{SJ}}$ allele in DP thymocytes have qualitative and quantitative differences (Fig. 5A). These differences are probably caused by $\text{TCR}\alpha^{\text{SJ}}$ allele modifications that place the $\text{TCR}\alpha$ enhancer 63 kb closer to the TEA promoter and remove regions of the $\text{J}\alpha$ gene cluster where lower molecular weight TEA-hybridizing germ-line transcripts normally terminate (32). It is important to note that germ-line $\text{TCR}\alpha$ transcripts were not detected in $\text{TCR}\alpha^{\text{SJSJ}}\cdot\text{RAG-2}^{-/-}$ DN thymocytes, demonstrating that transcription is not prematurely activated on the $\text{TCR}\alpha^{\text{SJ}}$ allele in DN thymocytes (Fig. 5A).

Fig. 5. DN to DP transition in $\text{TCR}\alpha^{\text{sl/sl}}$ mice. (A) Northern blot analysis of RNA isolated from $\text{RAG-2}^{-/-}$ thymocytes (DN) or $\text{RAG-2}^{-/-}$ TCR β transgenic thymocytes (DP) homozygous for the wild-type $\text{TCR}\alpha^{+/+}$ or $\text{TCR}\alpha^{\text{sl/sl}}$ alleles. RNA was probed with probe 8 (TEA), the $C\alpha$ probe, and the GAPDH probe as an RNA-loading control. RNA isolated from $\text{RAG-2}^{-/-}$ splenocytes (RS) was also analyzed as a negative control. The positions of the 28S and 18S RNA are indicated. (B) The number of BrdUrd⁺ DP thymocytes in individual $\text{TCR}\alpha^{+/+}$ (open bar) and $\text{TCR}\alpha^{\text{sl/sl}}$ (filled bar) mice at 3, 6, 16, and 48 h after BrdUrd injection. Three $\text{TCR}\alpha^{+/+}$ and $\text{TCR}\alpha^{\text{sl/sl}}$ mice were analyzed at each time point.

To directly assess the rate of generation of $\text{TCR}\alpha^{\text{sl/sl}}$ DP thymocytes from DN thymocytes, wild-type and $\text{TCR}\alpha^{\text{sl/sl}}$ mice were pulsed with BrdUrd, and thymocytes were harvested at 3, 6, 16, and 48 h after injection (Fig. 5B). Because BrdUrd incorporation occurs primarily in proliferating DN thymocytes, the rate of accumulation of BrdUrd⁺ DP thymocytes will be directly related to the rate of transition of DN cells to the DP compartment. These analyses revealed that the kinetics of BrdUrd⁺ DP thymocyte accumulation was remarkably similar in wild-type and $\text{TCR}\alpha^{\text{sl/sl}}$ mice (Fig. 5B). Thus, the increased numbers of DP thymocytes in $\text{TCR}\alpha^{\text{sl/sl}}$ mice is probably not caused by a significant increase in the generation of these cells from DN thymocytes.

Accumulation of $\text{TCR}\beta^{\text{low}}$ DP Thymocytes in $\text{TCR}\alpha^{\text{sl/sl}}$ Mice. Flow cytometric analyses revealed that the increase in DP thymocytes in $\text{TCR}\alpha^{\text{sl/sl}}$ mice is primarily caused by an increase in pre-TCR-expressing $\text{TCR}\beta^{\text{low}}$ DP thymocytes (Fig. 6A and B). Because the kinetics of generation of these cells is not altered (Fig. 5B), this accumulation probably reflects a defect in the ability of $\text{TCR}\alpha^{\text{sl/sl}}$ $\text{TCR}\beta^{\text{low}}$ DP thymocytes to generate an $\alpha\beta$ TCR and move to the $\text{TCR}\beta^{\text{int}}$ DP stage. Northern blot analyses revealed similar levels of $C\alpha$ -hybridizing mature TCR α transcripts in wild-type and $\text{TCR}\alpha^{\text{sl/sl}}$ thymocytes (Fig. 6C). Southern blot analyses of EcoRV-digested genomic DNA from $\text{TCR}\alpha^{\text{sl/sl}}$ thymocytes by using the TEA exon probe (probe 8) revealed $\approx 10\%$ retention of the 7-kb germ-line band and the generation of many nongerm-line size bands caused by the formation of reciprocal products from $V\alpha$ to $J\alpha$ rearrangements (Fig. 6D). These findings demonstrate that most of the $\text{TCR}\alpha^{\text{sl}}$ alleles in

Fig. 6. Accumulation of DP $\text{TCR}\beta^{\text{low}}$ thymocytes in $\text{TCR}\alpha^{\text{sl/sl}}$ mice. (A) Flow cytometric analysis of TCR β chain expression by wild-type $\text{TCR}\alpha^{+/+}$ (+/+; thin line) and $\text{TCR}\alpha^{\text{sl/sl}}$ (sl/sl; thick line) DP thymocytes. (B) Number of $\text{TCR}\beta^{\text{low}}$ DP thymocytes in $\text{TCR}\alpha^{+/+}$ ($n = 5$) and $\text{TCR}\alpha^{\text{sl/sl}}$ ($n = 4$) mice ($P < 0.05$). (C) Northern blot analysis of RNA isolated from $\text{TCR}\alpha^{+/+}$ (+/+) and $\text{TCR}\alpha^{\text{sl/sl}}$ (sl/sl) thymocytes and $\text{RAG-2}^{-/-}$ splenocytes (RS) by using $C\alpha$ and GAPDH probes. (D) Southern blot analysis of EcoRV-digested thymus and kidney DNA from $\text{TCR}\alpha^{\text{sl/sl}}$ mice probed with probe 8 and a RAG-1 probe (RAG) as a DNA-loading control. The positions of 9.4- and 6.5-kb molecular weight markers are indicated. A schematic of the $\text{TCR}\alpha^{\text{sl}}$ locus showing the relative position of the EcoRV (E) sites and probe 8 is shown.

$\text{TCR}\alpha^{\text{sl/sl}}$ thymocytes had undergone a $V\alpha$ to $J\alpha$ rearrangement. Moreover, Southern blot and PCR analyses of mature $\text{TCR}\alpha^{\text{sl/+}}$ $\alpha\beta$ T cells revealed that essentially all of these cells had undergone a $V\alpha$ to $J\alpha 56$ rearrangement on the $\text{TCR}\alpha^{\text{sl}}$ allele (data not shown). Taken together, these data demonstrate that $\text{TCR}\alpha^{\text{sl/sl}}$ mice have a diminished capacity to generate $\alpha\beta$ TCR-expressing DP thymocytes, which is caused neither by inefficient $V\alpha$ to $J\alpha$ rearrangement on the $\text{TCR}\alpha^{\text{sl}}$ allele nor by a reduction in the level of mature TCR α transcripts templated by this allele.

Diminished SP Thymocyte Generation in $\text{TCR}\alpha^{\text{sl/sl}}$ Mice. Paradoxically, the increase in $\text{TCR}\beta^{\text{low}}$ DP thymocytes in $\text{TCR}\alpha^{\text{sl/sl}}$ mice does not lead to a decreased number of $\text{TCR}\beta^{\text{int}}$ DP thymocytes in these mice (Fig. 7A). If $\text{TCR}\alpha^{\text{sl/sl}}$ mice had an isolated defect in the $\text{TCR}\beta^{\text{low}}$ DP to $\text{TCR}\beta^{\text{int}}$ DP thymocyte transition, they would be expected to have reduced numbers of $\text{TCR}\beta^{\text{int}}$ DP thymocytes. However, a concomitant reduction in the efficiency of positive selection could lead to normalization of $\text{TCR}\beta^{\text{int}}$ DP numbers in $\text{TCR}\alpha^{\text{sl/sl}}$ mice.

To investigate this possibility, $\text{TCR}\alpha^{\text{sl/sl}}$ and $\text{TCR}\alpha^{+/+}$ mice were injected with BrdUrd every 12 h, and the thymocytes were harvested at 1, 2, 3, 4, 5, and 7 days. Flow cytometric analyses revealed that maximal BrdUrd labeling of DP thymocytes ($\approx 80\%$) occurred by day 3 in $\text{TCR}\alpha^{\text{sl/sl}}$ and $\text{TCR}\alpha^{+/+}$ mice (data not shown). Therefore, the accumulation of BrdUrd⁺ SP thymocytes after day 3 should primarily reflect the rate of transition of DP thymocytes into the CD4^{+} and CD8^{+} SP compartments. The product (BrdUrd⁺ CD4^{+} or CD8^{+} SP thymocytes) to precursor (BrdUrd⁺ DP thymocytes) ratio was determined for each of the mice analyzed (Fig. 7B). These analyses revealed a progressive accumulation of BrdUrd⁺ CD4^{+} and BrdUrd⁺

Fig. 7. Diminished generation of SP thymocytes in $\text{TCR}\alpha^{\text{SJSJ}}$ mice. (A) The number of $\text{TCR}\beta^{\text{int}}$ DP thymocytes in $\text{TCR}\alpha^{+/+}$ ($n = 5$) and $\text{TCR}\alpha^{\text{SJSJ}}$ ($n = 4$) mice. (B) The ratio of $\text{BrdUrd}^+ \text{CD4}^+$ to BrdUrd^+ DP thymocytes (CD4/DP) (Upper) and $\text{BrdUrd}^+ \text{CD8}^+$ to BrdUrd^+ DP thymocytes (CD8/DP) (Lower) was determined for each $\text{TCR}\alpha^{+/+}$ (open square) and $\text{TCR}\alpha^{\text{SJSJ}}$ (filled triangle) mouse analyzed at 3, 4, 5, and 7 days of continuous BrdUrd labeling. Three $\text{TCR}\alpha^{+/+}$ and $\text{TCR}\alpha^{\text{SJSJ}}$ mice were analyzed at each time point. Linear regression analysis was performed on data from analysis of $\text{TCR}\alpha^{+/+}$ (solid line) and $\text{TCR}\alpha^{\text{SJSJ}}$ (dashed line) mice.

CD8^+ SP thymocytes between days 3 and 7 in the wild-type mice (Fig. 7B). In striking contrast, very little accumulation of $\text{BrdUrd}^+ \text{CD4}^+$ and $\text{BrdUrd}^+ \text{CD8}^+$ SP thymocytes was observed in $\text{TCR}\alpha^{\text{SJSJ}}$ mice during the same time interval (Fig. 7B). These data demonstrate that there is a significant reduction in the generation of SP thymocytes from DP thymocytes in $\text{TCR}\alpha^{\text{SJSJ}}$ mice.

Discussion

We have generated and analyzed mice with a modified $\text{TCR}\alpha$ locus ($\text{TCR}\alpha^{\text{SJSJ}}$) that has a limited capacity to undergo $\text{TCR}\alpha$ gene revision. Although the wild-type $\text{TCR}\alpha$ locus has 61 $J\alpha$ gene segments, the $\text{TCR}\alpha^{\text{SJSJ}}$ allele has just two $J\alpha$ gene segments of which only one, $J\alpha 56$, is capable of generating a productive $\text{VJ}\alpha$ rearrangement. The reduction in mature $\alpha\beta$ T cells in $\text{TCR}\alpha^{\text{SJSJ}}$ mice demonstrates that T cell development depends on the process of $\text{TCR}\alpha$ gene revision. Furthermore, this reduction is caused by the compound effects of a requirement for $\text{TCR}\alpha$ gene revision for both efficient generation of $\alpha\beta$ TCR-expressing DP thymocytes and the positive selection of these cells into the CD4^+ and CD8^+ SP thymocyte compartments.

$\text{TCR}\alpha^{\text{SJSJ}}$ mice have an increased number of pre-TCR-expressing ($\text{TCR}\beta^{\text{low}}$) DP thymocytes (Fig. 6A and B). This increase is not caused by either an increased generation of these

cells from the DN compartment or the proliferative expansion of these cells in the DP compartment (Fig. 5B; data not shown). Furthermore, annexin V staining did not reveal significant differences in the fraction of apoptotic DP thymocytes in $\text{TCR}\alpha^{\text{SJSJ}}$ and wild-type mice (data not shown). Thus, the increase in $\text{TCR}\beta^{\text{low}}$ DP thymocytes probably reflects a diminished capacity of pre-TCR-expressing $\text{TCR}\alpha^{\text{SJSJ}}$ DP thymocytes to generate an $\alpha\beta$ TCR and move to the $\text{TCR}\beta^{\text{int}}$ DP compartment. This diminished capacity could, in part, reflect a requirement for $\text{TCR}\alpha$ gene revision to optimize for the generation of a productive $\text{TCR}\alpha$ chain gene. In this regard, $\approx 44\%$ ($2/3 \times 2/3$) of $\text{TCR}\beta^{\text{low}}$ DP thymocytes in $\text{TCR}\alpha^{\text{SJSJ}}$ mice would rearrange both $\text{TCR}\alpha$ alleles nonproductively and, thus, would be expected to remain at the $\text{TCR}\beta^{\text{low}}$ stage until death. In contrast, through the process of $\text{TCR}\alpha$ gene revision, wild-type DP thymocytes could replace a nonproductive $\text{VJ}\alpha$ rearrangement with a new, potentially productive ($1/3$) $\text{VJ}\alpha$ rearrangement on the same allele.

The accumulation of $\text{TCR}\beta^{\text{low}}$ DP thymocytes in $\text{TCR}\alpha^{\text{SJSJ}}$ mice may also reflect the diminished capacity of these cells to generate a productive $\text{VJ}\alpha$ rearrangement that encodes a $\text{TCR}\alpha$ chain capable of pairing with the specific $\text{TCR}\beta$ chain (33–35). Thus, $\text{TCR}\alpha$ chain gene revision may be important at the $\text{TCR}\beta^{\text{low}}$ to $\text{TCR}\beta^{\text{int}}$ DP thymocyte checkpoint to optimize for the generation of a $\text{VJ}\alpha$ rearrangement that both is productive and encodes a $\text{TCR}\alpha$ chain capable of pairing with the $\text{TCR}\beta$ chain to generate an $\alpha\beta$ TCR.

Most randomly generated $\alpha\beta$ TCRs are unable to mediate positive selection of DP thymocytes (36–38). The notion that efficient positive selection of DP thymocytes relies on $\text{TCR}\alpha$ gene revision is supported by the significant reduction in the generation of CD4^+ and CD8^+ SP thymocytes from DP thymocytes in $\text{TCR}\alpha^{\text{SJSJ}}$ mice (Figs. 3C and 7B). $\text{TCR}\alpha$ gene revision would permit DP thymocytes with a productive but nonselectable $\text{VJ}\alpha$ rearrangement the opportunity to generate a new $\text{VJ}\alpha$ rearrangement, which may lead to the generation of an $\alpha\beta$ TCR capable of positive selection (20, 21). In addition, $\text{TCR}\alpha$ gene revision may rescue DP thymocytes from negative selection by excising and replacing $\text{VJ}\alpha$ rearrangements that encode the $\text{TCR}\alpha$ chains of autoreactive $\alpha\beta$ TCRs (19, 22).

Our findings demonstrate that most developing T cells generate more than two complete $\text{VJ}\alpha$ rearrangements in an attempt to successfully traverse the checkpoints of normal thymocyte development. The maximum number of $\text{VJ}\alpha$ rearrangements that can be generated through $\text{TCR}\alpha$ gene revision in developing T cells is not known but seems limited primarily by the survival parameters of DP thymocytes (39). In this regard, $\text{ROR}\gamma^{-/-}$ DP thymocytes, which exhibit diminished survival, seem to undergo a limited number of $\text{VJ}\alpha$ rearrangements compared with wild-type DP thymocytes or DP thymocytes from BclXL transgenic mice, which exhibit increased survival (39).

The requirement for $\text{TCR}\alpha$ gene revision during T cell development is consistent with the notion that many complete $\text{TCR}\alpha$ chain genes fail to fully satisfy the requirements imposed by thymocyte developmental checkpoints. Thus, mechanisms may be in place to allow newly generated $\text{VJ}\alpha$ rearrangements sufficient time to be “tested” to determine whether they encode suitable $\text{TCR}\alpha$ chains before the process of $\text{TCR}\alpha$ gene revision replaces them with another, likely nonfunctional, $\text{VJ}\alpha$ rearrangement. Moreover, once a functional $\text{VJ}\alpha$ rearrangement has been generated, mechanisms must be in place to prevent further $\text{V}\alpha$ to $J\alpha$ rearrangements on the same allele that would excise the functional $\text{VJ}\alpha$ rearrangement. In this regard, it is notable that RAG-1/-2 gene expression in DP thymocytes is regulated by a cohort of cis-acting elements that may have evolved, in part, to permit rapid cessation of RAG-1/-2 gene expression and $\text{V}\alpha$ to $J\alpha$ rearrangement on the positive selection of developing thymocytes (12, 18, 40, 41).

The reliance of developing T cells on their ability to generate more than two complete TCR α chain genes stands in marked contrast to the constraints imposed by the development on TCR β chain gene generation. Two complete VDJ β rearrangements (one to each of the two DJ β gene clusters) can be generated on each TCR β allele; thus, developing T cells can generate up to four complete VDJ β rearrangements. However, mice with modified TCR β loci such that developing T cells can generate only a single complete VDJ β rearrangement have no measurable defect in T cell development (42). Perhaps this normal development is because of the requirement that TCR β chain gene rearrangements need only be productive and encode a TCR β chain that forms a pre-TCR. In contrast, TCR α chain genes must be productive, must encode a TCR α chain capable of pairing with the TCR β chain, and must generate an $\alpha\beta$ TCR

that can be positively selected. The process of TCR α gene revision increases the likelihood that a developing T cell will generate a VJ α rearrangement that meets these three criteria. It is tempting to speculate that the requirement for this process may have been a driving force behind the expansion of the V α and J α gene segment clusters during evolution.

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