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## TCR $\beta$ Feedback Signals Inhibit the Coupling of Recombinationally Accessible V $\beta$ 14 Segments with DJ $\beta$ Complexes

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### Abstract

Antigen receptor allelic exclusion is thought to occur through mono-allelic initiation and subsequent feedback inhibition of recombinational accessibility. However, our previous analysis of mice containing a V(D)J recombination reporter inserted into V $\beta$ 14 (V $\beta$ 14<sup>Rep</sup>) indicated that V $\beta$ 14 chromatin accessibility is bi-allelic. To determine whether V $\beta$ 14 recombinational accessibility is subject to feedback inhibition, we analyzed TCR $\beta$  rearrangements in V $\beta$ 14<sup>Rep</sup> mice containing a pre-assembled in frame transgenic V $\beta$ 8.2D $\beta$ 1J $\beta$ 1.1 or an endogenous V $\beta$ 14D $\beta$ 1J $\beta$ 1.4 rearrangement on the homologous chromosome. Expression of either pre-assembled V $\beta$ DJ $\beta$ C $\beta$  chain accelerated thymocyte development due to enhanced cellular selection, demonstrating that the rate-limiting step in early  $\alpha\beta$  T cell development is the assembly of an in-frame V $\beta$ DJ $\beta$  rearrangement. Expression of these pre-assembled V $\beta$ DJ $\beta$  rearrangements inhibited endogenous V $\beta$ 14-to-DJ $\beta$  rearrangements as expected. However, in contrast to results predicted by the accepted model of TCR $\beta$  feedback inhibition, we found that expression of these pre-assembled TCR $\beta$  chains did not down-regulate recombinational accessibility of V $\beta$ 14 chromatin. Our findings suggest that TCR $\beta$  mediated feedback inhibition of V $\beta$ 14 rearrangements depends upon inherent properties of V $\beta$ 14, D $\beta$ , and J $\beta$  recombination signal sequences.

### Keywords

T cells; T cell receptor; gene rearrangements; molecular biology; transgenic/knockout mice

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## Introduction

Adaptive immunity relies on the ability of an organism to generate large numbers of lymphocytes that express antigen receptors of unique specificity. During lymphocyte development, the exons that encode T cell receptor (TCR) and immunoglobulin (Ig) variable regions are assembled through the rearrangement of germline variable (V), diversity (D), and joining (J) gene segments (1). V(D)J recombination is initiated by the lymphocyte-specific RAG1/RAG2 (RAG) endonuclease, which cleaves DNA strands between gene segments and their flanking recombination signal sequences (RSs), and completed by the RAG proteins, TdT, and generally expressed non-homologous end-joining proteins, which together repair these DNA ends to form V(D)J coding joins and signal joins (2,3). This site-specific recombination process generates a vast repertoire of antigen specificities through the large combination of possible joining events and the inherent imprecision in coding join formation. The majority of lymphocytes assemble and express functional TCR or Ig chains from only a single allele, a phenomenon referred to as allelic exclusion that limits development of lymphocytes expressing antigen receptors of multiple specificities (4). Current dogma is that antigen receptor allelic exclusion is regulated through allele-specific modulation of recombinational accessibility (5,6), which is defined as the chromatin structure of a gene segment being open and available to, but not necessarily bound by, the V(D)J recombinase (7,8). However, additional mechanisms, such as RAG deposition/binding onto RSs (9–11) and changes in locus topology or location (12,13), may contribute to the regulation of allelic exclusion.

The assembly of TCR  $\beta$  and  $\alpha$  variable region exons is regulated within the context of thymocyte development (6,14). TCR $\beta$  genes recombine in an ordered fashion with D $\beta$ -to-J $\beta$  rearrangement occurring before the joining of a V $\beta$  segment to an assembled DJ $\beta$  complex on one allele in CD4<sup>-</sup>/CD8<sup>-</sup> (double negative, DN) thymocytes (15,16). V $\beta$ -to-DJ $\beta$  rearrangements proceed through the coupling of RAG accessible V $\beta$  segments and DJ $\beta$  complexes (17–19), likely mediated by TCR $\beta$  locus contraction and chromosome looping that juxtapose V $\beta$  and D $\beta$ -J $\beta$ -C $\beta$  segments (13). Due to imprecise joining, approximately one-third of V $\beta$ DJ $\beta$  rearrangements is assembled productively (in-frame) and encodes a functional TCR $\beta$  chain. TCR $\beta$  chains pair with pT $\alpha$  molecules to form pre-TCR complexes that signal  $\beta$ -selection, which involves allelic exclusion, survival, expansion, and further differentiation to the CD4<sup>+</sup>/CD8<sup>+</sup> (double positive, DP) stage (14). In cells that assemble a non-productive (out-of-frame) V $\beta$ DJ $\beta$  rearrangement on the first allele, V $\beta$ -to-DJ $\beta$  recombination can initiate on the second allele, which, if productive, signals survival, expansion and differentiation (14). TCR $\alpha$  variable region exons are assembled from V $\alpha$  and J $\alpha$  segments in DP thymocytes (20). Productive V $\alpha$ J $\alpha$  rearrangements generate TCR $\alpha$  chains that can associate with TCR $\beta$  chains to form  $\alpha\beta$  TCRs, which, upon positive selection, promote further differentiation to CD4<sup>+</sup> or CD8<sup>+</sup> (single positive, SP) thymocytes (14). These cells exit the thymus and migrate to the peripheral lymphoid compartments as naive  $\alpha\beta$  T cells.

Allelic exclusion is thought to occur through asynchronous initiation of V-to-(D)J rearrangement between homologous loci, followed by inhibition of recombination on the second allele through feedback signals generated by expression of an in-frame antigen receptor gene assembled on the first allele (5,21–23). Molecular mechanisms by which V $\beta$  rearrangements initiate asynchronously in DN thymocytes have not been demonstrated, but several have been proposed from experimental observations. The asynchronous replication of TCR $\beta$  loci may promote epigenetic changes that render the earlier replicating allele preferentially accessible to RAG (24). In addition, the association of TCR $\beta$  alleles with repressive nuclear environments could limit recombinational accessibility to one allele in the time-window during which newly assembled V $\beta$ DJ $\beta$  rearrangements can be expressed and signal (12,13). Moreover, in DN cells exhibiting bi-allelic V $\beta$  chromatin accessibility (17,

25), the inherent low recombination potential of V $\beta$  RSSs might limit V $\beta$ -to-DJ $\beta$  rearrangements to one allele at a time (9).

Expression of productive TCR $\beta$  chains in DN thymocytes activates distinct signaling pathways for preventing V $\beta$ -to-DJ $\beta$  rearrangements and for promoting proliferation, expansion, and differentiation (26–28). TCR $\beta$  mediated down-regulation of E47 transcription factor binding to the locus leads to decreased V $\beta$  chromatin accessibility in DN cells (29). DN to DP differentiation leads to epigenetic changes in V $\beta$  chromatin that render V $\beta$  segments inaccessible to RAG (30,31), and de-contraction of TCR $\beta$  loci such that germline V $\beta$  segments are no longer juxtaposed with D $\beta$ -J $\beta$  segments (13). These latter two mechanisms may cooperate to maintain TCR $\beta$  allelic exclusion by preventing the rearrangement of germline upstream V $\beta$  segments to DJ $\beta$  complexes in DP cells (12,13). Unique among V $\beta$  segments, germline V $\beta$ 14 segments remain recombinationally accessible in DP thymocytes (32) and DP cells attempting V $\beta$ 14-to-DJ $\beta$  rearrangements can be eliminated by apoptosis to maintain TCR $\beta$  allelic exclusion (33). However, the mechanisms by which TCR $\beta$  mediated feedback signals inhibit the rearrangement of V $\beta$ 14 and other V $\beta$  segments in DN cells remains to be determined.

To date, all studies of antigen receptor allelic exclusion or feedback regulation have been conducted using correlative measures of recombinational accessibility, such as germline transcription, nuclease sensitivity, histone modifications, DNA methylation, DNA replication, and/or expression of an inserted reporter gene (24,25,30,31,33–37). To elucidate mechanisms that regulate V $\beta$  rearrangement, we previously generated and analyzed mice with replacement of the endogenous V $\beta$ 14 RS with a V(D)J recombination reporter consisting of germline D $\beta$ 1-J $\beta$ 1.1 segments (the V $\beta$ 14<sup>Rep</sup> allele)(17). Since D $\beta$ 1-to-J $\beta$ 1.1 rearrangement depends upon promoter-driven recombinational accessibility of D $\beta$ -J $\beta$  chromatin (38,39), we hypothesized V $\beta$ 14<sup>Rep</sup> D $\beta$ 1-to-J $\beta$ 1.1 rearrangements would occur only if the V $\beta$ 14 promoter and other potential *cis* elements rendered V $\beta$ 14 chromatin accessible to the RAG proteins. We proved that V $\beta$ 14 chromatin environment imparts lineage and developmental-stage specific recombinational accessibility upon V $\beta$ 14<sup>Rep</sup> (17). Notably, despite the presence of a functional TATA box in the 5'D $\beta$ 1 RS (40) and the influence of the J $\beta$ 1.1 RS upon steady-state D $\beta$ 1-J $\beta$ 1.1 transcripts (41), the frequency of V $\beta$ 14<sup>Rep</sup> recombination events was similar to the frequency of V $\beta$ 14 rearrangements on alleles containing specific replacement of the V $\beta$ 14 RS with the 3'D $\beta$ 1 RS (19). Collectively, these observations indicated that the higher intrinsic recombination potential of the 3'D $\beta$ 1 RS compared to the V $\beta$ 14 RS (42), and likely the ability of the 3'D $\beta$ 1 RS to bind c-fos/RAG complexes (11), enables the minimal frequency at which V $\beta$ 14 chromatin is rendered accessible and RSs within this region available for RAG binding to be quantified by V $\beta$ 14<sup>Rep</sup> rearrangement events (17).

Unexpectedly, we found that V $\beta$ 14<sup>Rep</sup> D $\beta$ -to-J $\beta$  recombination occurred on both TCR $\beta$  alleles in the majority of developing thymocytes, demonstrating that regulation of V $\beta$ 14 recombinational accessibility and V $\beta$ 14-to-DJ $\beta$  rearrangements are not mechanistically linked (17). These data also could indicate that V $\beta$ 14 recombinational accessibility is not subject to TCR $\beta$  mediated feedback inhibition. Alternatively, V $\beta$ 14<sup>Rep</sup> may simply rearrange efficiently and on both alleles during the time window required for the assembly and expression of V $\beta$ DJ $\beta$  rearrangements to signal inhibition of V $\beta$ 14 accessibility. To distinguish between these possibilities and determine whether undiscovered mechanisms might contribute to inhibition of V $\beta$ 14 rearrangements, we sought to directly evaluate the effect that TCR $\beta$  mediated feedback signals have upon V $\beta$ 14 recombinational accessibility by analyzing TCR $\beta$  rearrangements in  $\alpha\beta$  T lineage cells of V $\beta$ 14<sup>Rep</sup> mice that express an in-frame V $\beta$ DJ $\beta$  rearrangement prior to initiation of V $\beta$ 14 accessibility.

## Materials and Methods

### Mice

Generation and characterization of DO11.10 TCR $\beta$  transgenic mice (43) and V $\beta$ 14<sup>Rep/Rep</sup> mice (17) were previously described. Generation and characterization of the LN2 embryonic stem cells containing the pre-assembled V $\beta$ 14D $\beta$ 1J $\beta$ 1.4 rearrangement also were previously characterized (44). All experiments in mice were performed in accordance relevant institutional and national guidelines and regulations and approved by the Children's Hospital of Philadelphia IACUC committee.

### Analysis of $\alpha\beta$ T cell development

Single cell suspensions were prepared from the thymuses and spleens of 4–6 week old mice of each genotype. Cell numbers were obtained by counting trypan blue excluded cells using a hemocytometer. Cells were stained with the combinations of FITC-conjugated anti-CD8, anti-V $\beta$ 8, or anti-V $\beta$ 14 antibodies and PE-conjugated anti-CD4 or anti-C $\beta$  antibodies (BD Pharmingen). To analyze DN thymocyte populations, cells were stained with a cocktail of PE-conjugated anti-C $\beta$ , anti-C $\delta$ , anti-CD8, anti-CD45R, anti-CD19, anti-CD11c, anti-CD11b, anti-Ter119, anti-NK.1, and PE-Cy7-conjugated anti-CD25 and APC-conjugated anti-CD117 antibodies (BD Pharmingen). A BD FACSCalibur equipped with BD CellQuest Pro was used to acquire data and FlowJo software (Tree Star) was used to analyze data. All experiments were performed at least three separate times on independent mice of each genotype.

### FACS analysis of $\beta$ selection

Small versus large cells were distinguished after FACS analysis by plotting CD117 versus forward scatter and gating on small (forward scatter low) and large (forward scatter high) cells. BrdU incorporation into thymocyte populations was determined using the FITC BrdU Flow Kit (BD Pharmingen). Mice were injected i.p. with 100  $\mu$ L of kit-provided BrdU according to manufacturer instructions for *in vivo* labeling of mouse cells. After 1.5 hours, mice were sacrificed and thymuses were removed for FACS analysis. The number of cells was modified from manufacturer instructions by increasing to  $20 \times 10^6$  cells in 50  $\mu$ L staining buffer. The time of staining was also increased to 1 hour at 4°C. The rest of the procedure was followed exactly as if the cell amounts were not modified.

### Western blots

Primary thymocytes from specified genotypes were lysed in Tween 20 buffer (50 mM HEPES [pH 8.0], 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 0.1% Tween 20) containing a cocktail of protease inhibitors (Roche 11697498001). Samples were run on a denaturing 10% Tris-Glycine gel and transferred to nitrocellulose. Membranes were first incubated with 1:1000 dilution of an anti-cyclin D3 antibody (Santa Cruz Biotechnology sc-182) in 5% milk overnight at 4°C and washed for 10 minutes 4x with PBS containing 0.01% Tween. Secondary antibody was diluted 1:5000 in 5% milk and incubated for 2 hours at room temperature. Membranes were then stripped and probed in the same manner with an anti-tubulin antibody (Fischer 05829MI) to control for loading.

### Analysis of V $\beta$ 14<sup>Rep</sup> and TCR $\beta$ rearrangements

Cell sorting of DN and DP thymocytes was conducted with a MoFlow cell sorter (DakoCytomation) following staining of thymocytes with FITC-conjugated anti-CD8 $\alpha$  and PE-conjugated anti-CD4. PCR analysis of V $\beta$ 14<sup>Rep</sup> D $\beta$ -to-J $\beta$  rearrangements were conducted using 20 ng of genomic DNA as previously described (17).  $\alpha\beta$  T cell hybridomas were generated as previously described (45). Genomic DNA was isolated, digested with *EcoRI*, and subject to Southern blot analysis as described (45). Endogenous D $\beta$ 1-to-J $\beta$ 1 rearrangements

were characterized using the 5'D $\beta$ 1, inter-genic D $\beta$ 1-J $\beta$ 1, and 3'J $\beta$ 1 probes previously described (46). Endogenous V $\beta$ 14 and V $\beta$ 14<sup>Rep</sup> rearrangements were characterized using the 5'V $\beta$ 14 and 3'V $\beta$ 14 probes previously described (17).

## Results

### Generation of germline mice expressing a pre-assembled endogenous V $\beta$ 14D $\beta$ 1J $\beta$ 1.4 rearrangement

Due to the lack of a C $\beta$  allotypic marker, TCR $\beta$  allelic exclusion studies are often conducted using mice that express an in-frame V $\beta$ DJ $\beta$  rearrangement from a transgene randomly integrated into the genome (28,29,47–51). Thus, to directly evaluate the effect that TCR $\beta$  mediated feedback signals have upon recombinational accessibility of V $\beta$ 14 chromatin, we sought to analyze TCR $\beta$  rearrangements in  $\alpha\beta$  T cells of V $\beta$ 14<sup>Rep</sup> mice that also express a transgenic pre-assembled in-frame V $\beta$ DJ $\beta$  rearrangement. Since the D011.10 TCR $\beta$  transgene has been used to study TCR $\beta$  allelic exclusion and  $\alpha\beta$  T cell development (28,29,43), we decided to use this classical model of TCR $\beta$  mediated feedback inhibition as part of our analysis. This transgene consists of an in-frame pre-assembled V $\beta$ 8.2D $\beta$ 1J $\beta$ 1.1 rearrangement integrated into mouse chromosome 6 near the endogenous TCR $\beta$  locus (43)(Figure 1A; A.C.C. and C.H.B., unpublished observations). Germline mice carrying this transgene are hereafter referred to as V $\beta$ 8<sup>Tg</sup> mice. As previously reported, greater than 99% of  $\alpha\beta$  T lineage cells isolated from V $\beta$ 8<sup>Tg</sup> mice express V $\beta$ 8 as part of cell surface TCR $\beta$  chains (Figure 1B).

We were concerned that transgenic V $\beta$ DJ $\beta$  rearrangements integrated at chromosomal locations outside of endogenous TCR $\beta$  loci might not recapitulate physiological TCR $\beta$  mediated feedback signals. Nuclei of V $\beta$ 14<sup>+</sup>  $\alpha\beta$  T cells have been used in nuclear transfer (NT) experiments to generate embryonic stem (ES) cells containing an endogenous in-frame V $\beta$ 14D $\beta$ 1J $\beta$ 1.4 rearrangement on one TCR $\beta$  allele (the V $\beta$ 14<sup>NT</sup> allele; Figure 1A), an endogenous DJ $\beta$  rearrangement on the other TCR $\beta$  allele, and endogenous V $\alpha$ J $\alpha$  rearrangements on both TCR $\alpha$  alleles (44). We used these ES cells to generate mice with the V $\beta$ 14<sup>NT</sup> allele transmitted through the germline and bred these mice with wild-type 129SvEv mice for several generations to separate the V $\beta$ 14<sup>NT</sup> allele from the DJ $\beta$  allele and the two rearranged TCR $\alpha$  alleles. Through this approach, we generated V $\beta$ 14<sup>NT/WT</sup> mice that carry the V $\beta$ 14<sup>NT</sup> allele, a wild-type un-rearranged TCR $\beta$  allele, and two wild-type un-rearranged TCR $\alpha$  alleles. As expected, greater than 99% of  $\alpha\beta$  T lineage cells isolated from V $\beta$ 14<sup>NT/WT</sup> mice express V $\beta$ 14 as part of cell surface TCR $\beta$  chains (Figure 1B).

### Accelerated early thymocyte development in V $\beta$ 8<sup>Tg</sup> and V $\beta$ 14<sup>NT/WT</sup> mice

Despite the previous use of V $\beta$ 8<sup>Tg</sup> mice to study TCR $\beta$  feedback inhibition and  $\alpha\beta$  TCR selection (28,29,43), a thorough analysis of  $\alpha\beta$  T cell development in V $\beta$ 8<sup>Tg</sup> mice has never been published. In addition, thymocyte development in V $\beta$ 14<sup>NT/WT</sup> mice has never been reported. Thus, we first sought to investigate the potential influence of V $\beta$ 8<sup>Tg</sup> or V $\beta$ 14<sup>NT</sup> expression on gross  $\alpha\beta$  T cell development. Both V $\beta$ 8<sup>Tg</sup> and V $\beta$ 14<sup>NT/WT</sup> mice exhibited similar numbers of thymocytes and splenocytes as wild-type mice (Figure 2A). FACS analysis of thymocytes with anti-CD4, and anti-CD8 antibodies revealed a similar distribution of DN, DP, and SP populations among wild-type, V $\beta$ 8<sup>Tg</sup>, and V $\beta$ 14<sup>NT/WT</sup> mice (Figure 2B,C). However, we did observe statistically significant increases in the percentages of CD4<sup>+</sup> SP thymocytes and decreases in the percentages and CD8<sup>+</sup> SP thymocytes in V $\beta$ 8<sup>Tg</sup> and V $\beta$ 14<sup>NT/WT</sup> mice, as compared to in wild-type mice (Figure 2C). FACS analysis of splenocytes with anti-CD4 and anti-CD8 antibodies also revealed substantial increases in the percentages of CD4<sup>+</sup>  $\alpha\beta$  T cells and decreases in the percentages of CD8<sup>+</sup>  $\alpha\beta$  T cells in V $\beta$ 8<sup>Tg</sup> and V $\beta$ 14<sup>NT/WT</sup> mice, as compared to in wild-type mice (Figure 2B, D). Since the numbers of thymocytes and splenocytes were comparable among wild-type, V $\beta$ 8<sup>Tg</sup>, and V $\beta$ 14<sup>NT/WT</sup> mice, these data

indicate that expression of either one of these pre-assembled V $\beta$ DJ $\beta$  rearrangements directs development of CD4<sup>+</sup>  $\alpha\beta$  T cells at the expense of CD8<sup>+</sup>  $\alpha\beta$  T cells. The latter effect is most likely due to preferential binding of  $\alpha\beta$  TCR containing the V $\beta$ 8<sup>Tg</sup> and V $\beta$ 14<sup>NT</sup> TCR $\beta$  chains with class II MHC chains (52).

Development of DN thymocytes proceeds through four stages that can be defined by cell surface expression of CD117 or CD44 and CD25. TCR $\beta$  locus D $\beta$ -to-J $\beta$  rearrangements initiate in CD117<sup>+</sup>CD25<sup>-</sup> early T lineage progenitors (ETPs, or DNI cells) and continue in CD117<sup>+</sup>CD25<sup>+</sup> DNII thymocytes, while V $\beta$ -to-DJ $\beta$  rearrangements occur in CD117<sup>-</sup>CD25<sup>+</sup> DNIII thymocytes (16,53,54). The differentiation of ETP/DNI cells into DNII thymocytes and DNII cells into DNIII thymocytes occur independent of V(D)J recombination (55,56). In contrast, the development of DNIII cells into DNIV and then DP thymocytes requires the assembly and expression of an in-frame V $\beta$ DJ $\beta$  rearrangement (57). To determine whether expression of these pre-assembled V $\beta$ DJ $\beta$  rearrangements alters early thymocyte development, we analyzed the DN populations of thymocytes in wild-type, V $\beta$ 8<sup>Tg</sup>, and V $\beta$ 14<sup>NT/WT</sup> mice. FACS analysis with lineage markers and anti-CD117 and anti-CD25 antibodies revealed similar percentages of ETP/DNI and DNII cells in thymuses isolated from wild-type, V $\beta$ 8<sup>Tg</sup>, or V $\beta$ 14<sup>NT/WT</sup> mice (Figure 3A, B). In contrast, we observed significant decreases in the frequencies of DNIII thymocytes in V $\beta$ 8<sup>Tg</sup> and V $\beta$ 14<sup>NT/WT</sup> mice, as compared to in wild-type mice (Figure 3A,B). In addition, the frequencies of CD117<sup>-</sup>CD25<sup>low</sup> cells transitioning between the DNIII and DNIV populations were increased in V $\beta$ 8<sup>Tg</sup> and V $\beta$ 14<sup>NT/WT</sup> mice, as compared to in wild-type mice (Figure 3A). Since V $\beta$ 8<sup>Tg</sup> and V $\beta$ 14<sup>NT/WT</sup> mice contain similar numbers of total thymocytes as wild-type mice, our data indicate that the DNIII to DNIV developmental transition is accelerated in these mice. Similar observations have been found in mice expressing other transgenic V $\beta$ DJ $\beta$  rearrangements and in mice expressing a different endogenous V $\beta$ DJ $\beta$  rearrangement (58,59). Collectively, these observations indicate that the acceleration of developing thymocytes through the DNIII stage is a general phenomenon in mice expressing a pre-assembled TCR $\beta$  chain.

### **Assembly of an in-frame V $\beta$ DJ $\beta$ rearrangement is the rate-limiting step in early $\alpha\beta$ T cell development**

Expression of TCR $\beta$  chains in DNIII thymocytes activates  $\beta$ -selection signaling pathways that promote differentiation into DNIV cells and also promote cyclin D3 expression to stimulate cellular proliferation (14,60). To evaluate whether the accelerated thymocyte development in mice expressing a pre-assembled in-frame V $\beta$ DJ $\beta$  rearrangement is due to enhanced  $\beta$ -selection, we conducted further phenotypic analysis of DN thymocyte populations among wild-type, V $\beta$ 8<sup>Tg</sup>, and V $\beta$ 14<sup>NT/WT</sup> mice. FACS analysis with anti-V $\beta$ 8, and anti-V $\beta$ 14 antibodies demonstrated that V $\beta$ 8 and V $\beta$ 14 TCR $\beta$  chains are expressed on the cell surface of all DNIII thymocytes (Figure 4A). Western blot analysis revealed higher cyclin D3 protein levels in total thymocytes of V $\beta$ 8<sup>Tg</sup> and V $\beta$ 14<sup>NT/WT</sup> mice, as compared to wild-type mice (Figure 4B). In addition, BrdU incorporation experiments demonstrated a greater than two-fold increase in the percentage of S phase cells in the DNIII thymocytes of V $\beta$ 8<sup>Tg</sup> and V $\beta$ 14<sup>NT/WT</sup> mice, as compared to wild-type mice (Figure 4C). These data indicate that  $\beta$ -selection is enhanced in the DNIII thymocyte populations of mice expressing pre-assembled TCR $\beta$  chains, most likely because the time ordinarily required to assemble and express an in-frame endogenous V $\beta$ DJ $\beta$  rearrangement is bypassed. These observations formally demonstrate that the rate-limiting step in  $\beta$  selection is the assembly of an in-frame V $\beta$ DJ $\beta$  rearrangement.

Despite intracellular expression of pre-assembled V $\beta$ DJ $\beta$  rearrangements initiating in bone marrow lymphoid progenitor cells (58), TCR $\beta$  chains are not expressed on the cell surface of developing thymocytes until down-regulation of CD25 correlating with DNII to DNIII differentiation (14). This restricted expression is due to developmental-stage specific

expression of the CD3 subunits required for cell surface expression of pT $\alpha$ -TCR $\beta$  heterodimers. Unexpectedly, we also observed a greater percentage of large and S phase cells in the ETP/DNI and DNII populations of V $\beta$ 8<sup>Tg</sup> and V $\beta$ 14<sup>NT/WT</sup> mice, as compared to wild-type mice (Figure 4C). These findings suggest that pre-assembled TCR $\beta$  chains expressed in the cytoplasm or at undetectable levels on the cell surface of ETP and DNII cells might activate intracellular signaling cascades that regulate cellular size and proliferation. Alternatively, the decreased numbers of DNIII cells in the V $\beta$ 8<sup>Tg</sup> and V $\beta$ 14<sup>NT/WT</sup> mice could create a niche of physical space and/or cytokine exposure that leads to increased proliferation of ETP/DNI and DNII cells. However, further investigation of the molecular or cellular basis this phenomenon is beyond the scope of our current study.

The assembly and expression of TCR $\delta$  and TCR $\gamma$  chains in DNIII thymocytes leads to the development of  $\gamma\delta$  T cells (61). FACS analysis with anti-C $\beta$  and anti-C $\delta$  antibodies revealed significant decreases in the frequencies of  $\gamma\delta$  T cells in the thymuses and spleens of V $\beta$ 8<sup>Tg</sup> and V $\beta$ 14<sup>NT/WT</sup> mice, as compared to in wild-type mice (Figure 4D). Since the numbers of thymocytes and splenocytes are equivalent among mice of the three genotypes, these data indicate that the numbers of  $\gamma\delta$  T cells that develop is reduced in V $\beta$ 8<sup>Tg</sup> and V $\beta$ 14<sup>NT/WT</sup> mice. This observation, which also has been published for mice expressing other pre-assembled transgenic or endogenous TCR $\beta$  chains (58,62,63), indicates that the more efficient  $\beta$ -selection of thymocytes in V $\beta$ 8<sup>Tg</sup> and V $\beta$ 14<sup>NT/WT</sup> mice occurs at the expense of  $\delta$ -selection.

The goal of our current study is to evaluate the effect that TCR $\beta$  mediated feedback signals have upon V $\beta$ 14 recombinational accessibility by analyzing TCR $\beta$  rearrangements in  $\alpha\beta$  T lineage cells of V $\beta$ 14<sup>Rep</sup> mice containing a pre-assembled in-frame V $\beta$ DJ $\beta$  rearrangement. Our experimental approach depends upon expression of pre-assembled TCR $\beta$  chains in developing thymocytes prior to or concurrent with initiation of V $\beta$ 14 recombinational accessibility. We previously detected V $\beta$ 14<sup>Rep</sup> D $\beta$ -to-J $\beta$  rearrangements in DNIII thymocytes, but not in DNII cells, indicating that V $\beta$ 14 recombinational accessibility predominantly initiates in DNIII thymocytes (17). Here, we have shown that  $\beta$ -selection is enhanced and DNIII to DNIV thymocyte differentiation accelerated in V $\beta$ 8<sup>Tg</sup> and V $\beta$ 14<sup>NT/WT</sup> mice due to the premature and invariant expression of the pre-assembled TCR $\beta$  chains in DNIII cells. Collectively, these data demonstrate that the pre-assembled TCR $\beta$  rearrangements used in this study are expressed prior to or concurrent with initiation of V $\beta$ 14 accessibility, thereby validating our experimental approach.

### Expression of pre-assembled TCR $\beta$ chains inhibits endogenous V $\beta$ 14-to-DJ $\beta$ and D $\beta$ -to-J $\beta$ rearrangements

Before analyzing the effects of TCR $\beta$  mediated feedback signals upon modulation of V $\beta$ 14 RAG accessibility, we needed to determine whether expression of the pre-assembled V $\beta$ DJ $\beta$  rearrangements inhibits endogenous V $\beta$ 14-to-DJ $\beta$  rearrangements. To this aim, we established panels of  $\alpha\beta$  T cell hybridomas from V $\beta$ 8<sup>Tg</sup> and V $\beta$ 14<sup>NT/WT</sup> mice and analyzed V $\beta$  rearrangements by Southern blot analysis using a V $\beta$ 14 probe on *Eco*RI-digested genomic DNA isolated from these cells. We found no V $\beta$ 14-to-DJ $\beta$  rearrangements on the wild-type alleles in all 77 V $\beta$ 8<sup>Tg</sup> and all 86 V $\beta$ 14<sup>NT/WT</sup>  $\alpha\beta$  T cell hybridomas (Table I). V $\beta$ 14-to-DJ $\beta$  rearrangements normally occur on non-selected alleles in approximately 7% of wild-type  $\alpha\beta$  T cell hybridomas (17). Consequently, our data demonstrate that expression of either pre-assembled TCR $\beta$  chain prevents V $\beta$ 14-to-DJ $\beta$  rearrangements at the frequency they ordinarily occur in wild-type thymocytes.

We also sought to quantify the effect that expression of the transgenic V $\beta$ 8.2D $\beta$ 1J $\beta$ 1.1 rearrangement or endogenous V $\beta$ 14D $\beta$ 1J $\beta$ 1.4 rearrangement has upon the levels of endogenous D $\beta$ -to-J $\beta$  rearrangements. For this purpose, we conducted Southern blot analysis using an inter-genic D $\beta$ 1-J $\beta$ 1 probe on *Eco*RI-digested genomic DNA isolated from our panels

of  $V\beta 8^{Tg}$  and  $V\beta 14^{NT/WT}$   $\alpha\beta$  T cell hybridomas. We found that 5 of 77 (6%) of  $V\beta 8^{Tg}$  and 5 of 86 (6%)  $V\beta 14^{NT/WT}$  cells contained germline D $\beta$ 1-J $\beta$ 1 segments and, thus, lacked D $\beta$ 1-to-J $\beta$ 1 rearrangements on at least one allele (Table I). In wild-type  $\alpha\beta$  T cells, endogenous D $\beta$ 1-to-J $\beta$ 1 rearrangements occur to completion on both alleles (10,46). Consequently, these data demonstrate that expression of either the transgenic or endogenous pre-assembled  $V\beta$ DJ $\beta$  rearrangement leads to a reduction in the overall frequency of D $\beta$ 1-to-J $\beta$ 1 rearrangements. Importantly, since  $V\beta$ 14-to-DJ $\beta$ 1 rearrangements only occur on alleles that have already assembled DJ $\beta$ 1 complexes (19), these data further demonstrate that the pre-assembled TCR $\beta$  rearrangements used in this study are expressed prior to initiation of  $V\beta$ 14 recombinational accessibility and endogenous  $V\beta$ 14-to-DJ $\beta$ 1 rearrangement.

### **$V\beta$ 14 recombinational accessibility is maintained in thymocytes expressing pre-assembled $V\beta$ DJ $\beta$ rearrangements**

To directly evaluate the effect that TCR $\beta$  mediated feedback signals have upon  $V\beta$ 14 recombinational accessibility, we analyzed  $V\beta$ 14<sup>Rep</sup> rearrangements in DN and DP cells sorted from the thymuses of germline mice containing  $V\beta$ 14<sup>Rep</sup> on one allele and expressing  $V\beta$ 14<sup>NT</sup> from the other allele ( $V\beta$ 14<sup>Rep/NT</sup> mice). PCR with a primer that hybridizes to TCR $\beta$  locus sequences upstream of  $V\beta$ 14<sup>Rep</sup> and a primer that hybridizes to J $\beta$ 1.1 sequences amplifies an 850 bp product from germline  $V\beta$ 14<sup>Rep</sup> alleles and a 200 bp product from alleles with  $V\beta$ 14<sup>Rep</sup> D $\beta$ 1-to-J $\beta$ 1.1 rearrangements (Figure 5A). We observed amplification of the germline and DJ $\beta$  rearranged  $V\beta$ 14<sup>Rep</sup> products to similar extents in  $V\beta$ 14<sup>Rep/WT</sup> and  $V\beta$ 14<sup>Rep/NT</sup> DN thymocytes (Figure 5B), indicating that  $V\beta$ 14<sup>Rep</sup> recombinational accessibility is not down-regulated in DN cells expressing a pre-assembled TCR $\beta$  chain. In DP thymocytes, we observed amplification of the DJ $\beta$  rearranged  $V\beta$ 14<sup>Rep</sup> product to similar levels in  $V\beta$ 14<sup>Rep/WT</sup> and  $V\beta$ 14<sup>Rep/NT</sup> cells, but amplification of the germline  $V\beta$ 14<sup>Rep</sup> product to a lower level in  $V\beta$ 14<sup>Rep/WT</sup> cells as compared to  $V\beta$ 14<sup>Rep/NT</sup> cells (Figure 5B). Considering that  $V\beta$ , D $\beta$ , and J $\beta$  segments rearrange to  $V\beta$ 14<sup>Rep</sup> in approximately 10% of thymocytes (Figure 5C)(17), these data suggest that TCR $\beta$  feedback signals inhibit the rearrangement of endogenous  $V\beta$ , D $\beta$ , and/or J $\beta$  segments to  $V\beta$ 14<sup>Rep</sup>, but do not down-regulate  $V\beta$ 14 recombinational accessibility.

To quantify the effect that TCR $\beta$  mediated feedback signals have upon  $V\beta$ 14 recombinational accessibility and the rearrangement of endogenous  $V\beta$ , D $\beta$ , and/or J $\beta$  segments to  $V\beta$ 14<sup>Rep</sup>, we sought to analyze  $V\beta$ 14<sup>Rep</sup> rearrangements in  $\alpha\beta$  T lineage cells of germline mice containing  $V\beta$ 14<sup>Rep</sup> on one allele and expressing either  $V\beta 8^{Tg}$  or  $V\beta 14^{NT}$  from the homologous chromosome. For this purpose, we bred germline  $V\beta$ 14<sup>Rep/Rep</sup> mice with  $V\beta 8^{Tg}$  mice or  $V\beta 14^{NT/WT}$  mice to generate  $V\beta 8^{Tg}:V\beta 14^{Rep/WT}$  and  $V\beta 14^{Rep/NT}$  mice. Analyses of  $V\beta 8^{Tg}:V\beta 14^{Rep/WT}$  and  $V\beta 14^{Rep/NT}$  mice demonstrated that they exhibited  $\alpha\beta$  T cell development and populations identical to  $V\beta 8^{Tg}$  and  $V\beta 14^{NT/WT}$  mice, respectively (data not shown). To analyze TCR $\beta$  rearrangements, we established panels of  $\alpha\beta$  T cell hybridomas from  $V\beta 14^{Rep/WT}$ ,  $V\beta 8^{Tg}:V\beta 14^{Rep/WT}$ , and  $V\beta 14^{Rep/NT}$  mice. The  $V\beta 14^{Rep}$  allele contains a unique *Eco*RI restriction site that distinguishes between  $V\beta 14^{Rep}$  recombination events and  $V\beta 14$ -to-DJ $\beta$  rearrangements on the  $V\beta 14^{WT}$  allele (17). Thus, we conducted Southern blot analysis using a  $V\beta 14$  probe on *Eco*RI-digested genomic DNA isolated from these cells to analyze  $V\beta 14^{Rep}$  and  $V\beta 14$  rearrangements. Of the 67  $V\beta 14^{Rep/WT}$   $\alpha\beta$  T cell hybridomas analyzed, 47 (73%) contained  $V\beta 14^{Rep}$  recombination events (Table II). This observation is consistent with our earlier findings obtained from chimeric mice that  $V\beta 14$  is RAG accessible in a substantially higher percentage of thymocytes than the approximately 7% in which  $V\beta 14$ -to-DJ $\beta$  rearrangements occur (17,19,64). Unexpectedly, 52 of the 66 (79%)  $V\beta 8^{Tg}:V\beta 14^{Rep/WT}$   $\alpha\beta$  T cell hybridomas and 105 of the 185 (58%)  $V\beta 14^{Rep/NT}$   $\alpha\beta$  T cell hybridomas contained  $V\beta 14^{Rep}$  recombination events (Table II). These data indicate that expression of neither pre-



assembled V $\beta$ DJ $\beta$  rearrangement prevented V $\beta$ 14 recombinational accessibility during thymocyte development.

V $\beta$ 14<sup>Rep</sup> recombination events include V $\beta$ 14<sup>Rep</sup> D $\beta$ -to-J $\beta$  rearrangements that require V $\beta$ 14 recombinational accessibility, and rearrangement of endogenous V $\beta$ , D $\beta$ , and J $\beta$  segments to V $\beta$ 14<sup>Rep</sup> that also require accessibility of the participating TCR $\beta$  gene segments and their juxtaposition with V $\beta$ 14<sup>Rep</sup> (Figure 5C) (17). To quantify the level of each specific V $\beta$ 14<sup>Rep</sup> recombination event in the V $\beta$ 14<sup>Rep/WT</sup>, V $\beta$ 8<sup>Tg</sup>:V $\beta$ 14<sup>Rep/WT</sup>, and V $\beta$ 14<sup>Rep/NT</sup>  $\alpha\beta$  T cell hybridomas, we conducted additional Southern blot analysis using a series of TCR $\beta$  locus probes on *Eco*RI-digested genomic DNA isolated from these cells. The V $\beta$ 14<sup>Rep</sup> allele lacks endogenous D $\beta$ 2-J $\beta$ 2 segments (17), which limits the number of possible recombination events and simplifies analyses (10). We also confirmed the identity of rearrangements by sequence analysis of PCR amplified joins (data not shown). Of the 67 V $\beta$ 14<sup>Rep/WT</sup>  $\alpha\beta$  T cell hybridomas analyzed, 36 (54%) contained V $\beta$ 14<sup>Rep</sup> D $\beta$ -to-J $\beta$  rearrangements, seven (10%) harbored endogenous D $\beta$  or J $\beta$  segments rearranged to V $\beta$ 14<sup>Rep</sup>, and four (6%) carried endogenous V $\beta$  segments rearranged to V $\beta$ 14<sup>Rep</sup> (Table II). These data are consistent with our previous observations from chimeric mice that revealed RAG accessible V $\beta$ 14 segments are juxtaposed with RAG accessible V $\beta$ , D $\beta$ , and J $\beta$  segments in a greater percentage of thymocytes than the 7% in which V $\beta$ 14-to-DJ $\beta$  rearrangements normally occur (17, 19, 64). Of the 66 V $\beta$ 8<sup>Tg</sup>:V $\beta$ 14<sup>Rep/WT</sup>  $\alpha\beta$  T cell hybridomas analyzed, 37 (56%) contained V $\beta$ 14<sup>Rep</sup> D $\beta$ -to-J $\beta$  rearrangements, seven (11%) harbored endogenous D $\beta$  or J $\beta$  segments rearranged to V $\beta$ 14<sup>Rep</sup>, and eight (12%) carried endogenous V $\beta$  segments rearranged to V $\beta$ 14<sup>Rep</sup> (Table II). However, of the 185 V $\beta$ 14<sup>Rep/NT</sup>  $\alpha\beta$  T cell hybridomas analyzed, 82 (44%) contained V $\beta$ 14<sup>Rep</sup> D $\beta$ -to-J $\beta$  rearrangements, 19 (10%) harbored endogenous D $\beta$  or J $\beta$  segments rearranged to V $\beta$ 14<sup>Rep</sup>, and four (2%) carried endogenous V $\beta$  segments rearranged to V $\beta$ 14<sup>Rep</sup> (Table II).

These data reveal that the predominant manner by which expression of the endogenous V $\beta$ 14D $\beta$ 1J $\beta$ 1.4 rearrangement decreased V $\beta$ 14<sup>Rep</sup> recombination events is through the reduction of V $\beta$ 14<sup>Rep</sup> D $\beta$ -to-J $\beta$  rearrangements, which require V $\beta$ 14 recombinational accessibility but not necessarily juxtaposition of V $\beta$ 14<sup>Rep</sup> with endogenous D $\beta$ -J $\beta$  segments. The expression of either pre-assembled TCR $\beta$  chain also had no effect upon the level at which endogenous D $\beta$ -J $\beta$  segments rearranged with V $\beta$ 14<sup>Rep</sup>. For reasons detailed in the discussion, our collective data are consistent with the notion that TCR $\beta$  mediated feedback inhibition of V $\beta$ 14-to-DJ $\beta$  rearrangements depends upon the inherent properties of V $\beta$ 14, D $\beta$ , and J $\beta$  RSs.

## Discussion

Here, we have analyzed the effect that expression of a classical TCR $\beta$  transgene (V $\beta$ 8<sup>Tg</sup>) or a pre-assembled endogenous TCR $\beta$  gene (V $\beta$ 14<sup>NT</sup>) has upon recombinational accessibility of V $\beta$ 14 chromatin. We first showed that greater than 99% of  $\alpha\beta$  T lineage cells isolated from V $\beta$ 8<sup>Tg</sup> or V $\beta$ 14<sup>NT/WT</sup> mice expressed V $\beta$ 8 or V $\beta$ 14, respectively, as part of their cell surface TCR $\beta$  chains. We then demonstrated that expression of each pre-assembled TCR $\beta$  chain accelerated the DNIII to DNIV step of early thymocyte development due to enhanced  $\beta$ -selection in the DNIII population. Our analyses of TCR $\beta$  rearrangements revealed that expression of these pre-assembled V $\beta$ DJ $\beta$  rearrangements inhibited endogenous V $\beta$ 14-to-DJ $\beta$  rearrangements as expected. However, in contrast to results predicted by the accepted model of TCR $\beta$  feedback inhibition, we found that expression of these pre-assembled TCR $\beta$  chains neither blocked recombinational accessibility of V $\beta$ 14 chromatin nor inhibited rearrangements between V $\beta$ 14<sup>Rep</sup> and endogenous D $\beta$ -J $\beta$  segments.

One concern related to our study is that the V $\beta$ 14<sup>Rep</sup> V(D)J recombination reporter might alter recombinational accessibility of V $\beta$ 14 chromatin. Although we have previously proved that

local chromatin environment imparts lineage and developmental-stage specific recombinational accessibility upon  $V\beta 14^{Rep}$  (17), it is conceivable that replacement of the  $V\beta 14$  RS with  $V\beta 14^{Rep}$  increases the frequency at which  $V\beta 14$  chromatin is rendered recombinationally accessible and the RSs within this region available for RAG binding (17). However, the frequency of  $V\beta 14^{Rep}$  recombination events is similar to the frequency of  $V\beta 14$  rearrangements on alleles containing specific replacement of the  $V\beta 14$  RS with the 3'D $\beta 1$  RS (17,19), demonstrating that intrinsic properties of the 5'D $\beta 1$  and J $\beta 1.1$  RSs have negligible ability to promote  $V\beta 14$  recombinational accessibility. The ability of the 3'D $\beta 1$  RS to recombine with J $\beta$  RSs, and by extension potential c-fos mediated RAG deposition, requires prior recombinational accessibility of the 3'D $\beta 1$  RS. In this context, gene-targeted insertion of the 3'D $\beta 1$  RS into  $V\beta 14$  chromatin would be expected to increase the frequency of RAG binding without affecting  $V\beta 14$  recombinational accessibility (19), which was our logic behind using  $V\beta 14^{Rep}$  to quantify  $V\beta 14$  recombinational accessibility independent of  $V\beta 14$  synapsis/cleavage with endogenous D $\beta$ -J $\beta$  segments (17).

Recombinational accessibility involves removal of nucleosomes from RSs (65–69). Consensus RSs repress recombinational accessibility *in vivo* by positioning nucleosomes over themselves, while RSs that lack a consensus nonamer fail to position nucleosomes and exhibit higher rearrangement potential (66). Considering that the 3'D $\beta 1$  RS, but not the  $V\beta 14$  RS, contains a consensus nonamer, it seems unlikely that intrinsic properties of the 3'D $\beta 1$  RS would position nucleosomes in a manner that promotes recombinational accessibility more so than the  $V\beta 14$  RS. Thus, we interpret our findings within this and prior studies to accurately reflect  $V\beta 14$  recombinational accessibility.

Our findings here and before (17) that endogenous  $V\beta$  segments can rearrange directly to  $V\beta 14^{Rep}$  D $\beta 1$  segments that have not recombined with  $V\beta 14^{Rep}$  J $\beta 1.1$  segments has important implications for mechanisms that direct ordered TCR $\beta$  gene rearrangements. We had previously demonstrated that the assembly of endogenous TCR $\beta$  variable region exons through DJ $\beta$  intermediates is not driven solely by intrinsic properties of D $\beta$ /J $\beta$  versus  $V\beta$ /D $\beta$  RSSs, indicating that chromosomal factors such as the location or distance between participating gene segments influences their recombination *in vivo* (19). Since then two groups have shown *in vitro* and *in vivo* that RAG proteins bind to 3'D $\beta$  RSs and hinder RAG binding to the adjacent 5'D $\beta$  RSs until deletion of the 3'D $\beta$  RS RAG binding site through D $\beta$ -to-J $\beta$  rearrangement (11,70). One group also demonstrated that c-fos binds 3'D $\beta$  RSs and recruits the RAG proteins, which may help suppress  $V\beta$  rearrangements *in vivo* prior to D $\beta$ -to-J $\beta$  rearrangement (11). Notably, each of these *in vivo* studies was conducted with endogenous TCR $\beta$  loci or chromosomally-integrated TCR $\beta$  mini-loci in which the 3'D $\beta$  RSs were in their native location relative to the germline D $\beta$  promoters that function with the TCR $\beta$  (or heterologous) enhancer to direct D $\beta$ -J $\beta$  recombinational accessibility. In contrast, our *in vivo* study analyzes TCR $\beta$  rearrangements involving D $\beta 1$ -J $\beta 1.1$  genomic sequences outside of their native location. Our data reveal that completion of D $\beta$ -to-J $\beta$  recombination before  $V\beta$ -to-DJ $\beta$  rearrangement depends upon the chromosomal location of D $\beta 1$ -J $\beta 1.1$  and reveals that neither c-fos dependent nor c-fos independent RAG deposition upon 3'D $\beta$  RSs are sufficient to direct ordered TCR $\beta$  rearrangements. Thus, the location of D $\beta$ -J $\beta$  segments relative to their germline D $\beta$  promoters and/or the TCR $\beta$  enhancer may be critical for directing the assembly of endogenous TCR $\beta$  variable region exons through DJ $\beta$  intermediates.

The observation that expression of transgenic pre-assembled  $V\beta$ DJ $\beta$  rearrangements prevented endogenous  $V\beta$ -to-DJ $\beta$  rearrangements, but not D $\beta$ -to-J $\beta$  rearrangements, led to the hypothesis that TCR $\beta$  mediated feedback signals inhibit recombinational accessibility of  $V\beta$  segments (47,71). Consistent with this notion, TCR $\beta$  mediated down-regulation of E47 transcription factor binding to the locus leads to decreased  $V\beta$  chromatin accessibility in DN cells (29). However, a clear mechanistic link between recombinational accessibility and general

chromatin accessibility or transcriptional accessibility is lacking for V $\beta$  segments. Our study, which directly investigates the effect that TCR $\beta$  mediated feedback signals have upon V $\beta$  recombinational accessibility *in vivo*, reveals that RAG access to V $\beta$ 14 chromatin is maintained in thymocytes expressing pre-assembled V $\beta$ DJ $\beta$  rearrangements. It had previously been demonstrated that germline V $\beta$ 14 segments remain recombinationally accessible in DP thymocytes expressing a classical TCR $\beta$  transgene and that DP cells attempting V $\beta$ 14-to-DJ $\beta$  rearrangements are eliminated by apoptosis to maintain TCR $\beta$  allelic exclusion (33). Yet, the mechanisms by which TCR $\beta$  mediated feedback signals inhibit V $\beta$ 14 rearrangements in DN cells were never explored. As discussed below, our current observation indicates that TCR $\beta$ -mediated feedback signals must inhibit endogenous V $\beta$ 14-to-DJ $\beta$  rearrangements in DN thymocytes through mechanisms other than down-regulation of RAG access to V $\beta$ 14 chromatin.

The physiological relevance of conclusions reached from studies of TCR $\beta$  feedback regulation using mice that express transgenic V $\beta$ DJ $\beta$  rearrangements has been questioned due to their accelerated early thymocyte development (28). Rapid progression through the DNIII stage could be due to aberrantly high and/or premature expression of TCR $\beta$  chains driven by multiple integrated copies of the transgenic V $\beta$ DJ $\beta$  rearrangement (28,47). Such accelerated development could prevent normal initiation of V $\beta$  recombinational accessibility or TCR $\beta$  locus looping. We have shown here that  $\beta$ -selection is enhanced in the DNIII thymocyte populations of mice containing pre-assembled V $\beta$ DJ $\beta$  rearrangements due to the premature and invariant expression of TCR $\beta$  chains, even those expressed from a single copy of an endogenous V $\beta$ 14D $\beta$ 1J $\beta$ 1.4 rearrangement. However, expression of either pre-assembled V $\beta$ DJ $\beta$  rearrangements prior to initiation of endogenous V $\beta$ 14-to-DJ $\beta$  rearrangements enabled us evaluate the effect that TCR $\beta$  mediated feedback signals have upon V $\beta$ 14 recombinational accessibility. Despite the five-fold decrease in the numbers of DNIII cells where V $\beta$ -to-DJ $\beta$  rearrangements occur, the overall level of V $\beta$ 14 recombinational accessibility in DN cells was maintained in cells expressing the TCR $\beta$  transgene or the endogenous V $\beta$ 14D $\beta$ 1J $\beta$ 1.4 rearrangement. These data indicate that the initiation of V $\beta$ 14 recombinational accessibility occurs rapidly, efficiently, and on both TCR $\beta$  alleles in the majority of thymocytes as they differentiate into the DNIII stage. Whether these V $\beta$ 14 segments remain continually accessible until  $\beta$ -selection/death or cycle through periods of open and shut chromatin conformations, perhaps mediated by repositioning between euchromatin and heterochromatin, remains to be determined.

Why does expression of neither pre-assembled TCR $\beta$  chain lower the level of rearrangements between V $\beta$ 14<sup>Rep</sup> and endogenous D $\beta$ 1-J $\beta$ 1 segments? TCR $\beta$  locus RS beyond 12/23 joining restrictions mediated by RAG-RS interactions enable functional synapses between 3'D $\beta$ /J $\beta$ , 3'D $\beta$ /5'D $\beta$ , and V $\beta$ /5'D $\beta$  RSs, but not between V $\beta$ /J $\beta$  RSs (10,42,72–74). These RS constraints permit only a single rearrangement between endogenous D $\beta$ 1-J $\beta$ 1 and V $\beta$ 14 segments on wild-type TCR $\beta$  alleles. Replacement of the V $\beta$ 14 RS with 5'D $\beta$ 1, 3'D $\beta$ 1, and J $\beta$ 1.1 RSs on the V $\beta$ 14<sup>Rep</sup> allele enables nine possible recombination events between V $\beta$ 14<sup>Rep</sup> and the endogenous D $\beta$ 1-J $\beta$ 1 segments. The *in vitro* recombination efficiency of each of the nine functional RS pairs on the V $\beta$ 14<sup>Rep</sup> allele is higher than that of the single compatible V $\beta$ 14/5'D $\beta$ 1 RS pair on the wild-type allele (42). In this context, the overall efficiency of recombination between V $\beta$ 14<sup>Rep</sup> and endogenous D $\beta$ -J $\beta$  segments on the V $\beta$ 14<sup>Rep</sup> allele would be at least one order of magnitude greater than the rate of V $\beta$ 14-to-DJ $\beta$  rearrangement on the wild-type allele. Thus, one interpretation of our finding that expression of neither pre-assembled TCR $\beta$  chain inhibits rearrangements between V $\beta$ 14<sup>Rep</sup> and endogenous D $\beta$ 1-J $\beta$ 1 segments is that recombination events between these segments occur at such high efficiency that they are refractory to TCR $\beta$  mediated feedback inhibition. Due to the orientation and location of the V $\beta$ 14<sup>Rep</sup> 3'D $\beta$ 1 RS inserted in place of the V $\beta$ 14 RS, rearrangements between V $\beta$ 14<sup>Rep</sup> and endogenous D $\beta$ -J $\beta$  segments cannot assemble V $\beta$ 14(D)J $\beta$  exons that encode

functional TCR $\beta$  chains. Consequently, analysis of V $\beta$ 14-to-DJ $\beta$  rearrangements and V $\beta$ 14 expression in  $\alpha\beta$  T cells containing specific replacement of the endogenous V $\beta$ 14 RS with the 3'D $\beta$ 1 RS is required to determine whether TCR $\beta$  mediated feedback inhibition of V $\beta$ 14-to-DJ $\beta$  rearrangements, and possibly V $\beta$ 14 allelic exclusion, depends upon the inherent properties of V $\beta$ 14, D $\beta$ , and J $\beta$  RSs.

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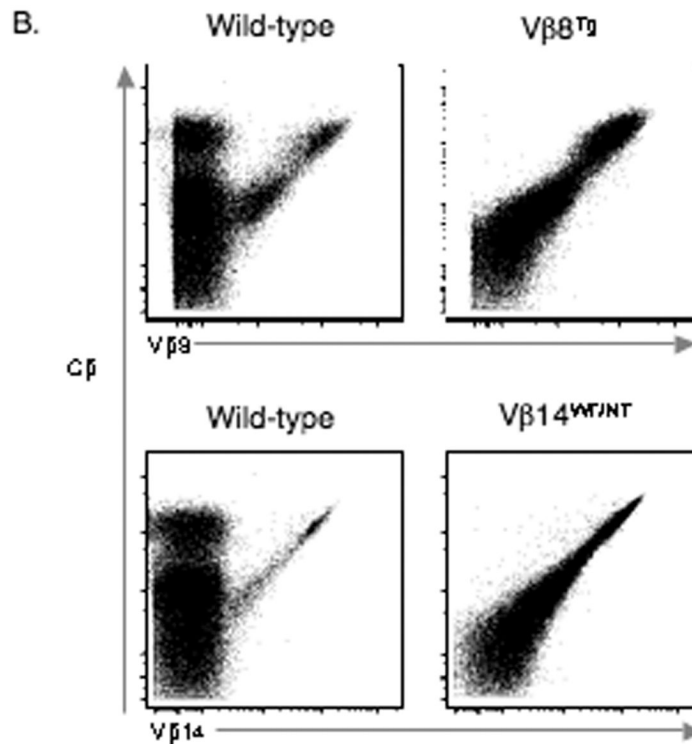
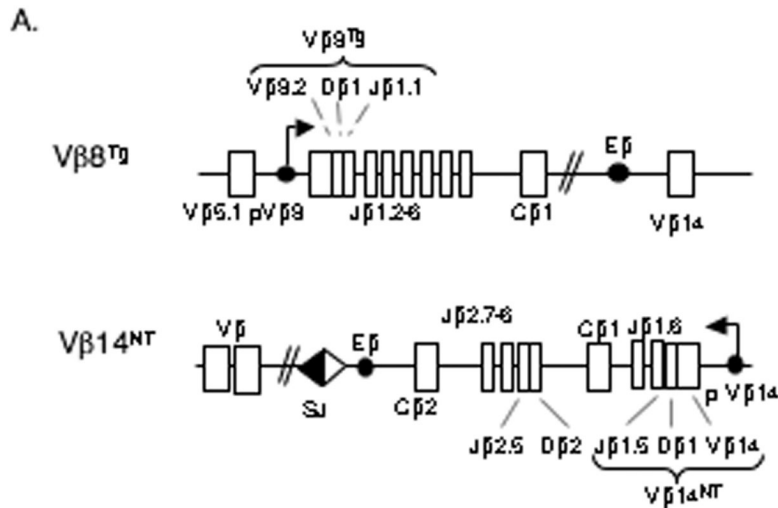
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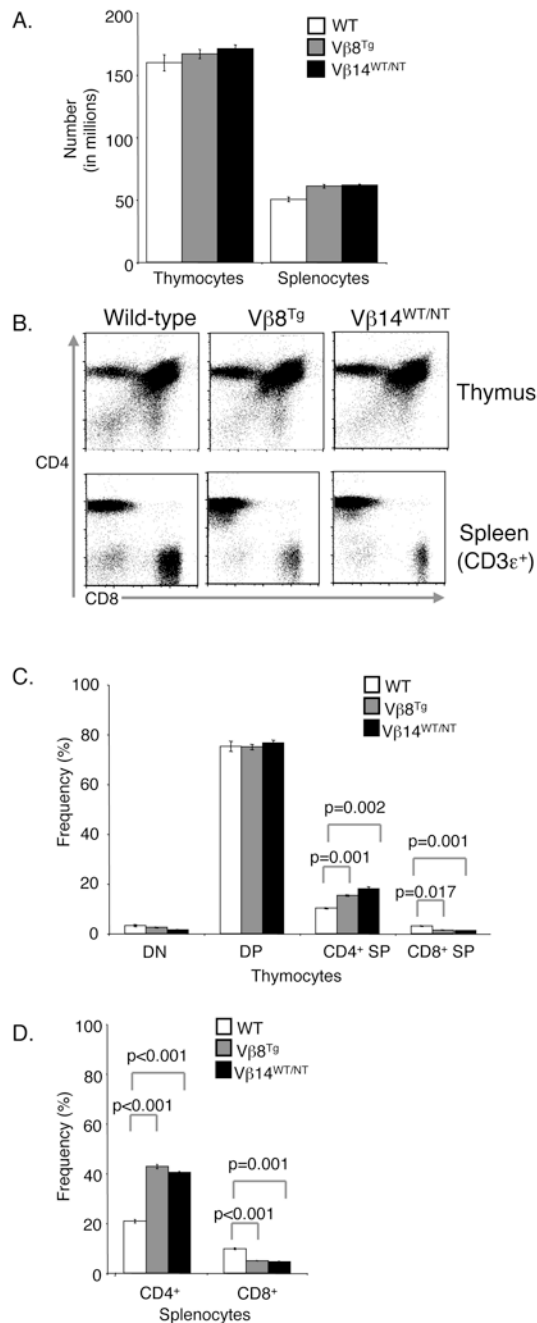
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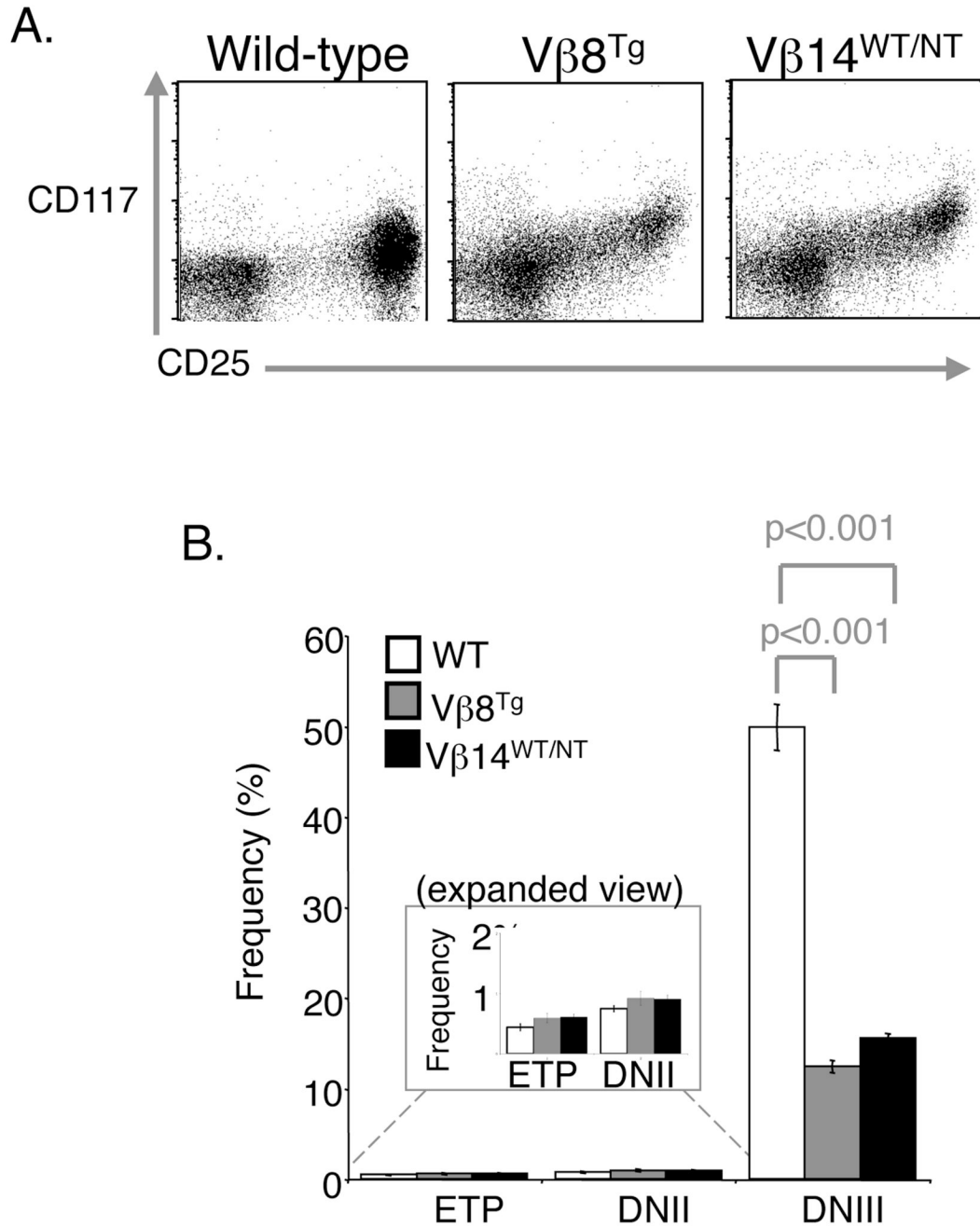
**Figure 1.  $V\beta 8^{Tg}$  and  $V\beta 14^{NT/WT}$  mice exhibit TCR $\beta$  allelic exclusion**

(A) Schematic representation of the transgenic  $V\beta 8^{Tg}$  and endogenous  $V\beta 14^{NT}$  loci. Open boxes depict  $V\beta$ ,  $D\beta$ ,  $J\beta$ , and  $C\beta$  segments. Closed circles depict the TCR $\beta$  enhancer ( $E\beta$ ) and the promoters of the pre-assembled  $V\beta D\beta J\beta$  rearrangements, with arrows indicating the direction of sense  $V\beta D\beta J\beta$  transcripts. Triangles show the 5'D $\beta 1/V\beta 14$  RSS join. (B) Shown are representative anti-C $\beta$ , by anti-V $\beta 8$  or anti-V $\beta 14$  FACS analysis of cells isolated from the thymuses and spleens of wild-type,  $V\beta 8^{Tg}$ , and  $V\beta 14^{NT/WT}$  mice.



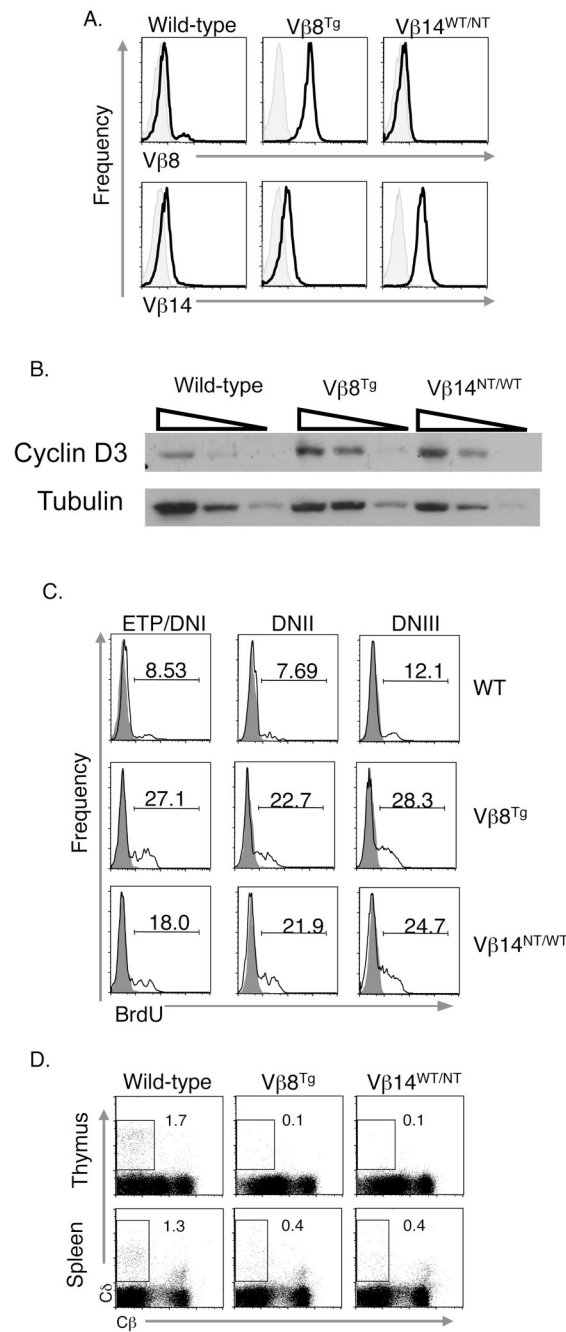
**Figure 2. Grossly normal  $\alpha\beta$  T cell development in  $V\beta 8^{Tg}$  and  $V\beta 14^{NT/WT}$  mice**

(A) Bar graphs showing the average number of thymocytes and splenocytes from at least five mice of each genotype. The error bars are standard error of the mean. (B) Shown are representative anti-CD4 and anti-CD8 FACS analysis of cells isolated from the thymuses and spleens of wild-type,  $V\beta 8^{Tg}$ , and  $V\beta 14^{NT/WT}$  mice. The percentage of DN, DP, CD4<sup>+</sup> SP, and CD8<sup>+</sup> SP thymocytes and CD4<sup>+</sup> and CD8<sup>+</sup>  $\alpha\beta$  T cells is depicted. (C–D) Bar graphs showing the average frequency of (C) DN, DP, CD4<sup>+</sup> SP, and CD8<sup>+</sup> SP thymocytes and (D) CD4<sup>+</sup> and CD8<sup>+</sup>  $\alpha\beta$  T cells from at least five mice of each genotype. The error bars are standard error of the mean. Significant differences have been calculated using a two-tailed Student's t-test.



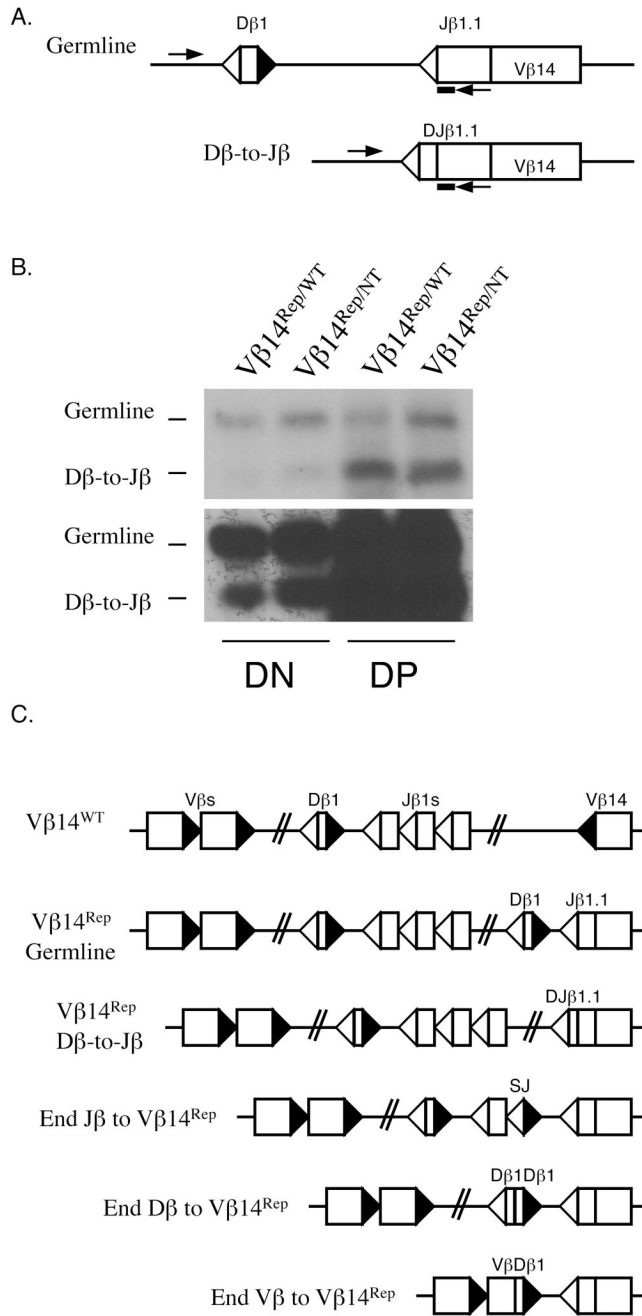
**Figure 3. Accelerated early thymocyte development in Vβ8<sup>Tg</sup> and Vβ14<sup>NT/WT</sup> mice**

(A) Shown are representative anti-CD117 and anti-CD25 FACS analysis of thymocytes gated on cells negative for mature cells markers (TCRβ, TCRδ, CD4, CD8α, CD19, CD11c, CD11b, B220 and NK1.1). (B) Bar graphs showing the average frequency of ETP, DNII, DNIII, DNIII/DNIV, and DNIV thymocytes at least five mice of each genotype. The error bars are standard error of the mean. Significant differences have been calculated using a two-tailed Student's t-test.



**Figure 4. β-selection is enhanced in Vβ8<sup>Tg</sup> and Vβ14<sup>NT/WT</sup> mice**

(A) Shown are representative histograms of anti-Vβ8 and anti-Vβ14 FACS analysis of DNIII thymocytes isolated from wild-type, Vβ8<sup>Tg</sup>, and Vβ14<sup>NT/WT</sup> mice. (B) Shown are representative Western blot analysis of cyclin D3 and tubulin protein expression in total thymocytes of wild-type, Vβ8<sup>Tg</sup>, and Vβ14<sup>NT/WT</sup> mice. (C) Shown are representative BrdU FACS analysis of ETP, DNII, DNIII, and DNIV thymocytes of wild-type, Vβ8<sup>Tg</sup>, and Vβ14<sup>NT/WT</sup> mice. The percentage of BrdU<sup>+</sup> cells is indicated. (D) Shown are representative anti-Cδ and anti-Cβ FACS analysis of cells isolated from the thymuses and spleens of wild-type, Vβ8<sup>Tg</sup>, and Vβ14<sup>NT/WT</sup> mice. The percentage of Cδ<sup>+</sup> T cells is depicted.



**Figure 5. V $\beta$ 14 recombinational accessibility is maintained in thymocytes expressing a pre-assembled TCR $\beta$  gene**

(A) Schematic representation of germline and DJ $\beta$  rearranged V $\beta$ 14<sup>Rep</sup> alleles indicating the positions of the PCR primers and probe used analysis of V $\beta$ 14<sup>Rep</sup> D $\beta$ -to-J $\beta$  recombination events. The sizes of PCR products amplified from germline and DJ $\beta$  rearranged V $\beta$ 14<sup>Rep</sup> alleles are indicated. (B) PCR analysis of V $\beta$ 14<sup>Rep</sup> D $\beta$ -to-J $\beta$  recombination events conducted on equal amounts of genomic DNA isolated from sort-purified DN and DP thymocytes of V $\beta$ 14<sup>Rep</sup>/WT and V $\beta$ 14<sup>Rep</sup>/NT mice. Shown are short and long exposures with the PCR product identities indicated. (C) Schematic representations of germline wild-type TCR $\beta$  (V $\beta$ 14<sup>WT</sup>) and V $\beta$ 14<sup>Rep</sup> loci, as well as the configurations of V $\beta$ 14<sup>Rep</sup> alleles that have undergone V $\beta$ 14<sup>Rep</sup>

D $\beta$ -to-J $\beta$  rearrangements or the rearrangement of endogenous (End) V $\beta$ , D $\beta$ , or J $\beta$  segments to the V $\beta$ 14<sup>Rep</sup> D $\beta$ 1 segment. Open boxes depict V $\beta$ , D $\beta$ , J $\beta$ , and C $\beta$  segments. Black triangles indicate 23-RSs and open triangles indicate 12-RSs. Also labeled are the signal join (SJ) formed during endogenous J $\beta$  rearrangements to V $\beta$ 14<sup>Rep</sup>, the D $\beta$ 1-D $\beta$ 1 coding join formed during endogenous D $\beta$  rearrangements to the V $\beta$ 14<sup>Rep</sup> D $\beta$ 1 segment, and the V $\beta$ 1-D $\beta$ 1 coding join formed during endogenous V $\beta$  rearrangements to the V $\beta$ 14<sup>Rep</sup> D $\beta$ 1 segment.

**Table I**

<i>A. Endogenous V<math>\beta</math>14 rearrangements in V<math>\beta</math>8<sup>Tg</sup> and V<math>\beta</math>14<sup>NT/WT</sup> <math>\alpha\beta</math> T cell hybridomas</i>			
<u>Genotype</u>	<u>Total Number Cells</u>	<u>V<math>\beta</math>14D<math>\beta</math>1J<math>\beta</math>1 Rearrangements</u>	
V $\beta$ 8 <sup>Tg</sup>	77	0 (0%)	
V $\beta$ 14 <sup>NT/WT</sup>	86	0 (0%)	
<i>B. Endogenous D<math>\beta</math>1-J<math>\beta</math>1 rearrangements in V<math>\beta</math>8<sup>Tg</sup> and V<math>\beta</math>14<sup>NT/WT</sup> <math>\alpha\beta</math> T cell hybridomas</i>			
<u>Genotype</u>	<u>Total Number Cells</u>	<u>D<math>\beta</math>1-J<math>\beta</math>1 Germline</u>	<u>D<math>\beta</math>1J<math>\beta</math>1 Rearranged</u>
V $\beta$ 8 <sup>Tg</sup>	77	5 (6%)	72 (94%)
V $\beta$ 14 <sup>NT/WT</sup>	86	5 (6%)	81 (94%)

**Table II**

A.  $V\beta 14^{Rep}$  rearrangements in  $V\beta 14^{Rep/WT}$ ,  $V\beta 8^{Ts}:V\beta 14^{NT/WT}$ , and  $V\beta 14^{Rep/NT}$   $\alpha\beta$  T cell hybridomas

Genotype	Total Number Cells	$V\beta 14^{Rep}$ Rearrangements
$V\beta 14^{Rep/WT}$	67	47 (73%)
$V\beta 8^{Ts}:V\beta 14^{Rep/WT}$	66	52 (79%)
$V\beta 14^{Rep/NT}$	185	105 (58%)

B.  $V\beta 14^{Rep}$  recombination events in  $V\beta 14^{Rep/WT}$ ,  $V\beta 8^{Ts}:V\beta 14^{NT/WT}$ , and  $V\beta 14^{Rep/NT}$   $\alpha\beta$  T cell hybridomas

Genotype	Total Number Cells	$V\beta 14^{Rep}$ Recombination Events	
$V\beta 14^{Rep/WT}$	67	$V\beta 14^{Rep}$ D $\beta$ -to-J $\beta$	36 (54%)
		D $\beta$ -J $\beta$ to $V\beta 14^{Rep}$	7 (10%)
		V $\beta$ to $V\beta 14^{Rep}$	4 (6%)
$V\beta 8^{Ts}:V\beta 14^{Rep/WT}$	66	$V\beta 14^{Rep}$ D $\beta$ -to-J $\beta$	37 (56%)
		D $\beta$ -J $\beta$ to $V\beta 14^{Rep}$	7 (11%)
		V $\beta$ to $V\beta 14^{Rep}$	8 (12%)
$V\beta 14^{Rep/NT}$	185	$V\beta 14^{Rep}$ D $\beta$ -to-J $\beta$	82 (44%)
		D $\beta$ -J $\beta$ to $V\beta 14^{Rep}$	19 (10%)
		V $\beta$ to $V\beta 14^{Rep}$	4 (2%)