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TCRβ Feedback Signals Inhibit the Coupling of Recombinationally Accessible Vβ14 Segments with DJβ 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 Vβ14 ($V\beta14^{Rep}$) indicated that Vβ14 chromatin accessibility is bi-allelic. To determine whether Vβ14 recombinational accessibility is subject to feedback inhibition, we analyzed TCRβ rearrangements in $V\beta14^{Rep}$ mice containing a pre-assembled in frame transgenic Vβ8.2Dβ1Jβ1.1 or an endogenous Vβ14Dβ1Jβ1.4 rearrangement on the homologous chromosome. Expression of either pre-assembled VβDJβCβ chain accelerated thymocyte development due to enhanced cellular selection, demonstrating that the rate-limiting step in early αβ T cell development is the assembly of an in-frame VβDJβ rearrangement. Expression of these pre-assembled VβDJβ rearrangements inhibited endogenous Vβ14-to-DJβ rearrangements as expected. However, in contrast to results predicted by the accepted model of TCRβ feedback inhibition, we found that expression of these pre-assembled TCRβ chains did not down-regulate recombinational accessibility of Vβ14 chromatin. Our findings suggest that TCRβ mediated feedback inhibition of Vβ14 rearrangements depends upon inherent properties of Vβ14, Dβ, and Jβ 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 assembles and expresses 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 β and α variable region exons is regulated within the context of thymocyte development (6,14). TCRβ genes recombine in an ordered fashion with Dβ-to-Jβ rearrangement occurring before the joining of a Vβ segment to an assembled DJβ complex on one allele in $CD4^{-}/CD8^{-}$ (double negative, DN) thymocytes (15,16). Vβ-to-DJβ rearrangements proceed through the coupling of RAG accessible Vβ segments and DJβ complexes (17–19), likely mediated by TCRβ locus contraction and chromosome looping that juxtapose Vβ and Dβ-Jβ-Cβ segments (13). Due to imprecise joining, approximately one-third of VβDJβ rearrangements is assembled productively (in-frame) and encodes a functional TCRβ chain. TCRβ chains pair with pTα molecules to form pre-TCR complexes that signal β-selection, which involves allelic exclusion, survival, expansion, and further differentiation to the $CD4^{+}/CD8^{+}$ (double positive, DP) stage (14). In cells that assemble a non-productive (out-of-frame) VβDJβ rearrangement on the first allele, Vβ-to-DJβ recombination can initiate on the second allele, which, if productive, signals survival, expansion and differentiation (14). TCRα variable region exons are assembled from Vα and Jα segments in DP thymocytes (20). Productive VαJα rearrangements generate TCRα chains that can associate with TCRβ chains to form αβ TCRs, which, upon positive selection, promote further differentiation to $CD4^{+}$ or $CD8^{+}$ (single positive, SP) thymocytes (14). These cells exit the thymus and migrate to the peripheral lymphoid compartments as naive αβ 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 Vβ rearrangements initiate asynchronously in DN thymocytes have not been demonstrated, but several have been proposed from experimental observations. The asynchronous replication of TCRβ loci may promote epigenetic changes that render the earlier replicating allele preferentially accessible to RAG (24). In addition, the association of TCRβ alleles with repressive nuclear environments could limit recombinational accessibility to one allele in the time-window during which newly assembled VβDJβ rearrangements can be expressed and signal (12,13). Moreover, in DN cells exhibiting bi-allelic Vβ chromatin accessibility (17,

25), the inherent low recombination potential of Vβ RSSs might limit Vβ-to-DJβ rearrangements to one allele at a time (9).

Expression of productive TCRβ chains in DN thymocytes activates distinct signaling pathways for preventing Vβ-to-DJβ rearrangements and for promoting proliferation, expansion, and differentiation (26–28). TCRβ mediated down-regulation of E47 transcription factor binding to the locus leads to decreased Vβ chromatin accessibility in DN cells (29). DN to DP differentiation leads to epigenetic changes in Vβ chromatin that render Vβ segments inaccessible to RAG (30,31), and de-contraction of TCRβ loci such that germline Vβ segments are no longer juxtaposed with Dβ-Jβ segments (13). These latter two mechanisms may cooperate to maintain TCRβ allelic exclusion by preventing the rearrangement of germline upstream Vβ segments to DJβ complexes in DP cells (12,13). Unique among Vβ segments, germline Vβ14 segments remain recombinationally accessible in DP thymocytes (32) and DP cells attempting Vβ14-to-DJβ rearrangements can be eliminated by apoptosis to maintain TCRβ allelic exclusion (33). However, the mechanisms by which TCRβ mediated feedback signals inhibit the rearrangement of Vβ14 and other Vβ 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 Vβ rearrangement, we previously generated and analyzed mice with replacement of the endogenous Vβ14 RS with a V(D)J recombination reporter consisting of germline Dβ1-Jβ1.1 segments (the $V\beta14^{Rep}$ allele)(17). Since Dβ1-to-Jβ1.1 rearrangement depends upon promoter-driven recombinational accessibility of Dβ-Jβ chromatin (38,39), we hypothesized $V\beta14^{Rep}$ Dβ1-to-Jβ1.1 rearrangements would occur only if the Vβ14 promoter and other potential *cis* elements rendered Vβ14 chromatin accessible to the RAG proteins. We proved that Vβ14 chromatin environment imparts lineage and developmental-stage specific recombinational accessibility upon $V\beta14^{Rep}$ (17). Notably, despite the presence of a functional TATA box in the 5'Dβ1 RS (40) and the influence of the Jβ1.1 RS upon steady-state Dβ1-Jβ1.1 transcripts (41), the frequency of $V\beta14^{Rep}$ recombination events was similar to the frequency of Vβ14 rearrangements on alleles containing specific replacement of the Vβ14 RS with the 3'Dβ1 RS (19). Collectively, these observations indicated that the higher intrinsic recombination potential of the 3'Dβ1 RS compared to the Vβ14 RS (42), and likely the ability of the 3'Dβ1 RS to bind c-fos/RAG complexes (11), enables the minimal frequency at which Vβ14 chromatin is rendered accessible and RSs within this region available for RAG binding to be quantified by $V\beta14^{Rep}$ rearrangement events (17).

Unexpectedly, we found that $V\beta14^{Rep}$ Dβ-to-Jβ recombination occurred on both TCRβ alleles in the majority of developing thymocytes, demonstrating that regulation of Vβ14 recombinational accessibility and Vβ14-to-DJβ rearrangements are not mechanistically linked (17). These data also could indicate that Vβ14 recombinational accessibility is not subject to TCRβ mediated feedback inhibition. Alternatively, $V\beta14^{Rep}$ may simply rearrange efficiently and on both alleles during the time window required for the assembly and expression of VβDJβ rearrangements to signal inhibition of Vβ14 accessibility. To distinguish between these possibilities and determine whether undiscovered mechanisms might contribute to inhibition of Vβ14 rearrangements, we sought to directly evaluate the effect that TCRβ mediated feedback signals have upon Vβ14 recombinational accessibility by analyzing TCRβ rearrangements in αβ T lineage cells of $V\beta14^{Rep}$ mice that express an in-frame VβDJβ rearrangement prior to initiation of Vβ14 accessibility.

Materials and Methods

Mice

Generation and characterization of DO11.10 TCRβ transgenic mice (43) and Vβ14$^{Rep/Rep}$ mice (17) were previously described. Generation and characterization of the LN2 embryonic stem cells containing the pre-assembled Vβ14Dβ1Jβ1.4 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 αβ 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-Vβ8, or anti-Vβ14 antibodies and PE-conjugated anti-CD4 or anti-Cβ antibodies (BD Pharmingen). To analyze DN thymocyte populations, cells were stained with a cocktail of PE-conjugated anti-Cβ, anti-Cδ, 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 β 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 μL 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 20×10^6 cells in 50 μL 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 Vβ14^{Rep} and TCRβ rearrangements

Cell sorting of DN and DP thymocytes was conducted with a MoFlow cell sorter (DakoCytomation) following staining of thymocytes with FITC-conjugated anti-CD8α and PE-conjugated anti-CD4. PCR analysis of Vβ14^{Rep} Dβ-to-Jβ rearrangements were conducted using 20 ng of genomic DNA as previously described (17). αβ T cell hybridomas were generated as previously described (45). Genomic DNA was isolated, digested with *Eco*RI, and subject to Southern blot analysis as described (45). Endogenous Dβ1-to-Jβ1 rearrangements

were characterized using the 5'Dβ1, inter-genic Dβ1-Jβ1, and 3'Jβ1 probes previously described (46). Endogenous Vβ14 and $V\beta14^{Rep}$ rearrangements were characterized using the 5'Vβ14 and 3'Vβ14 probes previously described (17).

Results

Generation of germline mice expressing a pre-assembled endogenous Vβ14Dβ1Jβ1.4 rearrangement

Due to the lack of a Cβ allotypic marker, TCRβ allelic exclusion studies are often conducted using mice that express an in-frame VβDJβ rearrangement from a transgene randomly integrated into the genome (28,29,47–51). Thus, to directly evaluate the effect that TCRβ mediated feedback signals have upon recombinational accessibility of Vβ14 chromatin, we sought to analyze TCRβ rearrangements in αβ T cells of $V\beta14^{Rep}$ mice that also express a transgenic pre-assembled in-frame VβDJβ rearrangement. Since the D011.10 TCRβ transgene has been used to study TCRβ allelic exclusion and αβ T cell development (28,29,43), we decided to use this classical model of TCRβ mediated feedback inhibition as part of our analysis. This transgene consists of an in-frame pre-assembled Vβ8.2Dβ1Jβ1.1 rearrangement integrated into mouse chromosome 6 near the endogenous TCRβ locus (43)(Figure 1A; A.C.C. and C.H.B., unpublished observations). Germline mice carrying this transgene are hereafter referred to as $V\beta8^{Tg}$ mice. As previously reported, greater than 99% of αβ T lineage cells isolated from $V\beta8^{Tg}$ mice express Vβ8 as part of cell surface TCRβ chains (Figure 1B).

We were concerned that transgenic VβDJβ rearrangements integrated at chromosomal locations outside of endogenous TCRβ loci might not recapitulate physiological TCRβ mediated feedback signals. Nuclei of $V\beta14^{+}$ αβ T cells have been used in nuclear transfer (NT) experiments to generate embryonic stem (ES) cells containing an endogenous in-frame Vβ14Dβ1Jβ1.4 rearrangement on one TCRβ allele (the $V\beta14^{NT}$ allele; Figure 1A), an endogenous DJβ rearrangement on the other TCRβ allele, and endogenous VαJα rearrangements on both TCRα alleles (44). We used these ES cells to generate mice with the $V\beta14^{NT}$ allele transmitted through the germline and bred these mice with wild-type 129SvEv mice for several generations to separate the $V\beta14^{NT}$ allele from the DJβ allele and the two rearranged TCRα alleles. Through this approach, we generated $V\beta14^{NT/WT}$ mice that carry the $V\beta14^{NT}$ allele, a wild-type un-rearranged TCRβ allele, and two wild-type un-rearranged TCRα alleles. As expected, greater than 99% of αβ T lineage cells isolated from $V\beta14^{NT/WT}$ mice express Vβ14 as part of cell surface TCRβ chains (Figure 1B).

Accelerated early thymocyte development in $V\beta8^{Tg}$ and $V\beta14^{NT/WT}$ mice

Despite the previous use of $V\beta8^{Tg}$ mice to study TCRβ feedback inhibition and αβ TCR selection (28,29,43), a thorough analysis of αβ T cell development in $V\beta8^{Tg}$ mice has never been published. In addition, thymocyte development in $V\beta14^{NT/WT}$ mice has never been reported. Thus, we first sought to investigate the potential influence of $V\beta8^{Tg}$ or $V\beta14^{NT}$ expression on gross αβ T cell development. Both $V\beta8^{Tg}$ and $V\beta14^{NT/WT}$ 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, $V\beta8^{Tg}$, and $V\beta14^{NT/WT}$ mice (Figure 2B,C). However, we did observe statistically significant increases in the percentages of $CD4^{+}$ SP thymocytes and decreases in the percentages and $CD8^{+}$ SP thymocytes in $V\beta8^{Tg}$ and $V\beta14^{NT/WT}$ 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 $CD4^{+}$ αβ T cells and decreases in the percentages of $CD8^{+}$ αβ T cells in $V\beta8^{Tg}$ and $V\beta14^{NT/WT}$ mice, as compared to in wild-type mice (Figure 2B, D). Since the numbers of thymocytes and splenocytes were comparable among wild-type, $V\beta8^{Tg}$, and $V\beta14^{NT/WT}$ mice, these data

indicate that expression of either one of these pre-assembled VβDJβ rearrangements directs development of $CD4^+$ αβ T cells at the expense of $CD8^+$ αβ T cells. The latter effect is most likely due to preferential binding of αβ TCR containing the $Vβ8^{Tg}$ and $Vβ14^{NT}$ TCRβ 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. TCRβ locus Dβ-to-Jβ rearrangements initiate in $CD117^+CD25^-$ early T lineage progenitors (ETPs, or DNI cells) and continue in $CD117^+CD25^+$ DNII thymocytes, while Vβ-to-DJβ rearrangements occur in $CD117^-CD25^+$ 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 VβDJβ rearrangement (57). To determine whether expression of these pre-assembled VβDJβ rearrangements alters early thymocyte development, we analyzed the DN populations of thymocytes in wild-type, $Vβ8^{Tg}$, and $Vβ14^{NT/WT}$ 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, $Vβ8^{Tg}$, or $Vβ14^{NT/WT}$ mice (Figure 3A, B). In contrast, we observed significant decreases in the frequencies of DNIII thymocytes in $Vβ8^{Tg}$ and $Vβ14^{NT/WT}$ mice, as compared to in wild-type mice (Figure 3A,B). In addition, the frequencies of $CD117^-CD25^{low}$ cells transitioning between the DNIII and DNIV populations were increased in $Vβ8^{Tg}$ and $Vβ14^{NT/WT}$ mice, as compared to in wild-type mice (Figure 3A). Since $Vβ8^{Tg}$ and $Vβ14^{NT/WT}$ 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 VβDJβ rearrangements and in mice expressing a different endogenous VβDJβ 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 TCRβ chain.

Assembly of an in-frame VβDJβ rearrangement is the rate-limiting step in early αβ T cell development

Expression of TCRβ chains in DNIII thymocytes activates β-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 VβDJβ rearrangement is due to enhanced β-selection, we conducted further phenotypic analysis of DN thymocyte populations among wild-type, $Vβ8^{Tg}$, and $Vβ14^{NT/WT}$ mice. FACS analysis with anti-Vβ8, and anti-Vβ14 antibodies demonstrated that Vβ8 and Vβ14 TCRβ 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 $Vβ8^{Tg}$ and $Vβ14^{NT/WT}$ 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 $Vβ8^{Tg}$ and $Vβ14^{NT/WT}$ mice, as compared to wild-type mice (Figure 4C). These data indicate that β-selection is enhanced in the DNIII thymocyte populations of mice expressing pre-assembled TCRβ chains, most likely because the time ordinarily required to assemble and express an in-frame endogenous VβDJβ rearrangement is bypassed. These observations formally demonstrate that the rate-limiting step in β selection is the assembly of an in-frame VβDJβ rearrangement.

Despite intracellular expression of pre-assembled VβDJβ rearrangements initiating in bone marrow lymphoid progenitor cells (58), TCRβ 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 pTα-TCRβ heterodimers. Unexpectedly, we also observed a greater percentage of large and S phase cells in the ETP/DNI and DNII populations of $V\beta8^{Tg}$ and $V\beta14^{NT/WT}$ mice, as compared to wild-type mice (Figure 4C). These findings suggest that pre-assembled TCRβ 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 $V\beta8^{Tg}$ and $V\beta14^{NT/WT}$ 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 TCRδ and TCRγ chains in DNIII thymocytes leads to the development of γδ T cells (61). FACS analysis with anti-Cβ and anti-Cδ antibodies revealed significant decreases in the frequencies of γδ T cells in the thymuses and spleens of $V\beta8^{Tg}$ and $V\beta14^{NT/WT}$ 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 γδ T cells that develop is reduced in $V\beta8^{Tg}$ and $V\beta14^{NT/WT}$ mice. This observation, which also has been published for mice expressing other pre-assembled transgenic or endogenous TCRβ chains (58,62,63), indicates that the more efficient β-selection of thymocytes in $V\beta8^{Tg}$ and $V\beta14^{NT/WT}$ mice occurs at the expense of δ-selection.

The goal of our current study is to evaluate the effect that TCRβ mediated feedback signals have upon Vβ14 recombinational accessibility by analyzing TCRβ rearrangements in αβ T lineage cells of $V\beta14^{Rep}$ mice containing a pre-assembled in-frame VβDJβ rearrangement. Our experimental approach depends upon expression of pre-assembled TCRβ chains in developing thymocytes prior to or concurrent with initiation of Vβ14 recombinational accessibility. We previously detected $V\beta14^{Rep}$ Dβ-to-Jβ rearrangements in DNIII thymocytes, but not in DNII cells, indicating that Vβ14 recombinational accessibility predominantly initiates in DNIII thymocytes (17). Here, we have shown that β-selection is enhanced and DNIII to DNIV thymocyte differentiation accelerated in $V\beta8^{Tg}$ and $V\beta14^{NT/WT}$ mice due to the premature and invariant expression of the pre-assembled TCRβ chains in DNIII cells. Collectively, these data demonstrate that the pre-assembled TCRβ rearrangements used in this study are expressed prior to or concurrent with initiation of Vβ14 accessibility, thereby validating our experimental approach.

Expression of pre-assembled TCRβ chains inhibits endogenous Vβ14-to-DJβ and Dβ-to-Jβ rearrangements

Before analyzing the effects of TCRβ mediated feedback signals upon modulation of Vβ14 RAG accessibility, we needed to determine whether expression of the pre-assembled VβDJβ rearrangements inhibits endogenous Vβ14-to-DJβ rearrangements. To this aim, we established panels of αβ T cell hybridomas from $V\beta8^{Tg}$ and $V\beta14^{NT/WT}$ mice and analyzed Vβ rearrangements by Southern blot analysis using a Vβ14 probe on *Eco*RI-digested genomic DNA isolated from these cells. We found no Vβ14-to-DJβ rearrangements on the wild-type alleles in all 77 $V\beta8^{Tg}$ and all 86 $V\beta14^{NT/WT}$ αβ T cell hybridomas (Table I). Vβ14-to-DJβ rearrangements normally occur on non-selected alleles in approximately 7% of wild-type αβ T cell hybridomas (17). Consequently, our data demonstrate that expression of either pre-assembled TCRβ chain prevents Vβ14-to-DJβ rearrangements at the frequency they ordinarily occur in wild-type thymocytes.

We also sought to quantify the effect that expression of the transgenic Vβ8.2Dβ1Jβ1.1 rearrangement or endogenous Vβ14Dβ1Jβ1.4 rearrangement has upon the levels of endogenous Dβ-to-Jβ rearrangements. For this purpose, we conducted Southern blot analysis using an inter-genic Dβ1-Jβ1 probe on *Eco*RI-digested genomic DNA isolated from our panels

of $V\beta8^{Tg}$ and $V\beta14^{NT/WT}$ αβ T cell hybridomas. We found that 5 of 77 (6%) of $V\beta8^{Tg}$ and 5 of 86 (6%) $V\beta14^{NT/WT}$ cells contained germline Dβ1-Jβ1 segments and, thus, lacked Dβ1-to-Jβ1 rearrangements on at least one allele (Table I). In wild-type αβ T cells, endogenous Dβ1-to-Jβ1 rearrangements occur to completion on both alleles (10,46). Consequently, these data demonstrate that expression of either the transgenic or endogenous pre-assembled VβDJβ rearrangement leads to a reduction in the overall frequency of Dβ1-to-Jβ1 rearrangements. Importantly, since Vβ14-to-DJβ1 rearrangements only occur on alleles that have already assembled DJβ1 complexes (19), these data further demonstrate that the pre-assembled TCRβ rearrangements used in this study are expressed prior to initiation of Vβ14 recombinational accessibility and endogenous Vβ14-to-DJβ1 rearrangement.

Vβ14 recombinational accessibility is maintained in thymocytes expressing pre-assembled VβDJβ rearrangements

To directly evaluate the effect that TCRβ mediated feedback signals have upon Vβ14 recombinational accessibility, we analyzed $V\beta14^{Rep}$ rearrangements in DN and DP cells sorted from the thymuses of germline mice containing $V\beta14^{Rep}$ on one allele and expressing $V\beta14^{NT}$ from the other allele ($V\beta14^{Rep/NT}$ mice). PCR with a primer that hybridizes to TCRβ locus sequences upstream of $V\beta14^{Rep}$ and a primer that hybridizes to Jβ1.1 sequences amplifies an 850 bp product from germline $V\beta14^{Rep}$ alleles and a 200 bp product from alleles with $V\beta14^{Rep}$ Dβ1-to-Jβ1.1 rearrangements (Figure 5A). We observed amplification of the germline and DJβ rearranged $V\beta14^{Rep}$ products to similar extents in $V\beta14^{Rep/WT}$ and $V\beta14^{Rep/NT}$ DN thymocytes (Figure 5B), indicating that $V\beta14^{Rep}$ recombinational accessibility is not down-regulated in DN cells expressing a pre-assembled TCRβ chain. In DP thymocytes, we observed amplification of the DJβ rearranged $V\beta14^{Rep}$ product to similar levels in $V\beta14^{Rep/WT}$ and $V\beta14^{Rep/NT}$ cells, but amplification of the germline $V\beta14^{Rep}$ product to a lower level in $V\beta14^{Rep/WT}$ cells as compared to $V\beta14^{Rep/NT}$ cells (Figure 5B). Considering that Vβ, Dβ, and Jβ segments rearrange to $V\beta14^{Rep}$ in approximately 10% of thymocytes (Figure 5C)(17), these data suggest that TCRβ feedback signals inhibit the rearrangement of endogenous Vβ, Dβ, and/or Jβ segments to $V\beta14^{Rep}$, but do not down-regulate Vβ14 recombinational accessibility.

To quantify the effect that TCRβ mediated feedback signals have upon Vβ14 recombinational accessibility and the rearrangement of endogenous Vβ, Dβ, and/or Jβ segments to $V\beta14^{Rep}$, we sought to analyze $V\beta14^{Rep}$ rearrangements in αβ T lineage cells of germline mice containing $V\beta14^{Rep}$ on one allele and expressing either $V\beta8^{Tg}$ or $V\beta14^{NT}$ from the homologous chromosome. For this purpose, we bred germline $V\beta14^{Rep/Rep}$ mice with $V\beta8^{Tg}$ mice or $V\beta14^{NT/WT}$ mice to generate $V\beta8^{Tg}$:$V\beta14^{Rep/WT}$ and $V\beta14^{Rep/NT}$ mice. Analyses of $V\beta8^{Tg}$:$V\beta14^{Rep/WT}$ and $V\beta14^{Rep/NT}$ mice demonstrated that they exhibited αβ T cell development and populations identical to $V\beta8^{Tg}$ and $V\beta14^{NT/WT}$ mice, respectively (data not shown). To analyze TCRβ rearrangements, we established panels of αβ T cell hybridomas from $V\beta14^{Rep/WT}$, $V\beta8^{Tg}$:$V\beta14^{Rep/WT}$, and $V\beta14^{Rep/NT}$ mice. The $V\beta14^{Rep}$ allele contains a unique *Eco*RI restriction site that distinguishes between $V\beta14^{Rep}$ recombination events and Vβ14-to-DJβ rearrangements on the $V\beta14^{WT}$ allele (17). Thus, we conducted Southern blot analysis using a Vβ14 probe on *Eco*RI-digested genomic DNA isolated from these cells to analyze $V\beta14^{Rep}$ and Vβ14 rearrangements. Of the 67 $V\beta14^{Rep/WT}$ αβ T cell hybridomas analyzed, 47 (73%) contained $V\beta14^{Rep}$ recombination events (Table II). This observation is consistent with our earlier findings obtained from chimeric mice that Vβ14 is RAG accessible in a substantially higher percentage of thymocytes than the approximately 7% in which Vβ14-to-DJβ rearrangements occur (17,19,64). Unexpectedly, 52 of the 66 (79%) $V\beta8^{Tg}$:$V\beta14^{Rep/WT}$ αβ T cell hybridomas and 105 of the 185 (58%) $V\beta14^{Rep/NT}$ αβ T cell hybridomas contained $V\beta14^{Rep}$ recombination events (Table II). These data indicate that expression of neither pre-

assembled VβDJβ rearrangement prevented Vβ14 recombinational accessibility during thymocyte development.

$V\beta14^{Rep}$ recombination events include $V\beta14^{Rep}$ Dβ-to-Jβ rearrangements that require Vβ14 recombinational accessibility, and rearrangement of endogenous Vβ, Dβ, and Jβ segments to $V\beta14^{Rep}$ that also require accessibility of the participating TCRβ gene segments and their juxtaposition with $V\beta14^{Rep}$ (Figure 5C) (17). To quantify the level of each specific $V\beta14^{Rep}$ recombination event in the $V\beta14^{Rep/WT}$, $V\beta8^{Tg}$:$V\beta14^{Rep/WT}$, and $V\beta14^{Rep/NT}$ αβ T cell hybridomas, we conducted additional Southern blot analysis using a series of TCRβ locus probes on *Eco*RI-digested genomic DNA isolated from these cells. The $V\beta14^{Rep}$ allele lacks endogenous Dβ2-Jβ2 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 $V\beta14^{Rep/WT}$ αβ T cell hybridomas analyzed, 36 (54%) contained $V\beta14^{Rep}$ Dβ-to-Jβ rearrangements, seven (10%) harbored endogenous Dβ or Jβ segments rearranged to $V\beta14^{Rep}$, and four (6%) carried endogenous Vβ segments rearranged to $V\beta14^{Rep}$ (Table II). These data are consistent with our previous observations from chimeric mice that revealed RAG accessible Vβ14 segments are juxtaposed with RAG accessible Vβ, Dβ, and Jβ segments in a greater percentage of thymocytes than the 7% in which Vβ14-to-DJβ rearrangements normally occur (17, 19, 64). Of the 66 $V\beta8^{Tg}$:$V\beta14^{Rep/WT}$ αβ T cell hybridomas analyzed, 37 (56%) contained $V\beta14^{Rep}$ Dβ-to-Jβ rearrangements, seven (11%) harbored endogenous Dβ or Jβ segments rearranged to $V\beta14^{Rep}$, and eight (12%) carried endogenous Vβ segments rearranged to $V\beta14^{Rep}$ (Table II). However, of the 185 $V\beta14^{Rep/NT}$ αβ T cell hybridomas analyzed, 82 (44%) contained $V\beta14^{Rep}$ Dβ-to-Jβ rearrangements, 19 (10%) harbored endogenous Dβ or Jβ segments rearranged to $V\beta14^{Rep}$, and four (2%) carried endogenous Vβ segments rearranged to $V\beta14^{Rep}$ (Table II).

These data reveal that the predominant manner by which expression of the endogenous Vβ14Dβ1Jβ1.4 rearrangement decreased $V\beta14^{Rep}$ recombination events is through the reduction of $V\beta14^{Rep}$ Dβ-to-Jβ rearrangements, which require Vβ14 recombinational accessibility but not necessarily juxtaposition of $V\beta14^{Rep}$ with endogenous Dβ-Jβ segments. The expression of either pre-assembled TCRβ chain also had no effect upon the level at which endogenous Dβ-Jβ segments rearranged with $V\beta14^{Rep}$. For reasons detailed in the discussion, our collective data are consistent with the notion that TCRβ mediated feedback inhibition of Vβ14-to-DJβ rearrangements depends upon the inherent properties of Vβ14, Dβ, and Jβ RSs.

Discussion

Here, we have analyzed the effect that expression of a classical TCRβ transgene ($V\beta8^{Tg}$) or a pre-assembled endogenous TCRβ gene ($V\beta14^{NT}$) has upon recombinational accessibility of Vβ14 chromatin. We first showed that greater than 99% of αβ T lineage cells isolated from $V\beta8^{Tg}$ or $V\beta14^{NT/WT}$ mice expressed Vβ8 or Vβ14, respectively, as part of their cell surface TCRβ chains. We then demonstrated that expression of each pre-assembled TCRβ chain accelerated the DNIII to DNIV step of early thymocyte development due to enhanced β-selection in the DNIII population. Our analyses of TCRβ rearrangements revealed that expression of these pre-assembled VβDJβ rearrangements inhibited endogenous Vβ14-to-DJβ rearrangements as expected. However, in contrast to results predicted by the accepted model of TCRβ feedback inhibition, we found that expression of these pre-assembled TCRβ chains neither blocked recombinational accessibility of Vβ14 chromatin nor inhibited rearrangements between $V\beta14^{Rep}$ and endogenous Dβ-Jβ segments.

One concern related to our study is that the $V\beta14^{Rep}$ V(D)J recombination reporter might alter recombinational accessibility of Vβ14 chromatin. Although we have previously proved that

local chromatin environment imparts lineage and developmental-stage specific recombinational accessibility upon Vβ14^{Rep} (17), it is conceivable that replacement of the Vβ14 RS with Vβ14^{Rep} increases the frequency at which Vβ14 chromatin is rendered recombinationally accessible and the RSs within this region available for RAG binding (17). However, the frequency of Vβ14^{Rep} recombination events is similar to the frequency of Vβ14 rearrangements on alleles containing specific replacement of the Vβ14 RS with the 3'Dβ1 RS (17,19), demonstrating that intrinsic properties of the 5'Dβ1 and Jβ1.1 RSs have negligible ability to promote Vβ14 recombinational accessibility. The ability of the 3'Dβ1 RS to recombine with Jβ RSs, and by extension potential c-fos mediated RAG deposition, requires prior recombinational accessibility of the 3'Dβ1 RS. In this context, gene-targeted insertion of the 3'Dβ1 RS into Vβ14 chromatin would be expected to increase the frequency of RAG binding without affecting Vβ14 recombinational accessibility (19), which was our logic behind using Vβ14^{Rep} to quantify Vβ14 recombinational accessibility independent of Vβ14 synapsis/cleavage with endogenous Dβ-Jβ 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 nuclesomes and exhibit higher rearrangement potential (66). Considering that the 3'Dβ1 RS, but not the Vβ14 RS, contains a consensus nonamer, it seems unlikely that intrinsic properties of the 3'Dβ1 RS would position nucleosomes in a manner that promotes recombinational accessibility more so than the Vβ14 RS. Thus, we interpret our findings within this and prior studies to accurately reflect Vβ14 recombinational accessibility.

Our findings here and before (17) that endogenous Vβ segments can rearrange directly to Vβ14^{Rep} Dβ1 segments that have not recombined with Vβ14^{Rep} Jβ1.1 segments has important implications for mechanisms that direct ordered TCRβ gene rearrangements. We had previously demonstrated that the assembly of endogenous TCRβ variable region exons through DJβ intermediates is not driven solely by intrinsic properties of Dβ/Jβ versus Vβ/Dβ 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'Dβ RSs and hinder RAG binding to the adjacent 5'Dβ RSs until deletion of the 3'Dβ RS RAG binding site through Dβ-to-Jβ rearrangement (11,70). One group also demonstrated that c-fos binds 3'Dβ RSs and recruits the RAG proteins, which may help suppress Vβ rearrangements *in vivo* prior to Dβ-to-Jβ rearrangement (11). Notably, each of these *in vivo* studies was conducted with endogenous TCRβ loci or chromosomally-integrated TCRβ mini-loci in which the 3'Dβ RSs were in their native location relative to the germline Dβ promoters that function with the TCRβ (or heterologous) enhancer to direct Dβ-Jβ recombinational accessibility. In contrast, our *in vivo* study analyzes TCRβ rearrangements involving Dβ1-Jβ1.1 genomic sequences outside of their native location. Our data reveal that completion of Dβ-to-Jβ recombination before Vβ-to-DJβ rearrangement depends upon the chromosomal location of Dβ1-Jβ1.1 and reveals that neither c-fos dependent nor c-fos independent RAG deposition upon 3'Dβ RSs are sufficient to direct ordered TCRβ rearrangements. Thus, the location of Dβ-Jβ segments relative to their germline Dβ promoters and/or the TCRβ enhancer may be critical for directing the assembly of endogenous TCRβ variable region exons through DJβ intermediates.

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

chromatin accessibility or transcriptional accessibility is lacking for Vβ segments. Our study, which directly investigates the effect that TCRβ mediated feedback signals have upon Vβ recombinational accessibility *in vivo*, reveals that RAG access to Vβ14 chromatin is maintained in thymocytes expressing pre-assembled VβDJβ rearrangements. It had previously been demonstrated that germline Vβ14 segments remain recombinationally accessible in DP thymocytes expressing a classical TCRβ transgene and that DP cells attempting Vβ14-to-DJβ rearrangements are eliminated by apoptosis to maintain TCRβ allelic exclusion (33). Yet, the mechanisms by which TCRβ mediated feedback signals inhibit Vβ14 rearrangements in DN cells were never explored. As discussed below, our current observation indicates that TCRβ-mediated feedback signals must inhibit endogenous Vβ14-to-DJβ rearrangements in DN thymoctyes through mechanisms other than down-regulation of RAG access to Vβ14 chromatin.

The physiological relevance of conclusions reached from studies of TCRβ feedback regulation using mice that express transgenic VβDJβ 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 TCRβ chains driven by multiple integrated copies of the transgenic VβDJβ rearrangement (28,47). Such accelerated development could prevent normal initiation of Vβ recombinational accessibility or TCRβ locus looping. We have shown here that β-selection is enhanced in the DNIII thymocyte populations of mice containing pre-assembled VβDJβ rearrangements due to the premature and invariant expression of TCRβ chains, even those expressed from a single copy of an endogenous Vβ14Dβ1Jβ1.4 rearrangement. However, expression of either pre-assembled VβDJβ rearrangements prior to initiation of endogenous Vβ14-to-DJβ rearrangements enabled us evaluate the effect that TCRβ mediated feedback signals have upon Vβ14 recombinational accessibility. Despite the five-fold decrease in the numbers of DNIII cells where Vβ-to-DJβ rearrangements occur, the overall level of Vβ14 recombinational accessibility in DN cells was maintained in cells expressing the TCRβ transgene or the endogenous Vβ14Dβ1Jβ1.4 rearrangement. These data indicate that the initiation of Vβ14 recombinational accessibility occurs rapidly, efficiently, and on both TCRβ alleles in the majority of thymocytes as they differentiate into the DNIII stage. Whether these Vβ14 segments remain continually accessible until β-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 TCRβ chain lower the level of rearrangements between $V\beta14^{Rep}$ and endogenous Dβ1-Jβ1 segments? TCRβ locus RS beyond 12/23 joining restrictions mediated by RAG-RS interactions enable functional synapses between 3'Dβ/Jβ, 3'Dβ/5'Dβ, and Vβ/5'Dβ RSs, but not between Vβ/Jβ RSs (10,42,72–74). These RS constraints permit only a single rearrangement between endogenous Dβ1-Jβ1 and Vβ14 segments on wild-type TCRβ alleles. Replacement of the Vβ14 RS with 5'Dβ1, 3'Dβ1, and Jβ1.1 RSs on the $V\beta14^{Rep}$ allele enables nine possible recombination events between $V\beta14^{Rep}$ and the endogenous Dβ1-Jβ1 segments. The *in vitro* recombination efficiency of each of the nine functional RS pairs on the $V\beta14^{Rep}$ allele is higher than that of the single compatible Vβ14/5'Dβ1 RS pair on the wild-type allele (42). In this context, the overall efficiency of recombination between $V\beta14^{Rep}$ and endogenous Dβ-Jβ segments on the $V\beta14^{Rep}$ allele would be at least one order of magnitude greater than the rate of Vβ14-to-DJβ rearrangement on the wild-type allele. Thus, one interpretation of our finding that expression of neither pre-assembled TCRβ chain inhibits rearrangements between $V\beta14^{Rep}$ and endogenous Dβ1-Jβ1 segments is that recombination events between these segments occur at such high efficiency that they are refractory to TCRβ mediated feedback inhibition. Due to the orientation and location of the $V\beta14^{Rep}$ 3'Dβ1 RS inserted in place of the Vβ14 RS, rearrangements between $V\beta14^{Rep}$ and endogenous Dβ-Jβ segments cannot assemble Vβ14(D)Jβ exons that encode

functional TCRβ chains. Consequently, analysis of Vβ14-to-DJβ rearrangements and Vβ14 expression in αβ T cells containing specific replacement of the endogenous Vβ14 RS with the 3'Dβ1 RS is required to determine whether TCRβ mediated feedback inhibition of Vβ14-to-DJβ rearrangements, and possibly Vβ14 allelic exclusion, depends upon the inherent properties of Vβ14, Dβ, and Jβ RSs.

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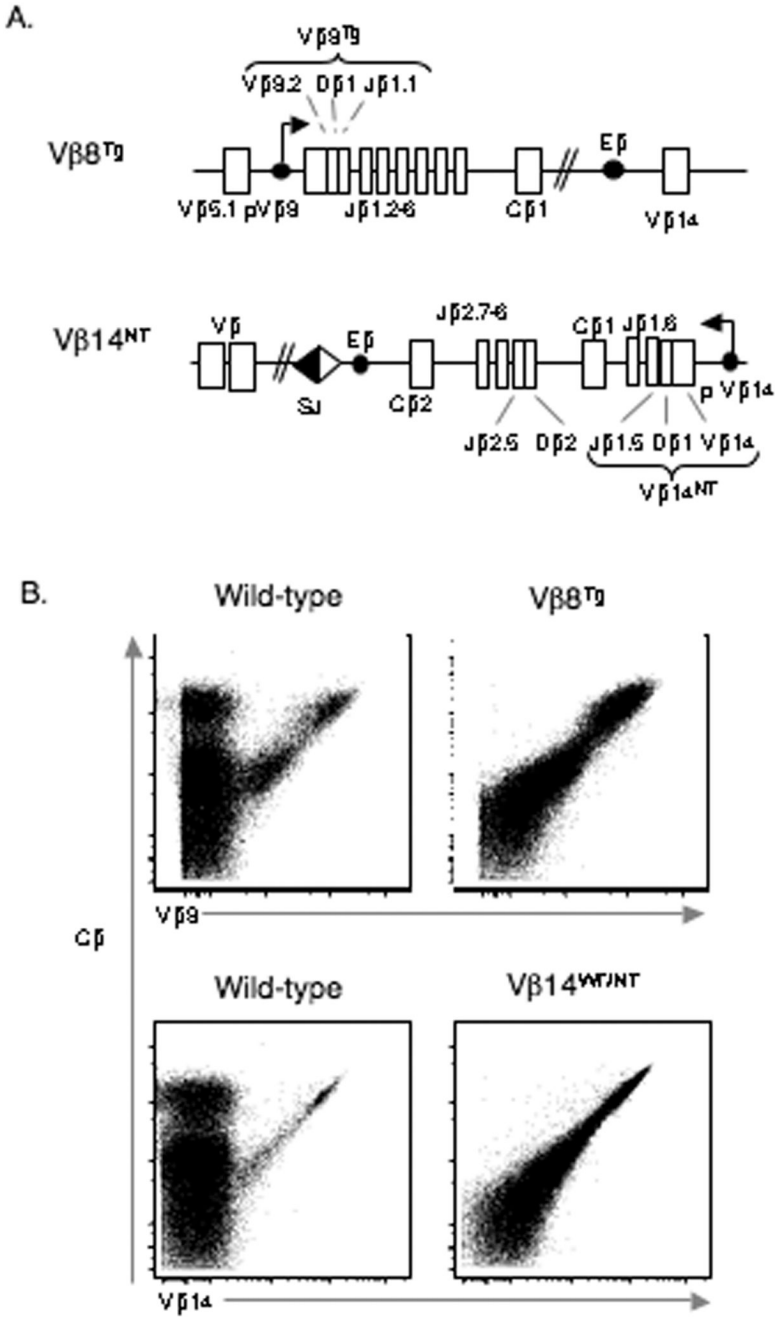

Figure 1. Vβ8^{Tg} and Vβ14$^{NT/WT}$ mice exhibit TCRβ allelic exclusion

(A) Schematic representation of the transgenic Vβ8^{Tg} and endogenous Vβ14^{NT} loci. Open boxes depict Vβ, Dβ, Jβ, and Cβ segments. Closed circles depict the TCRβ enhancer (Eβ) and the promoters of the pre-assembled VβDβJβ rearrangements, with arrows indicating the direction of sense VβDβJβ transcripts. Triangles show the 5'Dβ1/Vβ14 RSS join. (B) Shown are representative anti-Cβ, by anti-Vβ8 or anti-Vβ14 FACS analysis of cells isolated from the thymuses and spleens of wild-type, Vβ8^{Tg}, and Vβ14$^{NT/WT}$ mice.

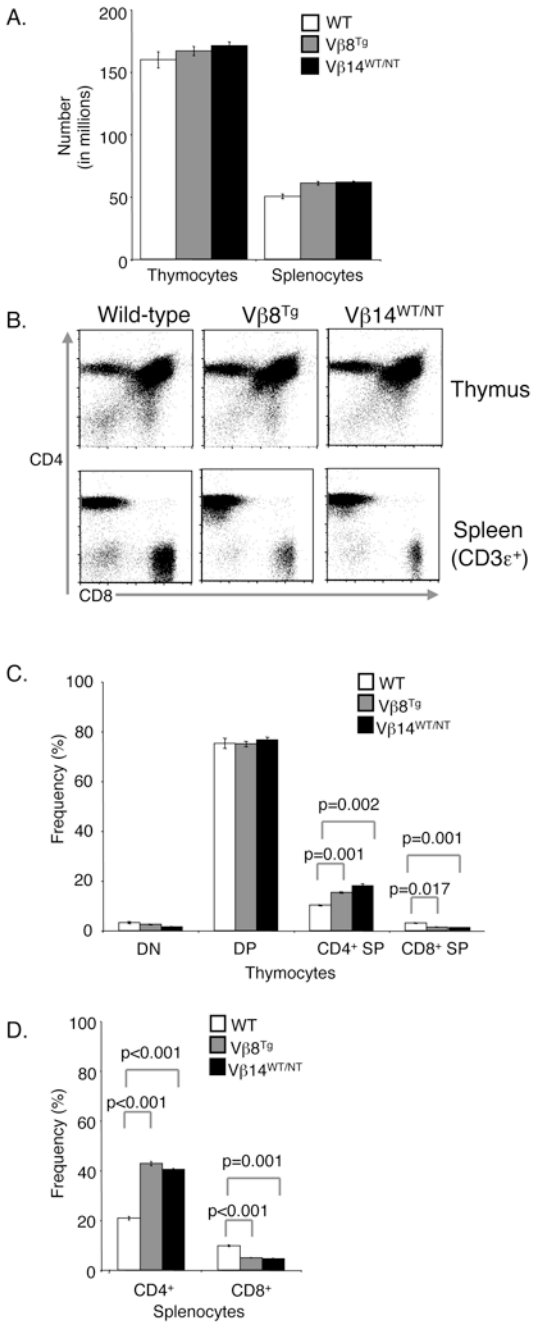

Figure 2. Grossly normal αβ T cell development in Vβ8^{Tg} and Vβ14$^{NT/WT}$ mice

(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, Vβ8^{Tg}, and Vβ14$^{NT/WT}$ mice. The percentage of DN, DP, CD4$^+$ SP, and CD8$^+$ SP thymocytes and CD4$^+$ and CD8$^+$ αβ T cells is depicted. (C–D) Bar graphs showing the average frequency of (C) DN, DP, CD4$^+$ SP, and CD8$^+$ SP thymocytes and (D) CD4$^+$ and CD8$^+$ αβ 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.

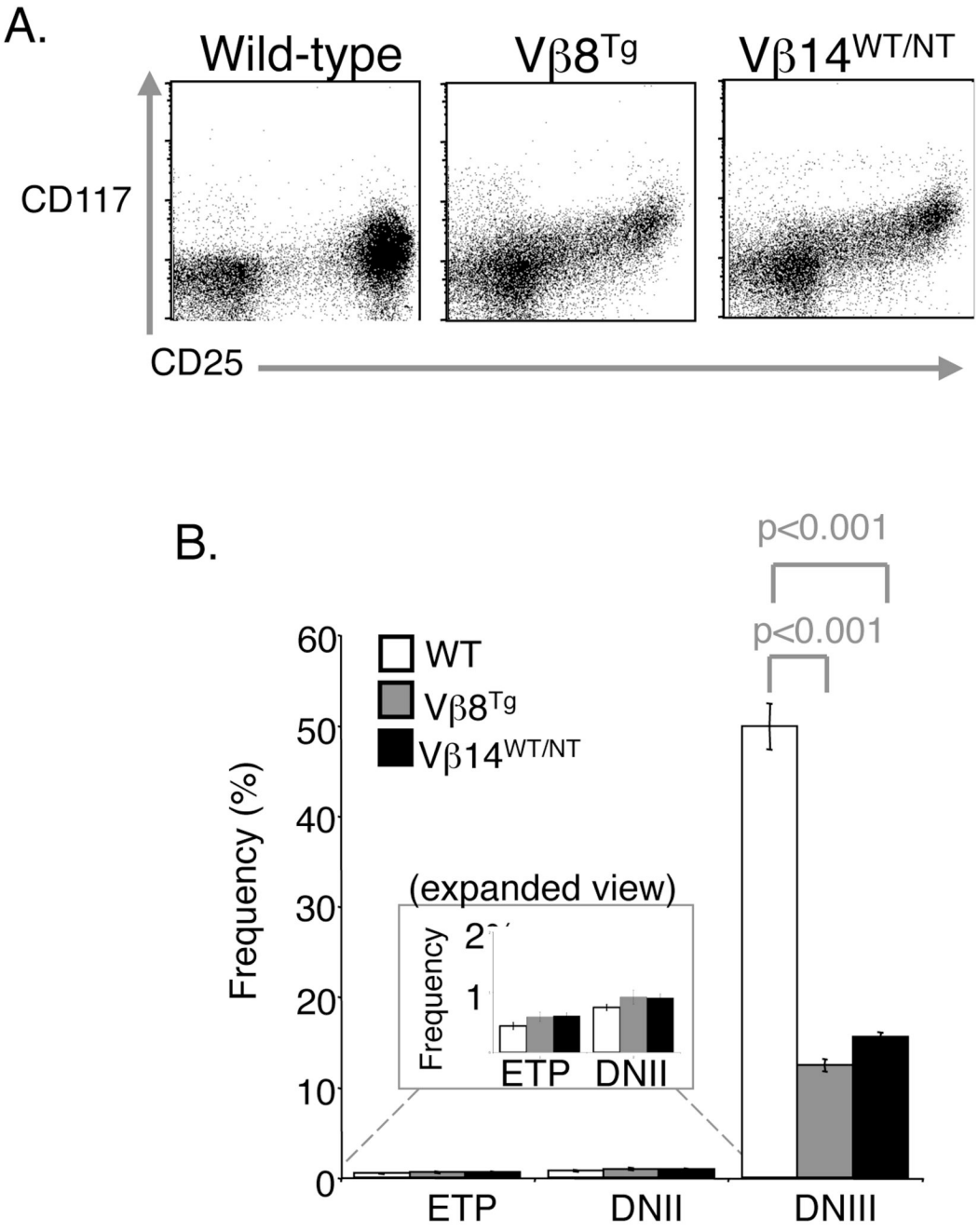

Figure 3. Accelerated early thymocyte development in Vβ8^{Tg} and Vβ14$^{NT/WT}$ mice

(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.

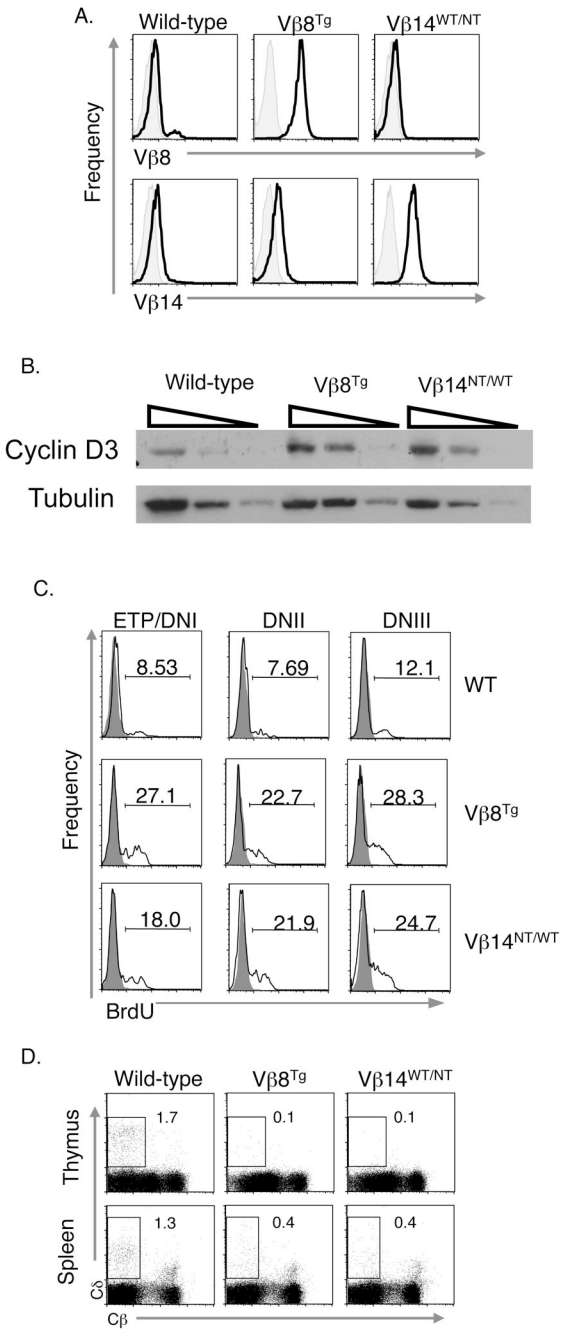

Figure 4. β-selection is enhanced in Vβ8^{Tg} and Vβ14$^{NT/WT}$ mice

(A) Shown are representative histograms of anti-Vβ8 and anti-Vβ14 FACS analysis of DNIII thymocytes isolated from wild-type, Vβ8^{Tg}, and Vβ14$^{NT/WT}$ mice. (B) Shown are representative Western blot analysis of cyclin D3 and tubulin protein expression in total thymocytes of wild-type, Vβ8^{Tg}, and Vβ14$^{NT/WT}$ mice. (C) Shown are representative BrdU FACS analysis of ETP, DNII, DNIII, and DNIV thymocytes of wild-type, Vβ8^{Tg}, and Vβ14$^{NT/WT}$ mice. The percentage of BrdU^{+} 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^{Tg}, and Vβ14$^{NT/WT}$ mice. The percentage of Cδ^{+} T cells is depicted.

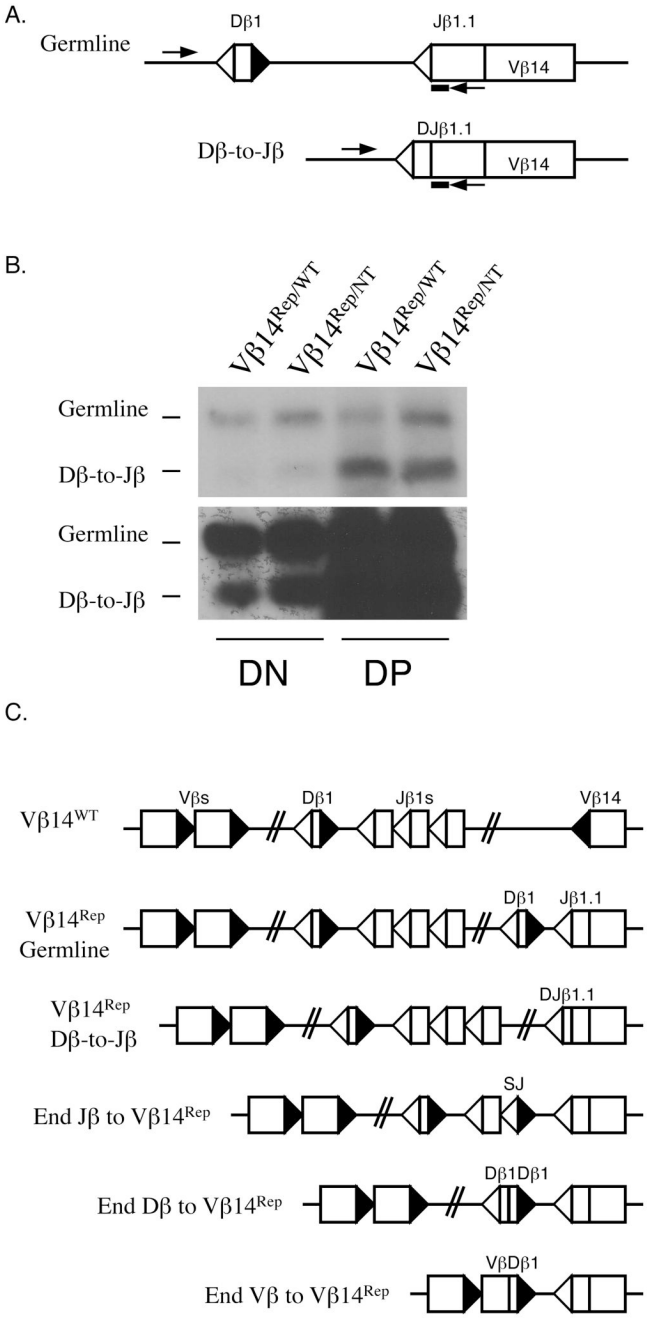

Figure 5. Vβ14 recombinational accessibility is maintained in thymocytes expressing a pre-assembled TCRβ gene

(A) Schematic representation of germline and DJβ rearranged Vβ14^{Rep} alleles indicating the positions of the PCR primers and probe used analysis of Vβ14^{Rep} Dβ-to-Jβ recombination events. The sizes of PCR products amplified from germline and DJβ rearranged Vβ14^{Rep} alleles are indicated. (B) PCR analysis of Vβ14^{Rep} Dβ-to-Jβ recombination events conducted on equal amounts of genomic DNA isolated from sort-purified DN and DP thymocytes of Vβ14$^{Rep/WT}$ and Vβ14$^{Rep/NT}$ mice. Shown are short and long exposures with the PCR product identities indicated. (C) Schematic representations of germline wild-type TCRβ (Vβ14^{WT}) and Vβ14^{Rep} loci, as well as the configurations of Vβ14^{Rep} alleles that have undergone Vβ14^{Rep}

Dβ-to-Jβ rearrangements or the rearrangement of endogenous (End) Vβ, Dβ, or Jβ segments to the $V\beta14^{Rep}$ Dβ1 segment. Open boxes depict Vβ, Dβ, Jβ, and Cβ segments. Black triangles indicate 23-RSs and open triangles indicate 12-RSs. Also labeled are the signal join (SJ) formed during endogenous Jβ rearrangements to $V\beta14^{Rep}$, the Dβ1-Dβ1 coding join formed during endogenous Dβ rearrangements to the $V\beta14^{Rep}$ Dβ1 segment, and the Vβ1-Dβ1 coding join formed during endogenous Vβ rearrangements to the $V\beta14^{Rep}$ Dβ1 segment.

Table I

A. Endogenous Vβ14 rearrangements in $Vβ8^{Tg}$ and $Vβ14^{NT/WT}$ αβ T cell hybridomas

Genotype	Total Number Cells	Vβ14Dβ1Jβ1 Rearrangements
$Vβ8^{Tg}$	77	0 (0%)
$Vβ14^{NT/WT}$	86	0 (0%)

B. Endogenous Dβ1-Jβ1 rearrangements in $Vβ8^{Tg}$ and $Vβ14^{NT/WT}$ αβ T cell hybridomas

Genotype	Total Number Cells	Dβ1-Jβ1 Germline	Dβ1Jβ1 Rearranged
$Vβ8^{Tg}$	77	5 (6%)	72 (94%)
$Vβ14^{NT/WT}$	86	5 (6%)	81 (94%)

Table II

A. $V\beta14^{Rep}$ rearrangements in $V\beta14^{Rep/WT}$, $V\beta8^{Tg}$:$V\beta14^{NT/WT}$, and $V\beta14^{Rep/NT}$ αβ T cell hybridomas

Genotype	Total Number Cells	$V\beta14^{Rep}$ Rearrangements
$V\beta14^{Rep/WT}$	67	47 (73%)
$V\beta8^{Tg}$:$V\beta14^{Rep/WT}$	66	52 (79%)
$V\beta14^{Rep/NT}$	185	105 (58%)

B. $V\beta14^{Rep}$ recombination events in $V\beta14^{Rep/WT}$, $V\beta8^{Tg}$:$V\beta14^{NT/WT}$, and $V\beta14^{Rep/NT}$ αβ T cell hybridomas

Genotype	Total Number Cells	$V\beta14^{Rep}$ Recombination Events	
$V\beta14^{Rep/WT}$	67	$V\beta14^{Rep}$ Dβ-to-Jβ	36 (54%)
		Dβ-Jβ to $V\beta14^{Rep}$	7 (10%)
		Vβ to $V\beta14^{Rep}$	4 (6%)
$V\beta8^{Tg}$:$V\beta14^{Rep/WT}$	66	$V\beta14^{Rep}$ Dβ-to-Jβ	37 (56%)
		Dβ-Jβ to $V\beta14^{Rep}$	7 (11%)
		Vβ to $V\beta14^{Rep}$	8 (12%)
$V\beta14^{Rep/NT}$	185	$V\beta14^{Rep}$ Dβ-to-Jβ	82 (44%)
		Dβ-Jβ to $V\beta14^{Rep}$	19 (10%)
		Vβ to $V\beta14^{Rep}$	4 (2%)