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Assembled DJβ Complexes can Influence TCRβ Chain Selection and Peripheral Vβ Repertoire

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Abstract

TCRβ chain repertoire of peripheral αβ T cells is generated through the step-wise assembly and subsequent selection of TCRβ variable region exons during thymocyte development. To evaluate the influence of a two-step recombination process on Vβ rearrangement and selection, we generated mice with a pre-assembled Dβ1Jβ1.1 complex on the $J\beta1^{\omega}$ allele, an endogenous TCRβ allele that lacks the Dβ2-Jβ2 cluster, creating the $J\beta1^{DJ\beta}$ allele. As compared to $J\beta1^{\omega/\omega}$ mice, both $J\beta1^{DJ\beta/\omega}$ and $J\beta1^{DJ\beta/DJ\beta}$ mice exhibited grossly normal thymocyte development and TCRβ allelic exclusion. In addition, Vβ rearrangements on $J\beta1^{DJ\beta}$ alleles occurred at the same frequency as Vβ rearrangements on $J\beta1^{\omega}$ alleles, and were similarly regulated by TCRβ mediated feedback regulation. However, thymocytes with VβDJβ rearrangements assembled on $J\beta1^{DJ\beta}$ alleles overall were preferentially selected and the Vβ repertoire of αβ T cells was significantly altered during αβ TCR selection in $J\beta1^{DJ\beta/\omega}$ and $J\beta1^{DJ\beta/DJ\beta}$ mice, as compared to in $J\beta1^{\omega/\omega}$ mice. Our data indicate that the diversity of DJβ complexes assembled during thymocyte development influences TCRβ chain selection and peripheral Vβ repertoire.

Keywords

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

Introduction

Generation and expression of a diverse repertoire of antigen receptors on the cell surface of lymphocytes is essential for adaptive immunity. TCR and Ig chains consist of variable

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regions that bind antigen and constant (C) regions. During lymphocyte development, TCR and Ig variable region exons ("genes") 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 induces DNA double strand breaks between participating gene segments and their flanking recombination signal sequences (RSSs), generating blunt signal ends and hairpin-sealed coding ends (2). Signal ends are repaired by generally expressed core non-homologous end-joining (NHEJ) proteins to form signal joins, while coding ends are opened by additional NHEJ proteins and then repaired by the core NHEJ factors to form V(D)J coding joins (3, 4). This process generates antigen receptor chain diversity through the combination of joining events, the inherent imprecision of NHEJ, and the random addition of non-template (N) nucleotides by the lymphocyte-specific terminal TdT protein (1).

In humans and mice, αβ T lymphocytes develop through a differentiation program that involves the assembly, expression, and selection of TCR genes (5). TCRβ genes are assembled through an ordered process in which Dβ-to-Jβ rearrangements are detectable in CD117(c-kit)$^+$CD44$^+$CD25$^-$ early T lineage progenitors (ETPs) and CD117$^+$CD44$^+$CD25$^+$ (stage II) CD4$^-$/CD8$^-$ (double negative or DN) thymocytes, and Vβ rearrangements initiate in CD117$^-$CD44$^-$CD25$^+$ (stage III) DN cells (6, 7). Although Dβ-to-Jβ recombination is not required for Vβ rearrangement, the deletion of sequences between Dβ and Jβ segments, such as the 3'Dβ RSSs, may facilitate Vβ-to-DJβ recombination (Sleckman, c-fos, Khor). The assembly and expression of a productive (in-frame) VβDJβ rearrangement on the first allele generates TCRβ chains that pair with pTα molecules to form pre-TCRs that select DNIII cells for further development (5). This β-selection process involves signals that rescue DNIII thymocytes from apoptosis, promote rapid cellular proliferation, direct differentiation into c-kit$^-$CD44$^-$CD25$^-$ DN IV cells and then CD4$^+$/CD8$^+$ (double positive, DP) thymocytes, and prevent Vβ-to-DJβ rearrangements on the second allele to ensure TCRβ chains are expressed from only one allele (5). In DP thymocytes, TCRα genes are assembled on both alleles from Vα and Jα segments (8). Productive VαJα rearrangements generate TCRα chains that can associate with TCRβ chains to form αβ TCR receptors, which are then subject to selection (9). Negative selection of αβ TCRs leads to cell death, while positive selection rescues DP cells from apoptosis and promotes their further development to CD4$^+$ or CD8$^+$ (single positive, SP) thymocytes, which exit the thymus as αβ T cells (9). Due to the imprecision of V(D)J joining, only one-third of VβDJβ rearrangements are assembled in-frame. Thymocytes that assemble a non-productive (out-of-frame) VβDJβ rearrangement on the first allele can undergo Vβ rearrangement on the second allele, which drives differentiation if assembled in-frame (10). Thus, in addition to their selected VβDJβ rearrangements, ~60% of normal αβ T cells contain DJβ rearrangements and ~40% contain out-of-frame VβDJβ rearrangements on their non-selected alleles, a phenomenon referred to as the 60/40 ratio (10, 11). This pattern of TCRβ rearrangements indicates that Vβ-to-DJβ rearrangements occur on one allele at a time in DNIII cells (Mostoslavasky), however there is no evidence that distinguishes whether Dβ-to-Jβ rearrangements occur asynchronously between alleles or on both alleles simultaneously.

The TCRβ chain repertoire of peripheral αβ T lymphocytes is generated through the assembly and selection of VβDJβ rearrangements during thymocyte development.

Generation of primary TCRβ repertoires is not random since rearrangements between both Dβ and Jβ segments and Vβ segments and DJβ complexes occur at varying relative levels in DN thymocytes as determined, at least in part, by genetic variations of their flanking RSSs (12–16). The relative frequency at which Vβs are expressed in DN thymocytes prior to β-selection and in DP cells prior to αβ TCR selection are similar, suggesting that Vβ repertoire is not substantially altered during β-selection (17). In contrast, the relative frequency at which Vβs are expressed in DP thymocytes versus SP thymocytes and peripheral αβ T cells can be dramatically different, indicating that the Vβ repertoire of TCRβ chains is shaped during αβ TCR selection (18–24). The length of VβDJβ joins also is modulated during thymocyte development with shorter sequences selected for during DP to SP differentiation (25, 26).

There are many gaps in our understanding of the mechanisms that evolved to control the regulated assembly and selection of TCRβ chains. The tri partite joining of Vβ, Dβ, and Jβ segments is required for generation of TCRβ chains with normal-sized third complementarity determining regions, which are involved in antigen binding (27). Accordingly, selective pressure enforced by antigens may have caused TCRβ loci to evolve Vβ, Dβ, and Jβ segments such that TCRβ chains include amino acids encoded by Dβ nucleotides and gain the increased junctional diversity of two joining events. The assembly of Ig heavy (H) chain genes from V_H, D_H, and J_H segments occurs through DJ_H intermediates and is subject to allelic exclusion (11), while the assembly of TCRδ genes occurs through both DJδ and VDδ intermediates and exhibits allelic inclusion (28). In this context, an ordered two-step recombination process also could have evolved to provide an additional level of regulatory control important for preventing Vβ to DJβ rearrangements on both alleles and enforcing TCRβ allelic exclusion (15).

The mouse TCRβ locus consists of 20 functional Vβ segments and two Dβ-Jβ clusters (Dβ1-Jβ1 and Dβ2-Jβ2) that each contains a single Dβ segment (Dβ1 or Dβ2) and six functional Jβ segments (Jβ1.1-Jβ1.6 or Jβ2.1-Jβ2.7) (Accession Numbers). In a population of DN thymocytes, Dβ2 rearranges to all six functional Jβ2 segments and Dβ1 rearranges to all 12 functional Jβ segments, creating DβJβ complexes of 18 Dβ-Jβ joining combinations (reference). In the DNIII population, all Vβ segments rearrange to these Dβ1Jβ1, Dβ1Jβ2, and Dβ2Jβ2 complexes (reference), with deletion of Dβ1Jβ1 complexes and germline Jβ1 segments upon joining to either Dβ1Jβ2 or Dβ2Jβ2 complexes. Moreover, in DNIII cells with primary Vβ rearrangements to Dβ1Jβ1 complexes, secondary Vβ rearrangements theoretically can occur to Dβ2Jβ2 complexes, resulting in deletion of the VβDβ1Jβ1 complexes. Since the number and complexity of TCRβ rearrangements present obstacles for the investigation of mechanisms that regulate the assembly of TCRβ variable region exons, we previously used gene targeting to delete the endogenous Dβ2-Jβ2 cluster and create the $Jβ1^{ω}$ allele on which Vβ rearrangements only can be targeted to DβJβ1 complexes of six Dβ-Jβ joining combinations (Bassing). Heterozygous and homozygous $Jβ1^{ω}$ mice exhibit αβ T cell development, TCRβ rearrangement, Vβ repertoire, and TCRβ allelic exclusion indistinguishable from wild-type mice with un-modified TCRβ loci (15). Thus, to evaluate the influence of Dβ-to-Jβ rearrangement on Vβ rearrangement and TCRβ selection, we generated and analyzed mice with a pre-assembled Dβ1Jβ1.1 complex on the $Jβ1^{ω}$ allele. Due to practical considerations, our analysis was unfortunately limited to one particular Jβ1

segment and one DβJβ coding join of a defined sequence and length, which could introduce biases in Vβ rearrangement and TCRβ selection. Yet, such potential biases also would indicate unequivocally that DJβ complexes assembled in DN thymocytes influences these downstream processes.

Materials and Methods

Targeting Construct and Probes

The DJβ targeting vector was constructed in pLNTK (29). The 5' homology arm is a 2.8 kb *Kpn*I/*Bam*HI fragment containing a pre-assembled Dβ1Jβ1.1 complex PCR-cloned from splenocytes, which was then blunted and ligated into the *Sal*I site of pLNTK. The 3' homology arm is a 1.8 kb *Bam*HI/*Sac*I genomic fragment containing Jβ1.2 through Jβ1.6, which was blunted and ligated into the *Xho*I site of pLNTK. This fragment also contained a *Hind*III site that was inserted just inside the *Bam*HI site prior to subcloning. The completed targeting vector was sequenced to confirm the integrity of the pre-assembled Dβ1Jβ1.1 complex. The 5'KO probe is a 300 bp PCR product amplified with primers 5'-GGATCCTGAGAACTGGACATAAGGG-3' and 5'-TTTAATCACTGTGTACTTCC-3'. The 3'KO probe is a 546 bp *Eco*RI/*Kpn*I fragment.

Gene Targeting and Generation of ES Cells

The DJβ targeting vector was electroporated into $J\beta1^{\omega/\omega}$ ES cells (15) as previously described (30) to generate $J\beta1^{DJ\beta Neo/\omega}$ ES cells. Targeted clones were identified by Southern blot analysis with the 5'Dβ1 probe on *Eco*RI digested genomic DNA (5.5 kb $J\beta1^{DJ\beta Neo}$, 9.3 kb $J\beta1^{\omega}$) and confirmed with the 3'Jβ1 probe on *Hind*III digested DNA (5.7 kb $J\beta1^{DJ\beta Neo}$, 8.9 kb $J\beta1^{\omega}$). Targeted ES cells were infected with recombinant AdenoCre and subcloned. Cre-deleted subclones were identified by Southern blot analysis with the 5'Dβ1 probe on *Eco*RI digested genomic DNA (5.5 kb $J\beta1^{DJ\beta Neo}$, 8.7 kb $J\beta1^{DJ\beta}$, 9.3 kb $J\beta1^{\omega}$) and confirmed with the 3'Jβ1 probe on *Bam*HI digested DNA (8.7 kb $J\beta1^{DJ\beta Neo}$, 13.5 kb $J\beta1^{DJ\beta}$, 8.3 kb $J\beta1^{\omega}$).

Mice

Generation of $J\beta1^{\omega/\omega}$ mice was previously described (15). DO11.10 TCRβ transgenic mice (31) were bred with $J\beta1^{DJ\beta/DJ\beta}$ mice to generate $V\beta8^{Tg}J\beta1^{DJ\beta/DJ\beta}$ mice. Germline $V\beta14^{NT/+}$ mice were generated from LN2 ES cells (32) as described (33).

FACS

Cells from single cell suspensions of the thymuses and spleens of 4–6 week-old mice were counted and then stained with indicated combinations of FITC-conjugated anti-CD8, anti-Vβ5, anti-Vβ8, anti-Vβ10b, and anti-Vβ14 antibodies; PE-conjugated anti-CD4, anti-CD8, anti-Cβ, and anti-Vβ10b antibodies; APC- conjugated anti-Cβ and anti-CD4; biotin-conjugated anti-Vβ14 and anti-Vβ12; and SA-PE-Cy7, SA-FITC, and SA-APC reagents (BD Pharmingen). For analysis of DN subsets, thymocytes were stained with a cocktail of PE-conjugated antibodies for TCRβ, TCRδ, CD8, CD45R, CD19, CD11c, CD11b, Ter119, and NK.1, as well as PE-Cy7-conjugated anti-CD25 and APC-conjugated anti-CD117 antibodies (BD Pharmingen). Data acquisition was conducted on a BD FACSCalibur

equipped with BD CellQuest Pro and data analysis performed with FlowJo software (Tree Star). Each FACS experiment was done at least three separate times on independent mice of each genotype.

Hybridoma Analysis

Hybridomas were generated by fusion of the BW-1100.129.237 thymic lymphoma cell line (34) with concanavalin A and IL-2 stimulated αβ T cells as previously described (30). Genomic DNA was isolated and subjected to Southern blot analysis. The Southern blot analysis of TCRβ rearrangements was conducted with the 5'Dβ1 and 3'Jβ1 probes on either *Eco*RI or *Hind*III digested genomic DNA isolated from the hybridomas. The 5'Dβ1 probe is a 400 bp *Nhe*I fragment. The 3'Jβ1 probe is a 777 bp *Drd*I fragment.

Results

Generation of Mice with a Pre-Assembled DβJβ1.1 Complex

Our previous gene-targeted modification of the endogenous Dβ1Jβ1 cluster demonstrated that a *loxP* site and a novel *Bam*HI site inserted just 3' of Jβ1.2 on one allele could be used to distinguish between VβDJβ1.1 and VβDJβ1.2 rearrangements and had no discernable effects on thymocyte development, Vβ rearrangement, or VβDJβ1.1 and VβDJβ1.2 expression (15). Thus, we used gene-targeted mutation to replace the germline sequences spanning the Dβ1 and Jβ1.1 segments with a pre-assembled Dβ1Jβ1.1 complex (Figure 1) on a single allele of $Jβ1^{ω/ω}$ embryonic stem (ES) cells. This Dβ1Jβ1.1 complex was isolated by PCR amplification and subcloning of Dβ1Jβ1.1 joins on DJβ rearranged alleles of wild-type splenic αβ T cells. The isolated Dβ1Jβ1.1 complex does not contain N or P nucleotides and compared to the full sequences of Dβ1 and Jβ1.1 is missing one C nucleotide. The initial targeting event resulted in the replacement of the endogenous sequences with the Dβ1Jβ1.1 complex and insertion of a neomycin resistant gene (Neo^r) flanked by *loxP* sites just 3' of the endogenous Jβ1.2 segment, creating the $Jβ1^{DJβNeo}$ allele (Figure 1). Next, we deleted the Neo^r gene through transient expression of the Cre recombinase in $Jβ1^{DJβNeo/ω}$ ES cells to leave a single *loxP* site inserted just 3' of the endogenous Jβ1.2 segment, creating the $Jβ1^{DJβ}$ allele (Figure 1). We also inserted a novel *Hind*III site next to the *loxP* site to distinguish between TCRβ rearrangements on the $Jβ1^{DJβ}$ and $Jβ1^{ω}$ alleles (Figure 1). Finally, we used $Jβ1^{DJβ/ω}$ ES cells to generate germline $Jβ1^{DJβ/ω}$ mice and then bred these with $Jβ1^{ω/ω}$ mice and also with each other to generate $Jβ1^{DJβ/ω}$ and $Jβ1^{DJβ/DJβ}$ mice, respectively. The comparative analysis of $Jβ1^{ω/ω}$ and $Jβ1^{DJβ/DJβ}$ mice will allow us to evaluate whether complete subversion of the tri partite TCRβ recombination process influences αβ T cell development, TCRβ rearrangement, Vβ repertoire, and/or TCRβ allelic exclusion. In addition, the analysis of TCRβ rearrangements in $Jβ1^{DJβ/ω}$ will reveal whether the assembly of a DJβ complex could affect Vβ rearrangement or influence TCRβ selection.

Normal αβ T Cell Development in $Jβ1^{DJβ/ω}$ and $Jβ1^{DJβ/DJβ}$ Mice

To evaluate the potential influence of Dβ-to-Jβ rearrangement on αβ T cell development, we analyzed thymocytes and splenocytes isolated from $Jβ1^{ω/ω}$, $Jβ1^{DJβ/ω}$, and $Jβ1^{DJβ/DJβ}$ mice. Both $Jβ1^{DJβ/ω}$ and $Jβ1^{DJβ/DJβ}$ mice exhibited similar numbers of thymocytes and splenocytes as $Jβ1^{ω/ω}$ mice (data not shown). FACS analysis of $Jβ1^{DJβ/ω}$ and $Jβ1^{DJβ/DJβ}$

thymocytes with anti-CD4 and anti-CD8 antibodies revealed a distribution of DN, DP, and SP populations similar to those in $J\beta1^{\omega/\omega}$ thymocytes (Figure 2A,D,E). In addition, FACS analysis of $J\beta1^{\omega/\omega}$, $J\beta1^{DJ\beta/\omega}$ and $J\beta1^{DJ\beta/DJ\beta}$ splenocytes with anti-CD4 and anti-CD8 antibodies revealed similar percentages of $CD4^+$ and $CD8^+$ αβ T cells in $J\beta1^{DJ\beta/\omega}$ and $J\beta1^{DJ\beta/DJ\beta}$ mice, as compared to in $J\beta1^{\omega/\omega}$ mice (Figure 2B,E). FACS analysis of $J\beta1^{DJ\beta/\omega}$ and $J\beta1^{DJ\beta/DJ\beta}$ thymocytes with anti-CD117 and anti-CD25 antibodies showed a similar distribution of ETPs, stage II, stage III, and stage IV DN cells as $J\beta1^{\omega/\omega}$ thymocytes (Figure 2C,D). Despite these similarities, there were detectable differences in the DN III and DN IV populations and statistically significant differences in SP thymocytes and $CD4^+$ and $CD8^+$ splenic αβ T cell populations among $J\beta1^{\omega/\omega}$, $J\beta1^{DJ\beta/\omega}$ and $J\beta1^{DJ\beta/DJ\beta}$ mice (Figure 2D,E). These data demonstrate that, although neither a single fixed DJβ rearrangement nor the sequence of the particular DJβ complex used has any substantial effect on thymocyte development, the inability to assemble a diverse DJβ repertoire has subtle or significant influences on different stages of αβ T cell differentiation. The decreased ratios of DNIII to DNIV cell populations that correlate with increased copy number of the $J\beta1^{DJ\beta}$ allele may reflect that Vβ rearrangements occur at a higher frequency on the $J\beta1^{DJ\beta}$ allele in DNIII thymocytes and/or DNIII cells expressing VβDJβ chains from $J\beta1^{DJ\beta}$ alleles are preferentially selected. The statistically significant differences in SP thymocytes and $CD4^+$ and $CD8^+$ splenic αβ T cell populations among $J\beta1^{\omega/\omega}$, $J\beta1^{DJ\beta/\omega}$ and $J\beta1^{DJ\beta/DJ\beta}$ mice suggest that selection of αβ TCR containing VβDJβ chains with the pre-assembled Dβ1Jβ1.1 complex may be altered.

$J\beta1^{DJ\beta/\omega}$ and $J\beta1^{DJ\beta/DJ\beta}$ αβ T Cells Exhibit TCRβ Allelic Exclusion

To evaluate the contribution of a two-step recombination process on enforcement of TCRβ allelic exclusion, we assayed for the expression of two distinct TCRβ chains on the cell surface of $J\beta1^{DJ\beta/DJ\beta}$ αβ T cells. Since an allotypic marker has not been reported for TCRβ chains, we conducted FACS analysis with antibodies specific for two different Vβ segments. FACS analysis of $J\beta1^{DJ\beta/DJ\beta}$ splenocytes with anti-Vβ5/anti-Vβ10 or anti-Vβ8/anti-Vβ14 antibodies failed to reveal any obvious αβ T lymphocyte populations that expressed two Vβs on the cell surface (Figure 3A). Many studies of TCRβ allelic exclusion have been conducted using transgenic mice that express a pre-rearranged, in-frame VβDJβ rearrangement randomly integrated into the genome (10, 37). Thus, we also generated and analyzed TCRβ allelic exclusion in $J\beta1^{DJ\beta/DJ\beta}$ mice expressing a transgenic in-frame Vβ8DJβ rearrangement ($V\beta8^{Tg}$). FACS analysis of $V\beta8^{Tg}$ and $V\beta8^{Tg}J\beta1^{DJ\beta/DJ\beta}$ splenocytes with an anti-Cβ antibody and an anti-Vβ5, anti-Vβ8, anti-Vβ10, anti-Vβ12, or anti-Vβ14 antibody revealed the absence of αβ T cells expressing any Vβ on the cell surface without expression of Vβ8 in either mouse (Figure 3B). In addition, FACS analysis of $V\beta8^{Tg}$ and $V\beta8^{Tg}J\beta1^{DJ\beta/DJ\beta}$ splenocytes with an anti-Vβ8 antibody and either an anti-Vβ12 or anti-Vβ14 antibody did not detect any difference in αβ T cells expressing both Vβ8 and another Vβ on the cell surface (Figure 3C). Collectively, these data indicate that a two-step recombination process is not required for enforcement of TCRβ allelic exclusion, at least at the level of detection of FACS using anti-Vβ antibodies specific for TCRβ chains with two different Vβs.

Normal TCRβ Feedback Regulation in $J\beta1^{DJ\beta/\omega}$ and $J\beta1^{DJ\beta/DJ\beta}$ and αβ T Cells

The observed enforcement of TCRβ allelic exclusion in $J\beta1^{DJ\beta/\omega}$ and $J\beta1^{DJ\beta/DJ\beta}$ mice suggests that Vβ-to-DJβ rearrangements on $J\beta1^{DJ\beta}$ alleles are subject to TCRβ-mediated feedback inhibition. However, despite impaired TCRβ-mediated feedback inhibition in pTα deficient thymocytes, dual Vβ expressing αβ T cells are not observed in either pTα deficient mice or pTα deficient mice containing a TCRβ transgene, indicating that TCRβ allelic exclusion also can be enforced by post Vβ-to-DJβ recombination mechanisms (Fred, von Boehmer). The 60/40 ratio of αβ T cells with VβDJβ/DJβ and VβDJβ/VβDJβ rearrangements reflects the regulation of Vβ rearrangement by TCRβ-mediated feedback inhibition (10). Therefore, to evaluate the influence of a two-step recombination process on TCRβ-mediated feedback regulation, we generated in a panel of 88 $J\beta1^{DJ\beta/DJ\beta}$ αβ T cell hybridomas and analyzed TCRβ rearrangements by Southern blot analysis. We first assayed TCRβ rearrangements on *Eco*RI-digested genomic DNA using the 5'Dβ1 and 3'Jβ1 probes, each of which hybridizes to the same 5.5 kb *Eco*RI germline fragment from the $J\beta1^{DJ\beta}$ allele (Figure 1). We found that 55 of 88 (62%) $J\beta1^{DJ\beta/DJ\beta}$ αβ T cell hybridomas retained 5.5 kb 5'Dβ1 and 3'Jβ1 bands, indicating they contained only one VβDJβ rearrangement (Table IA). The remaining 33 of 88 (38%) lacked the 5.5 kb 5'Dβ1 and 3'Jβ1 bands, but contained two novel-sized 3'Jβ1 bands, revealing they carried VβDJβ rearrangements on both $J\beta1^{DJ\beta}$ alleles (Table IA). These data demonstrate that $J\beta1^{DJ\beta/DJ\beta}$ αβ T cells exhibit the normal 60/40 ratio of VβDJβ/DJβ and VβDJβ/VβDJβ rearrangements. Thus, Vβ rearrangements on $J\beta1^{DJ\beta}$ alleles are subject to normal TCRβ feedback regulation, consistent with the observed enforcement of TCRβ allelic exclusion in $J\beta1^{DJ\beta/DJ\beta}$ and $V\beta8^{Tg}J\beta1^{DJ\beta/DJ\beta}$ αβ T cells.

VβDJβ Rearrangements are Preferentially Selected on $J\beta1^{DJ\beta}$ Alleles

To evaluate whether the presence of the pre-assembled Dβ1Jβ1.1 complex might either enhance or impair Vβ-to-DJβ rearrangement, we first sought to determine whether overall Vβ rearrangements occur at a similar level on the $J\beta1^{DJ\beta}$ and $J\beta1^{\omega}$ alleles. Unfortunately, due to the inherent biases of amplifying VβDβJβ1 rearrangements of one fixed size on $J\beta1^{DJ\beta}$ alleles versus of six different sizes on $J\beta1^{\omega}$ alleles, solid conclusions are not possible from PCR-based analysis of Vβ-to-DJβ rearrangements in non-selected DNIII thymocytes. The novel *Hind*III site inserted just downstream of the pre-assembled DJβ1.1 complex enables us to distinguish between TCRβ rearrangements on the $J\beta1^{DJ\beta}$ and $J\beta1^{\omega}$ alleles in $J\beta1^{DJ\beta/\omega}$ αβ T cells. Thus, we generated a panel of 247 $J\beta1^{DJ\beta/\omega}$ αβ T cell hybridomas and analyzed VβDJβ rearrangements in these cells by Southern blot analysis and by PCR amplification and sequencing. We first assayed TCRβ rearrangements on *Eco*RI-digested genomic DNA using the 5'Dβ1 and 3'Jβ1 probes, each of which hybridizes to the same 9.4 kb germline *Eco*RI fragment on the $J\beta1^{\omega}$ allele (Figure 1). We found that all 247 hybridomas lacked the 9.4 kb 3'Jβ1 band, but contained a novel-sized 3'Jβ1 band, revealing they had DJβ or VβDJβ rearrangements on the $J\beta1^{\omega}$ allele. In addition, we found that 144 (58%) contained a single 5'Dβ1 band and 103 (42%) lacked any 5'Dβ1 bands. These data indicate that $J\beta1^{DJ\beta/\omega}$ αβ T cells also exhibit the normal ratio of VβDJβ/DJβ and VβDJβ/VβDJβ rearrangements.

If Vβ-to-DJβ rearrangements occur at equal frequency on the $J\beta1^{DJ\beta}$ and $J\beta1^{\omega}$ alleles and VβDJβ rearrangements involving the single pre-assembled Dβ1Jβ1.1 complex and the

normal repertoire of rearranged Dβ1Jβ1 complexes are similarly selected, $J\beta1^{DJ\beta/\omega}$ αβ T cells of the VβDJβ/DJβ configuration should contain an equal frequency of VβDJβ rearrangements on the $J\beta1^{DJ\beta}$ and $J\beta1^{\omega}$ alleles. Thus, we next assayed TCRβ rearrangements using the 5'Dβ1 probe on *Hind*III-digested genomic DNA of the 144 $J\beta1^{DJ\beta/\omega}$ αβ T cell hybridomas with VβDJβ rearrangements on a single allele. The 5'Dβ1 probe hybridizes to an 8.9 kb germline *Hind*III fragment on the $J\beta1^{\omega}$ allele and to a 2.5 kb germline fragment on the $J\beta1^{DJ\beta}$ allele (Figure 1). We found that 45 of 144 (31%) retained and 99 of 144 (69%) lost the 2.5 kb 5'Dβ1 band (Table IB), revealing that the majority of $J\beta1^{DJ\beta/\omega}$ αβ T cells contain VβDJβ rearrangements on the $J\beta1^{DJ\beta}$ allele. This observation indicates that either Vβ rearrangements occur at a higher frequency on the $J\beta1^{DJ\beta}$ allele in DN cells or thymocytes expressing VβDJβ chains from $J\beta1^{DJ\beta}$ alleles are preferentially selected during development.

In addition to their in-frame and selected VβDJβ rearrangements, ~60% of normal αβ T cells contain DJβ rearrangements and ~40% contain out-of-frame VβDJβ rearrangements on their non-selected alleles (10, 11). Since $J\beta1^{DJ\beta/\omega}$ αβ T cell hybridomas exhibit this normal 60/40 ratio, the frequency at which out-of-frame VβDJβ rearrangements occur on the $J\beta1^{DJ}$ allele of $J\beta1^{DJ\beta/\omega}$ αβ T cells with VβDJβ/VβDJβ rearrangements can be used to distinguish between increased recombination versus preferential selection. If Vβ-to-DJβ rearrangements occurred at an increased frequency on $J\beta1^{DJ\beta}$ alleles and were selected equally as those on $J\beta1^{\omega}$ alleles, two-thirds (66%) of VβDJβ joins on $J\beta1^{DJ\beta}$ alleles would be out-of-frame; whereas, if VβDJβ rearrangements were preferentially selected on the $J\beta1^{DJ\beta}$ allele, greater than one-third of these VβDJβ joins would be in-frame. Thus, we cloned and sequenced VβDJβ1.1 coding joins on the $J\beta1^{DJ\beta}$ allele of representative $J\beta1^{DJ\beta/\omega}$ αβ T cell hybridomas with VβDJβ rearrangements on both alleles. We conducted PCR reactions on the genomic DNA isolated from 25 of these hybridomas using primers that hybridize to each specific Vβ and a primer that hybridizes 3' of the *Hind*III site on the $J\beta1^{DJ\beta}$ allele (Figure 1). PCR products were digested with either *Hind*III or *Bam*HI to distinguish between amplified Vβ rearrangements to the pre-assembled Dβ1Jβ1.1 complex on the $J\beta1^{DJ\beta}$ allele versus to Dβ1Jβ1.1 or Dβ1Jβ1.2 complexes on the $J\beta1^{\omega}$ allele. Sequence analysis of 25 PCR products representing Vβ rearrangements to the pre-assembled DJβ1.1 complex revealed that 13 (52%) were out-of-frame and 12 (48%) were in-frame (Table II). This data suggests that cells expressing VβDJβ chains from $J\beta1^{DJ\beta}$ alleles overall are preferentially selected during thymocyte development, which is consistent with the decreased ratios of DNIII to DNIV cell populations that correlate with increased $J\beta1^{DJ\beta}$ copy number (Figure 1C, D). However, these experiments do not address whether the rearrangement frequencies of particular Vβ segments to the pre-assembled DJβ1.1 complex on the $J\beta1^{DJ\beta}$ allele are increased or decreased.

Altered Vβ Repertoire in $J\beta1^{DJ\beta/\omega}$ and $J\beta1^{DJ\beta/DJ\beta}$ Mice

Although the Vβ repertoire is not substantially altered during β-selection (17), the relative frequency at which Vβs are expressed in DP versus SP thymocytes can be dramatically different, indicating that the Vβ repertoire of TCRβ chains is shaped during αβ TCR selection (18–24). The statistically significant differences in SP cells among $J\beta1^{\omega/\omega}$, $J\beta1^{DJ\beta/\omega}$ and $J\beta1^{DJ\beta/DJ\beta}$ mice suggest that incorporation of the pre-assembled Dβ1Jβ1.1

complex in productive VβDJβ rearrangements may influence αβ TCR selection of DP thymocytes. To investigate this issue, we sought to evaluate whether the repertoire of TCRβ chains is altered upon DP to SP differentiation in $Jβ1^{DJβ/ω}$ or $Jβ1^{DJβ/DJβ}$ mice, as compared to in $Jβ1^{ω/ω}$ mice. TCRβ chains are expressed at "low/intermediate" levels on DP thymocytes undergoing αβ TCR selection and at "high" levels on positively selected SP cells (9). Thus, we conducted FACS analysis of $Jβ1^{ω/ω}$, $Jβ1^{DJβ/ω}$, and $Jβ1^{DJβ/DJβ}$ thymocytes with an anti-Cβ antibody and an anti-Vβ5, anti-Vβ8, anti-Vβ10, anti-Vβ12, or anti-Vβ14 antibody. As we have done previously to evaluate αβ TCR selection (Wu), we quantified the percentages of cells expressing particular Vβ segments in TCRβ "low/intermediate" (DP) and TCRβ "high" (SP) thymocytes. We found similar percentages of $Vβ10^+$ and $Vβ14^+$ TCRβ "intermediate/low" and "high" thymocytes in $Jβ1^{ω/ω}$, $Jβ1^{DJβ/ω}$, and $Jβ1^{DJβ/DJβ}$ mice (Figure 4A,B). In contrast, we detected similar percentages of $Vβ8^+$ and $Vβ5^+$ TCRβ "low/intermediate" thymocytes in $Jβ1^{ω/ω}$, $Jβ1^{DJβ/ω}$, and $Jβ1^{DJβ/DJβ}$ mice, but significant decreases in the percentages of $Vβ8^+$ and $Vβ5^+$ TCRβ "high" thymocytes in $Jβ1^{DJβ/ω}$ and $Jβ1^{DJβ/DJβ}$ mice, as compared to in $Jβ1^{ω/ω}$ mice (Figure 4A,B). We also observed a similar percentage of $Vβ12^+$ TCRβ "low/intermediate" thymocytes in $Jβ1^{ω/ω}$, $Jβ1^{DJβ/ω}$, and $Jβ1^{DJβ/DJβ}$ mice, and a subtle decrease in the percentage of $Vβ12^+$ TCRβ "high" thymocytes in $Jβ1^{DJβ/ω}$ and $Jβ1^{DJβ/DJβ}$ mice, as compared to in $Jβ1^{ω/ω}$ mice (Figure 4A,B). These data demonstrate that incorporation of the pre-assembled Dβ1Jβ1.1 complex in productive VβDJβ rearrangements influences αβ TCR selection of DP thymocytes expressing particular Vβs.

The relative frequency at which Vβs are expressed in peripheral αβ T cells also can be dramatically different due to αβ TCR selection in DP thymocytes (references). The statistically significant differences in $CD4^+$ and $CD8^+$ splenic αβ T cells among $Jβ1^{ω/ω}$, $Jβ1^{DJβ/ω}$ and $Jβ1^{DJβ/DJβ}$ mice suggest that incorporation of the pre-assembled Dβ1Jβ1.1 complex in productive VβDJβ rearrangements also may influence peripheral Vβ repertoire. To investigate this issue, we also evaluated whether the repertoire of TCRβ chains is altered in $Jβ1^{DJβ/ω}$ or $Jβ1^{DJβ/DJβ}$ splenic αβ T lymphocytes, as compared to in $Jβ1^{ω/ω}$ cells. To this aim, we conducted FACS analysis of $Jβ1^{ω/ω}$, $Jβ1^{DJβ/ω}$, and $Jβ1^{DJβ/DJβ}$ splenocytes with an anti-Cβ antibody and an anti-Vβ5, anti-Vβ8, anti-Vβ10, anti-Vβ12, or anti-Vβ14 antibody. We found a similar percentage of $Vβ14^+$ and $Vβ10^+$ splenic αβ T cells as in $Jβ1^{ω/ω}$ mice (Figure 4C,D). In contrast, we detected significant decreases in the percentage of $Vβ8^+$ and $Vβ5^+$ splenic αβ T cells in $Jβ1^{DJβ/ω}$ and $Jβ1^{DJβ/DJβ}$ mice, as compared to in $Jβ1^{ω/ω}$ mice (Figure 4C,D). We also observed an increase in the percentage of $Vβ12^+$ splenic αβ T cells in $Jβ1^{DJβ/ω}$ and $Jβ1^{DJβ/DJβ}$ mice, as compared to in $Jβ1^{ω/ω}$ mice (Figure 4C,D), however, these differences were not statistically significant due to variation in the percentage of $Vβ12^+$ cells in mice among experiments. These data demonstrate that incorporation of the pre-assembled Dβ1Jβ1.1 complex in productive VβDJβ rearrangements also influences the Vβ repertoire of peripheral αβ T cells.

Discussion

To evaluate the influence of a two-step recombination process on Vβ rearrangement and selection, we generated mice with a pre-assembled Dβ1Jβ1.1 complex on an endogenous TCRβ allele that also lack Dβ2-Jβ2 segments, creating the $Jβ1^{DJβ}$ allele on which Vβ

rearrangements only can be targeted to this one particular Dβ1Jβ1 complex of a defined sequence and length. Our comparative analysis of $Jβ1^{DJβ/DJβ}$ mice and $Jβ1^{ω/ω}$ mice in which Vβ rearrangements can be targeted to Dβ1Jβ1 complexes of six possible Dβ-Jβ joining combinations demonstrated that complete subversion of the tri partite TCRβ recombination process did not detectably alter Vβ rearrangement or TCRβ allelic exclusion. However, our analysis of TCRβ rearrangements in $Jβ1^{DJβ/ω}$ revealed that cells expressing VβDJβ chains from $Jβ1^{DJβ}$ alleles overall are preferentially selected during thymocyte development. In addition, we found that incorporation of the pre-assembled Dβ1Jβ1.1 complex in productive VβDJβ rearrangements influences αβ TCR selection of DP thymocytes expressing particular Vβs and also the Vβ repertoire of peripheral αβ T cells. Collectively, our findings indicate that a two-step recombination process is not essential for normal regulation of Vβ rearrangement, but the sequence of DJβ complexes assembled during thymocyte development can influence TCRβ chain selection and peripheral Vβ repertoire.

TCRβ genes are assembled in an ordered fashion such that Dβ-to-Jβ and Vβ-to-DJβ, but not Vβ-to-Dβ, rearrangements occur during thymocyte development. This ordered assembly of TCRβ genes is controlled, at least in part, through the developmental stage-specific initiation of Dβ/Jβ in ETP and DNII cells and Vβ recombinational accessibility in DNIII thymocytes (12, 38, 39), most likely directed by activation of germline Dβ-Jβ and Vβ promoters independent of TCRβ gene recombination events (Sikes, Sikes, Vb reference). Recent data demonstrating that c-fos deposits RAG onto 3'Dβ RSSs and that Vβ-to-Dβ rearrangements occur in *c-fos*$^{-/-}$ thymocytes (40) supports the model that RAG occupancy of 3'Dβ RSSs prevents synaptic complex formation between the RAG proteins and Vβ RSSs and 5'Dβ RSSs until Dβ-to-Jβ rearrangement and deletion of 3'Dβ RSSs (40, 41). The lack of a detectable increase in the level of overall Vβ-to-DJβ rearrangements on $Jβ1^{DJβ}$ alleles as compared to on $Jβ1^{ω}$ alleles in $Jβ1^{DJβ/ω}$ αβ T cell hybridomas argues against a predominant role of such a steric hindrance mechanism for enforcement of ordered TCRβ gene rearrangements. However, the genomic deletion associated with the pre-assembled Dβ1Jβ1.1 complex could alter germline Dβ1-Jβ1 transcription (Khor) and/or nucleosome positioning over the 5'Dβ1 RSS (Baumann, Golding, Kwon, and Nightingale) in a manner that reduces RAG accessibility and Vβ-to-DJβ recombination on the $Jβ1^{DJβ}$ allele. In addition, chromatin changes associated with RAG-mediated cleavage during Dβ-to-Jβ rearrangement (Chen), which would not occur on the $Jβ1^{DJβ}$ allele, also may facilitate RAG accessibility and Vβ-to-DJβ rearrangement. Finally, it is conceivable that Dβ-to-Jβ rearrangements may occur asynchronously between alleles where RAG-cleavage activates DNA damage signals that prevent recombination events on the other allele. If so, Dβ-to-Jβ rearrangements that occur first on $Jβ1^{ω}$ alleles could inhibit Vβ-to-DJβ rearrangements on $Jβ1^{DJβ}$ alleles.

TCRβ genes are also regulated such that in-frame VβDJβ rearrangements form only on a single allele in the majority of developing thymocytes. Despite intense efforts, the manner by which Vβ rearrangements are restricted to one allele at a time is completely unknown; though evidence for both stochastic and directed control mechanisms has been provided (10, 42, 43). Our current observations that Vβ rearrangements occur at a similar level on $Jβ1^{DJβ}$ and $Jβ1^{ω}$ alleles in $Jβ1^{DJβ/ω}$ αβ T cell hybridomas and TCRβ allelic exclusion is maintained

in Jβ1$^{DJβ/DJβ}$ mice have implications for the potential mechanisms that restrict Vβ rearrangement to one allele at a time. First, our data formally shows that Dβ-to-Jβ rearrangement *per se* is not required for mono-allelic assembly and expression of TCRβ genes, and TCRβ allelic exclusion is achieved exclusively through regulation of the Vβ-to-DJβ rearrangement step in developing αβ T cells. Second, within the context of stochastic models of TCRβ allelic exclusion that invoke low recombination efficiency of the Vβ rearrangement step as the underlying mechanism (10), our data demonstrate that deletion of 3'Dβ RSSs upon Dβ-to-Jβ rearrangement to relieve potential steric hindrance of 5'Dβ RSSs is not the sole rate-limiting mechanism that restricts Vβ rearrangements to only one allele at a time.

The assembly and expression of TCRβ genes and the selection of TCRβ chains associated with pTα molecules is required for the differentiation of DNIII thymocytes into DNIV and then DP cells (5). Expression of transgenic in-frame VβDJβ rearrangements leads to a reduction in the percentage of DNIII cells and a concomitant increase in the percentage of DNIV thymocytes, demonstrating that the assembly and expression of TCRβ chains is the rate-limiting step in early thymocyte development (44, 45). In this study, we demonstrate that Jβ1$^{DJβ/ω}$ and Jβ1$^{DJβ/DJβ}$ mice exhibit slight reductions in the frequency of DNIII thymocytes and concomitant increases in the frequency of DNIV cells. The magnitude of these changes corresponds to the number of Jβ1^{DJβ} alleles, suggesting that the DNIII to DNIV transition and β-selection are slightly enhanced by the presence of the pre-assembled DJβ complex. The lack of a detectable increase in the level of overall Vβ-to-DJβ rearrangements on Jβ1^{DJβ} alleles as compared to on Jβ1^{ω} alleles in Jβ1$^{DJβ/ω}$ αβ T cell hybridomas suggests that VβDJβ rearrangements involving the particular DJβ join used in this study are better able to pair and/or signal with pTα chains than VβDJβ rearrangements involving the population of DJβ joins normally assembled on Jβ1^{ω} alleles. However, we cannot rule out the possibility that increased rearrangement frequencies of particular Vβ segments to the pre-assembled DJβ1.1 complex on Jβ1^{DJβ} alleles as compared to the Dβ1Jβ1 complexes of six Dβ-Jβ joining possibilities on Jβ1^{ω} alleles in DNIII thymocytes contributes to this slightly "accelerated" early thymocyte development. In this regard, recombination efficiencies can be influenced by the nucleotide composition of coding sequences flanking participating RSSs (references). Unfortunately, firm conclusions require the accurate quantification of VβDJβ rearrangements in non-selected DNIII thymocytes, which is not possible due to the inherent biases of amplifying VβDβJβ1 rearrangements of one fixed size on Jβ1^{DJβ} alleles versus of six different sizes on Jβ1^{ω} alleles.

The generation and expression of a broad repertoire of antigen receptors on the surface of lymphocytes is critical for development and function of an effective adaptive immune system. For example, under representation of a particular Vκ segment (VκA2) in the peripheral Igκ repertoire of humans due to allelic polymorphisms is associated with an increased susceptibility to *Haemophilus influenzae* type b (Hib) since VκA2 segments are often used in anti-Hib antibodies (46). It has been known for quite some time that the Vβ repertoire assembled in DN thymocytes can be substantially shaped during αβ TCR selection in the DP thymocytes of mice expressing super-antigens (18–24). Our current observations that incorporation of the pre-assembled Dβ1Jβ1.1 complex in productive VβDJβ rearrangements affects αβ TCR selection of DP thymocytes expressing particular

Vβs and the Vβ repertoire of peripheral αβ T cells demonstrates that the sequence of DJβ complexes assembled during thymocyte development can influence TCRβ chain selection and peripheral Vβ repertoire, even in the absence of super-antigens. In mice, restriction of endogenous Vα rearrangements to a single functional Jα segment substantially impairs positive selection of αβ TCR in DP thymocytes and leads to a marked reduction in peripheral αβ T cell numbers (Huang). Moreover, mice containing a single endogenous D_H segment exhibit reduced numbers of bone marrow B cells and defective immune responses to a particular T-independent antigen (Schelonka). Furthermore, we previously demonstrated that the frequency of Vβ2 and Vβ14 rearrangements is reduced by the presence of other Vβ segments that compete with Vβ2 and Vβ14 for the productive coupling with DJβ1 complexes (Bassing). Thus, we hypothesize that the two Dβ-Jβ clusters and the six Jβ segments within each cluster may have evolved under selective pressure to ensure the most beneficial representation of Vβ segments expressed in the peripheral TCRβ repertoire of αβ T cells.

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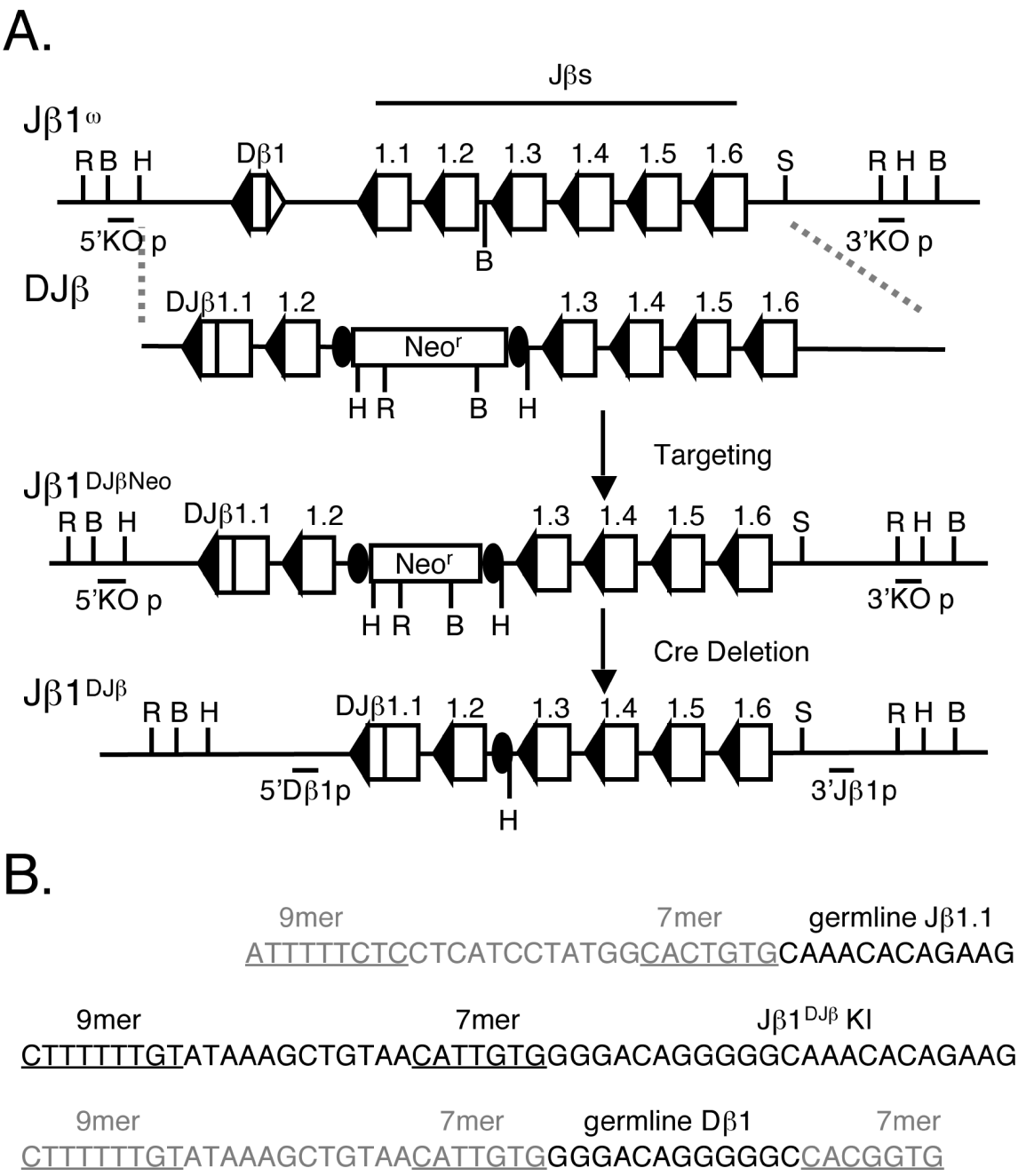

Figure 1. Gene-targeted generation of Jβ1$^{DJβ/ω}$ ES cells

(A) This schematic diagram illustrates the Jβ1^{ω} allele that lacks the Dβ2-Jβ2.7 segments, positioning of the DJβ targeting construct, and the resulting Jβ1^{DJβNeo} and Jβ1^{DJβ} alleles. Open boxes depict the Dβ1 and Jβ1 segments; their adjacent RSSs are triangles. Upon gene-targeting, the pre-assembled Dβ1Jβ1.1 complex replaced the germline Dβ1 and Jβ1.1 segments and a neomycin resistance gene (neo^{r}, rectangle) flanked by *loxP* sites (filled ovals) was inserted between the Jβ1.2 and Jβ1.3 segments. Following Cre-mediated deletion of the neo^{r} gene, the remaining segments align approximately to the position of an endogenous Dβ1Jβ1.1 complex. The solid horizontal bars indicate the relative locations of

the 5'KO, 3'KO, 5'Dβ1, and 3'Jβ1 probes. Restriction site designations: B, *Bam*HI; H, *Hind*III; R, *Eco*RI; S, *Sac*I. (B) Shown are the sequences of the germline Dβ1 segment with the 5'Dβ1 RSS and the heptamer of the 3'Dβ1 RSS, (top sequence), the pre-assembled Dβ1Jβ1.1 complex (middle sequence), and the germline Jβ1.1 segment with the Jβ1.1 RSS (bottom sequence).

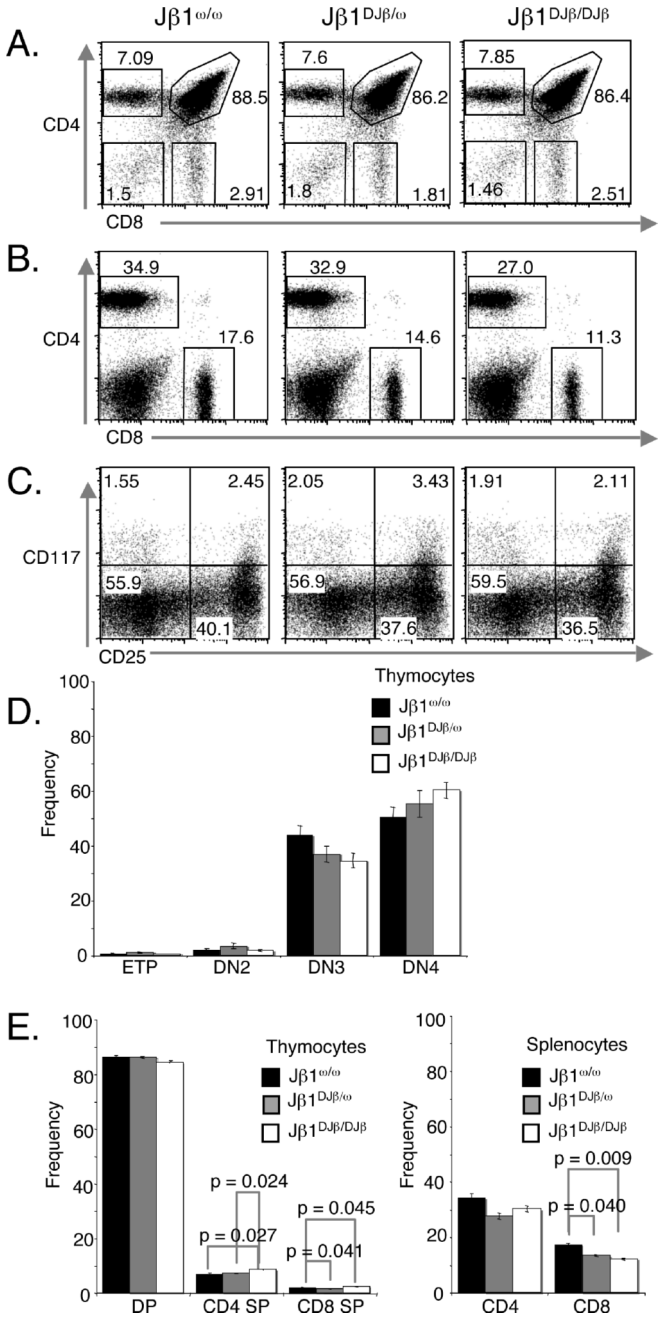

Figure 2. Normal αβ T cell development in Jβ1$^{DJβ/ω}$ and Jβ1$^{DJβ/DJβ}$ mice

(A–B) Shown are representative anti-CD4 and anti-CD8 FACS analysis of cells isolated from the (A) thymuses and (B) spleens of Jβ1$^{ω/ω}$, Jβ1$^{DJβ/ω}$ and Jβ1$^{DJβ/DJβ}$ mice. The percentage of (A) DN, DP, CD4$^+$ SP, and CD8$^+$ SP thymocytes and (B) CD4$^+$ and CD8$^+$ αβ T cells is indicated. (C) Shown are representative anti-CD117 and anti-CD25 FACS analysis of thymocytes negative for mature cells markers (TCRβ, TCRδ, CD4, CD8α, CD19, CD11c, CD11b, B220 and NK1.1). (D,E) Bar graphs showing the average frequency of cells within each thymocyte developmental stage and peripheral T cell population 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 t-test.

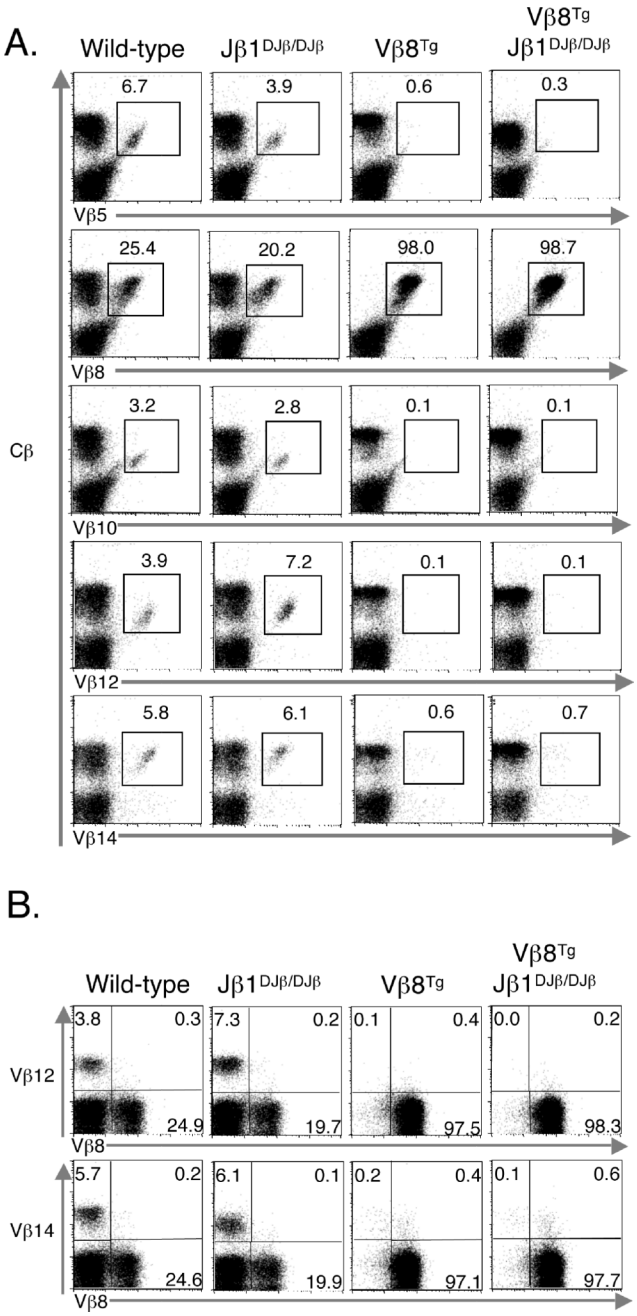

Figure 3. Jβ1$^{DJβ/DJβ}$ αβ T cells exhibit TCRβ allelic exclusion

(A) Shown are representative anti-Vβ5 and anti-Vβ10 or anti-Vβ8 and anti-Vβ14 FACS analysis of Cβ$^+$ cells isolated from the spleens of Jβ1$^{ω/ω}$ and Jβ1$^{DJβ/DJβ}$ mice. The percentage of Vβ5$^+$, Vβ10$^+$, and Vβ5$^+$Vβ10$^+$ or Vβ8$^+$, Vβ14$^+$, and Vβ8$^+$Vβ14$^+$ cells is indicated. (B) Shown are representative anti-Cβ and anti-Vβ5, anti-Vβ8, anti-Vβ10, anti-Vβ12, or anti-Vβ14 FACS analysis of cells isolated from the spleens of wild-type, Jβ1$^{DJβ/DJβ}$, Vβ8^{Tg}, and Vβ8^{Tg}Jβ1$^{DJβ/DJβ}$ mice. The percentage of Cβ$^+$ cells that express Vβ5, Vβ8, Vβ10, Vβ12, or Vβ14 is indicated. (C) Shown are representative anti-Vβ12 and anti-Vβ8 or anti-Vβ14 and anti-Vβ8 FACS analysis of Cβ$^+$ cells isolated from the spleens of

wild-type, $J\beta1^{DJ\beta/DJ\beta}$, $V\beta8^{Tg}$, and $V\beta8^{Tg}J\beta1^{DJ\beta/DJ\beta}$ mice. The percentage of $V\beta12^+$, $V\beta8^+$, and $V\beta12^+V\beta8^+$ or $V\beta14^+$, $V\beta8^+$, and $V\beta14^+V\beta8^+$ cells is indicated.

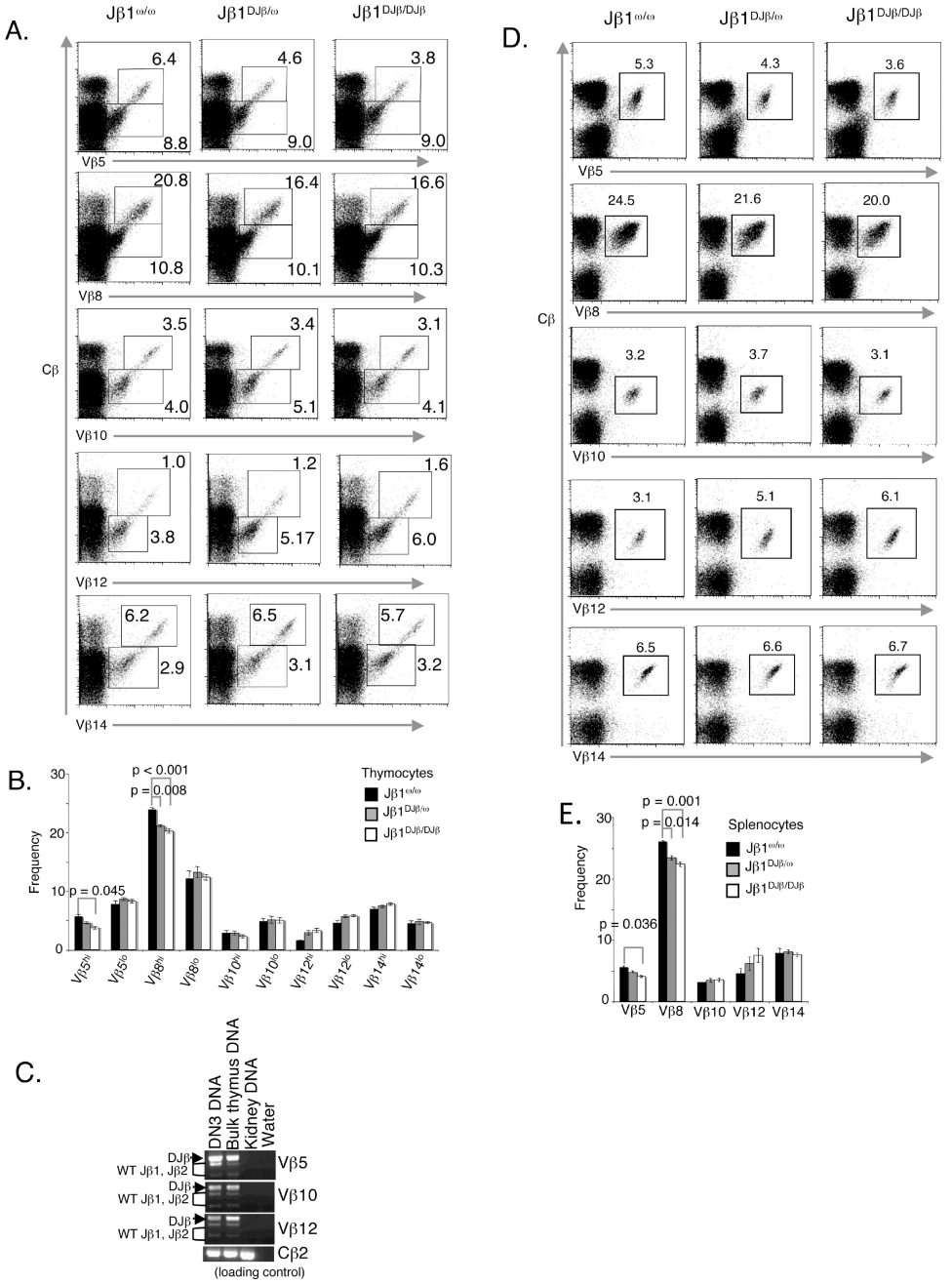

Figure 4. Altered αβ TCR selection and Vβ repertoire in Jβ1$^{DJβ/ω}$ and Jβ1$^{DJβ/DJβ}$ mice

(A) Shown are representative anti-Cβ and anti-Vβ5, anti-Vβ8, anti-Vβ10, anti-Vβ12, or anti-Vβ14 FACS analysis of cells isolated from the thymuses of Jβ1$^{ω/ω}$, Jβ1$^{DJβ/ω}$ and Jβ1$^{DJβ/DJβ}$ mice. The percentage of Cβ^{+} "low/intermediate" and "high" cells that express Vβ5, Vβ8, Vβ10, Vβ12, or Vβ14 is indicated. (B) Bar graphs showing the average percentage Cβ^{+} "low/intermediate" and "high" thymoctyes that express Vβ5, Vβ8, Vβ10, Vβ12, or Vβ14 is indicated. These values were obtained from three mice of each genotype. The error bars are standard error of the mean. Significant differences have been calculated using a two-tailed student t-test. (C) Shown are representative anti-Cβ and anti-Vβ5, anti-

Vβ8, anti-Vβ10, anti-Vβ12, or anti-Vβ14 FACS analysis of cells isolated from the spleens of $J\beta1^{\omega/\omega}$, $J\beta1^{DJ\beta/\omega}$ and $J\beta1^{DJ\beta/DJ\beta}$ mice. The percentage of $C\beta^{+}$ cells that express Vβ5, Vβ8, Vβ10, Vβ12, or Vβ14 is indicated. (D) Bar graphs showing the average percentage $C\beta^{+}$ splenic αβ T cells that express Vβ5, Vβ8, Vβ10, Vβ12, or Vβ14 is indicated. These values were obtained from three mice of each genotype. The error bars are standard error. Significant differences have been calculated using a two-tailed student t-test.

Table I

A. ***TCRβ rearrangement phenotypes in Jβ1$^{DJβ/DJβ}$ and Jβ1$^{DJβ/ω}$ αβ T cell hybridomas***[1]

Genotype	Total	VβDJβ/DJβ	VβDJβ/VβDJβ
Jβ1$^{DJβ/DJβ}$	88	55 (62.5%)	33 (37.5%)
Jβ1$^{DJβ/ω}$	247	144 (58.0%)	103 (42.0%)

B. ***Frequency of complete VβDJβ exon rearrangements on the DJβ allele versus the ω allele in heterozygous VβDJβ/DJβ Jβ1$^{DJβ/ω}$ αβ T cell hybridomas (144 total)***[2]

Genotype	Allele	Vβ-to-DJβ
Jβ1$^{DJβ/ω}$	DJβ	99 (69.0%)
	ω	45 (31.0%)

[1] Results for sections A and B were collected from hybridomas grown from at least three independent fusions. See *Materials and Methods* for details.

[2] VβDJβ rearrangements in Jβ1$^{DJβ/ω}$ mice could be linked to the different alleles using a unique restriction digest pattern introduced in the DJβ targeting. See *Materials and Methods* and FIGURE 1 for details.

Table II

Sequence analysis of VβDJβ coding joins on the Jβ1^{DJβ} alleles of Jβ1$^{DJβ/ω}$ αβ T cell hybridomas with bi-allelic VβDJβ rearrrangements

Vβ	Sequence (Codons)	P	N	P	DJβ Sequence (Codons)	Frame
Vβ16	AGC TTA GCC				AG GGG GCA	Out
Vβ11	AGC AGC CTC				AG GGG GCA	Out
Vβ8.2	AGC GGT GA		ATA		A CAG GGG GCA	In
Vβ10	GCC AGC AGC		TAT G		G GGG GCA	Out
Vβ13	AGC AGT TTC				CCG GGA CCA	Out
Vβ12	GCC AGC AGG				CAG GGG GCA	In
Vβ9	AGC AGT AGA				G GGG GCA	Out
Vβ15	TGT GGT GCT		CC	TC	GA CAG GGG GCA	In
Vβ8.2	GCC AGA GGT		GAA A		GA CAG GGG GCA	In
Vβ7	AGC AGT TTA		TAC		CAG GGG GCA	In
Vβ6	GCC AGC AGT A		AC		CAG GGG GCA	In
Vβ3	AGC AGT CTC		CTA		A CAG GGG GCA	Out
Vβ8.2	AGC GGT GAT		G	C	G GGG GCA	In
Vβ1	AGC AGC CAA				GA CAG GGG GCA	Out
Vβ1	AGC AGC		CA		A CAG GGG GCA	In
Vβ11	AGC AGC CT				A CAG GGG GCA	In
Vβ11	AGC AGC		TTA GG	T	GGG GCA	Out
Vβ12	AGC AGT C		A	CCC	G GGA CAG GGG GCA	In
Vβ10	AGC AGC		TA		GA CAG GGG GCA	Out
Vβ11	AGC AGC CT		T CAA CCC T		GA CAG GGG GCA	In
Vβ9	AGC AGC C		CA		GA CAG GGG GCA	Out
Vβ16	AGC TTA G		TG	C	G GGG GCA	Out
Vβ1	AGC AGC CA				GA CAG GGG GCA	Out
Vβ11	AGC AGC		TT		G GGA CAG GGG GCA	In
Vβ11	AGC AGC C		CA		GA CAG GGG GCA	Out