Posttranscriptional Silencing of VβDJβCβ Genes Contributes to TCR β Allelic Exclusion in Mammalian Lymphocytes

Natalie C. Steinel, Brenna L. Brady, Andrea C. Carpenter, Katherine S. Yang-Iott and Craig H. Bassing

J Immunol 2010; 185:1055-1062; Prepublished online 18 June 2010; doi: 10.4049/jimmunol.0903099
http://www.jimmunol.org/content/185/2/1055

References
This article cites 42 articles, 16 of which you can access for free at: http://www.jimmunol.org/content/185/2/1055.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Posttranscriptional Silencing of $V_\beta DJ_\beta C_\beta$ Genes Contributes to TCR$\beta$ Allelic Exclusion in Mammalian Lymphocytes

Natalie C. Steinel,*†,1 Brenna L. Brady,*†,1 Andrea C. Carpenter,* Katherine S. Yang-Iott,† and Craig H. Bassing*†

Feedback inhibition of V(D)J recombination enforces Ag receptor allelic exclusion in mammalian lymphocytes. Yet, in-frame $V_\beta DJ_\beta$ exons can assemble on both alleles in human and mouse $\alpha/\beta$ T lineage cells. To elucidate mechanisms that enforce TCR$\beta$ allelic exclusion in such cells, we analyzed $V_\beta$ expression and rearrangement in mice containing a functional $V_\beta 14 DJ_\beta 1.5 C_\beta 1$ gene ($V_\beta 14^{NT}$) and/or $V_\beta 8.2 DJ_\beta 1.1 C_\beta 1$ transgene ($V_\beta 8^{tg}$). The majority of $V_\beta 14^{NT}$ and $V_\beta 8^{tg}$ $\alpha/\beta$ T lineage cells expressed only $V_\beta 14^+$ or $V_\beta 8^+$ TCR$\beta$-chains, respectively, and lacked $V_\beta$ rearrangements on wild-type TCR$\beta$ loci. However, endogenous $V_\beta$ rearrangements and $\alpha/\beta$ T lineage cells expressing endogenous $V_\beta$s from wild-type alleles alone or with the prerearranged $V_\beta$ in cell surface TCR$\beta$-chains were observed in $V_\beta 14^{NT}$ and $V_\beta 8^{tg}$ mice. Although nearly all $V_\beta 8^{tg}$; $V_\beta 14^{NT}$ thymocytes and splenic $\alpha/\beta$ T cells expressed $V_\beta 8^+$ TCR$\beta$-chains, only half of these lymphocytes expressed $V_\beta 14^+$ TCR$\beta$-chains, even though similar steady-state levels of $V_\beta 14^{NT}$ mRNA were expressed in $V_\beta 8^+ V_\beta 14^+$ and $V_\beta 8^+ V_\beta 14^+$ populations. Our data demonstrated that posttranscriptional silencing of functionally assembled endogenous $V_\beta DJ_\beta C_\beta$ genes can enforce TCR$\beta$ allelic exclusion and reveal another mechanism that contributes to the development of lymphocytes with monospecific Ag receptors. The Journal of Immunology, 2010, 185: 1055–1062.

The adaptive immune systems of jawed vertebrates consist of T and B lymphocytes that express cell surface T cell Ag receptor (TCR) or B cell Ag receptor complexes, TCR and Ig V region exons are assembled in developing lymphocytes through the recombination of germline V(D)J gene segments (1). In mammals, the combination of possible rearrangement events within single genetic loci encoding each TCR and Ig chain contributes to diversification of Ag receptor binding specificities. However, in cartilaginous fish, each individual type of Ig chain is encoded by fully preassembled, partially preassembled, or unassembled germline gene segments located within hundreds of independent genetic loci (2). Most lymphocytes in jawed vertebrates express cell surface Ag receptor chains from a single allele or locus, a phenomenon that is referred to as Ag receptor allelic or haplotypic exclusion. For example, ~99% of mouse and human $\alpha/\beta$ T cells express cell surface TCR$\beta$-chains from a single allele (3–5). The majority of lymphocytes in mice and humans assemble a single in-frame exon within TCR$\beta$, IgH, and IgL loci due to feedback inhibition of variable, diversity, and joining [V(D)J] recombination, which is signaled by the expression of functional TCR or Ig chains and enforces Ag receptor allelic exclusion (6). In contrast, restricted expression of functional Ig genes from a single genetic locus seems to be the major mechanism that mediates haplotype exclusion in lymphocytes of cartilaginous fish (7).

In humans and mice, $\alpha/\beta$ T lymphocytes develop through a differentiation program that involves the assembly, expression, and selection of a functional $V_\beta DJ_\beta C_\beta$ gene from one allele (8). TCR$\beta$ genes are assembled through DJ$\beta$ intermediates in CD4$^+$CD8$^-$ (double-negative [DN]) thymocytes (9). Transcription through a functional $V_\beta DJ_\beta$ rearrangement generates TCR$\beta$-chains that can pair with pTc molecules to form pre-TCRs (8). These receptors signal feedback inhibition of further V$\beta$ rearrangement to enforce TCR$\beta$ allelic exclusion and select DN cells for differentiation into CD4$^+$CD8$^-$ (double-positive [DP]) thymocytes (8). DN cells that assemble an out-of-frame $V_\beta DJ_\beta$ rearrangement on the first allele can attempt V$\beta$ rearrangement on the second allele (9). In DP cells, TCR$\gamma$ genes are assembled on both alleles from $\gamma_\delta$ and $\alpha\beta$ segments (10). In-frame V$\alpha\delta$ rearrangements generate TCR$\alpha$-chains that can associate with TCR$\beta$ molecules to form $\alpha\beta$ TCRs (8). Positive selection of $\alpha\beta$ TCRs promotes further differentiation of DP cells into CD4$^+$ or CD8$^+$ (single-positive) thymocytes (8). These cells exit the thymus and migrate to the spleen and other peripheral locations as naive mature $\alpha/\beta$ T cells. However, DP thymocytes expressing autoreactive $\alpha\beta$ TCRs are frequently eliminated by apoptosis (8). TCR$\beta$ allelic exclusion has been hypothesized to prevent autoimmunity by facilitating the development and selection of cells with $\alpha\beta$ TCRs of a single specificity (11).

Generation and analysis of mice containing different preassembled $V_\beta DJ_\beta C_\beta$ transgenes demonstrated that expression of a functional TCR$\beta$-chain can inhibit rearrangement and expression of endogenous V$\beta$ segments (12). Enforcement of allelic exclusion by such feedback inhibition predicts that ~60% of $\alpha/\beta$ T cells...
contain DJb intermediates and ∼40% contain out-of-frame VβDJb rearrangements on their nonselected alleles (13). Yet, sequence analyses of TCRβ joins or mRNA revealed the presence of two in-frame VβDJb rearrangements in 5–10% of mouse and human αβ T cells that exhibit allelic exclusion (14, 15). In addition, in-frame endogenous Vβ14b rearrangements were found, but not expressed, in ∼10% of αβ T cell hybridomas generated from mice with a modified TCRβ locus that permits direct Vβ14- to-Jβ rearrangement (16). Moreover, VβDJbCβ genes that were assembled in-frame within a transgenic TCRβ mini-locus were not expressed on αβ T cells of mice containing a preassembled TCRβ transgene (17). Furthermore, although TCRβ-mediated feedback inhibition is blocked in pTα−/− thymocytes, TCRβ allelic exclusion is maintained in the αβ T lineage cells of pTα−/− mice (18, 19). Collectively, these data indicate that additional mechanisms must restrict the cell surface expression of functionally assembled TCRβ genes; however, the absence of an allotopic Cβ marker in humans and mice has prevented definitive conclusions. Thus, to elucidate mechanisms that enforce allelic exclusion in cells with two functional VβDJbCβ genes, we analyzed Vβ expression and rearrangement in αβ T lineage cells of mice containing one allelic copy of a preassembled functional endogenous TCRβ gene and/or classical TCRβ transgene.

Materials and Methods

Mice

Generation and characterization of Vβ8TG mice and LN2 embryonic stem cells containing the preassembled Vβ14DJb1.5Cβ gene were previously described (20, 21). Vβ8TG mice were bred onto a 129SvEv background and mated with one another to maintain one allelic copy of a preassembled functional endogenous Vβ14b gene and/or classical TCRβ transgene.

Flow cytometry

Single-cell suspensions of lymphocytes from thymuses and spleens were incubated with RBC lysis buffer (0.7 M NaCl and 17 mM Tris HCl). Cells were stained with the following Abs from BD Pharmingen (San Diego, CA): allophycocyanin–anti-Cβ (553174), allophycocyanin–cy7–anti-B220 (552094), FITC–anti-Vβ8 (553258), PE–anti-Vβ8 (553862), PE–anti-Vβ10 (553285), biotin–anti-Vβ8 (553192), biotin–anti-Vβ5 (553188), and PE–Cy7–strepavidin (557598). Cells were stained in FACS staining buffer. Live cells were gated on the basis of forward or side scatter and DAPI exclusion (D1306, Invitrogen). Data were collected on an LSR II and were analyzed using FlowJo; 500,000 events were collected for each sample file. All displayed events were gated on single DAPI+ B220+ TCRβ+ cells.

PCR analysis of Vβ rearrangements

Total thymocytes or spleenocytes were lysed in rapid lysis buffer (0.1 M Tris [pH 8.5], 0.2% SDS, 0.005 M EDTA, 0.2 M NaCl, and 250 μg/ml protease K). Genomic DNA was isolated by isopropanol precipitation. PCR conditions for a final volume of 25 μl were 10× PCR Buffer (Qiagen, Valencia, CA), 0.2 mM 2′-deoxyadenosine 5′-triphosphate (Applied Biosystems, Foster City, CA), 0.2 mM each primer, 5 U HotStarTaq polymerase (Qiagen), and 500 ng DNA. PCR cycles were 94°C for 3 min; 40 cycles of 94°C for 45 s, 60°C for 1 min, 30 s, and 72°C for 2 min, 30 s; and 72°C for 10 min. The Vβ-specific primers and the 3′ Jβ1.2 primer (P2) were described previously (22). The 3′ Jβ2.2 primer was 5′-CTC-CACCCCTGACTGATCCCTGCCACC-3′. The Cβ2 primers were 5′-CA-AACAAAAAGGCTACCTCCCTG-3′ and 5′-GCAGACAGACACCCCCCTG-ATGATAG-3′.

Generation and analysis of hybridomas

The generation and analysis of TCRβ gene rearrangements in Vβ14NT/− and Vβ14NT/NT αβ T cell hybridomas were conducted as previously described (16, 23, 24).

Analysis of Vβ14DJb1.5Cβ1 mRNA expression

The sort purification of Vβ14V8B8+ and Vβ14V8B8− thymocytes from Vβ8TG/Vβ14NT mice was conducted on a FACS Aria, with staining and gating strategy identical to that described above for flow cytometry. RNA was isolated using TRIzol, and poly-A cDNA was generated using the NEB Protoscript II cDNA synthesis kit. Expression levels of Vβ14DJb1.5Cβ1 and GAPDH mRNAs were determined by quantitative PCR (qPCR) on an ABI 7500 Fast Real-Time PCR machine using the following primer pairs: Vβ14F 5′-AAGCCCAATGCCTAGTATTGTG-3′ and Vβ14R 5′-TGGAGGTTGGGACACTTGAG-3′ and GAPDH 5′-CTTCACCACCATGGAGAGCC-3′ and GAPDH 5′-GGCATGACTGGTGCTGATGAG-3′.

Results

Expression of endogenous Vβ segments in αβ T lineage cells of mice containing a preassembled functional VβDJbCβ gene

Most investigations of TCRβ allelic exclusion have been conducted through analyses of mice expressing preassembled functional VβDJbCβ transgenes. The physiologic relevance of such studies has been questioned because of the varying extents to which transgenes enforce allelic exclusion, the high copy number of transgenes often required for allelic exclusion, and other potential transgenic artifacts (19, 25, 26). Thus, we sought to study TCRβ allelic exclusion in mice containing a single allelic copy of a preassembled functional endogenous VβDJbCβ gene. Chimeric mice containing preassembled in-frame endogenous TCR genes have been generated through the transfer of αβ T cell nuclei into embryonic stem cells (20). We used stem cells reconstituted with the nucleus of a Vβ14+ αβ T cell to establish mice containing a preassembled functional endogenous Vβ14DJb1.5Cβ1 gene (Vβ14NT) within their germline. These mice were bred with WT mice to separate the Vβ14NT allele from the other prearranged TCR alleles, and their offspring were intercrossed to establish mice containing the Vβ14NT gene on one (Vβ14NT/−) or two (Vβ14NT/NT) alleles (Fig. 1A). FACS analysis of Vβ14NT/− and Vβ14NT/NT thymocytes and splenocytes with anti-Vβ14 and anti-Cβ Abs revealed that most Vβ14NT/− cells and all Vβ14NT/NT cells expressed Vβ14 within surface TCRβ-chains (Fig. 1B). Notably, cell populations lacking Vβ14 within surface TCRβ-chains (Vβ14−) were detectable in Vβ14NT/−, but not Vβ14NT/NT, mice (Fig. 1B). These data indicated that expression of the preassembled functional Vβ14DJb1.5Cβ1 gene within cell-surface TCRβ-chains can be silenced in Vβ14NT/− mice.

In mice, Vβ8 is the most highly presented Vβ within cellsurface αβ TCR because three individual Vβ segments (Vβ8.1, Vβ8.2, and Vβ8.3) exist (27). The presence of Vβ8V8− and Vβ8−Vβ8+ splenic T cells was observed in mice containing a Vβ3+ TCRβ transgene that prevents the expression of other endogenous Vβ segments (5). Thus, in an initial attempt to characterize the Vβ14+ Cβ+ αβ T cell populations in Vβ14NT/− mice, we conducted FACS analysis of Vβ14NT/− and Vβ14NT/NT thymocytes and splenocytes with combinations of anti-Vβ8, anti-Vβ14, and anti-Cβ Abs. In Vβ14NT/− mice, we detected populations expressing only Vβ14, only Vβ8, or Vβ14 and Vβ8 within surface TCRβ-chains (Fig. 1C). The frequencies of Vβ8+ αβ T lineage cells were significantly lower in Vβ14NT/− mice compared with WT mice (107-fold lower in thymocytes and 57-fold lower in splenocytes) (Fig. 2B, 2C). Yet, we found only Vβ14+Vβ8− Cβ− populations in Vβ14NT/− mice (Fig. 1C), indicating that the Vβ14+ Cβ− populations in Vβ14NT/− mice represent bona fide αβ T cells rather than staining artifacts. These data demonstrated
that can be expressed as part of cell-surface TCRs. We used FACS analysis of V8-2 locus to evaluate whether endogenous V8 segments other than V88 are expressed in TCRb-chains on αβ T cells of V8b14NT/NT mice, we conducted FACS analysis of V8b14NT/NT and V8b14NT/NT transgenic mice, we conducted the same FACS analyses on thymocytes and splenocytes with combinations of anti-V85, anti-V86, anti-V810, anti-V812, and anti-Cb Abs. We found cell populations expressing V85 or V86 segments within surface TCRb-chains on V8b14 and V8b14- cells in V8b14NT/NT (Fig. 2A). Yet, we observed only V8b14-Cb+ populations in V8b14NT/NT mice (Fig. 2A), indicating that the V85-Cb+ and V86-Cb+ cells in V8b14NT/NT mice also represent bona fide αβ T cells rather than staining artifacts. We were unable to detect bona fide αβ T lineage cells expressing V810 or V812 within TCRb-chains on V8b14NT/NT or V8b14NT/NT mice (Fig. 2C). These data demonstrated that a limited repertoire of endogenous V8 segments is expressed within TCRb-chains on V8b14NT/NT αβ T lymphocytes, which results in TCRb allelic inclusion or occurs in association with silenced cell-surface expression of the preassembled functional V8b14DJ1.5Cb1 gene.

For purposes of comparison between V8b14NT and TCRb transgenic mice, we conducted the same FACS analyses on thymocytes and splenocytes of mice expressing a preassembled functional V8b8.2Db1.1Cb1 transgene from one allele (V8b8Tg) (21). V8b8Tg mice were shown to exhibit feedback inhibition of Vb rearrangement and TCRb allelic exclusion (25, 29). Consistent with these published findings, we observed that almost all V8b8Tg αβ T cells expressed V88 within cell-surface TCRb-chains (Fig. 3A). We failed to detect significant populations of αβ T cells expressing V810 in V8b8Tg mice (Fig. 3B). Although we observed cell populations expressing V85, V86, and V810 segments within surface TCRb-chains of V8b8Tg αβ T lineage cells (Fig. 3B), the frequencies of cells expressing these V8 segments in V8b8Tg mice were significantly lower than those in WT mice (V85 was 23-fold lower in thymocytes and 47-fold lower in splenocytes; V86 was...
Our observations that endogenous Vβ segments are expressed within TCRβ-chains on the surface of Vβ14^{NT/4} and Vβ8^{Tg} αβ T cells indicate that Vβ rearrangements must have occurred on WT alleles in developing Vβ14^{NT/4} and Vβ8^{Tg} thymocytes. Because surface expression of preassembled functional VβDJβCβ-chains can be silenced, FACS analysis with anti-Vβ-specific Abs cannot be used as an accurate readout of Vβ-to-DJβ rearrangements. Thus, to ascertain the repertoire of Vβ rearrangements in αβ T lineage cells of Vβ14^{NT/4} and Vβ8^{Tg} mice, we conducted PCR-based analysis of VβDJβ joins in WT, Vβ8^{Tg}, Vβ14^{NT/4}, and Vβ14^{NT/NT} thymocytes. The murine TCRβ locus contains 20 functional Vβ segments and two DJb-Jβ clusters (DJb1-Jβ1 and DJb2-Jβ2), each with one DJβ segment and six functional Jβ segments (Fig. 3A). We used combinations of Vβ- and Jβ-specific primers to amplify potential rearrangements of each functional endogenous Vβ segment to DJβ complexes involving Jβ1.1/Jβ1.2 or Jβ2.1/Jβ2.2 segments (Fig. 3A). We found that the levels of rearrangements involving many Vβ segments to DJβ1.1/DJβ1.2 and DJβ2.1/DJβ2.2 complexes were undetectable or substantially reduced in Vβ8^{Tg} and Vβ14^{NT/4} cells compared with in WT cells (Fig. 3B). In contrast, we found that the levels of rearrangements involving Vβ5, Vβ6, Vβ7, Vβ8, Vβ14, Vβ15, Vβ16, and Vβ17 segments to DJβ1.1/DJβ1.2 complexes were unchanged or slightly reduced in Vβ8^{Tg} and Vβ14^{NT/4} cells compared with in WT cells (Fig. 3B). These data demonstrated that a limited repertoire of endogenous Vβ segments can rearrange at appreciable levels in Vβ14^{NT/4} and Vβ8^{Tg} thymocytes, despite the presence of a preassembled functional VβDJβCβ gene/transgene.

Our PCR data seem to be in conflict with previous studies concluding that the levels of rearrangements involving all endogenous Vβ segments are substantially reduced in αβ T lineage cells of Vβ8^{Tg} mice (25, 29). These previous experiments quantified Vβ rearrangements only by PCR amplification of VβDJβ joins because, theoretically, VβDJβ joins can form on extra-chromosomal excision circles that might not be subject to feedback inhibition. To our knowledge, the direct quantification of chromosomal VβDJβ rearrangements in αβ T cells of mice containing preassembled TCRβ transgenes/genomes has not been reported. Therefore, we generated panels of Vβ14^{NT/4} and Vβ8^{Tg} αβ T cell hybridomas and quantified chromosomal TCRβ rearrangements by Southern blot analysis on EcoRI-digested genomic DNA using a series of TCRβ locus probes. Of the 82 Vβ14^{NT/4} hybridomas analyzed, 66 (81%) contained DJβ1 joins and 10 (12%) contained VβDJβ rearrangements on the WT TCRβ allele. Similarly, of the 129 Vβ8^{Tg} hybridomas analyzed, 102 (79%) contained DJβ1 rearrangements on one or both WT alleles, and 12 (9.3%) contained VβDJβ rearrangements on one or both TCRβ alleles (Table I). The remaining 6 (7%) Vβ14^{NT/4} and 15 (11%) Vβ8^{Tg} αβ T cell hybridomas contained germline TCRβ loci, Vβ-to-DJβ rearrangement, or rearranged loci with Southern blot patterns suggesting aberrant DJβ-to-Jβ rearrangements that deleted Jβ-coding sequences (Table I). In addition to VβDJβ joins on selected alleles, ~60% of normal αβ T cells contained DJβ joins, and ~40% contained VβDJβ joins on nonselected alleles. Accordingly, the overall level of chromosomal VβDJβ rearrangements was reduced only ~4-fold in Vβ14^{NT/4} and Vβ8^{Tg} αβ T lineage cells compared with in WT cells.

This modest reduction in the overall level of chromosomal Vβ rearrangements, compared with the substantial decrease in the numbers of cells expressing endogenous Vβ segments, indicates that not all VβDJβCβ genes assembled in-frame on WT alleles. A larger repertoire of endogenous Vβ segments rearranges in thymocytes containing preassembled functional VβDJβCβ genes.
are expressed within TCRβ-chains on Vβ14\textsuperscript{NT/NT} and Vβ8\textsuperscript{Tg} αβ T lineage cells. To further demonstrate this point, we conducted PCR analysis on serially diluted thymocyte DNA to quantify the levels of endogenous Vβ5, Vβ6, Vβ8, Vβ10, and Vβ14 rearrangements to DJβ1.1/DJβ1.2 and DJβ2.1/DJβ2.2 complexes in Vβ8\textsuperscript{Tg} and Vβ14\textsuperscript{NT/NT} cells compared with in WT cells. We found that the levels of Vβ6 and Vβ14 rearrangements to DJβ1.1/DJβ1.2 complexes were comparable among Vβ8\textsuperscript{Tg}, Vβ14\textsuperscript{NT/NT}, and WT cells, whereas Vβ6 and Vβ14 rearrangements to DJβ2.1/DJβ2.2 complexes were reduced ∼5-fold in Vβ8\textsuperscript{Tg} cells and ∼25-fold in Vβ14\textsuperscript{NT/NT} cells (Fig. 4C). The levels of Vβ5 rearrangements to DJβ1.1/DJβ1.2 complexes were reduced ∼5-fold in Vβ8\textsuperscript{Tg} cells and ∼25-fold in Vβ14\textsuperscript{NT/NT} cells, and the levels of Vβ5 rearrangements to DJβ2.1/DJβ2.2 complexes were reduced ∼25-fold in Vβ8\textsuperscript{Tg} and Vβ14\textsuperscript{NT/NT} cells (Fig. 4C). Vβ8 rearrangements to DJβ1.1/DJβ1.2 and DJβ2.1/DJβ2.2 complexes were reduced ∼25-fold and ∼100-fold, respectively, in Vβ14\textsuperscript{NT/NT} cells (Fig. 4C). Because of the genomic organization of Vβ8\textsuperscript{Tg}, we were unable to amplify endogenous Vβ8 rearrangements to DJβ1.1/DJβ1.2 complexes in Vβ8\textsuperscript{Tg} cells; Vβ8 rearrangements to DJβ2.1/DJβ2.2 complexes were reduced ∼25-fold (Fig. 4C). Consistent with our ability to detect only Vβ14*Vβ8* Cβ\* populations in Vβ14\textsuperscript{NT/NT} mice, we observed no PCR amplicons of Vβ-to-DJβ rearrangements in Vβ14\textsuperscript{NT/NT} cells (Fig. 4C). Our data indicated that the levels of chromosomal Vβ6 and Vβ14 rearrangements in Vβ14\textsuperscript{NT/NT} and Vβ8\textsuperscript{Tg} αβ T lineage cells are reduced to a lesser extent than are the numbers of cells expressing Vβ6 and Vβ14 within surface TCRβ-chains.

Posttranscriptional silencing of functionally assembled endogenous VβDJβCβ genes contributes to TCRβ allelic exclusion

Because αβ T lineage cells expressing two functional TCRβ genes are not selected against (30), our observations are consistent with the notion that not all VDJBCβ genes assembled in-frame on WT alleles are expressed on Vβ14\textsuperscript{NT/NT} and Vβ8\textsuperscript{Tg} αβ T cells. The inability of VαDJαCα chains to form functional pre-BCRs and promote differentiation can result in their lack of expression on the surface of B cells, ensuring IgH allelic exclusion (31). Yet, the silencing of Vβ14 and Vβ8 expression on Vβ14\textsuperscript{NT/NT} and Vβ8\textsuperscript{Tg} cells, respectively, cannot be due to defects in pairing with pTα because the Vβ14DJβ1.5Cβ1 and Vβ8.2DJα1.1Cβ1 genes were isolated from selected TCRβ alleles. Mature αβ T lineage cells frequently express intracellular TCRα-chains from both alleles but exhibit TCRα allelic exclusion through posttranslational mechanisms that seem to include competition between TCRα-chains for a single TCRα-chain or inability of one TCRα-chain to pair with the expressed TCRβ-chain (13, 32). Thus, we next conducted intracellular FACS analysis of Vβ14\textsuperscript{NT/NT}, Vβ8\textsuperscript{Tg}, and Vβ14\textsuperscript{NT/NT} αβ T lineage cells with anti-Vβ14 and anti-Vβ8 Abs to evaluate whether analogous mechanisms may restrict cell-surface expression of TCRβ-chains. We found Vβ8\* TCRβ-chains inside Vβ14\textsuperscript{NT/NT}, but not Vβ14\textsuperscript{NT/NT}, thymocytes and Vβ14* TCRβ-chains inside Vβ8\textsuperscript{Tg} thymocytes (Fig. 5A). The percentages of Vβ14\textsuperscript{NT/NT} and Vβ8\textsuperscript{Tg} thymocytes with intracellular

Table I. Analysis of TCRβ rearrangements in Vβ14\textsuperscript{NT/NT} and Vβ8\textsuperscript{Tg} αβ T cell hybridomas

<table>
<thead>
<tr>
<th></th>
<th>Total (n)</th>
<th>DJβ-to-Jβ (n [%])</th>
<th>Vβ-to-DJβ (n [%])</th>
<th>Other (n [%])</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vβ14\textsuperscript{NT/NT}</td>
<td>82</td>
<td>66 (80.5)</td>
<td>10 (12.2)</td>
<td>6 (7.3)</td>
</tr>
<tr>
<td>TCRβTg</td>
<td>129</td>
<td>102 (79.1)</td>
<td>12 (9.3)</td>
<td>15 (11.6)</td>
</tr>
</tbody>
</table>

Southern blot analysis using a series of TCRβ locus probes was used to characterize and quantify TCRβ rearrangements on WT alleles in panels of Vβ14\textsuperscript{NT/NT} and Vβ8\textsuperscript{Tg} αβ T cell hybridomas.
and extracellular Vβ8+ and Vβ14+ TCRβ-chains, respectively (compare Figs. 1C, 5A). These data suggested that not all VββDJβCβ and Vβ14DJβCβ genes assembled in-frame on WT alleles are expressed as TCRβ-chains within or on the surface of Vβ14NT- and Vβ8+ cells, respectively.

To demonstrate that the expression of functionally assembled endogenous VβDJβCβ genes within TCRβ-chains can be silenced, we bred Vβ14NT- and Vβ8+ mice to generate Vβ8+Vβ14NT+s mice. Extracellular FACS analysis of Vβ8+Vβ14NT+s thymocytes and splenocytes with anti-Vβ14, anti-Vβ8, and anti-Cβ Abs revealed substantial populations of Vβ8Vβ14Cβ3 and Vβ8+Vβ14Cβ- cells and a minor population of Vβ8Vβ14Cβ+ cells (Fig. 5B). Nearly all Vβ8+Vβ14NT+s cells expressed Vβ8, but only half expressed Vβ14, within cell-surface TCRβ-chains. Intracellular FACS analysis of Vβ8+Vβ14NT+s thymocytes and splenocytes with anti-Vβ14, anti-Vβ8, and anti-Cβ Abs showed populations of Vβ8Vβ14Cβ3, Vβ8Vβ14Cβ-, and Vβ8+Vβ14Cβ- cells (Fig. 5A), which were present at similar numbers as those observed with extracellular FACS analyses (compare Fig. 5A, 5B). Notably, almost all Vβ8+Vβ14NT+s cells expressed Vβ8, but only half expressed Vβ14, as part of intracellular TCRβ-chains. These data indicated that the expression of functionally assembled endogenous VβDJβCβ genes within TCRβ-chains can be silenced in mouse lymphocytes.

The silenced expression of functionally assembled VβDJβCβ genes within TCRβ-chains could occur at the level of mRNA or protein expression. To determine the level at which the preassembled functional endogenous Vβ14DJβ1.5Cβ1 gene is silenced, we used qPCR to quantify the steady-state levels of mature Vβ14+ mRNA in sort-purified Vβ8+Vβ14+ and Vβ8+Vβ14+ splenic αβ T cells of Vβ8+Vβ14NT+s mice. We found that the steady-state levels of Vβ14+ transcripts were comparable between each population of cells (Fig. 5C). These data demonstrated that expression of the Vβ14NT gene can be silenced at the level of protein. Thus, we conclude that posttranscriptional silencing of functionally assembled endogenous VβDJβCβ genes can contribute to the enforcement of TCRβ allelic exclusion in mammalian lymphocytes.

**Discussion**

In this study, we investigated VβDJβCβ gene/transgene expression and the rearrangement and expression of endogenous Vβ segments in αβ T lineage cells of mice containing the Vβ14NT gene and/or Vβ8+ transgene. We found that most Vβ14NT+s αβ T lineage cells isolated from 4-6-wk-old mice express only Vβ14 within cell-surface TCRβ-chains. These data provide direct evidence that expression of a functionally assembled VβDJβCβ gene from one allele can enforce TCRβ allelic exclusion, as would be expected from prior analyses of TCRβ transgenic mice. Yet, despite TCRβ feedback inhibition of Vβ rearrangements, a limited repertoire of endogenous Vβ segments is expressed within TCRβ-chains on Vβ14NT+s αβ T lymphocytes. Expression of these Vβ segments can result in TCRβ allelic inclusion or correlate with silenced expression of the functional Vβ14NT gene within cell-surface TCRβ-chains. We obtained analogous data through the analysis of Vβ8 mice. Similar observations have been published for mice containing a Vβ3+ TCRβ transgene (5). Thus, our current study indicated that incomplete downregulation of endogenous Vβ expression and silenced cell-surface expression of preassembled TCRβ-chains are general phenomena in αβ T lineage cells containing functional VβDJβCβ genes/transgenes. However, as discussed below, the expression of endogenous Vβ segments within such mice might be attributable to a common nonphysiologic aspect of V(D)J recombination in DN thymocytes with pre-assembled functional TCRβ genes/transgenes.

Consistent with the incomplete downregulation of endogenous Vβ expression, we found chromosomal VβDJβ1 joints on WT TCRβ alleles in ~10% of Vβ14NT+s and Vβ8+ αβ T lineage cells. Because ~40% of normal αβ T cells contain VβDJβ1 joints on nonselected TCRβ alleles, our data revealed that the overall level of endogenous Vβ rearrangements may be only 4-fold lower in Vβ14NT+s and Vβ8+ αβ T lineage cells compared with WT cells. A precise quantification cannot be made because VβDJβ2 joints in WT cells could arise through primary or secondary Vβ rearrangements. This modest reduction seems at odds with published studies demonstrating that TCRβ transgenes, such as Vβ8+, inhibit endogenous Vβ rearrangements to an apparently greater extent (25, 29). However, analyses of Vβ rearrangements in TCRβ transgenic mice predominantly have been conducted by PCR amplification of VβDJβ2 joints because, theoretically, VβDJβ1 joints can assemble on extrachromosomal circles that might not be subject to feedback inhibition. Considering the results of our analysis of TCRβ rearrangements in αβ T cell hybridomas generated from mice containing a preassembled TCRβ gene or transgene, reappraisals of conclusions gained from some previous studies of TCRβ-mediated feedback inhibition may be warranted. Still, the frequency of Vβ rearrangements on WT alleles in Vβ14NT+s and Vβ8+ cells is greater than we expected, considering the accepted model of TCRβ-mediated feedback inhibition. DNA cleavage during V(D)J recombination activates Ataxia TELangiectasia Mutated-dependent responses that may regulate lymphocyte differentiation and Ag receptor gene rearrangements (33, 34). We recently found that Amt−/− αβ T lineage cells exhibit a greater frequency of TCRβ allelic inclusion than WT cells (N. Steinel and C.H. Bassing,
unpublished observations). In this context, the ability of TCRβ genes/transgenes to bypass the necessity of assembling VβDJβCβ genes through DNA cleavage might prevent the activation of Ataxia Telangiectasia Mutated-dependent signals that inhibit endogenous Vβ rearrangements.

We also discovered that TCRβ allelic exclusion in mouse lymphocytes can be enforced through silencing the expression of functionally assembled VβDJβCβ genes within cell-surface TCRβ-chains. In Vβ14NT and Vβ8Tg αβ T lineage cells, TCRβ allelic exclusion of some endogenous Vβs mainly occurs through silencing of assembled VβDJβCβ genes involving these Vβ segments. Differential regulation of Vβ expression by inhibition of rearrangement versus silencing in Vβ14NT and Vβ8Tg αβ T lineage cells reinforces previous conclusions that germline transcription and recombinational accessibility of each Vβ segment is regulated individually (35, 36), and distinct cis-acting elements control Vβ rearrangement and TCRβ allelic exclusion (37). The endogenous Vβ segments that are regulated by feedback inhibition versus silencing in Vβ14NT and Vβ8Tg cells are interspersed throughout the TCRβ locus and reside proximal and distal from Dβ-Iβ segments. Our comparison of Vβ promoter, coding, and recombination signal sequences failed to reveal any similarities within or differences between these two groups of Vβs that could provide insight into the mechanistic basis for their distinct regulation. Yet, we did observe a low density of transposons and repetitive genomic sequences directly upstream or downstream of Vβ segments. To enforce allelic exclusion of more than TCR complexes (14, 15), posttranscriptional silencing of the Vβ14NT gene contributes to TCRβ allelic exclusion in approximately half of Vβ14NT, Vβ8Tg αβ T lineage cells. We demonstrated that this silencing occurs at the protein level, indicating that the Vβ14NT mRNA is not translated or the Vβ14NT chain is rapidly degraded in ~50% of αβ T cells expressing the Vβ8.2DJβ1.1Cβ1 transgene. In Vβ14NT Vβ8 cells, the Vβ8Tg and Vβ14NT proteins must each form stable αβ TCR complexes with the TCRα-chains expressed in Vβ14NT/Vβ8 cells. Perhaps intrinsic properties or high expression of the Vβ8Tg protein outcompetes the Vβ14NT protein for association with TCRα-chains in Vβ14NT/Vβ8 cells, leading to the rapid degradation of free Vβ14NT chains. Transcripts of in-frame VβDJβCβ genes from both alleles have been isolated from WT αβ T cells that exhibit TCRβ allelic exclusion (15); however, experiments to determine the potential expression of both genes within intracellular TCRβ have not been reported. Consequently, future experiments are needed to evaluate whether the regulation of TCRβ allelic exclusion at the protein level is a general mechanism that extends to other endogenous VβDJβCβ genes and occurs in cells lacking preassembled TCRβ transgenes. Although Vβ14NT can be silenced at the protein level in Vβ14NT/Vβ8Tg cells, our data cannot exclude contributions of other mechanisms, such as transcriptional or translational silencing, to enforce TCRβ allelic exclusion in αβ T lineage cells that have assembled in-frame VβDJβCβ genes on both alleles. Consistent with this notion, TCRβ allelic exclusion of a Vβ13 gene segment inserted upstream of the endogenous Dβ1 segment can be mediated through transcriptional downregulation postrecombination (37).

Thus, our findings suggest that to reach unequivocal conclusions, future studies of TCRβ allelic exclusion and feedback inhibition might need to include assays that quantify chromosomal Vβ-to-DJβ rearrangements, VβDJβCβ mRNA, and intracellular TCRβ protein.

Disclosures
The authors have no financial conflicts of interest.

References


