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Dramatically Increased Rearrangement and Peripheral Representation of Vβ14 Driven by the 3′Dβ1 Recombination Signal Sequence

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Summary

V(D)J recombination is targeted by short recombination signal (RS) sequences that are relatively conserved but exhibit natural sequence variations. To evaluate the potential of RS sequence variations to determine the primary and peripheral TCRβ repertoire, we generated mice containing specific replacement of the endogenous Vβ14 RS with the 3′Dβ1 RS (Vβ14/3′DβRS). These mice exhibited a dramatic increase in Vβ14$^+$ thymocyte numbers at the expense of thymocytes expressing other Vβs. In addition, the percentage of peripheral Vβ14$^+$ αβ T lymphocytes was similarly increased. Strikingly, this altered Vβ repertoire resulted predominantly from a higher relative level of primary Vβ14/3′DβRS rearrangement to DβJβ complexes, despite the ability of the 3′Dβ1 RS to break B12/23 restriction and allow direct rearrangement of Vβ14/3′DβRS to Jβ segments.

Introduction

During lymphocyte development, the exons that encode the T cell receptor (TCR) and the immunoglobulin (Ig) variable regions are assembled from germline variable (V), diversity (D), and joining (J) gene segments (Bassing et al., 2002; Gellert, 2002; Hesslein and Schatz, 2001). The lymphoid-specific RAG-1 and RAG-2 proteins initiate this process, referred to as V(D)J recombination, through the introduction of DNA double-strand breaks (DSBs) between a pair of participating V, D, or J gene segments and their flanking short recombination signal (RS) sequences (Fugmann et al., 2000; Gellert, 2002). The RAG-liberated coding ends are joined to each other to form coding joins and the RS ends are joined to each other to form RS joins; both joining events employ the actions of the ubiquitously expressed nonhomologous DNA end-joining (NHEJ) proteins (Bassing et al., 2002). This site-specific recombination process is essential for the generation of diverse antigen receptor repertoires, both through the multiple combinations of V, D, and J segments and the imprecision inherent in the joining process.

The RAG proteins are targeted to gene segments by flanking RS sequences. RSs are composed of relatively conserved heptamer (CACAGTG) and nonamer (ACAA AAACC) elements separated by generally nonconserved 12 or 23 base pair spacers (Max et al., 1979; Sakano et al., 1979). V(D)J recombination occurs only between V, D, and J segments flanked by RSs that contain 12 and 23 bp spacers (12- and 23-RSs), a restriction referred to as the 12/23 rule (Early et al., 1980). This restriction in the reaction, mediated at the level of RAG recognition and cutting (Eastman et al., 1996; Sawchuk et al., 1997; van Gent et al., 1996), ensures proper assembly of variable region genes. An additional restriction in rearrangement based on properties beyond those of simple 12/23 compatibility exists in the TCRβ locus as the 5′Dβ1 12-RS, but not the Jβ1 12-RSs, specifically targets rearrangement of a diverse repertoire of endogenous Vβ gene segments (Bassing et al., 2000). This "beyond 12/23 (B12/23) restriction" could be enforced by either RS sequence determinants that support functional synapses between only Vβ/5′Dβ RSs or RS-specific *trans*-acting factors that promote RAG accessibility. Such additional restrictions may also direct joining events in other antigen receptor loci and thus have important implications for variable region gene assembly and repertoire development.

The generation of antigen receptor repertoires is not random, as primary rearrangements between chromosomal V, D, and J segments occur at varying relative levels in developing lymphocytes (Alt et al., 1987; Feeney et al., 2000). Although generally conserved among all RSs, heptamer and nonamer elements exhibit slight sequence variations that can influence V(D)J recombination efficiency when assayed in extrachromosmal plasmid substrates (Akamatsu et al., 1994; Akira et al., 1987; Connor et al., 1995; Hesse et al., 1989; Nadel et al., 1998b; Ramsden and Wu, 1991; Williams et al., 2001). In addition, nucleotide sequences of spacers and flanking coding segments can also influence recombination efficiencies (Akira et al., 1987; Boubnov et al., 1995; Ezekiel et al., 1995; Fanning et al., 1996; Gerstein and Lieber, 1993; Larijani et al., 1999; Nadel et al., 1998a; Wei and Lieber, 1993). Therefore, the relative levels of primary rearrangements within a family of gene segments could be substantially influenced by natural variations in RS sequences that affect recombination efficiency. In this context, the relative levels of mouse Jβ and human V_H and Vκ primary rearrangements appear to reflect the recombination efficiency of their flanking RS sequences (Livak et al., 2000; Nadel et al., 1998a; Yu et al., 2002).

To date, the in vivo evidence that RS sequence variations can account for differences in the relative levels of primary rearrangement within endogenous loci is limited to the characterization of allelic polymorphisms in human Vβ and Vκ RSs (Feeney et al., 1996; Nadel et al., 1998b; Posnett et al., 1994). First, the reduced peripheral expression of Vβ3 in a family of individuals was found to correlate with an allelic polymorphism associated with a single nucleotide change in the Vβ3 23-RS spacer (Posnett et al., 1994). Some Native Americans exhibit an increased susceptibility to *Haemophilus influenzae* type b (Hib) disease that is associated with allelic poly-

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morphisms in the predominant Vκ (VκA2) expressed in anti-Hib antibodies (Feeney et al., 1996). One of these polymorphisms is a nucleotide change in the heptamer of the VκA2 12-RS that results in a significant decrease in recombination efficiency when assayed in plasmid substrates (Nadel et al., 1998b). However, the ability of gene segment RS sequence variations to influence the relative level of primary rearrangement and representation in the peripheral repertoire has not been formally tested in vivo.

In developing T lymphocytes, the 3′Dβ1 23-RS, but not the Vβ 23-RSs, recombines with Jβ1 12-RSs (Bassing et al., 2000). Given the marked effect of 5′Dβ versus Jβ1 12-RS sequence on the specific targeting of Vβs, sequence differences between Vβ 23-RSs and the 3′Dβ1 23-RS may also have significant influence on primary Vβ (to Dβ) rearrangement. Thus, to formally evaluate whether Vβ RS sequence variations can influence primary rearrangement and expression in the peripheral repertoire in vivo, we generated and analyzed mice containing specific replacement of the chromosomal Vβ14 23-RS with the 3′Dβ1 23-RS.

Results

Gene-Targeted Replacement of the Endogenous Vβ14 23-RS with the 3′Dβ1 23-RS

To formally evaluate whether particular Vβ RSs can influence the relative level of primary Vβ rearrangements, we generated ES cells and normal lymphocytes in mice in which the endogenous Vβ14 23-RS was replaced with the 3′Dβ1 23-RS. We used gene targeting to specifically replace the Vβ14 23-RS with the 3′Dβ1 23-RS in $J\beta1^{M3/\omega}$ ES cells without alteration of any immediate flanking sequence (Figure 1A). $J\beta1^{M3/\omega}$ ES cells contain deletion of the D-Jβ2 region on both TCRβ alleles, as well as deletion of Dβ1 and its flanking RSs on a single allele (the $J\beta1^{M3}$ or M3 allele) (Bassing et al., 2000). The $J\beta1^{M3}$ allele is incapable of rearranging and supporting αβ T cell development due to the B12/23 restriction of direct Vβ to Jβ rearrangement; however, the $J\beta1^{\omega}$ allele and thus $J\beta1^{M3/\omega}$ ES cells support the development of αβ T cells expressing a normal Vβ repertoire (Bassing et al., 2000). To facilitate rapid analysis, we generated $J\beta1^{M3/\omega}$ ES clones with this RS replacement targeted on the $J\beta1^{\omega}$ allele (Figures 1A and 1B; Supplemental Figure S1 at http://www.immunity.com/cgi/content/full/18/1/75/DC1). In this context, thymocytes and αβ T lymphocytes derived from these ES cells will be functionally haploid with respect to the modified Vβ14 gene segment (referred to as Vβ14/3′DβRS).

Because expression of drug resistance genes inserted into chromosomal loci can affect both transcription and V(D)J recombination, we utilized a *loxP*-Neo^r cassette to permit excision of the neomycin resistance gene. Thus, we expressed the Cre recombinase in targeted cells to excise the Neo^r cassette, leaving a single *loxP* site inserted 172 bp 5′ of the RS replacement, to create the $J\beta1^{M7}$ or M7 allele (Figures 1A and 1B). We also inserted a unique BglII site adjacent to the *loxP* site to distinguish between Vβ14 rearrangements on the $J\beta1^{M7}$ and $J\beta1^{M3}$ alleles (Figures 1A and 1B). Finally, to account for potential effects of the inserted BglII and *loxP* sites, we also used Cre-*loxP*-mediated gene targeting to introduce these sequences with no Vβ14 RS substitution on the $J\beta1^{\omega}$ allele, creating the control $J\beta1^{M8}$ or M8 allele (Figure 1B; data not shown).

Replacement of the Vβ14 23-RS with the 3′Dβ1 23-RS Promotes a Dramatic Increase in Vβ14$^+$ Thymocyte Numbers

To characterize the influence of the 3′Dβ1 23-RS on chromosomal Vβ14 rearrangement, $J\beta1^{M3/M7}$ ES cells were used to generate chimeric mice by RAG-2-deficient blastocyst complementation (RDBC) (Chen et al., 1993). We first conducted flow cytometric (FACS) analysis on thymocytes isolated from chimeric mice to analyze the effect of this RS replacement. $J\beta1^{M3/M7}$ mice exhibited numbers of thymocytes comparable to those of 129 control mice ($J\beta1^{M3/M7}$, $226 \pm 44 \times 10^6$; 129, $153 \pm 64 \times 10^6$). FACS analysis of $J\beta1^{M3/M7}$ thymocytes with anti-CD4 and anti-CD8 antibodies showed a normal distribution of DN, DP, and SP populations compared to 129 control mice (Figure 2A). In addition, thymocytes from $J\beta1^{M3/M7}$ mice exhibited a normal distribution of cells expressing "intermediate" and "high" levels of cell surface TCRβ representing, respectively, DP and SP thymocytes (Figure 2C). Furthermore, $J\beta1^{M3/M8}$ mice, as well as $J\beta1^{\omega/\omega}$ and $J\beta1^{M3/\omega}$ mice, exhibited normal numbers of thymocytes, a normal distribution of DN, DP, and SP populations, and a normal distribution of cells expressing intermediate and high levels of cell surface TCRβ (data not shown). Therefore, neither the Vβ14 RS replacement nor the inserted BglII and *loxP* sites had a discernable effect upon gross αβ T cell development.

Strikingly, FACS analysis with anti-Vβ14-specific antibodies revealed a profound increase in the numbers of $J\beta1^{M3/M7}$ thymocytes expressing Vβ14 (Figures 2B and 2D). We analyzed three $J\beta1^{M3/M7}$ and 129 control mice each and found, on average, 56% ($56.1 \pm 3.3\%$) of $J\beta1^{M3/M7}$ TCRβ high (SP) thymocytes express Vβ14 on the cell surface versus only 8% ($7.5 \pm 0.8\%$) of 129 control TCRβ high (SP) thymocytes (Figure 2D). As the number of thymocytes was comparable between $J\beta1^{M3/M7}$ and 129 control mice, $J\beta1^{M3/M7}$ mice contain, on average, approximately 7-fold more TCRβ high (SP) thymocytes that express Vβ14. In contrast, $J\beta1^{M3/M8}$ mice, as well as $J\beta1^{\omega/\omega}$ and $J\beta1^{M3/\omega}$ mice, exhibited normal numbers of TCRβ high (SP) thymocytes expressing Vβ14 (Supplemental Figure S2A at http://www.immunity.com/cgi/content/full/18/1/75/DC1; data not shown). Therefore, the increased numbers of Vβ14$^+$ TCRβ high (SP) thymocytes in $J\beta1^{M3/M7}$ mice is specifically due to sequence differences between the 3′Dβ1 23-RS and the Vβ14 23-RS that it replaced, rather than an unanticipated effect of the inserted BglII and *loxP* sites.

Despite increased numbers of $J\beta1^{M3/M7}$ thymocytes expressing Vβ14, the number of TCRβ-expressing thymocytes and the level of TCRβ expression was normal in $J\beta1^{M3/M7}$ mice; therefore, we also conducted FACS analysis with anti-Vβ5- and anti-Vβ8-specific antibodies. We analyzed three $J\beta1^{M3/M7}$ and 129 control mice each and found, on average, 4% ($3.5 \pm 1.5\%$) of $J\beta1^{M3/M7}$ TCRβ high (SP) thymocytes express Vβ5 on the cell surface versus 6% ($6 \pm 0.7\%$) of 129 control TCRβ high (SP) thymocytes (Figure 2D). Similarly, on average, 9%

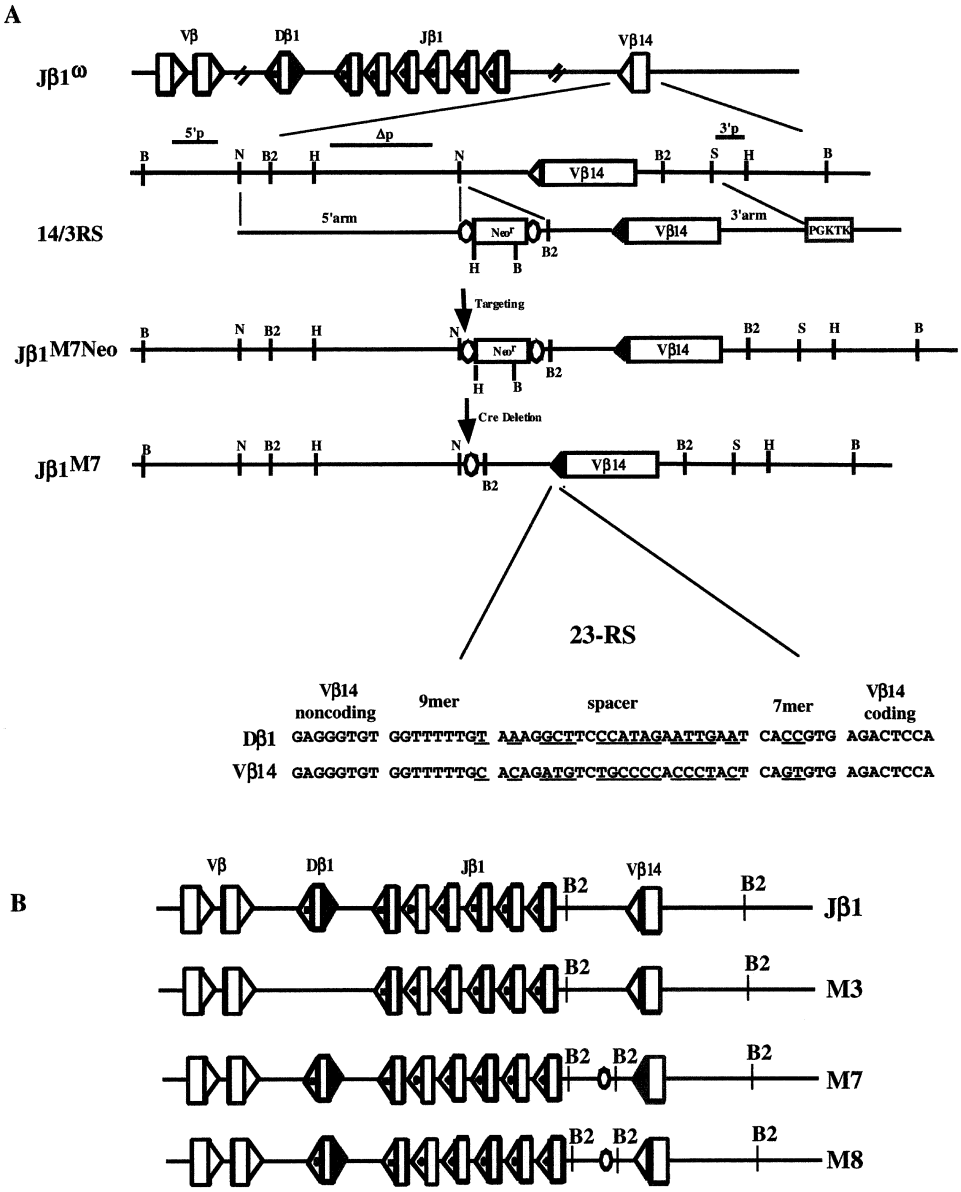

Figure 1. Generation of Jβ1$^{M3/M7}$ and Jβ1$^{M3/M8}$ ES Cells

(A) Schematic diagram of the TCRβ locus, 14/3RS targeting vector, and Jβ1^{M7Neo} and Jβ1^{M7} alleles. Representative upstream Vβs, the Dβ1Jβ1 cluster, and Vβ14 are depicted as open boxes. RSs are depicted as triangles with 12-RSs indicated with black circles. The sequences of the Vβ14 and 3′Dβ1 23-RSs are shown with nucleotide differences between the two underlined. Bars indicate the relative locations of the 5′, 3′, and Δp probes. Restriction site designations: B, BamHI; N, NdeI; B2, BglII; and H, HindIII. (B) Schematic representation of the Jβ1$^{\omega}$, Jβ1^{M3}, Jβ1^{M7}, and Jβ1^{M8} alleles.

(8.5 ± 0.3%) of Jβ1$^{M3/M7}$ TCRβ high (SP) thymocytes express Vβ8 on the cell surface versus 21% (20.6 ± 0.8%) of 129 control TCRβ high (SP) thymocytes (Figure 2D). Again, as the number of thymocytes was comparable between Jβ1$^{M3/M7}$ and 129 control mice, Jβ1$^{M3/M7}$ mice contain, on average, about a 50% decrease in the relative level of TCRβ high (SP) thymocytes that express Vβ5 and Vβ8 as compared to controls. Thus, the increased numbers of Vβ14^{+} thymocytes in Jβ1$^{M3/M7}$ mice correlates with decreased numbers of thymocytes expressing other Vβs.

Although the TCRβ repertoire is significantly influenced by positive and negative selection (Sebzda et al., 1999), the relative representation of specific Vβs is not altered during DN to DP thymocyte development (Wilson et al., 2001) and thus remains the same in spite of positive and negative selection (Aifantis et al., 1997, 1999). We could accurately quantify the numbers of TCRβ high (SP) thymocytes expressing Vβ14, Vβ5, and Vβ8, but we could not clearly distinguish thymocytes with "no" cell surface expression of TCRβ due to the wide range of intermediate TCRβ expression (Figure 2C). Thus, although we could not absolutely quantify TCRβ intermediate thymocytes expressing Vβ14, the numbers of

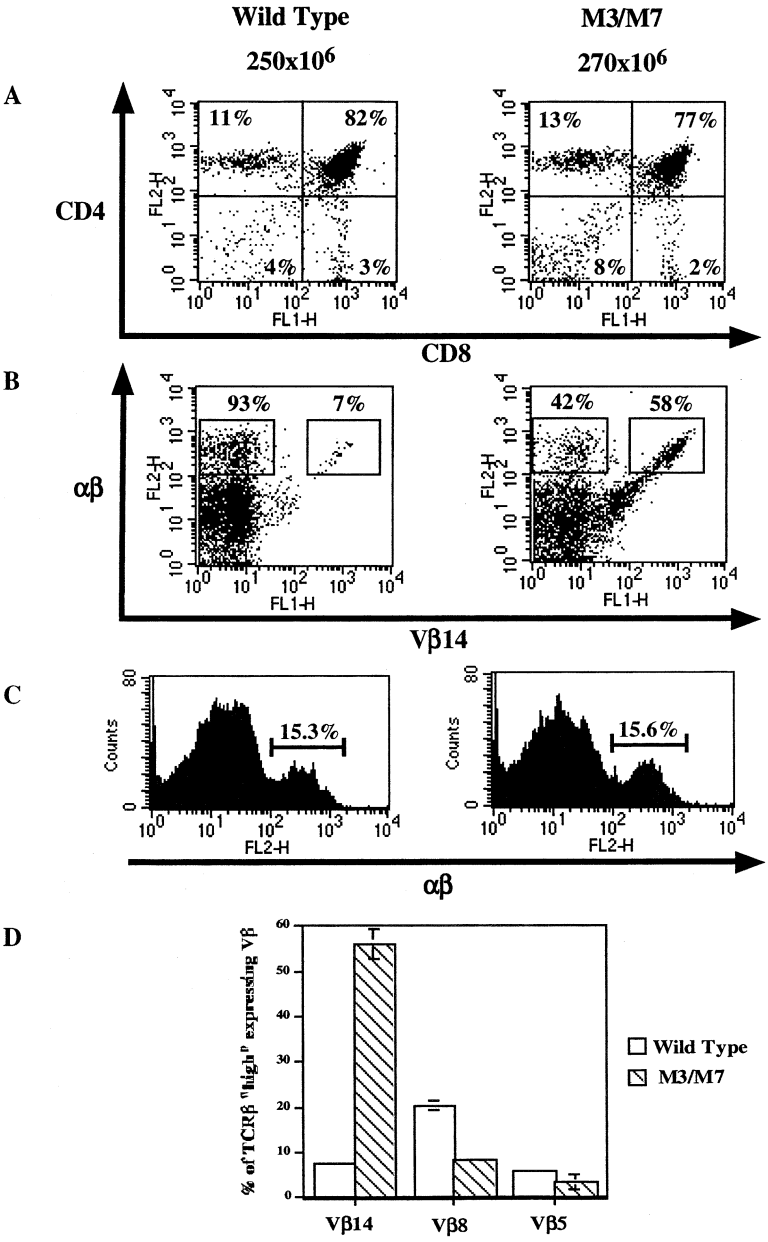

Figure 2. Flow Cytometric Analysis of Thymocytes from 4- to 8-Week-Old Jβ1$^{M3/M7}$ and Wild-Type Mice

(A) Shown is a representative CD4-PE and CD8-FITC staining. The percent of DN, DP, and SP thymocytes as well as the total numbers of thymocytes are indicated for these representative mice. (B) Shown is a representative TCRβ-PE and Vβ14-FITC staining. The percent of TCRβ high (SP) (see panel C) thymocytes that express Vβ14 or other Vβs are indicated. (C) Histogram plot of cell surface TCRβ expression with TCRβ high thymocytes marked and the percent of these cells indicated. (D) The percent of TCRβ high (SP) thymocytes expressing Vβ14, Vβ8, and Vβ5. The values were calculated from three Jβ1$^{M3/M7}$ and wild-type mice each.

these cells are also significantly elevated compared to 129 control mice (Figure 2B). Consequently, we conclude the 3′Dβ1 23-RS, when attached to Vβ14, promotes a higher relative level of Vβ14 rearrangement in developing thymocytes.

The 3′Dβ1 23-RS Promotes a Dramatic Increase in Vβ14$^+$ Peripheral αβ T Cells

Since the peripheral TCRβ repertoire is influenced by cellular selection (Sebzda et al., 1999), we next evaluated the effect of the Vβ14 RS replacement upon the Vβ repertoire of peripheral αβ T lymphocytes. Jβ1$^{M3/M7}$ and 129 control mice exhibit comparable numbers of cells in the spleen (Jβ1$^{M3/M7}$, 94 ± 43 × 10^6; 129, 93 ± 44 × 10^6) and lymph nodes (Jβ1$^{M3/M7}$, 20 ± 8 × 10^6; 129, 12 ± 1 × 10^6). FACS analysis of cells isolated from the lymph nodes and spleens of Jβ1$^{M3/M7}$ mice with anti-CD4 and anti-CD8 antibodies showed a normal distribution of CD4$^+$ and CD8$^+$ peripheral lymphocytes (Figure 3A; data not shown). However, FACS analysis with anti-Vβ14-specific antibodies revealed a strikingly higher percentage of Vβ14$^+$ peripheral αβ T lymphocytes in both the lymph nodes and spleens of Jβ1$^{M3/M7}$ mice, compared to 129 control mice (Figure 3B; data not shown). We analyzed three Jβ1$^{M3/M7}$ and 129 control mice each and found, on average, 65% (65.3 ± 0.5%) of αβ T cells in the lymph nodes of Jβ1$^{M3/M7}$ mice express Vβ14 on the cell surface versus only 7% (7.2 ± 1.0%) in 129 control mice (Figure 3C) and 7% in Jβ1$^{M3/M8}$ control mice (Supplemental Figure S2B at http://www.immunity.com/cgi/content/full/18/1/75/DC1). Thus, lymph nodes of Jβ1$^{M3/M7}$ mice contain, on average, an approximate 9-fold increase in the percentage of αβ T cells that express Vβ14. Therefore, there is no obvious selection against

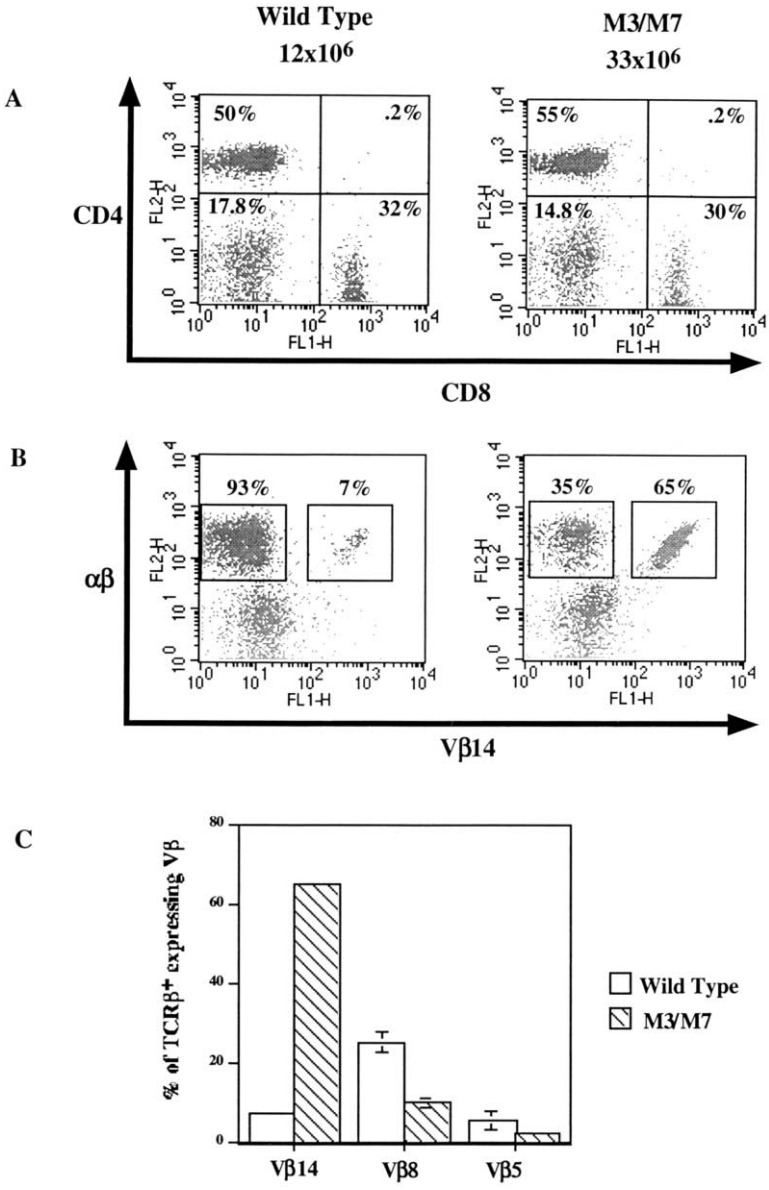

Figure 3. Flow Cytometric Analysis of the Lymph Nodes from 4- to 8-Week-Old Jβ1$^{M3/M7}$ and Wild-Type Mice

(A) Shown is a representative CD4-PE and CD8-FITC staining. The percentage of CD4$^+$, CD8$^+$, and non-T cells are indicated. The total numbers of cells isolated from the lymph nodes of these representative mice are also indicated. (B) Shown is a representative TCRβ-PE and Vβ14-FITC staining. The percent of αβ positive cells that express Vβ14 or other Vβs are indicated.

(C) The percent of αβ positive cells that express Vβ14, Vβ8 and Vβ5. The values were calculated from three Jβ1$^{M3/M7}$ and wild-type mice each.

the dramatically elevated levels of primary Vβ14 rearrangements that occur in developing Jβ1$^{M3/M7}$ thymocytes with respect to their representation in the peripheral repertoire.

FACS analysis with Vβ8-specific antibodies demonstrated, on average, 10% (10.3 ± 1.2%) of αβ T cells in the lymph nodes of Jβ1$^{M3/M7}$ mice express Vβ8 on the cell surface versus 25% (25.5 ± 2.6%) in 129 control mice (Figure 3C). Furthermore, FACS analysis with Vβ5-specific antibodies demonstrated, on average, 3% (2.7 ± 1.1%) of αβ T cells in the lymph nodes of Jβ1$^{M3/M7}$ mice express Vβ5 on the cell surface versus 6% (5.7 ± 2.4%) in 129 control mice (Figure 3C). Thus, the increased percentage of peripheral αβ T cells expressing Vβ14 in Jβ1$^{M3/M7}$ mice correlates with decreased percentages of αβ T cells expressing other Vβs. Consequently, when attached to Vβ14 on the Jβ1$^{\omega}$ allele of Jβ1$^{M3/\omega}$ cells, the 3′Dβ1 23-RS functions as a major determinant in setting the relative representation of Vβ14 in the peripheral αβ TCR repertoire.

The 3′Dβ1 23-RS Directs Vβ14 Gene Rearrangement Preferentially to the 5′Dβ1 12-RS

Because rearrangement of the 3′Dβ1 23-RS is not B12/23 restricted in extrachromosomal substrates (Jung et al., 2003), both Vβ14/3′DβRS to Dβ1Jβ1 and direct Vβ14/3′DβRS to Jβ1 rearrangement could, theoretically, occur on the Jβ1^{M7} allele. Consequently, the increase in relative Vβ14 rearrangement could be due to a greater number of acceptor RSs for Vβ14/3′DβRS (the 5′Dβ1 12-RS and 6 Jβ1 12-RSs) versus the beyond 12/23 restricted upstream Vβs (only the 5′Dβ1 12-RS). Alternatively, the 3′Dβ1 23-RS could preferentially direct highly efficient Vβ14 rearrangement to a single acceptor 12-RS (the 5′Dβ1 12-RS or one of the Jβ1 12-RSs). In addition, Vβ to Dβ1Jβ1 rearrangements involving upstream Vβs and

Vβ14/3′DβRS to Jβ1 rearrangements involving more 3′ germline Jβ1 segments could, theoretically, both occur on the same Jβ1^{M7} allele (Supplemental Figures S3A and S3D at http://www.immunity.com/cgi/content/full/18/1/75/DC1). However, as Vβ14 rearranges by inversion due to its orientation relative to the Dβ, Jβ, and Cβ segments (Supplemental Figure S3 at http://www.immunity.com/cgi/content/full/18/1/75/DC1) (Malissen et al., 1986), Vβ14/3′DβRS to Jβ1 rearrangements would render the upstream assembled VβDβ1Jβ1 complexes and Cβ1 in the opposite relative transcriptional orientation and thus only allow for development of cells expressing Vβ14 (Supplemental Figure S3D at http://www.immunity.com/cgi/content/full/18/1/75/DC1). Also, because Vβ14 rearranges by inversion, both coding and RS joins resulting from a Vβ14 rearrangement will be retained on the chromosome in Vβ14$^+$ αβ T cells (Supplemental Figures S3B, S3C, and S3D).

In developing αβ T cells, Vβ14 rearrangements normally occur to Dβ1Jβ1 complexes but not directly to Jβ1 segments (Supplemental Figure S4 at http://www.immunity.com/cgi/content/full/18/1/75/DC1) (Bassing et al., 2000). Notably, nonquantitative PCR-based analysis of TCRβ rearrangements in various tissues of Jβ1$^{M3/M7}$ mice demonstrated the presence of chromosomal signal joins between Vβ14/3′DβRS and Jβ1 segments in Jβ1$^{M3/M7}$ thymocytes (data not shown) and rearranged Dβ1Jβ1, Vβ5Dβ1Jβ1, and Vβ8Dβ1Jβ1 complexes in Jβ1$^{M3/M7}$ Vβ14$^+$ peripheral αβ T cells (Supplemental Figures S4A, S4B, and S4C), both of which could occur only if Vβ14/3′DβRS rearranged directly to a Jβ1 segment. Consequently, replacement of the Vβ14 23-RS with the 3′Dβ1 23-RS confers on Vβ14 the ability to rearrange directly to Jβ1 segments.

To quantify the relative level of Vβ14/3′DβRS rearrangements to Dβ1Jβ1 complexes versus Jβ1 segments, we analyzed TCRβ rearrangements in a panel of 46 Vβ14$^+$ Jβ1$^{M7/M3}$ αβ T cell hybridomas. Southern blot analysis of EcoRI digested genomic DNA with a probe that hybridizes 3′ of the Jβ1 segments (3′Jβ1) indicated each contained Vβ14/3′DβRS to Dβ1Jβ1 or Jβ1 rearrangement on the Jβ1^{M7} allele and that all Jβ1 segments were utilized (Table 2; Supplemental Figures S5A and S5B at http://www.immunity.com/cgi/content/full/18/1/75/DC1). Additional Southern blotting with probes that hybridize 5′ of Vβ14 (5′Vβ14) and 5′ of Dβ1 (5′Dβ1) demonstrated 37 of the 46 Vβ14$^+$ Jβ1$^{M7/M3}$ αβ T cell hybridomas contained a Vβ14/Dβ1 chromosomal RS join on the Jβ1^{M7} allele (Table 1; Supplemental Figures S3A, S3B, S5C, and S5D at http://www.immunity.com/cgi/content/full/18/1/75/DC1), consistent with Vβ14 to Dβ1Jβ1 rearrangement (see below). Of the other nine hybridomas, four contained Vβ14/3′DβRS to Dβ1Jβ1 or Jβ1 rearrangement with an aberrant rearrangement (#50, 89, 167, 192) (Table 1; Supplemental Figures S3A, S3E, S5B, S5C, and S5D at http://www.immunity.com/cgi/content/full/18/1/75/DC1; data not shown), most likely involving an upstream Vβ (VβX) to one of several cryptic RSs located between Eβ and Vβ14 (GenBank AE000665; Supplemental Figure 3A, E), as we have previously observed Dβ1 rearrangements into this region (Bassing et al., 2000). The other five contained Vβ14/3′DβRS to Jβ1 rearrangement as well as either Dβ1 to Jβ1 rearrangement (#216) or VβX to Dβ1Jβ1 rearrangement (#200, 253, 325, 372) on the same Jβ1^{M7} allele (Table 1; Supplemental Figures S3A, S3C, S3D, S5B, S5C, and S5D at http://www.immunity.com/cgi/content/full/18/1/75/DC1; data not shown). Therefore, in agreement with our PCR based analysis of cell populations, 5 out of 46 Vβ14$^+$ Jβ1$^{M7/M3}$ αβ T cell hybridomas contain direct Vβ14/3′DβRS to Jβ1 rearrangements, clearly demonstrating this RS replacment breaks the TCRβ locus B12/23 restriction. In addition, we have confirmed this conclusion with an independent line of experimentation (see below). However, despite the striking ability of Vβ14/3′DβRS to rearrange directly to Jβ1 segments, equally striking is the observation that the vast majority of Vβ14/3′DβRS appears to rearrange to prerearranged Dβ1Jβ1 complexes, a finding that warranted further analysis.

Table 1. Analysis of TCRβ Rearrangements in Jβ1$^{M3/M7}$ Vβ14$^+$ T Cell Hybridomas

Number of Hybridomas	Rearrangement of M7
37	Vβ14(Dβ1)Jβ1
4	VβXDβ1Jβ1 + Vβ14Jβ1
1	Dβ1Jβ1 + Vβ14Jβ1
4	VβXcRS + Vβ14(Dβ1)Jβ1

TCRβ allele configurations in T cell hybridomas were determined by Southern blotting of EcoRI digested DNA with the 5′Dβ1, 5′Vβ14, and 3′Jβ1 probes (Supplemental Figures S3 and S5). One of the hybridomas (#299) also had a VβX to cryptic RS rearrangement on the Jβ1^{M3} allele.

Although the Southern blotting analysis performed strongly suggested that the Vβ14/3′DβRS rearranges preferentially to Dβ1Jβ1 complexes versus Jβ1 segments, the observed Vβ14/Dβ1 chromosomal RS joins could also have formed through Vβ14/3′DβRS to Jβ1 rearrangement followed by recombination between the 5′Dβ1 12-RS and the 3′Dβ1 23-RS of the resultant Vβ14/Jβ1 chromosomal RS join. However, we could not detect by PCR Jβ1^{M7} alleles with these intermediate rearrangement events in Jβ1$^{M7/M3}$ thymoctyes (data not shown). To more rigorously investigate this issue, we used PCR to isolate and sequence 35 of the putative Vβ14Dβ1Jβ1 rearrangements from the hybridomas. We could clearly identify at least two, and frequently many more, Dβ1 nucleotides in 26 of 35 coding joins, with an additional 5 joins containing a single potential Dβ1 nucleotide (Table 2). Moreover, we note the presence of Dβ1 nucleotides is not due to selection since the rearrangement of Vβ14/3′DβRS directly to Jβ1 segments when located on the Jβ1^{M3} allele (from which Dβ1 and its flanking RSs are deleted) competes with Vβ to Dβ1Jβ1 rearrangements on the wild-type allele to also promote a dramatic increase in the numbers of Vβ14$^+$ thymocytes and the percentage of Vβ14$^+$ αβ T cells (C.W., C.H.B., and F.W.A., unpublished data). Consequently, the increased expression of Vβ14 in Jβ1$^{M7/M3}$ mice is due predominantly to a higher relative level of primary Vβ14 rearrangement to Dβ1Jβ1 complexes, rather than a greater number of acceptor RSs for Vβ14/3′DβRS versus Vβ14.

Table 2. Sequence Analysis of Vβ14(Dβ1)Jβ1 Rearrangements in Jβ1$^{M3/M7}$ Vβ14^{+} T Cell Hybridomas

Vβ14	N/P	Dβ1	N/P	Jβ	
TGGAGTCT		GGGACAGGGGGC		CAAACACAGAA	1.1
TGGAGTCT		GGGGG	GACAAT	ACACAGAA	
TGGAGTC	A	GGGACAGGG	CGG	CACAGAA	
TGGAGTCT	T	GGGGG		AAACACAGAA	
TGGAGTC		GGGACAGGGG	TGG	CACAGAA	
TGGAGTCT	T			AACACAGAA	
				CAAACTCCGAC	1.2
TGGAGTCT		AGGGGG	G	CCGAC	
TGGAGTC	CG	ACAGGG	G	CAAACTCCGAC	
TGGAGTCT		ACAGGG	AAGG	TCCGAC	
				TTCTGGAAATA	1.3
TGGA	ACCCC	GGGACA	C	CTGGAAATA	
TGGAGTCT	ATT	GGGG		TCTGGAAATA	
				TTTCCAACGAA	1.4
TGGAGTCT	A	GGGACAGGG		AACGAA	
TGGAGTC	CCC	ACAGGGG	TG	CCAACGAA	
TGGAG	CTC	GGGGG	G	TCCAACGAA	
TGGA	CTC	GGACAG		TTTCCAACGAA	
TGGAG	CCC	CAGGG		TTTCCAACGAA	
TGGAG		AC	CC	TCCAACGAA	
TGGAG	CCCA	AC		TTCCAACGAA	
TGGAGTCT	TTTT	G		CCAACGAA	
				TAACAACCAGG	1.5
TGGAGTCT	AGAATC	GGGACA	A	AACCAGG	
TGGAG	CCCAA	AC	G	ACCAGG	
TGGAG		GG		ACAACCAGG	
TGGA	CCCC	C		AACAACCAGG	
TGGAGT		A	A	TAACAACCAGG	
TGGAGTCT				TAACAACCAGG	
				TTCCTATAATT	1.6
TGGAGTC	GA	GGACAGGGGGC	T	TATAATT	
TGGAGT	TTCCTT	ACAGGGGG	ATTA	TATAATT	
TGGAGTC		AGGGGG	G	CTATAATT	
TGGAGTC		AGGG	T	TATAATT	
TGGAGTCT	AG	AGG		ATAATT	
TGGAGTCT		AG		ATAATT	
TGGAGTCT		AG		ATAATT	
TGGAGTC	GTC	C		TAATT	
TGGAG	CC	C		TTCCTATAATT	
TGGAGT				TCCTATAATT	
TGGAGTCT				CTATAATT	

Vβ14(Dβ1)Jβ1 coding joins from 35 of the T cell hybridomas with only Vβ14(Dβ1)Jβ1 rearrangement on the Jβ1^{M7} allele were PCR-amplified with the Vβ14 and P4 primers (Bassing et al., 2000). PCR products were directly sequenced with the Vβ14 primer. The nucleotides corresponding to Vβ14, Dβ1, and Jβ1 segments, as well as N/P nucleotides are indicated. Potential P nucleotides are underlined.

Discussion

Vβ RS Sequences Can Markedly Influence the Relative Level of Chromosomal Vβ Rearrangement

Here, we have demonstrated that chromosomal Vβ14 rearrangement can be dramatically influenced by its flanking 23-RS sequence. Specific replacement of the endogenous Vβ14 23-RS with the 3′Dβ1 23-RS causes a large increase in the number of thymocytes expressing Vβ14, with a corresponding decrease in those expressing other Vβs. As this altered TCRβ repertoire is specifically due to the Vβ14 23-RS substitution, the 3′Dβ1 23-RS drives a higher level of Vβ14 rearrangement, relative to that of other chromosomal Vβs. The increased numbers of cells expressing Vβ14 is predominantly due to a higher relative level of primary Vβ14 rearrangement to Dβ1Jβ1 complexes, rather than a greater number of acceptor RSs for Vβ14/3′DβRS versus Vβ14. Therefore, the 3′Dβ1 23-RS, when attached to Vβ14, must possess a greater intrinsic ability than the other Vβ 23-RSs to form functional synaptic complexes with the 5′Dβ1 12-RS. In this context, the Vβ14 23-RS replacement also results in a corresponding increase in the percentage of peripheral αβ T cells expressing Vβ14, formally demonstrating that an individual Vβ 23-RS can function to set the relative representation of that Vβ in the peripheral αβ TCR repertoire.

Vβ RS Sequences Contribute to the TCRβ Locus B12/23 Restriction

In developing T cells, the 5′Dβ1 12-RS, but not the Jβ1 12-RSs, specifically targets rearrangement of a diverse repertoire of endogenous Vβ gene segments, while the 3′Dβ1 23-RS, but not the Vβ 23-RSs, recombines with Jβ1 12-RSs (Bassing et al., 2000). We demonstrate here that the 3′Dβ1 23-RS, when attached to Vβ14, promotes

Vβ14 rearrangements directly to Jβ1 segments in developing T cells. Thus, the specific targeting of Vβ14 rearrangement to the 5′Dβ1 12-RS can be influenced by nucleotide differences between the Vβ14 23-RS and the 3′Dβ1 23-RS. Furthermore, in the absence of the 5′Dβ1 12-RS, the 3′Dβ1 23-RS promotes direct Vβ14 to Jβ1 rearrangement with a frequency approximately that predicted for normal overall Vβ to DJβ rearrangement, resulting in the development of, on average, a 6-fold increase over normal in the percentage of thymocytes and αβ T cells that express Vβ14 (C.W., C.H.B., and F.W.A., unpublished data). Therefore, the Vβ14 RS sequence alone clearly prevents direct Vβ14 to Jβ1 rearrangement and thus contributes to enforcement of the TCRβ locus B12/23 restriction.

However, despite B12/23 compatibility between the 3′Dβ1 23-RS and the Jβ1 12-RSs, the 3′Dβ1 23-RS, when attached to Vβ14, preferentially targets chromosomal Vβ14 rearrangement to the 5′Dβ1 12-RS. Thus, following Dβ1 to Jβ1 rearrangement, either the chromosomal 5′Dβ1 12-RS is more accessible than the Jβ1 12-RSs or the 5′Dβ1 12-RS has a significantly higher recombination potential than the Jβ 12-RSs. In extrachromosomal substrates transfected into nonlymphoid cells, the 3′Dβ1 23-RS directs Vβ14 rearrangement 5- to 10-fold preferentially to the 5′Dβ1 12-RS versus the Jβ1.4 RS (Jung et al., 2003). Consequently, the preference for Vβ14/3′DβRS rearrangements to the 5′Dβ1 12-RS versus the Jβ1 12-RSs can be mechanistically explained based predominantly on RS sequences, at least for these V, D, and J segments and their RSs and thus differential accessibility and/or additional T cell-specific factors need not be invoked to explain this particular bias. Therefore, the preferential in vivo rearrangement of Vβ14 to the 5′Dβ1 12-RS versus the Jβ1 12-RSs is consistent with the 3′Dβ1 23-RS, when attached to Vβ14, possessing a higher intrinsic ability to recombine with the 5′Dβ1 12-RS than with the Jβ 12-RSs. The nucleotide sequences of 5′Dβ 12-RSs and 3′Dβ 23-RSs are much more conserved between humans and mice, in both the spacers and heptamer/nonamer elements, than most Jβ 12-RSs and Vβ 23-RSs (Glusman et al., 2001; Ramsden et al., 1994). In this context, both heptamer/nonamer elements of 5′Dβ 12-RSs and 3′Dβ 23-RSs are closer to consensus than those of most Jβ 12-RSs and Vβ 23-RSs. Thus, our observations suggest 5′Dβ 12-RSs and 3′Dβ 23-RSs evolved to have greater recombination potential than Vβ 23-RSs and Jβ 12-RSs that, at least in part, could contribute to enforcement of the TCRβ locus B12/23 restriction.

Replacement of the Vβ14 23-RS with the 3′Dβ1 23-RS Increases the Efficiency of Vβ14 Rearrangement

The increased efficiency of Vβ14 to Dβ1Jβ1 rearrangement on the $J\beta1^{M7}$ allele is mediated by sequence differences between the 3′Dβ1 23-RS and the Vβ14 23-RS. The sequence of these RSs differs by only three nucleotides within the heptamer and nonamer elements and 15 nucleotides within the spacers (Figure 1A). In extrachromosomal substrates, the 3′Dβ1 23-RS spacer increases the recombination efficiency of several different Vβ RSs (Jung et al., 2003). Thus, the sequences of individual Vβ 23-RS spacers may be a major determinant of the relative level of primary Vβ rearrangement. In this context, a single nucleotide difference in the human Vβ3 23-RS spacer is associated with a 4-fold decrease in the representation of Vβ3 in the peripheral repertoire (Posnett et al., 1994). Furthermore, the sequences of human Vκ 12-RS spacers and the mouse V_H81X 23-RS spacer appear to affect, respectively, the relative level of primary Vκ rearrangements and the recombination efficiency of V_H81X (Larijani et al., 1999; Nadel et al., 1998a). Consequently, natural sequence variations in the RS spacers flanking V, D, and J segments may significantly influence recombination efficiency and thus generally function to shape the primary antigen receptor repertoire.

Recent data suggest that synapsis proceeds by the binding of RAGs to one RS, followed by capture of the other RS (Jones and Gellert, 2002; Mundy et al., 2002) and that the initial assembly of RAG may occur on 12-RSs to enforce the 12/23 rule (Jones and Gellert, 2002). Thus, RAGs may first assemble on 5′Dβ and Jβ 12-RSs, promote Dβ to Jβ rearrangement through the capture of 3′Dβ 23-RSs, and then allow for RAG-bound 5′Dβ and Jβ 12-RSs to capture individual Vβ 23-RSs for recombination. In this context, the TCRβ locus B12/23 restriction would prevent Vβ to Jβ rearrangement, while the relative level of Vβ rearrangement could be influenced by both the frequency with which individual Vβs are juxtaposed with DβJβ segments and the recombination potential of the participating Vβ 23-RSs.

The mechanisms that direct functional synapsis of Vβ and 5′Dβ RSs over large distances are unknown; however, chromosomal factors that actively promote the juxtaposition of Vβ and DβJβ segments seem more likely than random collision (Bassing et al., 2002; Hesslein and Schatz, 2001). In either scenario, Vβ rearrangement would be specifically targeted to 5′Dβ RSs, and not the immediately 3′ germline Jβ RSs, due to the B12/23 restriction of Vβ/Jβ synaptic complex formation. Given that the 3′Dβ12-RS evolved to rearrange to the Jβ1 segments, it seems unlikely that replacement of the natural Vβ14 23-RS with the 3′Dβ1 23-RS would lead to increased frequency of juxtaposition between Vβ14 and the Dβ1Jβ1 complex, as opposed to the flanking Jβ1 segments. Therefore, it seems more likely that this RS replacement increases the likelihood of juxtaposed Vβ14/3′Dβ23-RS and 5′Dβ1 12-RS complexes generating productive synapsis through increased RAG binding, cleavage, or joining mediated by the 3′Dβ1 23-RS. If so, this would further imply that a significant proportion of normal Vβ14 to Dβ1Jβ1 juxtaposition events do not lead to productive synapsis (allowing the 7-fold increased efficiency via the RS replacement) and that repeated cycles of juxtaposition and release of the normal Vβ14 23-RS with the 5′Dβ1 12-RS usually occur before productive synapsis. While this hypothetical scenario might be applied to other Vβs, it possible that additiional mechanisms may have evolved to modulate the representation of, for example, more distal Vβs.

Implications for Ordered TCRβ Rearrangement

In developing αβ T cells, assembly of TCRβ variable region genes is ordered with Dβ to Jβ rearrangement

occurring on both alleles prior to Vβ to DβJβ rearrangement (Born et al., 1985). Ordered rearrangement could be effected at the level of temporal accessibility of Dβ versus Vβ gene segments; alternatively, "order" might be established via differential efficiencies of Dβ to Jβ versus Vβ to Dβ rearrangement. Despite the dramatic increase in efficiency of Vβ14 rearrangement promoted by the 3′Dβ1 23-RS, we could not detect Vβ14 to Dβ1 rearrangement before Dβ1 to Jβ1 rearrangement by PCR in $J\beta1^{M7/M3}$ thymocytes (Supplemental Figures S4B and S4C at http://www.immunity.com/cgi/content/full/18/1/75/DC1). Therefore, differential accessibility of Dβ and Vβ segments and possibly other chromosomal factors, rather than RS efficiency alone, likely determine the ordered assembly of TCRβ variable region genes. However, the high recombination potential of the 3′Dβ1 23-RS may have evolved to ensure Dβ to Jβ rearrangement proceeds to completion before Vβ rearrangement. In this context, the excision of 3′Dβ 23-RSs from the TCRβ locus upon Dβ to Jβ rearrangement may facilitate formation of synaptic complexes between Vβ/Dβ RS pairs (Sleckman et al., 2000).

Natural Variations in Vβ RSs May Influence the Peripheral Vβ Repertoire

Assembly and expression of a broad antigen receptor repertoire is essential for the development of an effective immune system. In humans, a minor change in the RS sequence of a particular Vκ gene segment has a profound immunological consequence (Nadel et al., 1998b). Thus, particular RS sequences may have evolved to bias primary rearrangements in favor of assembled V(D)J complexes that generate a more effective peripheral antigen receptor repertoire (Livak et al., 2000; Yu et al., 2002). In mice, primary Vβ rearrangements are biased in a manner that does not reflect chromosomal organization (Aifantis et al., 1997, 1999; Wilson et al., 2001), suggesting RS efficiency and/or differential RS accessibility may influence the frequency of particular Vβ rearrangements. Here, we have unequivocally demonstrated in vivo that the flanking Vβ 23-RS sequence can be a major determinant of the relative level of primary Vβ rearrangement and thus function to set the representation of a particular Vβ in the peripheral repertoire.

The generation of effective peripheral antigen receptor repertoires is also influenced by the cellular selection of primary rearrangements during subsequent stages of lymphocyte development. For example, the primary V_H repertoire, with respect to the relative representation of V_H families, is significantly shaped during the pro- to pre-lymphocyte transition since many of the assembled V_HDJ_H complexes fail to pair with the surrogate light chain and signal further developmental progression (ten Boekel et al., 1997). In this context, although primary rearrangements of V_H81X occur in as many as 50% of developing pre-B cells, only approximately 5% of mature B cells express V_H81X (Marshall et al., 1996), most likely due to the inability of $V_H81XD_HJ_H$ chains to form a pre-B cell receptor (ten Boekel et al., 1997). In contrast, the primary Vβ repertoire, with respect to the relative representation of Vβ families, is not appreciably selected during the expansion of DN thymocytes due to the apparent lack of a similar preferential pairing with pTα (Aifantis et al., 1997, 1999). As such, the dramatically elevated levels of primary Vβ14 rearrangements that occur in developing $J\beta1^{M7/M3}$ thymocytes are not selected for or against and, as a result, approximately 65% of $J\beta1^{M7/M3}$ peripheral αβ T lymphocytes express Vβ14. Therefore, natural sequence variations in particular Vβ 23-RSs may have evolved under selective pressure to ensure the most advantageous relative representation of Vβ segments in the peripheral repertoire.

Experimental Procedures

Targeting Constructs and Probes

The 14/3RS targeting vector was constructed in pLNTK (Gorman et al., 1996). The 5′ homology arm is a 2.3 kb NdeI genomic fragment. The 3′ homology arm is a 2.4 kb SphI/NdeI fragment containing specific replacement of the Vβ14 23-RS with the 3′Dβ1 23-RS. The RS replacement was introduced via overlapping PCR. The primer pairs for the first stage reactions were 5′-GCGCGGTACCGTCGACA GATCTATGTGAGTCACTGATAACG-3′/5′-TAGAATTGAATCACCGT GAGACTCCAGGCACAGAGG-3′ and 5′-GAAGCCTTTACAAAAACC ACACCCTCTCTTTAGTCC-3′/5′-GCACGGAGAAGCTGCTTCTC-3′. The second stage reactions were performed with the outside primers using a mixture of the first stage reactions as the DNA template. The PCR reaction also introduced unique BglII and SalI sites for analysis and a unique KpnI site to facilitate subcloning. The second stage PCR product was digested with KpnI/BglI and subcloned into pBSK (Stratagene) containing the SphI/NdeI genomic fragment spanning Vβ14. The control 14/loxp targeting vector lacking the Vβ14 23-RS replacement was constructed the same way except only the outside primers were used. Both vectors were sequenced to verify the integrity of the subcloned PCR product. The 5′ probe is a 1.4 kb PstI/NdeI fragment. The 3′ probe is a 0.7 kb SphI/HindIII fragment. The Δp probe is a 1.5 kb HindIII/NdeI fragment.

Gene Targeting and Generation of Embryonic Stem Cells

The 14/3RS and 14/loxp targeting vectors were electroporated into $J\beta1^{M3/\omega}$ ES cells (Bassing et al., 2000) as described (Sleckman et al., 1997) to generate $J\beta1^{M3/M7Neo}$ and $J\beta1^{M3/M8Neo}$ ES cells, respectively. Targeted clones were identified by Southern blotting with the 5′ probe on BamHI-digested DNA (8 kb $J\beta1^{M7Neo}$, $J\beta1^{M8Neo}$; 18 kb $J\beta1^{M3}$) and confirmed with the 3′ probe on HindIII-digested DNA (5.1 kb $J\beta1^{M7Neo}$, $J\beta1^{M8Neo}$; 4.5 kb $J\beta1^{M3}$). To remove the PGK-*neo*^{r} gene, cells of independent targeted clones were infected with recombinant AdenoCre. $J\beta1^{M3/M7}$ and $J\beta1^{M3/M8}$ ES cells were identified via Southern blotting with the Δp probe on BglII-digested DNA (4 kb $J\beta1^{M7Neo}$, $J\beta1^{M8Neo}$; 2 kb $J\beta1^{M7}$, $J\beta1^{M8}$; 3.6 kb $J\beta1^{M3}$). The RS substitution was confirmed as targeted on the $J\beta1^{\omega}$ allele by the lack of a Cre-mediated deletion between the $J\beta1^{M3}$ and $J\beta1^{M7}/J\beta1^{M8}$ *loxP* sites (data not shown).

RDBC and the Generation of $J\beta1^{M3/M7}$ and $J\beta1^{M3/M8}$ Lymphocytes

$J\beta1^{M3/M7}$ and $J\beta1^{M3/M8}$ lymphocytes were generated through RAG-2-deficient blastocyst complementation as described (Chen et al., 1993). Three $J\beta1^{M3/M7}$ mice and one $J\beta1^{M3/M8}$ mouse were analyzed.

FACS Analysis

Cells from the thymus, spleen, and lymph nodes of RAG chimeric mice were isolated and stained with FITC-conjugated anti-CD8, anti-Vβ14 TCR, and anti-TCRβ chain as well as phycoerythrin-conjugated anti-CD4, anti-Vβ5.1, 5.2 TCR, anti-Vβ8, and anti-TCRβ chain antibodies (Pharmingen). Data acquisition and analysis was performed on a FACS Calibur flow cytometer equipped with CellQuest software (Becton Dickinson).

Hybridoma Analysis

Hybridoma clones were produced by fusion of concanavalin A and IL-2 stimulated T cells with the thymoma cell line BW-1100.129.237 (White et al., 1989) as previously described (Sleckman et al., 1997). Genomic DNA was isolated and subjected to Southern blotting and PCR. The Southern analysis of TCRβ rearrangements was conducted on EcoRI digested DNA with the 5′Vβ14, 5′Dβ1, and 3′Jβ1

probes. The 5′Vβ14 probe is the same fragment as the Δp probe. The 5′Dβ1 probe is a 0.743 kb AflII/HaeII fragment. The 3′Jβ1 probe is a 0.777 kb DrdI fragment.

PCR Analysis of Vβ14 Rearrangements

Vβ14 to (Dβ1)Jβ1.1 and (Dβ1)Jβ1.2 rearrangements were analyzed by PCR assay as previously described (Bassing et al., 2000). Vβ14/Dβ1 chromosomal signal joins were amplified by PCR with primers 5′-GGTTTCTTCCAGCCCTCAAGGGG-3′ and 5′-TGTGAGTCACTGATAACG-3′ and then detected with an oligo probe 5′-TTGGTGACTTCTGACTTG-3′.

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