Poor quality Vβ recombination signal sequences stochastically enforce TCRβ allelic exclusion

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The monoallelic expression of antigen receptor (AgR) genes, called allelic exclusion, is fundamental for highly specific immune responses to pathogens. This cardinal feature of adaptive immunity is achieved by the assembly of a functional AgR gene on one allele, with subsequent feedback inhibition of V(D)J recombination on the other allele. A range of epigenetic mechanisms have been implicated in sequential recombination of AgR alleles; however, we now demonstrate that a genetic mechanism controls this process for Tcrb. Replacement of V(D)J recombine targets at two different mouse Vβ gene segments with a higher quality target elevates Vβ rearrangement frequency before feedback inhibition, dramatically increasing the frequency of T cells with TCRβ chains derived from both Tcrb alleles. Thus, TCRβ allelic exclusion is enforced genetically by the low quality of Vβ recombinase targets that stochastically restrict the production of two functional rearrangements before feedback inhibition silences one allele.

Introduction

Monoallelic expression is an essential process that limits the dosage of numerous genes. Important examples include genetic imprinting and X-chromosome inactivation, as well as the tissue-specific allelic exclusion of olfactory neuron receptors and lymphocyte antigen receptors (AgR). While genetic imprinting and X-inactivation are vital for normal development and physiology, monoallelic expression of olfactory and AgR is fundamental for highly specific recognition and responses to diverse odors or pathogens. To date, mechanisms enforcing monoallelic gene expression programs have been shown to involve epigenetic-based transcriptional activation of an expressed allele and silencing of the nonexpressed allele (Khamlichi and Fell, 2018). Lymphocyte AgR allelic exclusion requires an additional level of regulation due to the obligate assembly of AgR genes by variable (diversity) joining (V(D)J) recombination. The germline TCR and Ig AgR loci are comprised of noncontiguous variable (V), joining (J), and in some instances diversity (D) gene segments. In developing T and B lymphocytes, the RAG1/RAG2 endonuclease cleaves at recombination signal sequences (RSSes) flanking these segments to assemble V(D)J exons, which encode the antigen-binding variable regions of Ig and TCR proteins (Bassing et al., 2002; Schatz and Swanson, 2011). Due to imprecise repair of RAG DNA double-strand breaks (DSBs), only one-third of V(D)J rearrangements occur in-frame to create functional genes. In the absence of other regulatory mechanisms, the frequent assembly of out-of-frame rearrangements and the requirement for AgR protein expression to drive T and B cell development dictate that biallelic expression of any TCR or Ig gene should occur in 20% of lymphocytes (Brady et al., 2010; Mostoslavsky et al., 2004). However, most AgR loci exhibit stringent allelic exclusion, presumably the result of mechanisms that enforce sequential assembly of their two respective alleles (Brady et al., 2010; Levin-Klein and Bergman, 2014; Mostoslavsky et al., 2004; Outters et al., 2015; Vettermann and Schlissel, 2010). At least for Igk loci, RAG DSBs on one allele signal down-regulation of RAG expression and inhibition of Vκ recombination on the other allele (Steinel et al., 2013). In addition, expression of a given AgR protein after the in-frame assembly of one allele often signals permanent feedback inhibition of V rearrangements on the opposing allele, at least in part via epigenetic changes that halt V recombination (Brady et al., 2010; Levin-Klein and Bergman, 2014; Mostoslavsky et al., 2004; Outters et al., 2015; Vettermann and Schlissel, 2010).

The mechanisms by which immature lymphocytes ensure sequential assembly of the two TCRβ, IgH, or Igκ alleles before feedback inhibition remain unknown (Brady et al., 2010; Levin-Klein and Bergman, 2014; Mostoslavsky et al., 2004; Outters et al., 2015; Vettermann and Schlissel, 2010). Indeed, there is considerable disagreement about whether these mechanisms are deterministic or stochastic (Brady et al., 2010; Levin-Klein and Bergman, 2014; Mostoslavsky et al., 2004; Outters et al., 2015; Vettermann and Schlissel, 2010).
Poor Vβ RSSs enforce TCRβ allelic exclusion

Wu et al., 2003). The low qualities of Vβ RSSs prevent their recombination with Jβ RSSs and thereby focus Vβ rearrangements to DJβ complexes (Banerjee and Schatz, 2014; Drejer-Teel et al., 2007; Jung et al., 2003; Tillman et al., 2003; Wu et al., 2003). At least for the atypical Vβ segment (Vβ1) that resides near Dβ-Jβ segments and rearranges by inversion, Vβ RSS identity rather than Vβ transcription and chromatin accessibility is a major factor that limits its recombination frequency (Wu et al., 2003; Yang-lovt et al., 2010).

Here, we demonstrate that low Vβ RSS quality provides a major underlying genetic, rather than epigenetic, mechanism to enforce TCRβ allelic exclusion by minimizing initiation of Vβ recombination on both alleles. We show that replacement of two different Vβ RSSs with a better-quality DJβ RSS in mice increases Vβ rearrangement frequency before TCRβ-signaled feedback inhibition, thereby elevating the fraction of T cells that expresses TCRβ protein from both alleles. In addition, we show that each Vβ RSS replacement allele competes with its homologous Tcrb allele for recombination, indicating that both alleles can be active for Vβ recombination within the same time frame. We conclude that TCRβ allelic exclusion is enforced genetically by the poor qualities of Vβ RSSs, which stochastically limit the assembly of functional Tcrb genes on each allele before feedback inhibition can epigenetically silence Vβ recombination.

Results

Generation of Vβ RSS replacement mice with grossly normal αβ T cell development

The Tcrb locus contains 23 functional Vβs positioned 250–735 kb upstream of the DJβ1-Cβ1 and DJβ2-Jβ2-Cβ2 clusters, each of which has one DJβ and six functional Jβs (Fig. 1 A). Tcrb has another Vβ (Trbv31, hereafter called Vβ31) located 10 kb downstream of Cβ2 and in the opposite transcriptional orientation from all other Tcrb coding sequences (Malissen et al., 1986). To determine potential roles of low-quality Vβ RSSs in governing TCRβ allelic exclusion, we made C57BL/6 mice carrying germ-line replacement of the Vβ31 and/or Trbv2 (V2) RSS with the stronger 3’Dβ1 RSS, referred to as the V2α or V31α modifications (Fig. 1 A and B; and Fig. S1). We created and studied mice with each distinct modification on one (V2αR, V2αR), both (V2αR, V31αR), or opposite alleles (V2αR/V31αR). All of these mutant mice have normal numbers and frequencies of mature αβ T cells and thymocytes at each developmental stage (Fig. 1 C and Fig. S2, A–F). In thymocytes, Dβ-Jβ rearrangement initiates at the DN1 stage and continues in DN2 and DN3 stages, while Vβ-Jβ rearrangement occurs only at the DN3 stage (Godfrey et al., 1993). To study rearrangements of V2α and V31α alleles without the influence of an opposing Tcrb allele, we introduced the WT, V2α, or V31α alleles opposite an allele lacking the Tcrb enhancer (Eβ), whose loss blocks all Tcrb rearrangements in cis (Bories et al., 1996; Bouvier et al., 1996). We found that V2α and V31α rearrangements initiate in DN3 cells but occur at much greater levels than V2 and V31 rearrangements (Fig. S3, A–C). Thus, replacement of the V2 or V31 RSS with the 3’Dβ1 RSS substantially increases the frequency of V2 or V31 recombination without altering normal development of αβ T cells.
RSS-replaced Vβ segments outcompete unmodified segments in the TCR repertoire

In WT C57BL/6 mice, Vβ representation is similar in αβ TCR repertoires of CD4+ or CD8+ single positive (SP) T cells and mirrors relative levels of Vβ rearrangement in DN3 cells (Wilson et al., 2001). We performed flow cytometry on SP thymocytes and naive splenic αβ T cells to determine whether RSS substitutions impact recombination and resultant usage of Vβ segments.
individual Vβs within the αβ TCR repertoire. We used a Cβ-specific antibody along with antibodies that bind a single Vβ (V2, TrbV4 [V4], TrbV9 [V9], or V3i) or a family of Vβs (TrbV12.1 and TrbV12.2 [V12] or TrbV3.1, TrbV3.2, and TrbV3.3 [V3i]). In WT mice, 7.0% of SP cells express V2+ or V3i+ TCRβ chains on their surface (Fig. 1, D–F). For mice with each Vβ RSS replacement on one or both alleles, we detected a 6–11-fold elevated representation of the modified Vβ on SP thymocytes (Fig. 1, D–F). Specifically, 40.9% of V2R/+ cells and 61.4% of V2R/+ cells expressed V2+ TCRβ chains, while 50.0% of V3iR/+ cells and 77.1% of V3iR/+ cells expressed V3i+ TCRβ chains (Fig. 1, D–F). As all genotypes have similar numbers of SP cells (Fig. 1 C), the increased usage of each modified Vβ must be at the expense of other Vβ segments. Indeed, there were fewer V3i+ cells in V2R/+ and V2R/R mice relative to WT mice (5.1% and 3.8%, versus 7.0%; Fig. 1, D–F) and fewer V2+ cells in V3iR/+ and V3iR/R mice compared with WT mice (4.3% and 2.3%, versus 7.0%; Fig. 1, D–F). Additionally, the percentage of cells expressing V4+, V12+, V13+, or V19+ TCRβ protein was reduced in V2R/+ and V3iR/+ mice, and more so in V2R/R and V3iR/R mice (Fig. 1, D and G; Fig. S4, A–E; and data not shown). The altered repertoires of Vβ RSS replacement mice show that the 3’Dβ1 RSS empowers V2 and V3i to outcompete normal Vβ segments for recombination and resultant usage in the αβ TCR repertoire.

We noted that each homozygous RSS-replaced genotype used its modified Vβ segment ~1.5 times more than their heterozygous counterpart (Fig. S4 F). This less-than-additive effect implies that both Tcrb alleles compete with each other for rearrangement and usage in the αβ TCR repertoire. The repertoires of V2R/+, V3iR/+, and V2R+/V3iR/+ mice yield further evidence for allelic competition, as each modified Vβ is less represented in V2R+/V3iR/+ mice relative to V2R/+ or V3iR/+ mice (Fig. 1, D–F; and Fig. S4, A–C). These differences imply that the overall Vβ recombination efficiency of each RSS-replaced allele is elevated such that it outcompetes the homologous allele. To further test this possibility, we analyzed WT/ETβ2, V2R/ETβ2, and V3i/ETβ2 mice in which the Eβ-deleted (ETβ) allele cannot compete with the functional allele. The percentages of V2+ and V3i+ SP cells each are similar between WT/ETβ2 and WT mice (Fig. 2, A–D). In contrast, each RSS-replaced Vβ is used ~1.5 times more in V2R/ETβ2 or V3i/ETβ2 mice compared with V2R/+ or V3iR/+ mice, respectively (Fig. 2, A–D), and the percentages of V2+ and V3i+ cells are similar to V2R/R and V3iR/R mice (compare Fig. 1, D–F, with Fig. 2, A–D). To our knowledge, the only demonstrated function of the endogenous Eβ element is to drive Tcrb recombination. Thus, while we cannot rule out the contribution of another function of Eβ, our data suggest that each RSS-replaced Vβ segment outcompetes for rearrangement the other Vβs on both alleles.

Vβ RSS replacements increase biallelic assembly and expression of functional TCRβ genes

To determine potential effects of Vβ RSS replacements on TCRβ allelic exclusion, we performed flow cytometry to quantify αβ T cells that stain with two different anti-Vβ antibodies because of a lack of allotopic markers that identify TCRβ chains encoded by each allele. We used this approach to determine the percentages of αβ T cells expressing two different types of TCRβ chains, first with an antibody for V2 or V3i in combination with V4, V12, V13, or V19 antibodies. For each combination, we observed that 0.05–0.21% of SP cells stained with both antibodies in WT mice (Fig. 3, A–D). We detected increased frequencies of SP cells that stained for V3i and each other Vβ tested in V3iR/+ and V3iR/R mice (Fig. 3, C and D), and likewise, for V2 and each other Vβ assayed in V2R/+ and V2R/R mice (Fig. 3, A and B). In WT mice, 0.09% of SP thymocytes were V2+V3i+, which increased to 0.3–0.68% of cells in mice carrying V2R or V3iR on one or both alleles (Fig. 3, E and F). Strikingly, the frequency of V2+V3i+ SP cells is increased 27-fold in V2R+/V3iR/+ mice compared with WT mice (2.47% versus 0.09%, Fig. 3, E and F). The 3.5-fold greater frequency of V2+V3i+ cells in V2R+/V3iR/+ mice compared with mixed V2R/R and V3iR/R cells provides firm evidence for αβ T cells expressing both V2+ and V3i+ TCRβ chains. The sum of double-staining cells for all Vβ combinations tested indicates that the fraction of αβ T cells expressing two distinct TCRβ proteins in V2R+/V3iR/+ mice is 4.5-fold greater than normal (Fig. 3 G). We note similar findings in splenic αβ T cells (Fig. S5, A–G). Collectively, these data demonstrate that replacement of a single Vβ RSS with the 3’Dβ1 RSS on one or opposite alleles elevates the frequencies of αβ T cells expressing two different types of TCRβ protein.

To determine if Vβ RSS replacements increase biallelic assembly of functional TCRβ genes, we created 102 αβ T cell hybridomas from V2R+/V3iR/+ mice and analyzed Tcrb rearrangements. We compared our data to a prior study of 212 WT hybridomas, where 56.6% contained a single Vβ rearrangement on one allele and 3Dβ1 rearrangement on the other allele (V(D)/DJ; Table 1), and 43.4% contained an in-frame and an out-of-frame Vβ rearrangement on opposite alleles (V(D)/V(D); Table 1; Khor and Sleckman, 2005). Of our V2R+/V3iR/+ hybridomas, 45.1% were V(D)/DJ and 31.4% were V(D)/V(D) (Table 1). Unexpectedly, 23.5% of V2R+/V3iR/+ hybridomas had two Vβ rearrangements (V3i and another Vβ) on one allele, which has never been observed in WT cells (23.5% versus 0%; Table 1 and Fig. S5 H; Khor and Sleckman, 2005). We also observed V3i recombination directly to 3Dβ segments in 23.5% of V2R+/V3iR/+ hybridomas (Table 1). Such direct Vβ-to-Jβ rearrangements rarely occur and have only been reported in hybridomas from mice carrying replacement of the V3i RSS with the better 3’Dβ1 RSS or the βJ1.2 RSS with the stronger 3’Dβ1 RSS (Bassing et al., 2000; Sleckman et al., 2000; Wu et al., 2003, 2007). Finally, we found that eight (7.8%) of the V2R+/V3iR/+ hybridomas had recombination of both V2R and V3iR (Table 1). Notably, two of these contained an in-frame V2Dββ rearrangement on one allele and an in-frame V3Dββ rearrangement on the other allele (Table 1 and Table 2), mirroring the 2.47% of V2+V3i+ cells detected by flow cytometry. While our hybridoma analysis provides unequivocal evidence that Vβ RSS replacements on opposite alleles increase the overall frequency of Vβ recombination, our sample size precludes concrete evidence for an elevated frequency of biallelic in-frame Vβ rearrangements. However, by considering our hybridoma and flow cytometry data together, we conclude that replacement of a Vβ RSS with the 3’Dβ1 RSS on opposite alleles increases the overall frequency...
of Vβ rearrangements, leading to greater incidence of biallelic assembly and expression of functional Tcrb genes.

**The ability of the 3’ Dβ1 RSS to elevate Vβ recombination does not require c-Fos binding**

The increased Vβ recombination and biallelic TCRβ expression in Vβ RSS replacement mice can be explained by the greater strength of the 3’ Dβ1 RSS for recombining to Dβ and Jβ RSSs. However, 3’ Dβ RSSs, but not Vβ RSSs, can bind the c-Fos transcription factor, which in turn can enhance 3’ Dβ1 RSS recombination activity on plasmid substrates (Wang et al., 2008). Therefore, to address potential contributions of c-Fos on the ability of 3’ Dβ1 RSS substitutions to increase Vβ recombination, we established mice carrying V2 or V31 RSS replacements with a two-nucleotide variant 3’ Dβ1 RSS that cannot bind c-Fos (the V2rc or V31rc modification; Fig. 4, A and B; Wang et al., 2008). We analyzed mice with each variant Vβ RSS replacement on one or opposite alleles and observed normal αβ T cell development (data not shown). We found a 4.9-fold increase of V2rc cells in V2r/αβ mice and a 5.4-fold increase of V31rc cells in V31r/αβ mice (Fig. 4, C–E). In V2rc/V31r/αβ mice, we found greater than normal frequencies of V2αβ and V31αβ cells, which were less than twice the levels in V2r/αβ or V31r/αβ mice, respectively (Fig. 4, C–E). Finally, we detected greater than normal frequencies of V2α’/V31α’ T cells in V2r/αβ, V31r/αβ, and V2rc/V31rc/αβ mice (Fig. 4, F and G). Critically, these data indicate that the effects of the 3’ Dβ1 RSS replacements at increasing Vβ recombination and biallelic TCRβ expression do not require c-Fos binding.

**Vβ RSS replacements increase the initiation of Vβ recombination before feedback inhibition**

The elevated incidences of biallelic TCRβ expression in Vβ RSS replacement mice could arise from increased Vβ recombination before feedback inhibition and/or continued Vβ recombination after feedback inhibition. We found V2 or V31 rearrangements are greater than normal in DN3 thymocytes of V2r/Eβα and V31r/Eβα mice, respectively (Fig. 5, C). As Vβ segments recombine independent of competition and feedback inhibition from the Eβα allele in these mice, these data indicate that the 3’ Dβ1 RSS replacements increase Vβ recombination before feedback inhibition. To determine whether TCRβ-mediated feedback inhibition prevents V2r and V31r rearrangements, we generated and analyzed V2r/Δβ and V31r/Δβ mice expressing a preassembled functional TCRβ transgene (TcrbTg). Expression of the transgenic V13 TCRβ protein signals feedback inhibition of Vβ rearrangements in DN3 thymocytes (Steinel et al., 2010). However, ~3% of TcrbTg αβ T cells express TCRβ protein from VDJβ rearrangements that occur before TcrbTg-mediated feedback inhibition (Steinel et al., 2010). By flow cytometry, we found that the TcrbTg more effectively reduces usage of V2 than V31 when each is flanked by their own RSS or the 3’ Dβ1 RSS (Fig. 5, A–D). To quantify V2r and V31r rearrangements, we made hybridomas from V2r/Δβ, TcrbTgV2r/Δβ, V31r/Δβ, and TcrbTgV31r/Δβ mice. We detected V2r rearrangement in 50% of V2r/Δβ cells but not in any TcrbTgV2r/Δβ cells (P = 2.68 × 10−5, Pearson’s χ² test with Yates’ correction), and V31r rearrangement in 50% of V31r/Δβ cells and 15% of TcrbTgV31r/Δβ cells (P = 1.63 × 10−5, Pearson’s χ² test with Yates’ correction; Table S1). Our previous analysis of 129 TcrbTg αβ T cell hybridomas showed that 2.3% had a V31 rearrangement and an additional 7% carried recombination of a different Vβ (Table S1 and data not shown; Steinel et al., 2010). These data demonstrate that TcrbTg-mediated feedback inhibition suppresses recombination of V2r and V31r and does so more effectively for V2r. One potential mechanism of feedback inhibition could involve blocking RAG access to 5’ Dβ RSSs in double negative (DN) thymocytes (Bassing et al., 2000). In this scenario, recombination of V2r or V31r directly to Jβ segments would continue as TCRβ signals initiate DN–to–double positive (DP) thymocyte development. However, in hybridomas where V31r is the only Vβ that rearranged, V31r recombined with Jβ segments in 38% of V31r/Δβ cells and 14% of TcrbTgV31r/Δβ cells (Table S1), revealing that TCRβ-mediated feedback also inhibits V31r-to-Jβ rearrangements.
recombination. Therefore, the increased frequency of Vβ recombination before TCRβ-mediated feedback inhibition is the mechanistic basis for the higher than normal frequencies of biallelic TCRβ expression in Vβ RSS replacement mice.

**Discussion**

Our study answers a longstanding question in immunology: How do developing lymphocytes assemble only one allele of any AgR locus into a functional gene before feedback inhibition permanently halts further V recombination? In contrast to prevailing epigenetic models of sequential V rearrangements between alleles, we demonstrate that a genetic mechanism governs monoallelic gene assembly and expression at Tcrb before TCRβ-signaled feedback inhibition. Specifically, improving the quality of only a single Vβ RSS on each allele increases Vβ recombination frequency before enforcement of feedback inhibition. The modified Vβ segments initiate recombination in DN3 thymocytes and are subject to TCRβ-signaled feedback inhibition, indicating that they still behave like Vβ segments and do not gain rearrangement properties of Dβ segments. These targeted alterations also reveal that the two Tcrb alleles compete with each other for Vβ recombination and, when high-efficiency Vβ-RSSs are present on both alleles, a dramatic increase in cells...
expressing TCRβ proteins from both alleles occurs. We conclude that Tcrb has evolved to possess poor-quality Vβ-RSSs, which stochastically limit the incidence of productive Vβ rearrangements on both alleles before feedback inhibition terminates their recombination in subsequent stages of thymocyte development. This stochastic genetic mechanism may cooperate with additional epigenetic processes that have been implicated in asynchronous V recombination between alleles. For example, if asynchronous V recombination between alleles. For example, if transcriptionally repressive nuclear structures to reduce the probability of Vβ recombination on the second allele after DSB repair allows Vβ recombination. If this recombination is out-of-frame, RAG re-expression after DSB repair allows Vβ recombination on the second allele, or the first allele when a Dβ2β2 complex is available. In the latter case, poor Vβ RSSs again limit the chance for Vβ recombination on both alleles. Upon an in-frame rearrangement on either allele, the encoded TCRβ protein activates cyclin D3 to move cells into S phase (Sicinska et al., 2003), where RAG2 is degraded (Lin and Desiderio, 1994). Based on data from pro-B cells (Powers et al., 2012), cyclin D3 also could repress Vβ recombination on the other allele. We propose that stochastic interactions of Tcrb alleles with the nuclear lamina that inhibit RAG access, Vβ accessibility, and chromosome looping between Vβ and Dβ-Jβ segments (Chan et al., 2013; Chen et al., 2018; Schlimgen et al., 2008) cooperate with poor Vβ RSSs to restrain biallelic Tcrb gene assembly during the time DN3 cells can attempt Vβ recombination. After an in-frame VDJβ rearrangement, TCRβ-signaled transcriptional silencing of RAG expression during DN-to-DP thymocyte development prevents Tcrb recombination as cells rapidly proliferate and differentiate. Finally, TCRβ signals that drive differentiation of DP thymocytes activate epigenetic mechanisms that block Vβ recombination.

Table 1. Analysis of Tcrb rearrangements in αβ T cell hybridomas

| Genotype | Number | % Total | WT* No. | % Total | P value |
|----------|--------|---------|---------|---------|---------|
| V(D)/D   | 102    | 45.1    | 212     | 56.6    |         |
| V(D)/V(D)| 46     | 45.1    | 120     | 56.6    | 1.842–10|
| V(D)-V(D)/D| 32    | 31.4    | 92      | 43.4    | 0.9597  |
| V(D)/V(D)/D| 10   | 9.8     | 0       | 0       | 0.8995  |
| V(D)/V(D)/V(D)| 14 | 13.7    | 0       | 0       | 1.039–08|
| Monoallelic V(D)| 56 | 54.9    | 120     | 56.6    | 0.3305  |
| Biallelic V(D)| 46 | 45.1    | 92      | 43.4    | 0.9455  |
| 1 V(D)| 46     | 45.1    | 120     | 56.6    | 0.3305  |
| 2 V(D)| 42     | 41.2    | 92      | 43.4    | 0.8995  |
| 3 V(D)| 14     | 13.7    | 0       | 0       | 1.039–08|
| V2(D)| 13     | 12.7    |         |         |         |
| V31(D)| 58     | 56.9    |         |         |         |
| V31-to-D| 33    | 32.4    |         |         |         |
| V31-to-J| 24    | 23.5    |         |         |         |
| V2(D)/31(D)| 8  | 7.8     |         |         |         |
| V2(D)/V31(D)| 2  | 2.0     |         |         |         |

P values were generated by Pearson’s χ² test with Yates’s continuity correction. IF, in-frame.

*Khor and Sleckman, 2005.
when Tcra genes assemble (Agata et al., 2007; Jackson and Krangel, 2005; Majumder et al., 2015; Skok et al., 2007). The loss of expression of the E47 transcription factor in DP thymocytes silences Vβ chromatin and recombination to maintain TCRβ allelic exclusion during Tcra recombination (Agata et al., 2007). In addition to down-regulation of Vβ accessibility, diminished contacts between Vβ and Dβ-Jβ segments and additional factors that prevent RAG-mediated synopsis, cleavage, and joining of Vβ and Dβ-Jβ segments could maintain TCRβ allelic exclusion in DP thymocytes (Jackson and Krangel, 2005; Majumder et al., 2015; Skok et al., 2007). Notably, as Vβ 23-RSSs share features with Vγ, 23-RSSs, but not other 23-RSSs (Liang et al., 2002), and Vβ and Vγ rearrangements are similarly activated sequentially between alleles and feedback inhibited by epigenetic mechanisms (Brady et al., 2010), all aspects of this model could apply to IgH allelic exclusion.

The field has strived to elucidate mechanisms that drive AgR locus V rearrangements across large genomic distances, with focus on factors that promote broad usage of V gene segments and enforce allelic exclusion. In vivo studies have demonstrated that V accessibility and V contact with D-J segments can influence relative V rearrangement frequency (Fuxa et al., 2004; Jain et al., 2018; Ryu et al., 2004). Computational analyses based on correlations conclude V accessibility is the predominant factor for V usage at Tcrb and Igh, while V RSS quality and contact with D-J segments each function as a binary switch to prevent or allow recombination (Bolland et al., 2016; Gopalakrishnan et al., 2013). On the contrary, our data show that V2 and V31 RSSs function far beyond reaching a minimal threshold for recombination with Dβ RSSs. The increased usage of V2R and V31R at the expense of other Vβ segments on the same allele indicates that most, if not all, Vβs dynamically compete with each other for productive synopsis with Dβ complexes. On a normal allele, RAG protein bound to Dβ RSSs likely repeatedly captures and releases different Vβ RSSs (Wu et al., 2003), analogous to recombination between RSSs in vitro (Lovely et al., 2015). This sampling of Vβs could occur via diffusional-based synopsis of Vβ RSSs positioned in a cloud of spatial proximity (Ji et al., 2010) or by chromosomal-loop scanning-based synopsis (Jain et al., 2018). To determine RSS quality, the field typically uses an algorithm that calculates a recombination information content (RIC) score based on statistical modeling of how each nucleotide diverges from an averaged RSS (Cowell et al., 2003). The RIC scores of the RSSs that we manipulated in vivo predict the 3′Dβ1 RSS replacement would decrease V2 recombination and the variant 3′Dβ1 RSS substitution would replace both V2 and V31 rearrangements (Fig. S5 I). The differences between predicted and empirical data could be due to several possibilities, including that the RIC algorithm does not address pairwise effects of RSSs. Regardless, the discrepancies between predictions of machine-generated associations and our in vivo data highlight the vital need to test computational-based models of V(D)J recombination. In addition to elucidating mechanisms that enforce allelic exclusion, the field has worked to identify physiological roles for monoallelic assembly and expression of Tcrb, Igh, and Igk genes (Brady et al., 2010). Our in vivo RSS replacement approach provides an unprecedented opportunity to test these models of biallelic assembly and expression of diverse genes, at least for Tcra.

Table 2. Sequence analysis of the V2 and V31 rearrangements on opposite alleles in V2R+/V31R- T cell hybridomas

| Clone | Vβ     | N/P | Potential Dβ | N/P | Jβ       | Status     |
|-------|--------|-----|-------------|-----|----------|------------|
| 1     | V2     | A   |             |     | CCGGGCAG | 2.2 Out-of-frame |
| V31   | GCTTAGGCTCT | AC  |             |     | CCCAAGGAAGA | 1.4 In-frame |
| 23    | V2     | T   |             | TGG | GAACAG   | 2.7 In-frame |
| V31   | The V31 rearrangement was not identified |
| 54    | V2     | CAA | GAC         |     | AACACGGGCA | 2.2 In-frame |
| V31   | GGTCAGGCTCT | T   |             |     | TGAAAGA  | 2.7 Out-of-frame |
| 55    | V2     | T   |             | I   | CTCTATGAACAG | 2.7 In-frame |
| V31   | GGTCAGGCTCT | GG   |             |     | GAACAG   | 2.7 In-frame |
| 59    | V2     | TC  |             | SS  | AGTCAAACACCTTG | 2.4 In-frame |
| V31   | GGCT  |     |             | GGC | CACCTTG  | 2.4 Out-of-frame |
| 62    | V2     | The V2(Dβ)β2 rearrangement was not cloned and sequenced |
| V31   | V31-to-5′Dβ1 RSS hybrid join, deleting all subsequent β1-Cβ1 and Dβ2/β2-Cβ2 sequences |
| 90    | V2     | TT  |             | CT  | C       | 2.7 In-frame |
| V31   | GCTTAGGCTCT | CCCCT |             |     | TGGAAAGA | 2.3 In-frame |
| 105   | V2     | C   |             | G   | CAG    | 2.7 In-frame |
| V31   | V31 rearranged to the Vβ coding sequence of an out of frame V29(Dβ)β2.7 rearrangement |     |             |     |         |            |

Sequences are underlined every three nucleotides to orient the reading frame. Dβ nucleotides in bold are from Dβ1, italicized nucleotides are from Dβ2, and for V2 rearrangements, unformatted nucleotides could be from either.
Materials and methods

Mice

All experimental mice assayed in this study were 4–6 wk old, of mixed sex, and housed under specific pathogen-free conditions at the Children’s Hospital of Philadelphia (CHOP). Animal husbandry, breeding, and experiments were performed in accordance with national guidelines and regulations and approved by the CHOP Institutional Animal Care and Use Committee. We used CRISPR/Cas9-mediated genomic editing in C57BL/6 zygotes to create mice carrying replacement of a V2 RSS with the normal 3\Dβ1RSS(V2R allele) or variant 3\Dβ1RSS(V2F allele) or a V31 RSS with the variant 3\Dβ1RSS(V31F allele).

To replace the V2 RSS, we identified a suitable target protoscaler adjacent motif 5’-AGG-3’ located on the antisense strand of the V2 RSS spacer. We subcloned the 20-mer “Trbv2 gRNA target” sequence (Table S1) into the pSpCas9(BB)-2A-Puro vector and in vitro transcribed a single-stranded guide RNA using described methods (Ran et al., 2013). The CHOP Transgenic Core microinjected zygotes with a mixture of the single-stranded guide RNA (8 μM), Cas9 protein (8 μM), and a single-strand oligonucleotide (ssDNA) repair template (10 μM; Chen et al., 2016; Wang et al., 2013). To generate the V2F allele, we used the ssDNA 5’-GGACTACTGAGTACCTGAGCTCAGGTAGACCAGTTACATCAACAGTTTCCTGGATCCACTGAGGAGGTTTTTGTAAGGCTTCCCATAGAATTGAATCACCGTGTCTTGGCTGCTGGCACAGAAGTATGTGGCCGAGTCATCAGGCTTTAGAGCTGTGATCTGAAGG-3’ (Integrated DNA Technologies). To generate the V31F allele, we used the ssDNA 5’-GGACTACTGAGTGCCCAGGCTCAGGTAGACCAGTTACATCAACAGTTTCCTGGATCCACTGAGGAGGTTTTTGTAAGGCTTCCCATAGAATTGGAGCACCGTGTCTTGGCTGCTGGCACAGAAGTATGTGGCCGAGTCATCAGGCTTTAGAGCTGTGATCTGAAGG-3’ (Integrated DNA Technologies). For both V2R and V2F alleles, founders were identified by PCR on tail DNA using the 5’V2 and 3’Dβ1RSSRev primers and/or 3’V2 and 3’Dβ1RSS primers. Each RSS replacement was then verified in homozygous mice by PCR sequencing using the 5’V2 and 3’V2 primers. For subsequent genotyping, primers 3’V2 and V2Fos were used to identify the V2R allele, and primers 3’V2 and V2Fos were used to identify the V2F allele (see Table S2 for a list of all primers).

To generate the V31F allele, we identified a suitable target protoscaler adjacent motif 5’-AGG-3’ located on the sense strand of the V31 RSS spacer. As outlined above, we used the 20-mer “Trbv3 sl gRNA target” (Table S1) and the ssDNA 5’-CAGGCC...
GAAGGACGACCAATTCTATCTAAGACCGAGAACGCTGCTTCTCAGCCACTCTGCTTTACCTGCGTGAATGCTGCTTTACAAAAACCACACCCCTCTCTTTAGTCCTTCCCTCCTACTAGGAACCCCTACTAGGGATG GGTGGAGGGGGTTTGCCACTGAATTT-3' 9

3

CTTTAGTCCTTCCTCCCTCACTAGGAACCCTCACTAGGGATG

9

V2R/+

GGTGAGGGGGTTTGGCACTGAATTT-3'

CD8+ T cell development, thymocytes were stained with anti-CD4, anti-CD8α, anti-TCRβ, anti-c-Kit, anti-CD25, and a lineage (Lin) panel composed of anti-TCRγδ, CD11b, CD11c, CD19, B220, TER119, and NK1.1 antibodies. Differential expression of c-Kit and CD25 in Lin−CD4−CD8− TCRβ− cells identifies DN1−4 thymocytes. Gross thymocyte development was assessed based on the expression of CD4 and CD8. Peripheral αβ T cell numbers were determined by staining splenocytes with anti-CD4, anti-CD8α, and anti-TCRβ antibodies and identifying CD4+ TCRβ+ or CD8+ TCRβ− cells. To monitor TcRβ allelic exclusion, we wished to avoid potential background staining artifacts that can result as a consequence of using biotinylated primary antibodies and streptavidin secondaries. Thus, we ordered directly conjugated antibodies, most of which are available in only FITC and PE, respectively. These are anti-TCRα, anti-TCRβ, anti-c-Kit, anti-CD25, and a lineage (Lin) panel composed of anti-TCRγδ, CD11b, CD11c, CD19, B220, TER119, and NK1.1 antibodies. Differential expression of c-Kit and CD25 in Lin−CD4−CD8− TCRβ− cells identifies DN1−4 thymocytes. Gross thymocyte development was assessed based on the expression of CD4 and CD8. Peripheral αβ T cell numbers were determined by staining splenocytes with anti-CD4, anti-CD8α, and anti-TCRβ antibodies and identifying CD4+ TCRβ+ or CD8− TCRβ− cells. To monitor TcRβ allelic exclusion, we wished to avoid potential background staining artifacts that can result as a consequence of using biotinylated primary antibodies and streptavidin secondaries. Thus, we ordered directly conjugated anti-TCRβ antibodies, most of which are available in only FITC and PE. We stained cells in PBS containing 3% FCS and 0.1% NaN₃ with the following antibodies: anti-CD4 APC-eFluor780, anti-CD8α Pacific Blue, and anti-TCRβ APC. In addition to the aforementioned antibodies, we stained cells with anti-Vδ4 (V2) PE or anti-Vβ14 (V31) FITC, and a corresponding antibody in either FITC or PE, respectively. These are anti-Vβ10b (V4) PE, anti-Vβ5.1, 5.2 (V12) FITC or PE, anti-Vβ6 (V19) FITC or PE, and anti-Vδ8 (V13) FITC or PE. Surface TCRβ expression was assayed on singlet and SP (CD4+ and CD8+ ) cells. Data were collected on an LSR Fortessa and analyzed with FlowJo software (Tree Star). Single cells were gated on the basis of forward and side scatter.

Generating and analyzing αβ T cell hybridomas
We generated a panel of αβ T cell hybridoma clones using two independent splenocyte cultures from two different V2R+/V31+/R mice (Shinkai et al., 1993), to establish experimental mice as a consequence of using biotinylated primary antibodies and streptavidin secondaries. Thus, we ordered directly conjugated antibodies, most of which are available in only FITC and PE, respectively. These are anti-TCRα, anti-TCRβ, anti-c-Kit, anti-CD25, and a lineage (Lin) panel composed of anti-TCRγδ, CD11b, CD11c, CD19, B220, TER119, and NK1.1 antibodies. Differential expression of c-Kit and CD25 in Lin−CD4−CD8− TCRβ− cells identifies DN1−4 thymocytes. Gross thymocyte development was assessed based on the expression of CD4 and CD8. Peripheral αβ T cell numbers were determined by staining splenocytes with anti-CD4, anti-CD8α, and anti-TCRβ antibodies and identifying CD4+ TCRβ+ or CD8− TCRβ− cells. To monitor TcRβ allelic exclusion, we wished to avoid potential background staining artifacts that can result as a consequence of using biotinylated primary antibodies and streptavidin secondaries. Thus, we ordered directly conjugated anti-TCRβ antibodies, most of which are available in only FITC and PE. We stained cells in PBS containing 3% FCS and 0.1% NaN₃ with the following antibodies: anti-CD4 APC-eFluor780, anti-CD8α Pacific Blue, and anti-TCRβ APC. In addition to the aforementioned antibodies, we stained cells with anti-Vδ4 (V2) PE or anti-Vβ14 (V31) FITC, and a corresponding antibody in either FITC or PE, respectively. These are anti-Vβ10b (V4) PE, anti-Vβ5.1, 5.2 (V12) FITC or PE, anti-Vβ6 (V19) FITC or PE, and anti-Vδ8 (V13) FITC or PE. Surface TCRβ expression was assayed on singlet and SP (CD4+ and CD8+ ) cells. Data were collected on an LSR Fortessa and analyzed with FlowJo software (Tree Star). Single cells were gated on the basis of forward and side scatter.

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Flow cytometry
Single-cell suspensions were prepared from the thymuses and spleens of mice and depleted of red blood cells, and Fc receptors were blocked using anti-CD16/CD32. All antibody stains were performed in PBS containing 3% FCS and 0.1% NaN₃ (see Table S3 for a list of all antibodies). To determine effects on gross αβ T cell development, thymocytes were stained with anti-CD4, anti-CD8α, anti-TCRβ, anti-c-Kit, anti-CD25, and a lineage (Lin) panel composed of anti-TCRγδ, CD11b, CD11c, CD19, B220, TER119, and NK1.1 antibodies. Differential expression of c-Kit and CD25 in Lin−CD4−CD8− TCRβ− cells identifies DN1−4 thymocytes. Gross thymocyte development was assessed based on the expression of CD4 and CD8. Peripheral αβ T cell numbers were determined by staining splenocytes with anti-CD4, anti-CD8α, and anti-TCRβ antibodies and identifying CD4+ TCRβ+ or CD8− TCRβ− cells. To monitor TcRβ allelic exclusion, we wished to avoid potential background staining artifacts that can result as a consequence of using biotinylated primary antibodies and streptavidin secondaries. Thus, we ordered directly conjugated anti-TCRβ antibodies, most of which are available in only FITC and PE. We stained cells in PBS containing 3% FCS and 0.1% NaN₃ with the following antibodies: anti-CD4 APC-eFluor780, anti-CD8α Pacific Blue, and anti-TCRβ APC. In addition to the aforementioned antibodies, we stained cells with anti-Vδ4 (V2) PE or anti-Vβ14 (V31) FITC, and a corresponding antibody in either FITC or PE, respectively. These are anti-Vβ10b (V4) PE, anti-Vβ5.1, 5.2 (V12) FITC or PE, anti-Vβ6 (V19) FITC or PE, and anti-Vδ8 (V13) FITC or PE. Surface TCRβ expression was assayed on singlet and SP (CD4+ and CD8+ ) cells. Data were collected on an LSR Fortessa and analyzed with FlowJo software (Tree Star). Single cells were gated on the basis of forward and side scatter.

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mice, using established methods and reagents (Sleckman et al., 1997). We characterized Tcrb rearrangements of each clone by Southern blot analyses using strategies and probes previously described (Bassing et al., 2000, 2008; Khor and Sleckman, 2005; Wu et al., 2003). The V3β8 allele contains an additional 101-bp sequence that distinguishes it from an unmodified V3β allele, and using primers S’V3β and 3’V3β permits us to determine in clones with V3β rearrangements which V3β rearranged (Horowitz and Bassing, 2014; Wu et al., 2003). We used the S’β2 and 3’Dβ1RSSRev primers to PCR-identify which V2 rearranged in clones with V2 rearrangements. For clones with recombination of the RSS-replaced V2 and V3β segments, we PCR-sequenced each rearrangement using the S’β2 or S’V3β primer in combination with each of the Jβ reverse primers and PCR conditions previously reported (Wu et al., 2003; see Table S2 for a list of all primers).

Cell sorting
Single-cell suspensions of total thymocytes were stained and sorted to isolate DN1/2 and DN3 thymocytes. Following red blood cell depletion, thymocytes were stained with anti-CD4, anti-CD8α, anti-TcRβ, anti-c-Kit, anti-CD25, and the Lin panel. Thymocytes were first gated on Lin‘CD4‘CD8‘TcRβ‘ cells and then sorted c-Kit+ cells to isolate DN1/2 cells or c-Kit‘CD25+ cells to isolate DN3 cells.

Real-time quantitative PCR (qPCR) analysis
TagMan qPCR assays were performed on DNA isolated from sorted DN1/2 and DN3 thymocytes to detect Vβ(Dβ1)β1.1, Vβ(Dβ1)β2.1, and Dβ2β2.1 rearrangements using previously described reagents and methods (Gopalakrishnan et al., 2013). Total Vβ(Dβ1)β1.1, Vβ(Dβ1)β2.1, and Dβ2β2.1 rearrangements were normalized to an unarranged region of the genome (CD19).

Quantification and statistical analysis
Data are reported as mean ± SD. Statistical analyses were done with Prism 8.

Online supplemental material
Fig. S1 provides sequence validation for the V2 and V3β RSS replacement mice. Fig. S2 provides analysis of thymocyte and αβ T-cell development. Fig. S3 provides analysis of V2 and V3β rearrangements in DN thymocytes. Fig. S4 shows similar changes in the Vβ repertoire of RSS replacement mice in peripheral αβ T cells. Fig. S5 shows peripheral αβ T cells with biallelic Tcrb gene expression. Table S2 provides analysis of Tcrb rearrangements in Tcrbβ7γ hybridomas. Table S3 is a list of oligonucleotides used to generate, genotype, and sequence the mouse models as well as to perform the TaqMan qPCR. Table S3 is a list of all the key reagents to perform flow cytometry and create hybridomas, and additional mouse strains used.

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Author contributions: C.H. Bassing conceived and supervised this study. C.H. Bassing and K.S. Yang-Iott designed the V2β and V3β modifications. G.S. Wu designed the V2β and V3β modifications. G.S. Wu and C.H. Bassing designed the research plan. G.S. Wu, with assistance from K.D. Lee, conducted and analyzed all mouse experiments. K.S. Yang-Iott and M.A. Klink made and analyzed hybridomas, and worked with G.S. Wu and C.H. Bassing to identify Tcrb rearrangements. K.E. Hayer performed all statistical analyses. G.S. Wu and C.H. Bassing worked together to prepare the manuscript.

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References
Agata, Y., N. Tamaki, S. Sakamoto, T. Ikawa, K. Masuda, H. Kawamoto, and C. Murre. 2007. Regulation of T cell receptor beta gene rearrangements and allelic exclusion by the helix-loop-helix protein, E47. Immunity. 27: 871–884. https://doi.org/10.1016/j.immuni.2007.11.015
Akira, S., K. Okazaki, and H. Sakano. 1997. Two pairs of recombination signals are sufficient to cause immunoglobulin V-(D)-J joining. Science. 238:1134–1138. https://doi.org/10.1126/science.2381032
Banerjee, J.K., and D.G. Schatz. 2014. Synapsis alters RAG-mediated nicking at Tcrb recombination signal sequences: implications for the “beyond 12/23” rule. Mol. Cell. Biol. 34:2566–2580. https://doi.org/10.1128/MCB.00411-14
Bassing, C.H., F.W. Alt, M.M. Hughes, M. D’Auteuil, T.D. Wehrly, B.B. Woodman, F. Gärtner, J.M. White, L. Davidson, and B.P. Sleckman. 2000. Recombination signal sequences restrict chromosomal V(D)J recombination beyond the 12/23 rule. Nature. 405:583–586. https://doi.org/10.1038/35046435
Bassing, C.H., W. Swat, and F.W. Alt. 2002. The mechanism and regulation of chromosomal V(D)J recombination. Cell. 109(2, Suppl):S45–S55. https://doi.org/10.1016/S0092-8674(02)00675-X
Bassing, C.H., S. Whitlow, R. Mostoslavsky, K. Yang-Iott, S. Ranganath, and F.W. Alt. 2008. Vbeta cluster sequences reduce the frequency of primary Vbeta2 and Vbeta14 rearrangements. Eur. J. Immunol. 38:2554–2572. https://doi.org/10.1002/eji.200838347
Bolland, D.J., H. Koohy, A.L. Wood, L.S. Matheson, F. Krueger, M.J. Stubbington, A. Baizan-Edge, P. Chovanec, B.A. Stubbs, K. Tabbada, et al. 2016. Two Mutually Exclusive Local Chromatin States Drive Efficient V(D)J Recombination. Cell Rep. 15:2475–2487. https://doi.org/10.1016/j.celrep.2016.05.020
Bories, J.C., J. Demengeot, L. Davidson, and F.W. Alt. 1996. Deletion of the mouse T-cell receptor beta gene enhancer blocks alphabeta T-cell development. Proc. Natl. Acad. Sci. USA. 93:7867–7871. https://doi.org/10.1073/pnas.93.15.7871
Bouvier, G., F. Watrin, M. Naspetti, C. Verthuy, P. Naquet, and P. Ferrier. 1996. Deletion of the mouse T-cell receptor beta gene enhancer blocks alpha beta T-cell development. Proc. Natl. Acad. Sci. USA. 93:7871–7881. https://doi.org/10.1073/pnas.93.15.7877
Brady, B.L., N.C. Steinel, and C.H. Bassing. 2010. Antigen receptor allelic exclusion: an update and reappraisal. J. Immunol. 185:3801–3808. https://doi.org/10.4049/jimmunol.1001158
Chan, E.A., G. Teng, E. Corbett, K.R. Choudhury, C.H. Bassing, D.G. Schatz, and M.S. Krangel. 2013. Peripheral subnuclear positioning suppresses Tcrb recombination and segregates Tcrb alleles from...
Ryu, C.J., B.B. Haines, H.R. Lee, Y.H. Kang, D.D. Draganov, M. Lee, C.E. Whitehurst, H.J. Hong, and J. Chen. 2004. The T-cell receptor beta variable gene promoter is required for efficient V beta rearrangement but not allelic exclusion. Mol. Cell. Biol. 24:7015–7023. https://doi.org/10.1128/MCB.24.16.7015-7023.2004

Schatz, D.G., and P.C. Swanson. 2011. V(D)J recombination: mechanisms of initiation. Annu. Rev. Genet. 45:167–202. https://doi.org/10.1146/annurev-genet-110410-132552

Schlimmen, R.J., K.L. Reddy, H. Singh, and M.S. Krangel. 2008. Initiation of allelic exclusion by stochastic interaction of Tcrb alleles with repressive nuclear compartments. Nat. Immunol. 9:802–809. https://doi.org/10.1038/ni.1624

Shih, H.Y., and M.S. Krangel. 2013. Chromatin architecture, CCCTC-binding factor, and V(D)J recombination: managing long-distance relationships at antigen receptor loci. J. Immunol. 190:4915–4921. https://doi.org/10.4049/jimmunol.1300918

Shinkai, Y., S. Koyasu, K. Nakayama, K.M. Murphy, D.Y. Loh, E.L. Reinherz, and F.W. Alt. 1993. Restoration of T cell development in RAG-2-deficient mice by functional TCR transgenes. Science. 259:822–825. https://doi.org/10.1126/science.8430336

Sicinska, E., I. Alfantis, L. Le Cam, W. Swat, Q. Yu, A.A. Ferrando, S.D. Levin, Y. Geng, H. von Boehmer, et al. 2003. Requirement for cyclin D3 in lymphocyte development and T cell leukemias. Proc. Natl. Acad. Sci. USA. 100:8225–8230. https://doi.org/10.1073/pnas.0700081104

Slekman, B.P., C.H. Bassing, M.M. Hughes, A. Okada, M. Draganov, L. Davidson, J. Chen, and F.W. Alt. 2000. Mechanisms of V(D)J recombinase action at T cell receptor loci in rearranging thymocytes. Nat. Immunol. 1:837–847. https://doi.org/10.1038/ni1448

Sleckman, B.P., C.G. Bardon, R. Ferrini, L. Davidson, and F.W. Alt. 1997. Function of the TCR alpha enhancer in alphabeta and gammadelta T cells. Immunity. 7:505–515. https://doi.org/10.1016/S1074-7613(00)80372-6

Sleckman, B.P., C.H. Bassing, M.M. Hughes, A. Okada, M. Dauteuil, T.D. Wei, and J. Chen. 2000. Mechanisms that direct ordered assembly of T cell receptor beta locus V, D, and J gene segments. Proc. Natl. Acad. Sci. USA. 97:7975–7980. https://doi.org/10.1073/pnas.103019097

Steinle, N.C., S.B. Lee, A.T. Tubbs, J.J. Bednarski, E. Schulte, K.S. Yang-lott, D.G. Schatz, B.P. Sleckenman, and C.H. Bassing. 2013. The ataxia telangiectasia mutated kinase controls Igk allelic exclusion by inhibiting secondary Vk-to-Jk rearrangements. J. Exp. Med. 210:233–239. https://doi.org/10.1084/jem.20121605

Tillman, R.E., A.L. Wooley, B. Khor, T.D. Wehrly, C.A. Little, and B.P. Sleckenman. 2003. Cutting edge: targeting of V beta to D beta rearrangement by RSSs can be mediated by the V(D)J recombination in the absence of additional lymphoid-specific factors. J. Immunol. 170:5–9. https://doi.org/10.4049/jimmunol.170.1.5

VanDyk, L.F., T.W. Wise, B.B. Moore, and K. Meek. 1996. Immunoglobulin D(H) recombination signal sequence targeting: effect of D(H) coding and flanking regions and recombination partner. J. Immunol. 157:4005–4015.

Vettermann, C., and M.S. Schlissel. 2010. Allelic exclusion of immunoglobulin genes: models and mechanisms. Immunol. Rev. 237:22–42. https://doi.org/10.1111/j.1600-065X.2010.00935.x

Wang, X., C. Xiao, Y. Zhang, X. Wen, X. Gao, S. Okada, and X. Liu. 2008. Regulation of Tcra recombination ordering by c-Fos-dependent RAG deposition. Nat. Immunol. 9:794–801. https://doi.org/10.1038/ni.1614

Wei, Z., and M.R. Lieber. 1993. Lymphoid V(D)J recombination. Functional analysis of the spacer sequence within the recombination signal. J. Biol. Chem. 268:3180–3183.

Wilson, A., C. Marechal, and H.R. MacDonald. 2001. Biased V beta usage in immature thymocytes is independent of DJ beta proximity and pT alpha pairing. J. Immunol. 166:51–57. https://doi.org/10.4049/jimmunol.166.1.51

Wu, C., C.H. Bassing, D. Jung, B.B. Woodman, D. Foy, and F.W. Alt. 2003. Dramatically increased rearrangement and peripheral representation of Vbeta14 driven by the 3’Dbeta1 recombination signal sequence. Immunology. 18:75–85. https://doi.org/10.1046/j.1365-2567.2002.01830.x

Wu, C., S. Ranganath, M. Gleason, B.B. Woodman, T.M. Borjeson, F.W. Alt, and C.H. Bassing. 2007. Restriction of endogenous T cell antigen receptor beta rearrangements to Vbeta14 through selective recombination signal sequence modifications. Proc. Natl. Acad. Sci. USA. 104:4002–4007. https://doi.org/10.1073/pnas.0700008104

Yang-lott, K.S., A.C. Carpenter, M.A. Rowh, N. Steinle, B.L. Brady, K. Hchedlinger, R. Jaenisch, and C.H. Bassing. 2010. TCR beta feedback signals inhibit the coupling of recombinationally accessible V beta 14 segments with DJ beta complexes. J. Immunol. 184:1369–1378. https://doi.org/10.4049/jimmunol.0900723

Wu et al.

Poor Vß RSSs enforce TCRß allelic exclusion

Journal of Experimental Medicine

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13 of 13
Figure S1. Validation of V2 and V31 RSS replacement mice. Sequence validation of the V2 or V31 RSS replacement with the 3′Dβ1 RSS. The 3′Dβ1 RSSs are highlighted in blue.
Figure S2. **Normal gross αβ T cell development in V2 and V31 RSS replacement mice.** (A and B) DN thymocyte development. Representative plots (A) and quantification (B) of DN cells. Gated on Lin−CD4−CD8−TCRβ− thymocytes (n ≥ 5 mice per group). (C and D) Global thymocyte development. Representative plots (C) and quantification (D) of DN, DP, CD4+, and CD8+ thymocytes (n = 5 mice per group). (E and F) Representative plots (E) and quantification (F) of SP αβ T cells in the spleen. Gated on TCRβ+ cells (n = 5 mice per group). (B, D, and F) Two-way ANOVA followed by Dunnett’s post-tests for multiple comparisons. All quantification plots show mean ± SD. Data in B, D, and F are compiled from at least five experiments.
Figure S3. The 3’Dβ1 RSS replacement increases V2 and V31 recombination in DN3 thymocytes. (A) Quantification of Dβ2-Jβ2.1 rearrangements by TaqMan qPCR in DN1/2 thymocytes (n = 3 mice per group). (B and C) Quantification of indicated Vβ rearrangements by TaqMan qPCR in DN1/2 (B) or DN3 (C) thymocytes (n = 3 mice per group, two-way ANOVA followed by Bonferroni’s post-tests for multiple comparisons). These data are compiled from three experiments. N.D., not determined; *, P < 0.05; ****, P < 0.0001.
Peripheral αβ T cells exhibit similar shifts in the Vβ repertoire in RSS replacement mice. (A) Representative plots of SP splenocytes expressing V2⁺, V31⁺, or V19⁺ TCRβ chains. (B–D) Quantification of V2⁺ (B), V31⁺ (C), or V19⁺ (D) SP thymocytes (n = 5 mice per group, one-way ANOVA followed by Tukey’s post-tests for multiple comparisons). (E) Quantification of SP splenocytes expressing V4⁺, V12⁺, or V13⁺ TCRβ chains (n = 5 mice per group, two-way ANOVA followed by Tukey’s post-tests for multiple comparisons). (F) Ratio of the V2⁺ and V31⁺ Vβ repertoires. The fold change calculates the frequency of V2⁺ cells from V2R/R mice divided by V2R/+ mice. A similar calculation was made for V31⁺ cells from V31R/R and V31R/+ mice. All quantification plots show mean ± SD. Multiple post-tests are compared with WT unless indicated by bars, and P values are corrected for multiple tests. ns, not significant; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data in B–F are compiled from five experiments.
αβ T cells exhibiting biallelic Tcrb gene expression seed the periphery. (A, C, and E) Representative plots of SP splenocytes expressing both V2+ and V19+ (A), V31+ and V19+ (C), or V2+ and V31+ (E) TCRβ chains. (B, D, and F) Quantification of SP splenocytes expressing the two indicated TCRβ chains. n = 5 mice per group, two-way ANOVA followed by Tukey’s post-tests for multiple comparisons (B and D), one-way ANOVA followed by Dunnett’s post-tests comparing each RSS mutant to WT (F). (G) Quantification of double-staining SP splenocytes for each Vβ combination tested (n = 5 mice per group, two-way ANOVA). (H) Depiction of the recombination events that could result in two TCRβ chains expressed from one allele. RSSs indicated as triangles. (I) RIC scores of RSSs in this study, generated by RSSSite (https://www.itb.cnr.it/rss/). All quantification plots show mean ± SD. Multiple post-tests are compared with WT unless indicated by bars, and P values are corrected for multiple tests. ns, not significant, *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data in B, D, F, and G are compiled from five experiments.

Figure S5. αβ T cells exhibiting biallelic Tcrb gene expression seed the periphery. (A, C, and E) Representative plots of SP splenocytes expressing both V2+ and V19+ (A), V31+ and V19+ (C), or V2+ and V31+ (E) TCRβ chains. (B, D, and F) Quantification of SP splenocytes expressing the two indicated TCRβ chains. n = 5 mice per group, two-way ANOVA followed by Tukey’s post-tests for multiple comparisons (B and D), one-way ANOVA followed by Dunnett’s post-tests comparing each RSS mutant to WT (F). (G) Quantification of double-staining SP splenocytes for each Vβ combination tested (n = 5 mice per group, two-way ANOVA). (H) Depiction of the recombination events that could result in two TCRβ chains expressed from one allele. RSSs indicated as triangles. (I) RIC scores of RSSs in this study, generated by RSSSite (https://www.itb.cnr.it/rss/). All quantification plots show mean ± SD. Multiple post-tests are compared with WT unless indicated by bars, and P values are corrected for multiple tests. ns, not significant, *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Data in B, D, F, and G are compiled from five experiments.

Wu et al. Poor Vβ RSSs enforce TCRβ allelic exclusion

Journal of Experimental Medicine
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Tables S1–S3 are provided online as separate files. Table S1 is an analysis of Tcrβ rearrangements in TcrβTg T cell hybridomas. Table S2 shows oligonucleotides used for generating mouse lines, genotyping, sequencing, and TaqMan qPCR. Table S3 shows key reagents.