Inefficient V(D)J recombination underlies monogenic T cell receptor β expression

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Edited by Peter Cresswell, Yale University, New Haven, CT, and approved July 1, 2020 (received for review May 24, 2020)

The assembly of T cell receptor (TCR) and immunoglobulin (Ig) genes by V(D)J recombination generates the antigen receptor (AgR) diversity that is vital for adaptive immunity. At most AgR loci, V(D)J recombination is regulated so that only one allele assembles a functional gene, ensuring that nearly every T and B cell expresses a single type, or specificity, of AgR. The genomic organizations of some AgR loci permit the assembly and expression of two distinct genes on each allele; however, this is prevented by undetermined mechanisms. We show that the poor qualities of recombination signal sequences (RSSs) flanking Vβ gene segments suppress the assembly and expression of two distinct TCRβ genes from a single allele. Our data demonstrate that an intrinsic genetic mechanism that stochastically limits Vβ recombination efficiency governs monogenic TCRβ expression, thereby restraining the expression of multiple AgRs on αβ T cells.

V(D)J recombination | allelic exclusion | monogenic expression | lymphocyte development | recombination signal sequence

The vast diversity of antigen receptors (AgRs) expressed by T and B lymphocytes is essential for effective adaptive immunity. A T cell antigen receptor (TCR) is composed of TCR β and α or γ and δ proteins, whereas a B cell receptor (or antibody) consists of immunoglobulin H (IgH) and Igκ or Igλ proteins. Developing T and B cells assemble TCR or Ig genes, respectively, through lymphocyte lineage- and developmental stage-specific recombination of variable (V), diversity (D), and joining (J) gene segments (1, 2). Each V(D)J rearrangement and downstream constant (C) region exons comprise a TCR or Ig gene, which encodes protein only if the V and J segments are recombined in-frame. The number of possible joining combinations and imprecise repair of V(D)J coding joins produce enormous AgR gene diversity.

V(D)J recombination poses a challenge for highly specific adaptive immune responses because both alleles of a locus could assemble an in-frame gene, producing a lymphocyte with two distinct AgRs. To achieve monospecificity, V gene segment rearrangements at TCR and Ig loci are regulated between alleles to enforce monoallelic AgR expression (3, 4). This allelic exclusion is achieved by sequential initiation of V recombination between alleles and subsequent feedback inhibition of V recombination signaled by protein expressed from the in-frame V(D)J rearrangement (3, 4). Notably, the genomic organizations of mammalian TCRβ, TCRγ, TCRδ, and Igλ loci, as well as Ig loci of cartilaginous fish, permit the assembly and expression of multiple genes from a single allele, providing an additional obstacle to achieve monospecificity. Currently, there are no reported mechanisms by which V rearrangements are regulated on individual alleles to achieve monogenic protein expression.

TCRβ loci consist of 23 functional Vβs that recombine by deletion to either one of two downstream Dβ–Jβ–Cβ clusters, and another Vβ (*V31*) located further downstream that rearranges through inversion to either Dβ–Jβ–Cβ cluster (Fig. 1*A*) (5). Theoretically, each TCRβ allele can assemble and express two distinct genes—one involving an upstream Vβ and another involving *V31*. However, this has not been observed at any detectable level (6, 7), indicating that mechanisms control Vβ recombination on each allele to ensure monogenic TCRβ recombination and expression. The semiconserved recombination signal sequences (RSSs) that flank AgR locus gene segments target V(D)J recombinase activity and direct specific V(D)J rearrangements (1). For TCRβ, the poor qualities of Vβ RSSs stochastically restrain the number of Vβ rearrangements before TCRβ protein-signaled feedback inhibition, thereby allowing for monoallelic assembly and expression of functional TCRβ genes (8). To determine whether poor Vβ RSSs also limit the assembly and expression of two different TCRβ genes from the same allele, we generated C57BL/6 mice carrying replacements of both a *V2* RSS and a *V31* RSS on the same allele with the stronger 3′Dβ1 RSS (the $V2^{R}V31^{R}$ allele).

Results

We studied wild-type (*WT*), heterozygous $V2^{R}V31^{R}/WT$, and homozygous $V2^{R}31^{R}/V2^{R}31^{R}$ mice. The mutant mice had normal numbers and frequencies of mature splenic αβ T cells and thymocytes at each developmental stage. Due to the lack of congenic markers, TCRβ proteins cannot be identified by the allele that encodes them, nor whether they include Cβ1 versus Cβ2 regions. Thus, we performed flow cytometry using anti-V2 and anti-V31 antibodies to quantify cells expressing $V2^{+}$ and $V31^{+}$ TCRβ proteins. We assayed $CD4^{+}$ and $CD8^{+}$ single-positive (SP) thymocytes as they are mature and naive αβ T cells. Reflecting published data (8, 9), we detected a small fraction (0.11%) of cells that stained with both antibodies in *WT* mice (Fig. 1 *B* and *C*), which is consistent with a small population of $V2^{+}V31^{+}$ αβ T cells. We observed a 12.4-fold increased fraction of these cells in $V2^{R}31^{R}/WT$ mice, and a 32.8-fold increase in $V2^{R}31^{R}/V2^{R}31^{R}$ mice (Fig. 1 *B* and *C*). These elevated frequencies of dual-$TCR\beta^{+}$ cells corresponded with the greater utilizations of V2 and V31 in expressed TCRβ chains (Fig. 1 *D*–*F*). These data demonstrate that enhancing RSS quality of two Vβs on the same allele increases their rearrangement and consequently the fraction of T cells expressing two distinct types of TCRβ proteins. As the Vβ repertoire of SP thymocytes reflects the relative levels that individual Vβ segments recombine (10), the preferential usage of V31 over V2 reveals that $V31^{R}$ outcompetes $V2^{R}$ for rearrangement. This could be due to greater accessibility of *V31* (11) or interaction of *V31* with Dβ–Jβ segments before TCRβ locus contraction places *V2* near Dβ–Jβ segments. Notably, the higher than twofold increase of these dual-$TCR\beta^{+}$ cells in $V2^{R}31^{R}/V2^{R}31^{R}$ mice compared to $V2^{R}31^{R}/WT$ mice implies that two distinct V(D)Jβ rearrangements can contribute to TCRβ expression from the same allele.

To determine whether a single TCRβ allele can indeed support expression of TCRβ proteins from two different V(D)Jβ rearrangements, we analyzed mice where one TCRβ allele is inactivated

Author contributions: G.S.W. designed research; G.S.W. performed research; G.S.W. analyzed data; and G.S.W. and C.H.B. wrote the paper.

The authors declare no competing interest.

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First published July 20, 2020.

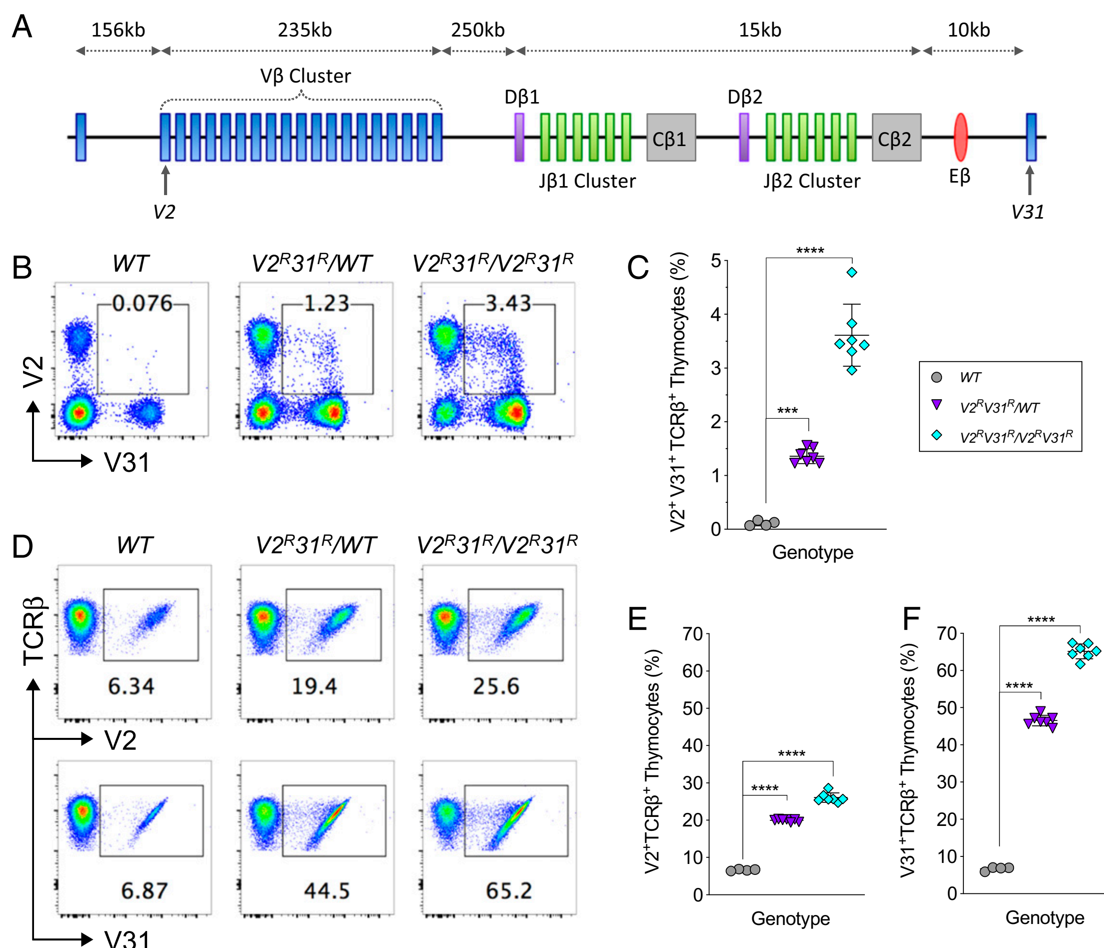

Fig. 1. Increased frequency of dual-TCRβ+ cells and altered Vβ repertoire in mice with two Vβ RSS replacements on the same allele. (*A*) Schematic of the TCRβ locus and relative positions of Vβ, Dβ, and Jβ segments, Cβ exons, and the Eβ enhancer. (*B* and *C*) Representative plots (*B*) and quantification (*C*) of SP thymocytes expressing both V2+ and V31+ TCRβ chains. (*D*–*F*) Representative plots (*D*) and quantification of SP thymocytes expressing V2+ (*E*) or V31+ (*F*) TCRβ chains. $n \geq 4$ mice per group; one-way ANOVA, Dunnett's multiple posttests comparing each RSS mutant to *WT*; $***P < 0.001$, $****P < 0.0001$. Data in *B*–*F* are compiled from five experiments.

by deletion of the TCRβ enhancer (Eβ) (12, 13). We assayed mice carrying the Eβ-deleted allele opposite a *WT* allele, an allele with an RSS replacement of either *V2* ($V2^R$) or *V31* ($V31^R$), or both (8). We detected a small percentage (0.094%) of $V2^+V31^+$ SP thymocytes in *WT/EβΔ* mice (Fig. 2 *A* and *B*), potentially representing a rare population of cells expressing two different TCRβ proteins from the same *WT* allele. Regardless, we observed $V2^+V31^+$ cells at a 1.9-fold greater frequency in $V2^R$/*EβΔ* mice and at a 4.8-fold greater frequency in $V31^R$/*EβΔ* mice (Fig. 2 *A* and *B*). Thus, enhancing the quality of either Vβ RSS elevates the fraction of cells expressing both $V2^+$ and $V31^+$ TCRβ proteins. Notably, we detected a 14.4-fold increased frequency of $V2^+V31^+$ cells in $V2^RV31^R$/*EβΔ* mice relative to *WT/EβΔ* mice (Fig. 2 *A* and *B*), indicating that enhancing quality of two Vβ RSSs synergistically increases the percentage of cells expressing both $V2^+$ and $V31^+$ TCRβ proteins. Indeed, deleting part of the *V31* RSS on the $V2^R$ allele (the $V2^R31Δ$ allele; Fig. 2*C*) dramatically reduces the frequency of $V2^+V31^+$ cells to levels that are equivalent or less than that in $V2^R$/*EβΔ* mice (0.178% versus 0.135%; Fig. 2 *A* and *B*). Collectively, these data confirm that the $V2^R31^R$ allele promotes expression of two distinct TCRβ proteins from two different V(D)Jβ rearrangements on a single allele.

Our study demonstrates that an intrinsic genetic mechanism governs monogenic TCRβ assembly and expression. We show that poor-quality Vβ RSSs cooperate to limit assembly and expression of two distinct TCRβ genes from one allele. We previously showed that poor-quality Vβ RSSs stochastically restrain Vβ recombination frequency before feedback inhibition to decrease biallelic assembly and expression of TCRβ genes (8). We now further conclude that low-quality Vβ RSSs also lower the incidence that both *V31* and an upstream Vβ recombine on the same allele. These rearrangements could involve either 1) a deletional *V2* rearrangement to the Dβ1–Jβ1–Cβ1 cluster and an inversional *V31* rearrangement to the Dβ2–Jβ2–Cβ2 cluster, or 2) an inversional *V31* rearrangement to the Dβ1–Jβ1–Cβ1 cluster, which inverts a portion of the locus that contains the Dβ2–Jβ2–Cβ2 cluster, and then an inversional *V2* rearrangement to the Dβ2–Jβ2–Cβ2 cluster (7) (Fig. 2*D*). To achieve monogenic TCRβ assembly and expression, this RSS-based genetic mechanism might function with epigenetic processes that have been implicated to enforce monoallelic Vβ recombination. For example, it has been proposed that dynamic interactions of Vβ segments with the nuclear lamina lowers Vβ recombination efficiency by repressing Vβ chromatin accessibility and chromosome looping between Dβ–Jβ clusters and upstream Vβ segments (14, 15). In this context, poor-quality Vβ RSSs could lower the likelihood that two Vβ rearrangements occur on an allele when *V31* and an upstream Vβ segment are both accessible and the upstream Vβ is looped in proximity with Dβ–Jβ segments. Thus, the properties of RSSs may underlie monogenic assembly and expression of mammalian

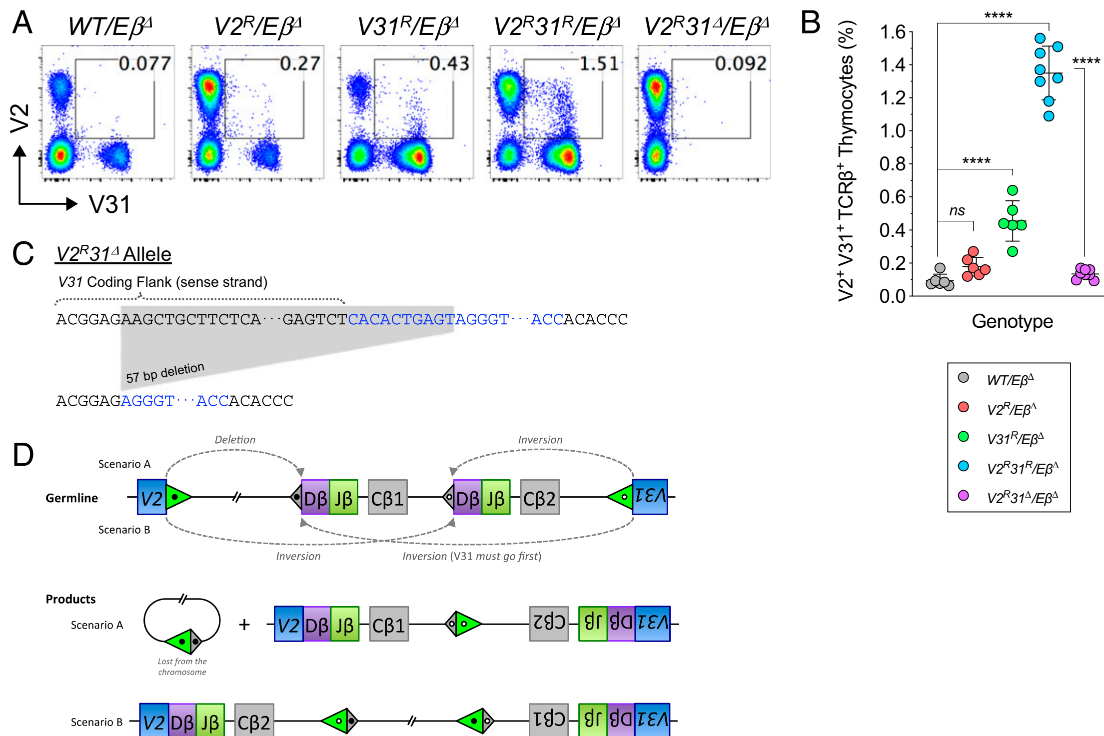

Fig. 2. Expression of two different TCRβ chains from the $V2^{R}V31^{R}$ allele. (*A* and *B*) Representative plots (*A*) and quantification (*B*) of SP thymocytes expressing both V2^{+} and V31^{+} TCRβ chains ($n \geq 6$ mice per group; one-way ANOVA, Tukey's multiple posttests; ns, not significant; ****$P < 0.0001$). (*C*) Schematic of the sense strand and truncation of the *V31* region of the $V2^{R}31\Delta$ allele, with the *V31* RSS indicated in blue. (*D*) Depiction of the recombination events that could result in two TCRβ chains expressed from one allele. RSSs are indicated as triangles. Data in *A* and *B* are compiled from four experiments.

TCRγ, TCRδ, and Igλ proteins in mammals, and Ig proteins in cartilaginous fish. Additionally, V RSSs may similarly contribute to Igκ and Igλ isotypic exclusion in B cells.

Methods

Mice. All experimental mice assayed in this study were 4 to 6 wk old, of mixed sex, and housed under specific pathogen-free conditions at the Children's Hospital of Philadelphia (CHOP). Mouse 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 homozygous $V2^{R}$ zygotes (8) to either replace the *V31* RSS with the 3′Dβ1 RSS (the $V2^{R}31^{R}$ allele) or to truncate the *V31* RSS (the $V2^{R}31\Delta$ allele) using methods previously described (8). Founding mice were backcrossed to C57BL/6 mice for two to five generations and then crossed to *WT*, *EβΔ/Δ*, or to each other to generate the experimental animals used in this study.

Flow Cytometry. Single-cell suspensions from thymuses and spleens were prepared as previously described (8). Cells were stained in PBS containing 3% FCS and 0.1% NaN_3 with the following antibodies: anti-CD4 APC-eFluor780, anti-CD8α Pacific Blue, anti-TCRβ APC, anti-*V2* PE, and anti-*V31* FITC (8). Data were collected on an LSR Fortessa and analyzed with FlowJo (Tree Star).

Statistical Analysis. Data are reported as mean ± SD. Statistical analyses were done with Prism 8.

Data Availability.

All study data are included in the article.

ACKNOWLEDGMENTS. We thank Adele Harman and Jennifer Dunlap of the CHOP Transgenics Core for help establishing mice with the $V2^{R}31^{R}$ and $V2^{R}31\Delta$ alleles. National Institutes of Health Grants T32 AI055428 (G.S.W.) and R01 AI 130231 (C.H.B.) supported this work.

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