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Regulation of TCRβ Allelic Exclusion by Gene Segment Proximity and Accessibility

Hrisavigi D. Kondilis-Mangum,* Han-Yu Shih,* Grace Mahowald,† Barry P. Sleckman,† and Michael S. Krangel*

Ag receptor loci are regulated to promote allelic exclusion, but the mechanisms are not well understood. Assembly of a functional TCR β-chain gene triggers feedback inhibition of Vβ-to-DJβ recombination in double-positive (DP) thymocytes, which correlates with reduced Vβ chromatin accessibility and a locus conformational change that separates Vβ from DJβ gene segments. We previously generated a Tcrb allele that maintained Vβ accessibility but was still subject to feedback inhibition in DP thymocytes. We have now further analyzed the contributions of chromatin accessibility and locus conformation to feedback inhibition using two novel TCR alleles. We show that reduced Vβ accessibility and increased distance between Vβ and DJβ gene segments both enforce feedback inhibition in DP thymocytes. The Journal of Immunology, 2011, 187: 000–000.

A defining characteristic of T and B lymphocytes is their ability to create and express unique Ag receptors that can recognize a vast array of foreign pathogens. To achieve receptor diversity, Ag receptor variable domains are encoded by multiple V, D, and J gene segments that are joined by a process known as V(D)J recombination (1). RAG1/2 mediates V(D)J recombination by: 1) binding to recombination signal sequences (RSSs) that flank Ag receptor gene segments; 2) bringing two RSSs (one with a 12- and one with a 23-bp spacer) into a synaptic complex; and 3) generating DNA double-strand breaks between the coding sequences and RSSs. Hairpin-sealed coding ends are subsequently opened by the Artemis endonuclease and ligated by nonhomologous end joining proteins to form Ag receptor coding joints. Because RAG1/2-generated double-strand breaks are potentially toxic, V(D)J recombination is highly regulated.

BCR and TCR genes undergo stepwise recombination in developing B and T lymphocytes, respectively (2–4). Igh rearranges in pro-B cells, and Igk and Igl rearrange in pre-B cells; Tcrb, Tcrg, and Tcrd rearrange in CD4+CD8+ double-negative (DN) thymocytes, and Tcra rearranges in CD4+CD8+ double-positive (DP) thymocytes. Moreover, Igh and Tcrb rearrangements are ordered such that D-to-J recombination precedes V-to-DJ recombination. This regulation is achieved, in part, by cis-elements such as enhancers and promoters that alter the chromatin landscape to make RSSs accessible to RAG1/2 (5). Accessible chromatin is characterized by active transcription, histone H3 and H4 acetylation, histone H3 lysine 4 trimethylation (H3K4me3), and removal of nucleosomes, and hypomethylation of CpG dinucleotides (2, 4). H3K4me3-modified nucleosomes also stimulate V(D)J recombination by docking RAG2 (6, 7) and enhancing the catalytic activity of the RAG1/2 complex (8).

Ag receptor loci also undergo changes in their conformation during lymphocyte development (9). A contracted locus conformation is thought to promote V(D)J recombination by facilitating the interaction between RSSs separated by great distances (e.g., Vβ and Dβ RSSs, Vβ and Dβ RSSs). Detailed analysis of contracted Igh loci revealed that Vβ segments spanning 2.5 megabases are all situated proximal to Dβ RSSs, presumably affording them all an opportunity for recombination (10). This interpretation is supported by the behavior of Pax5 deficient pro-B cells, in which Igh contraction and distal Vβ recombination are both impaired (11).

Ag receptor loci are also regulated to enforce allelic exclusion (12–14). For Igh and Tcrb, allelic exclusion is manifest at the V-to-DJ step and thought to occur in two phases: 1) an initiation phase, in which V-to-DJ recombination is regulated so that it is not attempted simultaneously on the two alleles; and 2) a maintenance phase, in which V-to-DJ recombination is terminated by a feedback mechanism once an in-frame rearrangement is produced. Feedback inhibition of Igh recombination in pre-B cells of Tcrb recombination in DP thymocytes is associated with epigenetic and locus conformational changes. Thus, whereas Igh and Tcrb alleles are by multiple criteria accessible in pre-B cells and DN thymocytes, respectively, their V gene segments display reduced accessibility in pre-B cells and DP thymocytes (2, 13, 14). In addition, unrearranged Igh and Tcrb alleles, although contracted in pre-B and DN thymocytes, respectively, become decontracted in pre B-cells and DP thymocytes (15, 16). These changes could inhibit recombination by limiting RAG1/2 binding to V segment RSSs and the likelihood of RSS synapses.

Several genetically modified Igh and Tcrb alleles have been created to assess the significance of these changes for feedback inhibition. Two Tcrb alleles with large deletions (β10 and Vβ1 NT) (17, 18) moved the otherwise distant Vβ10 gene segment into proximity of DJβ gene segments and increased its accessibility in DP thymocytes. Disruption of allelic exclusion was detected on...
**Mechanism of Tcrb Allelic Exclusion**

Vp1 NT alleles only, but no data evaluated whether altered Vp1 10 recombination reflected a loss of feedback inhibition in DP thymocytes as opposed to dysregulated rearrangement in DN thymocytes. Another study simply inserted a Vp gene segment just upstream of Dfr gene segments (19). Although allelic exclusion was perturbed at the level of Vp recombination, whether this reflected a loss of feedback inhibition in DP thymocytes was not evaluated in this study either. Bates et al. (20) generated a modified IgH allele in which a Vp gene segment was introduced just upstream of Dfr gene segments. This allele clearly displayed a disruption of feedback inhibition in pre-B cells. However, as the genetic manipulation moved the Vp4 into an accessible chromatin domain and also modulated distance, the individual effects of accessibility and distance could not be distinguished.

Jackson et al. (21) previously generated a Tcrb allele in which Vp accessibility was maintained in DP thymocytes by introducing the Tera enhancer (Ea) into the middle of the Vp array (ExaKI allele). Despite accessible Vp9 chromatin, feedback inhibition of Vp-to-Dfr recombination was maintained in DP thymocytes, indicating that parameters other than chromatin accessibility must be essential to enforce feedback inhibition in DP thymocytes. We have now further analyzed contributions of gene segment accessibility and proximity to feedback inhibition through the generation of two novel TCR alleles. Our results establish that reduced RSS accessibility and increased distance between RSSs both contribute to feedback inhibition of Vp-to-Dfr recombination in DP thymocytes.

**Materials and Methods**

**Mice and gene targeting**

Wild-type 129, Rag2-deficient (Rag2<sup>−/−</sup>) mice and Rag2<sup>−/−</sup> mice containing a rearranged Tcrb transgene (tg) were purchased from Taconic Farms. ExaKI, ExaKI Rag2<sup>−/−</sup> and ExaKI Rag2<sup>−/−</sup> Tcrb<sup>−/−</sup> mice were previously described (21). All mice were used in accordance with protocols approved by the Duke University and Washington University Animal Care and Use Committees.

DJEaKI mice were generated as follows: Dfr<sub>9</sub> and homology arms were PCR amplified using Pfu Turbo (Stratagene) and cloned using a TOPO Cloning kit (Invitrogen, Carlsbad, CA). DJE<sub>9</sub> arm was cloned between the NheI and the SalI sites. ES cells derived from Dfr<sub>9</sub> mice (21) were used for homologous recombination, whether this recombination reflected a loss of feedback inhibition in DP thymocytes was not evaluated in this study either. Bates et al. (20) generated a modified IgH allele in which a Vp gene segment was introduced just upstream of Dfr gene segments. This allele clearly displayed a disruption of feedback inhibition in pre-B cells. However, as the genetic manipulation moved the Vp4 into an accessible chromatin domain and also modulated distance, the individual effects of accessibility and distance could not be distinguished.

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**Germline transcription**

Chromatin immunoprecipitation

Chromatin was prepared from primary thymocytes of 2- to 3-wk-old mice by small-scale micrococcal nuclease digestion as previously described (27). Immunoprecipitations were performed using anti-H3K4me3 (04-745, clone MC315; Millipore) and normal rabbit IgG (AB-105-C; R&D Systems). All samples were resuspended in 2 μl sample by SYBR Green Real-Time PCR using a LightCycler 480 (Roche). All quantitative PCR amplifications used a touchdown strategy (touchdown quantitative PCR [TD-qPCR]) in which annealing temperature was reduced gradually from 65 to 58°C over 10 cycles followed by 55 cycles at 58°C. Primers used are listed in Supplemental Table I.

**Coding joints**

Genomic DNA isolated from sorted DN3 and DP thymocytes was amplified by touchdown PCR as follows: 5 min at 94°C, 31–35 cycles of 30 s at 94°C, 10 s at 62°C, and 0.1 mM EDTA; input samples were further diluted 1:50. Bound and input samples were quantified using 2 μl sample by SYBR Green Real-Time PCR using the TD-qPCR program. Actb was amplified for 40 cycles at 62°C. Primers are listed in Supplemental Table I.

**RSS retention**

DP thymocyte genomic DNA was amplified by TD-qPCR as described above. Primers are listed in Supplemental Table I.

**Genomic Southern blot**

Whole thymus genomic DNA was digested with restriction enzyme, subjected to 0.7% agarose gel electrophoresis, and transferred to a nylon membrane. Blots were hybridized with γ<sup>32</sup>P-labeled oligonucleotide probes. CdH1 was amplified for 23 cycles at 62°C. Primers and probes are listed in Supplemental Table I.

**Results**

**EaKI locus conformation**

Previous studies of mice carrying Tcrb alleles with an introduced E<sub>a</sub> (EaKI; Fig. 1A) indicated that elevation of Vp<sub>B</sub> accessibility, by itself, could not subvert feedback inhibition of Vp<sub>B</sub>-to-Dfr recom-
were contracted in DN thymocytes and decontracted in DP thymocytes (16), we found that, on average, wild-type alleles deficient DN and DP thymocytes (Fig. 2). Consistent with previous experiments (16), we used 3D-FISH to measure the distance between wild-type alleles, they were decontracted in DP thymocytes. To assess this, we used 3D-FISH to measure the distance between the V, D, J, and C gene segments and cis-elements. Bottom panel, EαKI allele, targeting construct, and DJEαKI allele. Right panel, Southern blot analysis of genomic DNA from wild-type and DJEαKI fragments are 7.1 and 3.5 kb, respectively.

FIGURE 2. TCR loci and gene targeting strategies. A, Generation of the DJEαKI allele. Top panel, Tcrb locus, including relative positions of and distances between the V, D, J, and C gene segments and cis-elements. Bottom panel, EαKI allele, targeting construct, and DJEαKI allele. Right panel, Southern blot analysis of genomic DNA from wild-type, heterozygous (+/DJEαKI), and homozygous (DJEαKI/DJEαKI) mice. DNA was digested with EcoRV and hybridized with a Vα13 probe. Expected wild-type and DJEαKI fragments are 7.1 and 3.5 kb, respectively.

B, Generation of the β-in-α allele. Top panel, Tcrb locus, including relative positions of and distances between the V, D, J, and C gene segments and cis-elements. Bottom panel, TCRα-in-α allele, targeting construct, and β-in-α allele. Right panel, Southern blot analysis of genomic DNA from wild-type, heterozygous (+/β-in-α), and homozygous (β-in-α/β-in-α) mice. DNA was digested with EcoRI and hybridized with a Vα13 probe. Expected wild-type and β-in-α fragments are 3.8 and 5.9 kb, respectively. Bent arrow, promoter; open and filled large triangles, 23- and 12-bp RSSs, respectively; small triangles, loxp sites. B, BamHI; DT, diphtheria toxin; E, EcoRI; V , TCR loci and gene targeting.

FIGURE 3. Regulation of the DJEαKI allele. We used homologous recombination to introduce a cassette containing PDα1 and a rearranged Dβ1(Uβ1.1(DJβ1KI)~1.0 kb 3’ of the Vβ13 RSS on the EαKI allele (DJEαKI allele; Fig. 1A). Mice homozygous for the DJEαKI allele displayed normal thymocyte development as assessed by cell number and expression of cell-surface markers CD4, CD8, CD25, and CD44 (data not shown).

We addressed chromatin accessibility on the DJEαKI allele by introducing it onto Rag2-deficient (Rag2−/−) and Rag2−/−× Tcrb tg backgrounds for analysis of steady-state germline transcripts and histone modifications in DN and DP thymocytes. Like Dα1 transcripts on wild-type alleles, DJβ1 transcripts on DJEαKI alleles were of comparable abundance in DN and DP thymocytes (Fig. 3A, right panel). Moreover, like nucleosomes at the Dα1 RSS on wild-type alleles, those at the DJβ1 RSS on DJEαKI alleles were H3K4me3 modified in DN thymocytes and displayed increased H3K4me3 in DP thymocytes (Fig. 3B, right panel). Hence, the DJβ1 RSS appears to reside in accessible chromatin in both DN and DP thymocytes of DJ EαKI mice.
As documented previously (21), germline transcription of \( V_\beta 13 \) is downregulated on transition from DN to DP on wild-type alleles but is upregulated on EozKI alleles (Fig. 3A, center panel). Unexpectedly, we found that introduction of the DJ\(_{EaKI}\) blunted the effect of Ea on \( V_\beta 13 \) in DJEozKI DP thymocytes, such that \( V_\beta 13 \) transcripts were upregulated as compared with wild-type DP thymocytes, but were no more abundant in DJEozKI DP than in DJEozKI DN thymocytes. This might reflect a suppression of transcription due to competition between PDJ\(_{b1}\) and the DJ\(_{b13}\) promoter or an effect of the DJ\(_{EaKI}\) on \( V_\beta 13 \) transcript stability. The DJ\(_{EaKI}\) also unexpectedly suppressed H3K4me3 at the \( V_\beta 13 \) RSS in both DN and DP thymocytes (Fig. 3B, center panel). Given these results, we also analyzed accessibility at \( V_\beta 8.1 \), which lies 5 kb upstream of \( V_\beta 13 \). Unlike \( V_\beta 13 \), the upregulation of \( V_\beta 8.1 \) transcription in EozKI DP thymocytes was maintained in DJEozKI DP thymocytes (Fig. 3A, left panel). Moreover, the upregulation of \( V_\beta 8.1 \) H3K4me3 in EozKI DP thymocytes was only partly suppressed by the DJ\(_{EaKI}\) (Fig. 3B, left panel). Taken together, the transcription and chromatin data suggest that \( V_\beta 13 \) is moderately accessible and that \( V_\beta 8.1 \) is highly accessible in DJEozKI DN and DP thymocytes.

To analyze \( V_\beta \)-to-DJ\(_{b}\) recombination, we prepared genomic DNA from purified DN3 and DP thymocytes of EozKI and DJEozKI mice, amplified with \( V_\beta 13 \) and \( J_\beta 1.1 \) primers, and distinguished DJ\(_{b}\)-IgK from endogenous DJ\(_{b1}\)IgK1 rearrangement using a DJ\(_{b}\)-IgK-specific probe (Fig. 4A). \( V_\beta 13 \)-to-DJ\(_{b}\)IgK rearrangement was readily detected in sorted DN3 and DP thymocytes of DJEozKI mice but not in EozKI controls. To measure the frequency of DJ\(_{b}\)KI recombination, we quantified residual rearranged \( V_\beta 13 \) and DJ\(_{b}\)IgK RSSs in DP thymocyte genomic DNA (Fig. 4B, center and right panels). We found that \( \sim 70\% \) of the DJ\(_{b}\)IgK and \( V_\beta 13 \) RSSs were lost in DJEozKI DP thymocytes. These losses likely reflect recombination to DJ\(_{b}\)IgK as well as recombination to the endogenous D\(_{b}\) gene segments that would delete \( V_\beta 13 \) and DJ\(_{b}\)IgK.

We also analyzed DJ\(_{b}\)IgK recombination by genomic Southern blot of EcoRI-digested whole thymus DNA (Fig. 4C). As compared with DJEozKI kidney (lane 2), a \( V_\beta 13 \) probe detected substantial loss of DNA carrying unrearranged \( V_\beta 13 \) and DJ\(_{b}\)IgK and detected two major and several minor rearranged fragments (lane 3). However, the predicted 8.0 kb fragment representing \( V_\beta 13\)-DJ\(_{b}\)IgK rearrangement was not detected. The additional rearranged fragments may represent excision circles carrying signal joints generated by rearrangement of upstream \( V_\beta \) segments to DJ\(_{b}\)IgK (e.g., \( V_\beta 8.1 = 6.7 \) kb, \( V_\beta 8.2 = 5.5 \) kb, \( V_\beta 8.3 = 3.0 \) kb, \( V_\beta 5.1 = 4.2 \) kb, and \( V_\beta 5.2 = 4.6 \) kb), as well as DJ\(_{b}\)IgK signal end (SE) recombination intermediates (2.5 kb), all of which would hybridize to the \( V_\beta 8.1 \) probe. Consistent with the former, we detected \( V_\beta 8.1\)-to-DJ\(_{b}\)IgK recombination using a PCR strategy (Fig. 4A). Excision circles and SE intermediates generated in DN thymocytes should be undetectable by genomic Southern blot of whole thymus because they would be diluted by the proliferative burst that accompanies the DN to DP transition. The apparent abundance of \( V_\beta 13 \)-containing excision circles and DJ\(_{b}\)IgK SE intermediates in DJEozKI thymocytes suggested that they were generated by recombination events occurring in DP rather than in DN thymocytes.

To directly test for \( V_\beta \) and DJ\(_{b}\)IgK recombination in DP thymocytes, we used LM-PCR to detect SE recombination intermediates in sorted thymocyte subpopulations (Fig. 4D). Because this assay cannot distinguish DJ\(_{b}\)IgK from endogenous D\(_{b}\)IgK SEs, we evaluated DJ\(_{b}\)IgK SEs by comparison of DJEozKI to EozKI samples. As expected, 5‘ D\(_{b}\)1, \( V_\beta 13 \) and \( V_\beta 8.1 \) SEs were readily detected in EozKI DN thymocytes but were barely detected in EozKI DP thymocytes. However, these SE intermediates were all readily detected in DJEozKI DP thymocytes (Fig. 4D). In contrast, control 5‘ D\(_{b}\)2 SEs were undetectable in DJEozKI DP thymocytes, indicating a selective loss of feedback inhibition involving DJ\(_{b}\)IgK and upstream \( V_\beta \) gene segments.

To formally demonstrate that DJ\(_{b}\)IgK rearrangements in DP thymocytes occurred chromosomally rather than on excision circles generated by \( V_\beta \)-to-endogenous D\(_{b}\) recombination in DN thymocytes, we analyzed \( V_\beta \)-to-DJ\(_{b}\)IgK recombination in thymocytes of DJEozKI mice that express a Tcrb tg. Feedback inhibition by such transgenes specifically suppresses \( V_\beta \)-to-DJ\(_{b}\)IgK recombination and the excision circles generated by these recombination events (21). Indeed, increased retention of a DNA segment situated 5‘ of D\(_{b}\)1, normally lost during \( V_\beta \)-to-endogenous D\(_{b}\) recombination, was apparent in wild-type, EozKI, and DJEozKI Tcrb tg DP thymocytes (Fig. 4B, left panel). However, suppression of \( V_\beta 13 \) and DJ\(_{b}\)IgK RSS loss was only partial in DJEozKI Tcrb tg DP thymocytes and for \( V_\beta 13 \) was much diminished as compared with the complete suppression in EozKI Tcrb tg DP thymocytes (Fig. 4B, center and right panels). This indicates continued chromosomal recombination of \( V_\beta 13 \) and upstream \( V_\beta \) to DJ\(_{b}\)IgK in DP thymocytes, despite feedback inhibition of endogenous \( V_\beta \)-to-DJ\(_{b}\)IgK recombination by the Tcrb tg. Consistent with this interpretation,
recombination events detected by the V$b\_13$ probe were more abundant on Southern blots of DEJEs Ki Tcrb tg thymus DNA (Fig. 4C, compare lanes 3 and 4), and V$b\_13$ and DJ$b\_1$Ki SE intermediates were more abundant in DEJEs Ki Tcrb tg thymus DNA (Fig. 4D). We conclude that by reducing the distance between accessible gene segments, the DJ$b\_1$Ki promotes chromosomal V$b\_13$ recombination in DP thymocytes and thereby subverts the process of feedback inhibition.

**Regulation of the β-in-α allele**

To further assess constraints on Tcrb gene segment recombination in DP thymocytes, we introduced a Tcrb recombination substrate into the Tcra locus, because this locus normally undergoes recombination in DP thymocytes (Tcrb-in-Tcra; β-in-α allele). The Tcrb substrate contained the same DJ$b\_1$Ki as in the DEJEs Ki allele, with the V$b\_13$ promoter and gene segment (V$b\_13$KI) situated just upstream (Fig. 1B). A BamHI site introduced ~90 bp 3′ of the V$b\_13$RSS was used in some experiments to distinguish the V$b\_13$KI from the endogenous V$b\_13$. We used homologous recombination to introduce this Tcrb recombination substrate into a previously generated Tcra allele [TcRαs], which contains only the J$a\_61$ and J$a\_56$ gene segments (24), so that it replaces the TEA promoter and the entire J$a\_1$ array of the wild-type Tcra locus. In this way, the Tcrb recombination substrate carries V$b\_13$ and DJ$b\_1$ segments that are in close physical proximity and that will be accessible in DP thymocytes due to the activity of the endogenous E$a\_α$. We generated heterozygous β-in-α mice, which were then intercrossed to produce β-in-α homozygous mice (Fig. 1B). These mice displayed normal DN thymocyte development and efficient differentiation to the DP stage, but were blocked in their development beyond the DP stage (data not shown). We presume that the chimeric TcRα proteins encoded by β-in-α alleles (which would include D$b\_β$ and J$b\_β$, rather than J$a\_1$ sequences) are either unstable, cannot assemble with TCRβ proteins, or cannot create a TCRαβ complex that can support positive selection.

We analyzed germline transcription and H3K4me3-modified nucleosomes on the β-in-α allele after introducing it onto Rag2$^{-/-}$ and Rag2$^{-/-}$ × Tcrb tg backgrounds. As expected, transcription of the DJ$b\_1$Ki was low in DN thymocytes and substantially upregulated in DP thymocytes (Fig. 5A, left panel). Specific amplification of V$b\_13$KI revealed it to behave similarly (Fig. 5A, center panel). We also directly compared V$b\_13$KI transcription to endogenous V$b\_13$ transcription using a PCR strategy that amplified both equally (Fig. 5B, right panel). V$b\_13$KI transcripts were more abundant in β-in-α DP thymocytes than were endogenous V$b\_13$ transcripts in wild-type DN thymocytes. Similar conclusions were drawn from analysis of H3K4me3 on β-in-α alleles (Fig. 5B). These data suggest that the Tcrb substrate, like J$a\_1$ gene segments on a wild-type allele, is regulated by E$a\_α$ and is highly accessible in β-in-α DP thymocytes.

We assayed V$b\_13$KI-DJ$b\_1$Ki recombination in wild-type and β-in-α DN3 and DP thymocytes by amplification with V$b\_13$ and J$a\_1$1.1 primers followed by hybridization with a DJ$b\_1$Ki-specific probe (Fig. 6A). This strategy detected the germline substrate in β-in-α DN and DP thymocytes (Fig. 6A), but detected abundant V$b\_13$KI-DJ$b\_1$Ki rearranged alleles selectively in β-in-α DP thymocytes (Fig. 6A). On the basis of germline RSS loss, fully 60% of the DJ$b\_1$Ki had undergone recombination in β-in-α DP thymocytes (Fig. 6A). However, the same analysis indicated that only ~10% of the V$b\_13$KI had undergone recombination (Fig. 6B), indicating that DJ$b\_1$Ki could rearrange to RSSs other than V$b\_13$KI. To further address this, we hybridized EcoRI-digested whole thymus genomic DNA to V$b\_13$ and Ca probe. In addition to unrearranged V$b\_13$ (7.8 kb) and unrearranged V$b\_13$KI (5.9 kb),
substrate rather than the endogenous gene segments because thymocytes (Fig. 6).

However, consistent with low-frequency V_{b}13KI recombination, we could not detect V_{b}13KI-to-DJ_{b}KI rearranged alleles (4.3 kb) (Fig. 6C, lane 3). The C_{a} probe also detected a 5.9-kb unrearranged fragment, but in addition detected a large number of additional recombination events involving the DJ_{b}KI, including a potential 4.3 kb V_{b}13KI-to-DJ_{b}KI rearrangement (Fig. 6C, lane 6). However, the majority of DJ_{b}KI rearrangements did not involve V_{b}13KI but, presumably, upstream V_{b} alleles instead. Thus, V_{b} RSSs appear to outcompete the V_{b}13K SE for the DJ_{b}E RSS in vivo. This result is consistent with previous studies demonstrating that proximal V_{a} segments are contracted and accessible in DP thymocytes (25, 29) and that V_{a} RSSs are generally superior to V_{b} RSSs as recombinnase substrates (30).

To confirm that V_{b}-in-α alleles undergo recombination and are not subject to feedback inhibition in DP thymocytes, we sorted DN and DP thymocytes from wild-type and V_{b}-in-α mice and detected recombination intermediates by LM-PCR (Fig. 6D). As previously described (21), wild-type thymocytes displayed 5’ DJ_{1, 5’, DJ_{2}} and V_{b}13 SEs that were abundant in DN but not in DP thymocytes. In contrast, 5’ DJ_{1} and V_{b}13 SEs were at least as abundant in β-in-α DP thymocytes as they were in β-in-α DN thymocytes (Fig. 6D). This represents dysregulation of the β-in-α substrate rather than the endogenous gene segments because 5’ DJ_{2} SEs were reduced in abundance in DP as compared with DN thymocytes. We conclude that β-in-α alleles undergo both V_{b}13KI-to-DJ_{b}KI and endogenous V_{c}-to-DJ_{b}KI recombination in DP thymocytes.

Discussion

Numerous studies have correlated reduced Ag receptor locus accessibility and an extended Ag receptor locus conformation with the feedback inhibition of V(D)J recombination that mediates allelic exclusion (2, 13, 14). We previously forced V_{b} accessibility in DP thymocytes but could not overcome the inhibition of V_{b}-to-DJ_{b} recombination that normally characterizes this compartment (21). In this study, we found that, like wild-type alleles, those accessible EozKI alleles are extended in DP thymocytes. We therefore generated two new alleles (DJEozKI and β-in-α) to formally test whether gene segment proximity and accessibility are both critical effects of feedback inhibition. By comparing the behavior of DJEozKI to EozKI alleles, we varied the proximity of accessible V_{b} and DJ_{b} segments; in DP thymocytes, these gene segments are accessible on both alleles, but they are in physical proximity on DJEozKI alleles only. We found that DJEozKI but not EozKI alleles supported V_{b}-to-DJ_{b} recombination in DP thymocytes. By comparing β-in-α alleles in DN and DP thymocytes, we varied the accessibility of proximal V_{b} and DJ_{b} segments; these gene segments are in physical proximity in both compartments, but become accessible due to developmental activation of EozKI in DP thymocytes only. We found that β-in-α alleles supported V_{b}-to-DJ_{b} recombination in DP but not in DN thymocytes. Based on
the data from both models, we conclude that gene segment accessibility and gene segment proximity are both essential for chromosomal V(D)J recombination and that feedback inhibition of $V_{\beta}$-to-$DJ_{\beta}$ recombination on wild-type $Tcrb$ alleles in DP thymocytes is normally enforced by both a loss of RSS accessibility to RAG1/2 and a decontracted locus conformation that inhibits RSS synthesis.

Pre-TCR signals initiate feedback inhibition and promote $Tcrb$ epigenetic changes that enforce feedback inhibition, but the critical signaling pathways and downstream effector proteins are only partially understood (14). To the best of our knowledge, the only signaling pathway or downstream effector that has clearly been shown to impact $Tcrb$ allelic exclusion through effects in DP thymocytes is the transcription factor E47. E47 supports $Tcrb$ locus accessibility and recombination in DN thymocytes and is downregulated in response to pre-TCR signaling in DP thymocytes (31). Notably, its overexpression was shown to override feedback inhibition and promote $V_{\beta}$-to-$DJ_{\beta}$ recombination in DP thymocytes (31). However, $Tcrb$ locus accessibility and conformation were not evaluated in E47-overexpressing DP thymocytes, leaving the basis for this override of feedback inhibition undefined. We predict that E47 must support $V_{\beta}$ accessibility and $Tcrb$ locus contraction to account for the described effects on $Tcrb$ recombination.

Although modulation of gene segment proximity appears to represent an important component of the feedback inhibition program, the mechanisms of locus contraction and decontraction are poorly understood. Recent studies have implicated architectural proteins cohesin and CTCF as regulators of long-distance interactions and V(D)J recombination at the $Tcrb$ and Igh loci (32–34), but it is not known whether these proteins regulate overall locus conformation. E47 (35) and transcription factors Pax5 (11, 36), YY1 (37), and Ikaros (38) have all been implicated in $Igh$ locus contraction, but whether and how they might trigger $Igh$ locus decontraction is uncertain. Much less is known about the roles of architectural proteins and transcriptional regulators in $Tcrb$ locus contraction and decontraction events. This will certainly be an important avenue for future studies.

Our data argue that gene segment proximity and accessibility are critical determinants of the $Tcrb$ locus feedback inhibition program. Moreover, our results suggest that there are not likely any additional constraints imposed on the rearrangement of most $V_{\beta}$ gene segments to $D_{\beta}1$ in DP thymocytes; for example, specific factors that regulate the usage of $V_{\beta}5$ and $5’$ $D_{\beta}1$ RSSs. Were such constraints to exist, they should have been unperturbed by our genetic manipulations, and feedback would have remained intact on both the DJEaK1 and $\beta$-in-$\alpha$ alleles. We caution that we cannot formally eliminate the possibility that what we interpret to result from a change in physical distance could actually reflect the loss of an intervening regulatory element that is intrinsically inhibitory to $V_{\beta}$-to-$DJ_{\beta}$ recombination. The identity of that element would be a matter of speculation. However, we imagine that it would function, like a change in physical proximity, to limit synopsis of $V_{\beta}5$ and $D_{\beta}1$ RSSs.

Despite the conclusions outlined above, additional layers of regulation may be required to explain the suppression of certain types of $Tcrb$ locus recombination events in DP thymocytes. A particularly vexing issue is $V_{\beta}14$-to-$DJ_{\beta}$ recombination, because, unlike all other $V_{\beta}$ gene segments, $V_{\beta}14$ is located near $D_{\beta}9$ and $J_{\beta}2$ gene segments, and its accessibility is not downregulated by pre-TCR signaling and is apparently high in DP thymocytes (17, 39–41). Because $D_{\beta}9$ and $J_{\beta}2$ segments are also accessible and support RAG1/2 binding in DP thymocytes (42), the suppression of $V_{\beta}14$ rearrangement may depend on unique features of inversional rearrangement (40) or of the $V_{\beta}14$ RSS (41).

A second issue is the problem of secondary rearrangements. Reduced accessibility and locus decontraction can account for inhibition of $V_{\beta}$-to-$DJ_{\beta}1$ or $-DJ_{\beta}2$ rearrangement on a $Tcrb$ allele that had not yet undergone $V_{\beta}$ rearrangement. However, because the $V_{\beta}$ segments immediately upstream of a rearranged $V_{\beta}$ are accessible in DP thymocytes (43, 44) and proximal to accessible downstream $D_{\beta}2$ and $J_{\beta}2$ segments (42), it is not clear what would suppress secondary $V_{\beta}$-to-$DJ_{\beta}2$ rearrangement on an allele that had already undergone primary $V_{\beta}$-to-$DJ_{\beta}1$ rearrangement. Recent work has demonstrated that secondary rearrangements can occur on these alleles and that they can replace even an in-frame $VDJ_{\beta}1$ rearrangement (18), but there was no indication that this occurred in DP as opposed to DN thymocytes. Indeed, analysis of DP thymocytes failed to detect SE intermediates at the accessible $V_{\beta}$ segments upstream of a rearranged $V_{\beta}$ gene segment (43). Moreover, SEs at $5’$ $D_{\beta}2$ RSSs are strongly suppressed in DP thymocytes (21 and this study). Thus, DP thymocytes appear not to be permissive for secondary $Tcrb$ recombination.

Because we found that accessible $V_{\beta}$s can rearrange to the $DJ_{\beta}K1$ in DJEaK1 DP thymocytes, it seems unlikely that any additional mechanism that might be required to suppress secondary recombination would be directed at $V_{\beta}$ RSSs. However, it remains possible that there is a specific regulatory mechanism directed at the $5’$ $D_{\beta}2$ RSS. Indeed, $D_{\beta}2$ regulation appears to be unusually complex, with promoters both upstream and downstream of $D_{\beta}2$ (45, 46). The downstream promoter is preferentially active on unrearranged alleles and presumably directs $D_{\beta}2$-to-$J_{\beta}2$ rearrangement; the upstream promoter only becomes active once the downstream promoter is eliminated by $D_{\beta}2$-to-$J_{\beta}2$ rearrangement and is likely important to direct $V_{\beta}$-to-$DJ_{\beta}2$ rearrangement. Activity of the $5’$ promoter suggests that the $5’$ $D_{\beta}2$ RSS resides in accessible chromatin on $D_{\beta}2$-to-$J_{\beta}2$ rearranged alleles in DP thymocytes. However, it is unclear whether these alleles support RAG1/2 binding, because the only assays of RAG1/2 binding at $D_{\beta}2$ in DP thymocytes were conducted on alleles that were in germline configuration and in which only the downstream promoter should have been active (42). Therefore, it is not known whether RAG1/2 can bind to the $5’$ $D_{\beta}2$ RSS in DP thymocytes, and it remains possible that secondary rearrangements could be suppressed in DP thymocytes by a specific mechanism that occludes RAG1/2 binding to the $5’$ $D_{\beta}2$ RSS. Our results demonstrate conclusively that, for most $V_{\beta}$ gene segments, accessibility and conformational constraints alone can fully account for the suppression of $V_{\beta}$-to-$DJ_{\beta}1$ recombination in DP thymocytes. However, additional work will be required to clarify the mechanisms, beyond accessibility and conformational constraints, that impart feedback inhibition to $V_{\beta}14$ recombination and secondary recombination events involving $D_{\beta}2$.

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### Disclosures

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### References


MECHANISM of Tcrb ALLELE EXCLUSION


