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Regulation of TCRβ Allelic Exclusion by Gene Segment Proximity and Accessibility

Hrisavgi 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.

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Abbreviations used in this article: 3D-FISH, three-dimensional fluorescence in situ hybridization; DN, double-negative; DP, double-positive; Eα, Tcra enhancer; ES, embryonic stem; H3K4me3, histone H3 lysine 4 trimethylation; LM-PCR, ligation-mediated PCR; neoβ, neomycin-resistance; PDβ1, promoter PDβ1; Rag2−/−, Rag2-deficient; RSS, recombination signal sequence; SE, signal end; TD-qPCR, touch-down quantitative PCR; Ig, transgene.

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Vpx1 NT alleles only, but no data evaluated whether altered Vpx1 recombination reflected a loss of feedback inhibition in DP thymocytes as opposed to dysregulated rearrangement in DN thymocytes. Another study simply inserted a Vpx gene segment just upstream of DJb gene segments (19). Although allelic exclusion was perturbed at the level of Vpx1 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 Vpx1 gene segment was introduced just upstream of DJb gene segments. This allele clearly displayed a disruption of feedback inhibition in pre-B cells. However, as the genetic manipulation moved the Vpx1 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 Vβ accessibility was maintained in DP thymocytes by introducing the Tcra enhancer (Eαb) into the middle of the Vβ array (EαbKI allele). Despite accessible Vβ chromatin, feedback inhibition of Vβ-to-DJb 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 Vβ-to-DJb recombination in DP thymocytes.

Materials and Methods

**Mice and gene targeting**

Wild-type 129, Rag2-deficient (Rag2<sup>2−/−</sup>) (22), and Rag2<sup>2−/−</sup> mice containing a rearranged Tcrb transgene (tg) (23) were purchased from Taconic Farms. EαbKI, EαbKI Rag2<sup>2−/−</sup>, and EαbKI Rag2<sup>2−/−</sup> Tcrb tg 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.

DJEαbKI mice were generated as follows: DJb1 homology arms and PCR were cloned using Pfu Turbo (Stratagene) and cloned using a KpnI/AatII site. Embryonal stem (ES) cells were derived from EαbKI mice (21) were used for homologous recombination, which was verified by Southern blot of SacI-digested genomic DNA analyzed with a 5′ Tcrb probe and of EcoRI-digested genomic DNA analyzed with a 3′ Tcrb probe (Supplemental Table I). The neo<sup>+</sup> cassette was removed by transient transfection of ES cells with Cre recombinase.

β-inteins were generated as follows: the Tcrb substrate was amplified from the DJEαbKI targeting construct and extends from nt 3251 to 11810 and 11815 to 12960 of GenBank file MMAE000655. 5′ and 3′ homology arms extended from nt 154066 of GenBank file MMAE000665, 5′ and 3′ homology arms extended from nt 152528 of GenBank file MMAE000664 (upstream of the Vβ1.1 probe) and 203H5 (3′ homology arm) were cloned between the NotI and XmaI sites. The α2 promoter was cloned into the SalI site. Embryonal stem (ES) cells were stained with Texas Red-conjugated streptavidin. Cells were collected from the CD4<sup>+</sup> and CD8<sup>−</sup> populations and 5′ and DJb1 were cloned into the XhoI site, the 3′ arm was cloned into the Sall site. Embryonal stem (ES) cells derived from EαbKI mice (21) were used for homologous recombination, which was verified by Southern blot of SacI-digested genomic DNA analyzed with a 5′ Tcrb probe and of EcoRI-digested genomic DNA analyzed with a 3′ Tcrb probe (Supplemental Table I). The neo<sup>−</sup> cassette was removed by transient transfection of ES cells with Cre recombinase.

**Three-dimensional fluorescence in situ hybridization**

Bacterial artificial chromosome clones 75PS (5′ Tcrb probe) and 203HS (3′ Tcrb probe) were directly labeled and used for three-dimensional fluorescence in situ hybridization (3D-FISH) as previously described (25). Probe-to-probe distances were calculated as previously described (25). Only nuclei with two distinguishable signals for both alleles were analyzed. Statistical tests were performed using Prism 3.0 (GraphPad).

**Cell sorting**

DN3 thymocytes were isolated as previously described (26). Cells were stained with the following Abs for 30 min on ice: CD45- conjugated anti-CD3e (clone 145-2C11), anti-CD4 (clone GK1.5), and anti-CD8 (clone 53-6.7); and biotinylated anti-CD24 (clone M1-69), PE-conjugated anti-CD44 (clone 1H7), and FITC-conjugated anti-CD25 (clone 7D4). After washing, cells were stained with Texas Red-conjugated streptavidin. Cells were collected from the CD24<sup>−</sup>CD3<sup>+</sup>CD4<sup>−</sup>CD8<sup>−</sup> and CD25<sup>−</sup>CD44<sup>+</sup> gates. DP thymocytes were isolated as previously described (26). Cells were stained with FITC-conjugated anti-CD4 (clone GK1.5) and PE-conjugated anti-CD8 (clone 53-6.7) for 30 min on ice. Cells were collected from the CD4<sup>+</sup>CD8<sup>−</sup> gate. All Abs were purchased from eBioscience. Samples were sorted to at least 95% purity using a DiVa cell sorter (BD Biosciences); analysis was with CellQuest software (BD Biosciences).

**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 DC315; Millipore) and normal rabbit IgG (AB-105-C; R&D Systems). All samples were resuspended in 200 µl 10 mM Tris- HCl (pH 8) 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 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 35 cycles at 58°C. Primers used are listed in Supplemental Table I.

**Germline transcription**

Approximately 2 × 10<sup>7</sup> primary thymocytes were resuspended in 1 ml TRizol (Invitrogen), and RNA was isolated according to the manufacturer’s instructions. cDNA was synthesized using the Super Script III kit (Invitrogen) using up to 2 µg purified RNA. Transcripts were quantified 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.

**Coded 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, 30 s at annealing temperature, and 30–60 s at 72°C. Annealing temperature was held at 68, 66, and 64°C for five cycles each and at 62°C for the remaining cycles. Amplicons were resolved on a 2.0% agarose gel, transferred onto a nylon membrane, and detected by hybridization with γ<sup>32</sup>P-labeled oligonucleotide probes. CAD4 was amplified for 23 cycles at 62°C. Primers and probes 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 α<sup>32</sup>P-labeled probes listed in Supplemental Table I.

**Signal ends**

Genomic DNA (1.5 µg) of DN3 and DP thymocytes was analyzed by ligation-mediated PCR (LM-PCR) as previously described (21, 28). Amplification by touchdown PCR and detection of amplicons were as described above. Supplemental Table I lists linker sequences, primers, and probes.

**Results**

**EαbKI locus conformation**

Previous studies of mice carrying Tcrb alleles with an introduced EαbKI (Fig. 1A) indicated that elevation of Vβ accessibility, by itself, could not subvert feedback inhibition of Vβ-to-DJb recom-
We hypothesized that recombination might remain suppressed on accessible EαKI alleles if, like wild-type alleles, they were decontracted in DP thymocytes. To assess this, we used 3D-FISH to measure the distance between the 5′ and 3′ ends of wild-type and EαKI Tcrb alleles in recombinase-deficient DN and DP thymocytes (Fig. 2). Consistent with previous experiments (16), we found that, on average, wild-type alleles were contracted inDN thymocytes and decontracted inDP thymocytes. The behavior of EαKI alleles was indistinguishable from wild-type, indicating that they decontract in DP thymocytes despite the presence of Eα and an accessible chromatin configuration. Therefore, to formally test whether the distance between accessible Vβ and DJβ gene segments limited Vβ-to-DJβ recombination inDP thymocytes, we generated and characterized two novel TCR locus alleles that approximated accessible Vβ and DJβ gene segments inDP thymocytes.

**Regulation of the DJEαKI allele**

We used homologous recombination to introduce a cassette containing PDα1 and a rearranged Dα1-Uα1.1 (DJαKI) ~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, DJαKI 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 DJαKI RSS on DJEαKI alleles were H3K4me3 modified in DN thymocytes and displayed increased H3K4me3 in DP thymocytes (Fig. 3B, right panel). Hence, the DJαKI RSS appears to reside in accessible chromatin in both DN and DP thymocytes of DJ EαKI mice.
FIGURE 3. Chromatin accessibility of DJEαKI alleles. A. Germline transcription in wild-type (WT), EoKI, and DJEαKI DN (all Rag22/−/−) and DP (all Rag22/−/− × Tcrb tg) thymocytes analyzed by quantitative real-time PCR. Results were normalized to those for Actb and represent the mean ± SEM of two to six independent experiments using cDNA diluted 1:10 for Dβ1 and Actb PCRs and undiluted cDNA for Vβ13 and Vβ8.1 PCRs. B. Chromatin immunoprecipitation of H3K4me3-modified nucleosomes of wild-type, EoKI, and DJEαKI DN (all Rag22/−/−) and DP (all Rag22/−/− × Tcrb tg) thymocytes. Ratios of bound to input were normalized to those for β2-microglobulin and represent the mean ± SEM of two to four independent experiments.

As documented previously (21), germline transcription of Vβ13 is downregulated on transition from DN to DP on wild-type alleles but is upregulated on EoKI alleles (Fig. 3A, center panel). Unexpectedly, we found that introduction of the DJbKI blunt the effect of Eo on Vβ13 in DJEαKI DP thymocytes, such that Vβ13 transcripts were upregulated as compared with wild-type DP thymocytes, but were no more abundant in DJEαKI DP than in DJEαKI DN thymocytes. This might reflect a suppression of transcription due to competition between PDJb and the Vβ13 promoter or an effect of the DJbKI on Vβ13 transcript stability. The DJbKI also unexpectedly suppressed H3K4me3 at the Vβ13 RSS in both DN and DP thymocytes (Fig. 3B, center panel). Given these results, we also analyzed accessibility at Vβ8.1, which lies 5 kb upstream of Vβ13. Unlike Vβ13, the upregulation of Vβ8.1 transcription in EoKI DP thymocytes was maintained in DJEαKI DP thymocytes (Fig. 3A, left panel). Moreover, the upregulation of Vβ8.1 H3K4me3 in EoKI DP thymocytes was only partly suppressed by the DJbKI (Fig. 3B, left panel). Taken together, the transcription and chromatin data suggest that Vβ13 is moderately accessible and that Vβ8.1 is highly accessible in DJEαKI DN and DP thymocytes.

To analyze Vβ-to-DJβ recombination, we prepared genomic DNA from purified DN3 and DP thymocytes of EoKI and DJEαKI mice, amplified with Vβ13 and Jb1.1 primers, and distinguished DJbKI from endogenous DJb1.1 rearrangement using a DJbKI-specific probe (Fig. 4A). Vβ13-to-DJbKI rearrangement was readily detected in sorted DN3 and DP thymocytes of DJEαKI mice but not in EoKI controls. To measure the frequency of DJβKI recombination, we quantified residual unrearranged Vβ13 and DJbKI RSSs in DP thymocyte genomic DNA (Fig. 4B, center and right panels). We found that ~70% of the DJbKI and Vβ13 RSSs were lost in DJEαKI DP thymocytes. These losses likely reflect recombination to DJbKI as well as recombination to the endogenous Dβ gene segments that would delete Vβ13 and DJbKI.

We also analyzed DJbKI recombination by genomic Southern blot of EcoRI-digested whole thymus DNA (Fig. 4C). As compared with DJEαKI kidney (lane 2), a Vβ13 probe detected substantial loss of DNA carrying unrearranged Vβ13 and DJbKI and detected two major and several minor rearranged fragments (lane 3). However, the predicted 8.0 kb fragment representing Vβ13-DJbKI rearrangement was not detected. The additional rearranged fragments may represent excision circles carrying signal joints generated by rearrangement of upstream Vβ segments to DJbKI (e.g., Vβ8.1 = 6.7 kb, Vβ8.2 = 5.5 kb, Vβ8.3 = 3.0 kb, Vβ5.1 = 4.2 kb, and Vβ5.2 = 4.6 kb), as well as DJbKI signal end (SE) recombination intermediates (2.5 kb), all of which would hybridize to the Vβ13 probe. Consistent with the former, we detected Vβ8.1-to-DJbKI 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 Vβ13-containing excision circles and DJbKI SE intermediates in DJEαKI thymocytes suggested that they were generated by recombination events occurring in DP rather than in DN thymocytes.

To test directly for Vβ and DJbKI recombination in DP thymocytes, we used LM-PCR to detect SE recombination intermediates in sorted thymocyte subpopulations (Fig. 4D). Because this assay cannot distinguish DJbKI from endogenous Dβ1 SEs, we evaluated DJbKI SEs by comparison of DJEαKI to EoKI samples. As expected, 5′ Dβ1, Vβ13 and Vβ8.1 SEs were readily detected in EoKI DN thymocytes but were barely detected in EoKI DP thymocytes. However, these SE intermediates were all readily detected in DJEαKI DP thymocytes (Fig. 4D). In contrast, control 5′ Dβ2 SEs were undetectable in DJEαKI DP thymocytes, indicating a selective loss of feedback inhibition involving DJbKI and upstream Vβ gene segments.

To formally demonstrate that DJbKI rearrangements in DP thymocytes occurred chromosomally rather than on excision circles generated by Vβ-to-endogenous Dβ recombination in DN thymocytes, we analyzed Vβ-to-DJbKI recombination in thymocytes of DJEαKI mice that express a Tcrb tg. Feedback inhibition by such transgenes specifically suppresses Vβ-to-DJbKI recombination and the excision circles generated by these recombination events (21). Indeed, increased retention of a DNA segment situated 5′ of Dβ1, normally lost during Vβ-to-endogenous Dβ recombination, was apparent in wild-type, EoKI, and DJEαKI Tcrb tg DP thymocytes (Fig. 4B, left panel). However, suppression of Vβ13 and DJbKI RSS loss was only partial in DJEαKI Tcrb tg DP thymocytes and for Vβ13 was much diminished as compared with the complete suppression in EαKI Tcrb tg DP thymocytes (Fig. 4B, center and right panels). This indicates continued chromosomal recombination of Vβ13 and upstream Vβs to DJbKI in DP thymocytes, despite feedback inhibition of endogenous Vβ-to-DJb recombination by the Tcrb tg. Consistent with this interpretation,
FIGURE 4. Recombination of DJEaKI alleles. A. Coding joint analysis. Genomic DNAs from sorted EcoKI and DJEaKI DN3 and DP thymocytes without (−) or with (+) a Tcra tg were serially 3-fold diluted (wedges) and analyzed by PCR. Blots of PCRs using Vβ and Jβ1.1 primers were hybridized with a DJβ1KI-specific probe. *Cd14* amplification was used to control for DNA loading. Data are representative of two independent experiments. B. RSS usage. Genomic DNAs from DJEaKI kidney and from sorted wild-type (WT), EcoKI, and DJEaKI DP thymocytes, without (−) or with (+) a Tcra tg, were analyzed by quantitative real-time PCR. Percent amplicon remaining was calculated as: ([experimental amplicon in thymus/experimental amplicon in kidney]/[β2-microglobulin in thymus/β2-microglobulin in kidney]) × 100. Data are the mean ± SEM of two to three samples for each genotype. C, Genomic Southern blot. Top panel, Unfractionated thymus (Th) and kidney (K) genomic DNAs were digested with EcoRI and analyzed by Southern blot using a Vβ13 probe. DNA loading was assessed using a control (Ctrl) trypsinogen probe. Bottom panel, Schematic of expected EcoRI fragments, including a diagram of predicted excision circles containing Vβ(×)-DJβaKI signal joint (SJ) recombination products. D, SE analysis. Thymocyte genomic DNA samples were linker-ligated, serially 3-fold diluted (wedges), and analyzed by PCR. *Cd14* amplification was used to control for DNA loading. The data are representative of two independent experiments. −, no DNA.

recombination events detected by the Vβ13 probe were more abundant on Southern blots of DJEaKI Tcra tg thymus DNA (Fig. 4C, compare lanes 3 and 4), and Vβ13 and DJβaKI SE intermediates were more abundant in DJEaKI Tcra tg thymus DNA (Fig. 4D). We conclude that by reducing the distance between accessible gene segments, the DJβaKI promotes chromosomal Vβ recombination in DP thymocytes and thereby subverts the process of feedback inhibition.

**Regulation of the β-in-α allele**

To further assess constraints on Tcra gene segment recombination in DP thymocytes, we introduced a Tcra recombination substrate into the Tcra locus, because this locus normally undergoes recombination in DP thymocytes (Tcra-in-Tcra; β-in-α allele). The Tcra substrate contained the same DJβaKI as in the DJEaKI allele, with the Vβ13 promoter and gene segment (Vβ13KI) situated just upstream (Fig. 1B). A BamHI site introduced ~90 bp 3′ of the Vβ13RSS was used in some experiments to distinguish the Vβ13KI from the endogenous Vβ13. We used homologous recombination to introduce this Tcra recombination substrate into a previously generated Tcra allele [TcraKI] (24), which contains only the Jα61 and Jα56 gene segments (24), such that it replaces the TEA promoter and the entire Jα array of the wild-type Tcra locus. In this way, the Tcra recombination substrate carries Vβ and DJβ segments that are in close physical proximity and that will be accessible in DP thymocytes due to the activity of the endogenous Eα. 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 DJβ and Jβ, rather than Jα 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+/− × Tcra tg backgrounds. As expected, transcription of the DJβaKI was low in DN thymocytes and substantially upregulated in DP thymocytes (Fig. 5A, left panel). Specific amplification of Vβ13KI revealed it to behave similarly (Fig. 5A, center panel). We also directly compared Vβ13KI transcription to endogenous Vβ13 transcription using a PCR strategy that amplified both equally (Fig. 5B, right panel). Vβ13KI transcripts were more abundant in β-in-α DP thymocytes than were endogenous Vβ13 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 Tcra substrate, like Jα gene segments on a wild-type allele, is regulated by Eα and is highly accessible in β-in-α DP thymocytes.

We assayed Vβ13KI-to-DJβaKI recombination in wild-type and β-in-α DN3 and DP thymocytes by amplification with Vβ13 and Jβ1.1 primers followed by hybridization with a DJβaKI-specific probe (Fig. 6A). This strategy detected the germline substrate in β-in-α DN and DP thymocytes (Fig. 6A), but detected abundant Vβ13KI-to-DJβaKI rearranged alleles selectively in β-in-α DP thymocytes (Fig. 6A). On the basis of germline RSS loss, fully 60% of the DJβaKI had undergone recombination in β-in-α DP thymocytes (Fig. 6A). However, the same analysis indicated that only ~10% of the Vβ13KI had undergone recombination (Fig. 6B), indicating that DJβaKI could rearrange to RSSs other than Vβ13. To further address this, we hybridized EcoRI-digested whole thymus genomic DNA to Vβ13 and Cα probes. In addition to unrearranged Vβ13 (7.8 kb) and unrearranged Vβ13KI (5.9 kb),
the V_β13 probe detected a potential DJ_β13KI SE intermediate (2.5 kb) and one or two additional minor species (Fig. 6C, lane 3). However, consistent with low-frequency V_β13KI recombination, we could not detect V_β13KI-to-DJ_β13KI rearranged alleles (4.3 kb) (Fig 6C, lane 3). The C_α probe also detected a 5.9-kb unrearranged fragment, but in addition detected a large number of additional recombination events involving the DJ_β13KI, including a potential 4.3 kb V_β13KI-to-DJ_β13KI rearrangement (Fig. 6C, lane 6). However, the majority of DJ_β13KI rearrangements did not involve V_β13KI but, presumably, upstream V_α gene segments instead. Thus, V_α RSSs appear to compete the V_β13KI RSS for the DJ_β13KI RSS in vivo. This result is consistent with previous studies demonstrating that proximal V_α segments are contracted and accessible in DP thymocytes (25, 29) and that V_α RSSs are generally superior to V_β RSSs as recombination substrates (30).

To confirm that β-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 β-in-α mice and detected SE recombination intermediates by LM-PCR (Fig. 6D). As previously described (21), wild-type thymocytes displayed 5’ DJ_β1, 5’ DJ_β2 and V_β13 SEs that were abundant in DN but not in DP thymocytes. In contrast, 5’ DJ_β1 and V_β13 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_β2 SEs were reduced in abundance in DP as compared with DN thymocytes. We conclude that β-in-α alleles undergo both V_β13KI-to-DJ_β13KI and endogenous V_α-to-DJ_β13KI 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_β accessibility in DP thymocytes but could not overcome the inhibition of V_β-to-DJ_β recombination that normally characterizes this compartment (21). In this study, we found that, like wild-type alleles, those accessible EoKI alleles are extended in DP thymocytes. We therefore generated two new alleles (DJEoKI and β-in-α) to formally test whether gene segment proximity and accessibility are both critical effectors of feedback inhibition. By comparing the behavior of DJEoKI to EoKI alleles, we varied the proximity of accessible V_β and DJ_β segments; in DP thymocytes, these gene segments are accessible on both alleles, but they are in physical proximity on DJEoKI alleles only. We found that DJEoKI but not EoKI alleles supported V_β-to-DJ_β recombination in DP thymocytes. By comparing β-in-α alleles in DN and DP thymocytes, we varied the accessibility of proximal V_β and DJ_β segments; these gene segments are in physical proximity in both compartments, but become accessible due to developmental activation of EoKI in DP thymocytes only. We found that β-in-α alleles supported V_β-to-DJ_β 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(DJ) recombination and that feedback inhibition of V_{μ}-to-D_{Jμ} 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 signaling initiates feedback inhibition and promotes Tcrb epigenetic changes that enforce feedback inhibition, but the critical signaling pathways and downstream effectors 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_{μ}-to-D_{Jμ} recombination in DP thymocytes (31). However, Tcrb locus accessibility and conformation were not evaluated in E47-overexpressing DP thymocytes, leaving open the possibility 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(DJ) recombination at the Tcra 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 conformation, 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_{μ} gene segments to D_{Jμ} in DP thymocytes; for example, specific factors that regulate the usage of V_{μ} and 5' D_{Jμ} RSSs. Were such constraints to exist, they should have been unperturbed by our genetic manipulations, and feedback would have remained intact on both the DJExaKI and β-in-α 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_{μ}-to-D_{Jμ} 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_{μ} and D_{Jμ} 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_{μ}14-to-D_{Jμ} recombination, because, unlike all other V_{μ} gene segments, V_{μ}14 is located near D_{μ} and J_{μ} gene segments, and its accessibility is not downregulated by pre-TCR signaling and is apparently high in DP thymocytes (17, 39–41). Because D_{μ} and J_{μ} segments are also accessible and support RAG1/2 binding in DP thymocytes (42), the suppression of V_{μ}14 rearrangement may depend on unique features of inversionsal rearrangement (40) or of the V_{μ}14 RSS (41).

A second issue is the problem of secondary rearrangements. Reduced accessibility and locus decontraction can account for inhibition of V_{μ}-to-D_{Jμ} or -D_{Jμ}2 rearrangement on a Tcrb allele that had not yet undergone V_{μ} rearrangement. However, because the V_{μ} segments immediately upstream of a rearranged V_{μ} are accessible in DP thymocytes (43, 44) and proximal to accessible downstream D_{Jμ} and J_{μ} segments (42), it is not clear what would suppress secondary V_{μ}-to-D_{Jμ}2 rearrangement on an allele that had already undergone primary V_{μ}-to-D_{Jμ}1 rearrangement. Recent work has demonstrated that secondary rearrangements can occur on these alleles and that they can replace even an in-frame VDJ_{μ}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_{μ} segments upstream of a rearranged V_{μ} gene segment (43). Moreover, SEs at 5' D_{μ} RSS 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_{μ}s can rearrange to the DJExaKI in DJExaKI DP thymocytes, it seems unlikely that any additional mechanism that might be required to suppress secondary recombination would be directed at V_{μ} RSSs. However, it remains possible that there is a specific regulatory mechanism directed at the 5' D_{μ} RSS. Indeed, D_{μ}2 regulation appears to be unusually complex, with promoters both upstream and downstream of D_{μ}2 (45, 46). The downstream promoter is preferentially active on unrearranged alleles and presumably directs D_{μ}2-to-J_{μ}2 rearrangement; the upstream promoter only becomes active once the downstream promoter is eliminated by D_{μ}2-to-J_{μ}2 rearrangement and is likely important to direct V_{μ}-to-D_{Jμ}2 rearrangement. Activity of the 5' promoter suggests that the 5' D_{μ}2 RSS resides in accessible chromatin on D_{μ}2-to-J_{μ}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_{μ}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_{μ}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_{μ}2 RSS. Our results demonstrate conclusively that, for most V_{μ} gene segments, accessibility and conformational constraints alone can fully account for the suppression of V_{μ}-to-D_{Jμ}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_{μ}14 recombination and secondary recombination events involving DI_{μ}2.

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References
Mechanism of TCR Allelic Exclusion


