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Domain-Specific and Stage-Intrinsic Changes in Tcrb Conformation during Thymocyte Development

Kinjal Majumder,*1 Levi J. Rupp,†‡1 Katherine S. Yang-Iott,†‡ Olivia I. Koues,* Katherine E. Kyle,* Craig H. Bassing,†‡ and Eugene M. Oltz*

Considerable cross-talk exists between mechanisms controlling genome architecture and gene expression. AgR loci are excellent models for these processes because they are regulated at both conformational and transcriptional levels to facilitate their assembly by V(D)J recombination. Upon commitment to the double-negative stage of T cell development, Tcrb adopts a compact conformation that promotes long-range recombination between Vβ gene segments (Trbvs) and their DβJβ targets. Formation of a functional VβDβJβ join signals for robust proliferation of double-negative thymocytes and their differentiation into double-positive (DP) cells, where Trbv recombination is squelched (allelic exclusion). DP differentiation also is accompanied by decontraction of Tcrb, which has been thought to separate the entire Trbv cluster from DβJβ segments (spatial segregation-based model for allelic exclusion). However, DP cells also repress transcription of unarranged Trbv, which may contribute to allelic exclusion. We performed a more detailed study of developmental changes in Tcrb topology and found that only the most distant portion of the Trbv cluster separates from DβJβ segments in DP thymocytes, leaving most Trbv spatially available for rearrangement. Preferential dissociation of distal Trbv is independent of robust proliferation or changes in transcription, chromatin, or architectural factors, which are coordinately regulated across the entire Trbv cluster. Segregation of distal Trbv also occurs on alleles harboring a functional VβDβJβ join, suggesting that this process is independent of rearrangement status and is DP intrinsic. Our finding that most Trbv remain associated with DβJβ targets in DP cells revises allelic exclusion models from their current conformation-dominant to a transcription-dominant formulation. The Journal of Immunology, 2015, 195: 1262–1272.

The assembly and expression of AgR genes is controlled at multiple levels, including chromatin accessibility, transcription, and changes in three-dimensional (3D) conformation (1–4). Because AgR genes span large regions of mammalian genomes, which are independently activated and repressed during lymphocyte development, these loci serve as excellent models to study long-range mechanisms that coordinate gene expression (5). In addition to gene expression, regulatory mechanisms shared among AgR loci orchestrate the process of V(D)J recombination (4), which assembles exons encoding their variable regions from large arrays of V, D, and J gene segments. The availability of gene segments for recombination within each AgR locus is modulated during lymphocyte development to guide their ordered and tissue-specific assembly, as well as to enforce allelic exclusion, a process that ensures the production of only a single, functional allele for each AgR gene in most B and T cells (6). Proper regulation of V(D)J recombination is essential for generating a diverse repertoire of AgRs, driving lymphocyte development, and avoiding chromosomal translocations that characterize many forms of leukemia and lymphoma (7–9).

All AgR loci have at least one powerful enhancer element that activates promoters associated with the most proximal clusters of gene segments, revising the chromatin landscape to facilitate the deposition of the histone modification H3K4me3, an epigenetic mark recognized by RAG2 (10–12), which, together with RAG1, are lymphocyte-specific components of the V(D)J recombinase. Because the enhancer-proximal gene segments are decorated with such a high density of RAG proteins, these regions have been coined recombination centers (RCs) (13). As an example, the Tcrb enhancer, called Eβ, is activated upon commitment of multipotent progenitors to the

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The 4C-seq data presented in this article have been submitted to the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE68955.
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Abbreviations used in this article: BE, boundary element; 3C, chromosome conformation capture, 4C, circular chromosome conformation capture; CHIP, chromatin immunoprecipitation; CTCF, CCCTC binding factor; 3D, three-dimensional; DN, double-negative; DP, double-positive; Eβ, Tcrb enhancer; FISH, fluorescence in situ hybridization; 5′PC, 5′ Prxs2CCCTC binding factor site; qPCR, quantitative PCR; RC, recombination center; -seq, coupled with high-throughput sequencing.

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T cell lineage in the thymus (14, 15). In turn, Eβ associates with promoters located in each of the proximal DJβJ clusters, triggering 1) robust transcription, 2) chromatin accessibility at the unarranged gene segments, 3) RAG-1/2 deposition, and 4) DJβ-to-βJ recombination, which occurs over short distances (16).

Complete assembly of V region exons for Tcrb, Tcrd, and Igα requires a second round of recombination between joined DJ elements in the RC and one of many V segments spayed out over large genomic distances. Numerous studies have shown that the second, long-range V-to-DJ recombination event is facilitated by conformational changes at these loci (17–19). As an example, upon commitment to the double-negative (DN) stage of T cell development, the most distal ends of Tcrb, which are separated by >500 kb in the linear genome, come together in 3D space, a process coined locus contraction (20, 21). Tcrb contraction coincides with the folding of its Vβ cluster into two spatially distinct domains, spanning proximal and distal portions of the Trbv array (22). Each of the more compact Trbv domains also folds into the RC, presumably via the process of locus contraction, endowing the Vβ segments with spatial access to DJβJ substrates (7, 22).

Indeed, we have shown that Trbv usage is largely limited by the activities of their associated promoters rather than by their absolute proximity to the RC (7), suggesting that all Vβ gene segments have crossed a spatial threshold required for RAG-mediated recombination.

Interactions between the distal Trbv domain and the RC appear focused on a site called 5’PC, which binds the architectural protein CCCTC binding factor (CTCF) and is located ~25 kb upstream of the DJβJ cluster (22). Genome-wide studies have revealed that when CTCF is bound to pairs of sites with convergent orientations, CTFC–CTFC dimerization can generate structural loops (23, 24). In many cases, such chromosomal loops are stabilized via association of CTCF dimers with cohesin, a ring-like complex that locks the loop bases into place (25). Of note, the numerous CTCF sites scattered throughout both Trbv domains are all in the same orientation, which favors their association with the 5’PC site near the RC. A similar mechanism of long-range tethering appears to be at play for other AgR loci, with V segments forming distinct domains that harbor multiple CTCF sites in a convergent orientation relative to those near the RC (26, 27). In what may be a related finding, ablation of CTCF or its key binding sites in AgR loci disrupts spatial interactions and long-range V/DJ recombination (26–30).

Although locus contraction promotes long-range recombination at nearly all AgR loci, this process is developmentally dynamic. For example, when DN thymocytes generate a productive Tcrb allele, pre–TCR signaling induces at least 10 rounds of rapid cell division (31). These proliferating cells ultimately differentiate into the resting CD4+CD8+ (double-positive [DP]) subset, in which division (31). These proliferating cells ultimately differentiate into allele, pre–TCR signaling induces at least 10 rounds of rapid cell

Materials and Methods

Mice

Thymocytes were harvested from Rag1−/−/C57BL/6 mice directly (DN) after injection of anti-CD3ε (DP) (35), or those expressing a Tcrb transgene (DP) (36, 37). For certain experiments (indicated in Results), thymocytes were isolated from mice of the following genotypes, all of which were on a mixed 129SvE/JC57BL/6 background: RAG1−/−/Tcrb−/−; Cdcl3−/−; (DN and DP), Vβ1NTNT/Rag1−/− (DP) (38), or Vβ1NTNT; Lat−/−/Rag1−/− (DN) (39). All experiments were conducted on mice that were between 4 and 6 wk of age. Animal procedures and experimental protocols were approved by the Institutional Animal Care and Use Committees at Washington University School of Medicine in St. Louis and Children’s Hospital of Philadelphia.

Chromosome conformation capture assays

Chromosome conformation capture (3C) assays were performed precisely as described (22) using 107 thymocytes or pro–B cells from Rag1−/− mice or from cultured 3T3 fibroblasts. Cross-linking efficiencies were measured using Taqman–quantitative PCR (qPCR) assays with primers and probes shown in Supplemental Table I.

Circular chromosome conformation capture coupled with high-throughput sequencing

Circular chromosome conformation capture (4C) assays were performed with HindIII as the primary restriction enzyme for cutting cross-linked chromatin. The HindIII-digested DNA was ligated using the 3C protocol and resuspended in buffer EB (100 μl, Qiagen). Secondary enzyme digestion was performed with NsilI (100 U, overnight). After heat inactivation, the digested DNA was religated to generate circularized products of interaction partners, purified by phenol/chloroform extraction, precipitated in isopropanol, resuspended in buffer EB (100 μl), and quantified as described previously (40). Inverse PCR was performed on the circularized DNA using primers within HindIII–NsilI fragments at Eβ or at Trbv5 (see Supplemental Table I). Inverse PCR products were diluted 1:100 in TE buffer and used as templates for nested inverse PCRs (see Supplemental Table II), yielding the 4C DNA libraries.

Purified 4C DNA (100 ng, PCR purification kit, Qiagen) was used for indexed library preparation. On average, eight indexed libraries were pooled and subjected to 42-bp single-end sequencing according to the manufacturer’s protocol (HiSeq2000 from Illumina, San Diego, CA). Sequence tags were aligned to the reference genome (build MM9) with Bowtie (41). The rC-seq package (42) was used to calculate reads per million for each sample and identify anchor interaction regions. EMBoss (43) was used to generate a genome-wide map of the HindIII restriction fragments for assignment of reads. To compare between samples, reads per million values for each fragment were quantile normalized. For visualization of the 4C coupled with high-throughput sequencing (4C-seq) data, a running mean was calculated using a window size of three contiguous HindIII fragments (40).

Public data sources

Chromatin immunoprecipitation (ChIP)–seq data were obtained from the Gene Expression Omnibus repository for CTCF (accession number GSE41739, see ref. 36), as well as H3K4me1 and H3K4me3 (accession number GSE55635, see ref. 44) (http://www.ncbi.nlm.nih.gov/genbank).
3D fluorescence in situ hybridization

3D fluorescence in situ hybridization (3D-FISH) assays were performed with Tcrb bacterial artificial chromosome probes spanning Trbvl (RP23-7SP5) and trypsinogen (RP23-203HS) precisely as described (22). Imaged data were analyzed using ImageJ as described (22).

ChIP assays

ChIP assays were performed as described previously (22) using the following Abs: CTCF (Rockland Immunocchemicals, 600-401-C42), RAD21 (Abcam, ab9902), H3Ac (EMD Millipore, 06-599), H3K4me2 (Abcam, ab32356), and IgG (Santa Cruz Biotechnology, sc-2027). ChIP assays were analyzed by qPCR with primer combinations shown in Supplemental Table I.

RNA extraction and germline transcription

RNA was prepared from 0.5 to 1 million cells using TRIzol (Invitrogen). cDNA was generated from 1 µg RNA using murine leukemia virus reverse transcriptase (New England Biolabs) and analyzed by qPCR with primer combinations provided in Supplemental Table I.

Results

Only distal Trbv segments dissociate from the RC in DP thymocytes

Repression of Tcrb gene assembly in DP cells correlates with significantly attenuated expression of unarranged Trbv segments (45, 46) and spatial segregation of the extreme 5′ and 3′ ends of the locus, that is, deconcentration (20–22). The latter cell imaging data have been extrapolated into models suggesting that Tcrb reverts to an extended conformation in DP thymocytes, with a loss of all RC–Trbv interactions (10, 20). To study developmental changes in Tcrb conformation at a higher resolution, we performed 4C-seq, an approach that allows us to measure the cross-linking efficiency of a given restriction fragment (viewpoint) with the entire genome (accession number GSE68955). The 4C-seq assays were performed with cross-linked chromatin from RAG-deficient DN thymocytes or RAG-deficient animals injected with STaLAb (22). Additional 4C experiments using the distal Trbv5 (distal) and Trbv9 (proximal) segments in their germline configuration due to a lack of recombinase activity (35). As shown in Fig. 1A, the Tcrb enhancer region (Eββ) associates with the entire Trbv cluster in DN thymocytes, which is consistent with prior findings (7, 20, 22). Surprisingly, many of these long-range interactions are maintained in DP thymocytes, despite Tcrb deconcentration, as ascertained by 3D-FISH using probes for the 5′ and 3′ ends of the locus (Fig. 1B). Notably, only the distal region of the Trbv cluster appears to dissociate uniformly from this RC viewpoint upon transition to the DP stage, suggesting that deconcentration does not involve the entire Tcrb locus in DP thymocytes.

To further validate the compartmentalized changes in Tcrb conformation during thymocyte development, we performed 3C-qPCR to focus on specific interactions between pairs of restriction fragments. We find that Eββ association with Trbv segments spread over the proximal half of the cluster is unperturbed when comparing DN to DP thymocytes generated by anti-CD3ε injection (Fig. 1C). In contrast, Eββ association with the more distal Trbv segments diminishes significantly in these DP cells (Fig. 1C). The changes in long-range Trbv–RC interactions, as well as reversal of locus contraction, are recapitulated in RAG-deficient DP thymocytes when their development is driven by the expression of a Tcrb transgene (Fig. 1B, 1D) (36, 37). The latter data preclude the possibility that superphysiologic signaling by anti-CD3ε is responsible for our observations.

3C analyses using complementary viewpoints in the Trbv cluster further confirmed our findings from 4C-seq. For example, robust cross-linking between the distal Trbv5 region and two restriction fragments in the Dβββ cluster is observed in DN thymocytes, but it is reduced to near background levels in DP cells (Fig. 1E, left). As expected, Trbv5 association with 5′PC, the CTCF binding element that serves as a tether for long-range interactions between the RC and distal Vβ region (22), is also lost in DP thymocytes (Fig. 1E, left). In sharp contrast, associations between the more proximal Trbv23 and the 5′PC-RC portion of the locus are unaffected by the DN to DP transition (Fig. 1E, right), even though these regions are separated by >200 kb in the linear genome. Preferential dissociation of the distal Trbv region from the RC does not correlate with domain-specific changes in transcription, which is reduced in DP thymocytes for Vβ segments within both the proximal and distal domains, regardless of the stimulus employed to generate DP cells (Fig. 1F). As expected, transitioning to the DP stage has no effect on or augments transcription of the RC and the proximal Trbv31 gene segment, which is located adjacent to the RC (39, 47). Similar to transcription, histone modifications associated with active promoters (H3K4me3), or simply open chromatin (H3K4me1) (44), are coordinately regulated within the entire Trbv cluster when comparing ChIP-seq data from DN and DP thymocytes (Fig. 1G). We conclude that dissociation of Vβ segments from the RC is highly restricted to the distal region of the Trbv cluster and is independent of changes in transcription and chromatin, which occur over the entire Trbv cluster.

Trbv domains remain folded in DP thymocytes

In DN thymocytes, distal and proximal regions of the Trbv cluster fold into separate spatial domains that associate with the RC in 5′PC-dependent and -independent manners, respectively (22). Additional 4C experiments using the distal Trbv5 region as a viewpoint revealed that its association with other distal Vβ segments is retained in DP thymocytes (Fig. 2A). However, Trbv5 becomes spatially segregated from Trbv segments in the RC-proximal portion of the cluster during this developmental transition (Fig. 2A).

3C data using distal and proximal Trbv viewpoints confirmed that each domain retains its internal, folded conformation, but only the distal Vβ domain strays from the RC in DP thymocytes (Figs. 1E, 2B). For example, association between Trbv5 and Trbvl within the distal domain is unchanged in DN versus DP cells, whereas interaction between Trbv5 (distal) and Trbv12-2 (proximal) diminishes significantly (Fig. 2B, left), despite a nearly equal linear distance between Trbv5 and each of these two gene segments. Conversely, Trbv23, which is in the proximal domain, cross-links with equal efficiencies to the proximal Trbv12-2 segment in DN and DP cells, but with a reduced efficiency to the distal Trbvl segment in DP thymocytes (Fig. 2B, right). These conformational data indicate that the developmental transition of DN to DP thymocytes disrupts long-range interactions between the RC and the distal Vβ domain, which remains in a folded conformation. In contrast, RC interactions with the folded cluster of proximal Trbv segments are fully retained upon differentiation to the DP stage. Thus, we conclude that repression of recombination at proximal Trbv segments in DP cells is independent of spatial dissociation from their target substrates in the Dβββ cluster.

Binding of architectural proteins and chromatin boundaries are largely unaltered in DN and DP thymocytes

The architectural protein CTCF facilitates the formation of structural loops in metazoan genomes, including those found in AgR loci (23, 24). Many loops are stabilized via the association of
CTCF with the ring-like cohesin complex, which is thought to ensnare the bases of chromatin loops (25). To determine whether the loss of distal Trbv–RC contacts in DP thymocytes is mechanistically related to reduced binding of supporting architectural complexes, we performed ChIP-qPCR assays using chromatin from DN (RAG1<sup>−/−</sup>) and DP subsets (RAG1<sup>−/−</sup>:anti-CD3ε).
FIGURE 2. Interactions within each Trbv domain are unchanged in DN and DP thymocytes. (A) Top, Schematic of Trcb, as described for Fig. 1A. Bottom, Long-range interactions using a Trbv5 viewpoint (anchor symbol in Trcb schematic). The data are presented as an average of three independent experiments, with y-axes (reads per million [rpm]) ranging from 1 to 6500. (B) 3C analysis was performed with Trbv5 and Trbv23 viewpoints in DN and DP thymocytes, using 3T3 fibroblasts as negative controls. Significant differences between DN and DP samples are denoted as *p < 0.05 (Student t test).

CTCF binding sites in Tcrb are shown in Fig. 3A (top) as established by ChiP-seq data from RAG-deficient DN thymocytes (36). Remarkably, levels of CTCF and the cohesin subunit RAD21 are not altered significantly in DN and DP thymocytes at the vast majority of tested sites, including those in distal and proximal Trbv domains, as well as RC flanking sites (Fig. 3A, middle and bottom). The one exception is a modest loss of CTCF at the 5'PC site, which functions as a tether for the distal Trbv domain in DN cells. However, levels of RAD21 at this tethering site remain unchanged in DP when compared with DN thymocytes.

We have recently shown that the tethering function of 5'PC can be compromised, despite retention of CTCF and RAD21 binding, by deletion of a chromatin boundary element (BE) located upstream of the RC (APDf1, Fig. 3C, top) (22). Removal of the natural BE permits a spread of highly active chromatin from the RC to 5'PC, which becomes a new boundary and, in some manner, acquisition of this function compromises its ability to serve as a long-range tether (22). Thus, one potential mechanism for specific dissociation of distal Trbv segments would be inactivation of the RC-proximal chromatin boundary in DP thymocytes, which would in turn disarm the 5'PC tether.

Initially, we tested this possibility by monitoring RNA expression from the normally silent tryptsinogen gene Prss2, which is activated in DN thymocytes upon deletion of the RC-proximal BE (Fig. 3B). However, Prss2 remains transcriptionally silent in DP thymocytes, suggesting that RC-proximal boundary function remains intact in these cells. This conclusion is bolstered by ChiP assays that monitored the spread of active chromatin upstream of the RC. The invasion of active chromatin is significant in DN cells lacking the native BE (APDf1), but not in DP thymocytes (Fig. 3C). Thus, dissociation of distal Trbv segments in DP thymocytes is not simply due to a loss of CTCF-cohesin within this structural domain, nor is it due to a disruption of chromatin boundary function upstream of the RC, which would impair distal Trbv tethering to 5'PC.

Stage-specific changes in Tcrb conformation are independent of the DN to DP proliferative burst

Prior studies have shown that most structural loops within the genome are disrupted during mitosis and reform in resting daughter cells (48). Developmental progression of DN thymocytes to the DP stage is associated with a robust proliferative burst. Indeed, a TCRβ+ DN thymocyte, on average, undergoes 10–11 rounds of division before coming to rest at the DP stage (31). Accordingly, we hypothesized that changes in Tcrb conformation during the DN to DP transition may require this robust proliferation, which would dissolve the DN architecture and allow its reconfiguration into the DP conformation.

To test this hypothesis, we used mice that lacked the gene encoding a CDK4/6 regulatory subunit, cyclin D3 (Ccnd3<sup>−/−</sup>), a defect that severely compromises thymocyte proliferation (31). When RAG1<sup>−/−</sup>:Tcrb transgenic mice are crossed into the Ccnd3-deficient background (RAG1<sup>−/−</sup>:Ccnd3<sup>−/−</sup>:Tcrb), thymocytes progress to the DP stage of development without the normal proliferative burst (31). These cellular defects are highlighted in Fig. 4A. Compared with RAG1<sup>−/−</sup>:Tcrb mice, in which the TCRβ-driven proliferative burst generates large numbers of DP thymocytes (1.2 × 10<sup>9</sup>) (31, 36), RAG1<sup>−/−</sup>:Ccnd3<sup>−/−</sup>:Tcrb thymuses also contain primarily DP cells, but at dramatically reduced numbers (1.5 × 10<sup>7</sup> cells). When probing endogenous Tcrb loci, RAG1<sup>−/−</sup>:Ccnd3<sup>−/−</sup>:Tcrb DP thymocytes exhibit no defects in short-range interactions between Eβ and the DB1 region compared with their proliferation-competent RAG1<sup>−/−</sup>:Tcrb DP counterparts (Fig. 4B). Importantly, preferential dissociation of the distal Trbv domain is unaffected in Ccnd3<sup>−/−</sup> DP thymocytes when these interactions are measured from two independent viewpoints within the RC (Fig. 4C, 4D).

We also examined global conformational changes at Tcrb in RAG1<sup>−/−</sup>:Ccnd3<sup>−/−</sup>:Tcrb thymocytes using 3D-FISH. We first sorted DN and DP cells from these animals to remove contaminating CD8<sup>+</sup> cells (Fig. 4A), which represent an intermediate between the DN and DP stages (49). Fixed cells were hybridized to fluorescent probes for regions at the very 5' end of Tcrb and near the RC (Fig. 4B, top), with probe separation used as a metric for locus contraction. As shown in Fig. 4E, Tcrb is contracted in RAG1<sup>−/−</sup>:Ccnd3<sup>−/−</sup>:Tcrb DN thymocytes compared with DP cells. In fact, locus decontraction is statistically indistinguishable in DP thymocytes derived from Ccnd3-deficient and -sufficient animals. Taken together, the low- (FISH) and high-resolution (3C) data indicate that dissociation of the distal Trbv and RC domains is independent of the massive proliferative burst that precedes DP thymocyte differentiation and, instead, may be mediated by DP-intrinsic mechanisms.

The hinge region for dissociation of distal Trbvs is unaltered epigenetically and transcriptionally during thymocyte development

To gain a better understanding of mechanisms that control specific dissociation of the distal Trbv domain in DP thymocytes, we used 3C to map the inflection point between retained and lost RC interactions. Our initial 3C analyses (Fig. 1C) suggested that the transition occurred in a 40-kb window upstream of Trbv12-1 (retained RC interaction) and Trbv5 (lost RC interaction). A more refined 3C “walk,” using Eβ as the viewpoint, revealed a transition between two adjacent restriction fragments, which have similar (Trbv11) or substantially diminished (5'V11) interaction with Eβ when comparing DN to DP thymocytes (Fig. 5A, left). All additional restriction fragments tested upstream of 5'V11, including those spanning distal Trbv segments, exhibit diminished association with Eβ in DP relative to DN cells (Figs. 1A, 1C, 5A).

The topological transition in DP thymocytes is also evident when two independent viewpoints are used near the RC (DB2 and 5'PC, Fig. 5B, right). Importantly, complementary 3C experiments, using viewpoints at this topological transition within the Trbv cluster,
supported our conclusions. Association between a restriction fragment spanning Trbv11 and a fragment within the RC is indistinguishable in DN and DP thymocytes (Fig. 5C, right), whereas 3C data from the 5′9V11 viewpoint reflects its dissociation from the RC during this developmental transition (Fig. 5C, left). Therefore, the region located directly upstream of Trbv11 serves as a "hinge" for developmentally regulated dissociation of distal Vβ gene segments from the RC in DP thymocytes.

We next explored potential mechanisms controlling this developmental hinge, focusing on specific changes to chromatin that may occur in this region during the DN to DP transition. First, binding of CTCF and RAD21 to a site nearest the hinge region is unaltered in DP compared with DN thymocytes (Fig. 5D). Second, transcription of two long interspersed element repetitive elements located 3′ of Trbv11 is repressed in DP thymocytes, which is similar to reduced expression of all germline Trbv segments in these cells (Fig. 5E). Third, similar to transcription, histone acetylation at sites within the hinge region also diminishes during transition to the DP subset (Fig. 5F). Thus, the hinge region exhibits no obvious epigenetic or transcriptional characteristics distinguishing it from developmental changes that occur over the entire Trbv cluster.

Stage-specific dissociation of distal Trbv segments on functionally rearranged alleles

Our data suggest that, on germline Tcrb alleles in RAG-deficient mice, the distal Trbv domain separates spatially from the RC in DP thymocytes via mechanisms independent of 5′PC function. During normal T cell development, the DN to DP transition is driven by functional Tcrb rearrangements, which are usually restricted to a single allele in each cell (6). All long-range recombination events between upstream Vβ segments and the RC will delete 5′PC. Moreover, a large subset of these rearrangements also deletes the inflection point for dissociation of distal Vβ segments from the RC, positioned upstream of Trbv11. Thus, examination of Tcrb conformation on a more physiologic allele, harboring a distal Trbv rearrangement, would provide an independent test for whether 5′PC or the 5′V11 hinge region is involved in stage-specific separation of distal Trbv segments.

For this purpose, we performed 3C analyses on thymocytes from a mouse strain that harbors two functional Vβ5Dβ1Jβ1.4 alleles in their germline (Fig. 6A), termed Vβ1NT mice (38). Whereas the remaining Trbvs on this allele can rearrange to Dβ2Jβ2 segments in DN thymocytes, recombination of these upstream Vβ segments is repressed in DP cells (39).
FIGURE 4. Alterations in Tcrb conformation do not require extensive thymocyte proliferation. (A) Representative FACS analysis of thymocytes from mice with the indicated genotypes. Cells were stained with CD4-PE and CD8-FITC. A representative of duplicate experiments is shown with percentages of thymocyte subsets highlighted. (B) 3C analyses show that short-range interactions between the Eβ viewpoint and DJβ1 region. Anchor symbols denote the viewpoints for 3C analyses. (C and D) Long-range interactions between either the DJβ1 (C) or Eβ (D) viewpoint and gene segments within the proximal (V11) and distal (V1 and V4–5) domains of the Tcrb cluster. Thymocytes from RAG1-deficient (DN), RAG1Δ−/−:Tcrb (DP), and RAG1Δ−/−:Tcrb: Cond3Δ−/− (DP) mice were used for this analysis. Statistically significant differences are denoted as *p < 0.05 (Student t test). (E) 3D-FISH with Trbv1 and Tsp probes (see (B), schematic) was used to determine the contraction status of Tcrb in DN or DP cells from the indicated genotypes. Significant differences in the 3D-FISH assays are denoted as ****p < 0.0001 (one-way ANOVA, Tukey post hoc test), and ns represents nonsignificant differences in median distances. All mice were deficient for RAG1.

the Vβ1NT allele drives developmental progression of thymocytes to the DP stage (39). Thus, to assess Tcrb conformation in DN thymocytes, pre–TCR signaling was crippled in RAG1Δ−/−:Vβ1NT mice by making them homozygous for a null mutation in the LAT adaptor molecule (38, 39). As shown in Fig. 6A, the Eβ enhancer associates not only with the rearranged V11 and distal (V1 and V4–5) domains of the Tcrb cluster. Thymocytes from RAG1-deficient (DN), RAG1Δ−/−:Tcrb (DP), and RAG1Δ−/−:Tcrb: Cond3Δ−/− (DP) mice were used for this analysis. Statistically significant differences are denoted as *p < 0.05 (Student t test). (E) 3D-FISH with Trbv1 and Tsp probes (see (B), schematic) was used to determine the contraction status of Tcrb in DN or DP cells from the indicated genotypes. Significant differences in the 3D-FISH assays are denoted as ****p < 0.0001 (one-way ANOVA, Tukey post hoc test), and ns represents nonsignificant differences in median distances. All mice were deficient for RAG1.

To determine whether intra–Vβ interactions are maintained on the rearranged allele following differentiation to the DP stage, we performed 3C analyses using Trbv2 as the viewpoint. As shown in Fig. 6C, association between Trbv2 and Trbv1 on the rearranged Vβ1NT allele, observed in DN thymocytes, is largely lost upon transition to the DP stage. In contrast, Trbv1–2 association is maintained on DP thymocytes with germline Tcrb alleles, further confirming that the folded configuration of the distal Tcrb domain is retained in these cells (Fig. 2B). As expected, Trbv2 dissociates from the RC in DP thymocytes harboring either the rearranged or germline allele. Thus, although RC-distal Vβ interactions are disrupted on both types of alleles, the conformational impact of DP differentiation on the distal Vβ cluster appears to be distinct for Tcrb loci with a germline (remains folded) versus the Vβ1NT configuration (unravels). Underlying mechanisms for this distinction remain to be determined; however, multiple CTCF sites are deleted from the rearranged Vβ1NT allele, which may preclude intra–Vβ folding, once these segments dissociate from the RC. Future studies could address this issue by examining new Tcrb alleles that have rearranged to more RC-proximal Vβ segments, and thus retain a fuller complement of CTCF sites.

Diminished RC–Trbv looping upstream of the preassembled VβDβJβ exon in DP thymocytes does not correlate with reduced deposition of CTCF at any site along the Vβ1NT allele (Fig. 6D). Similarly, RAD21 levels are not significantly altered at any of these CTCF sites, with the exception of a site situated 5′ to the rearranged Trbv5 segment, where RAD21 binding is reduced (Fig. 6E). However, the functional significance of reduced cohesin deposition at this single site remains unclear. Notwithstanding, we show that spatial segregation of distal Trbv segments from the RC in DP thymocytes is a common feature of germline, as well as
rearranged, Tcrb alleles (Fig. 7). Dissociation of the distal Trbv domain in DP thymocytes occurs regardless of whether the 5’PC tether or the 5’V11 inflection regions are present. These data strongly suggest that separation of distal Vβ segments is independent of specific cis-acting elements and is a process inherent to thymocytes transitioning from the DN to DP stage.

Discussion

The assembly of large AgR loci during lymphocyte development is controlled by coordinated changes in transcription, chromatin, and conformation (1–5). At the appropriate developmental stage for assembly, recombinase targets within each AgR locus become transcriptionally active and long-range recombination is facilitated by locus contraction (10). For Igh and Tcrb, these features are reversed at subsequent stages of development to enforce allelic exclusion, despite continued expression of the V(D)J recombinase (17, 20, 45, 46). With regard to locus conformation, it has been thought that Igh and Tcrb decontraction is accompanied by the loss of spatial associations between their respective RCs and V clusters (20, 32). In this work, we provide the most rigorous examination to date of this model, using high-resolution 3C to probe spatial associations within Tcrb as thymocytes pass from the permissive DN stage of development to the DP subset, in which its allelic exclusion is enforced.

A major finding from our 3C studies is that most of the Trbv cluster remains associated with the RC after transition of thymocytes to the DP stage and after Tcrb locus decontraction (Fig. 7). Only the most distal portion of the Trbv domain, upstream from the Trbv11 gene segment, spatially segregates from the RC. These conformational and cell imaging data remain consistent with one another, because all previous 3D-FISH assays, including our own, employed probes for the most distal Trbv domain, which separates to a greater average distance from RC probes in DP thymocytes (20, 22). Although only distal Vβ segments dissociate from the RC, both the proximal and distal Trbv domains appear to remain folded in their thymocyte-specific conformations, which promotes domain-specific association of gene segments (Fig. 7).

Of note, our conclusions differ significantly from those of a published study that also used 3C to probe Tcrb conformations in DN and DP thymocytes (20). The authors of the previous study concluded that both Trbv–RC and Trbv–Trbv interactions are disrupted in DP cells over the entire Vβ cluster. The specific source of this discrepancy remains unclear; however, the previous study used DN thymocytes cultured on stromal cells and older methods for 3C analyses that are, at best, only semiquantitative.

Notwithstanding, our data clearly preclude a model in which Tcrb allelic exclusion is enforced primarily by topological dissociation of the Trbv cluster from its DβJβ targets in DP thy-
mocytes. Although this spatial mechanism may be dominant for Trb1–5, the only functional Vβ segments that separate from the RC, distinct factors must prevent long-range recombination of the other 16 Vβ segments, which remain associated with the RC in DP thymocytes. Thus, for this latter set of Vβ segments, transcriptional suppression in DP cells is the most likely mechanism for enforcing allelic exclusion. In this regard, we have shown that, in DN thymocytes, the relative levels of spatial proximity for each Trbv segment do not contribute in a measurable way to their usage in Vβ1NT alleles (e.g., V1) spatially segregate from the Eβ interactome in DP thymocytes.

We also assessed possible mechanisms that may drive Tcrb conformational changes during thymocyte development. Our analyses discount domain-specific changes in transcription or general chromatin features as a force for the preferential dissociation of distal Trbv segments from the RC. All of these features appear to be regulated coordinately over the entire Tcrb cluster, including the mapped inflection point for RC dissociation near Trbv11. Moreover, loss of distal Trbv–RC interactions in DP cells is independent of CTCF/cohesin deposition at specific sites within the Vβ cluster or at their tether near the RC. We also found that Tcrb deconcatenation and distal Trbv dissociation from the RC are both independent of the massive proliferative burst preceding DN to DP transition.

The latter finding suggests that Tcrb conformational remodeling occurs via a DP-intrinsic process. Indeed, this model is strongly supported by our topological analyses of a functionally rearranged Tcrb allele. In DN thymocytes, upstream Trbv segments remain associated with an RC that harbors a functional Vβ5Dβ1Jβ1.4 rearrangement (Fig. 7). Similar to germline Tcrb loci, the distal Trbv segments become transcriptionally repressed (38, 39, 45) and dissociate from the RC interactome in DP thymocytes. The spatial segregation occurs on a rearranged allele that lacks both the normal long-range tether for distal Vβ segments (5‘PC) and the

![FIGURE 6.](image)

![FIGURE 7.](image)
mapped inflection point for Trbv–RC associations in DP cells (5′V11). These findings indicate that, rather than specific cis-elements orchestrating Tcrb conformational changes, perhaps spatial segregation of distal Trbv segments occurs via a process that is intrinsic to DP thymocytes. In fact, a switch from RC association with entire V clusters at the earliest precursor stages to a loss of RC-distal V interactions at subsequent stages may be a general feature of lymphocyte development. In DN thymocytes, distal and proximal V segments associate with RCs at both Tcrb (this study) and Teraβd (33). In the latter case, a global V–RC interactome likely facilitates more diverse usage of V segments in assembled TcrbIg genes. However, upon transition to the DP stage, distal portions of both the Trbv and Trav clusters dissociate from the RC, which limits initial Tcrα recombinaton to the most proximal Vs segments (33). For Tcrb, DP-intrinsic dissociation of distal Vβ segments might curb their secondary rearrangement to the downstream Distβ2β cluster, if present, which would delete an existing VβDβ1ββ join. Likewise, in pro–B cells, distal V segments at both the Igh and Igk loci associate with their RCs (34, 50); however, whether these long-range interactions are maintained in pre–B cells remains an open question.

In the case of thymocyte development, future studies must focus on mechanisms driving the stage-specific changes in Tcrb conformation. One attractive possibility for an underlying mechanism is stage-specific alteration of transcriptional status over the entire Trbv cluster. Our prior studies have shown that the Trbv domains fold and interact with distant Dββ1β cluster in DN thymocytes, independent of RC transcription (22). Because many Vβ segments become transcriptionally active upon their differentiation to the DN stage, we speculate that the induced expression dictates land–V–RC interaction over the entire Trbv cluster. Our prior studies have shown that the Trbv domains fold and interact with distant Dββ1β cluster in DN thymocytes, independent of RC transcription (22). Because many Vβ segments become transcriptionally active upon their differentiation to the DN stage, we speculate that the induced expression dictates association with the RC (22). Because many Vβ segments become transcriptionally active upon their differentiation to the DN stage, we speculate that the induced expression dictates association with the RC. Likewise, dissociation of the distal Trbv domain is accompanied by a widespread loss of Vβ transcription (45, 46). Perhaps both of these expression-induced changes in conformation are governed by the activation or suppression of a key transcription factor, such as E2A, which has been implicated in the activation or suppression of a key transcription factor, such as E2A, which has been implicated.

The authors have no financial conflicts of interest.

Disclosures


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