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INSIGHTS

RSSs set the odds for exclusion

Michael S. Krangel

In this issue of *JEM*, Wu et al. (https://doi.org/10.1084/jem.20200412) provide new insights into allelic exclusion. They demonstrate that V_β-to-$D_\beta J_\beta$ rearrangement occurs stochastically on two competing *Tcrb* alleles, with suboptimal V_β recombination signal sequences limiting synchronous rearrangements and essential for allelic exclusion.

A fundamental organizing principle of adaptive immunity is the antigen-driven clonal selection of lymphocytes, each bearing a single, unique cell surface antigen receptor (AgR). Complete AgR genes are assembled by V(D)J recombination, a process that is initiated by the RAG recombinase (composed of RAG1 and RAG2) and imparts diversity through combinatorial and imprecise joining of variable (V), diversity (D), and joining (J) gene segments at AgR loci. Although individual developing lymphocytes have the potential to rearrange and express AgR genes from both alleles, this generally does not happen, due in part to a form of regulation known as allelic exclusion (Vettermann and Schlissel, 2010).

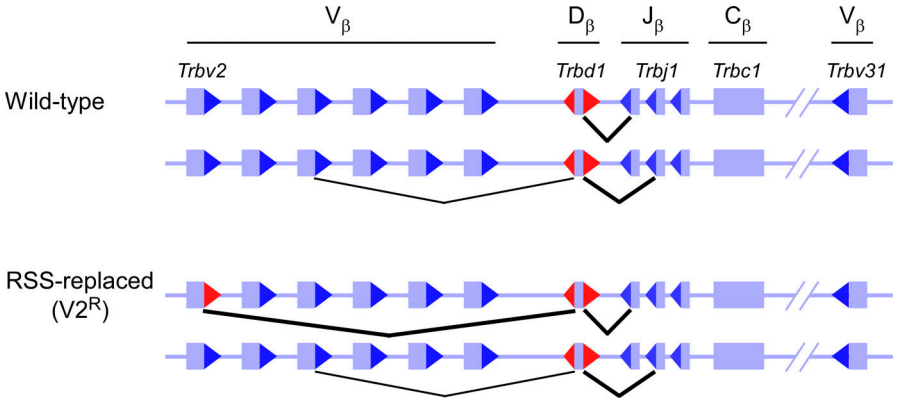
The mechanism of allelic exclusion has intrigued and eluded molecular immunologists for quite some time. Because V(D)J joining is imprecise, only one third of assembled AgR genes can encode a functional protein. One of the earliest applications of transgenesis to studies of lymphocyte development demonstrated the role of feedback inhibition, in which the functional AgR protein is sensed by its assembly into a signaling complex that drives developmental progression and suppresses further V gene segment rearrangement (Weaver et al., 1985). However, a protein product encoded by a functionally rearranged allele can only influence the course of events on the other allele if the two alleles attempt rearrangement in an asynchronous manner. How, then, is allelic asynchrony established? Models fall into two general categories: deterministic and stochastic (Vettermann and Schlissel, 2010). In deterministic models, the two alleles in any lymphocyte precursor would be intrinsically different, with one the initial choice to undergo rearrangement, and the other having an opportunity to rearrange only if the initial rearrangement were nonproductive. Stochastic models, by contrast, posit that there is no intrinsic difference between alleles, but rather that recombination is inefficient on both alleles, thereby distributing recombination attempts in time and making it unlikely that the two alleles would undergo recombination simultaneously. Regardless of model, there is the question of which molecular mechanisms suppress recombination to mediate these allelic programs. By and large, investigators have focused on epigenetic mechanisms, including those regulating chromatin accessibility and subnuclear localization. Indeed, at the *Igk* locus, the evidence strongly supports a deterministic model in which one allele per cell, randomly chosen, is early replicating and subsequently becomes demethylated and accessible for RAG binding (Farago et al., 2012). This biases V_κ-to-J_κ rearrangement to occur on one allele at a time. However, despite evidence for asynchronous replication, there is no similar evidence for deterministic epigenetic distinctions between the two *Tcrb* alleles in $CD4^-CD8^-$ double negative (DN) thymocytes. Rather, a prior study demonstrated an unrearranged V_β gene segment to be equally transcribed on both alleles in individual DN thymocytes (Jia et al., 2007). Moreover, analysis of *Tcrb* rearrangements in T cell hybridomas revealed that out-of-frame rearrangement of a V_β segment to the *Trbd1-Trbj1* cluster on one allele is often followed by V_β rearrangement on the second allele rather than rearrangement of V_β to the *Trbd2-Trbj2* cluster on the first (Khor and Sleckman, 2005). This argues against any intrinsic difference in rearrangement potential on the two alleles. Nevertheless, formal proof of the stochastic nature of V_β-to-$D_\beta J_\beta$ rearrangement has been lacking, as has an underlying mechanism.

Insights from Michael S. Krangel.

In this issue of *JEM*, Wu et al. (2020) now provide convincing evidence that V_β-to-$D_\beta J_\beta$ rearrangement occurs stochastically on the two *Tcrb* alleles in DN nuclei, with the two alleles in competition for successful rearrangement. They show as well that the suboptimal recombination signal sequences

Department of Immunology, Duke University Medical Center, Durham, NC.

Michael S. Krangel: krang001@mc.duke.edu.



RSS replacement increases the probability of synchronous V_β-to-$D_\beta J_\beta$ rearrangement and disrupts allelic exclusion. This image shows the *Tcrb* locus, depicting subsets of V_β and *Trbj1* gene segments; for simplicity, the *Trbd2-Trbj2-Trbc2* cluster (downstream of *Trbd1-Trbj1-Trbc1*) is not shown. Small triangles indicate 12-bp spacer RSSs; larger triangles indicate 23-bp spacer RSSs; red indicates optimal RSSs; and blue indicates suboptimal RSSs. *Tcrb* locus D_β-to-J_β rearrangement occurs first and is biallelic, whereas V_β-to-$D_\beta J_\beta$ rearrangement occurs subsequently, and in wild-type, occurs one allele at a time (top). Replacing the suboptimal *Trbv2* RSS with the highly efficient *Trbd1* 3′ RSS increases the frequency of *Trbv2* rearrangement and increases the odds that *Trbv2* will rearrange in synchrony with a V_β gene segment on the opposing allele (bottom). Synchronous allelic rearrangement cannot be suppressed by feedback inhibition and results in allelic inclusion.

(RSSs) that flank V_β gene segments are important to limit the frequency of V_β rearrangement events, thereby promoting allelic asynchrony and allelic exclusion.

All V, D, and J gene segments are flanked by RSSs that are recognized by RAG and define the sites of DNA cleavage that initiate V(D)J recombination. Wu et al. (2020) attacked the allelic exclusion problem by using CRISPR/Cas9 gene editing to replace the relatively low-quality RSSs flanking *Trbv2* and *Trbv31* with the high-quality RSS that flanks *Trbd1* on its 3′ side. It is typical for *Trbv2* and *Trbv31* to each be rearranged in-frame and expressed in ∼7% of mature T cells. However, in mice carrying a single *Trbv2* replacement allele ($V2^R$), *Trbv2* was used in 40% of T cells; in mice carrying a single *Trbv31* replacement allele ($V31^R$), *Trbv31* was used in 50% of T cells. Increased rearrangement of these V_β segments was restricted to the appropriate stage of DN thymocyte development. The authors then showed that the modified V_β segments were not only rearranging in competition with other V_β segments on the replacement allele, but were also competing with V_β segments on the opposing allele. This was made evident by comparing use of the RSS-replaced V_β in mice heterozygous for the replacement to (1) mice homozygous for the replacement, (2) mice in which the replacement allele was paired with a recombinationally inactive *Tcrb* allele, and (3) mice in which a $V2^R$ allele was paired with a $V31^R$ allele. Predictions are quite different in a stochastic scenario in which $V2^R$ and $V31^R$ rearrange in the same time window in competition with all V_β segments on both alleles, as opposed to a deterministic scenario in which the replacement allele is initially active in half of DN3 thymocytes, and the opposing allele is initially active in the other half.

Wu et al. (2020) then showed that high frequency rearrangement of *Trbv2* or *Trbv31* is associated with increased frequencies of allelically included T cells, which express these along with another V_β segment. Dual expression involving *Trbv31* and another V_β can theoretically occur as a result of two rearrangements on a single allele, the result of *Trbv31* being isolated from other V_β segments downstream of D_β and J_β segments in an inverted orientation. Indeed, single cell data confirmed the presence of two V_β rearrangements (one being *Trbv31*) on one allele in mice carrying one $V31^R$ and one $V2^R$ allele. However, the only explanation for dual expression of *Trbv2* with V_β segments other than *Trbv31*, or for increased frequency of dual *Trbv2* and *Trbv31* expression in T cells carrying both as compared with a single replacement allele, is a disruption of allelic exclusion. Consistent with this, the authors identified a hybridoma with in-frame rearrangement of *Trbv2* on one allele and *Trbv31* on the other. Wu et al. (2020) further investigated whether RSS strength or another RSS feature was the critical variable modulated by RSS replacement. The *Trbd1* 3′ RSS was previously shown to support binding of c-Fos (Wang et al., 2008). However, RSS replacements using a modified *Trbd1* RSS that does not bind c-Fos still disrupted allelic exclusion. Finally, the authors showed that rearrangement of the RSS replacement alleles was still subject to feedback inhibition, consistent with the notion that disruption to allelic exclusion occurred earlier, due to increased probability of synchronous allelic rearrangement in DN thymocytes.

Is this all there is to *Tcrb* allelic exclusion? Hardly. Will lessons from *Tcrb* be relevant to understand the regulation of other AgR loci? Yes, but only partially. As noted by the authors, a role for poor quality RSSs in promoting allelic asynchrony seems likely to apply to the *Igh* locus as well, since V_H and V_β RSSs likely share this particular feature (Liang et al., 2002). However, in other aspects, *Tcrb* is an odd bird. *Tcrb* alleles in DN thymocyte nuclei associate stochastically with two classically repressive nuclear compartments, pericentromeric heterochromatin and the nuclear lamina, with one, if not two, associated alleles in almost all DN thymocytes (Schlimgen et al., 2008). Such associations appear to reduce V_β-to-$D_\beta J_\beta$ rearrangement, perhaps by limiting exposure to RAG proteins (Chan et al., 2013). *Tcrb* is also unusual in its chromatin organization, with alternating regions of euchromatin and heterochromatin and most V_β segments separated from D_β and J_β segments by a heterochromatic region that interacts with the nuclear lamina (Chen et al., 2018). How this organization impacts V_β-to-$D_\beta J_\beta$ recombination in DN thymocytes is uncertain.

The Bassing laboratory previously demonstrated that the cellular response to double-strand breaks (DSBs) transiently suppresses continued attempts at V_κ-to-J_κ rearrangement on both alleles following initial RAG-mediated cleavage at the *Igk* locus (Steinel et al., 2013). This regulation has the effect of spacing recombination events in time and narrowing the window for those that might otherwise slip by as synchronous, or nearly so. An intact DSB response also facilitates *Tcrb* and *Igh* allelic exclusion (Steinel et al., 2014). The DSB response down-regulates RAG gene expression but

may transiently suppress V(D)J recombination in other ways as well.

Finally, there is the issue of feedback inhibition mediated by a functional TCRβ protein. Attention has long focused on changes to the locus in $CD4^+CD8^+$ thymocytes: (1) reduced transcription and chromatin accessibility of unrearranged V_β gene segments, and (2) a locus decontraction event thought to remove V_β segments from contacts with $D_\beta J_\beta$ segments (Majumder et al., 2015a). However, the latter is now understood to apply only to the more distal V_β gene segments (Majumder et al., 2015b). While the former is undoubtedly important, the "dirty little secret" is that *Trbv31* is allelically excluded despite proximity to $D_\beta J_\beta$ segments and increased transcription and chromatin accessibility in $CD4^+CD8^+$ thymocytes (Yang-Iott et al., 2010; Majumder et al., 2015b). There is clearly more to the story than we currently know.

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