INSIGHTS

RSSs set the odds for exclusion

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In this issue of JEM, Wu et al. (https://doi.org/10.1084/jem.20200412) provide new insights into allelic exclusion. They demonstrate that Vβ-to-Dββ rearrangement occurs stochastically on two competing Tcrb alleles, with suboptimal Vβ 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 Vε-to-Jε 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 Vβ 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 Vβ segment to the Trbd-Trbj cluster on one allele is often followed by Vβ rearrangement on the second allele rather than rearrangement of Vβ 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 Vβ-to-Dββ rearrangement has been lacking, as has an underlying mechanism.

In this issue of JEM, Wu et al. (2020) now provide convincing evidence that Vβ-to-Dββ 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...
RSSs set the odds for exclusion

(RSSs) that flank $\beta_\nu$ gene segments are important to limit the frequency of $\beta_\nu$ 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 $\beta_\nu$ segments. Increased rearrangement of these $\beta_\nu$ segments was restricted to the appropriate stage of DN thymocyte development. The authors then showed that the modified $\beta_\nu$ segments were not only rearranging in competition with other $\beta_\nu$ segments on the replacement allele, but were also competing with $\beta_\nu$ segments on the opposing allele. This was made evident by comparing use of the RSS-replaced $V_\beta$ 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 Trrb 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 $\beta_\nu$ 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 $V_\beta$ segment. Dual expression involving Trbv31 and another $V_\beta$ can theoretically occur as a result of two rearrangements on a single allele, the result of Trbv31 being isolated from other $\beta_\nu$ segments downstream of $D_\beta$ and $J_\beta$ segments in an inverted orientation. Indeed, single cell data confirmed the presence of two $\beta_\nu$ 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 $\beta_\nu$ 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 Trbd3’ RSS was previously shown to support binding of c-Fos (Wang et al., 2008). However, RSS replacements using a modified Trbd3 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 Trrb allelic exclusion? Hardly. Will lessons from Trrb 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 asynchronous seems likely to apply to the IgH locus as well, since $V_\gamma$ and $V_\beta$ RSSs likely share this particular feature (Liang et al., 2002). However, in other aspects, Trrb 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 (Schlingeman et al., 2008). Such associations appear to reduce $V_\beta$ to $D_\beta$J 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 $\beta_\nu$ segments separated from $D_\beta$ and $J_\beta$ segments by a heterochromatic region that interacts with the nuclear lamina (Chen et al., 2018). How this organization impacts $V_\beta$ to $D_\beta$J recombination in DN thymocytes is uncertain.

The Basset laboratory previously demonstrated that the cellular response to double-strand breaks (DSBs) transiently suppresses continued attempts at $V_\gamma$ to $J_\gamma$ rearrangement on both alleles following initial RAG-mediated cleavage at the IgH 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 Vβ gene segments, and (2) a locus decontraction event thought to remove Vβ segments from contacts with DβJβ segments (Majumder et al., 2015a). However, the latter is now understood to apply only to the more distal Vβ 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 DβJβ 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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