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Rebecca A. Glynn and Craig H. Bassing

Variable diversity joining recombination-mediated AgR gene assembly is the basis for adaptive immunity in jawed vertebrates. In this *Pillars of Immunology* article, Shinkai et al. (1) showed that RAG2 stimulates T cell development by promoting TCR gene assembly.

In 1993, evidence suggested a common recombinase assembled V region exons of all AgR genes from V, D, and J gene segments. This idea was supported by the observation that conserved recombination signal sequences (RSSs) flank and direct site-specific recombination of all *Ig* and *Tcr* gene segments (2). Furthermore, RSS-directed recombination was recognized to assemble the following: 1) complete TCR genes (*TCR β* and *TCR α* or *TCR γ* and *TCR δ*) only in immature T cells, 2) complete BCR genes (*IgH* and *Ig κ* or *Ig λ*) only in immature B cells, and 3) *TCR β* or *IgH* in prolymphocytes preceding *TCR α* or *Ig κ* or *Ig λ* in prelymphocytes (3). A screen for genes capable of recombining RSSs on plasmids in nonlymphoid cell lines identified *Rag1* and *Rag2*, which are expressed in immature T and B cells (4, 5). RAG1 weakly induced recombination, whereas RAG2 synergized with RAG1 to promote a 1000-fold increase in recombination activity (4, 5). The RAG proteins were named to encompass the uncertain potential function(s) of their products in activating versus being components of the recombinase. The inactivation of *Rag1* or *Rag2* was shown to fully block assembly of all AgR genes and development of T and B cells beyond prolymphocyte stages (6, 7). Yet, the potential cause/effect relationship between loss of V(D)J recombination and failure of lymphocyte differentiation remained unproven.

T and B cell development was known to proceed through discrete stages and transitions linked with assembly, expression, and signaling of AgR genes. In the case of $\alpha\beta$ T cell development, CD4⁻CD8⁻ (double-negative [DN]) thymocytes assemble TCR β genes first (8). Concomitant with TCR β expression, DN cells proliferate, expand, and differentiate into CD4⁺CD8⁺ (double-positive [DP]) thymocytes that then assemble TCR α genes (8). The expression of $\alpha\beta$ TCRs on DP thymocytes allows for positive selection on self-peptide/MHC

ligands that activates TCR-mediated signaling to drive development of CD4⁺ or CD8⁺ (single-positive [SP]) thymocytes, which then exit the thymus as mature $\alpha\beta$ T cells (8). Inactivation of *TCR β* or *TCR α* causes a block in $\alpha\beta$ T cell development at the DN or DP stage, respectively, suggesting that TCR β and TCR α proteins each signal differentiation (9, 10). Consistent with this view, a preassembled TCR β transgene partially rescued DN-to-DP thymocyte development in *Scid* mice, which have a mutation that impairs DNA double-strand break repair, completion of V(D)J recombination, and development of T and B cells (11). Additionally, it was recognized that disulfide-linked $\alpha\beta$ TCR dimers noncovalently associate with invariant CD3 proteins (CD3- ϵ , - δ , - γ , - ζ , and - η) to form complete $\alpha\beta$ TCR complexes (12). Studies in T cell lines indicated surface expression of $\alpha\beta$ TCR dimers or CD3 complexes were dependent on the association of TCR β , TCR α , and CD3 in the Golgi (13, 14). In line with this notion, TCR β was not detected on DP cells lacking TCR α (9). Collectively, these observations raised important questions about how TCR β -chains are expressed on DN cells and signal DN-to-DP cell development.

This *Pillars of Immunology* article yielded important insights into relationships between the following: 1) TCR gene assembly and T cell development, and 2) TCR β surface expression and signaling in DN cells (1). Shinkai et al. used flow cytometry to ascertain the effects of *TCR α* , *TCR β* transgenes, or both on $\alpha\beta$ T cell development in *Rag2*^{-/-} mice. Whereas TCR α alone had no effect, TCR β fully rescued the expansion and differentiation of DN into DP thymocytes but failed to develop SP cells. Coexpression of TCR α and TCR β fully rescued the following: 1) expansion and differentiation of DN cells into DP cells, 2) differentiation of DP cells into CD4⁺ SP cells, and 3) cellularity of CD4⁺ $\alpha\beta$ T cells because the transgenic $\alpha\beta$ TCR used was MHC class II-restricted and signaled development of only CD4⁺ cells (15). These data revealed the necessary and sufficient roles for expression of TCR β in early $\alpha\beta$ T cell development and of TCR α and TCR β in late $\alpha\beta$ T cell development. This work produced unequivocal evidence that the inability of *Rag2* deficient mice (*Rag2*^{-/-}) to assemble TCR β and TCR α genes causes the block of $\alpha\beta$ T cell development. Consequently, Shinkai et al. cemented a fundamental advance that the main, and possibly only, function of RAG2 is to promote initiation of V(D)J recombination.

Moving beyond the role of RAG2 in V(D)J recombination, Shinkai et al. assessed effects of the TCR β transgene on surface expression and formation of TCR/CD3 complexes. Unsurprisingly, flow cytometry failed to detect CD3 ϵ on *Rag2*^{-/-} DN cells (7). Conversely, transgenic TCR β induced CD3 ϵ and TCR β expression on *Rag2*^{-/-} DP cells. Next, Shinkai

Cell and Molecular Biology Graduate Group, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104; and Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104

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Address correspondence and reprint requests to Dr. Craig H. Bassing, Children's Hospital of Philadelphia, 4054 Colket Translational Research Building, 3501 Civic Center Boulevard, Philadelphia, PA 19104. E-mail address: bassing@email.chop.edu

Abbreviations used in this article: DN, double-negative; DP, double-positive; RSS, recombination signal sequence; SP, single-positive.

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et al. used two-dimensional gel electrophoresis of thymocyte proteins immunoprecipitated with anti-TCR β , anti-CD3 ϵ , or anti-CD3 ζ /anti-CD3 η Abs to identify complexes. As expected, they detected TCR $\alpha\beta$ -CD3 $\gamma\delta\epsilon\zeta$ complexes in TCR β^+ :Rag2^{+/+} DP cells. Interestingly, they found CD3 $\gamma\delta\epsilon$ and CD3 $\zeta\eta$ complexes in Rag2^{-/-} DN cells and TCR β -CD3 $\gamma\delta\epsilon$ complexes in TCR β^+ :Rag2^{-/-} DP cells. These data revealed the following: 1) expression and interaction of CD3 subunits does not require RAG2 or TCR β proteins, 2) TCR β proteins can bind CD3 $\gamma\delta\epsilon$ complexes independent of TCR α proteins, and 3) TCR α drives integration of CD3 ζ in TCR $\alpha\beta$ -CD3 complexes. Building on a model for B cells (16), Shinkai et al. proposed that TCR β -CD3 complexes may interact with a surrogate TCR α -chain to signal expansion and differentiation of DN thymocytes.

The knowledge and reagents of this *Pillars of Immunology* article provided an invaluable foundation for elucidating molecular mechanisms that promote lymphocyte development and control V(D)J recombination (1). Subsequent work of Shinkai et al. (17) and another group showed that CD3 proteins are expressed on DN cells before TCR β gene assembly and can be activated to signal DN-to-DP development (18). Thus, TCR β signals through CD3 proteins, a finding that led to identification of pre-T α as the surrogate TCR α -chain. This surrogate forms pre-TCRs with TCR β -CD3 $\gamma\delta\epsilon$ complexes to signal ligand-independent differentiation of TCR β^+ DN cells (19, 20). The approach of Shinkai et al. (1) was used to show that RAG2 drives B cell development by promoting assembly of *IgH* genes in pro-B cells and *Igk* or *Igl* genes in pre-B cells (21). These analogous studies gave unequivocal evidence for RAG2-dependent activity of a common recombinase for all AgR loci. This was proven by the demonstration that RAG1 and RAG2 are the only proteins required for cleavage of a V(D)J recombination substrate (22).

Since 1993, mice deficient for *Rag1* or *Rag2* alone and with AgR transgenes have been instrumental for identifying roles and elucidating functions of AgR locus *cis*-elements in regulating lymphocyte lineage- and developmental stage-specific V(D)J recombination (23). These mice also allowed discovery that RAG proteins bind to D and J segments, generating recombination centers that capture distal V segments to assemble complete AgR genes (24). Recently, such mice have revealed unexpected roles for RAG1 and RAG2 beyond simply initiating V(D)J recombination. RAG cleavage in developing T and B cells signals transcriptional activation of a genetic program that modulates cellular localization and includes genes important for proper lymphocyte selection (25). Moreover, RAG double-strand breaks induced in common lymphoid precursor cells cause heritable gene expression changes that facilitate the protective activities of adaptive and, surprisingly, innate lymphocytes (26). Overall, the observations planted by Shinkai et al. (1) continue to propel discoveries about the mechanisms that guide lymphocyte development and endow them with their unique functionalities.

Disclosures

The authors have no financial conflicts of interest.

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