Noncore RAG1 Regions Promote Vβ Rearrangements and αβ T Cell Development by Overcoming Inherent Inefficiency of V β Recombination Signal Sequences

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The RAG proteins are comprised of core endonuclease domains and noncore regions that modulate endonuclease activity. Mutation or deletion of noncore RAG regions in humans causes immunodeficiency and altered TCR repertoire, and mice expressing core but not full-length Rag1 (Rag1\(^{C/C}\)) or Rag2 (Rag2\(^{C/C}\)) exhibit lymphopenia, reflecting impaired V(D)J recombination and lymphocyte development. Rag1\(^{C/C}\) mice display decreased D-to-J and V-to-DJ rearrangements of TCRβ and IgH loci, whereas Rag2\(^{C/C}\) mice show decreased V-to-DJ rearrangements and altered Vβ/VH repertoire. Because Vβs/VHs only recombine to DJ complexes, the Rag1\(^{C/C}\) phenotype could reflect roles for noncore RAG1 regions in promoting recombination during only the D-to-J step or during both steps. In this study, we demonstrate that a preassembled TCRβ gene, but not a preassembled DβJβ complex or the prosurvival BCL2 protein, completely rescues αβ T cell development in Rag1\(^{C/C}\) mice. We find that Rag1\(^{C/C}\) mice exhibit altered Vβ utilization in V-to-DJβ rearrangements, increased usage of 3’α or 3’α segments in Vα-to-α rearrangements, and abnormal changes in Vβ repertoire during αβ TCR selection. Inefficient Vβ/VH recombination signal sequences (RSSs) have been hypothesized to cause impaired V-to-DJ recombination on the background of a defective recombinase as in core-Rag mice. We show that replacement of the Vβ14 RSS with a more efficient RSS increases Vβ14 recombination and rescues αβ T cell development in Rag1\(^{C/C}\) mice. Our data indicate that noncore RAG1 regions establish a diverse TCR repertoire by overcoming Vβ RSS inefficiency to promote Vβ recombination and αβ T cell development, and by modulating TCRβ and TCRα gene segment utilization. The Journal of Immunology, 2014, 192: 000–000.

The lymphocyte-specific RAG1/RAG2 (1) endonuclease generates Ag receptor diversity by recombining germline V, D, and J gene segments of TCR and Ig loci. RAG cleavage between two participating gene segments and their adjacent recombination signal sequences (RSSs) yields DNA double-strand breaks (DSBs) comprised of hairpin-sealed coding ends and blunt signal ends (1, 2). RAG proteins along with DNA damage response/repair proteins hold these DNA ends in a stable postcleavage complex and facilitate their repair by nonhomologous end-joining factors (3, 4). The combination of possible V(D)J joining events and the imprecise manner by which coding ends are processed cooperate to generate Ag receptor diversity.

The RAG1 and RAG2 proteins are each comprised of core endonuclease domains, defined as the minimal sequences required for DNA cleavage in vitro, and noncore regions that modulate this activity (2, 5). RAG1 or RAG2 mutations that alter or delete noncore RAG1 or RAG2 region amino acids and reduce overall V(D)J recombinase activity cause Omenn syndrome or other fatal severe combined immunodeficiencies associated with oligoclonal TCR repertoire and increased T cell–mediated autoimmunity (6–9). However, the contribution of diminished recombinase activity to aberrant TCR repertoire and autoreactive T cells in Omenn syndrome patients remains undetermined (6). Rag1\(^{C/C}\) and Rag2\(^{C/C}\) mice each display reduced numbers of mature T and B cells, reflecting impaired lymphocyte development beyond the progenitor stages associated with reduced TCRβ and IgH recombination (10–12). Whereas Rag1\(^{C/C}\) mice display reduced levels of both D-to-J and V-to-DJ recombination of TCRβ and IgH loci (11), Rag2\(^{C/C}\) mice show predominantly decreased V-to-DJ rearrangements associated with altered Vβ/VH usage (10, 12). Although no reductions in the levels of V-to-J recombination of Igk or TCRα loci were discovered in either Rag1\(^{C/C}\) or Rag2\(^{C/C}\) mice, potential changes in the utilization of individual Igk or TCRα gene segments have not been assayed (10–12). Considering that Vβs and VHβ only recombine to DJ complexes, the Rag1\(^{C/C}\) phenotype could reflect roles for noncore RAG1 regions in promoting recombination during only the D-to-J step or during both the D-to-J and V-to-DJ steps (11). Because Vβ and VHβ rearrangements are selectively impaired in Rag2\(^{C/C}\) mice, it has been hypothesized that the Rag2\(^{C/C}\) mouse phenotype is due to the interaction of a defective recombinase with unique features of Vβ/VH RSSs (10). In support of this notion, Vβ/VH RSSs that more closely resemble those of Dβ and Vα/Vβ/Vγ/Vδ segments are more efficiently cleaved by core-RAG proteins in vitro, and their associated Vβ/VH segments are recombined and expressed at higher frequencies in Rag2\(^{C/C}\) mice relative to normal mice (10). However, neither this decade-old model nor its prediction that Rag1\(^{C/C}\) mice also would exhibit altered Vβ and VHβ utilization has been tested.

In humans and mice, αβ T cells develop in the thymus through a differentiation program that involves the ordered assembly, ex-
pression, and selection of TCR genes. TCRβ genes assemble through DJβ-to-Jβ and then VBβ-to-DJββ rearrangements in CD4+ CD8+ double-negative (DN) thymocytes (13). The DJβ-to-Jββ recombination step initiates in c-KiitCD25+ DN1 cells and continues in c-KiitCD25+ DN2 cells, whereas VBβ-to-DJββ recombination occurs in c-KiitCD25+ DN3 cells (13). Because TCRβ loci contain 31 VB segments (Trb1–Trb31) and two DJβ-Jβ-Cβ clusters (Trbd1-Trb1-Trbc1 and Trbd2-Trb2-Trbc2) each with one DJβ and six functional Jβ segments, secondary VBβ rearrangements can occur to DJβ2Jβ2 (Trbd2Trb2) complexes on alleles with primary VBβ rearrangements to assembled DJββ1 (Trbd1Trb1) complexes (14). Utilization of individual VBβ segments in primary VBβ-to-DJββ1 rearrangements is biased independent of VBβ position (15). However, VBβ position relative to a preassembled VBβDJβ1CB1 gene can influence the usage of VBβ segments in secondary VBβ-to-DJββ2 rearrangements (14, 16).

Assembly and expression of a functional TCRβ gene generates TCRβ-chains that pair with pre–α-TCR proteins to form pre-TCRs, which promote survival and differentiation, downregulate RAG expression, and induce expression of the cyclin D3 (Cnd3) protein (17, 18). Cnd3 drives proliferation as DN3 cells downregulate RAG expression and differentiate into c-KiitCD25+ DN4 and then CD4+CD8+ double-positive (DP) thymocytes (17, 18). TCRα genes assemble through Vα-to-Jα rearrangements on both alleles in DP cells, where VBβ-to-DJββ recombinations is silenced (19, 20). The assembly and expression of a functional VαJα5C5 gene generates TCRα-chains that can pair with TCRβ-chains to generate αβ TCRs, which are selected based on interactions with thymic epithelial cells (17). Positive selection increases expression of αβ TCRs and promotes differentiation of DP thymocytes into CD4+CD8− or CD4+CD8+ single-positive (SP) thymocytes that emigrate from the thymus as mature αβ T cells (17). Because TCRα loci contain ~100 Vα and ~50 Jα segments, successive Vα-to-Jα rearrangements can occur until positive selection or until all Vα or Jα segments have been used (13, 19). Although pre-TCR selection and thymocyte expansion does not significantly change VBβ repertoire during DN-to-DP thymocyte differentiation (15), positive selection can substantially alter VBβ representation in αβ TCRs during DP-to-SP thymocyte development (21–27).

The mouse αβ T cell differentiation program provides a useful experimental model to elucidate roles for noncore RAG1 regions in promoting V(D)J recombination and controlling TCR gene repertoire. Rag1C/C mice exhibit reduced DJβ-Jβ and VBβ-to-DJββ recombination in DN3 thymocytes and impaired DN3-to-DP thymocyte development from accumulation of cells at the DN3 stage (11). These phenotypes may arise from impaired DJβ-to-Jβ and/or VBβ-to-DJββ recombination in the absence of noncore RAG1 regions. However, considering that RAG DSBs induce changes in the expression of proteins involved in cellular survival, lymphocyte differentiation, and Ag gene receptor selection (28), these phenotypes also may arise from impaired signaling in response to RAG DSBs induced in Rag1C/C DN3 thymocytes. To determine how noncore RAG1 regions promote TCRβ gene assembly and αβ T cell development, we have created and analyzed Rag1C/C mice containing a preassembled DJββ complex or preassembled functional TCRβ gene, expressing the prosurvival EμBCL2 transgene, or with the 3′DJβ RSS in place of the VBβ RSS. We demonstrate that this TCRβ gene, but not the DJββ complex or BCL2, completely rescues DN3-to-DP thymocyte development in Rag1C/C mice, indicating that the predominant function of noncore RAG1 regions in differentiating αβ T cells is to promote VBβ recombination. We show that Rag1C/C mice exhibit altered VBβ utilization in VBβ-to-DJββ rearrangements and that neither apoptosis of cells attempting VBβ recombination nor TCRβ-dependent expansion of DN3 thymocytes contributes to this abnormal VBβ repertoire. We detected increased usage of 3′Jα segments in Vα-to-Jα rearrangements and abnormal selection of the VBβ repertoire in Rag1C/C mice, revealing that noncore RAG1 regions also function during TCRα recombination in DP thymocytes. Finally, we show that the 3′DJβ RSS increases VBβ4 recombination and partially rescues αβ T cell development in Rag1C/C mice. Collectively, our data indicate that noncore RAG1 regions establish a diverse αβ TCR repertoire by overcoming VBβ RSS inefficiency to promote VBβ recombination and αβ T cell development and by modulating TCRβ and TCRα gene segment utilization.

**Materials and Methods**

**Mice**

Rag1C/C (11), EμBCL2 (29), Jβ1<sup>1/MO</sup> (22), VBβ<sup>INTENT</sup> (14), and Cnd3<sup>−/−</sup> (18) mice were obtained and used to generate the mice described in this study. The germline VBβ<sup>14</sup>DJββ<sup>RSS</sup> mice were generated by Cre-loxP-mediated gene-targeting using W4 mouse embryonic stem cells and the construct previously employed to make chimeric VBβ<sup>14</sup>DJββ<sup>RSS</sup> mice (21). All experimental mice were on a mixed 129SvEv and C57BL/6 background and were littermate or age-matched mice between 4 and 6 wks of age. All experiments were conducted in accordance with national guidelines and approved by the Institutional Animal Care and Use Committee of the Children’s Hospital of Philadelphia.

**Flow cytometry**

Single-cell suspensions were stained with Abs in PBS containing 2% BSA. All Abs were purchased from BD Pharmingen. CD4 and CD8 analysis was performed using anti-CD4 (553653), anti-CD8 (553031), and anti-TCRβ (553174) Abs. DN stage analysis was performed on lineage-negative cells stained with a mixture of PE-conjugated anti-CD4 (553653), anti-CD8a (553033), anti-TCRβ (553172), anti-TRCγ (553178), anti-B220 (553090), anti-CD19 (553786), anti-CD11b (553311), anti-CD11c (557401), anti-NK1.1 (553165), and anti-Ter119 (553673) Abs in addition to anti-CD25 (552880) and anti-CD117 (553356) Abs. Vβ analysis was performed using Abs against TCRβ (553174) as well as Vβ5 (553189), Vβ6 (553102), Vβ8 (553861), Vβ10 (553285), Vβ14 (553258), and streptavidin-FTTC (554060). Data was acquired on a FACSCalibur (BD Biosciences, San Jose, CA) using CellQuest software (BD Biosciences) and analyzed using FlowJo software (Tree Star).

**Polymerase chain reaction**

Genomic DNA from sorted thymocytes (on 100 ng and 1.5 serial dilutions) was subjected to long-range PCR with the use of primers and PCR conditions as described (30, 31).

**Results**

**Transgenic BCL2 expression partially rescues early αβ T cell development in Rag1C/C mice**

To determine whether impaired cellular survival in response to RAG DSBs causes impaired DN3-to-DP thymocyte development in Rag1C/C mice, we created and analyzed Rag1C/C mice containing the EμBCL2 transgene (EμBCL2:Rag1C/C mice) because expression of the prosurvival BCL2 protein increases survival of DN3 cells attempting TCRβ rearrangements (32). Consistent with the prosurvival effect of BCL2, we detected ~3-fold increases in the numbers of DN and DP thymocytes in EμBCL2:Rag1C/C mice compared with Rag1C/C mice, although the difference in DN thymocyte numbers did not reach statistical significance (Fig. 1A, 1B). Similarly, we found equivalent numbers of DN3 and DN4 thymocytes in EμBCL2:Rag1C/C mice (Fig. 1C, 1D). We also detected ~4.5-fold decreased numbers of total and DP thymocytes in EμBCL2:Rag1C/C mice relative to EμBCL2 mice (Fig. 1A, 1B), indicating that BCL2 expression does not completely rescue impaired DN-to-DP thymocyte development in Rag1C/C mice.
Rag1<sub>C</sub>C mice. These observations indicate that BCL2 expression does not substantially enhance the survival of Rag1<sub>C</sub>C DN3 thymocytes, yet it has a more pronounced effect on promoting survival of DP thymocytes or cells during DN-to-DP thymocyte expansion and differentiation in Rag1<sub>C</sub>C mice. Therefore, we conclude that reduced survival of DN3 thymocytes in response to RAG DSBs is not a major cause of the impaired TCRβ gene assembly and DN-to-DP thymocyte development in Rag1<sub>C</sub>C mice.

**FIGURE 1.** Transgenic BCL2 expression partially rescues αβ T cell development in Rag1<sub>C</sub>C mice. (A) Representative flow cytometry analysis of CD4 and CD8 expression on total thymocytes isolated from littermate or age-matched WT (n = 3), Rag1<sub>C</sub>C (n = 3), EmBCL2 (n = 3), or EmBCL2:Rag1<sub>C</sub>C (n = 3) mice. The average number of total thymocytes for mice of each genotype is indicated in parentheses, and the frequencies of cells in the DN, DP, CD4<sup>+</sup> SP, and CD8<sup>+</sup> SP quadrants are indicated on the plots. (B) Graphs showing the average numbers of DN and DP thymocytes from mice of the indicated genotypes. Error bars are SEM. *p ≤ 0.05. (A and B) This experiment was independently performed three times, each time on one mouse of each genotype. (C) Representative flow cytometry analysis of c-Kit and CD25 expression on DN thymocytes isolated from littermate or age-matched WT (n = 3), Rag1<sub>C</sub>C (n = 3), EmBCL2 (n = 3), or EmBCL2:Rag1<sub>C</sub>C (n = 3) mice. The frequencies of DN cells in the DN1, DN2, DN3, and DN4 quadrants are indicated on the plots. (D) Graphs showing the average numbers of DN3 and DN4 thymocytes from mice of the indicated genotypes. Error bars are SEM. *p ≤ 0.05. (C and D) This experiment was independently performed three times, each time on one mouse of each genotype.
A preassembled TCRβ gene completely rescues early αβ T cell development in Rag1C/C mice

To determine potential functions of noncore Rag1 regions in promoting Db-to-Jβ and Vβ-to-Dβ recombination, we generated and analyzed Rag1C/C mice that contain a preassembled DβJβ complex (Jβ1DJ) or preassembled functional TCRβ gene (Vβ1NT) on both TCRβ alleles. The Jβ1DJ allele contains a preassembled Dβ1Jβ1.1 (Trbd1Trbj1.1) complex, lacks Dβ2 (Trbd2) and Jβ2 (Trbj2) segments, and must recombine 1 of 35 Vβ segments to the Dβ1Jβ1.1 complex to promote αβ T lymphocyte development (22). The Vβ1NT allele contains a preassembled functional endogenous Vβ1DJβ1.4Cβ1 (Trbv5Trbd1Trbj1.4Trbc1) gene that promotes αβ T cell development independent of TCRβ recombination (14). We detected ~2-fold more DP thymocytes in Rag1C/C mice (Fig. 2A, 2B). However, the DP cell numbers in Rag1C/CJβ1DJ/DJ mice and Rag1C/C mice

**FIGURE 2.** A preassembled TCRβ gene completely rescues early αβ T cell development in Rag1C/C mice. (A) Representative flow cytometry analysis of CD4 and CD8 expression on total thymocytes isolated from littermate or age-matched WT (n = 3), Rag1C/C (n = 3), Jβ1DJ/DJ (n = 3), and Rag1C/CJβ1DJ/DJ mice (n = 3). The average number of total thymocytes for each genotype is indicated in parentheses, and the frequencies of cells in the DN, DP, CD4+ SP, and CD8+ SP quadrants are indicated on the plots. (B) Graphs showing the average numbers of DN and DP thymocytes from mice of the indicated genotypes. Error bars are SEM. *p < 0.05. (A and B) This experiment was independently performed three times, each time on one mouse of each genotype. (C) Representative flow cytometry analysis of c-Kit and CD25 expression on DN thymocytes isolated from littermate or age-matched WT (n = 3), Rag1C/C (n = 3), Jβ1DJ/DJ (n = 3), and Rag1C/CJβ1DJ/DJ (n = 3) mice. The frequencies of DN cells in the DN1, DN2, DN3, and DN4 quadrants are indicated. (D) Graphs showing the average numbers of DN3 and DN4 thymocytes from mice of the indicated genotypes. Error bars are SEM. *p < 0.05. (C and D) This experiment was independently performed three times, each time on one mouse of each genotype. (E) Representative flow cytometry analysis of CD4 and CD8 expression on total thymocytes isolated from littermate or age-matched WT (n = 3), Rag1C/C (n = 3), Vβ1NT/NT (n = 6) mice, and Vβ1NT/NTRag1C/C (n = 6) mice. The average number of total thymocytes for each genotype is indicated in parentheses, and the frequencies of cells in the DN, DP, CD4+ SP, and CD8+ SP quadrants are indicated on the plots. (F) Graphs showing the average numbers of DN and DP thymocytes from mice of the indicated genotypes. Error bars are SEM. *p < 0.05. (E and F) This experiment was independently performed three times, each time on at least mouse of each genotype. (G) Representative flow cytometry analysis of c-Kit and CD25 expression on DN thymocytes isolated from littermate or age-matched WT (n = 3), Rag1C/C (n = 3), Vβ1NT/NT (n = 3), and Vβ1NT/NTRag1C/C (n = 3) mice. The frequencies of DN cells in the DN1, DN2, DN3, and DN4 quadrants are indicated. (H) Graphs showing the average numbers of DN3 and DN4 thymocytes from mice of the indicated genotypes. Error bars are SEM. *p < 0.05. (G and H) This experiment was independently performed three times, each time on one mouse of each genotype.
were 2-fold or more lower as compared with EμBCL2 and wild-type (WT) mice, respectively (Fig. 2B versus Fig. 1B). We also detected ~8-fold more DN4 cells in Rag1$^{1\text{C/CJ}}$B1$^{D/DJ}$ mice relative to Rag1$^{1\text{C/C}}$ mice (Fig. 2C, 2D), with DN4 thymocyte numbers in Rag1$^{1\text{C/CJ}}$B1$^{D/DJ}$ mice and Rag1$^{1\text{C/C}}$ mice significantly lower than in EμBCL2 and WT mice, respectively (Fig. 2D versus Fig. 1E). These data demonstrate that a preassembled DbJB1 complex on both TCRβ alleles partially rescues DN3-to-DN4 and DN-to-DP thymocyte development in Rag1$^{1\text{C/C}}$ mice. In Rag1$^{1\text{C/C}}$B1$^{NT/NT}$ mice relative to Rag1$^{1\text{C/C}}$ mice, we observed greater numbers of DP (4-fold more) and DN4 (11-fold more) thymocytes (Fig. 2E–H). Notably, the numbers of DP and DN4 thymocytes in Rag1$^{1\text{C/CJ}}$B1$^{NT/NT}$ mice were similar to those in Vβ1$^{NT/NT}$ and WT mice (Fig. 2E–H), showing that a preassembled functional TCRβ gene completely rescues both DN3-to-DN4 and DN-to-DP thymocyte development in Rag1$^{1\text{C/C}}$ mice. Therefore, our data indicate that noncore RAG1 regions promote both DbJ-to-Jβ and Vβ-to-Jβ rearrangements and that reduced Vβ-to-Jβ recombination is the major cause of accumulation of cells at the DN3 stage and impaired DN-to-DP thymocyte development in Rag1$^{1\text{C/C}}$ mice.

**Rag1$^{1\text{C/C}}$ mice exhibit altered Vβ utilization in primary and secondary Vβ rearrangements**

The TCRβ locus architecture permits primary Vβ rearrangements to DbJ1DbJ complexes and then secondary Vβ rearrangements to DbJ2Db2 complexes, which occur on the Vβ1$^{NT}$ allele (14). Our current analysis of thymocyte development in Rag1$^{1\text{C/CJ}}$B1$^{D/DJ}$ mice and Rag1$^{1\text{C/CJ}}$VB1$^{NT/NT}$ mice and our previous analysis of Vβ8 (Trbv13.1, Trbv13.2, and Trbv13.3) and Vβ10 (Trbv4) rearrangements in DN3 cells of Rag1$^{1\text{C/C}}$ mice (11) demonstrate that primary Vβ-to-DJβ rearrangements are impaired in the absence of noncore Rag1 regions. However, these analyses cannot address potential function of noncore Rag1 regions in secondary Vβ rearrangements or quantify relative usage of individual Vβ segments in Vβ-to-DJβ rearrangements. Because DN-to-DP thymocyte differentiation does not significantly alter Vβ repertoire (15), the use of flow cytometry to monitor Vβ expression on TCRβ$^{int}$ DP thymocytes provides a more sensitive means than PCR to quantify relative Vβ usage in Vβ rearrangements (14, 15, 21). Thus, to determine the contributions of noncore Rag1 regions in Vβ utilization during primary Vβ-to-DJβ rearrangements, we assayed Vβ5 (Trbv12.1 and Trbv12.2), Vβ6 (Trbv19), Vβ8, Vβ10, and Vβ14 (Trbv31) expression on DP thymocytes of Rag1$^{1\text{C/CJ}}$B1$^{D/DJ}$ and Jβ1$^{D/DJ}$ mice, as only primary Vβ rearrangements occur on the Jβ1$^{D/DJ}$ allele (22). We observed lower percentages of Vβ5* (5-fold less) and Vβ10* (~3-fold less) DP thymocytes in Rag1$^{1\text{C/C}}$B1$^{D/DJ}$ mice as compared with Jβ1$^{D/DJ}$ mice (Fig. 3A, 3B). We detected increases in the percentages of Vβ6+ (2-fold more) and Vβ14+ (3-fold more) DP thymocytes, but no difference in the percentages of Vβ6* DP cells in Rag1$^{1\text{C/CJ}}$B1$^{D/DJ}$ mice relative to Jβ1$^{D/DJ}$ mice (Fig. 3A, 3B). Because only secondary Vβ-to-Jβ rearrangements involving Vβ10 occur on the Vβ1$^{NT}$ allele (14), we next quantified Vβ10 expression on DP thymocytes of Rag1$^{1\text{C/C}}$ VB1$^{NT/NT}$ and VB1$^{NT/NT}$ mice to evaluate whether noncore Rag1 regions promote such secondary Vβ rearrangements. We found a

**FIGURE 3.** Rag1$^{1\text{C/C}}$ mice exhibit altered Vβ utilization in primary and secondary Vβ rearrangements. (A) Representative flow cytometry analysis of TCRβ and Vβ expression shown for Vβ10 or Vβ14 on total thymocytes isolated from littermate or age-matched Jβ1$^{D/DJ}$ (n = 3) and Rag1$^{1\text{C/CJ}}$B1$^{D/DJ}$ (n = 3) mice. The frequencies of cells in the depicted TCRβ$^{int}$ gate are indicated. (B) Graph showing the average frequencies of TCRβ$^{int}$ cells expressing Vβ10, Vβ8, Vβ5, Vβ6, or Vβ14 in thymocytes from Jβ1$^{D/DJ}$ and Rag1$^{1\text{C/CJ}}$B1$^{D/DJ}$ mice. Error bars are SEM. *p ≤ 0.05. (A and B) This experiment was independently performed three times, each time on one mouse of each genotype. (C) Representative flow cytometry analysis of TCRβ and Vβ10 expression on total thymocytes isolated from littermate or age-matched Vβ1$^{NT/NT}$ (n = 6) and Vβ1$^{NT/NT}$Rag1$^{1\text{C/C}}$ (n = 6) mice. The frequencies of cells in the depicted TCRβ$^{int}$ gate are indicated. (D) Graph showing the average frequencies of TCRβ$^{int}$ cells expressing Vβ10 in thymocytes from Vβ1$^{NT/NT}$ and Vβ1$^{NT/NT}$Rag1$^{1\text{C/C}}$ mice. Error bars are SEM. *p ≤ 0.05. (C and D) This experiment was independently performed three times, each time on at least one mouse of each genotype. (E) Representative flow cytometry analysis of TCRβ and Vβ10 or Vβ14 expression on total thymocytes isolated from littermate or age-matched WT (n = 3), Rag1$^{1\text{C/C}}$ (n = 3), EμBCL2 (n = 3), or EμBCL2:Rag1$^{1\text{C/C}}$ (n = 3) mice. The frequencies of cells in the depicted TCRβ$^{int}$ gate are indicated. (F) Graph showing the average frequencies of TCRβ$^{int}$ cells expressing Vβ10, Vβ8, Vβ5, Vβ6, or Vβ14 in thymocytes from mice of the indicated genotypes. Error bars are SEM. *p ≤ 0.05. (E and F) This experiment was independently performed three times, each time on one mouse of each genotype.
7-fold lower percentage of Vβ10+ DP thymocytes in Rag1^{1/C} Vβ1^{1/NT}/Vβ1^{1/NT} mice as compared with Vβ1^{1/NT}/Vβ1^{1/NT} mice (Fig. 3C, 3D). Collectively, these data indicate that noncore Rag1 regions affect Vβ utilization in primary and secondary Vβ rearrangements, at least on Jβ1^{1/D} and Vβ1^{1/NT} alleles.

To evaluate whether noncore Rag1 regions affect Vβ utilization in total Vβ rearrangements on normal TCRβ alleles, we quantified expression of Vβ5, Vβ6, Vβ8, Vβ10, and Vβ14 on DP thymocytes of Rag1^{1/C} and WT mice. We detected lower percentages of Vβ5 (∼6-fold less) and Vβ10+ (∼2-fold less) DP cells in Rag1^{1/C} mice relative to WT mice (Fig. 3E, 3F). We also detected an ∼2-fold increased percentage of Vβ8* and an ∼3-fold increased percentage of Vβ14+ DP thymocytes, but no difference in the percentage of Vβ6+ DP cells, in Rag1^{1/C} mice as compared with WT mice (Fig. 3E, 3F). These data demonstrate that noncore Rag1 regions influence Vβ utilization in total Vβ rearrangements on normal TCRβ alleles.

Although we did not observe a major role for impaired survival of Rag1^{1/C} thymocytes in response to RAG DSBs during TCRβ recombination, altered survival of DN3 TCRβ segments during rearrangements of particular Vβ segments could influence Vβ repertoire in Rag1^{1/C} mice. To investigate this possibility, we quantified the expression of Vβ5, Vβ6, Vβ8, Vβ10, and Vβ14 on DP thymocytes of EμBCL2:Rag1^{1/C} and Rag1^{1/C} mice. We detected no significant differences in the frequencies of Vβ6*, Vβ8*, Vβ10*, or Vβ14+ DP thymocytes between EμBCL2:Rag1^{1/C} and Rag1^{1/C} mice (Fig. 3E, 3F), but we did observe a slightly higher frequency of Vβ8* DP cells in EμBCL2:Rag1^{1/C} mice as compared with Rag1^{1/C} mice (Fig. 3E, 3F). These data suggest that impaired survival of DN3 cells in response to RAG DSBS does not cause the altered relative representation of Vβ5, Vβ6, Vβ8, Vβ10, and Vβ14 on DP thymocytes of Rag1^{1/C} mice.

Whereas Vβ representation is not significantly altered during DN-to-DP thymocyte differentiation in mice with normal levels of Vβ rearrangements (15), altered expansion of DN cells expressing particular Vβ segments could contribute to the altered Vβ repertoire in DP thymocytes of Rag1^{1/C} mice that have reduced numbers of thymocytes caused by impaired Vβ-to-DβJββ recombination. To assess this possibility, we generated and analyzed Rag1^{1/C}Ccn3^{Δ/−} mice because expression of TCRβ-chains drive DN-to-DP thymocyte expansion through Ccn3, and Ccn3^{Δ/−} mice exhibit normal Vβ repertoire in DP thymocytes (18, 33). We found equivalent numbers of DN thymocytes, but ∼10-fold lower numbers of DP cells, in Rag1^{1/C}Ccn3^{Δ/−} mice as compared with Ccn3^{+/−} mice (Fig. 4A, 4B), indicating that TCRβ-mediated DN-to-DP thymocyte development is profoundly impaired in Rag1^{1/C}Ccn3^{Δ/−} mice relative to Ccn3^{+/−} mice. Notably, the numbers of DP cells in Rag1^{1/C}Ccn3^{Δ/−} mice were reduced ∼40-fold as compared with Rag1^{1/C} mice versus ∼17-fold for Ccn3^{+/−} mice relative to WT mice (compare Fig. 4B and Fig. 1B). We also found an ∼5-fold lower frequency of Vβ10* DP cells but an ∼2-fold increase frequency of Vβ14+ DP thymocytes in Rag1^{1/C}Ccn3^{Δ/−} mice as compared with Ccn3^{+/−} mice (Fig. 4C, 4D). Considering that we observed similar increased and decreased frequencies of Vβ10* and Vβ14+ DP thymocytes, respectively, in Rag1^{1/C} mice relative to WT mice (Fig. 3E, 3F), our data indicate that the altered representation of Vβ10 and Vβ14 on DP thymocytes of Rag1^{1/C} mice is not caused by differences in TCRβ-mediated proliferation of DN cells expressing particular Vβ segments. Therefore, based on our quantification of Vβ expression on DP thymocytes of mice expressing WT or core-Rag1 proteins in combination with other genetic modifications, we conclude that Rag1 noncore regions control Vβ repertoire at the level of relative Vβ usage in primary and secondary Vβ-to-DβJββ rearrangements.

Rag1^{1/C} mice exhibit altered Jα utilization in Vα-to-Jα rearrangements and abnormal changes in Vβ repertoire during aβ TCR selection

In DP thymocytes of WT mice, TCRα recombination occurs on both alleles and normally involves successive rounds of Vα-to-Jα rearrangements on each allele (19). Although both TCRα alleles are recombined in mature αβ T cells of Rag1^{1/C} mice (11), whether the absence of noncore Rag1 regions results in a modest reduction in Vα-to-Jα rearrangements in DP thymocytes is not known. Because reduced V(D)J recombinase activity in DP cells leads to increased representation of 5'Jαs and decreased representation of 3'Jαs in Vα-to-Jα rearrangements (34), we investigated whether Vα rearrangements in DP cells of Rag1^{1/C} mice are similarly biased. For this purpose, we used PCR primers that hybridize to the Vα3 (Traj9-4) family of gene segments or to Jα61 (Traj61), Jα42 (Traj42), Jα17 (Traj17), or Jα4 (Traj4) to amplify Vα3-to-Jα rearrangements involving these 5'Jαs (Jα61, Jα42) or 3' (Jα17, Jα4) Jα gene segments from sort-purified DP cells of WT or Rag1^{1/C} mice. The levels of PCR products representing Vα3 rearrangements to Jα61 and Jα42 were reduced in DP thymocytes from Rag1^{1/C} mice relative to WT mice (Fig. 5A). In contrast, the levels of PCR products representing Vα3 rearrangements to Jα17 and Jα4 were elevated in DP thymocytes from Rag1^{1/C} mice as compared with WT mice (Fig. 5A). These data reveal that loss of noncore Rag1 regions results in decreased usage of 5'Jαs and increased usage of 3'Jαs in Vα-to-Jα rearrangements. Although this biased targeting of Vα3 rearrangements toward 3'Jα gene segments is not consistent with diminished recombinase activity at the TCRα locus, our results demonstrate that noncore Rag1 regions control formation of the TCRα gene repertoire during Vα-to-Jα recombination in DP thymocytes.

Because positive selection of αβ TCRs expressed on DP thymocytes shapes TCRβ repertoire (22–27, 35) and Jα repertoire is altered in DP thymocytes of Rag1^{1/C} mice (Fig. 5A), we investigated the impact of noncore Rag1 regions on Vβ repertoire during DP-to-SP thymocyte development. We detected an ∼4-fold lower frequencies of Vβ6* TRCPβ^{high} SP thymocytes and higher frequencies of Vβ6* (∼2-fold more), Vβ8* (∼2-fold more), and Vβ14* (∼5-fold more) TRCPβ^{high} SP cells in Rag1^{1/C} mice relative to WT mice (Fig. 5B, 5C). Considering that we observed ∼2-fold lower frequencies of Vβ10+ DP thymocytes and equivalent frequencies of Vβ6+ DP cells in Rag1^{1/C} mice relative to WT mice (Fig. 3E, 3F), these data indicate that positive selection of DP thymocytes alters Vβ repertoire differently in αβ TCRs of Rag1^{1/C} and WT mice. To confirm this notion, we calculated the ratios of the frequencies of cells expressing Vβ5, Vβ6, Vβ8, or Vβ14 on TRCPβ^{high} versus TRCPβ^{int} thymocytes in Rag1^{1/C} and WT mice (Fig. 5D). This analysis indicated enhanced selection for Vβ6*, Vβ8*, Vβ10+, and Vβ14+ cells and increased selection against Vβ5* cells during positive selection of DP thymocytes in Rag1^{1/C} mice as compared with WT mice (Fig. 5D). Collectively, these data show that loss of noncore Rag1 regions leads to abnormal changes in Vβ repertoire during αβ TCR selection.

Replacement of the Vβ14 RSS with the more efficient 3'Dβ1 RSS increases Vβ14 recombination frequency and rescues αβ T cell development in Rag1^{1/C} mice

We have shown that Rag1^{1/C} mice exhibit impaired DN3-to-DP thymocyte development caused by reduced levels of Vβ-to-DβJββ rearrangements, and that this impaired Vβ recombination is associated with altered recombination frequencies of individual Vβ segments. A similar phenotype in Rag2^{1/C} mice led to the hypothesis that conserved sequence features of Vβ/Vα RSSs that make them inefficient relative to 3'Dβ and Vα/Vβ/Vα RSSs...
contributes to impaired V-to-DJ recombination on the background of a diminished recombinase (10). In support of this model, the Vβ8 RSS more closely resembles the consensus Vc/Vα/Vβ/Vα RSS than the canonical Vβ RSSs, and Vβ8 exhibits a 1.8-fold higher rearrangement and expression in Rag1/Ccnd3/Ccnd3−/− and Rag1/Ccnd3/Ccnd3−/− mice compared with WT mice (10) (Fig. 3E, 3F). Additionally, the Vβ5 and Vβ10 RSSs more closely resemble the consensus Vβ/VH RSS than canonical Vβ RSSs, and Vβ5 and Vβ10 exhibit ~2-fold lower rearrangement and expression in Rag1/Ccnd3/Ccnd3−/− mice relative to WT mice (Fig. 3E, 3F). However, the Vβ14 RSS more closely resembles the Vβ/VH RSS consensus than other Vβ RSSs, but Vβ14 exhibits 2-fold higher rearrangement and expression in Rag1/Ccnd3/Ccnd3−/− mice relative to WT mice (Fig. 3E, 3F). This latter finding suggests that factors in addition to Vβ RSS inefficiency may contribute the altered relative frequency of Vβ segments in Rag1/Ccnd3/Ccnd3−/− mice.

To directly test the prediction that Vβ RSS inefficiency causes impaired Vβ rearrangement in Rag1/Ccnd3/Ccnd3−/− mice, we sought to determine whether replacement of a Vβ RSS with a 3’Dβ or Vc/Vα/Vβ/Vα RSS increases recombination and expression of this Vβ to the same extent in Rag1/Ccnd3/Ccnd3−/− and WT mice. For this purpose, we established mice with gene-targeted replacement of the Vβ14 RSS with the 3’Dβ1 RSS on an otherwise normal TCRβ allele (Vβ143’Dβ1RSS+/+ mice) because we previously showed in chimeric mice that this RSS replacement increases the frequencies of Vβ14 rearrangement and expression on a WT RAG background (21). We then made and analyzed in parallel Vβ143’Dβ1RSS+/+ and Rag1/Ccnd3/Ccnd3−/− mice, as well as control Rag1/Ccnd3/Ccnd3−/− and WT mice. Consistent with our previous findings (21), we detected an ~20-fold increase in the frequency of Vβ14+ TCRβ−/− DP thymocytes in Vβ143’Dβ1RSS+/+ mice relative to WT mice (Fig. 6A, 6B). We observed a similar ~20-fold increase in the frequency of Vβ14+ TCRβ−/− DP thymocytes in Rag1/Ccnd3/Ccnd3−/− mice relative to Rag1/Ccnd3/Ccnd3−/− mice (Fig. 6A, 6B), revealing that sequence of the RSS attached to Vβ14 is a major determinant of the frequency of Vβ14 rearrangement and expression in both WT and Rag1/Ccnd3/Ccnd3−/− mice. Therefore, our data provide direct support for the decade-old model that sequence features of Vβ/VH

FIGURE 4. TCRβ recombination and TCRβ-mediated Ccnd3-dependent DN thymocyte proliferative expansion cooperate in αβ T cell development. (A) Representative flow cytometry analysis of CD4 and CD8 expression on total thymocytes isolated from littermate or age-matched Ccnd3/Ccnd3 mice relative to WT mice (n = 4) and Rag1/Ccnd3/Ccnd3−/− (n = 5) mice. The average number of total thymocytes for each genotype is indicated in parentheses, and the frequencies of cells in the depicted TCRβ−/− gate are indicated. (B) Graph showing the average numbers of DN and DP thymocytes from Ccnd3/Ccnd3 and Rag1/Ccnd3/Ccnd3−/− mice. Error bars are SEM. *p < 0.05. (A and B) This experiment was independently performed three times, each time on at least one mouse of each genotype. (C) Representative flow cytometry analysis of TCRβ and Vβ10 or Vβ14 expression on total thymocytes isolated from littermate or age-matched Ccnd3/Ccnd3 mice. The frequencies of cells in the depicted TCRβ−/− gate are indicated. (D) Graph showing the average frequencies of TCRβ−/− cells expressing Vβ10 or Vβ14 in thymocytes from Ccnd3/Ccnd3−/− (n = 4) and Rag1/Ccnd3/Ccnd3−/− (n = 5) mice. Error bars are SEM. *p < 0.05. (C and D) This experiment was independently performed three times, each time on at least one mouse of each genotype.
RSSs that render them less efficient than 3′DJb and Vc/Vα/Jα/Vβ/Vα RSSs contribute to impaired V rearrangements on the background of a defective recombinase as in Rag2C/C and Rag1C/C mice. However, we still detected ~2-fold greater frequencies of Vb14 rearrangements and expression in Rag1C/C > Vb14DJ9RSS+ mice as compared with Vb14DJ9RSS/+ mice (Fig. 6A, 6B) as we observed between Rag1C/C and WT mice (Fig. 3E, 3F), highlighting that noncore Rag1 regions may modulate Vβ usage in Vβ rearrangements through mechanisms in addition to their function with inefficient Vβ RSSs.

Because the 3′DJb1 RSS replacement drives increased Vb14 rearrangement in Rag1C/C DN3 cells, we next investigated the effect of this single RSS replacement on the impaired DN3-to-DP thymocyte development of Rag1C/C mice. We found ~5-fold greater numbers of total and DP thymocytes in Rag1C/C > Vb14DJ9RSS+ mice as compared with Rag1C/C mice (Fig. 6C), with no significant differences in total and DP cell numbers or the percentages of thymocytes at the DP stage among Rag1C/C > Vb14DJ9RSS+, Vb143DJ1RSS+, and WT mice (Fig. 6D, 6E). Whereas DN cell numbers were similar among all four genotypes (Fig. 6D), the percentages of thymocytes at the DN stage were lower in Rag1C/C > Vb143DJ1RSS+ mice compared with Rag1C/C mice (Fig. 6C) but higher in Rag1C/C > Vb143DJ1RSS+ mice relative to Vb143DJ1RSS+ and WT mice (Fig. 6C). Consistent with these observations, we detected increased numbers of DN4 cells and an increased percentage of DN thymocytes at the DN4 stage in Rag1C/C > Vb143DJ1RSS+ mice as compared with Rag1C/C mice (Fig. 6E), but lower values for these two parameters in Rag1C/C > Vb143DJ1RSS+ mice relative to Vb143DJ1RSS+ and WT mice (Fig. 6E). These data indicate that replacement of the RSS of only 1 of the 20 functional Vβ segments with the more efficient 3′DJb1 RSS partially rescues DN3-to-DN4 thymocyte development and completely rescues DN-to-DP thymocyte development in Rag1C/C mice. Therefore, we conclude that noncore Rag1 regions drive Vβ T cell development by overcoming inherent inefficiencies of Vβ RSSs to promote Vβ rearrangements.

Discussion

We have taken a genetic approach with Rag1C/C mice to determine how noncore RAG1 regions promote TCRβ gene assembly and αβ T cell development. The reduced levels of DB-to-JB and Vβ-to-DJbβ recombination in DN3 thymocytes and impaired DN-to-DP thymocyte development from accumulation of cells at the DN3 stage of Rag1C/C mice could arise from decreased efficiency of TCRβ recombination and/or survival of DN3 thymocytes in response to RAG DSBs. Our observation that expression of the anti-apoptotic BCL2 protein does not substantially enhance the survival of Rag1C/C DN3 cells and only minimally rescues thymocyte development in Rag1C/C mice indicates that preventing death of DN3 cells in response to RAG DSBs is not the major means through which noncore RAG1 regions promote TCRβ recombination and αβ T cell development. Our finding that a preassembled DJbβ complex partially rescues thymocyte development in Rag1C/C mice confirms the hypothesis that noncore RAG1 regions promote αβ T cell development in part by stimulating DB-to-JB recombination (11), and it also provides unequivocal evidence that noncore RAG1 regions drive Vβ-to-DJbβ recombination. Combined with these data, our observation that a preassembled TCRβ gene completely rescues thymocyte development in Rag1C/C mice proves that noncore RAG1 regions promote TCRβ gene assembly and αβ T cell development by stimulating Vβ recombination. However, because DB-to-JB recombination precedes Vβ-to-DJbβ recombination, our data cannot differentiate the relative contribution of noncore RAG1 regions in each of these steps of TCRβ gene assembly in driving αβ T cell development.

In contrast to Rag1C/C mice, which we have shown exhibit substantial defects in both DB-to-JB and Vβ-to-DJbβ recombination and altered Vβ repertoire, Rag2C/C mice have a major impairment in only Vβ-to-DJbβ recombination that is associated with altered Vβ usage (10). Because Rag2C/C mice also have a reduction in overall recombinase activity in thymocytes but no apparent defect in TCRα recombination, it was proposed that the interaction of a diminished recombinase with RSS sequences unique to Vβs may cause the Vβ-to-DJbβ recombination defect, rather than loss of specific properties of the noncore RAG2 region in stimulating Vβ rearrangements (10). Our findings that a preassembled DJbβ complex or functional TCRβ gene each rescues αβ T cell development to some extent cannot distinguish between impaired TCRβ gene assembly caused by di-
The average number of total thymocytes for each genotype is indicated in parentheses, and the frequencies of DN, DP, CD4+ SP, and CD8+ SP cell populations showing the average frequencies of TCR development in Rag1C/C mice. (c) Flow cytometry analysis of c-Kit and CD25 expression on DN thymocytes isolated from littermate or age-matched V0.05. (e) Graph showing the average number of DN and DP cells from mice of the indicated genotypes. Error bars are SEM. *p < 0.05. This experiment was independently performed three times, each time on at least one mouse of each genotype.

FIGURE 6. Replacement of the Vβ14 RSS with the more efficient 3′D81 RSS increases Vβ14 recombination frequency and rescues αβ T cell development in Rag1C/C mice. (A) Representative flow cytometry analysis of TCRβ and Vβ14 expression on total thymocytes isolated from littermate or age-matched Vβ14+D81RSS+ (n = 3) and Rag1C/C Vβ14+D1RSS+ (n = 3) mice. The frequencies of cells in the depicted TCRβ+ gates are indicated. (B) Graph showing the average frequencies of TCRβ+ cells expressing Vβ14 in thymocytes from mice of the indicated genotypes. *p < 0.05. (A and B) This experiment was independently performed three times, each time on at least one mouse of each genotype. (C) Representative flow cytometry analysis of CD4 and CD8 expression on total thymocytes isolated from littermate or age-matched Vβ14+D81RSS+ (n = 3) and Rag1C/C Vβ14+D1RSS+ (n = 3) mice. The average number of total thymocytes for each genotype is indicated in parentheses, and the frequencies of DN, DP, CD4+ SP, and CD8+ SP cell populations are indicated on the plots. (D) Graph showing the average number of DN and DP cells from mice of the indicated genotypes. Error bars are SEM. *p < 0.05. (C and D) This experiment was independently performed three times, each time on at least one mouse of each genotype. (E) Representative flow cytometry analysis of c-Kit and CD25 expression on DN thymocytes isolated from littermate or age-matched Vβ14+D81RSS+ (n = 3) and Rag1C/C Vβ14+D1RSS+ (n = 3) mice with the frequencies of DN1, DN2, DN3, and DN4 cell populations indicated. (F) Graph showing the average numbers of DN3 and DN4 cells from mice of the indicated genotypes. Error bars are SEM. *p < 0.05. This experiment was independently performed three times on a total of three mice of each genotype. (C and D) This experiment was independently performed three times, each time on at least one mouse of each genotype.

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diminished recombinase activity versus the loss of specific functions of Rag1 noncore regions that stimulate Dβ-to-Jβ and/or Vβ-to-DJβ recombination. However, our demonstration that replacement of the Vβ14 RSS with the more efficient 3′D81 RSS dramatically increases the frequency of Vβ14 rearrangement in Rag1C/C thymocytes and partially rescues thymocyte development proves that sequence features of the Vβ14 RSS that distinguish it from the 3′D81 RSS contribute to impaired Vβ-to-DJβ recombination in Rag1C/C mice. Notably, in the presence or absence of this Vβ14 RSS replacement, we have shown that loss of noncore Rag1 regions results in increased Vβ14 rearrangements, indicating that the interaction of a defective recombinase with inefficient Vβ RSSs is not the sole cause of impaired Vβ recombination in Rag1C/C mice. Furthermore, our discovery that Vα3 rearrangements are biased toward 3′Ja segments in DP thymocytes of Rag1C/C mice is opposite the prior observation of increased usage of 5′Ja segments in mice with diminished recombinase activity in DP thymocytes (34). Therefore, we conclude that specific activities of noncore RAG1 regions modulate Vβ and Ja gene segment utilization during V(D)J recombination.

How might noncore RAG1 regions modulate Vβ and Ja usage? Both Vβ-to-DJβ and Vα-to-Ja recombination are driven by TCR enhancers and promoters that enable RAG2 binding to histone H3 proteins trimethylated on lysine 4 located over DJ segments and also render DJ RSSs nucleosome-free and accessible for RAG cleavage (36–38). The RAG1/RAG2 proteins in DJ recombination centers capture accessible V RSSs to mediate V-to-(D)J rearrangements (39). Changes in locus topology that place V gene segments near DJ segments are likely critical for the formation of synaptic complexes between V and DJ RSSs (39, 40), which may dissociate more often than they are cleaved by RAG to assemble V(D)J joins (21, 39). The relative usage of individual Vβ and Ja gene segments in rearrangements should be determined by accessibility of their RSSs, frequencies at which they enter synaptic complexes, efficiencies of their flanking RSSs, and physical forces that destabilize precleavage synaptic complexes (21, 39, 41).
Because differences in TCRα locus conformation have been observed between DP cells that lack Rag1 protein or express a cleavage-defective mutant Rag1 protein (42), noncore Rag1 regions may promote or preserve changes in TCRβ and TCRα locus topology that, respectively, help assemble or stabilize synaptic complexes for some Vβ and Jα segments over others. The noncore Rag1 regions contain a really interesting new gene (RING) domain with E3 ubiquitin ligase activity (43, 44) and other sequences that bind the RING E3 ubiquitin ligase complex VprBP/DDB1/Cul4A/Roc1 (45). The RING1 domain catalyzes histone H3 monoubiquitylation, which reduces core histone binding and promotes transcription of genes (46–48), and RAG1 mutations that reduce this E3 ubiquitin ligase activity impair V(D)J recombination (46, 47, 49). Although targets of VprBP/DDB1/Cul4A/Roc1 in the context of V(D)J recombination are not known, deletion of VprBP initiating in pro-B cells causes a block in B cell development at this stage that correlates with greater impairment of Vα1-DJα recombination than Dα1-Jα recombination (45). Differences in accessibility and transcription among germline Vβ and Jα gene segments are observed in DN and DP thymocytes, respectively (13, 20, 50), whereas changes in TCRα locus topology control Jα usage in Vαo-Jα rearrangements (51). Thus, noncore Rag1 regions could modulate Vβ and Jα usage by ubiquitylating H3 histones or other proteins to remove nucleosomes from some Vβ and Jα RSSs more than others and/or similarly function to modify proteins that organize TCRβ and TCRα locus topology. The increased utilization of 3′Jα segments in Rag1C/C mice suggests that noncore Rag1 regions might inhibit successive Vαo-Jα rearrangements. Although transcription from Vα promoters stimulates such rearrangements to shape TCRα repertoire (50), mechanisms that suppress Vαo-Jα recombination and provide DP thymocytes time to express and select TCRα genes are not known. Considering that the RAG1 RING domain promotes RAG1 polyubiquitylation (43), and ubiquitylation targets proteins for proteasomal degradation and changes in cellular localization, our data are consistent with a regulatory role of the Rag1 noncore region in constraining Rag1 protein expression to ensure time for assembled TCRα genes to be expressed and selected before initiation of further Vαo-Jα recombination. Another possibility is that RAG1-mediated ubiquitylation of histones cooperates with ATM-dependent histone H2A ubiquitylation (52) to transiently inhibit accessibility and transcription of germline Vα gene segments in response to RAG DSBS induced during Vαo-Jα rearrangements. However, the biased utilization of 3′Jα segments in Rag1C/C mice could result from ability of the core-RAG1 recombine to directly recognize downstream Jα segments without proceeding through the normal 5′ to 3′ gradient of replacement rearrangements. Another possibility is that the altered TCRβ repertoire in Rag1C/C mice results in fewer αβ TCRs that can undergo positive selection, increasing successive TCRα rearrangements and resulting in increased representation of downstream Jα segments. In this later scenario, the altered Jα usage of Rag1C/C mice would be the result of altered Rag1 activity at the TCRβ locus rather than at the TCRα locus.

Generation, selection, and expression of a broad αβ TCR and IgH/IgL repertoires are critical for effective adaptive immunity. RAG1 or Rag2 mutations that diminish RAG endonuclease activity cause inefficient TCR gene assembly, reduced numbers of αβ T cells beyond the progenitor stage, restricted TCRβ and TCRα repertoires, and immunodeficiency (6–9), revealing that efficient V(D)J recombination is critical for generation of αβ TCR diversity. TCRβ-mediated, Ccnd3-dependent thymocyte expansion contributes to αβ TCR diversity by allowing multiple changes for each unique TCRβ gene assembled in DN thymocyte to be selected with a different TCRα-chain in DP cells (18). Our demonstration that impaired TCRβ recombination is the predominant cause of reduced DP thymocyte numbers in Rag1C/C mice and that DP cell numbers are lower in Rag1C/C and Ccnd3−/− mice than in Rag1C/C and Ccnd3−/− mice indicates that TCRβ recombination efficiency and TCRβ-mediated thymocyte expansion cooperate to generate αβ TCR diversity. A polymorphism in the human VαA2 RSS impairs VαA2 recombination efficiency, reduces VαA2 representation in the Vα repertoire, and confers susceptibility to Haemophilus influenzae (53), revealing that alterations to the mechanisms that control utilization of individual V gene segments can have deleterious consequences. Our study indicates that, similar to loss of the noncore Rag2 region (10), absence of noncore Rag1 regions decreases Vβ-to-Jβ recombination and alters the primary Vβ repertoire generated during Vβo-Jβ recombination. Our study also reveals that the loss of noncore Rag1 regions leads to abnormal changes in Vβ repertoire during αβ TCR selection, which could arise from altered Jα usage during Vαo-Jα rearrangements and/or impaired signaling of gene expression changes in response to RAG DSBS in DP thymocytes. Regardless, our findings suggest that noncore regions of Rag1 may have coevolved with the noncore Rag2 domain and Vβ sequences that bind the RING E3 ubiquitin ligase complex (RING) domain with E3 ubiquitin ligase activity (43, 44) and locus topology that, respectively, help assemble or stabilize synapses for each unique TCRα ensemble.

Disclosures

The authors have no financial conflicts of interest.

References
