

HyperIgE in hypomorphic recombination-activating gene defects

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Increased immunoglobulin-E (IgE) levels associated with eosinophilia represent a common finding observed in Omenn syndrome, a severe immunodeficiency caused by decreased V(D)J recombination, leading to restricted T- and B-cell receptor repertoire. V(D)J recombination is initiated by the lymphoid-restricted recombination-activating gene (*RAG*) recombinases. The lack of *RAG* proteins causes a block in lymphocyte differentiation, resulting in T^B severe combined immunodeficiency. Conversely, hypomorphic mutations allow the generation of few T and B cells, leading to a spectrum of immunological phenotypes, in which immunodeficiency associates to inflammation, immune dysregulation, and autoimmunity. Elevated IgE levels are frequently observed in hypomorphic *RAG* patients. Here, we describe the role of *RAG* genes in lymphocyte differentiation and maintenance of immune tolerance.

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Introduction

RAG1 and RAG2 structures

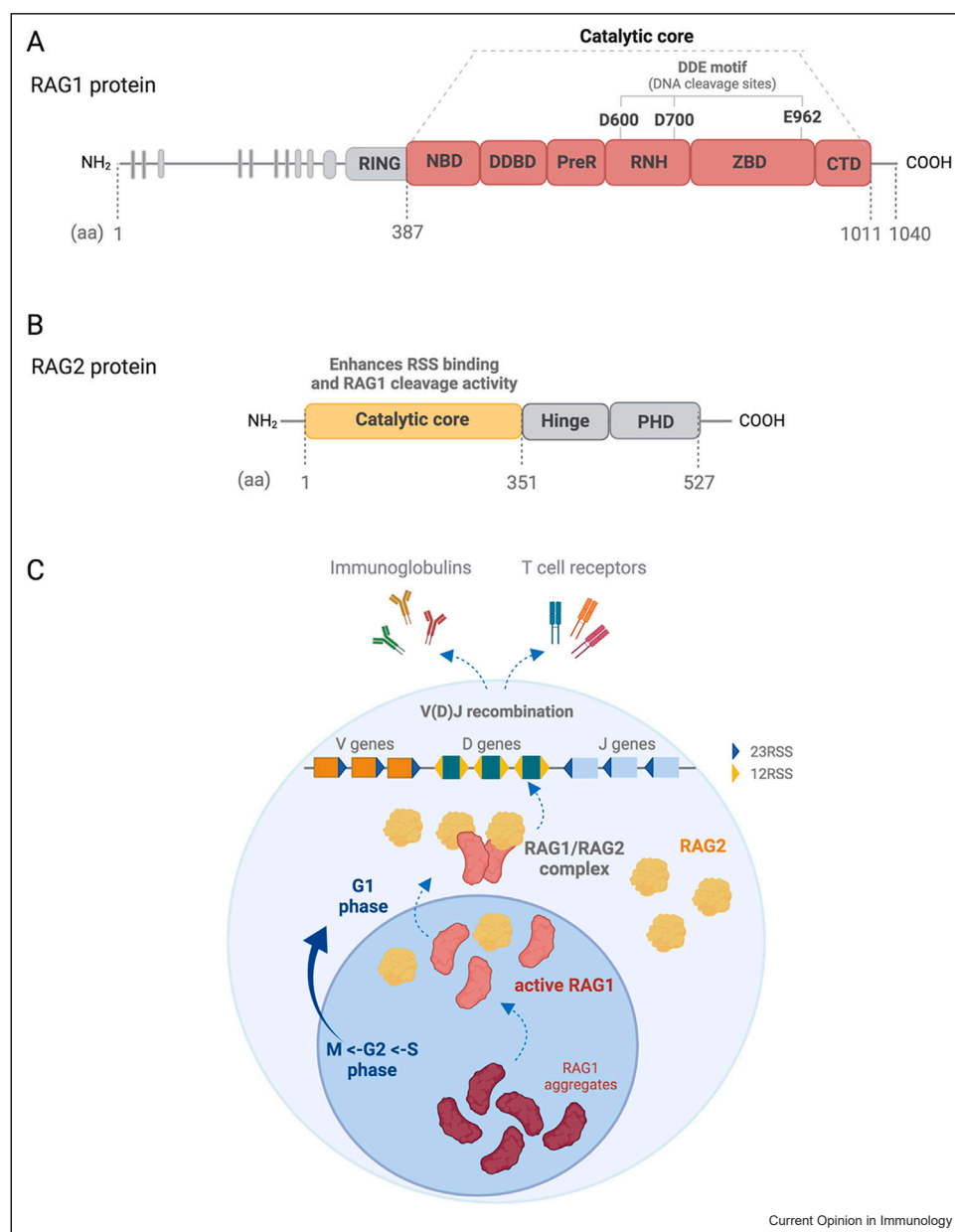
V(D)J recombination is the process responsible for the rearrangement of variable (V), diversity (D), and joining (J) coding elements of the immunoglobulin (Ig) and

T-cell receptor (TCR) genes. Master regulators of V(D)J recombination are recombination-activating gene (*RAG*) recombinases [1]. RAG1 and RAG2 proteins form a heterotetrameric RAG recombinase complex that cleaves V(D)J gene segments adjacent to recombination signal sequences (RSS), composed of conserved heptamer and nonamer elements separated by either 12- or 23-bp spacer DNA (termed 12 RSS and 23 RSS, respectively) (Figure 1). Evolved from a transposon ancestor, RAG1 protein includes the catalytic site for DNA cleavage and requires interaction with RAG2 [2] (Figure 1a). The human RAG1 contains 1043 residues. The catalytic core spans the amino acids (aa) 387-1011 and includes various domains. The N-terminus enables RAG1 to localize in the nucleolus and translocate outside in G1 phase. The highly conserved catalytic residues D600, D708, and E9626 form the so-called DDE motif, which has a critical role in the catalysis and formation of recombination intermediates.

Human RAG2 is composed of 527 aa with the minimal region required for the catalytic activity spanning 1-351 aa [3] (Figure 1b). The core region also enhances RSS binding and RAG1 interaction. RAG2 does not directly contribute to DNA cleavage but enhances RAG1 cleavage activity, being responsible for stabilization and specificity to the interaction of RAG1 with substrate DNA as well as contributing to protein-protein interaction within the RAG tetramer [4].

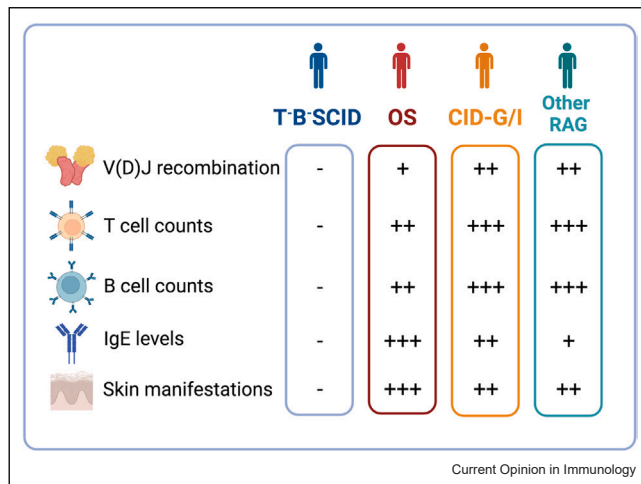
Post-transcriptional modification provides an additional control level, modulating catalytic activity during the cell cycle. V(D)J recombination occurs in G1 phase when classical nonhomologous end joining (NHEJ) dominates double-strand process. In the mouse system, Schatz's team demonstrated that RAG1 is stored in nucleoli and escapes in response to G1 arrest [5]. These data have been recently confirmed in the human counterpart, in which RAG1 protein aggregates in the nucleus, leading to very low levels of recombination activity in the absence of RAG2. In G1 phase, RAG2 is highly expressed and interacts with RAG1-disrupting aggregates and activating robust recombinase activity [6] (Figure 1c). This 'jail-break' model offers an additional level of restriction to NHEJ-driven repair and allows to further understand the

Figure 1



RAG proteins and V(D)J recombination. **(a)** Human RAG1 contains 1043 residues, with a catalytic core (aa 387-1011) that includes different domains: the nonamer-binding domain (NBD, aa 394-460), a dimerization and DNA-binding domain (DDBD, aa 461-517), pre-RNaseH (preR, aa 518-590), catalytic RNaseH (RNH, aa 591-721), a zinc-binding domain (ZBD, aa 722-965), and the carboxy-terminal domain (CTD, aa 966-1008). The highly conserved DDE motif (D600, D708, and E9626 residues) is also reported. The noncore N-terminal region is characterized by basic motifs followed by a C3HC4 RING finger domain required for RAG1 subnuclear localization and normal levels of V(D)J recombination, respectively. **(b)** Human RAG2 protein is characterized by 527 aa, with a catalytic region spanning from 1 to 351. The noncore region of RAG2 regulates chromatin accessibility and recombination activity throughout the plant homeodomain (PHD). **(c)** V(D)J recombination leads to the assembly of V, D, and J segments of Ig and TCR. RAG recombinase is a heterotetrameric RAG1/RAG2 complex that recognizes, binds, and cleaves V(D)J gene segments adjacent to RSS, which are composed of a conserved heptamer, a spacer of either 12 or 23 base pairs, and a conserved nonamer. The 'jailbreak' model has been proposed as a regulatory mechanism restricting RAG recombination activity to the G1 phase of the cell cycle. Robust V(D)J recombination is induced by RAG2-mediated disruption of RAG1 aggregates [6]. (a-c) Pictures created with BioRender.com.

Figure 2



Clinical manifestations in RAG patients. Overview of the spectrum of clinical manifestations caused by RAG mutations according to the levels of V(D)J recombination activity, lymphocyte counts, IgE, and skin manifestations. Picture created with BioRender.com.

effect of missense mutations mapping in the interacting RAG1/RAG2 domains on recombination activity [3].

Recombination-activating gene and lymphocyte differentiation

RAG genes are the first players of a sophisticated process, leading to the generation of polyclonal T and B cells with enormous antigen receptor diversity. *RAG* expression is tightly controlled during lymphocyte differentiation to mediate TCR and B-cell receptor (BCR) gene assembly [6,7], while ectopic *RAG*-mediated cleavage can lead to oncogenic translocations [8]. In thymocytes, two waves of expression have been described, with the first one during the double-negative stage (CD4⁻CD8⁻) to catalyze TCR β , TCR γ , and TCR δ rearrangements. In T cells that have rearranged $\gamma\delta$ and β chains, *RAG* genes are downregulated and re-expressed during progression toward double-positive (DP) stage (CD4⁺CD8⁺) to sustain TCR α gene recombination [9]. Two waves of expression also occur during pro-B and pre-B-cell progression supporting Ig heavy and light chain (Igk or Ig λ) rearrangements, respectively [10].

RAG expression regulation is the result of the interplay between lineage-specific transcription factors and chromatin architecture [11]. Several transcription factors are involved in lymphocyte differentiation and among these, E2A and HEB establish T-cell identity, while Pax5 and EBF1 are required for B-cell differentiation [12–14]. The antisilencer element (ASE) located 73 kb upstream *RAG2* promoter is an essential *cis*-regulator of *RAG* gene transcription in DP thymocytes engaging *RAG1* and

RAG2 promoters and forming an active chromatin hub. Additional transcriptional regulators such as GATA3, Runx1, and SATB1 contribute to the assembly of transcriptionally active conformation of the *RAG* locus [11].

In vitro experiments have demonstrated the co-occupancy of the intracellular domain of Notch and E2A in *RAG* T-cell enhancer (R-Ten) region, while *in vivo* models carrying deletions in R-Ten and in the B-cell-specific enhancers R1B and R2B, showed a block in thymocytes and B-cell development, respectively [15]. Overall, this evidence highlights the hierarchical assembly of transcriptionally active chromatin hub containing ASE and *RAG* promoters that confer complex activity during T- and B-cell differentiation and raise relevant questions on the feasibility and safety of novel therapeutic strategies based on gene correction approaches [16].

Recombination-activating gene deficiency and clinical manifestations

Identification of *RAG* mutations has been instrumental to dissect the role played by various domains of *RAG* proteins [17]. Biallelic null mutations that abrogate recombination activity have been described in patients with T- and B-cell absence and severe combined immunodeficiency (T⁻B⁻ SCID). Higher frequency of immature NK cells than healthy infants with higher amount of perforin and enhanced degranulation activity have been reported and might contribute to control viral infections [18,19]. T⁻B⁻ SCID patients present a severe disease, which is fatal if untreated by hematopoietic stem cell transplantation (HSCT). However, *RAG* mutations also lead to a spectrum of clinical presentations, including Omenn syndrome (OS), atypical SCID (AS), delayed-onset combined immunodeficiency with granulomas, and/or autoimmunity [20] (Figure 2). These forms have been reported in patients carrying mutations that allowed some residual recombination activity and resulted in the generation of oligoclonal and autoreactive T cells [21]. Novel recombination activity assays complemented by molecular and biochemical analysis have highlighted the correlation between protein level and severity of the immune and clinical phenotypes [17,22]. However, environmental factors, including microbiota and infections, contribute to the heterogeneity of the clinical phenotype adding a layer of complexity to the effect of genetic determinants [23,24].

Omenn syndrome

Hypomorphic mutations or frameshift mutations at the N-terminus of *RAG1* gene have been identified and characterized in OS patients [17,25]. This immunodeficiency is characterized by life-threatening infections, erythroderma, lymphadenopathy, hepatosplenomegaly, eosinophilia, and severe hypogammaglobulinemia with elevated IgE [26,27]. Oligoclonal and activated T lymphocytes infiltrate target organs, such as gut and skin, contributing to

tissue damage and leading to chronic diarrhea and severe erythroderma [23,28]. These clinical signs have similar features resembling maternal T-cell engraftment. OS pathogenesis has been extensively dissected by *in vitro* and *in vivo* studies. *In vitro* recombination assay is a useful tool to define the correlation between the level of residual recombination activity and the clinical phenotype [29,30]. Low levels of recombination activity lead to the generation of a more restricted T- and B-cell repertoire with severe and early-onset clinical manifestations, typically observed in OS patients. Defective negative selection process in OS thymus leads to the egression of very few autoreactive thymocytes containing central cysteine and increased frequency of hydrophobic aminoacids at positions 6–7 of the CDR3 [31,32]. Dramatic reduction of T cells expressing the products of distal TCR alpha variable (TRAV) genes, including TRAV1-2, which are part of the semi-invariant TCR expressed by human mucosa-associated invariant T cells, was observed [33]. Importantly, innate-like lymphocytes recognize microbial vitamin-B metabolites and have a role in preventing infection and immune dysregulation [34]. The restricted composition of TCR repertoire associated with activated T-cell phenotype contributes to predispose to the development of autoimmune signs [1].

Elevated IgE levels in the presence of barely detectable circulating B cells are commonly observed in OS. Unexpectedly, despite pre-B-cell-stage block, enzyme-linked immunospot assay performed in bone marrow OS samples showed Ig-secreting cells and autoantibodies [35]. This impaired B-cell maturation affects downstream antibody functionality by impacting IgH isotype preference. Immature B cells preferentially switch to IgE through a mechanism involving increased direct class switch recombination from C μ to C ϵ - [36]. This preferential bias accounts for the elevated levels of IgE in various primary immunodeficiencies characterized by perturbed B-cell development. Impaired receptor editing, the process responsible for purging autoreactive B cells during the early stages of maturation, and defective B-cell tolerance checkpoint from transitional to mature B cells provides an additional level of immune dysregulation, accounting for the presence of autoreactive B cells and autoantibodies [35,37]. Moreover, elevated B-cell activating factor (BAFF) produced by myeloid cells promoting survival of transitional B cells enriches the peripheral B pool of self-reactive B cells with preferential expansion of clones using IGHV4-34, IGHV3-9, IGH4-31, and IGHV3-23 genes that are usually associated with autoreactive B cells [20,38].

Mouse models forward back

More insights come from the analysis of *in vivo* models carrying hypomorphic mutations identified in OS patients. Two mouse models, the memory mutant mouse (MM) carrying R972Q *Rag1* mutation and the codon

change R229Q in *Rag2* gene, were generated showing elevated serum IgE levels and skin manifestations ranging from skin redness (MM mouse) to severe erythroderma (the *Rag2*^{R229Q} mouse) [39]. In both mutants, the low recombination activity resulted in partial block of T- and B-cell differentiation and increased Th2 cytokine profile. However, an independent mouse model successfully generated by CRISPR/Cas gene editing technology and carrying the same codon change of the MM model showed signs more similar to AS with detectable B cells and presence of IgM and IgG [40]. Thus, the *Rag2*^{R229Q} mouse represents a more reliable model to dissect immune dysregulation mechanisms in OS and confirmed data from human OS thymi [41,42]. Defects in thymic epithelial component maturation and defective AIRE expression by mature thymic epithelial cells (TECs) result in defective cross-talk between TECs and thymocytes, with the egression of very few lymphocytes able to overcome the differentiation block. Perturbed B-cell tolerance characterized by impaired receptor editing, elevated BAFF cytokine with survival of immature B cells and plasmacells, and consequent production of autoantibodies were also described in mouse models. Importantly, *in vivo* BAFF-R blockade reduced serum levels of nucleic acid-specific autoantibodies and significantly ameliorated inflammatory tissue damage, providing evidence of the contribution of B cells to immune dysregulation in hypomorphic RAG defects [35,37].

Availability of murine models was also instrumental to dissect the pathophysiology of cutaneous and gastrointestinal clinical manifestations [24]. Analysis of gut mucosa from *Rag2*^{R229Q} mice shows signs of intestinal disease. Defective IgA production contributes to mucosal barrier leakage and favors bacterial translocation, as shown by elevated levels of circulating lipopolysaccharide. Activated T cells express high levels of gut homing receptor markers (CCR9) and infiltrate lamina propria with increased expression of Th1 and Th17 cytokines. Analysis of the microbiota showed reduction in richness and diversity with an enrichment of the Proteobacteria phylum, a proteobacteria observed in chronic inflammatory conditions. The role of dysbiosis was highlighted by transfer fecal experiments from wild-type animals with dramatic decrease of inflammation. Conversely, fecal transfer from OS mice led to the development of inflammatory cells enriched in Th1/Th17 signature. Furthermore, antibiotic treatment-depleting microflora showed a dramatic impact not only on gut inflammation, but even on systemic signs with dramatic reduction in Th1/Th17 cytokines and lymphocyte infiltration in kidney, lung, and liver. Strikingly, the levels of IgE dramatically dropped [28].

Disruption of gut barrier homeostasis has an impact on tissue integrity [43]. The skin, primary interface with the environment, may be the target of pathogen invasions

and physical damage, particularly in case of lymphopenic conditions. Consistently, the cutaneous hallmark of OS is severe exfoliative dermatitis with consequent life-threatening bacterial infections. Increased expression of skin homing receptors was found in the mouse model and confirmed in OS patients, who showed increased cutaneous lymphocyte antigen and CCR4 in peripheral T cells together with high CCL17/CCL22, IL-13, and IL-5 serum levels. Unexpectedly, our group found increased levels of IFN- γ , CXCL9, and CXCL10 in the serum of OS patients and murine counterparts, supporting the hypothesis that OS is not a pure Th2-driven disease but rather an inflammatory disease. Analysis of cutaneous flora showed decreased abundance with loss of protective bacteria [23]. Based on these results, breaches in skin and gut barriers may favor systemic bacterial translocation with ectopic T-cell activation and induction of tissue homing receptors.

Overall, these data highlight the impact of dysbiosis and tolerance break in determining the clinical signs of OS and how commensal antigens and gut-derived pathogens contribute to systemic signs, including elevated IgE and skin manifestations.

Atypical severe combined immunodeficiency and combined immunodeficiency with granulomas, and/or autoimmunity forms

Molecular analysis allowed to identify novel forms of SCID during infancy. These patients present low count of circulating autologous T cells but higher than 300 cells/mL and a reduced functionality. Symptoms in common with OS are skin manifestations and elevated IgE, but in the absence of severe lymphoproliferation and hepatosplenomegaly typically observed in OS. A condition characterized by $\gamma\delta$ T-cell expansion, frequent occurrence of autoimmune cytopenia, disseminated cytomegalovirus infection, and increased risk to develop Epstein–Barr virus lymphoproliferation has been described [44]. Importantly, these forms may associate more frequently to cytopenia and autoimmune hemolytic anemia. Vasculitis may be the first sign of disease (50%) in some patients and accompanied by the presence of autoantibodies against cytokines (IFN- α , - ω , and IL-12) [45]. AS leads to early death, unless treated by HSCT. Of note, AS and OS represent the majority of RAG deficiencies.

Other SCID forms are characterized by later clinical onset and show a broad spectrum of autoimmune manifestations (myasthenia gravis, Guillain–Barré syndrome, vitiligo psoriasis, cytopenias, and vasculitis) and/or inflammatory manifestations such as granulomatous lesions in various organs and sterile chronic multifocal osteomyelitis [20]. These forms usually do not present elevated IgE and analysis of V(D)J indicates sufficient recombination activity that can sustain a broader

repertoire [20]. AS knock-in models (F971L, R972Q, and R972W *Rag1*) corresponding to mutations identified in RAG1 patients were generated by Notarangelo's team, showing incomplete T- and B-cell block, increased IgE in the presence of profound hypogammaglobulinemia (R972W), partial Ig production and antigen response, and broad spectrum of autoantibodies (R972Q). These models are important tools that help to dissect disease pathophysiology and test novel therapeutic strategies.

Conclusions

HyperIgE associated with clinical signs of immune dysregulation, including skin and gut cellular infiltration, is the hallmark of OS. Molecular and cellular analyses have indicated to which extent low levels of recombination activity shape TCR and BCR repertoires and influence the clinical signs. However, biochemical results can partially explain the immune phenotype, while other players act as disease modifiers, explaining the severity of autoimmune manifestations and inflammatory signs. In conclusion, evaluation of the various contributors is instrumental to indicate therapeutic strategies and undertake new paths toward a precision medicine approach.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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