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RAG deficiencies: Recent advances in disease pathogenesis and novel therapeutic approaches

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Abstract

The RAG1 and RAG2 proteins initiate the process of V(D)J recombination and therefore play an essential role in adaptive immunity. While null mutations in the *RAG* genes cause severe combined immune deficiency with lack of T and B cells ($T^{-}B^{-}$ SCID) and susceptibility to life-threatening, early-onset infections, studies in humans and mice have demonstrated that hypomorphic *RAG* mutations are associated with defects of central and peripheral tolerance resulting in immune dysregulation. In this review, we provide an overview of the extended spectrum of RAG deficiencies and their associated clinical and immunological phenotypes in humans. We discuss recent advances in the mechanisms that control RAG expression and function, the effects of perturbed RAG activity on lymphoid development and immune homeostasis, and propose novel approaches to correct this group of disorders.

Keywords

Genotype-phenotype correlation; Immune tolerance; RAG; VDJ recombination

Introduction

Adaptive immune responses require expression of receptors capable of specific antigen recognition. In jawed vertebrates, this function is accomplished through V(D)J recombination, in which the joining of variable (V), diversity (D), and joining (J) coding elements of the immunoglobulin and TCR genes, results in the generation of a diversified repertoire of antigen-specific TCRs and BCRs. The RAG1 and RAG2 proteins initiate the V(D)J recombination process [1–3]. Two molecules of RAG1 and two molecules of RAG2 form a heterotetramer that binds to recombination signal sequences (RSSs) flanking the V, D, and J genes and introduces double-strand breaks in the DNA, which are subsequentially repaired by the nonhomologous end-joining DNA repair pathway by joining the gene segments to form coding joints and the cleaved RSSs and intervening sequences to form

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signal joints [3]. RAG1 and RAG2 have distinct roles. In particular, RAG1 contains the RNaseH fold catalytic domain and regions that make direct contact with the RSS, and is responsible for the enzymatic activity of the RAG complex [4, 5], while RAG2 promotes DNA binding by scanning the genome for epigenetic signatures characterized by histone 3 trimethylated at lysine 4 (H3K4me3) and facilitates cleavage functions of RAG1 [6]. The lack of either RAG1 or RAG2 protein causes a very similar phenotype with complete lack of mature T and B cells resulting in a nonleaky severe combined immunodeficient (T^-B^-SCID) phenotype, both in mice and humans [7–9]. However, the phenotype associated with *RAG* mutations is much broader, with hypomorphic variants allowing various degrees of T- and B-cell development. Furthermore, while susceptibility to severe infections is the main manifestation of SCID, immune dysregulation is a prominent feature in patients with hypomorphic *RAG* variants [10]. Altogether, these data demonstrate that RAG1 and RAG2 are essential not only to control T- and B-cell development, but that their function is also required to maintain immune tolerance.

Role of RAG proteins in V(D)J recombination: Recent developments

RAG1 and *RAG2* expression is tightly controlled in a cell type and developmental stage-specific manner [11]. There are two waves of *RAG* gene expression in developing T and B lymphocytes [12]. In mice, the first wave of *Rag* expression in developing thymocytes occurs at the $CD4^-CD8^-$ double-negative (DN) stage to catalyze *Tcrg, Tcrb*, and *Tcrd* gene rearrangement. Thymocytes that successfully rearrange *Tcrg* and *Tcrd* to become γδ T cells will then permanently shutdown *Rag* expression, while cells that successfully rearrange *Tcrb* transiently downregulate *Rag* expression and differentiate along the αβ T-cell pathway. The second wave of *Rag* expression occurs at the $CD4^+CD8^+$ double-positive (DP) stage, and promotes rearrangements at the *Tcra* locus. If an αβ TCR heterodimer is successfully expressed, and DP thymocytes receives sufficient TCR signaling allowing positive selection, *Rag* gene expression is permanently downregulated [13–15]. Single-cell RNA sequencing studies in human thymic samples have confirmed that *RAG* gene expression is restricted to DN cells and DP cells; moreover, they have shown that RAG expression starts increasing from the late proliferative phase of each cell stage and peaks during quiescence, demonstrating that cell proliferation and V(D)J recombination need to be uncoupled [16].

Similarly to thymocytes, *Rag* gene expression in mice occurs at two distinct stages also during B-cell development, with expression in pro-B cells supporting *Igh* rearrangement and expression in pre-B cells supporting *Igk* and *Igl* rearrangement [17].

The mechanisms controlling *Rag* gene expression have been the object of extensive investigation. The *Rag1* and *Rag2* genes are juxtaposed on chromosome 11p12 and 2p in humans and mice, respectively, are separated by only approximately 8 kb and their promoters are distant approximately 25 kb. *Rag1* and *Rag2* are convergently transcribed, and for both the entire protein is encoded by a single exon. Transcriptional regulation of the *Rag* genes involves cis-regulatory elements that differ between B and T cells [11]; some of these elements have been recently identified in a mouse atlas of open chromatin regions [18]. In mice, *Rag* expression at DN stage of T-cell development is induced by sequences within 10 kb upstream of *Rag2* [19], while in DP thymocytes, when *Rag* expression reaches the

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highest levels, it is controlled by a distal antisilencer element (ASE), placed 73 kb upstream of *Rag2* [20], which acts as an enhancer through direct interaction with the *Rag1* and *Rag2* promoters [21]. In B cells, *Rag* promoters are controlled by proximal, distal, and *Erag* enhancers upstream of *Rag2* [22, 23]. Various transcription factors are also involved in *Rag* gene expression. In particular, by interacting with the *Erag* enhancer, Foxo1 serves as a positive regulator [24], while Gfi1b, Ebf1, and c-Myb act as negative regulators [25–28]. Other transcription factors binding directly to the *Rag2* promoter include PAX5, MYB, SP1, LEF1, NF-Y, C/EBP GATA3, and NFATc1 [29], whereas NF-Y and NFAT bind directly to the *Rag1* promoter [30, 31]. Furthermore, GATA3 and E2A are critical regulators of the ASE, while Runx1 and E2A regulate the *Rag1* promoter [32]. The hierarchical assembly of a transcriptionally active chromatin hub containing the ASE and *Rag* promoters, with *Rag2* recruitment and expression secondary to the assembly of a functional *ASE-Rag1* framework, has been demonstrated in DP thymocytes [32]. Additionally, it has been shown that downregulation of *Rag* expression in DP thymocytes depends on IKAROS and occurs with disassembly of the chromatin hub in the *Rag* locus [32]. Negative regulation of *RAG1* expression by IKAROS has been confirmed also in human cells [33].

The broad phenotype of RAG deficiency and genotype–phenotype correlation in humans

Severe combined immune deficiency—RAG deficiency may manifest with a broad range of phenotypes that reflect the degree of adaptive immunity compromise which in turn depends, at least in part, on the residual recombination activity of the mutant RAG protein(s). Null mutations that abrogate recombination activity are responsible for SCID, with lack or severe reduction of T and B cells and agammaglobulinemia; however, development of NK cells is not affected. SCID patients are prone to life-threatening and opportunistic infections since early in life [34, 35]. Immunization with live agents may lead to severe infection and should be strictly avoided [36]. SCID is fatal within the first few years of life unless immune reconstitution is achieved with hematopoietic stem cell transplantation (HSCT) [37, 38]. As compared to patients with typical SCID, those with atypical (or "leaky") forms of SCID have partially preserved T-cell count and/or function, although markedly lower than age-matched referenced values [39]. In these patients, CMV infection is often associated with expansion of γδ T cells and autoimmune cytopenias [40, 41].

Omenn syndrome—Omenn syndrome (OS) represents another severe presentation of RAG deficiency [34, 35]. It is characterized by prominent immune dysregulation, with erythroderma, lymphadenopathy, hepatosplenomegaly, eosinophilia, and elevated IgE (but otherwise low IgG, IgA, and IgM). T-lymphocytic infiltrates are present in target organs, contributing to tissue damage. Similar features may also be seen in patients with SCID and with maternal T-cell engraftment [42]. However, the T cells of OS patients are autologous and oligoclonal [43]. Both atypical SCID and OS are sustained by hypomorphic *RAG* variants with minimal levels of recombination activity [44–46] permitting a low number of successful recombination events. Frameshift mutations in the N-terminus of RAG1 are a common cause of atypical SCID and OS [47]. However, it should be noted that these variants with very low recombination activity may also cause typical SCID [48], in particular, when the stochastic process of V(D)J recombination does not yield productive

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rearrangements. Both OS and atypical SCID are fatal conditions, unless treated with HSCT. Availability of universal newborn screening for SCID has permitted to define that in the United States RAG deficiency (including both RAG1 and RAG2 defects) accounted for 11% of all cases of SCID and as many as 41.2% of cases of atypical SCID and OS observed in the period 2010-2018 [49].

Combined and common variable immunodeficiencies—The extended phenotype associated with hypomorphic *RAG* mutations includes also combined immune deficiency (CID), which is often associated with granulomas and/or autoimmunity (CID-G/AI) [34, 50–52]. At variance with SCID and OS, CID may have a delayed clinical onset, and may allow survival into adulthood, reflecting milder impairment of adaptive immunity [34]. In particular, T- and B-cell counts as well as immunoglobulin serum levels are often partially preserved [34], consistent with significantly higher levels of recombination activity supported by the *RAG* mutations, as compared to SCID and OS [34, 45, 46]. In some patients, the disease may manifest as CD4 lymphopenia [53]. Progressive development of hypogammaglobulinemia associated with normal or even increased B-cell count but reduced proportion of switched memory B cells has been also reported [54, 55]. These forms of late-onset CID may mimic common variable immunodeficiency (CVID) [55, 56]. However, at variance with CVID, there is a consistent numerical defect of naïve $CD4_+$ T cells. As in patients with atypical forms of SCID, viral infections, in particular due to CMV or EBV may act as modifiers of the immunological phenotype, inducing lymphoproliferation. Clonotypic T-cell expansions in patients with hypomorphic *RAG* mutations may also reflect immune tolerance breakdown, with self-reactive T cells infiltrating target organs. Whatever the mechanism, this chronic lymphoproliferation may occasionally progress to clonal disease [57, 58].

Treatment-refractory autoimmune cytopenias are a common manifestation of RAG-dependent CID [51]. Vasculitis and organ-specific autoimmunity have been also frequently reported [34, 51, 59]. Noncaseating granulomas involving the skin or any other organ often develop starting in late childhood or young adulthood. Presence of the rubella virus vaccine (RVV) strain has been documented in several RAG-deficient patients with CID [60]. Immunohistochemical analysis of the granulomatous lesions has revealed that the RV is harbored by M2 macrophages [61]. While immunization with live viral vaccines is contraindicated in patients with immunodeficiency, patients with RAG-dependent CID may not be diagnosed early enough to avoid exposure to the RVV.

Although there are significant immunological differences among SCID, OS, and CID, all conditions are characterized by a reduced proportion of naïve T cells as a result of impaired V(D)J recombination [34]. Furthermore, high-throughput sequencing of the TCR repertoire reveals restrictions and clonotypic expansions [43], and molecular signatures of self-reactivity are often present in the TCR CDR3 [62]. Finally, a unique feature of V(D)J recombination at the *TCRA* locus is that it happens in waves, with initial rearrangements involving the most downstream *TRAV* and most upstream *TRAJ* genes. If such rearrangements are nonproductive, thymocytes proceed with further rearrangements that ultimately involve the most upstream *TRAV* and most downstream *TRAJ* genes. In patients with hypomorphic *RAG* mutations, there is reduced frequency of rearrangements involving

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these distal *TCRA* elements, including *TRAV1-2*, which is expressed as invariant TCR-α chain by mucosa-associated invariant T cells. The low number of mucosa-associated invariant T cell detected in patients with hypomorphic *RAG* mutations further contributes to the immune dysregulation of this condition [63].

Animal models of RAG deficiency

Genetically engineered mouse models have been used to define the effects of individual gene disruption observed in patients with RAG deficiencies. Because of the inability of developing lymphocytes to initiate V(D)J recombination, *Rag1* and *Rag2* knockout mice show a block in B- and T-cell development at $B220_+CD43_+$ pro-B-cell stage (Fig. 1) and DN stage (Fig. 2), respectively, and complete absence of mature T and B cells [7, 8]. To confirm that this phenotype is not influenced by homologous recombination with introduction of a Neo cassette in embryonic stem cells, $Rag1^{-/-}$ and $Rag2^{-/-}$ pigs and $Rag2^{-/-}$ mice have been generated by TALEN and CRISPR/Cas9 gene editing, respectively [64, 65], confirming that RAG deficiency does not impact viability and development, but causes loss of mature T and B cells with preserved generation of NK cells.

Murine models for Omenn syndrome

The identification of patients with "leaky" forms of disease associated with hypomorphic *RAG* mutations has prompted the development of knockin mouse models, with the aim of better defining the pathophysiology of these conditions. Two mouse models of OS due to *Rag* mutations were reported at the same time. The first model, homozygous for a hypomorphic *Rag1* variant (R972Q), was the result of a spontaneous mutation and was identified because of the presence of a high proportion of memory $CD8_+$ T cells in the absence of any infection, and for this reason called the memory mutant (MM) mouse [66]. The R972Q Rag1 mutant protein was shown to have decreased recombination activity (about 15% of WT protein). Consistently, MM mice manifested a partial block in T- and B-cell development, reduced numbers of mature T and B cells and abnormal architecture of lymphoid tissues. Additionally, MM mice showed skin redness, hepatosplenomegaly, eosinophilia, oligoclonal T cells, and elevated serum IgE level. Their peripheral T cells showed an activated/memory phenotype and increased production of Th2 cytokines. Because of these features, the MM mouse was proposed to represent a model of OS. However, when another mouse model carrying the R972Q *Rag1* variant was generated by CRISPR/Cas gene editing [67], it became clear that only a proportion of these mice have the typical changes of OS. Additionally, $Rag1^{R972Q/R972Q}$ mice have detectable B cells, produce IgM and IgG and a broad range of autoantibodies. In this regard, the R972Q *Rag1* mutant mice are more a model of CID than of OS. The second mouse model of OS recapitulates more faithfully the patients' phenotype, and was generated by introducing the R229Q *Rag2* mutation previously found in patients with OS [68]. $Rag2^{R229Q/R229Q}$ mice present oligoclonal T cells, absence of circulating B cells and eosinophilia. In addition, they show infiltration of T cells and eosinophils in gut and skin, causing diarrhea, alopecia, and erythroderma in a significant proportion of the animals. These cell infiltrates contrast with the severe lymphopenia of thymus, spleen, and lymph nodes. In the thymus, the cortex/ medulla demarcation is lost, and the medullary compartment is severely reduced, with lack of autoimmune regulator (Aire) expression and low number of Treg and NKT cells [68–70].

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A detailed analysis of the B-cell compartment revealed that, despite a severe B-cell developmental block, the *Rag2*$^{R229Q/R229Q}$ mice present a normal or even enlarged compartment of immunoglobulin-secreting cells (ISC), sustained by elevated levels of T cell-derived homeostatic and effector cytokines [71]. Defects of receptor editing and elevated levels of BAFF were found to contribute to the B-cell tolerance breakdown and the development of B cells producing high affinity autoantibodies against target organs, highlighting a previously unidentified role for B cells in the pathogenesis of immune dysregulation of OS [71]. Similar to OS patients, *Rag2*$^{R229Q/R229Q}$ mice have prominent signs of gut inflammation, with $CD4_+$ Th1/Th17 cells infiltrates in the lamina propria [72]. T cells are directly responsible for these inflammatory changes, since their adoptive transfer in immunodeficient hosts is sufficient to cause colitis, and conversely depletion of $CD4_+$ cells ameliorates bowel inflammation. In these mice, the lack of B cells and consequent reduced amounts of fecal IgA and IgM results in mucosal abnormalities, with enhanced absorption of microbial products and altered composition of the microbiota, which in turn sustain the Th1/Th17 response [72]. Accordingly, use of broad-spectrum antibiotics reduced T-cell infiltrates and improved gut inflammation. Very recently, the study of *Rag2*$^{R229Q/R229Q}$ mice has provided support for an interplay between gut and skin in promoting cutaneous inflammation, a hallmark of OS [73]. In particular, a high proportion of peripheral T cells from *Rag2*$^{R229Q/R229Q}$ mice express skin-homing receptors, and the skin of these mice is infiltrated by Th1 cells. Similar abnormalities have been also detected in OS patients. Disruption of the epidermal barrier in *Rag2*$^{R229Q/R229Q}$ mice is associated with increased microbial challenge. Finally, induction of more severe colitis precipitates enhanced skin infiltration with Th1 cells [73].

Murine models for atypical SCID and combined immune deficiency

Another knockin mouse model was generated to investigate the functional consequences of a *Rag1* point mutation (S723C) that does not affect proficiency of DNA double-strand break formation but rather causes defects in postcleavage complex formation and end-joining in vitro [74]. This mutant manifested impaired T- and B-cell development, reminiscent of atypical SCID in humans. Analysis of thymic architecture and composition revealed severe reduction in size and cellularity affecting in particular medullary thymic epithelial cells, markedly reduced expression of Aire and several tissue restricted antigens (TRAs) and reduced number of Treg cells [75]. In the BM, there was a severe but incomplete block in development at the pro-B-cell stage, with virtually absent immature and mature recirculating B cells. In the periphery, profound B-cell lymphopenia was contrasted by residual levels of immunoglobulins and accumulation of ISCs [76]. While responses to T-independent and T-dependent antigens and production of high affinity antibodies were severely impaired in these mice, high amounts of low-affinity self-reactive antibodies were detected. These signs of autoimmunity were associated with defects of receptor editing in the BM and increased serum BAFF levels. Along with significant lymphocytic infiltrates in peripheral tissues, these data support the notion that hypomorphic *Rag* mutations are often associated with immune dysregulation, as a result of defects of central and peripheral T- and B-cell tolerance. In addition, *Rag1*-S723C mice presented aberrant DNA double-strand breaks within rearranging loci that, on a p53 mutant background, resulted in predisposition to development of thymic lymphomas [74].

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More recently, CRISPR/Cas gene editing has been used to introduce in mice hypomorphic mutations in the C-terminus domain (CTD) of RAG1 (F971L, R972Q, R972W) corresponding to those (F974L, R975Q, R975W) identified in patients with variable phenotypic severity, ranging from atypical SCID to CID-G/AI [67]. All three mouse models showed an incomplete block of T- and B-cell development. Thymic defects were more pronounced in R972W mice, with a marked reduction in size and cellularity and a nearly complete block at the $CD25^{+}CD44^{-}$ DN3 stage. By contrast, a significant proportion of DP and of $CD4^{+}$ and $CD8^{+}$ single-positive (SP) cells were detected in R972Q and F971L mice. In the BM, B-cell development was partially blocked at the pro-B and pre-B stages, with increased leakiness in particular in the R972Q mouse [67]. Reduced number of T and B cells were present in the periphery. In particular, T cells mainly expressed an activated/memory phenotype, although a significant fraction of naive cells was present in both F971L and R972Q mice. Partial preservation of immunoglobulin levels and response in particular to T-independent antigen were documented in R972Q and F971L mice, while profound hypogammaglobulinemia but increased serum IgE were present in R972W mice [67]. Broad-spectrum autoantibodies were documented, in particular in the R972Q mouse model, similarly to patients with hypomorphic mutations in the *RAG1* CTD domain [52]. However, anticytokine antibodies often found in patients were not observed in these mice. Finally, high-throughput sequencing revealed marked skewing of *Ighv* and *Trbv* gene usage in early lymphoid progenitors, with an increased frequency of productive rearrangements. Along with increased apoptosis of B-cell progenitors, these data suggest preferential survival of lymphoid progenitors that successfully rearrange TCR and immunoglobulin genes on the first allele. More recent analysis of the *Trb* repertoire in splenic T cells isolated from R972W and F971L mutant mice has revealed an enrichment in hydrophobic aminoacid doublets at the apex of the CDR3 in the TCR-β chain [62], a biomarker of self-reactivity [77], similarly to what found in patients with hypomorphic *RAG* mutations [43, 62].

Impact of RAG gene defects on lymphoid development and immune tolerance: Recent developments

B- and T-cell development in patients with RAG deficiencies—The rarity of the condition, difficulty in obtaining BM and thymus samples, and lack of reliable techniques to model human T-cell development in vitro have hampered the fine characterization of T- and B-cell developmental defects in patients with RAG deficiencies.

Noordzij et al. performed flow cytometric analysis of BM samples obtained from 5 RAG-deficient SCID pediatric patients and demonstrated a block at pro-B and pre-BI cell stage [78] (Fig. 1). However, the relative frequencies of pro-B and pre-BI cells varied, even for patients carrying the same mutation. These results were confirmed by Cassani et al. [71], who analyzed four patients with OS and reported that on average 92% of the B cells in the BM of these patients were represented by pro-B and pre-BI cells. Importantly, some ISCs escape developmental blocks as indicated by ELISpot analysis showing the presence of cells producing either IgM or IgG/A in patients with OS [71]. Ijspert et al. analyzed the BM of 22 patients with similar truncating mutations in the N-terminus of RAG1 [48]. In spite of the variable phenotype (SCID, OS, CID), these patients manifested a similar early block in B-cell development (Fig. 1), with a limited number of pre-BII cells being present only in

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patients with CID and in one of the OS patients. These data indicate that the nature and functional activity of the mutant alleles are not the only determinants of RAG deficiency phenotypic heterogeneity.

The study of early human T-cell development in patients with RAG deficiency has proven to be even more challenging, because of the very limited access to thymic samples from patients. Taking advantage of the development of methods allowing to model human T-cell development from induced pluripotent stem cells (iPSCs) [79], Braueret al. studied T-cell differentiation of human iPSCs generated from three *RAG1*-mutated patients with different clinical and immunologic phenotypes (SCID and OS) [80]. In all cases, T-cell development progressed up to immature CD4$^+$CD8$^-$ (immature single positive) and DP stages, but only transiently, as these cells were quickly lost over time, due to the accumulation of double-strand breaks and decreased cell survival [80]. The analysis of sorted ISP and DP cells revealed an overall restriction of TCR rearrangements, preferential usage of certain *V* and *J* genes, and skewing of CDR3 length. Some of these features had been previously noted in peripheral T cells from patients with OS [81]. Similar results were obtained by Themeli et al. who studied T-cell differentiation of iPSCs generated from a patient carrying a *RAG2* null mutation, with only few cells reaching the DP stage [82]. In this study, the impaired T-cell differentiation was accompanied by an increase in CD7$^-$CD56$^+$CD33$^+$ NK-like cells.

More recently, a very efficient protocol for in vitro T-cell differentiation of CD34$^+$ cells in a 3D artificial thymic organoid (ATO) system was devised by Seet et al. [83]. Using this technique, we analyzed the T-cell differentiation of BM or mobilized peripheral blood CD34$^+$ cells from six patients with *RAG* mutations, manifesting with a wide range of phenotypes [84]. Surprisingly, we observed generation of DP cells in all cases, including samples isolated from T$^-$B$^-$SCID patients, in contrast with what predicted by *Rag* knockout mouse models in which T-cell development is blocked at DN stage [7, 8] (Fig. 2). However, we noticed that severity of the disease inversely correlated with the persistence of DP cells in the ATOs, with more severe presentations associated with loss of these cells in culture over time [84]. Additionally, in vitro production of CD3$^-$CD56$^+$ NK cells was markedly increased in patients with severe *RAG* mutations, suggesting a more pronounced unbalance versus NK cell lineage development in T$^-$B$^-$SCID patients [84].

In parallel to our study, Bifsha et al. published a report proposing an alternative approach to study in vitro T-cell differentiation [85]. In this method, CD34$^+$ cells isolated from limited amount of peripheral blood are cocultured in a 3D system with OP9-DLL4 cells used in earlier methods to support T-cell differentiation [86]. In their report, they compared the T-cell differentiation potential of CD34$^+$ cells from a patient with a null *RAG2* mutation (the same used in [84]) and an OS patient carrying a hypomorphic *RAG1* mutation, and observed a different ability of the cells from these two patients to reach the DP stage, with the patient carrying the null mutation showing a more severe defect. Overall, these data indicate that RAG function is not strictly required to reach the DP stage of T-cell differentiation in humans; however, in the absence of normal RAG, survival of these cells is not sustained, presumably due also to illegitimate rearrangements.

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RAG mutations impact establishment of central tolerance in humans—Besides directly impacting on T-cell development, *RAG* mutations have important consequences also on the composition and maturation of TECs and on the establishment of central tolerance. Cavadini et al. were the first ones to demonstrate lack of AIRE and TRA expression in the thymi of two patients with OS [87]. These results have been subsequently confirmed in other patients with hypomorphic *RAG* mutations including CID-G/AI [88, 89]. Abnormalities of TEC development and maturation and impairment of AIRE expression in the thymus of patients with RAG deficiency arise from defects of cross-talk between developing T cells and stromal cells. For patients with hypomorphic mutations permitting residual development of T cells, defective AIRE expression in the thymus translates into escape of self-reactive T cells from negative selection and impaired generation of Treg cells, thereby favoring autoimmune manifestations. Similar to patients with Autoimmune Polyendocrinopathy-Enteropathy-Candidiasis-Ectodermal Dystrophy (APECED), a condition due to germline *AIRE* mutations, patients with hypomorphic *RAG* variants often produce autoantibodies targeting IFN-α, IFN-ω, IL-17, and IL-22, and some lung antigens such as KCNRG and BPIFB1 [52, 89]. In patients with RAG defects, the occurrence of neutralizing antitype I IFN antibodies is associated with a high risk of severe varicella infection [52, 55], whereas anti-KCNRG and anti-BPIFB1 antibodies may induce or aggravate development of chronic lung disease [90].

Treatment of RAG deficiency: Current status and perspectives

Allogeneic HSCT represents the only curative option currently available for RAG deficiency. For patients with SCID or OS, optimal results (>80% 10-year survival) are achieved with HSCT from HLA-matched related donors; transplantation from haploidentical donors is associated with a very high rate of graft rejection (up to 75%) if no conditioning regimen is used [37]. Furthermore, HSCT from donors other than HLA-matched siblings is often associated with poorer T- and B-cell reconstitution especially when no conditioning or nonmyeloablative regimens are used. These results may reflect competition between mutant and donor-derived cells which in humans may extend up to DP cells in the thymus and pre-B cells in the BM. On the other hand, it is well established that use of myeloablative regimens for HSCT carries the risk of increased toxicity and reduced survival. An alternative strategy would be to use nongenotoxic agents to deplete the endogenous HSCs. A clinical trial using humanized anti-CD117 monoclonal antibody is currently underway, and early data show safety and efficacy [91]. Alternatively, immunotoxins depleting $CD45_{+}$ cells would have the advantage of purging also lymphoid progenitor and mature cells, which can mediate immune dysregulation especially in patients with CID-G/AI. In this condition, HSCT with conventional conditioning regimens has led to suboptimal results, with 38% of the patients being deceased at a median age of 8.4 years [51]. Preclinical data using anti-CD45-Saporin in mice with null or hypomorphic *Rag1* mutations indicate that this agent can efficiently deplete the stem cell and lymphoid compartment, and promote robust and durable engraftment of donor-derived cells [92].

Gene therapy represents an alternative approach to correct RAG deficiency. Initial attempts with retroviral vectors have revealed the risk of lymphoma [93]. Depending on the nature of the promoter used, variable efficiency of T- and B-cell reconstitution has been achieved in

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Rag1$^{-/-}$ mice reconstituted with lentiviral vectors [94]. Partial immune reconstitution has been associated with development of lymphocytic infiltrates in target organs and autoantibodies [95]. More favorable results have been obtained in *Rag2*$^{-/-}$ mice [96]; however, when a similar approach was attempted in *Rag2*$^{R229Q/R229Q}$ mice, the T- and B-cell count of treated animals remained lower than normal [97].

Gene editing represents an alternative strategy, with the advantage of preserving endogenous regulation of gene expression. The size of the *RAG1* cDNA may represent a potential limitation, because of difficulties in packaging the entire donor template in adenoassociated viral vectors; however, because of the smaller length of *RAG2* cDNA, RAG2 deficiency is an attractive target for gene editing, and proof of concept has been provided in patient-derived iPSCs, with restoration of in vitro T-cell development and generation of a diverse TCR repertoire in gene edited cells [82, 98].

Conclusions

In recent years, important advances have been made in the characterization of the molecular mechanisms that control *RAG1* and *RAG2* gene expression. With the progressive implementation of whole genome sequencing, it is likely that improved knowledge of the molecular mechanisms controlling *RAG* expression may help interpret the significance of genetic variants affecting regulatory regions of the *RAG* genes in patients with defects of lymphoid development. Definition of the crystal structure of the RAG1/RAG2 complex may help predict the functional consequences of naturally occurring *RAG* variants [4, 5], which can be confirmed using in vitro recombination assays [45, 46]. However, observations in patients have shown that genotype–phenotype correlation in RAG deficiency is not absolute [48], possibly reflecting both the semistochastic nature of V(D)J recombination and the impact of epigenetic factors. An intriguing hypothesis that has yet to be tested is that because of the heterotetrameric nature of the RAG complex, double heterozygosity for hypomorphic variants in the *RAG* genes may limit the number of fully functional RAG complexes and interfere with normal T- and B-cell development.

Novel cellular and animal models, and more powerful methods to study human T-cell development have offered important insights into the broad immunological and clinical phenotype associated with *RAG* mutations (Fig. 3). In particular, studies in patients and mice have shown that hypomorphic *RAG* mutations that allow for subnormal levels of T- and B-cell development have important consequences on lymphostromal cross-talk in the thymus and on mechanisms of T-cell tolerance [75, 87], as well as on the efficiency of receptor editing in the BM and on survival of self-reactive peripheral B cells [71, 76]. Altogether, these abnormalities explain the high frequency of autoimmune manifestations observed in humans and mice with hypomorphic *RAG* mutations [51, 52, 71]. Use of novel in vitro methods to investigate human T-cell development has revealed important differences between humans and mice with *RAG* mutations [84], emphasizing the importance of performing such studies directly on human tissues and/or cells whenever possible. Finally, there is hope that novel therapeutic approaches based on use of nongenotoxic conditioning regimens and gene editing (Fig. 3) may improve outcome for patients with RAG deficiency

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including those with severe immune dysregulation. If successful, these strategies may inform similar developments also for patients with other forms of inborn errors of immunity.

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Abbreviations:

AIRE	autoimmune regulator
ASE	antisilencer element
ATO	artificial thymic organoid
CID	combined immune deficiency
CID-G/AI	combined immune deficiency with granulomas and/or autoimmunity
CTD	C-terminus domain
CVID	common variable immune deficiency
DN	double negative
DP	double positive
HSCT	hematopoietic stem cell transplantation
iPSC	induced pluripotent stem cells
ISC	immunoglobulin-secreting cell
ISP	immature single-positive
MM	memory mutant
OS	Omenn syndrome
RAG	Recombinase Activating Gene
RSS	recombination signal sequence
RVV	rubella virus vaccine
SCID	severe combined immune deficiency
TRAs	tissue restricted antigens

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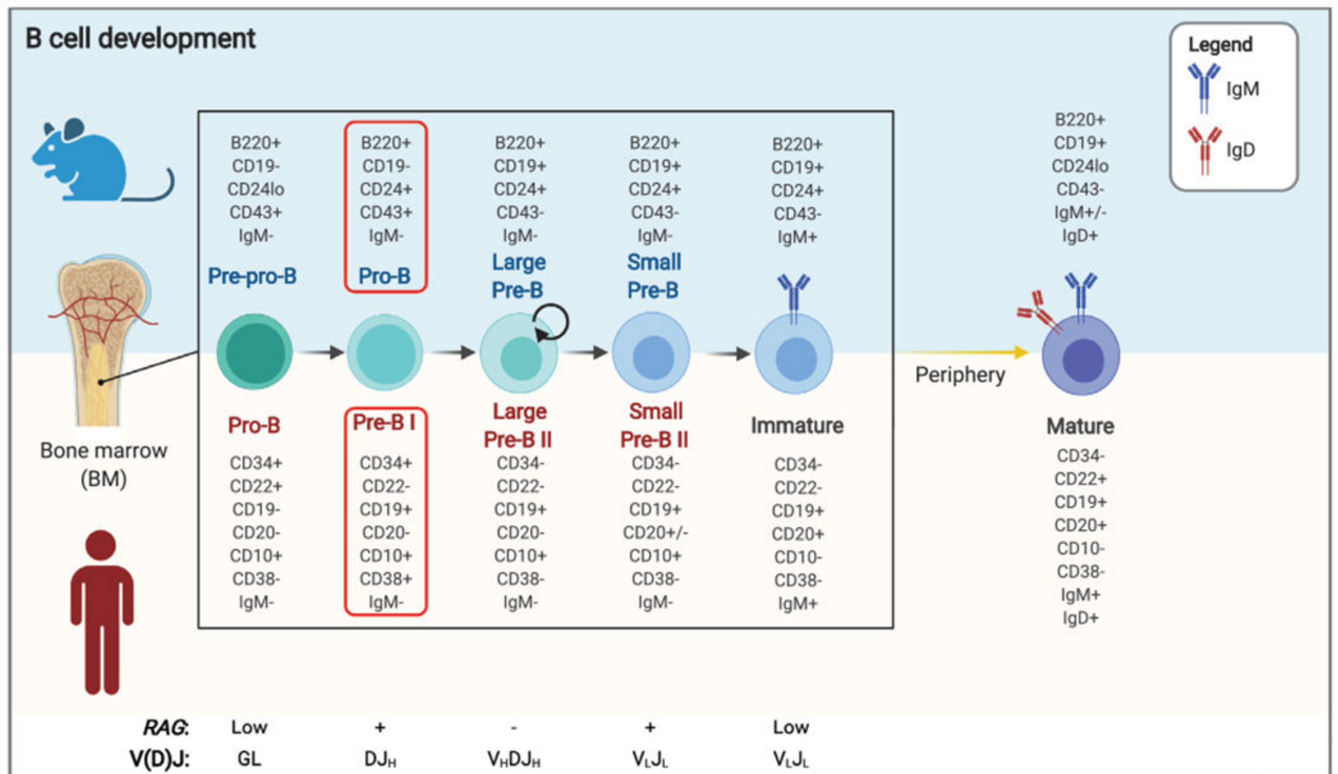

Figure 1.

B-cell development block in RAG-deficient patients and mice. Schematic representation of B-cell development in mice and humans, with the list of surface markers that can be used to identify various stages of maturation. Stages at which *RAG* genes are expressed, and the concurrent nature of VDJ recombination products detected at each stage, are indicated at the bottom. The red boxes indicate the stage (pro-B and pre-B I) at which development is blocked in RAG-deficient mice and patients. Proliferating cells are indicated by a circular arrow. GL, germline.

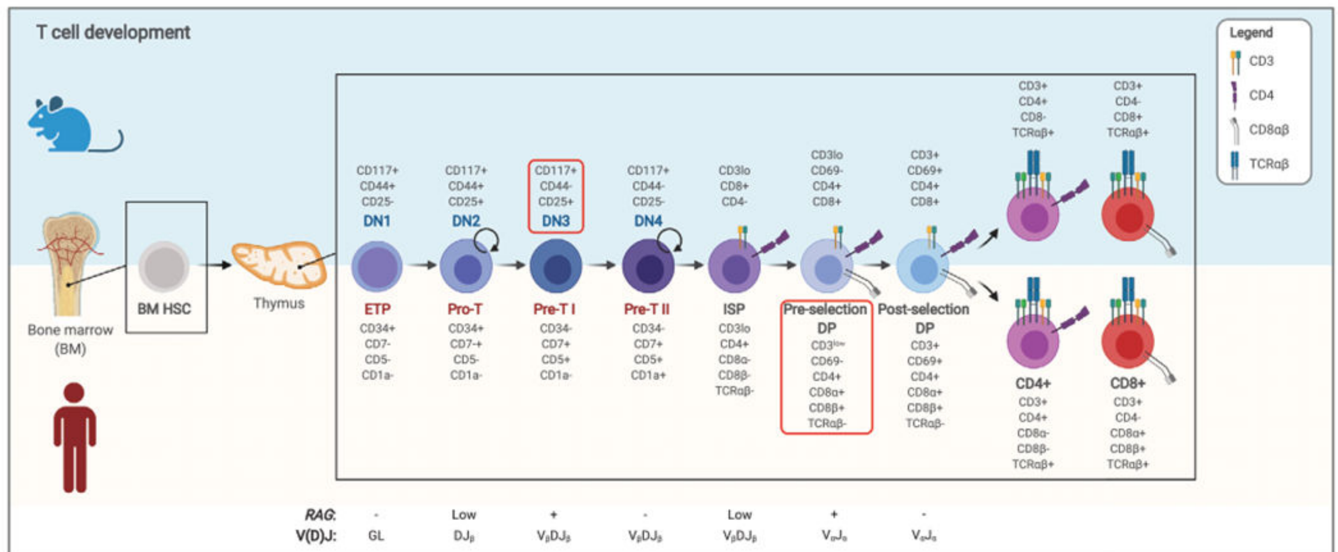

Figure 2.

T-cell differentiation blocks in RAG-deficient patients and mice. Representation of T-cell development in mice and humans, with the list of surface markers that can be used to identify the different stages of maturation. Stages at which *RAG* genes are expressed, and the concurrent nature of VDJ recombination products detected at each stage, are indicated at the bottom. Proliferating cells are indicated by a circular arrow. The red boxes indicate the stage at which development is blocked in RAG deficiency. Of note, use of artificial thymic organoids (ATOs) allowed to establish that T-cell development in *RAG*-mutated patients occurs at the preselection double-positive (DP) stage, unlike what was found in mice, where the block is present at the double-negative 3 (DN3) stage. DN, double negative; DP, double positive; ETP, early thymic progenitor; GL, germline; HSC, hematopoietic stem cell.

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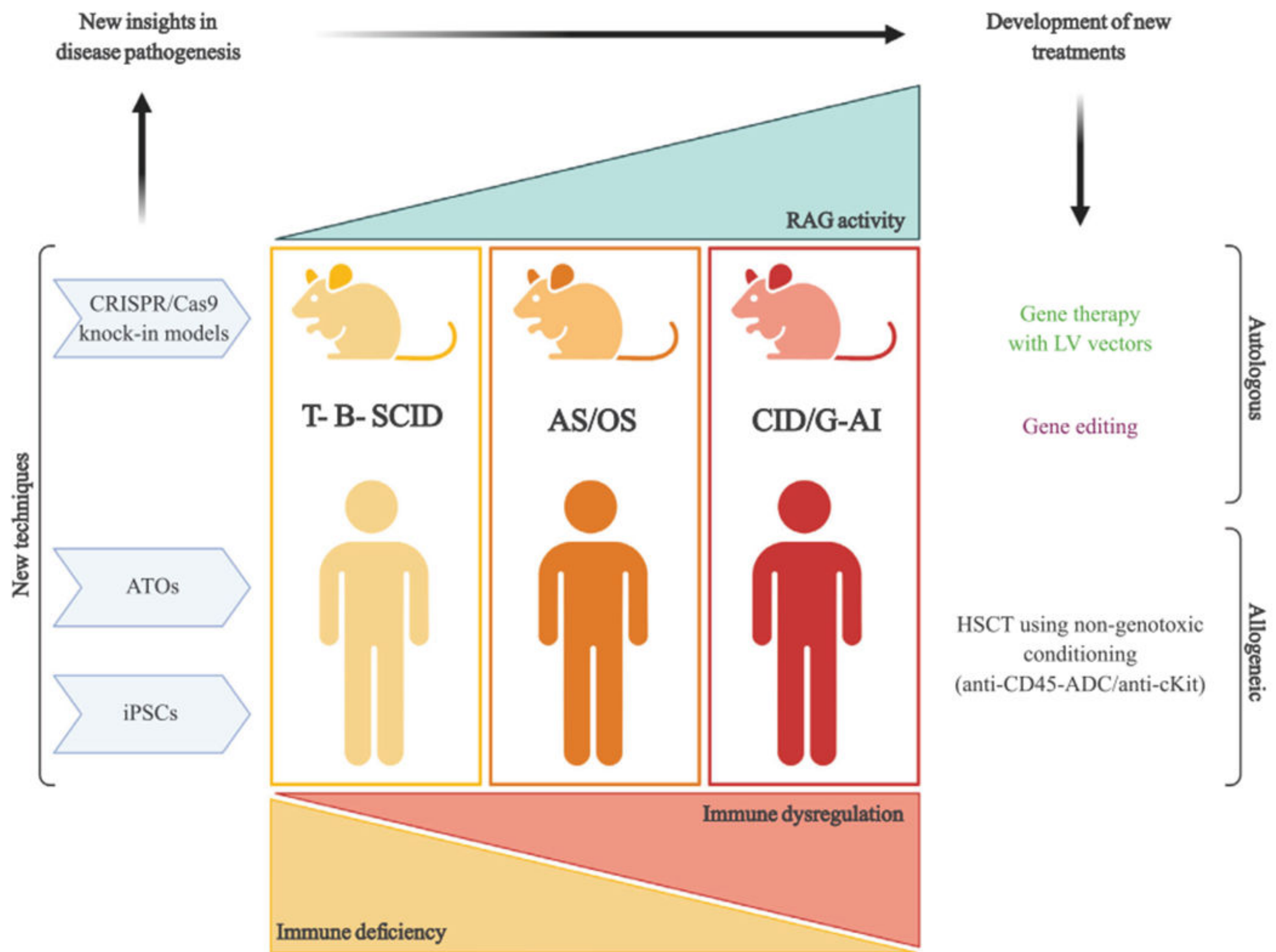

Figure 3.

Novel investigational techniques offer new insights into pathophysiology of RAG deficiency and open novel therapeutic perspectives. Depicted here are some of the new experimental techniques that have provided novel insights into RAG deficiencies. In particular, CRISPR/Cas9 technique has been used to generate novel knockin mouse models carrying *RAG* mutations found in patients, while artificial thymic organoids (ATOs) and induced pluripotent stem cells (iPSCs) have allowed in vitro modeling of faulty T-cell development using cells isolated from RAG-deficient patients. These novel tools have also made possible the development and testing of novel prospective therapeutic approaches such as lentiviral vector-mediated gene therapy, gene editing, and use of nongenotoxic conditioning regimens for hematopoietic stem cell transplantation (HSCT).