

ADDENDUM



RAGs and BUGS: An alliance for autoimmunity

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ABSTRACT

Hypomorphic *Rag* mutations in humans cause Omenn Syndrome (OS) a severe immunodeficiency associated with autoimmune-like manifestations mediated by oligoclonal activated T and B cells. The clinical and immunological spectrum of OS presentation is extremely broad. However, the role played by environmental triggers in the disease pathogenesis remains largely unknown. We have recently shown in a murine model that gut microbiota has a substantial role in determining the distinctive immune dysregulation of OS. Here, we describe how dysbiosis and loss of T cell tolerance to commensals influence the expression of autoimmunity at the barrier site and beyond, and the disease hallmark hyper-IgE. We discuss how commensal antigens and gut-derived pathogenic T cells could potentially modulate skin immunity to determine cutaneous degenerations in OS. These mechanisms may have broader implications for a deeper understanding of the role of gut microbes in influencing barriers integrity and host physiology.

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Introduction

Recombination-activating gene 1 (*RAG1*) and *RAG2* encode for lymphoid-specific proteins, expressed during the early stages of T and B cell development and required for the initiation of the V(D)J recombination process. The efficient completion of this process is required for the generation of a fully diversified repertoire of antigen-specific T- (TCRs) and B-cell receptors (BCRs). Disruption of these genes in mice and humans results in the abrogation of V(D)J recombination, blockade of lymphocyte development with consequent lack of mature T and B cells, which causes the clinical syndrome referred to as severe combined immunodeficiency (T-B- SCID).^{1–3} Hypomorphic RAGs mutations, that allow a residual recombination activity, were found in patients with Omenn Syndrome (OS) a peculiar disease in which immunodeficiency is associated with immune dysregulation and autoimmunity mediated by oligoclonal activated T cells.⁴ However, recent evidence indicate that *RAG* mutations constitute a more complex entity than

previously thought, associated with a broad spectrum of clinical and immunological phenotype.⁵

Immune dysregulation underlying partial RAG deficiency

Similar to T- B- SCID, OS generally arises during the first year of life with chronic diarrhea, skin eruptions, early-onset severe infections, and failure to thrive. This condition is inevitably fatal if not treated with haematopoietic stem cell transplantation.⁶ Unlike patients with complete RAG deficiency, individuals with OS exhibit enlarged lymph nodes and hepatosplenomegaly, associated either with normal or enhanced homeostatic expansion of oligoclonal T cells infiltrating multiple organs.^{7,8} The accumulation of T cells harbouring particular TCR specificities in different tissues is suggestive of autoantigen-driven selection and expansion.^{8,9} Circulating B cells are virtually absent. Nonetheless, in these patients high levels of serum IgE associate with eosinophilia. In the last years, advances in the comprehension of disease

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pathogenesis have clearly shown that immune dysregulation in OS is determined by defects of central and peripheral T cell and B cell tolerance. A broad spectrum of autoantibodies was reported in patients with OS¹⁰ and expansion of autoreactive immunoglobulin secreting cells was observed in mouse models of the disease.^{11,12} Furthermore, increased serum B-cell activating factor (BAFF) levels, reflecting B cell lymphopenia and inflammation in condition of RAG deficiency, has been proposed to promote the survival of self-reactive immature B cells both in humans and mouse models.^{11,12} In contrast to peripheral organs, the thymus is almost completely devoid of T cells and profound alterations in thymic architecture, epithelial cells development, expression of AIRE as well as of AIRE-dependent tissue restricted antigens have been described in patients with OS and mice with hypomorphic RAG mutation.¹³⁻¹⁵ Furthermore, impaired generation of thymic immunosuppressive Treg cells has been described both in patients¹³ and mice.¹⁴ Circulating FOXP3+ cells in OS patients co-express activation markers and fail to suppress proliferation of allogenic activated CD4+ T cells. These cells probably represent activated memory T rather than bona fide Treg cells.¹⁶ Finally, defects of invariant natural killer T (iNKT) cells may also contribute to OS immune dysregulation.¹⁷

Recently, in our mouse model of OS we found that mucosal B cell deficiency results in altered composition of intestinal commensal bacteria and their increased translocation across the intestinal epithelium. Loss of T cell tolerance to microbiota leads to Th17 and Th1-mediated intestinal inflammation. Depletion of gut bacteria, achieved by treatment with oral broad-spectrum antibiotics, reversed most of these abnormalities, ameliorated systemic autoimmunity and normalized serum hyperIgE. Here, we discuss these and other relevant findings suggesting that microbiota-derived signals may play a critical role in promoting immune dysregulation distinctive of partial RAG deficiency.

Commensal bacteria shape T cell responses and influence autoimmunity and hyper IgE

The availability of a homozygous *Rag2* knock in mouse carrying the R229Q mutation, originally described in several patients with OS and shown to reduce V(D)J recombination activity by over 150-

fold,¹⁴ offers a unique setting to evaluate the contributory roles of environmental triggers to the autoimmune manifestations of the disease. Hypomorphic *Rag2*^{R229Q} mice develop spontaneous intestinal disease with marked increased frequencies of IFN- γ and IL-17 secreting cells, which are implicated in the pathogenesis of colitis in mice as well as in inflammatory bowel disease (IBD) patients.¹⁸ Accordingly, *Rag2*^{R229Q} CD4 T cells, either isolated from apparently healthy or colitic mice, were able to transfer the disease into immunodeficient hosts. Regulatory T cells are known to accumulate to high frequency in the intestinal *lamina propria* to maintain immune tolerance to commensal bacteria.¹⁹ In *Rag2*^{R229Q} mice, we detected Treg cells in the intestinal mucosa, however they were unable to control the progression of the disease. This observation suggests that *Rag2*^{R229Q} Tregs may not be functionally competent. Alternatively, effector T cells may be more resistant to Tregs-mediated suppression. When equivalent number of Tregs isolated from spleen and mesenteric lymph nodes of wild-type or *Rag2*^{R229Q} mice were adoptively transferred into the severe combined immunodeficiency model of colitis induced by CD4⁺ CD45RB^{hi} T cells,²⁰ colitis was suppressed by transfer of wild-type Tregs, but not Tregs from mutant mice. Furthermore, we found that transfer of a limited number of wild-type Tregs into *Rag2*^{R229Q} mice resulted in decreased Th1/Th17 cells and amelioration of intestinal disease. Therefore, a skewed polarization of T cells toward Th1/Th17 differentiation pathway found in hypomorphic mice might be related to failure in the establishment of intestinal Treg mediated immunosuppression. Our results are also consistent with recent findings indicating that TCR repertoire diversity is required to ensure tolerance toward intestinal microbiota and avoid Th17 cells-mediated colitis.²¹ In addition, colitis can be driven by alterations in the gut bacteria composition.^{22,23} Because we found that *Rag2*^{R229Q} mice exhibit also evident intestinal B cells as well as IgA deficiency, which have profound effects on gut microbial ecology,²⁴ we analyzed microbiota composition in mutant mice and their wild-type littermates. *Rag2*^{R229Q} mice were characterized by diminished bacterial diversity and enrichment of several genera within the *Proteobacteria* phylum, previously associated with development of IBD in humans.²⁵

Additionally, serum lipopolysaccharide (LPS) concentrations were increased in *Rag2*^{R229Q} mice compared with wild-type mice, indicating that the hypomorphic defect in RAG genes results leakage of the gut-endothelial barrier.²⁶

Since an altered relationship between microbiota and intestinal immune system can drive Th17 cell-mediated inflammation²⁷ we examined the effect of long-term dosing of antibiotics (metronidazole, vancomycin, ampicillin) on the development of potentially pathogenic intestinal Th1/Th17 responses in mutant mice. After one month the oral delivery of this antibiotics reduced the cecal bacterial content by 1000-fold. Antibiotic-treated mice exhibited decreased frequency and numbers of intestinal Th1/Th17 cells, indicating that intestinal inflammation in *Rag2*^{R229Q} mice is mediated by microbial colonization. Interestingly, the altered microbial communities of *Rag2*^{R229Q} mice were able to promote an inflammatory immune phenotype into wild-type mice in fecal transplant experiments, supporting a causal role of pathobionts selected in *Rag2*^{R229Q} mice in immune dysregulation.

Remarkably, we found that also expansion of splenic Th1/Th17 cells¹² in mutant mice was attenuated by administration of antibiotics. This observation suggests that skewed T cell polarization at the intestinal mucosa by the altered microbiota in *Rag2*^{R229Q} mice might result in systemic dissemination of pathogenic T cells probably sustained by homeostatic cytokines and/or translocated microbial products. In fact, the vast majority of circulating mutant T cells expressed the gut homing receptor CCR9 and CCR9+ T cells markedly diminished following antibiotic administration. Since CCR9 expression is commonly acquired by T cells upon priming by GALT dendritic cells,²⁸ we speculated that it could be considered a marker of their intestinal origin. In this regard, we found that gut-derived CD103⁺ DCs were markedly increased in the mesenteric lymph nodes of *Rag2*^{R229Q} mice, suggesting that gut endothelial barrier leakage may eventually increase the propensity of antigen-presenting cells to take up antigens and present them to T cells in lymphoid organs. Alternatively, T cells might be activated in peripheral lymphoid organs by intestinal products absorbed through a leaky gut endothelial

barrier.²⁹ Whether peripheral *Rag2*^{R229Q} T cells bear TCRs specific for intestinal antigens or are activated by circulating microbial products to generate Th1/Th17 cells remain to be elucidated.

Of note, intestinal as well as splenic “apparently” Treg cell populations were also contracted in mutant mice upon antibiotic treatment. Intriguingly, commensal-specific Tregs were shown to lose suppressive ability and acquire effector function during homeostatic disruption.^{29,30} Based on our previous findings in OS patients, in whom we detected a residual population of peripheral Foxp3⁺ cells with altered phenotype and lack of suppressive function,¹⁶ it is tempting to speculate that conversion from a regulatory to effector phenotype may be important in the pathogenesis of autoimmunity in hypomorphic RAG conditions.

A number of studies have documented the ability of gut commensals to influence autoimmunity at distant sites, with inhibitory or enhancing effects.^{31–34} *Rag2*^{R229Q} mice recapitulate the autoimmune manifestations in humans at mucosal barriers and systemic target organs.¹⁵ Thus, we sought to determine the effect of flora depletion on autoimmune phenomena in different organs of mutant mice. A decreased number of infiltrating T cells was observed in the kidney, lung, and liver of treated *Rag2*^{R229Q} mice treated with antibiotics as compared with untreated controls, indicating a cooperative role of commensal microbiota in organ-specific autoimmunity.

Overall, our findings pose the important question of how commensal bacteria and/or commensal-derived signals might contribute to triggering systemic autoimmunity in hypomorphic RAG condition. The development of autoreactive lymphocytes is proposed to occur due to different, not mutually exclusive mechanisms, namely bystander activation, epitope spreading and molecular mimicry.^{35,36} Each of these 3 mechanisms applied to T cell activation by commensals could be implicated in the development of autoimmune manifestations in OS (Fig. 1). Concomitant productive presentation of self- and microbial-antigens might be favored in OS due to altered composition and homeostatic control of T cells (consequent to the genetic lesion) as well as mucosal barrier defects and dysbiosis leading to tissue damage. Alternatively, commensals could activate autoreactive T cells through the engagement of cross-reactive

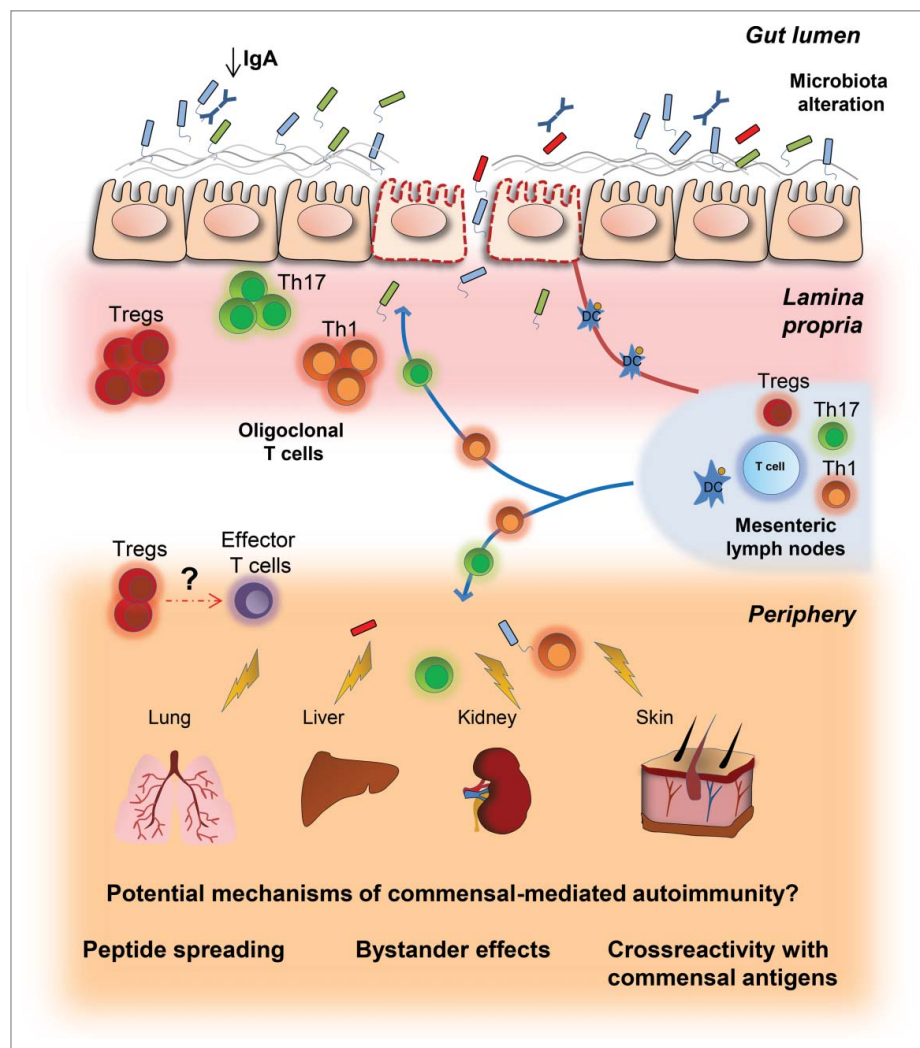


Figure 1. The proposed role of commensal bacteria in the pathogenesis of OS. Mucosal B cell deficiency and functional impairment in oligoclonal Tregs compromise gut barrier integrity and alter microbiota composition, favoring microbial access to the lamina propria and circulation. Antigen presentation by dendritic cells and priming of T cells in the mesenteric lymph nodes (MLN) lead to generation of gut tropic Th17 and Th1 cells, which expand in the lamina propria leading to gut chronic inflammation. From the MLN, Th17/Th1 T cells also circulate in the periphery and infiltrate target organs such as lung, liver, kidney and skin, causing autoimmunity. Tregs in periphery could acquire effector function under condition of altered homeostasis. Three potential mechanisms, not mutually exclusive, could concur to induce autoreactive T cells. Triggering of pattern-recognition receptors, such as Toll like receptors and Nod-like receptors, could sustain autoreactive T cell activation, induced by concomitant presentation of tissue and microbiota antigens (bystander activation). Peptide spreading could be caused by the altered immune response to microbiota, leading to tissue damage and release of multiple self-antigens. This event could in turn result in simultaneous presentation of bacterial and self-antigens. Finally, crossreactivity with commensal antigens (molecular mimicry) could act inducing activation of immune responses against self-antigens.

TCRs.^{31,37} Whereas commensal/self-reactive TCRs should be in physiologically conditions associated with T cells that regulate immune responses, a break in T cell tolerance to commensals might provoke a switch to a pathogenic T cell response.²⁹

Importantly, crossreactivity to commensals extends also to B cells. B-cell development can occur in the intestinal mucosa, where it is regulated by extracellular signals from commensal microbes that influence gut immunoglobulin repertoires.³⁸

Accordingly, the majority of IgA⁺ and IgG⁺ plasmablasts in the gut are directed to commensal and self-antigen, with a significant degree of polyreactivity.³⁹ Increased B cell polyreactivity caused by defects in central and peripheral B cell tolerance has been suggested in OS patients¹⁰ and represents a critical factor for autoimmunity. Furthermore, crossreactive T follicular helper (Tfh) cells could have a synergistic effect, supporting the B cell production of autoantibodies.

Production of IgE is also regulated by commensal microbiota. In particular, it has been shown that microbial exposure during early life and a critical level of bacterial diversity are required in order to inhibit generation of IgE secreting cells.⁴⁰ Consistent with this notion, markedly elevated IgE levels in *Rag2*^{R229Q} mice may reflect the low-diversity of microbiota that is likely defective in promoting immune regulation. Remarkably, we found that depleting gut bacteria with antibiotics was beneficial for normalizing serum IgE concentrations that were significantly reduced. Since our previous findings indicate that B cell abnormalities and plasma cells differentiation are dependent on the presence of hyperactivated CD4⁺ T cells,¹² we correlated these results with the diminished serum concentration of cytokines that are known to promote class switch recombination. These analyses showed that antibiotic administration results in substantial changes in the pool of circulating proinflammatory Th1/Th17 cells. Even though additional studies are required to carefully investigate the impact of microbial stimuli on *Rag2*^{R229Q} B lymphocyte development and function, these results indicate that microbiota can constitute a pharmacological target for this crucial pathogenetic aspect of the disease.

Skin degeneration in OS: The other side of gut inflammation?

OS patients present a spectrum of skin manifestations. Besides erythroderma, the clinical hallmark of the disease, psoriasis and alopecia are common features,⁴¹ while vitiligo has been recently reported as a unique clinical sign in the absence of overt immunological defects.⁴² Skin eruptions are present frequently in the OS murine counterpart.¹⁴ Mechanisms described for induction of gut autoimmunity and maintenance by commensal bacteria may operate in the generation of skin pathology in *Rag2*^{R229Q} mice. Most frequent skin disorders, such as atopic dermatitis and psoriasis, have all been associated with loss of cutaneous barrier function, dysbiosis of the skin flora and exaggerated immune responses to microbial antigens.⁴³ Nonetheless, the interesting question is whether gut inflammation (including barrier defect and dysbiosis) and skin disease are causally linked. The relationship between gut commensals and skin disorder is largely unknown.

Recent findings indicate that antibiotic-driven perturbation of gut microbiota during infancy increases the susceptibility and the severity of psoriasis, induced later in adults in 2 different experimental models.⁴⁴ This study further suggests infancy constitutes a critical time window when key immunological thresholds are established for life, as consequence of exposure to a diverse microbial population.^{40,45-47} Importantly, these authors additionally found that antibiotic administration early in life affected microbial composition and diversity not only in the gut but also in the skin, which remained altered till adult age, even when the mice were exposed to the normal environment for additional periods. OS and in general SCID patients are commonly treated with antimicrobial therapeutics to manage chronic or recurrent systemic infections, yet the impact of systemic antibiotics on skin homeostasis is incompletely understood. Host interactions with skin commensals are crucial in developing cutaneous immunity.⁴⁸ Interestingly, cutaneous exposure to food antigens was shown to reprogram gut homing effector cells in lymph nodes to express skin-homing receptors and to elicit allergic skin inflammation.⁴⁹ This observation raises the intriguing possibility that gut T cells may migrate to the skin and potentially modulate skin immunity. Furthermore, despite the authors showed that the reprogramming of T cell homing required cutaneous antigen challenge,⁴⁹ it is also possible that antigens delivered to peripheral LN from oral immunization might induce antigen specific T cells to express skin-homing receptors. It is tempting to speculate that systemically translocated commensal antigens in the presence of breaches of the gut barrier and/or genetic lesion predisposing to aberrant immune responses to microbiota can activate cross-reactive T cells with skin homing potential (Fig. 2). Testing these possibilities may help to shed light on the pathogenic mechanisms underlying skin autoimmunity in OS. More in general, it may be instrumental to reconcile the frequent observation of skin complications in IBD,⁵⁰ with important therapeutic implications.

Concluding remarks

Here we discussed our recent report showing for the first time that commensals and/or commensal bacteria-derived signals might play a key role in influencing immune dysregulation at barrier sites and beyond in

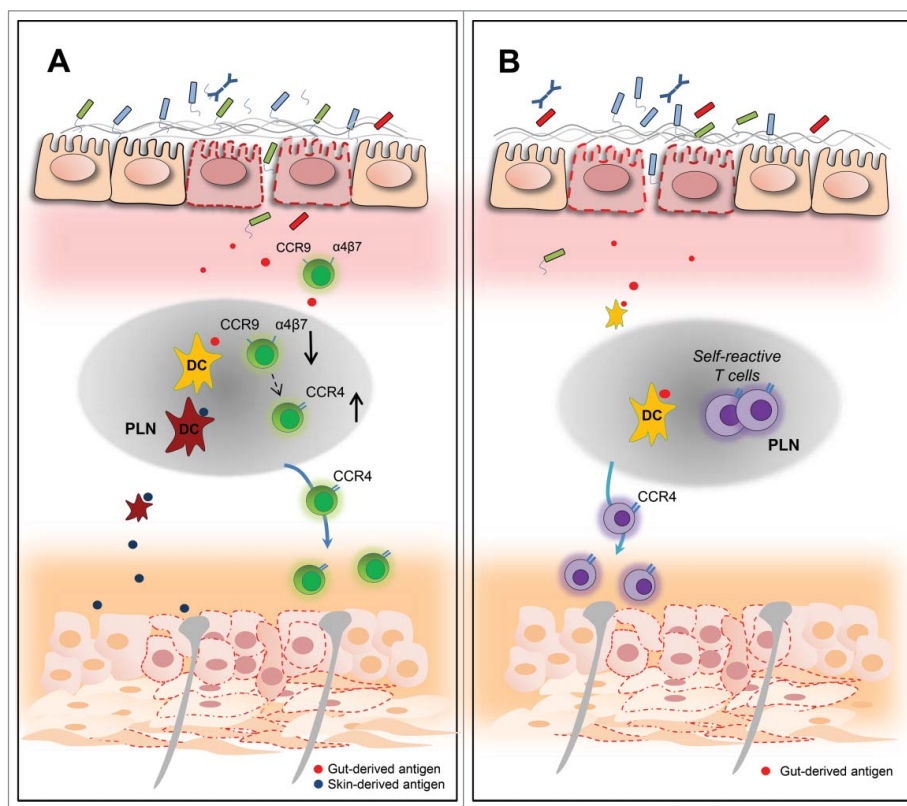


Figure 2. Potential mechanisms by which commensal bacteria and gut inflammation could promote skin degeneration in OS. In OS, the pathogenesis underlining skin manifestations still remains elusive. Two different not mutually exclusive mechanisms can be envisaged. Panel A: systemic dissemination of gut tropic pathogenic Th1/Th17 cells might occur in OS, sustained by homeostatic cytokines and/or translocated microbial products. In peripheral lymph nodes (PLN), presentation of gut and/or skin-derived antigens by dendritic cells results in reprogramming of pathogenic gut homing CCR9⁺ α4β7⁺ T cells to skin tropic CCR4⁺ T cells and in their migration to the skin compartment, where they might contribute to tissue inflammation and autoimmunity. Panel B: gut barrier disruption could induce translocation and presentation of bacterial antigens by dendritic cells to circulating self-reactive T cells. Activation of autoreactive T cells in PLN leads to upregulation of the skin homing receptor, CCR4, and migration of pathogenic self-reactive T cells toward skin compartment.

RAG-associated immune deficiency. We also elaborated on the hypothesis whereby gut microbiota may induce autoreactive T and B cells and sustain immune response to self-antigens. Recent advances in genomic analyses, protein arrays, immuno-bioinformatics, as well as the availability of suitable animal models would provide the tools for testing these hypothesis in future studies.

Abbreviations

OS	Omenn Syndrome
SCID	Severe Combined Immune deficiency
Th1	T helper Type1
Th17	T helper Type17
IgE	immunoglobulin E
LN	lymph node
IBD	Inflammatory Bowel Disease

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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