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Sulfate-reducing bacteria stimulate gut immune responses and contribute to inflammation in experimental colitis

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ABSTRACT

The intestinal microbiota is critical for mammalian immune system development and homeostasis. Sulfate-reducing bacteria (SRB) are part of the normal gut microbiota, but their increased levels may contribute to colitis development, likely in association with hydrogen sulfide (H_2S) production. Here, we investigated the effects of SRB in the gut immune response in germ-free mice, and in experimental colitis. After 7 days of colonization with *Desulfovibrio indonesiensis* or with a human SRB consortium (from patients with colitis), germ-free mice exhibited alterations in the colonic architecture, with increased cell infiltration in the lamina propria. SRB colonization upregulated the Th17 and Treg profiles of cytokine production/cell activation, in T cells from mesenteric lymph nodes. These alterations were more pronounced in mice colonized with the human SRB consortium, although *D. indonesiensis* colonization produced higher levels of H_2S. Importantly, the colon of C57BL/6 mice with colitis induced by TNBS or oxazolone had increased SRB colonization, and the administration of *D. indonesiensis* to mice with TNBS-induced colitis clearly exacerbated the alterations in colonic architecture observed in the established disease, and also increased mouse weight loss. We conclude that SRB contribute to immune response activation in the gut and play an important role in colitis development.

1. Introduction

In mammals, the microbial community regulates important host metabolic and physiological functions, playing key roles in the development and homeostasis of the host immune system. The microbiota is crucial for the development and differentiation of both local and systemic components of innate and adaptive immunity, through its interaction with the mucosal immune system [1]. In particular, the gut microbiota appears to direct the organization and maturation of lymphoid tissues [2]. In the gut, the recognition of microbial compounds is essential for host T cell maturation, and for these cells to be recruited, as well as induced to differentiate and eventually reside in the gut [3]. Additionally, the recognition of microbial components drives the shift to high affinity IgA in the Peyer's Patch and is essential for B-cell mediated immune responses [4].

The importance of the microbiota for the development of mucosal immune responses is particularly evident in germ-free mice, which lack commensal bacteria and, thus, exhibit extensive defects in the gut-associated lymphoid system and in antibody production [5]. Germ-free mice are, therefore, more susceptible to infections by certain pathogens [6], even when compared with specific pathogen-free (SPF) mice [7].

Changes in the composition of the gut microbiota can contribute to the development of different pathological conditions, including obesity, autoimmune diseases and colitis [8–10]. Altered immune responses and their relationship with commensal microbiota that inhabit gastrointestinal tract are key aspects of the development of inflammatory bowel diseases (IBD) - namely Crohn's disease (CD) and ulcerative colitis (UC) - characterized by excessive (i.e., non-protective) inflammation [11,12]. Both systemic and local immune responses against mucosal bacteria appear increased in IBD [13–15]. Also, in fecal samples

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from patients with IBD, *Escherichia coli* and *Bacteroides* spp. are increased to the detriment of probiotic bacteria such as *Lactobacillus* spp. and *Bifidobacterium* [13,14], showing that bacterial populations changes – or dysbiosis – occur in the gut of patients with IBD. Furthermore, CD patients respond to treatments that alter the gut flora, such as metronidazole administration and certain diets [16,17]. These data support the theory that IBD can develop as an exacerbated inflammatory response against the intestinal flora [18]. Studies with germ-free mice also support this notion; although these immune-impaired mice are totally resistant to colitis induction, even upon interleukin-10 deficiency (which leads to spontaneous colitis in non-germ free strains [19], the administration of a human bacterial consortium restores colitis development in germ-free/IL-10 deficient mice [20].

Sulfate-reducing bacteria (SRB) - a diverse anaerobic group of organisms with a variety of nutritional and morphological peculiarities [21] – represent a component of the microbiota of particular interest in the context of colitis. SRB numbers were consistently increased in biopsies of patients with active UC, when compared to those of patients with quiescent disease [22]. In particular, two species of SRB - *Desulfovibrio desulfuricans* and *D. vulgaris* - were correlated to UC disease activity, and found in higher quantities colonizing human colonic crypt mucous gel, when compared to healthy patients [23].

Sulfate reduction by SRB generates hydrogen sulfide (H_2S), which is toxic to colonic epithelial cells, by inhibiting butyrate metabolism in colonocytes [24]. Also, Ijssennagger and co-workers showed that H_2S breaks the mucus barrier and allows bacteria to be in close proximity to the colonic epithelium, leading to inflammation [25,26]. In mammals, endogenous H_2S is typically produced in nanomolar concentration, and works as a messenger in the central and peripheral nervous system, and also in the immune, endocrine, reproductive and gastrointestinal systems [27–30]. In the mouse colon, H_2S is present in millimolar concentrations, comparable to those observed in human feces [28,31], and higher than normal H_2S levels may disturb intestinal homeostasis and contribute to the development of colitis and colon-rectal cancer [26,32,33]. Feces from IBD patients have excessive levels of H_2S [24] and the levels of sulfide detoxification enzymes are increased in peripheral blood of patients with UC [34]; these data represent a possible link between H_2S production by SRB and UC progression. In agreement with this hypothesis, exposure to H_2S can alter gut homeostasis and immune response activities that are involved in UC development, such as neutrophil migration [35] and T cell activation [36].

Although increased SRB colonization and H_2S production have been associated with colitis severity/development, direct evidence for this link is still lacking. Here, we examined the effect of SRB colonization on the gut architecture and immune system activation in germ-free mice, to isolate the effect of SRB from that of other elements of the microbiota. Also, we used an experimental model of chemically-induced colitis to assess whether increased SRB colonization can contribute to colitis development.

2. Materials and methods

2.1. Mice

C57BL/6 were bred at the animal house for transgenic mice of the Federal University of Rio de Janeiro (UFRJ, Rio de Janeiro, RJ, Brazil), and maintained at 22 °C in a 12 h light/dark cycle. Germ-free Swiss/NIH mice were maintained at the Laboratório de Gnotobiologia e Imunologia from the Universidade Federal de Minas Gerais (UFMG, Belo Horizonte, MG, Brazil), according to standard procedures [37]. In all experiments, female 6–8-week-old mice weighing approximately 20 g were used. The experimental protocols involving animals were approved by the Commission for Ethical Use of Research Animals (CEUA, UFRJ; ref. 078/15).

2.2. Human SRB consortium production and SRB growth detection

A human SRB consortium was obtained from specimens of mucosa taken during colonoscopy of patients with colitis treated at the Clementino Fraga Filho University Hospital (HUCFF, UFRJ). The work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The study protocol was approved by the Ethical Committee of the HUCFF, and informed consent was obtained from all patients (approval number 188/08). Mucosal specimens were transferred to a sterile physiological saline solution, maintained in an atmosphere of oxygen-free nitrogen, and then completely dissociated to ensure release of bacterial cells from mucosa. Aliquots of mucosal samples were inoculated in 10 mL vials containing VM medium I, specifically designed to recover SRB [22]. SRB activity and growth were detected by a medium color change to black, due to the production of iron sulfide from H_2S released by active bacteria.

2.3. Colonization of germ-free mice with SRB

Human SRB consortium cultures or cultures of *Desulfovibrio indonesiensis* (isolated from a biofilm on a corroded ship off the Indonesian coast [38] were centrifuged at 2000*g* at 4 °C, resuspended in PBS (at a density of 5×10^8 bacteria/mL), and 200 μL of this suspension was inoculated intragastrically into germ-free mice (10^8 bacteria/animal). Control groups received PBS instead. To assess SRB colonization in germ-free mice, feces were collected daily until day 7 post-colonization and inoculated in 10 mL vials containing VM medium I. On day 7 post-colonization, animals were euthanized, and duodenum and colon samples were collected and incubated in VM medium I as performed for feces samples. Cultures were inspected for medium color change (indicative of SRB growth) at regular intervals over a period of 28 days.

2.4. Colitis induction

The colitis inducers oxazolone and 2,4,6-trinitrobenzenesulfonic acid (TNBS) (both from Sigma Aldrich) were diluted immediately before administration in mice. For colitis induction, wild type C57BL/6 mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg body weight) and xylazine (5 mg/kg body weight), and a no 4 intratracheal-type tube was carefully inserted into the colon (4 cm proximal to the anus) and used for the intrarectal administration of 100 μL of one of the following solutions: 2.5% (w/v) TNBS in 50% (v/v) ethanol [39]; 1.5% (w/v) TNBS in 35% (v/v) ethanol [40]; 1.0% (w/v) oxazolone in 50% (v/v) ethanol [39]; or 100 μL of 50% (v/v) ethanol only (all purchased from Sigma Aldrich, St. Louis, Missouri, USA). During intrarectal administration, mice were kept with their head down in a vertical position for 4 min to ensure full retention of injected solutions. The animals treated with 2.5% (w/v) TNBS in 50% (v/v) ethanol or 50% (v/v) ethanol were inoculated intragastrically with *D. indonesiensis* (10^8 bacteria/animal) or with saline (control groups) 5, 24, 48 and 72 h after intrarectal treatments. Mice were weighed before and up the four days after colitis induction. On day 4 post-colitis induction, animals were euthanized, fragments of colon (corresponding to the region from the cecum to the distal portion) were removed and measured, and then incubated in 10-mL vials containing × 10 mL VM medium I, to evaluate SRB growth, or fixed for histological evaluation.

2.5. Histological sample preparation and colitis severity evaluation

Fragments of 1.0 cm from the distal colon were fixed in 10% (v/v) formaldehyde in aqueous phosphate buffer pH 7.3, for 24 h. Then, fragments were dehydrated in ethanol, clarified in xylene and embedded in paraffin. Sections (5-μm-thick) were mounted onto poly-L-lysine-coated slides, stained by hematoxylin and eosin and examined on