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Research paper

Burn injury influences the T cell homeostasis in a butyrate-acid sphingomyelinase dependent manner

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ABSTRACT

Following burn injury, a key factor for patients susceptible to opportunistic infections is immune suppression. Butyrate levels are important in maintaining a functional immune system and these levels can be altered after injury. The acid sphingomyelinase (Asm) lipid signaling system has been implicated in a T cell actions with some evidence of being influenced by butyrate. Here, we hypothesized that burn-injury changes in butyrate levels would mediate Asm activity and, consequently, T cell homeostasis. We demonstrate that burn injury temporally decreases butyrate levels. We further determined that T cell Asm activity is increased by butyrate and decreased after burn injury. We additionally observed decreased T cell numbers in Asm-deficient, burn-injured, and microbiota-depleted mice. Finally, we demonstrate that butyrate reduced T cell death in an Asm-dependent manner. These data suggest that restoration of butyrate after burn injury may ameliorate the T cell lost observed in burn-injured patients by Asm regulation.

1. Introduction

Following burn injury, the development of bacterial infections and sepsis is the leading cause of mortality [1,2]. While a disrupted skin barrier contributes significantly to infectious complications after burn, a major underlying factor is thermal-injury induced immune dysfunction. With improvements in wound care, early burn excision, and antibiotic administration, wound infections have decreased while nosocomial infections have become the primary cause for mortality in burn-injured patients, particularly during the state of immunosuppression [2,3]. Consequently, there is a need to understand the factors that control burn-related immune dysfunction and susceptibility to nosocomial infections.

Multiple factors contribute to immunosuppression following burn-injury. These include altered frequency of regulatory T cells, increased levels of cytokines, immune cell apoptosis, and alteration in lipid mediators [4–6]. It is unknown whether these mechanisms have an underlying common mediator. Recent data has implicated the gut microbiota in playing a critical role in local (intestinal) [7,8] and systemic immune homeostasis [9–11]. Intestinal microbiota serves multiple functions, including the production of short chain fatty acids (SCFA), the primary nutrient of the colonocyte [12,13]. The SCFA butyrate has been shown to promote accumulation of regulatory T cells [14,15]. The role of butyrate in inflammation and immunity is further supported by a study demonstrating butyrate administration can protect against inflammatory bowel disease [15]. While alterations in the microbiota, and butyrate, can affect the systemic immune response, the reciprocal action occurs as well. Alterations in the immune response can shift the composition of the microbiota. It has previously been shown that loss of T-bet in innate immune cells alters the microbiota [16]. Similarly, loss of NLRP6 inflammasome, specifically in colonic epithelial cells, can increase inflammation and drive shifts in the composition of the microbiota [17]. In both of these instances, the shifts in the microbiota resulted in worsened autoimmune colitis. Taken a step further, a recent clinical study examined the fecal material from patients with systemic inflammatory response syndrome, due to infection, trauma, or burn, and demonstrated a loss of anaerobes and reduction in organic acids [18]. Further, burn injury has been shown to induce a dysbiosis of the intestinal microbiome, increase intestinal permeability and increase bacterial translocation [19,20], suggesting that the gut may be a potential source of bacterial

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infection. While it is known that trauma and burn injury alter the gut microbiota, it is unknown how these changes affect immune responsiveness. Specifically, it is unknown how changes in intestinal butyrate following burn injury affect the immune response.

In addition to altering intestinal microbiota, trauma and burn injury can alter the acid sphingomyelinase (Asm) lipid signaling system. Sphingolipids are a major component of the cell membrane. Asm is an enzyme that is ubiquitously expressed and converts the cell membrane phospholipid sphingomyelin to ceramide [21,22]. The Asm/ceramide system plays a role in cell adhesion, migration, and T cell apoptosis [23–29]. Additionally, Asm activity has been shown to be induced by butyrate in a colon cancer cell model [30]. Here, we sought to further explore the link between Asm and butyrate in the microbiota and the potential role in immune suppression.

A function of the gut microbiota is to produce short chain fatty acids (SCFA), the primary nutrient of the colonocyte [13]. Specifically, members from the *Firmicute* phylum and the *Clostridiale* family produce butyrate [31], a crucial SCFA for immune system regulation. We previously demonstrated that burn injury significantly reduces the *Firmicute* phylum, in particular the *gnavus* and *eutactus* species, both known butyrate producers [20], particularly on post burn day (PBD) 6. As it is known that the microbiota and Asm system are altered following burn injury, it is unknown how butyrate, may impact Asm activity. Further, it is unknown if alterations in Asm caused by butyrate alter the T cell homeostasis following burn injury. In this study, we hypothesized that potential burn-injury altered butyrate levels would mediate Asm activity and T cell numbers.

2. Methods and materials

2.1. Mice

Male CF-1 mice were obtained from Charles River Laboratories (Wilmington, MA) at five weeks of age and allowed to acclimate for one to two weeks prior to conducting experiments. For gene-targeted experiments, acid sphingomyelinase (Asm) knock-out homebred mice and their wild-type littermates were utilized as previously described [32,33]. All mice were housed in standard environmental conditions and fed with a pellet diet and water ad libitum. All murine experiments were conducted between 7 am and 10 am using protocols approved by the Institution Animal Care and Use Committee (IACUC number 08-09-19-01) of the University of Cincinnati.

2.2. Scald burn injury

Male CF-1 mice underwent a full-thickness scald injury on their dorsal surface as previously described [34]. Briefly, mice were weighed and subsequently anesthetized with 4.5% inhaled isoflurane in oxygen. Hair was clipped from their dorsal surface and they were placed in a template exposing 28% of their total body surface area. They were immersed in 90.0 °C water for 9 s, resulting in a full-thickness scald injury. Following injury, mice received intraperitoneal resuscitation with 1.5 mL sterile saline. The mice were allowed to recover on a 42.0 °C heating pad for 3 h following scald injury. Sham treated mice underwent the above procedure, except they were not immersed in water.

2.3. Butyric acid measurements

Mice were subjected to sham or scald injury as described above. On PBD 1–8, stool samples were collected from the cecum and butyric acid levels were analyzed as previously described [35]. Briefly, stool samples were weighed and diluted at a ratio of 1:5 in 25% *meta*-phosphoric acid. The samples were centrifuged at 16,000*g* for 15 min at 4.0 °C, filtered through 0.45 μm syringe tip filter (Thermo Fischer Scientific, Waltham, MA), and then stored at −80.0 °C until ready for analysis. Butyrate concentrations were performed by high performance liquid chromatography (HPLC) using a Rezex ROA-organic acid H+ (8%) 300 × 7.8 mm analytical column (Phenomenex, Torrance, CA) at 65.0 °C. A 0.01 N sulfuric acid mobile phase at a flow rate of 0.7 mL/min was used. A UV detector set at wavelength 210 nm monitored the effluent. Butyric acid levels were compared to standards ranging from 1 to 100 mM. Concentrations were corrected for dilution and fecal weight and expressed as μmol per gram of wet weight feces [35,36].

2.4. Asm activity

Spleens were harvested from uninjured mice and T cells were separated on an AutoMACS as described by the manufacturer (Miltenyi Biotec, Auburn, CA). The isolated T cells were incubated for 1 h at 37 °C *in vitro* with or without 100 μM sodium butyrate (Sigma-Aldrich, St. Louis, MO). Separately, spleens were harvested from sham or PBD 6 mice and T cells isolated by AutoMACS. All samples were frozen at −80 °C until ready for Asm analysis. Asm activity was quantified as previously described [37] in which the release of ^{14}C-phosphorylcholine from [^{14}C]-sphingomyelin was used to determine Asm activity.

2.5. Microbiota reduction via antibiotic treatment

Mice underwent oral gavage with a broad-spectrum antimicrobial cocktail of 200 μL for ten days as described previously [38]. For the first three days, mice were treated with 1 mg/kg amphotericin B (Sigma-Aldrich). Subsequently, on days four through ten, mice were gavaged with an antimicrobial cocktail consisting of 50 mg/kg vancomycin hydrochloride (Hospira, Inc., Lake Forest, IL), 100 mg/kg neomycin sulfate (Santa Cruz Biotechnology, Dallas, TX), 100 mg/kg metronidazole (Acros Organics, NJ) and 1 mg/kg amphotericin B. Additionally, 1 g/L of ampicillin (Sandoz Inc., Princeton, NJ) was added to their drinking water. Control mice were gavaged with equal volume of sterile saline.

2.6. Flow cytometry analysis of surface antigens

Analyses of cell surface antigen expression were performed as previously described [39] on harvested spleens. Flow cytometry data acquisition and analysis were performed on an Attune Acoustic Focusing Cytometer using Attune Cytometric Software v2.1. The following antibodies were used: CD4 (Clone: RM4-5, BD Biosciences, San Jose, CA), CD8 (Clone: 53-6.7, BD Biosciences), CD44 (Clone: IM7, BD Biosciences), and CD62L (Clone: MEL-14, BD Biosciences). Naïve T cells were identified on flow cytometry with $CD62L^{Hi}/CD44^{Lo}$ antigen expression.

2.7. T Cell apoptosis determination

For T cell apoptosis measurements, splenocytes were isolated from CF-1 mice and cultured for 24 h. Cells were incubated at 37 °C with 0 μM, 0.1 μM, 1 μM, 10 μM, 100 μM, or 1000 μM concentrations of butyrate (Sigma-Aldrich) and 1 nM dexamethasone (Sigma-Aldrich). To evaluate phosphatidyl serine exposure, cells were then labeled for Annexin V (BD Pharminogen) as previously described [40]. In a subsequent experiment, splenocytes were harvested from Asm WT or KO mice. These cells were incubated for 24 h at 37 °C with one of the following treatments: A) (−) butyrate/(−) dexamethasone, B) 100 μM butyrate, C) 1 nM dexamethasone or D) 100 μM butyrate and 1 nM dexamethasone. Again, cells were