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Short Communication

The commensal microbiota exacerbate infectious colitis in stressor-exposed mice

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ABSTRACT

Exposure to a prolonged restraint stressor disrupts the colonic microbiota community composition, and is associated with an elevated inflammatory response to colonic pathogen challenge. Since the stability of the microbiota has been implicated in the development and modulation of mucosal immune responses, we hypothesized that the disruptive effect of the stressor upon the microbiota composition directly contributed to the stressor-induced exacerbation of pathogen-induced colitis. In order to establish a causative role for stressor-induced changes in the microbiota, conventional mice were exposed to prolonged restraint to change the microbiota. Germfree mice were then colonized by microbiota from either stressor-exposed or non-stressed control mice. One day after colonization, mice were infected with the colonic pathogen, *Citrobacter rodentium*. At six days post-infection, mice that received microbiota from stressor-exposed animals had significant increases in colonic pathology and pro-inflammatory cytokine (e.g. IL-1β) and chemokine (e.g. CCL2) levels after *C. rodentium* infection in comparison with mice that received microbiota from non-stressed mice. 16S rRNA gene sequencing revealed that microbial communities from stressed mice did not have any detectable *Bifidobacterium* present, a stark contrast with the microbial communities from non-stressed mice, suggesting that stressor-induced alterations in commensal, immunomodulatory *Bifidobacterium* levels may predispose to an increased inflammatory response to pathogen challenge. This study demonstrates that the commensal microbiota directly contribute to excessive inflammatory responses to *C. rodentium* during stressor exposure, and may help to explain why gastrointestinal disorders are worsened during stressful experiences.

1. Introduction

The gastrointestinal (GI) tract is colonized by a consortium of microbes, including bacteria, fungi, viruses, and archaea, that are collectively termed the microbiota. Commensal bacteria that reside as part of the microbiota normally exhibit considerable stability under healthy host conditions. However, when disruption in the microbial communities that inhabit both the lumen and mucosa of the GI tract do occur (in a state known as dysbiosis), they can negatively feedback upon the host and lead to dysfunction in host physiology and immunology (Chen et al., 2015; De Minicis et al., 2014; Duboc et al., 2013; Qin et al., 2012). A number of external effectors can induce dysbiosis, including antibiotics and diet, leading to well-established changes in community structure and resultant feedback upon host health (Kim et al., 2012, 2014; Martinez-Medina et al., 2014). Perception of a psychological stressor has also been implicated in microbiota community structure alterations, but prior to this study it was not known whether stressor-induced changes in the microbiota also directly impact host health.

Previous studies have demonstrated that exposure to prolonged restraint stress significantly shifts the mucosal-associated microbiota and reduced the relative abundance of the immunomodulatory genus, *Lactobacillus* (Galley et al., 2014b). Similar findings have been extended to human and non-human primate hosts (Bailey and Coe, 1999; Knowles et al., 2008). Exposure to prolonged

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restraint stress also exacerbates the inflammatory response to enteric pathogen challenge in animal models (Bailey et al., 2010; Mackos et al., 2013) as evidenced by increases in inflammatory cytokine mRNA and colonic pathology. However, whether stressor-induced dysbiosis is directly linked to host physiological or immune function has not yet been elucidated. Thus, this study aimed to determine whether stressor-induced changes to the colonic microbiota lead to increased colonic inflammation upon challenge with the colonic pathogen *Citrobacter rodentium*.

Germ-free (GF) mice are commonly used for examining the effects the microbiota have upon host physiology and immunity. For example, through the use of GF models, the microbiota have been implicated in weight gain, wherein conventionalized mice had increased fat deposition compared to GF mice (Turnbaugh et al., 2006). Germfree mice have also been used to demonstrate that the microbiota contribute to the development of mucosal immune cell (e.g. Th17 cells, macrophages) maturation, abundance and activation (Ivanov et al., 2008; Niess and Adler, 2010; Souza et al., 2004). Additionally, transplanting the microbiota from donors with altered genotypes (e.g. NOD2$^{-/-}$ mice) or phenotypes (e.g. obese humans) into GF mice via fecal transplant has been used to analyze the complex interplay between host-mediated modeling of the microbiota compositional structure, and microbiota-mediated feedback on host function (Couturier-Maillard et al., 2013; Ridaura et al., 2013). We hypothesized that stressor-induced alterations to the microbiota were directly associated with a heightened inflammatory response to colonic pathogens, and we tested this hypothesis by colonizing GF mice with the microbiota from stressor-exposed mice and assessing the inflammatory response to challenge with *C. rodentium*. To our knowledge, this is the first study in which the microbiota from stressed mice were transplanted into naïve GF mice, and thus represents a significant step in the characterization of the effect of psychological stress upon the microbiota and the resultant impacts upon host physiology and immunity.

2. Materials and methods

2.1. Mice

Conventional male CD-1 mice (aged 6–8 weeks) were ordered from Charles River Laboratories (Raleigh, NC). Mice were housed 3 per cage and acclimated for a week in the vivarium before stressor exposure. Healthy male germ-free (GF) Swiss Webster (aged 6–8 weeks) mice were kept in sterile cages within a fully decontaminated BSL-2 level biosafety cabinet for the duration of the study. Mice were handled under aseptic conditions with sterile gloves as we have previously reported (Allen et al., 2012). Germfree mice were monitored by periodic fecal Gram-staining, 16 s PCR, and intestinal pathology tests performed by the Ohio State University Lab Animal Resource Department and by our own laboratory and were confirmed to be free of pathogens and other microbial contaminants. All mice were given sterilized food and water ad libitum and kept on a 12-h light:dark cycle (Conventional mice-0600-1800 lights on, Germ-free mice-0700-1900 on). All stressors, infection, and sacrifice protocols were approved by the Ohio State University Animal Care and Use Committee.

2.2. Stressor

Restraint (RST) stress was administered to conventional mice as previously published (Galley et al., 2014b). Briefly, mice were placed in a 50-mL conical tube for 16 consecutive hours, beginning at 1700 and concluding at 0900 the following morning. The RST stressor was continued each night for a total of seven cycles. Two control groups were used: a food and water deprivation group that had food and water removed during the restraint period, but were not otherwise restrained (FWD), and a control group that was undisturbed and kept in a separate room (HCC).

2.3. Germ-Free Reconstitution Experiments

Immediately following the final cycle of the respective stressors, conventional mice were euthanized via CO_2 asphyxiation. Colons were aseptically removed, and the tissue was bisected using sterile forceps and scissors. An equivalent amount of fecal pellets for each group was pooled in 3 mL of anaerobically pre-reduced PBS, in addition to a mucosal scraping from each colon. Each donor group was comprised of pooled fecal and mucosal slurry from three total mice per experiment. The fecal slurry was immediately placed in an anaerobic canister with a BD GasPak EZ until transplant into the GF mice. Fecal microbiota were transplanted to GF mice via oral gavage within two hours of conventional mouse sacrifice. GF mice were gavaged with 200 μL total fecal slurry, and then were food and water deprived for two hours. Germfree mice that received microbiota from stressor-exposed donors are labeled RST-GF (n = 7), those receiving microbiota from non-stressed control donors are labeled HCC-GF (n = 6), and those receiving microbiota from donors that were deprived of food and water during the periods that restrained animals were in the restraining tubes are labeled FWD-GF (n = 6). The slurries were kept at −80 °C until sequencing via Illumina at a later date.

2.4. Bacteria

Citrobacter rodentium strain DBS120 (pCRP1::Tn5) was grown overnight at 37 °C in tryptic soy broth. After growth, the pathogen culture was brought up to 1×10^9 CFU/mL in sterile water and 100 μL was given to RST-GF, HCC-GF, or FWD-GF mice 24 h after receiving the fecal microbiota transplant. Mice were monitored and sacrificed at day 6 post-infection. Total infectious burden was measured by plating shed fecal pellets from the GF mice, at days 3 through 6 post-infection, on MacConkey agar supplemented with 40 μg/mL kanamycin.

2.5. qRT-PCR

On Day 6 post-infection, colons were collected and bisected. Half was used for RNA isolation using the previously published TriZOL method (Mackos et al., 2013). Briefly, isolated RNA was normalized to 1 μg per sample and then reverse transcribed to cDNA via Promega Reverse Transcription System. Multiplex qRT-PCR was performed on the ABI Prism 7000 system using primers targeting mRNA for interleukin-1β (Forward: GGCCTCAAAGGAAAGAATCTATACC; Reverse: GTATTGCTTGGGATCCACACTCT, probe: ATGAAAGACGGCACACCCACCCTG), CCL2 (Forward: TTGGCTCAGCCAGATGCA; Reverse CCTACTCATTGGGATCATCTTGC; Probe: AACGCCCCACTCACCTGCTGCTACT), and inducible nitric oxide synthase (iNOS, Forward: CAGCTGGGCTGTACAAACCTT; Reverse: TGAATGTGATGTTTGCTTCGG, Probe: CGGGCAGCCTGTGAGACCTTTGA). Murine 18S (Forward: CGGCTACCACATCCAAGGAA; Reverse: GCTGGAATTACCGCGGCT; Probe: TGCTGGCACCAGACTTGCCCTC) was used as a housekeeper gene. The comparative threshold cycle method was used for data analysis as previously described, with HCC-GF set as baseline (Bailey et al., 2010).

2.6. Histopathology

Half of the colon collected from RST-GF, HCC-GF, and FWD-GF mice was fixed in neutral buffered formalin until paraffin embedding and hematoxylin and eosin staining. Total colonic pathology