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Indole-3-carbinol prevents colitis and associated microbial dysbiosis in an IL-22-dependent manner

Philip B. Busbee,1 Lorenzo Menzel,1 Haider Rasheed Alrafas,1 Nicholas Dopkins,1 William Becker,1 Kathryn Miranda,1 Chaunbing Tang,2 Saurabh Chatterjee,3 Udai P. Singh,1 Mitzi Nagarkatti,1 and Prakash S. Nagarkatti1

1Department of Pathology, Microbiology and Immunology, University of South Carolina School of Medicine, Columbia, South Carolina, USA. 2Department of Chemistry and Biochemistry, University of South Carolina College of Arts and Sciences, Columbia, South Carolina, USA. 3Environmental Health and Disease Laboratory, Department of Environmental Health Sciences, Arnold School of Public Health, University of South Carolina Columbia, South Carolina, USA.

Introduction

Colitis, an inflammatory bowel disease, is caused by a variety of factors, but luminal microbiota are thought to play crucial roles in disease development and progression. Indole is produced by gut microbiota and is believed to protect the colon from inflammatory damage. In the current study, we investigated whether indole-3-carbinol (I3C), a naturally occurring plant product found in numerous cruciferous vegetables, can prevent colitis-associated microbial dysbiosis and attempted to identify the mechanisms. Treatment with I3C led to repressed colonic inflammation and prevention of microbial dysbiosis caused by colitis, increasing a subset of gram-positive bacteria known to produce butyrate. I3C was shown to increase production of butyrate, and when mice with colitis were treated with butyrate, there was reduced colonic inflammation accompanied by suppression of Th17 and induction of Tregs, protection of the mucus layer, and upregulation in Pparg expression. Additionally, IL-22 was increased only after I3C but not butyrate administration, and neutralization of IL-22 prevented the beneficial effects of I3C against colitis, as well as blocked I3C-mediated dysbiosis and butyrate induction. This study suggests that I3C attenuates colitis primarily through induction of IL-22, which leads to modulation of gut microbiota that promote anti-inflammatory butyrate.

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of intestinal homeostasis, and Th17 cells, which are often associated with proinflammatory responses leading to colitis (10, 11). Thus, AhR is a potential therapeutic target against colitis, and known ligands for this receptor could serve as critical preventive and therapeutic agents.

Indole-3-carbinol (I3C), a natural plant product found in many cruciferous vegetables, is a promising candidate for the treatment of colitis because it is both a known ligand for AhR (12, 13) and was shown to affect bacteria and bacterial byproducts, key components of the gut microbiome (14, 15). Some early reports showed that I3C was able to reduce the severity of dextran sodium sulfate–induced (DSS-induced) colitis, and this effect was lost in AhR−/− mice, resulting in a more severe disease state (16). However, currently, there are no studies reported that focus on how I3C treatment of colitis affects the gut microbial environment and, consequently, the inflammation in the gut. In the current study, therefore, we investigated the effect of I3C on 2,4,6-trinitrobenzenesulfonic acid–induced (TNBS-induced) colitis with concurrent analysis of the gut microbiome and metabolites. We demonstrate that I3C can modulate the gut microbiome during colitis in such a way that it prevents the overgrowth of potential pathogenic bacteria while selectively allowing antiinflammatory butyrate production, thereby inducing an antiinflammatory state. However, induction of IL-22, which has been shown to promote host defense against microbes (17), was found to occur after I3C administration but not with butyrate supplementation. Neutralizing IL-22 negated I3C-mediated protective effects against colitis, particularly by preventing alterations in the gut microbiome and increases in butyrate levels, suggesting that this cytokine was essential in regulating colitis-associated microbial dysbiosis.

**Results**

**I3C treatment reduces colitis symptoms and alters CD4+ Th17 and Treg subsets in the mesenteric lymph node.** In order to test the efficacy of I3C treatment in the TNBS colitis model, mice with colitis were treated with an I3C regime previously reported by us to be effective in other types of inflammatory disorders (14, 18, 19), as illustrated in Figure 1A. We used 4 groups of mice: Vehicle alone, I3C alone, TNBS + Vehicle, and TNBS + I3C. We observed a 15%–20% reduction in weight of TNBS + Vehicle–treated mice (Figure 1B). However, in the TNBS + I3C group, this percentage of weight loss was significantly reduced. Mice induced with TNBS colitis also had significant reduction (~2 cm) in the overall length of the colon compared with control groups (Figure 1C). However, colitis mice treated with I3C prevented this colon-shortening effect. For the macroscopic colitis score, TNBS + Vehicle mice had significantly higher scores compared with controls, which were reduced in the TNBS + I3C group (Figure 1D). FITC-dextran gut permeability assay was also performed, and TNBS + Vehicle mice showed increased levels of detectable fluorescein in the circulating serum compared with controls, indicative of increased intestinal permeability (Figure 1E). In contrast, TNBS + I3C mice had much lower levels of FITC-dextran circulating in the blood, comparable with the baseline levels of controls. Next, we measured circulating levels of serum amyloid A (SAA), a common inflammatory biomarker found to be increased in patients with Crohn’s disease and ulcerative colitis (20). TNBS + Vehicle mice had significantly higher concentrations of SAA when compared with control mice, but these levels were reduced upon treatment with I3C (Figure 1F). To test if I3C was also effective against another model of colitis, we used DSS-induced colitis and found that I3C was able to significantly reduce disease parameters in the DSS model as it did in TNBS model, to include effects in weight loss, colon length, macroscopic colitis score, and SAA levels (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.127551DS1).

Previous reports — including results from our laboratory — have shown that, in the TNBS and DSS models of colitis, there is a significant increase in the inflammatory Th17 subset in the colon and colonic-associated mesenteric lymph node (MLN) (21–24), which plays a role in pathogenesis. To that end, we studied Th17/Treg subsets in the MLN and found that, in the TNBS + Vehicle group, there was a significant increase in the number of Th17 cells (Figure 1G). However, treatment with I3C prevented Th17 expansion and significantly increased Tregs in the MLN (Figure 1H).

Colonoscopy images revealed that the TNBS + Vehicle group had evidence of ulcerations and tissue sloughing in the lining of the colon, whereas these observations were far less in colonic linings of controls or colitis mice treated with I3C, resulting in a less severe colonoscopy score (Figure 1I). In support of these endoscopic observations, histological evaluation revealed overall tissue destruction in mucosa, submucosa, and LP layers; loss of crypts; and increased evidence of cellular infiltration in diseased mice when compared with controls (Figure 1J). Colons from TNBS-induced colitis mice treated with I3C, however, maintained crypt formation and normal colonic tissue architecture, and they showed reduced signs of
cellular infiltration. Representative colonoscopy and H&E colon stains are depicted in Figure 1, K and L. Collectively, these data effectively demonstrate that I3C was able to protect mice from TNBS-mediated colitis, which was associated with a decrease in Th17 and increase in Tregs.

Treatment with I3C prevents TNBS colitis-associated microbial dysbiosis. Next, we studied microbial dysbiosis induced by TNBS and the potential protective effect of I3C. We performed bacterial 16S rRNA gene sequencing studies using the MiSeq platform. Analysis of the sequenced data obtained from the colonic flushes was performed using the standardized online NIH-based analysis tool Nephele (25) — specifically, the 16S paired-end QIIME option — in order to determine differences in gut microbiome diversity, phylogeny, and function. In terms of the α diversity, or overall species richness within each sample, there were no significant differences detected, as illustrated by the chao1 rarefaction measurement (Figure 2A). However, in terms of the β diversity, which measures the similarity or dissimilarity between the experimental groups, there was evidence of clear separation of the disease group (TNBS + Vehicle) from the control (Vehicle) and treatment (TNBS + I3C) groups (Figure 2B). While the colitis disease samples clustered and separated from the other 2 groups, the control and I3C treatment samples clustered together much closer to one another. This suggested that the microbiome composition in the TNBS + I3C group resembled more closely the controls than the TNBS + Vehicle mice.

Phylogenetic analysis of the gut microbiome from the phylum to the species level was performed on the samples using standard operational taxonomic unit (OTU) classification (Supplemental Figures 2–7). A distinct bacterial signature was found in the TNBS + Vehicle mice that differed from the I3C-treated and controls — particularly in the enrichment of mostly gram-negative bacteria in the Bacteroidetes phylum. At the phylum level, mice with colitis had increased abundance in both Proteobacteria and the more recently discovered TM7 bacteria compared with control or I3C-treated mice (Supplemental Figure 3). A similar lineage pattern was seen at the class level, as Gammaproteobacteria and TM7-3 were enriched in TNBS + Vehicle mice, as well as at the order level with increased abundance of Pseudomonadales, a subset of Gammaproteobacteria, and CW040, a subset of TM7-3 (Supplemental Figures 4 and 5). At the family to genus level, TNBS + Vehicle mice were enriched in gram-negative bacteria (Bacteroides, Odoribacter, Prevotella, and Pseudomonas) compared with the other groups (Supplemental Figures 6 and 7). Of those disease-enriched bacteria, only Bacteroides and Prevotella were found to be significantly increased in the TNBS + Vehicle group when compared with controls or TNBS + I3C (Supplemental Figure 8, A and B). At the species level, Bacteroides acidifaciens (B. acidifaciens) was shown to be significantly more abundant in TNBS + Vehicle mice but was effectively reduced with I3C treatment (Supplemental Figure 8C). In almost all the bacterial taxa, the TNBS + I3C group had very similar microbial gut composition as the controls, except for 2 gram-positive Firmicutes enriched only in the TNBS + I3C treatment group (Planococaceae and Roseburia) (Supplemental Figure 7). Among these 2, Roseburia was the only one found to be significantly enriched in the TNBS + I3C–treated mice compared with TNBS + Vehicle and control groups (Supplemental Figure 8D). Linear discriminant analysis effect size (LefSe) (26) was performed to identify any key microbial biomarkers that might differentiate both the disease and I3C-treated states. In comparisons of the Vehicle versus TNBS + Vehicle groups, linear discriminant analysis (LDA) scores (Figure 2C) and the corresponding cladogram (Supplemental Figure 9A) showed that colitis mice were most enriched in gram-negative Odoribacter, Prevotella, and Bacteroides, including B. acidifaciens. However, the Vehicle control mice had higher enrichment of gram-positive Firmicutes to include Bacilli (e.g., Lactobacillus) and Clostridium microbes. When comparing the TNBS + Vehicle with TNBS + I3C groups, Roseburia was found to have the highest LDA score and enrichment in the I3C-treated mice (Figure 2D and Supplemental Figure 9B). Collectively, these data show that a combination of enriched gram-negative bacteria (Odoribacter, Prevotella, and Bacteroides) could serve as potential biomarkers of colitis, whereas effective treatment with I3C was characterized by an enrichment in gram-positive bacteria in the genus Roseburia.

In order to further confirm results from the Nephele and LefSe data outputs, PCR was performed with primers specific for the species B. acidifaciens (Figure 2E) and genus Roseburia (Figure 2F). Results from the PCR validation studies confirmed observations from the sequencing data. Specifically, B. acidifaciens was found to be significantly increased in the TNBS + Vehicle mice (Figure 2E), while Roseburia was significantly higher in the TNBS + I3C group (Figure 2F). Taken together, the phylogenetic data reveal that I3C treatment effectively prevented TNBS-induced microbial dysbiosis, which was characterized by increases in gram-negative bacteria, but I3C also selectively increased gram-positive Roseburia, which are known to increase production of SCFA butyrate.

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[154x107]and treatment (TNBS + I3C) groups (Figure 2B). While the colitis disease samples clustered and separat-

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[154x119]Roseburia was found to be significantly increased in the TNBS + Vehicle mice (Figure 2E), while

[154x120]PCR validation studies confirmed observations from the sequencing data. Specifically,

[154x123]Bacteroides acidifaciens

[154x126]was found to be significantly more abundant in

[154x129]TNBS + Vehicle mice but was effectively reduced with I3C treatment (Supplemental Figure 8C). In almost

[154x132]all the bacterial taxa, the TNBS + I3C group had very similar microbial gut composition as the controls,

[154x135]except for 2 gram-positive Firmicutes enriched only in the TNBS + I3C treatment group (Planococaceae and

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[154x141]enriched in the TNBS + I3C–treated mice compared with TNBS + Vehicle and control groups (Supplemental Figure 8D). Linear discriminant analysis effect size (LefSe) (26) was performed to identify any key microbial biomarkers that might differentiate both the disease and I3C-treated states. In comparisons of the Vehicle versus TNBS + Vehicle groups, linear discriminant analysis (LDA) scores (Figure 2C) and the corresponding cladogram (Supplemental Figure 9A) showed that colitis mice were most enriched in gram-negative Odoribacter, Prevotella, and Bacteroides, including B. acidifaciens. However, the Vehicle control mice had higher enrichment of gram-positive Firmicutes to include Bacilli (e.g., Lactobacillus) and Clostridium microbes. When comparing the TNBS + Vehicle with TNBS + I3C groups, Roseburia was found to have the highest LDA score and enrichment in the I3C-treated mice (Figure 2D and Supplemental Figure 9B). Collectively, these data show that a combination of enriched gram-negative bacteria (Odoribacter, Prevotella, and Bacteroides) could serve as potential biomarkers of colitis, whereas effective treatment with I3C was characterized by an enrichment in gram-positive bacteria in the genus Roseburia.

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In addition to the phylogenetic results acquired from the 16S rRNA gene sequencing data, the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PiCRUSt) option in the Nephele analysis pipeline was used to determine how changes in the gut microbiome were affecting Level 2 (L2) and L3 KEGG pathways, a theoretical method used to determine potential changes in microbial functions based on bacterial OTU composition. At L2 pathways, TNBS mice had significant increases in percent abundances in the digestive system and amino acid metabolism, but these were reduced with I3C treatment (Supplemental Figure 10A). Of the 328 identified L3 pathways, 43 were found to be significantly altered either between the control and TNBS + Vehicle groups or between the TNBS + Vehicle and TNBS + I3C groups (Supplemental Figure 10B). Among those 43, 4 were found to be significantly enriched in the colitis samples compared with controls but were reduced after treatment with I3C. These pathways included riboflavin metabolism, tropane,
piperidine and pyridine alkaloid biosynthesis, α-linolenic acid (ALA) metabolism, and ubiquitin system. Pentose and glucoronate interconversions and electron transfer carriers were the only 2 L3 pathways found to be significantly lower in abundance in the TNBS + Vehicle mice compared with controls, but they were restored in the TNBS + I3C–treated groups. Among all aforementioned significantly altered pathways, LefSe analysis showed that riboflavin metabolism and the pentose and glucoronate interconversion pathways could serve as biomarkers for the TNBS + Vehicle groups (Figure 3A and Supplemental Figure 11).

Next, we measured SCFA concentrations in colonic flushes, as shown in representative GC plots in Figure 3B. Acetic acid (Figure 3C), propionic acid (Figure 3D), and i-butyric/isobutyric (Figure 3E) were not significantly altered compared with control TNBS + Vehicle, and TNBS + I3C treatment samples. However, I3C treatment was found to significantly increase the concentration of n-butyric acid (Figure 3F) compared with control; disease studies showed that alterations in microbial composition and function caused by TNBS-induced colitis can be effectively prevented with I3C treatment and that I3C is able to alter butyrate production, perhaps through the ability of this compound, to selectively increase bacterial species in the genus Roseburia, which are known to produce high levels of butyrate.
Treatment with sodium butyrate prevents TNBS-induced colitis–associated symptoms. Collectively, the results thus far showed that I3C was able to increase colonic butyrate levels in the TNBS model of colitis, which correlated with the observation that I3C treatment leads to an increase in butyrate-producing Roseburia spp. With this information, butyrate supplementation experiments were performed in lieu of standard microbiome transfer experiments to determine what I3C effects, if any, could be mediated through increased butyrate production. To that end, TNBS colitis mice were treated with sodium butyrate (NaB) as detailed in Figure 4A. The dose and route of NaB mimicked previous reports in other mouse models (27, 28) and correlated with similar colonic butyric acid concentrations seen in the I3C treatment experiments (29).
In the experiments replacing I3C with NaB, striking similarities in results and outcomes were observed. NaB treatment was able to significantly reduce weight loss (Figure 4B), colon shortening (Figure 4C), and macroscopic colitis scores (Figure 4D) compared with the disease controls, similar to I3C. Treatment of TNBS colitis mice with NaB also reduced colitis-associated gut permeability (Figure 4E) and SAA levels (Figure 4F). T cell subtyping in the MLNs showed that NaB treatment was also able to effectively reduce the increases in disease-associated Th17 cells (Figure 4G) and significantly induce antiinflammatory Tregs (Figure 4H). Colonoscopy images on day 3 revealed that the diseased mice had ulcerations and tissue sloughing, which were not present in the TNBS mice treated with NaB (Figure 4I), reflected in an increased colonoscopy score (Figure 4J). Also, in the TNBS-induced colitis mice, the colon histology preparations showed significant amounts of cellular infiltration and complete destruction of the colonic tissue normal architecture, whereas in diseased mice treated with NaB, there were minimal signs of damage, in the form of elongated cellular crypt formation indicative of tissue healing processes (Figure 4K), reflected in the histopathological scores (Figure 4L). All of these observations indicated that NaB was able to ameliorate colitis disease parameters, but more importantly, the fact that these results closely resembled the outcomes in the I3C experiments strongly suggested that butyrate-induction by I3C is a key mechanistic factor in the natural compound’s effectiveness in countering the detrimental effects caused by colitis induction.

I3C and NaB prevent bacterial localization into colonic crypts, maintain protective mucus production, and increase Pparg expression during TNBS-induced colitis, but only I3C increases IL-22 production. Because a continuous layer of mucus in the colon prevents luminal bacteria from coming into close contact with the epithelial surface, and the fact that increases in mucus-degrading bacteria play a role in ulcerative colitis (30), we investigated the effect of I3C on mucus production in the colon. To examine bacterial localization, transmitting electron microscopy (TEM) was performed on colon tissues extracted from all experimental groups. Collected TEM images show the relative proximity of bacteria in the mouse colon (Figure 5A). In the Vehicle, I3C, and NaB groups, clear separation of intestinal microbes away from the intestinal epithelium surface was maintained. In the TNBS + Vehicle control, the image is a colonic crypt in cross-section with bacteria entering the crypt lumen, which was not present in any of the colon sections collected. Treatment of TNBS-induced colitis by I3C or NaB treatment reestablished the distance between intestinal epithelium and gut microbes that was disrupted by TNBS-induced colitis. Such localization of the bacteria in the colonic crypt region is indicative of breakdown in the protective mucus layer. To examine this further, PAS staining was performed on colon tissue sections. Control tissues (Vehicle, I3C, NaB) showed normal staining with bright magenta highlighting goblet cells and mucus secretion in the crypt lumen (Figure 5B). However, in the disease tissue of TNBS + Vehicle mice, the presence of goblet cells and mucus production was greatly reduced. Treatment of colitis mice with I3C or NaB restored the presence of crypt-associated goblet cells and mucus, indicating that these treatments were maintaining the protective mucus layer of the colon, which could be preventing bacteria from coming into close contact to the epithelial surface. To further substantiate this, colonic expression of Muc2 was determined, as Muc2 serves as a key component of the mucus layer in the colon (31). Results of colonic Muc2 expression showed that treatment with I3C significantly increased expression of mRNA for this protein when compared with TNBS + Vehicle or normal control groups (Figure 5C), thus reinforcing results from the PAS stains. While NaB was shown to trend toward an increase in Muc2 expression, especially compared with the disease state, the results were not found to be significant.

More recent reports have shown that receptor PPARG and cell signaling IL-22 play significant roles in intestinal health, where both are implicated in regulating the immune response in the colon epithelial, particularly to gut microbes (17, 32, 33). There is also evidence that butyrate can act as an agonist to the PPARG (34). With this knowledge, expression of mouse Pparg mRNA was determined. Colitis mice treated with either I3C or NaB had significant increases in Pparg compared with either naive controls or TNBS + Vehicle mice (Figure 5D). Interestingly, when examining the expression of colonic IL-22, only I3C, and not NaB, was able to significantly increase this cytokine (Figure 5E). Taken together, these studies highlight how I3C, perhaps through butyrate-dependent and butyrate-independent manners, can affect important key regulators of host-microbe interactions in the colon.

IL-22 neutralization negates protective effects of I3C against TNBS-induced colitis. Because IL-22 expression levels were shown to increase after I3C treatment in the colon, secretion of this cytokine was evaluated during disease (TNBS + Vehicle) and treated (TNBS + I3C) states. Colon explants from experimental mice were excised and cultured overnight before detecting IL-22 secretion levels. As shown in Figure 6A, IL-22
Figure 4. Treatment with NaB resulted in amelioration of TNBS-induced colitis. (A) TNBS colitis was induced in mice to test the efficacy of treatment with NaB with the following experimental groups: Vehicle ($n = 7$), NaB ($n = 6$), TNBS + Vehicle ($n = 9$), and TNBS + NaB ($n = 9$), with data combined from 3 independent experiments unless otherwise stated. (B–D) Disease parameters accessed included percent weight loss (B), colon length (C), and macroscopic score (D). (E) FITC-dextran was given to experimental mice by oral gavage on day 3 of the TNBS model, and serum was collected 4 hours later to test gut permeability for Vehicle ($n = 5$), NaB ($n = 5$), TNBS + Vehicle ($n = 6$), and TNBS + NaB ($n = 6$). Data depicted are combined from 2 independent experiments. (F) On day 3, serum was also collected to determine the levels of circulating SAA for Vehicle ($n = 6$), NaB ($n = 5$), TNBS + Vehicle ($n = 7$), and TNBS + NaB ($n = 9$). Data are combined from 2 independent experiments. (G and H) Total cell numbers of T cell subsets in the MLN were determined for Th17 (G) and Tregs (H) for Vehicle ($n = 6$), NaB ($n = 5$), TNBS + Vehicle ($n = 9$), and TNBS + I3C ($n = 9$). (I and J) Representative colonoscopy images (I) taken during day 3 of the TNBS model and scored (J; $n = 5$). (K and L) Representative H&E stains (K) were scored appropriately (L; $n = 5$). Scale bars: 100 μm (original magnification, ×100). Images are combined representative samples from 3 independent experiments. Data are shown as mean ± SEM, and significance was determined using 1-way ANOVA and Tukey’s multiple comparisons test; *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; ****$P < 0.001$. 
Figure 5. Treatment with I3C or NaB prevents bacterial localization into the colonic crypts, maintains mucus production, and leads to upregulation in Pparg, but it only I3C increases IL-22 in the TNBS model. (A) Colonic tissue sections were fixed and imaged with TEM. Representative images are shown for Vehicle (n = 6), I3C (n = 6), NaB (n = 6), TNBS + Vehicle (n = 6), TNBS + I3C (n = 6), and TNBS + NaB (n = 6). Black arrows indicate bacteria present in proximity to the colonic luminal surface microvilli, with TNBS + Vehicle image being a cross-section showing bacteria in the colonic crypt area. Scale bars indicated within images. (B) Colonic tissue sections were fixed and subjected to PAS staining. Representative images are shown for Vehicle (n = 6), I3C (n = 6), NaB (n = 6), TNBS + Vehicle (n = 6), TNBS + I3C (n = 6), and TNBS + NaB (n = 6). Scale bars: 100 μm (original magnification, ×100). Images are representative from 1 experiment out of 2 independent experiments performed. (C–E) Real-time PCR detecting colonic mRNA expression for Muc2 (C), Pparg (D), and IL-22 (E) was performed on colonic tissue samples (Vehicle n = 9, I3C n = 6, NaB n = 6, TNBS + Vehicle n = 9, TNBS + I3C n = 6, and TNBS + NaB n = 6) run in triplicate for each experimental group, and data are combined from 2 independent experiments. Data are shown as mean ± SEM, and significance was determined using 1-way ANOVA and Tukey’s multiple comparisons test; **P < 0.01; ***P < 0.005; ****P < 0.001.
was found to be secreted at higher levels after treatment with I3C compared with disease controls. With this in mind, the source of increased IL-22 secretion was determined by phenotyping the colonic LP fraction using flow cytometry. T helper cells that produce IL-22 were not found to be significantly altered among the experimental groups (Figure 6B). However, IL-22 in innate lymphoid Type 3 cells (ILC3s) was found to be significantly higher after treating with I3C (Figure 6C).

In order to determine if IL-22 secretion was necessary for I3C-mediated protection during colitis, I3C treatment of TNBS colitis experiments was repeated to include a neutralizing antibody against IL-22. Neutralization of IL-22 prevents I3C-mediated protection against colitis and ability to increase butyric acid. (A) Washed colonic explanted tissue (1 cm from cecum) were cultured for 24 hours (n = 5 per group). Supernatants were collected for analysis of IL-22 secretion by ELISA. (B) Total number of IL-22-expressing Th cells (n = 5 per group). (C) Total number of ILC3s expressing IL-22 (n = 5 per group). (D) TNBS colitis was induced in mice as described in Methods, and the efficacy of treatment with I3C in the presence or absence of neutralizing IL-22 antibody (aIL-22) was tested with the following experimental groups: TNBS + Isotype (n = 5), TNBS + IL-22 (n = 5), TNBS + I3C + Isotype (n = 5), and TNBS + I3C + aIL-22 (n = 5). Vehicle + Isotype (n = 5) and Vehicle + aIL-22 (n = 5) were included to assess weight and colon length compared with disease controls. (E-G) Weight loss (E) and colon length (F and G) were assessed. (H) Representative colonoscopy images taken during day 3 of the TNBS model are depicted, along with colonoscopy scores (I, n = 5). (J and K) Fold change of bacteria from colonic flushes using real-time PCR were determined for _B. acidifaciens_ (J) and _Roseburia_ (K). (L) SCFA n-butyric concentration was determined using GC-FID. Data are representative of 2 independent experiments. Data within dot plots and box-and-whisker plots equal the mean ± SEM, and significance was determined using 1-way ANOVA and Tukey’s multiple comparison test; *P < 0.05; **P < 0.01; ***P < 0.005; ****P < 0.001.
IL-22 (aIL-22), as detailed in Figure 6D. Interestingly upon aIL-22 treatment, the ability of I3C to prevent colitis-associated weight loss was reversed when compared with the isotype controls (Figure 6E). In addition, I3C treatment with the neutralizing antibody was not able to prevent colitis-associated shortening of the colon (Figure 6, F and G) nor damage to the colon (Figure 6, H and I). Most interesting was the loss in the ability of I3C to alter the gut microbiome after IL-22 neutralization. *B. acidifaciens*, which was decreased after I3C treatment with the isotype control (TNBS + I3C + Isotype), was no longer decreased after neutralizing IL-22 (TNBS + I3C + aIL-22) (Figure 6J). Even in the untreated disease state with neutralization of IL-22 (TNBS + Vehicle + aIL-22), there was a significant increase in *B. acidifaciens* when compared with the isotype control (TNBS + Vehicle + Isotype), suggesting that IL-22 plays an important role in regulating this colitis-associated bacterial species. Additionally, butyrate-producing *Roseburia*, which increased after I3C treatment (TNBS + I3C + Isotype), was no longer increased upon I3C + aIL-22 treatment (Figure 6K). In correlation with this observation of *Roseburia* regulation, while butyrate levels increased in I3C isotype controls (TNBS + I3C + Isotype), it was decreased significantly after aIL-22 treatment (Figure 6L). Collectively, these data demonstrated that IL-22 is important in I3C-mediated protective effects during colitis, particularly in regulating the gut microbiome and butyrate levels.

**Discussion**

Research and development of novel and safe therapeutics to combat this colitis are of prominent importance. Studies show that intestinal inflammation is linked to the loss of balance maintained by regulatory mechanisms occurring at the complex interaction of the host intestinal barrier and the lumen-bound microbes. There is a growing consensus that alterations in the gut microbiota are directly linked to the disease; whether this alteration is causative of the disease or a product of the disease state is still an area of research that is being explored. However, there appears to be some consistency in a microbial profile that occurs during colitis, which is further reinforced by this study.

In TNBS-induced colitis in rats, microbiome analysis revealed that the disease groups had increases in overall Bacteriodetes and a decrease in Firmicutes at the phylum level (35). In a murine DSS-induced colitis model, researchers found a significant increase in Bacteroidales, which correlated with intestinal mucin degradation, whereas Clostridiales decreased during inflammation, correlating with a reduction in butyrate (36, 37). These microbial profiles are not limited to animal models, as similar results have been found in the human populations diagnosed with colitis. In human inflammatory bowel disease (IBD) patients, certain bacteria seem to become more prominent when compared with healthy controls, such as members of the family Enterobacteriaceae, Bacteriodaceae, and Bacteroides (38–42). 16S rRNA gene profiles of mucosal tissue samples from ulcerative colitis patients found that this patient population had higher abundances of *Prevotella* and *Bacteroides* (43). Reports also indicated that an expansion in Proteobacteria is consistently found in IBD patients (44). TM7, a recently defined and cultured bacterium, often resides almost exclusively in the oral cavity, with limited abundance in the gut microflora. However, *TM7* was found to be significantly higher in mucosal samples in Crohn’s disease and ulcerative colitis patients when compared with normal controls (45). Interestingly, 16S rRNA microbiome profiling in the current study identified these alterations in the TNBS-induced mouse model, as well, which demonstrates that the current colitis model and microbiota study closely mimics what is seen in the human disease. In view of this, highlighted in this report is the increase in *B. acidifaciens* during TNBS-induced colitis, which was also observed in a mouse DSS model (37). This bacterial species displays key characteristics that could very well classify this as a colitis-specific biomarker — or, at the very least, a species that promotes colitis development and progression. *B. acidifaciens* was recently shown to act as a mucin degrader in vivo (46). Mucin serves as a natural barrier in the normal intestinal environment, and the degradation of this layer would help promote microbial dysbiosis and intestinal inflammation. In addition, *B. acidifaciens* is known to produce SCFA end products acetate, propionate, and succinate (47). These SCFAs — particularly acetate — when absorbed and transported to the liver, can lead to a decrease in butyrate, which serves as an energy source for colonic epithelial cells (48, 49). However, it should be noted that propionate supplementation in the DSS-induced colitis model was found to be protective (50), suggesting that the combination and concentration of these SCFAs might be playing a role in overall disease severity or regulation. Interestingly, in a recent study, *B. acidifaciens* was inoculated into mice to prevent obesity and resulted in increased weight loss and reduction in fat mass (51) — an outcome seen in colitis, as well.
Phylogenetic bacterial profiles were not the only aspects of microbial dysbiosis that were shared between the current study and the human disease condition. Bacterial function in the disease state was altered similarly, which is more than likely due to the changes in microbial diversity and abundance. For example, the current report highlights that microbial functions such as riboflavin metabolism, ALA metabolism, and ubiquitin system were all increased in the TNBS-induced disease state. Riboflavin metabolism, which is indicative of an increase in gram-negative bacteria, is a hallmark of an inflammatory environment characterized by increases in oxidative stress, and increased metabolism of riboflavin was observed in colitis patients (40, 52, 53). ALA is not produced by the human host and has been consumed as part of the host diet. This omega fatty acid is known to reduce the inflammatory response by reducing NF-κB activation and oxidative stress, and ALA supplementation was shown to reduce colitis severity (54). As this process is increased in the disease state, it can be reasoned that the microbes are metabolizing ALA and competing with the host, therefore depleting a potential antiinflammatory source. Lastly, an increase in ubiquitin system is well documented as an exploitative mechanism by human pathogenic bacteria on the host (55, 56), and this process was found to be altered in colitis (57). The fact that these alterations in microbial composition and function in the current study matched reports in human colitis populations is encouraging, but even more important is the fact that treatment with I3C prevented TNBS colitis–induced microbial dysbiosis, which could be attributed to the antimicrobial activities of I3C presented in earlier reports (58).

Of particularly note was the fact that I3C was shown to almost selectively lead to an increase in abundance of gram-positive bacteria known to produce butyrate (Roseburia spp.). This is a very significant finding, especially considering that supplementation of microbiota from Crohn's disease patients with Roseburia spp. led to increased butyrate production and enhancement in the intestinal epithelial barrier integrity (59). In addition, a recent report shows how R. intestinalis is able to prevent IL-17 secretion and increase Treg differentiation in TNBS-induced colitis (60). There are many reports showing that butyrate is effective at alleviating the burdens inflicted by colitis, particularly through mechanisms that include increases in antimicrobial peptides (AMPs) and mucus production (61–64). More recently, the PPARG signaling pathways (e.g., NF-κB and MAPK) have become a target for amelioration of colitis, and findings suggest that this receptor activation negatively regulates key inflammatory mediators (65–67). In the current study, I3C and NaB were able to significantly increase transcript expression of this receptor in the colon. It will be interesting to determine in future studies if these mechanisms regulated by I3C, to include increases in mucus and Pparg, are strictly dependent on butyrate or if this natural indole compound implores butyrate-independent mechanisms to elicit these responses. Regardless, the data in the current study is extremely encouraging, as it adds to the currently scarce amount of research studies providing evidence that I3C is a potential therapeutic agent in combating the effects of colitis. It is also exciting to show that I3C, along with its other beneficial properties, can modulate the gut microbiome during colitis in such a way that it prevents the overgrowth of potential pathogenic bacteria while selectively allowing an antiinflammatory butyrate-producer to thrive in the microenvironment.

As noted previously, I3C is a well-known ligand of AhR. In several different mouse models of colitis, activation of AhR by various known ligands resulted in protection against colitis, most notably through alterations in Treg and Th17 generation (68–71). More recently, it was shown that AhR ligands can stimulate AhR-expressing ILC3s, intraepithelial lymphocytes of the mucosa that are known to strengthen the intestinal epithelial cell barrier through an IL-22–dependent epithelial cell regeneration mechanism and produce AMPs (8, 72–75). In this regard, I3C may be able to prevent the expansion of pathogenic gram-negative bacteria in colitis through activating AhR-expressing cells (T cells, ILCs, colonic epithelial cells) to produce IL-22, promote the release of host AMPs, or alter the immune response in other ways. As butyrate treatment alone did not result in an increase in IL-22 mRNA expression, perhaps due to the fact that this SCFA generally acts through GPRs and not AhR signaling (76), these findings suggested that I3C is able to incorporate mechanisms independently of increasing butyrate production to prevent the negative effects associated with colitis. In this particular report, IL-22 production by ILC3s seemed to be altered after I3C treatment. This would suggest that an increase in butyrate-producers by I3C is a beneficial consequence, particularly in shifting Th17 to Treg populations, but the host defense to microbial dysbiosis by way of an increase in IL-22 is dependent solely on I3C, an activator of AhR. IL-22, via a CARD9-dependent mechanism, was shown to play an important role in attenuating intestinal inflammation during colitis, and mice deficient in CARD9 and IL-22 had altered microbiomes in addition to being susceptible to colitis development (77). Future studies investigating the regulation of IL-22 by I3C, and whether or not it is dependent on AhR activation,
will have to be conducted. In addition, while male and female mouse microbiomes have been shown to be similar (78), sex differences have been observed in both susceptibility to TNBS-colitis induction (79, 80) and response to AhR ligands (81–83). Inasmuch as the current report focuses on findings in female mice only, disease outcomes and key findings (e.g., increased IL-22 production by ILC3s) will have to be investigated in male mice, as well. In summary, the current study has identified IL-22 as the key molecule through which I3C mediates its beneficial effects against colitis, including reversal of colitis-mediated dysbiosis, increased butyrate production, and antiinflammatory response.

Methods

Animals. Female BALB/cJ and C57BL/6 mice were purchased from the Jackson Laboratory, and experimental mice used were between 8 and 10 weeks of age. All mice were housed at the Association for Assessment and Accreditation of Laboratory Animal Care–accredited (AAALAC-accredited) animal facility at the University of South Carolina School of Medicine. Mice were housed in specific pathogen-free conditions, under 12-hour light/12-hour dark cycles.

Induction of colitis and treatment with I3C, NaB, or neutralization of IL-22. TNBS colitis was induced in susceptible BALB/cJ mice (84). Mice were given a total volume of 50 μL intrarectal injections of 1 mg of TNBS (Sigma-Aldrich) in 50% ethanol under light isoflurane anesthesia, as previously reported (84). For treatment groups, mice were given either 100 μL i.p. injections of I3C (40 mg/kg in 0.05% DMSO/corn oil) or oral gavage of NaB (200 mg/kg in water), both purchased from Sigma-Aldrich, 2 hours after injection of TNBS. Subsequent treatments were given daily throughout the entire experiment. The dose and route of I3C is consistent with our previous reports (14, 18), and the NaB dosage and route were determined based on other published reports (27–29, 85). All experimental groups in the TNBS model were given 50% ethanol intrarectal injections to ensure changes observed were not due to administration of the ethanol diluent. Vehicle controls in these experiments were also given i.p. injections of 0.05% DMSO/corn oil. For the DSS model, DSS was purchased from Chem-Impex International and introduced into drinking water (ab libitum) at a 3% concentration for 1 week, followed by a week of regular drinking water for female C57BL/6 mice, as previously described (86). For treatment groups in the DSS model, I3C treatment (i.p. injections of 40 mg/kg in 0.05% DMSO/corn oil) begins 1 hour after introduction of DSS into the drinking water and was given every other day until completion of the experiment. Vehicle groups in the DSS model were also given i.p. injections of 0.05% DMSO/corn oil. For neutralization of IL-22 experiments, functional grade IL-22 monoclonal antibody (IL-22JOP; 16-7222-85) was purchased from Thermo Fisher Scientific, along with rat IgG2aκ isotype control (eBR2a; 16-4321-85). To neutralize IL-22, the aIL-22 was given i.p. 24 hours prior to administration of TNBS or treatment with I3C at a 50-μg dose in 100 μL of PBS (Sigma-Aldrich), as well as during the middle of the colitis experiments (day 2). Dose and route of IL-22 neutralization were based on a previous report (87).

Assessment of colitis disease parameters. For both colitis models, mice were weighed daily, and colon lengths were measured at experimental end-points. To determine macroscopic colitis scores in the experimental groups, a slightly modified scoring system (Table 1) was used based on previous reports (88). To test in vivo gut permeability, the FITC-dextran assay was used as reported elsewhere (89). Briefly, on day 3, mice were given 600 mg/kg of 4kD FITC-dextran (Sigma-Aldrich) dissolved in 100 μL of PBS by oral gavage. Four hours later, blood was collected from mice by retroorbital bleeding, and FITC-dextran concentrations were determined using a PerkinElmer Life Sciences spectrophotometer with excitation wavelength at 480 nm. SAA levels, which have been used in the clinic to determine the severity of colitis in patients (90), were assessed using a SAA mouse ELISA purchased by Abcam. SAA levels were determined using a PerkinElmer Life Sciences spectrophotometer with excitation wavelength at 480 nm. SAA levels, which have been used in the clinic to determine the severity of colitis in patients (90), were assessed using a SAA mouse ELISA purchased by Abcam. SAA levels were determined from serum collected at day 3 of the TNBS model and day 10 of the DSS model. Colonoscopy images were taken on day 3 of the TNBS model using a Karl Storz Tele Pack Vet X LED endoscope designed for small animals. The colonoscopy scoring parameters were based on a methodology previously reported (91).

Microscopic evaluation of the colon tissues. Colon histology was obtained by harvesting proximal colon samples on day 4 of the TNBS model. Tissues were fixed in methanol-Carnoy’s fixative solution for 24 hours and then embedded in paraffin before cutting into 9-μm sections. For H&E staining, sections were stained as previously described (92). Histopathological scoring parameters were based on previously published reports (93). For periodic acid-schiff (PAS) staining, a staining kit purchased from Sigma-Aldrich was used following manufacturer’s instructions. Images of stained sections were taken using a Leica DM2500 with LASX image capture suite (Leica Microsystems Inc.). For TEM images, mouse colons were harvested.
and prepared as previously described (92). Images were captured on a JEOL 1400S TEM located in the Instrumentation Resource Facility at the University of South Carolina School of Medicine.

**Analysis of Treg and Th17 subsets in MLN during colitis.** MLNs were excised from mice during experimental end-points and prepared for staining as previously described (14). To assess and quantify Treg/Th17 subsets, samples were stained with fluorescently labeled antibodies purchased from BioLegend. To quantify Treg subsets, True-Nuclear Transcription Factor Buffer Set (BioLegend) was used as per the instructions from the manufacturer to label cells with antibodies for CD3-FITC (clone 17A2), CD4-APC (clone GK1.5), and FoxP3-PE (clone MF-14). For Th17 subsets, BD Cytofix/Cytoperm from BD Biosciences was used to fix and permeabilize the cells for staining using CD4-APC and IL-17-PE (clone TC11-18H10.1) antibodies. BD FACS Celesta and FlowJo analysis software (BD Biosciences) were used to determine percentage of these T cell subsets, and total Treg/Th17 numbers were determined by multiplying this percentage by total number of cells isolated from the MLNs. Flow cytometry gating strategy and representative plots from experimental groups for Th17/Treg subsets are depicted in Supplemental Figure 12. Gates were positioned according to unstained (negative control) and single-color antibody controls (positive controls).

**Examination of IL-22 secretion from colonic explants.** Explants of colon tissues were prepared as previously described (94). Briefly, colon tissue (length of 1 cm from the cecum) was excised from experimental mice (TNBS + Vehicle and TNBS + I3C) and washed 3 times with PBS. Tissues were placed in 24-well cell culture plates in complete RPMI 1640 media (Corning) (10% FBS, 1% penicillin/streptomycin, 1% HEPES; Sigma-Aldrich) and incubated at 37°C at 5% CO₂ for 24 hours. Supernatants were collected following centrifugation at 8000 × g at 4°C for 10 minutes and stored at −80°C for later analysis by ELISA. Mouse IL-22 ELISA kit was purchased from BioLegend for detection of IL-22 in the collected supernatants.

**LP isolation and IL-22–positive cell phenotyping.** Cells from the LP fraction were isolated as previously described (24). IL-22 production by immune cells in the LP were phenotyped using flow cytometry (BD FACs Celesta) based on a previous report (95). The gating strategy is summarized in Supplemental Figure 13. Gating and analysis of flow plots was performed using FlowJo version 10.5.3 (BD Biosciences). For phenotyping, the following antibodies from BioLegend were used: BV421-Lin (BioLegend, 133311), APC/Cyanine 7-CD45 (clone 30-F11), BV785-CD3 (clone:145-2C11), CD90.2-FITC (clone 53-21), Alexa-Fluor 647 IL-22 (clone Poly5164), and Rorγt-PE (clone Q31-378).

**Microbial 16S rRNA gene analysis and PiCRUST functional analysis.** To perform bacterial phylogenetic and functional analysis, genomic DNA was isolated from colonic flushes on day 4 of TNBS model and sequenced as previously described (96). For all downstream analysis after sequencing, this study used the Nephele platform from the National Institute of Allergy and Infectious Diseases (NIAID) Office of Research Resources.
of Cyber Infrastructure and Computational Biology (OCICB) in Bethesda, Maryland, USA (25). The LefSe Galaxy web application tool developed by the Huttenhower group was performed using default parameters as previously published (26).

**SCFA analysis from colonic flushes.** Quantification of SCFA from colonic flushes collected from experimental groups was performed as previously described (96, 97). Briefly, colon contents (100 mg) were extracted using 25% metaphosphoric acid, centrifuged (12,000 g for 15 minutes at 4°C), and filtered with Ultra-free MC columns from Thermo Fisher Scientific. HP 5890 gas chromatograph configured with flame-ionization detectors (GC-FID) was used for SCFA analysis, using 2-ethylbutyric acid as an internal standard (IS) for all samples analyzed.

**Quantitative PCR (qPCR).** Real-time PCR was performed using bacterial primers designed to recognize the 16S rRNA gene subunit of bacteria (Table 2). 16S rRNA sequences were obtained from the National Center for Biotechnology Information (NCBI) database, and primers were designed and ordered by Integrated DNA Technologies (IDT). Genomic DNA was isolated as described above. PCR reactions were performed on a CFX96 qPCR system from Bio-Rad. For gene transcript analysis in colonic tissue, RNA from colon tissues (25 mg of proximal colon) were ground up using Kontes RNase-free pellet pestle grinders from VWR International. RNA from colonic tissues was isolated using an RNAeasy Plus Mini kit from Qiagen following manufacturer’s instructions, and cDNA was synthesized as previously described (19). Primers for *Muc2*, *Pparg*, and *IL-22* (Table 2) were designed using IDT website primer design interface, and PCR reaction and analysis was performed using Bio-Rad CFX96 PCR platform. For bacteria validation studies, levels were normalized to universal eubacterial primer. For genes, expression levels were normalized to GAPDH. Fold changes from PCR analysis were obtained by using the ΔΔCT method with comparison to the control groups, as previously described (98).

**Availability of data and material.** The authors declare that all the data supporting the findings of this study are provided within the manuscript, additional information file, and provided links. Raw sequenced reads generated from the Illumina platform have been deposited into the NCBI Sequence Read Archive public database, with SRA accession number PRJNA497446.

**Statistics.** GraphPad Prism software was used for all statistical analysis unless otherwise indicated. In order to determine statistical significance, 1-way ANOVA and Tukey’s post hoc multiple comparisons tests were used when comparing 3 or more groups. For comparisons between only 2 groups, an unpaired, 2-tailed standard Student’s *t* test was used. A *P* value less than 0.05 was considered significant.

**Study approval.** All procedures involving animals were performed according to NIH guidelines under protocols approved by the IACUC at the University of South Carolina.

### Author contributions

PBB, MN, and PSN contributed to the overall research design and manuscript preparation. PBB performed the majority of experiments and the analysis of the data presented in the manuscript. MN and PSN were responsible for obtaining all the funding. LM contributed by preparing and providing TEM images of colon tissue from experimental groups. HA, ND, WB, KM, and US assisted with collecting data for the TNBS-induced colitis experiments to include performing flow cytometry, ELISA explorant, and preparation of samples for SCFA analysis. CT and SC assisted in statistical analysis and interpretation of the data. All authors read and approved the final manuscript.

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<th>Primer</th>
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<th>Reverse</th>
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<td>GTATGGGATGGGGATCGTT</td>
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<td><em>Roseburia</em></td>
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<td><em>Pparg</em></td>
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<td><em>IL-22</em></td>
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**Table 2. Primers for real-time PCR**
Acknowledgments

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Address correspondence to: Prakash Nagarkatti, 202 Osborne Administration Building, University of South Carolina, Columbia, South Carolina 29208, USA. Phone: 803.777.5458; Email: prakash@mailbox.sc.edu.

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