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ABOUT COVER

Editorial Board of *World Journal of Gastroenterology*, Dr. Conrado M Fernandez-Rodriguez is Chief of the Gastroenterology Unit at Alcorcon Foundation University Hospital and Associate Professor of Medicine at University Rey Juan Carlos. His main research interest is chronic liver diseases, for which he has authored more than 140 peer-reviewed publications, including in top gastroenterology and hepatology journals. He serves as Director of the Scientific Committee of the Spanish Society of Digestive Diseases, Associate Editor of Hepatology for *Spanish Journal of Gastroenterology*. He is also a member of the Spanish Steering Committee of Alcohol-Related Liver Disease National Registry (ReHalc) and Scientific Advisor of the Spanish Committee for Hepatitis C virus Elimination, and direct participant in several multicenter international clinical trials (Respond-2, REGENERATE, STELLAR-4) and national trials and registries (TRIC-1, HEPAMet, Hepa-C, ColHai). (L-Editor: Filipodia)

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ORIGINAL ARTICLE

Basic Study

Immune and microRNA responses to *Helicobacter muridarum* infection and indole-3-carbinol during colitis

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Author contributions: Alkarkoushi RR, Hui Y, Tavakoli AS, Testerman TL and Singh U assisted with conceptualization, performed experiments, analyzed data, and drafted the manuscript; Nagarkatti P and Nagarkatti M assisted with conceptualization and funding acquisition; Chatzistamou I performed histopathological analysis; Bam M analyzed microRNA data; Testerman TL conceptualized assisted with funding acquisition, and wrote and edited the manuscript.

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Abstract

BACKGROUND

Indole-3-carbinol (I3C) and other aryl hydrocarbon receptor agonists are known to modulate the immune system and ameliorate various inflammatory and autoimmune diseases in animal models, including colitis induced by dextran sulfate sodium (DSS). MicroRNAs (miRNAs) are also gaining traction as potential therapeutic agents or diagnostic elements. Enterohepatic *Helicobacter* (EHH) species are associated with an increased risk of inflammatory bowel disease, but little is known about how these species affect the immune system or response to treatment.

AIM

To determine whether infection with an EHH species alters the response to I3C and how the immune and miRNA responses of an EHH species compare with responses to DSS and inflammatory bowel disease.

METHODS

We infected C57BL/6 mice with *Helicobacter muridarum* (*H. muridarum*), with and without DSS and I3C treatment. Pathological responses were evaluated by histological examination, symptom scores, and cytokine responses. MiRNAs analysis was performed on mesenteric lymph nodes to further evaluate the regional immune response.

RESULTS

H. muridarum infection alone caused colonic inflammation and upregulated proinflammatory, macrophage-associated cytokines in the colon similar to changes seen in DSS-treated mice. Further upregulation occurred upon treatment with DSS. *H. muridarum* infection caused broad changes in mesenteric lymph node miRNA expression, but colitis-associated miRNAs were regulated similarly in *H. muridarum*-infected and uninfected, DSS-treated mice. In spite of causing colitis exacerbation, *H. muridarum* infection did not prevent disease amelioration by I3C. I3C normalized both macrophage- and T cell-associated cytokines.

CONCLUSION

Thus, I3C may be useful for inflammatory bowel disease patients regardless of EHH infection. The miRNA changes associated with I3C treatment are likely the result of, rather than the cause of immune response changes.

Key words: *Helicobacter muridarum*; MicroRNA; Immune; T regulatory cell; T helper 17 cell; Colitis; Cytokine

Core tip: The immune response to *Helicobacter muridarum* (*H. muridarum*), an enterohepatic *Helicobacter* species, mimics responses seen during chemically induced colitis and human inflammatory bowel disease (IBD) in terms of local and systemic cytokine responses and microRNA changes. Most microRNAs that are altered in IBD are also altered by *H. muridarum* infection with or without dextran sodium sulfate treatment. Furthermore, *H. muridarum* does not alter activity of an aryl hydrocarbon receptor agonist, indole-3-carbinol, a natural compound being explored as a treatment for IBD. Therefore, *H. muridarum* infection provides a viable model for predicting the effects of enterohepatic *Helicobacter* species on IBD.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic, idiopathic inflammatory bowel disease (IBD) characterized by inflammation of the colon. In the past decade, inflammatory bowel disease has emerged as a public health challenge and a global disease with increasing incidence in newly industrialized and industrialized countries worldwide, especially in North America, and Europe[1]. In 2015, 3.1 million adults in the United States were living with IBD[2], with direct and indirect costs estimated to be between $14.6 and $31.6 billion in 2014 alone[3]. The pathogenesis of IBD is complex and influenced by genetic susceptibility, dysregulation of the innate and adaptive immune systems, environmental factors, and intestinal dysbiosis; however, a crucial feature of UC is the imbalance between T regulatory (Treg) cells and T helper 17 (Th17) cells[4].

The best-known member of the *Helicobacter* genus is *Helicobacter pylori* (*H. pylori*), which colonizes the stomach, causing gastritis, gastric cancer, and a range of extragastric diseases[5]. Enterohepatic *Helicobacter* (EHH) species colonize the colon and sometimes the biliary tree. Some of these poorly studied organisms commonly cause persistent, asymptomatic infections, but occasionally cause intestinal diseases or even cancer in species ranging from rodents to primates[6-8]. Several studies suggest that EHH species are associated with IBD in humans[9-11]. The prevalence of EHH species in human populations is not clearly known, but one study found 9% infection in healthy control patients[11]. *Helicobacter muridarum* (*H. muridarum*) is an enterohepatic *Helicobacter* (EHH) species that was initially described as a member of the normal flora of conventional rodents[12]. Subsequent studies, however, showed that *H. muridarum*

experimental procedures were conducted in accordance with the guidelines for the use of experimental animals and were approved by the Institutional Review Committee on Animal Care and Use at the University of South Carolina.

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could induce colitis and gastritis in mice, suggesting a potential pathogenic role for the bacterium[13-15].

Dextran sodium sulfate (DSS)-induced colitis is the most widely used mouse colitis model for studying acute colitis and inflammation-associated colon cancer. DSS is a water-soluble, negatively charged, sulfated polysaccharide with a highly variable molecular weight. DSS-induced murine colitis, which most closely resembles human UC, employs DSS at a molecular weight of 40000 Da[16,17]. The mechanism by which DSS induces intestinal inflammation is disruption of tight junctions, allowing dissemination of proinflammatory intestinal contents[18]. In conjunction with colonic damage, DSS induces a range of proinflammatory cytokines and a Th1/Th17 response[17,19].

The aryl hydrocarbon receptor (AhR) regulates several signaling pathways relevant to intestinal health, including the balance between Tregs and Th17 cells[20,21]. AhR is needed for the survival of intraepithelial lymphocytes and also the organogenesis of lymphoid structures in the gastrointestinal tract[22]. Indole-3-carbinol (I3C), a dietary compound from cruciferous vegetables such as broccoli, activates AhR, as does its acidic condensation product, 3,3′-diindolylmethane (DIM)[23]. Both I3C and DIM have been investigated as treatments for a range of inflammatory diseases and cancers, including colitis[24-26], but the effects of *Helicobacter* infection on the response to I3C has not been studied. I3C and DIM are available for purchase as dietary supplements.

MicroRNAs (miRNAs) are being investigated as potential diagnostic and treatment tools. MiRNAs are highly conserved, noncoding, single-stranded, small ribonucleic acid molecules (17–27 nucleotides) that control gene expression post-transcriptionally. They typically bind at the 3′ untranslated region of the target gene messenger RNA (mRNA) leading to the degradation of the target RNA or inhibition of the translation of the RNA[27,28]. MiRNAs regulate genes involved in a wide range of cellular signaling pathways, including the Immune response. During the past ten years, much research has been done to uncover their roles in cellular proliferation, differentiation, maturation, and apoptosis[27]. Moreover, substantial scientific evidence underlines the functional roles and potential value of these tiny ribonucleic acid molecules for regulating autoimmunity and inflammation by affecting the differentiation, maturation, and functions of various immune cells in diseases including colitis[28,29]. Furthermore, many pieces of evidence show the participation of miRNAs in the regulation of T-cell development, differentiation, maturation, and activation[30]. Since the Treg/Th17 balance is crucial to intestinal health[31], understanding how miRNA expression is controlled by inflammatory and anti-inflammatory signals, such as I3C, could lead to identification of miRNAs capable of rebalancing the immune response in the inflamed colon.

The aims of this study were to examine the relative effects of *H. muridarum* and I3C on mouse colon pathology, immune response, and miRNA expression. We used the standard mouse model of DSS-induced colitis in C57BL/6 mice. Some groups were infected with *H. muridarum* and treated with I3C. Treatment responses were monitored in the colon and mesenteric lymph nodes.

MATERIALS AND METHODS

Animals

The research described in this manuscript (including the acquisition of animals and all protocols for their use) was approved by the University of South Carolina Institutional Animal Care and Use Committee prior to commencement of studies. University of South Carolina is an AALAC accredited institution and all animal care procedures followed the NIH Guide for the Care and Use of Laboratory Animals. Female C57BL/6J mice (aged 8-10 wk) were purchased from The Jackson Laboratory, Bar Harbor, Maine, United States. Animals were housed in a controlled environment (12 h light/dark cycle) with food and water ad libitum. After one week of acclimation on a normal chow diet, the mice were randomly divided into groups. Groups of 5-7 animals were used in each experiment. The experimental groups included control (Ctrl), *H. muridarum*, *H. muridarum* plus DSS, *H. muridarum* plus DSS plus I3C, DSS, and DSS plus I3C (DSS/I3C). Each experiment included either all male or all female mice, as indicated in the text.

Bacterial strains, cultivation, and infection

H. muridarum strain ATCC4982 was purchased from the American Type Culture Collection and was cultured in a humidified environment at 37 °C with 10% CO_2, 5%

O_2 in Ham's F-12 medium containing 20 mL/L fetal calf serum in tissue culture flasks. *H. muridarum* bacteria were passaged every 2 to 3 d. After microscopically verifying appropriate morphology and motility, the culture was centrifuged at 25°C at 4500 rpm for 20 min, then the pellet was suspended in 9 g/L sodium chloride to produce a suspension containing approximately 28465 to 142072 adenosine triphosphate (ATP) relative luminescence units per 200 μL, as determined using the luminescent BacTiter-Glo ATP viability assay (Promega Corp.,Madison, Wisconsin, United States). Mice were inoculated by orogastric gavage (200 μL) every other day for a total of four inoculations. Viability of the remaining bacterial suspension was reconfirmed using the luminescent BacTiter-Glo ATP assay.

Infection was confirmed by polymerase chain reaction (PCR) of stool DNA using *H. muridarum*-specific primers as follows. Fecal samples were collected and stored at -80°C until analysis. Fecal DNA was isolated using the EZNA stool DNA kit (Omega Bio-Tek, Inc., Norcross Georgia, United States) according to the manufacturer's recommendations. Fecal PCR was performed using *H. muridarum* 16S rRNA gene-specific primers (H. m. p30f, 5'-ATGGGTAAGAAAAAAAAAGATTGCAA-3', and H. m. p30r, 5'-CTATTTCATATCCGCTCTTGAGAATC-3'), which amplify an 800 bp conserved region of the 16S rRNA, as previously described[32].

Induction of colitis with DSS

DSS (MW 40 000, Chem-Impex International, Inc, Wood Dale, Illinois, United States) at a concentration of 1-30 g/L was provided in drinking water for 10-13 d. The volume of DSS consumed, animal weight, diarrhea score, and stool blood score were recorded daily. The disease activity index was calculated from weight, diarrhea, and stool blood scores as previously described[33,34]. Stool blood was detected using a colorimetric fecal occult blood test (Helena Laboratories, ColoScreen catalog No. 5083). Briefly, we determined the disease activity index using the following variables: Stool blood (0, negative; 1, weakly positive; 2, strongly positive; 3, rusty-colored stool and 4, gross bleeding), changes in weight (0, < 1%; 1, 1%-5%; 2, 6%-10%; 3, 11%-15%; and 4, > 15%), and stool consistency (0-1, normal; 2-3, loose stools; and 4, diarrhea).

I3C preparation and dosage

For treatment groups, I3C purchased from Chem-Impex International, Inc. was suspended in DMSO prior to dilution in corn oil. I3C was administered orogastrically at a dose of 40 mg/kg in a total volume of 100 μL, as described previously[24]. Animals were treated with either I3C or vehicle (20 mL/L DMSO in corn oil) daily, beginning on the first day of the DSS cycle.

Histopathological colitis score

Formalin-fixed colon tissue was embedded in paraffin and cut into 5 μm thick sections. Next, the colon sections were stained using hematoxylin and eosin (H and E). Four randomly chosen, non-overlapping fields of each stained section were analyzed and assigned a colitis severity score by a pathologist using methods described previously[34,35]. In short, the degree of colitis was scored on the basis of the following parameters: Extent of the injury (0, none; 1, mucosa; 2, mucosa and submucosa; and 3, transmural), inflammation severity (0, none; 1, mild; 2, moderate; and 3, severe), and crypt damage (0, none; 1, basal one-third damaged; 2, basal two-thirds damaged; 3, crypt loss and the presence of surface epithelium; and 4, loss of the entire crypt and epithelium). Then the degrees for each of these aforementioned parameters were multiplied by an extent score that represented the percentage of each parameter that had a given feature as follows: 1, 0-25%; 2, 26%-50%; 3, 51%-75%; and 4, 76%-100%. We defined the total score as the sum of the three parameters. The bottom limit total colitis score was 0; the upper limit total

Characterization of CD4+ T cells in the mesenteric lymph node and spleen

For flow cytometry, the mesenteric lymph nodes (MLN) and spleens were pooled from each group of mice and placed in ice-cold medium. These tissues were mechanically disrupted, teased into single-cell suspensions, filtered through a cell strainer (70 μm), and placed in complete medium (RPMI-1640 containing 100 mL/L of heat-inactivated fetal bovine serum). The isolated cell suspension was stimulated with a cell stimulation cocktail (eBioscience™) plus protein transport inhibitors (Invitrogen, catalog 00-4975), for 4-6 h. Stimulated cells were incubated with anti-CD4 mAb and anti-CD25 mAb for 15 min on ice (Biolegend, United States). For intracellular cytokine staining, the cell suspension was incubated with anti-IFNγ, Interleukin-17 after treating the cells with Fixation/permeabilization kit (BD Biosciences catalog 554714).

For Treg identification, we used FOXP3/Transcription Factor Staining Buffer set (eBioscience Invitrogen) before adding anti-FOXP3. Staining and washing were carried out in complete medium on ice. The stained cells were analyzed with a Beckman Coulter FC500 flow cytometer.

Enzyme linked immunosorbent assays

Interleukin-17 (IL-17), IL-6, IL-10, IL-4, IL-6, IL-21, IL-22, IL-23, IL-1β, transforming growth factor beta 1 (TGF-β1) , tumor necrosis factor-alpha (TNF-α) and interferon gamma (INF-γ), in the plasma and/or in colonic tissue lysates were quantified by ELISA kits (R and D Systems, Minneapolis, MN, United States) following the manufacturer's recommendations. Colon tissues were prepared for ELISA as described previously[33]. Briefly, the mouse colons where washed immediately with cold phosphate buffered saline and frozen at -70°C until use. The samples were homogenized in 200 μL protein analysis buffer [10 mL of 1 mol/L Tris-hydrochloric acid (pH 8.0), 6 mL of 5 mol/L sodium chloride and 2 mL of Triton X-100 to 182 mL of sterilized distilled water][33] in 2 mL microcentrifuge tubes with a 0.9-2.0 mm stainless steel bead blend and homogenized with a tissue homogenizer (MP FastPrep-24) at a speed of 0.4 m/s for 20 s. Samples were frozen and thawed, and homogenized three times, then centrifuged at 30000 *g* for 30 min at 4°C. The supernatant was collected, and the pellet was re-suspended in phosphate buffer. Protein concentrations were determined using a bicinchoninic acid assay (Bio-Rad). Samples were frozen until the ELISA assays were performed and 0.5-1.0 mg/mL of protein was used for each run, depending on the interleukin type.

Sample collection and RNA isolation

Mesenteric lymph nodes were collected from the groups on the day of the sacrifice and immediately frozen at -70°C prior to use. Mesenteric lymph nodes were ground with mortar and pestle in liquid nitrogen. QIAzol Lysis Reagent (Qiagen, catalog 217004) was added to the samples and they were then homogenized by MP FastPrep-24 with 0.9-2.0 mm stainless steel beads (0.4 m/s for 10 s). Total RNA, including mRNA, miRNA and other small RNA molecules, were isolated from all the mesenteric lymph node with the miRNeasy Kit (Qiagen, Germany), following the manufacturer's procedure. The concentration and purity of the isolated RNA was determined using a Beckman Coulter DU800 UV/visible spectrophotometer. RNA quality was assessed by measuring the absorbance (A260/A280, A260/A230) of isolated samples and by agarose gel electrophoresis.

MicroRNA array analysis

The microarray was performed at the University of South Carolina School of Medicine following the protocol described by Bam *et al*[36,37]. Briefly, total RNA isolated as described above was hybridized to an Affymetrix miRNA-v3 gene chip (Affymetrix, Sunnyvale, CA, United States) as directed by the manufacturer. Raw data was processed in the Transcriptome Analysis Console (Affymetrix). The heat map was generated in Genesis[38]. The data from Transcriptome Analysis Console were used to calculate the linear fold-change of the expression of miRNAs to compare the miRNA expression differences among treatment groups. A linear fold-change of at least ± 1.5 was used as a cutoff value for the inclusion of a miRNA for further analysis. Moreover, only the miRNAs which were significant on the basis of P value (< 0.05) calculated using student's t-test, were included in the analysis.

MiRNA-target gene prediction

Ingenuity Pathway Analysis (IPA, Qiagen, Redwood City, CA, United States) was used to predict the targets of the differentially expressed miRNAs. Networks relevant to regulatory T cells were generated to identify relevant miRNA species for testing. Other databases [TargetScan (http://www.targetscan.org/vert_72/), miRWalk (http://mirwalk.umm.uni-heidelberg.de/)] were also used to identify genes targeted by specific miRNAs.

Quantitative real-time PCR analysis of miRNA and gene expression

Total RNA from MLN was isolated and purified with the miRNeasy Kit (Qiagen, Valencia, CA, United States), following the manufacturer's procedure. The miScript II RT complementary DNA (cDNA) synthesis kit (Qiagen, Germany) was used according to the manufacturer's specifications to reverse-transcribe cDNA by taking 1 μg each of total RNA in a 20 μL total volume. The quantitative real-time (qRT-PCR) reactions were carried out using miScript Primer Assays or miScript Precursors (Qiagen,

Germany) according to manufacturer instructions. U6, SnorD96, Snor68, Snor234, and Snor202 were evaluated for stability among groups and SnorD96 was chosen for normalization. SnorD96 has also been used by others[39,40]. Primers were purchased from Qiagen, Maryland.

For mRNA expression analysis, cDNA was made from total RNA as described. A two-step amplification qRT-PCR was carried out using SsoAdvanced™ SYBR® green supermix from Bio-Rad (Hercules, CA, United States) with the mouse primers shown in Table 1. The real-time PCR conditions were as follows: Initial step at 95°C for 10 s followed by cycles (n = 40) consisting of 30 s at 95°C, followed by 30 s annealing/extension at 60°C and a final extension step for 30 s at 72°C. Data are normalized to expression of the reference gene encoding β-actin. Primers were purchased from Integrated Technologies and from Invitrogen. Melting temperatures ranged from 56.0°C to 64.5°C. Primer efficiency was measured for each primer set. All reactions were performed in triplicate. The qPCR experiments were carried out on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, United States). Fold changes were calculated using the $2^{-\Delta\Delta CT}$ (Livak) method.

Statistical analysis

Significance of differences between groups at single time points were determined using the Mann-Whitney U-test using GraphPad Prism software (Version 8.2). P values less than 0.05 were considered significant. Colitis symptom time course data were analyzed using a repeated measure analysis with 13 measures taken per animal (day one to day thirteen) randomly assigned to six groups (Ctrl, *H. muridarum*, *H. muridarum*/DSS, *H. muridarum*/DSS/I3C, DSS/I3C) and three experiments. Descriptive statistics were computed on the variables. For categorical variables, the univariate constructions will be included frequency distributions. For continuous variable statistics included measure of central tendency (mean and median) and measure of spread (standard deviation and range). Descriptive statistics for main variables were carried out for each group. In the analysis, expected mean squares were calculated and the appropriate combination used for hypothesis tests with specific functions of the repeated measures. General linear model analyses in SAS (MIXED procedure) were used to examine the effects of day, group, and day by group interaction. Post-hoc comparisons for the appropriate effects were examined. In addition, parameter estimates of the effects of covariate (experiment) and of the appropriate structure for the repeated observations was estimated. Adjusted Tukey-Kramer multiple comparison was used for significant effects. Significance levels are indicated as $^{a}P \leq 0.05$; $^{b}P \leq 0.01$; $^{c}P \leq 0.001$; $^{d}P \leq 0.0001$.

RESULTS

Exacerbation of colitis by H. muridarum is counteracted by I3C treatment

In three independent experiments, female wild-type C57BL/6 mice were infected with live *H. muridarum* bacteria seven, five, three, and one days prior to commencement of DSS treatment (day zero). Pathology in mice treated with 30 g/L DSS was so severe that one *H. muridarum*/DSS treated mouse required euthanasia. For this reason, 10 g/L DSS was used in subsequent experiments. Figure 1 shows average values from three independent experiments. Statistical analysis results are shown in Table 2 and Table S1. Overall disease activity was increased by each treatment except *H. muridarum* when compared to the control group. *H. muridarum* alone occasionally induced stool softening and a small amount of fecal occult blood, yet *H. muridarum* decreased diarrhea scores in DSS-treated mice (Figure 1A and Table 2). On the other hand, *H. muridarum* increased fecal occult blood and weight loss in DSS-treated mice. I3C was as effective in ameliorating colitis symptoms in *H. muridarum* mice as it was in uninfected mice. Significant shortening of the colon, an indicator of inflammation, occurred in *H. muridarum*-infected mice compared with control mice (Figure 1B). DSS treatment of *H. muridarum* mice caused additional shortening and colon length was similar to DSS mice. I3C significantly increased colon length in both infected and uninfected mice. It is evident from the pathology scores that the infection with *H. muridarum* alone can induce pathology such as dilatation of glandular crypts, edema, and destruction of epithelium and glands (Figure 1B). In some cases, pathology caused by *H. muridarum* alone is comparable to that caused by DSS treatment, yet damage to the mucosa was not reflected in symptom scores in these mice. Treatment of *H. muridarum*-infected mice with DSS further worsened pathology.

Table 1 Primers used for transcription analysis

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Actb (β-actin)	TCACCCACACTGTGCCCATCTACG	CAGCGGAACCGCTCATTGCCAATGG
Il17a	TCAGCGTGTCCAAACACTGAG	CGCCAAGGGAGTAAAGACTT
Foxp3	AGCAGTCCACTTCACCAAGG	GGATAACGCCAGAGGAGCTG
Rorc	CCGCTGAGAGGGCTTCAC	TGCAGGAGTAGGCCACATTACA
Il10	TGAATTCCCTGGGTGAGAAGC	ATCACTCTTCACCTCCAC
Il6	AGCCAGAGTCCTTCAGAGAGAT	AAAAAGTGCCGCTACCCTGA

Table 2 Colitis symptom score *P* values

Treatment groups[2]		% Weight	Stool blood	Diarrhea	DAI
Ctrl *vs*	Hm	0.2495	0.6142[1]	0.0248[1]	0.0659[1]
	Hm/DSS	< 0.0001[1]	< 0.0001[1]	< 0.0001[1]	< 0.0001[1]
	Hm/DSS/I3C	0.9574	< 0.0001[1]	< 0.0001[1]	< 0.0001[1]
	DSS	0.0552	< 0.0001[1]	< 0.0001[1]	< 0.0001[1]
	DSS/I3C	0.1381	< 0.0001[1]	< 0.0001[1]	< 0.0001[1]
Hm *vs*	DSS	0.9823	< 0.0001[1]	< 0.0001[1]	< 0.0001[1]
	Hm/DSS	< 0.0001[1]	< 0.0001[1]	< 0.0001[1]	< 0.0001[1]
Hm/DSS *vs*	DSS	< 0.0001[1]	0.0007[1]	0.0041[1]	< 0.0001[1]
	Hm/DSS/I3C	< 0.0001[1]	< 0.0001[1]	< 0.0001[1]	< 0.0001[1]
DSS *vs*	DSS/I3C	0.9977	0.0026[1]	< 0.0001[1]	< 0.0001[1]

[1]Significant *P* values.
[2]Ctrl: Control; Hm: *Helicobacter muridarum;* Hm/DSS: *Helicobacter muridarum* plus DSS; Hm/DSS/I3C: *Helicobacter muridarum* plus DSS plus I3C; DSS/I3C: DSS plus I3C.

Effects of H. muridarum, DSS, and I3C on miRNA expression

Since miRNAs contribute to immune cell differentiation, we sought to determine which miRNAs were regulated by I3C, which were regulated by *H. muridarum*, and whether *H. muridarum* affected the I3C response. To accomplish this, we performed miRNA analysis on total RNA isolated from the mesenteric lymph nodes of all groups from one of the experiments. A heat map was constructed highlighting the differences in miRNA abundance among the groups (Figure 2A). We found that each group had a pattern that was distinct from all others. For example, *H. muridarum* infection alone altered miRNA expression and miRNA expression in DSS, I3C treated mice was different depending on whether they were infected or uninfected.

We sought to determine whether I3C-regulated miRNAs are associated with regulation of the major Treg and Th17 transcriptional regulators, FOXP3 and RORC. To this end, we used *in silico* analysis of predicted miRNA targets and pathways as well as online databases to search for miRNAs induced by I3C in *H. muridarum* /DSS mice that could target *Foxp3* and *Rorc* genes. Among these potential miRNAs, we identified 3 candidates that had acceptable alignment scores and were highly predicted to target *Foxp3* or *Rorc*. These miRNAs included miR-let7a-5p and miR-29a-3p, which target RORC, and miR-874-5p and miR-6906-5p, which target *FoxP3*. It should be noted that other members of the let-7 family also target *Rorc* and some were similarly regulated by I3C in *H. muridarum*-infected mice. We performed qRT-PCR on cDNA samples reverse transcribed from total MLN RNA. As predicted, we found increased expression of miR-23a-3p and let-7a-2, which target *Rorc* (Figure 2B). Differences between untreated and I3C-treated groups were only significant for *H. muridarum*-infected mice, but the *Rorc*-targeted miRNAs miR-29a-3p and let-7a trended higher in uninfected mice. We also found that I3C decreased expression of *Foxp3*-targeting miR-874-5p and miR-6906-5p in *H. muridarum*-infected mice, but only

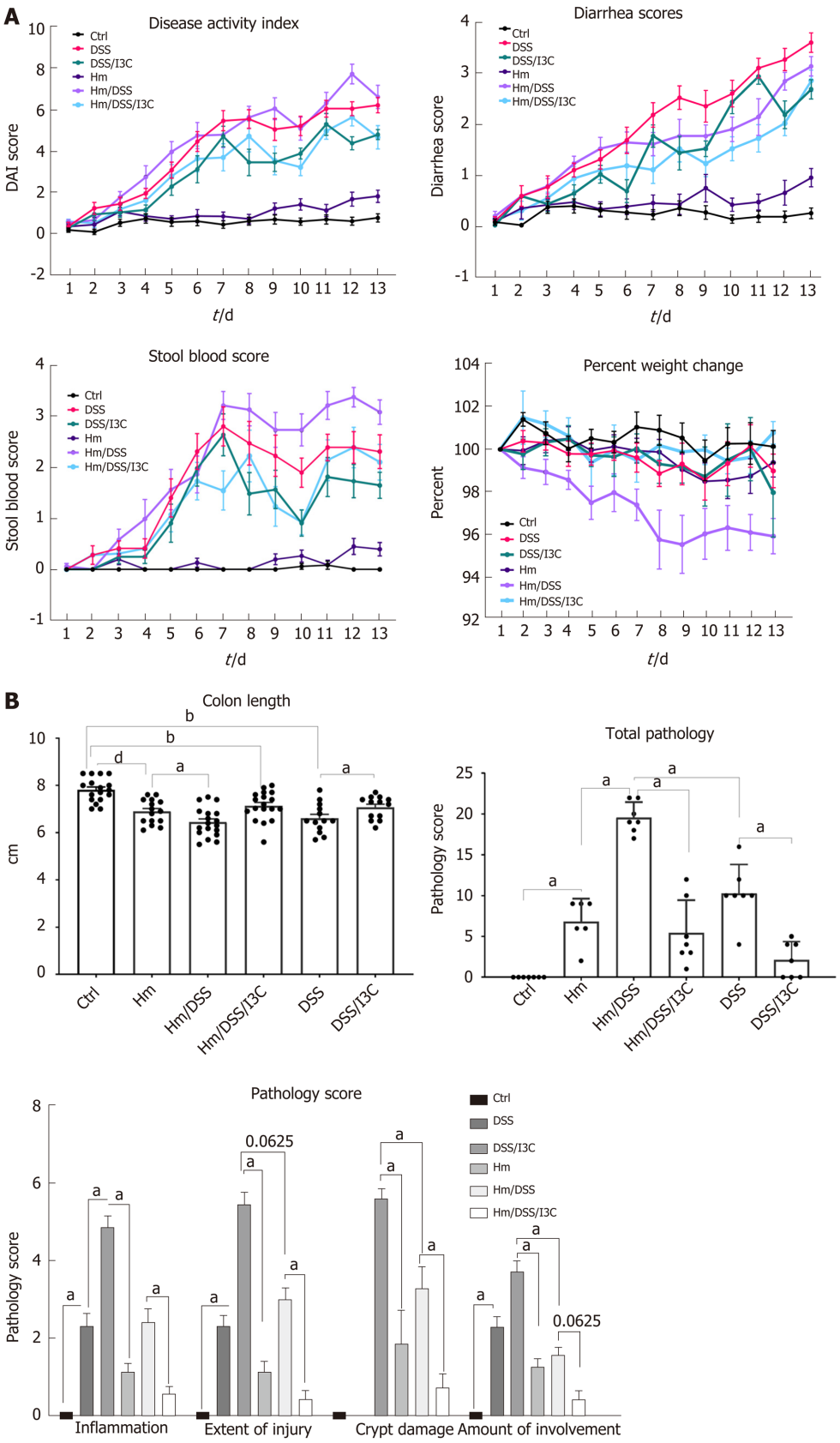


Figure 1 Symptom scores and histopathology. A: Colitis symptom scores (n = 17-21 per group); B: Colon length (n = 17-21 per group) and histopathology scores (n = 7 per group). $^{a}P \leq 0.05$; $^{b}P \leq 0.01$; $^{c}P \leq 0.001$; $^{d}P \leq 0.0001$. Ctrl: Control; Hm: *Helicobacter muridarum;* Hm/DSS: *Helicobacter muridarum* plus DSS; Hm/DSS/I3C: *Helicobacter muridarum* plus DSS plus I3C; DSS/I3C: DSS plus I3C.

miR-6906-5p was reduced in uninfected mice. This is not surprising since miR-874-5p was not predicted to be elevated by DSS in uninfected mice, but it highlights the different miRNA responses seen among groups. The miRNAs miR-15b and miR-16

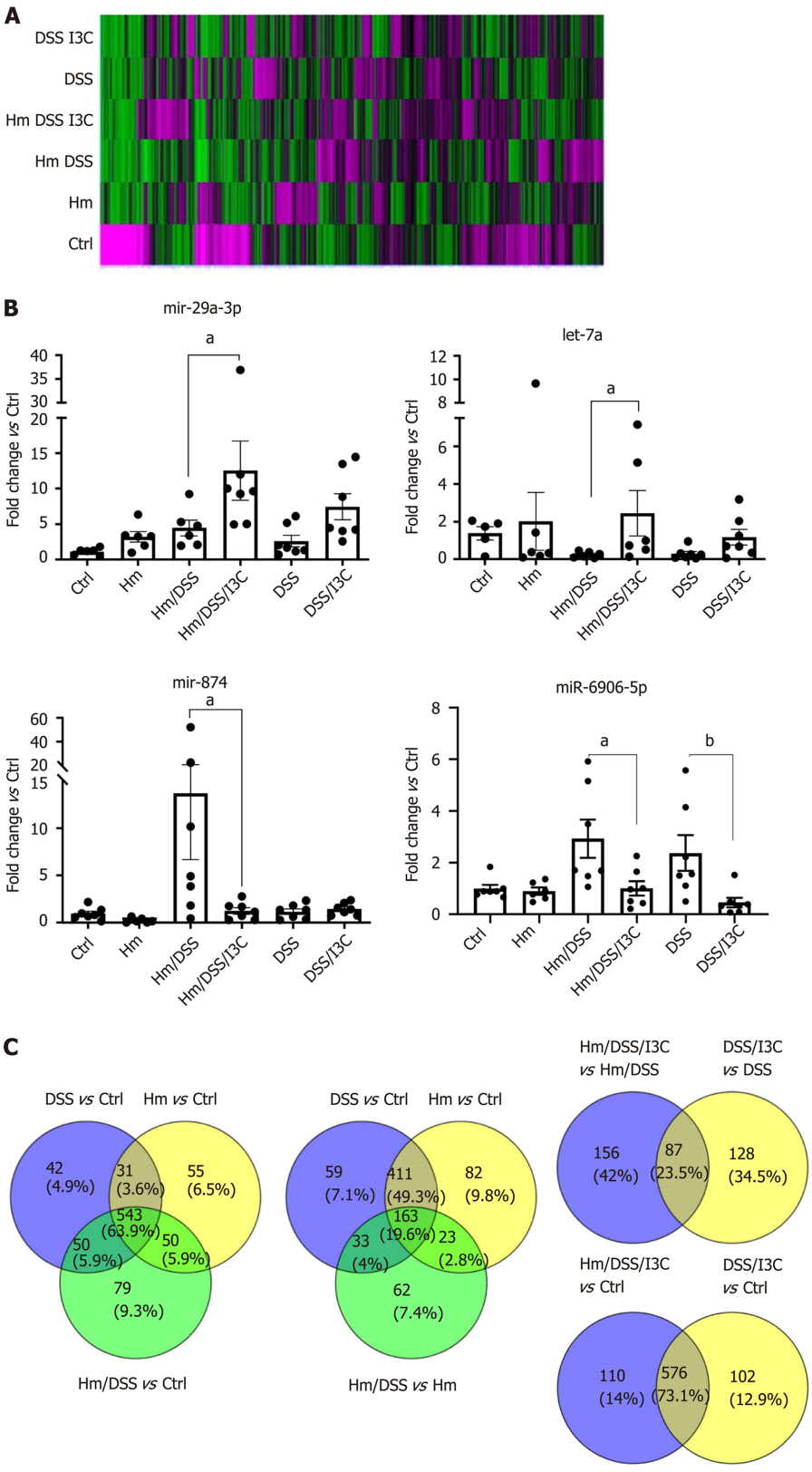


Figure 2 MicroRNA expression analysis. A: A heat map of expression intensities for each group was generated using Genesis software with red representing high expression and green representing low expression; B: Expression of microRNA as determined by quantitative real time PCR. All values are normalized to expression in the control group (n = 7); and C: Venn diagrams generated using Venny 2.1 demonstrate microRNA expression changes common to different treatment conditions. [a]$P \leq 0.05$; [b]$P \leq 0.01$. Ctrl: Control; Hm: *Helicobacter muridarum;* Hm/DSS: *Helicobacter muridarum* plus DSS; Hm/DSS/I3C: *Helicobacter muridarum* plus DSS plus I3C; DSS/I3C: DSS plus I3C.

support Treg development by targeting a suppressor of Treg development and miR-15b/16 previously have been shown to be induced by DIM[41,42]. We also found these miRNAs to be increased by I3C in *H. muridarum*-infected, DSS-treated mice (Figure S1

), but their expressions were predicted to be oppositely regulated in uninfected mice.

A closer look at miRNA microarray data was illuminating. The first Venn diagram shown in Figure 2C highlights miRNAs that displayed a greater than 2-fold change in the colitis groups compared with the control group. The *H. muridarum* group has 679 miRNAs up or down-regulated compared with the control, *vs* 666 miRNAs in the DSS group, and 722 miRNAs in the *H. muridarum* /DSS group. Interestingly, the majority (n = 574) of the miRNA changes are shared between the *H. muridarum* group and the DSS group and most of these (n = 543) are also shared with the *H. muridarum* /DSS group as well. This demonstrates that the miRNA response to *H. muridarum* infection is very similar to the response induced by DSS treatment. More importantly, miRNAs common between the *H. muridarum* group and the DSS group were almost all regulated in the same direction. All miRNA data are found in Table S2.

A second Venn diagram further highlights the effect of DSS treatment by substituting the *H. muridarum* /DSS *vs* *H. muridarum* comparison for the *H. muridarum* *vs* control comparison. Not surprisingly, the number of miRNAs changed between *H. muridarum* /DSS and *H. muridarum* (n = 281) is much smaller than the effect of *H. muridarum* /DSS compared to Ctrl (n = 722), but the majority of those miRNAs (69.8%) are also altered by DSS. It should be noted that 80.1% of the miRNAs altered by DSS treatment of *H. muridarum* mice are found within the *H. muridarum* /DSS *vs* Ctrl comparison. 85.7% if miRNAs common between DSS *vs* Ctrl and *H. muridarum*/DSS *vs* *H. muridarum* were concordant in the direction of change. This recapitulates the findings shown in the first Venn diagram, indicating that *H. muridarum* infection and DSS treatment have similar effects.

A third Venn diagram was constructed to study the effects of I3C treatment. In I3C-treated animals (Figure 2C), there was less overlap between *H. muridarum*-infected and –uninfected animals (87 miRNAs, or 23.5%). Oddly, most of the 87 common miRNAs (71.2%) were oppositely regulated in *H. muridarum*-infected and uninfected mice. In most cases, miRNAs were upregulated by I3C in *H. muridarum*-infected mice, but downregulated by I3C in uninfected mice. The predicted fold changes were also larger in *H. muridarum*-infected mice. The reason for this odd regulation pattern is discussed in the next paragraph. An alternative method for Identifying I3C effects is to compare *H. muridarum* /DSS/I3C *vs* control with DSS/I3C *vs* Ctrl (Figure 2C). These miRNA populations overlap heavily (73.1%). Most of the 87 previously identified miRNAs (56/87) are found in the overlap group. Since there is also heavy overlap between the putative I3C-regulated miRNAs and DSS-regulated miRNAs (529/666), it is not clear whether any of the miRNAs are strictly responsive to I3C; however, the overlap is consistent with the hypothesis that I3C normalizes miRNAs involved in colitis.

Examination of specific miRNAs provides a clearer demonstration of the effects of *H. muridarum*, DSS, and I3C and an explanation for the differential regulation of miRNAs by I3C in infected *vs* uninfected mice. We examined a list of 45 miRNAs that are altered in human IBD[43-45]. Almost all of the human IBD-associated miRNAs were altered by *H. muridarum* and/or DSS. Table 3 shows raw expression data and fold changes for the selected miRNAs. When compared with control values, these miRNAs were all downregulated, whereas many were upregulated compared to healthy controls in humans[43]. Possible reasons for this are discussed later. Expression decreases are mostly modest in *H. muridarum* *vs* Ctrl, but extreme in *H. muridarum* /DSS *vs* Ctrl- up to 3,646-fold decreased. The expression reductions were less extreme in the *H. muridarum* /DSS/I3C group compared to Ctrl. Expression of the selected miRNAs was lower in DSS/I3C group than the DSS group, but in many cases, the reductions were less than two-fold, which is why those miRNAs did not show up as common between infected and uninfected mice treated with I3C. It should be noted that there was not a global decrease in miRNA expression in any treated groups *vs* Ctrls; the decreases are specific to certain miRNAs. All 45 human IBD-associated miRNAs were among the 576 miRNAs in the putative I3C regulated group (Figure 2C) and all but two of the 45 were regulated by *H. muridarum* and/or DSS. Bian *et al*[46] also reported that many of these miRNAs are differentially regulated in DSS-treated mice, suggesting that a core set of miRNAs are relevant to colitis in both humans and mice[46].

H. muridarum infection alters T helper cell profiles.

Since miRNAs are pleotropic in their effects, we sought to confirm predicted effects on Treg and Th17 populations. MLN transcript analysis by qRT-PCR demonstrated that I3C decreased RORC and increased FOXP3 expression, consistent with a switch from Th17 to Treg (Figure 3). These results were mirrored by the decrease in IL17 and increase in IL10 expression. Expression of *Il6*, which is involved in Th17 induction, is also shown. RORC and IL17 were more strongly induced by DSS in *H. muridarum*-

Table 3 MicroRNA expression

	Raw expression data						Fold changes						
miRNA	Ctrl	Hm	Hm/DSS	DSS/Hm/ I3C	DSS	DSS/ I3C	Hm *vs* Ctrl	Hm/DSS *vs* Ctrl	DSS/Hm/I3C *vs* Ctrl	DSS *vs* Ctrl	DI *vs* Ctrl	DMI *vs*DHM	DI *vs* DSS
mmu-let-7a-5p	12.93	11.14	5.72	9.11	10.71	10.59	-3.45	-147.58	-14.06	-4.65	-5.07	10.5	-1.09
mmu-let-7b-5p	14.14	11.84	7.97	10.45	11.21	10.89	-4.92	-72.01	-12.96	-7.62	-9.56	5.56	-1.25
mmu-let-7d-5p	13.76	11.52	8.09	10.44	11.1	10.86	-4.71	-50.67	-9.97	-6.29	-7.42	5.08	-1.18
mmu-let-7e-5p	11.06	10.06	1.7	8.39	9.6	9.23	-2	-654.64	-6.34	-2.74	-3.54	103.31	-1.29
mmu-let-7g-5p	10.31	6.71	1.37	2.01	7.52	6.16	-12.19	-491.93	-317.1	-6.92	-17.81	1.55	-2.58
mmu-miR-103-3p	11.85	10.44	6.42	10.09	10.88	10.02	-2.66	-43.07	-3.39	-1.96	-3.57	12.7	-1.82
mmu-miR-106a-5p	9.29	5.45	1.15	2.03	5.51	2.78	-14.34	-282.96	-153.91	-13.78	-91.54	1.84	-6.64
mmu-miR-127-3p	7.9	4.55	0.95	2.8	2.43	1.91	-10.18	-123.98	-34.2	-44.34	-63.55	3.63	-1.43
mmu-miR-128-3p	5.81	0.79	0.94	1.04	0.77	1.3	-32.4	-29.23	-27.31	-33.04	-22.87	1.07	1.44
mmu-miR-135a-1-3p	3.64	1.3	1.8	1.26	1.43	2.12	-5.04	-3.57	-5.19	-4.62	-2.86	-1.45	1.62
mmu-miR-140-3p	10.2	9.92	1.83	7.91	9.62	8.3	-1.21	-329.16	-4.87	-1.49	-3.73	67.62	-2.51
mmu-miR-140-5p	6.33	1.38	0.79	1.02	1.38	0.87	-30.86	-46.56	-39.48	-30.86	-43.84	1.18	-1.42
mmu-miR-142-5p	2.9	0.99	1.35	0.99	1.16	1.01	-3.78	-2.93	-3.78	-3.34	-3.72	-1.29	-1.11
mmu-miR-145a-5p	12.99	11.51	6.79	10.2	11.15	10.28	-2.78	-73.5	-6.89	-3.57	-6.55	10.66	-1.83
mmu-miR-146a-5p	10.34	7.84	1.24	3.54	7.72	7.84	-5.65	-550.48	-111.6	-6.13	-22.7	4.93	-3.7
mmu-miR-150-5p	13.4	11.1	4.14	8.96	10.4	9.76	-4.92	-614.48	-21.78	-8.01	-12.51	28.22	-1.56
mmu-miR-155-5p	9.86	8.15	1.54	3.58	7.65	5.31	-3.27	-320.03	-77.72	-4.61	-23.48	4.12	-5.09
mmu-miR-15b-5p	11.17	10.17	1.48	8	9.92	8.84	-1.99	-824.5	-9.01	-2.37	-5.02	91.56	-2.12
mmu-miR-16-5p	13.18	10.38	1.35	9.1	10.8	9.11	-7	-3646.25	-16.92	-5.22	-16.9	215.51	-3.23
mmu-miR-17-5p	10.71	9.01	1.33	7.8	9.29	7.82	-3.25	-666.3	-7.53	-2.67	-7.43	88.54	-2.78
mmu-miR-185-5p	9.27	6.58	0.99	5.64	5.86	5.07	-6.45	-310.71	-12.33	-10.58	-18.36	25.19	-1.74
mmu-miR-18a-5p	7.32	1.37	0.84	1.37	1.41	1.74	-61.84	-89.41	-61.84	-60.29	-47.91	1.45	1.26
mmu-miR-195a-5p	10.59	8.03	1.91	5.03	8.22	7.39	-5.89	-409.38	-47.19	-5.17	-9.14	8.68	-1.77
mmu-miR-196b-5p	3.11	0.94	0.94	1.08	1.05	0.83	-4.49	-4.49	-4.09	-4.19	-4.86	1.1	-1.16
mmu-miR-199a-5p	9.5	4.56	1.77	2.01	7.72	1.77	-2.03	-101.88	-21.49	-3.71	-78.21	4.74	-21.08

mmu-miR-19a-3p	4.08	1.56	1.39	0.91	1.24	1.25	-5.73	-6.45	-9.02	-7.15	-7.13	-1.4	1
mmu-miR-19b-3p	9.43	6.13	1.18	5.87	7.97	5.61	-9.84	-303.99	-11.79	-2.74	-14.07	25.78	-5.14
mmu-miR-20b-5p	9.03	4.87	1.76	2.29	5.85	1.96	-17.77	-154.33	-106.75	-9.05	-133.96	1.45	-14.8
mmu-miR-221-3p	9.19	6.07	1.24	1.42	7.45	5.25	-8.65	-246.56	-218.06	-3.34	-15.34	1.13	-4.59
mmu-miR-222-3p	8.89	7.65	1.12	4.11	6.24	4.58	-2.36	-219.12	-27.5	-6.29	-19.86	7.97	-3.16
mmu-miR-223-3p	5.45	1.57	1.01	0.99	1.84	0.87	-14.69	-21.58	-21.99	-12.14	-23.85	-1.02	-1.96
mmu-miR-24-2-5p	12.74	10.84	1.78	9.51	10.8	10.12	-56.17	-101.73	-64.83	-72.38	-48.35	1.57	1.5
mmu-miR-27a-3p	9.96	5.94	1.35	1.57	8.22	5.2	-16.28	-390.62	-337.26	-3.34	-27.11	1.16	-8.12
mmu-miR-28a-3p	7.53	4.75	0.83	1.4	1.49	1.12	-6.86	-103.85	-69.7	-65.83	-84.8	1.49	-1.29
mmu-miR-29a-3p	10.97	8.84	2.38	7.73	9.45	7.38	-4.37	-386.19	-9.45	-2.86	-12.06	40.85	-4.21
mmu-miR-29c-3p	5.38	0.87	1.23	1.28	2.05	1.8	-22.8	-17.81	-17.22	-10.08	-12.01	1.03	-1.19
mmu-miR-30e-5p	7.8	1.13	1.64	1.64	3.46	1.32	-101.76	-71.46	-71.46	-14.68	-89.04	1	-4.42
mmu-miR-345-5p	5.41	2.46	2.85	3.65	2.81	1.18	-7.76	-5.93	-3.41	-6.1	-18.84	1.74	-3.09
mmu-miR-374b-5p	4.34	0.92	1.19	0.89	1.58	1.13	-10.7	-8.87	-10.91	-6.78	-9.23	-1.23	-1.36
mmu-miR-423-5p	7.07	3.13	1.29	4.25	2.56	1.26	-15.33	-54.69	-7.03	-22.66	-55.78	7.78	-2.46
mmu-miR-491-5p	2.95	1.39	1.15	1.01	1.34	1.07	-2.96	-3.49	-3.84	-3.06	-3.7	-1.1	-1.21
mmu-miR-532-5p	8.4	5.55	0.89	3.43	3.45	1.75	-7.22	-183.34	-31.45	-30.94	-101.04	5.83	-3.27
mmu-miR-760-3p	2.44	0.99	0.74	1.1	1.15	1.17	-2.73	-3.25	-2.54	-2.46	-2.42	1.28	1.02
mmu-miR-877-5p	4.05	3.56	1.93	2.14	3.28	1.8	-1.4	-4.35	-3.75	-1.7	-4.76	1.16	-2.79
mmu-miR-93-5p	10.41	8.67	1.86	7.64	8.66	6.85	-3.34	-373.43	-6.79	-3.35	-11.77	54.98	-3.52

Ctrl: Control; Hm: *Helicobacter muridarum;* Hm/DSS: *Helicobacter muridarum* plus DSS; Hm/DSS/I3C: *Helicobacter muridarum* plus DSS plus I3C; DSS/I3C: DSS plus I3C.

infected mice than in uninfected mice, consistent with the increased pathology. Although FOXP3 was less strongly induced by I3C in *H. muridarum*-infected mice, IL10 expression was similar to that of uninfected mice treated with I3C. These results were corroborated by flow cytometry (Figure S2). The Th17 cell population increased sharply following DSS treatment of *H. muridarum*-infected animals and decreased following I3C treatment while the Treg population increased.

Production of cytokines in colon tissue and plasma

Our gene expression and flow cytometry data from the mesenteric lymph nodes

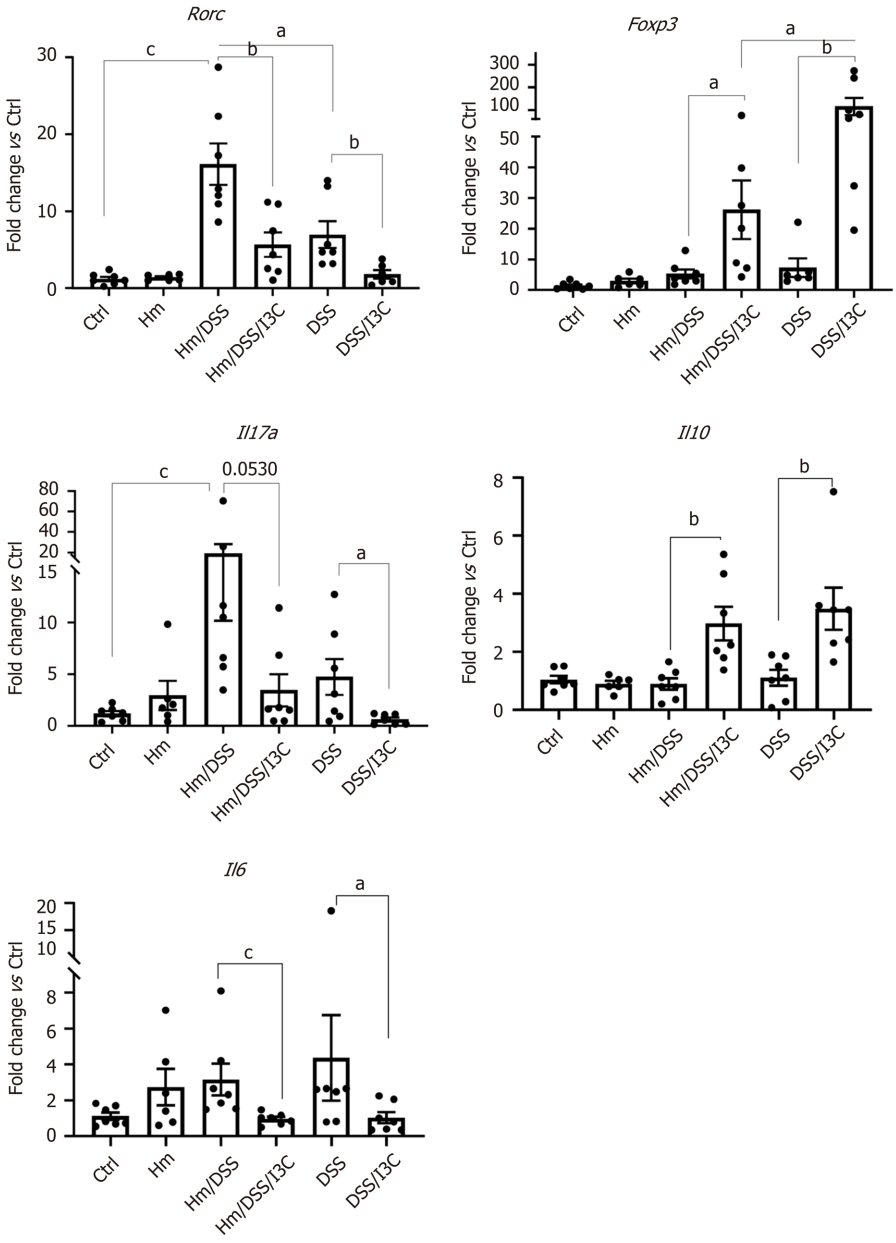
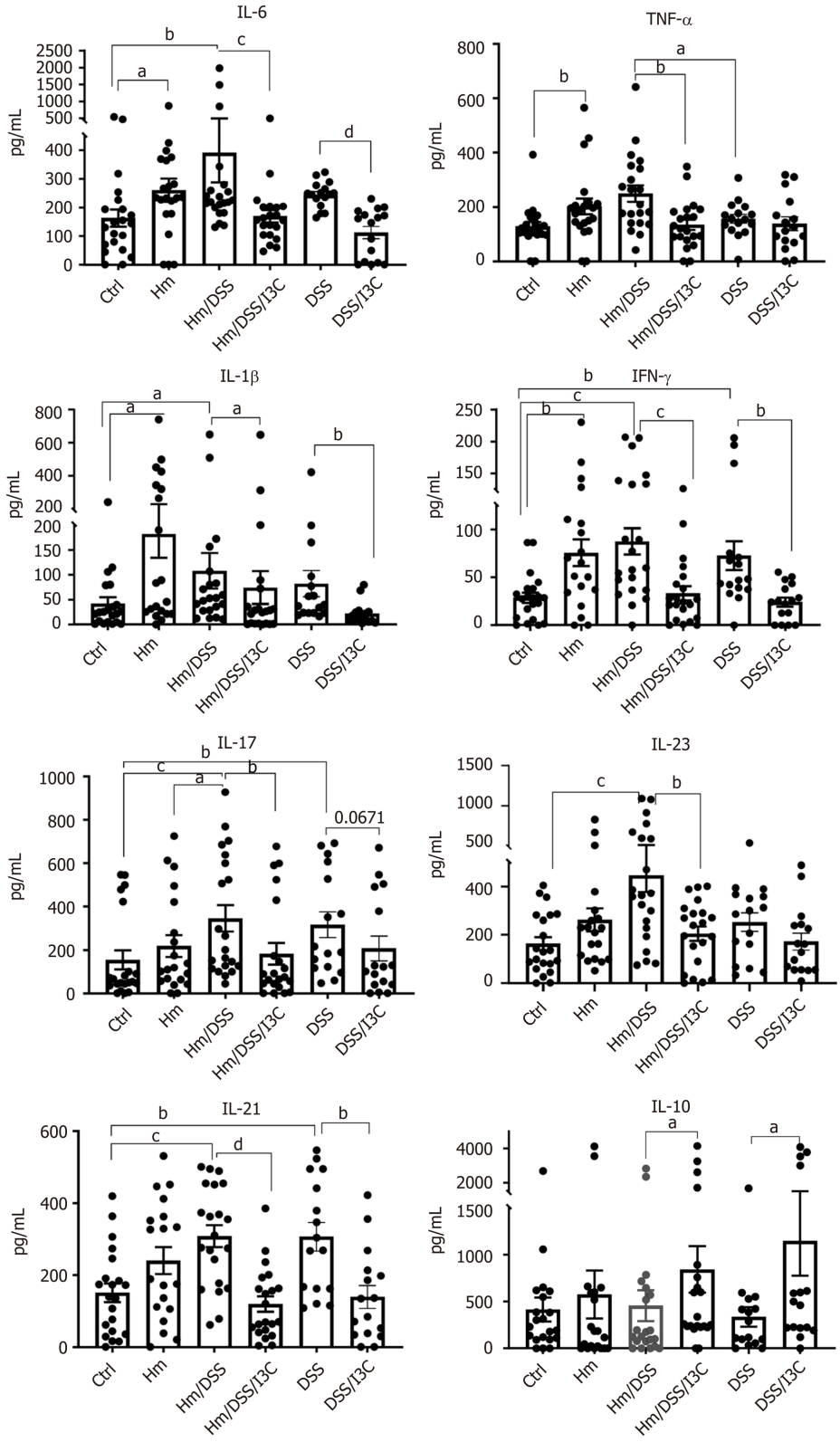


Figure 3 Expression of T helper 17 and Treg-associated genes in mesenteric lymph nodes. Gene expression was determined from total complementary DNA using qRT-PCR and values were normalized to the control group (n = 7). [a]$P \leq 0.05$; [b]$P \leq 0.01$; [c]$P \leq 0.001$. Ctrl: Control; Hm: *Helicobacter muridarum;* Hm/DSS: *Helicobacter muridarum* plus DSS; Hm/DSS/I3C: *Helicobacter muridarum* plus DSS plus I3C; DSS/I3C: DSS plus I3C.

clearly show that DSS and *H. muridarum* shift the T helper cell profile towards a Th17-dominated response, whereas I3C increases the Treg population. We next measured cytokine concentrations in colon homogenates to assess local immune cell and epithelial responses. These measurements encompass both epithelial cells and inflammatory cells. In most cases, production of pro-inflammatory cytokines was altered by multiple variables. Infection with *H. muridarum* alone increased all pro-inflammatory cytokines tested except IL-17 and IL-23 compared with control mice (Figure 4). In fact, cytokine levels in *H. muridarum*-infected mice were similar to those in uninfected, DSS-treated mice. Treatment of *H. muridarum*-infected mice with DSS caused trends towards further increases in most cytokines, but this was only significant in the case of IL-17. I3C treatment reduced secretion of all pro-inflammatory cytokines in DSS-treated, *H. muridarum*-infected and/or uninfected mice.

Levels of the anti-inflammatory cytokines IL-10 and TGFβ were only significantly altered by I3C, though TGFβ levels trended lower in DSS-treated mice (Figure 4). There were trends towards decreased IL-4 levels in uninfected mice treated with I3C, but not in *H. muridarum* infected mice treated with I3C. To summarize, I3C both decreases secretion of pro-inflammatory cytokines an increases secretion of anti-


IL-6
TNF-α
IL-1β
IFN-γ
IL-17
IL-23
IL-21
IL-10
pg/mL
Ctrl
Hm
Hm/DSS
Hm/DSS/I3C
DSS
DSS/I3C
0.0671

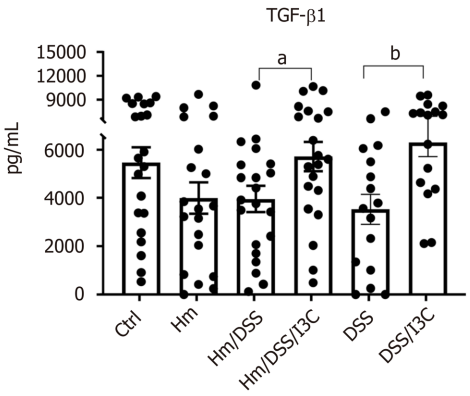


Figure 4 Colon cytokine production. Protein levels were determined by ELISA using colon homogenates (n = 17-21). [a]$P \leq 0.05$; [b]$P \leq 0.01$; [c]$P \leq 0.001$; [d]$P \leq 0.0001$. Ctrl: Control; Hm: *Helicobacter muridarum;* Hm/DSS: *Helicobacter muridarum* plus DSS; Hm/DSS/I3C: *Helicobacter muridarum* plus DSS plus I3C; DSS/I3C: DSS plus I3C.

inflammatory cytokines.

Plasma cytokines showed less dramatic changes than colon cytokines, the proinflammatory IL-17 and IL-6 cytokine concentrations were elevated in the *H. muridarum* /DSS group and to a lesser extent in the DSS group. I3C treatment reduced both to control levels. IL-10 was elevated by I3C in *H. muridarum*-infected mice and trended higher in the DSS/I3C group (Figure 5). TGFβ was reduced only in *H. muridarum*-infected mice and did not respond to I3C treatment. IL-4 and IL-22 were not significantly altered under any condition (Figure S3). Serum amyloid a levels were significantly increased only in *H. muridarum* /DSS mice, consistent with more severe pathology in that group. The neutrophil marker myeloperoxidase was strongly increased by *H. muridarum* infection, even in the absence of DSS treatment (Figure 5).

DISCUSSION

Effects of H. muridarum on susceptibility to DSS-induced colitis and treatment with I3C

Several models of inflammation and inflammatory bowel disease suggest that bacteria are necessary, but insufficient triggers of IBD[47] and several studies have reported that EHH species modulate IBD. As an example, *H. macacae* has been connected with chronic idiopathic colitis in young rhesus macaques and a study of children with CD reported PCR evidence for *Helicobacter* infection in 59% of patients *vs* 9% of healthy controls[11,48]. Similarly, Laharie *et al*[10] found that *H. pullorum* or *H. canadensis* infection was considerably related to CD in adults[10]. Finally, *H. canis*, another EHH species, has been detected in duodenal ulcerations associated with CD[49]. Therefore, certain *Helicobacter* species are almost certainly involved in IBD pathogenesis; however, the exact mechanism of EHH involvement remains undiscovered.

Th17 cells have a crucial role in colitis development in both humans and mouse models[50,51]. *H. pylori* is known to induce a Th17 response in the gastric mucosa[52-54], yet *H. pylori* infection is associated with a decreased risk of IBD[55]. *H. pylori* only colonizes the gastric mucosa, meaning that any effects of *H. pylori* on the colon are likely due to systemic effects of infection. Furthermore, EHH species lack the major *H. pylori* virulence factors *cagA* and *vacA*. Thus, one cannot assume that mucosal or immune effects of *H. pylori* infection will match those caused by EHH species. It is therefore necessary to use infection with EHH species to investigate potential mechanisms of EHH-mediated contributions to IBD.

The present study extends existing knowledge on *H. muridarum* pathogenesis. *H. muridarum* infection has been previously shown to induce colitis in C57BL/6 mice treated with DSS and in monoassociated severe combined immunodeficiency mice following the transfer of certain T cell populations[13,35,56]. We found increased weight loss and stool blood in *H. muridarum*-infected mice treated with DSS compared with DSS treatment alone, but diarrhea was actually lessened. Increased stool blood suggests damage to the mucosal barrier, potentially increasing exposure to other members of the gut microbiota. Though *H. muridarum* alone did not cause appreciable colitis symptoms, it caused modest colon shortening and inflammatory infiltrates.

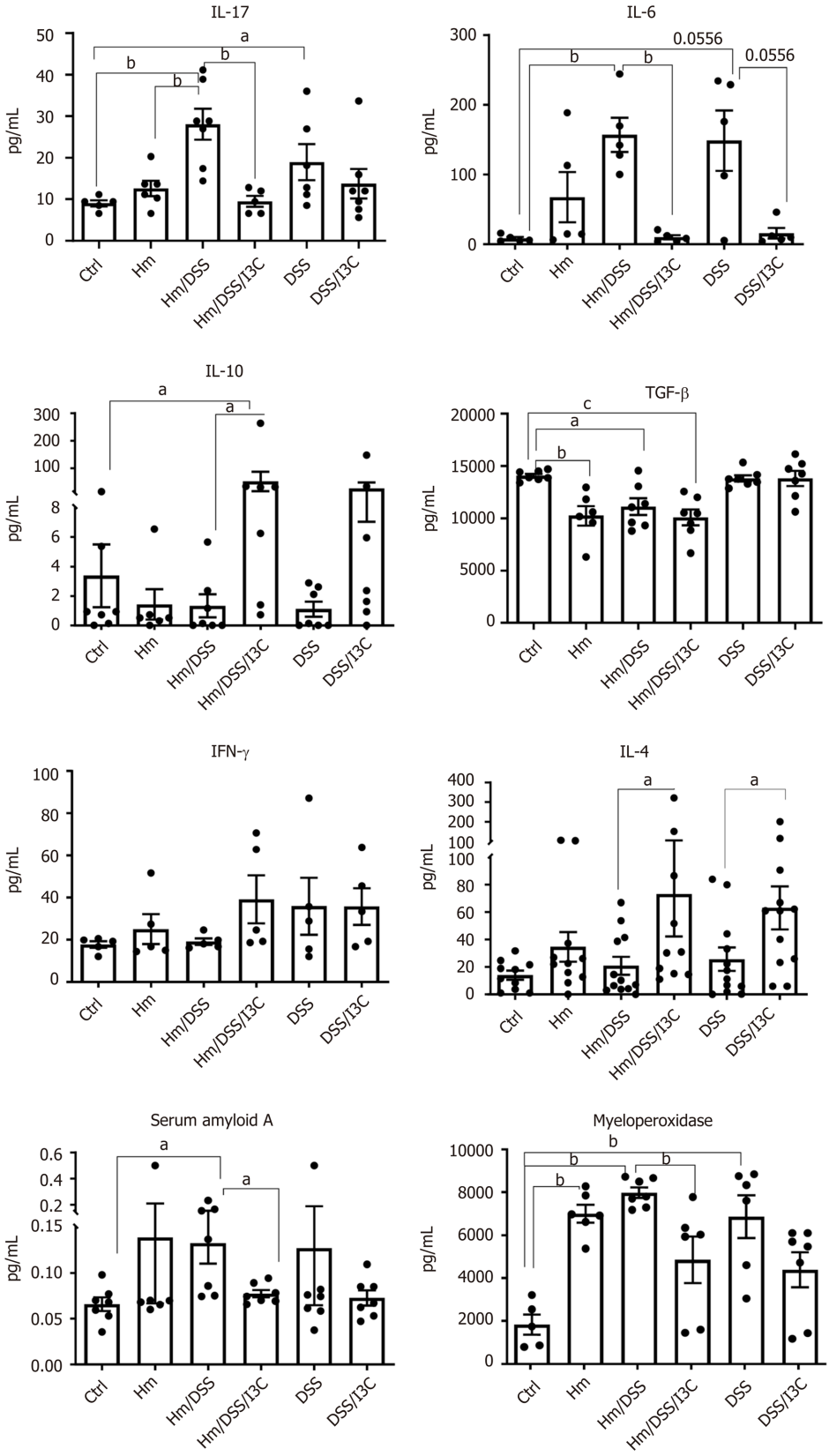


Figure 5 Plasma cytokine and inflammatory protein markers. Protein levels were determined by ELISA using plasma collected at the time of euthanasia (n = 5-7). $^{a}P \leq 0.05$; $^{b}P \leq 0.01$; $^{c}P \leq 0.001$. Ctrl: Control; Hm: *Helicobacter muridarum;* Hm/DSS: *Helicobacter muridarum* plus DSS; Hm/DSS/I3C: *Helicobacter muridarum* plus DSS plus I3C; DSS/I3C: DSS plus I3C.

Effects of H. muridarum, DSS, and I3C on microRNA expression

There is increasing interest in the use of miRNAs to diagnose and treat a wide variety of diseases and cancers. We examined mesenteric lymph node miRNA expression to

determine whether miRNA signatures explained the effects of *H. muridarum* and I3C. Many studies show that miRNAs participate in the regulation of crucial lymphocyte functions such as lymphocyte development, maturation, activation, and differentiation[19,28,57,58]. When considering the roles of miRNAs in Treg and Th17 function, it is necessary to distinguish between miRNAs involved in T cell differentiation and miRNAs involved in function. For example, miRNAs binding to the Treg transcriptional regulator gene FOXP3 prevent differentiation of Tregs and must be downregulated to allow Treg differentiation. Some miRNAs promote FoxP3 expression by downregulating expression of other genes, while others are induced by FoxP3 and influence Treg function. Still other miRNAs act in an autocrine manner, being both induced by FoxP3 and inducing FOXP3 expression[59]. Therefore, miRNA expression patterns can differ between naïve and mature T cells and between highly active and anergic T cells. A further complication of miRNA interpretation is that the same miRNA can have different effects depending on the cell type in which it is expressed. For example, miR-155 reportedly induces both Treg and Th17 cell differentiation[60] making it impossible to predict whether increased miR-155 in the lymph node, or even within the T cell population, favors a Treg or Th17 phenotype. Relative concentrations of miRNAs within each cell most likely dictate the cell phenotype.

These nuances explain the often disparate and contradictory results published in the literature. For example, Iborra *et al*[43] sought to compare serum and mucosal miRNAs profiles in human ulcerative colitis and Crohn's disease[43]. They found little overlap between serum and tissue miRNAs and many differences between their results and those published by others. Each miRNA has hundreds or thousands of targets[61] and each gene is likely controlled by dozens of miRNAs. In the context of IBD and colon cancer, miR-15b/16 is reported to regulate Tregs, macrophages, TP53, aquaporin 8, and the adenosine A2a receptor[41,62-65]. Surprisingly, miR-16 has even been suggested as a stable reference miRNA[66]. The multiple targets of miR-15b/16 may then explain why treatment of mice with miR-16 precursors ameliorates colitis, yet elevated miR-16 in human blood samples is associated with more severe IBD[62,67,68].

Interpretation of miRNA data in our study proved similarly challenging; however, there was remarkable overlap between the responses to *H. muridarum*, DSS, and I3C. Nonetheless, the direction of regulation was not always as expected. For example, when looking at a set of colitis-associated miRNAs, expression dropped following treatment with either *H. muridarum* or DSS, whereas human studies found increases in these miRNAs in IBD patients *vs* controls[43-45]. The human studies used either peripheral blood or colon biopsies as a source of miRNAs, whereas we used mesenteric lymph nodes. Lymph nodes differ in cellular composition compared to peripheral blood[69] and since the cell subsets change following *H. muridarum* infection or DSS treatment, the ratios of T cells to dendritic or other cell populations may change as well. Because colitis-associated miRNAs are likely expressed in multiple cell types, the meaning of a miRNA increase or decrease cannot be deciphered without knowing whether they rise or fall in each cell subtype. Presorting cells by flow cytometry would provide better data for understanding the effects of miRNA expression changes, but would not be feasible for most clinical samples due to the limited amounts of tissue available.

One would think that if DSS or *H. muridarum* decreases miRNA expression, then I3C treatment should increase it back to the control value. This was not necessarily the case for the same reasons mentioned above. I3C increased the number of cells found in MLN, and likely the ratios of cell types. The fact that I3C altered roughly the same subset of miRNAs as DSS or *H. muridarum* is consistent with its known anti-inflammatory effects, but does not shed light on the mechanism of I3C activity. Rather, the results are consistent with the hypothesis that miRNA changes due to I3C result from, rather than cause, immune response normalization.

We did not find measurable effects of *H. muridarum* infection alone on cytokine expression in the lymph nodes or on plasma cytokine levels; however, there were clear pro-inflammatory changes in the colon, which were further exacerbated by DSS administration. The cytokines induced by *H. muridarum* (TNFα, IL-1β, IL-6, and IFNγ) are typical of those induced by DSS treatment[17]. It is therefore not surprising that *H. muridarum* further increased production of inflammatory cytokines and worsened pathology following DSS treatment. In humans with IBD, increases in mucosal TNFα, IL-1β, IL-6, IL-23 and IFNγ are due to lamina propria monocytes or macrophages[70-72]. IFNγ is also produced by Th1 cells or potentially a new intraepithelial lymphocyte subtype, IL-17$^+$ IFNγ$^+$ T cells[73]. Regardless of cell source, IFNγ plays an important role in IBD pathology in humans and mice[74,75]. Thus, the effects of *H. muridarum* in our mouse model will likely be relevant to humans infected with enterohepatic species. In

general, *H. muridarum* infection more strongly influenced production of monocyte/dendritic cell-associated cytokines (TNFα, IL-1β, IL-6 , IL-23) compared to T cell-associated cytokines (IFNγ, IL-4, IL-10, IL-17, IL-21, IL-22), although IFNγ was increased by *H. muridarum* alone.

Cytokines secreted by monocytes or dendritic cells can drive T cell differentiation. TGFβ and IL-6 can drive several differentiation pathways depending upon which other cytokines are present[76]. The combination TGFβ, IL-6, and IL-23 efficiently induce Th17 differentiation[77]. In spite of local cytokine responses suggestive of a Th17-promoting milieu, we did not find evidence of a substantial T cell shift in mice infected with *H. muridarum* alone. DSS treatment was required for enhanced expression of RORC and IL17 in lymph nodes or increased plasma IL-17. In contrast, there was no apparent difference in Treg markers in lymph nodes or plasma between infected and uninfected mice. These data suggest that local effects of *H. muridarum* are conducive to, but not sufficient for Th17 polarization. This is consistent with a "two hit" hypothesis, although in this case, the two hits are *H. muridarum* and an irritant rather than host genetics and the microbiome.

I3C shifts the immune balance

The use of alternative medicine to treat inflammatory disorders is appealing to many patients. Numerous studies demonstrate that dietary indoles possess anti-cancer properties such as anti-oxidant activity, regulation of cell cycle and apoptosis, and control of endocrine metabolism[78-83]. DIM is sold over the counter as BioResponse DIM® with claims that it promotes breast health, prostate health, and weight management. Before such products can be confidently recommended, their mechanisms of action must be uncovered. In the case of IBD, it is prudent to investigate the effect of gut microbiota on response to treatment.

Several natural compounds, including I3C, are AhR ligands[84]. AhR is now known to govern differentiation and function of both T cells and macrophages[85-87]. Several studies have shown that AhR plays a vital role in regulation of immune responses specifically promoting the generation of Tregs while suppressing Th17 cells[88,89]. Previous studies have provided convincing proof that Foxp3-positive Treg cells are essential for gastrointestinal immune homeostasis[90] and that increased Th17 differentiation promotes colitis[50,51]. Consistent with other reports from various disease models, we found that I3C increases the Treg population and decreases the Th17 population[91-93]. The shift from Th17 to Treg was not inhibited by *H. muridarum* infection. Additionally, we found that I3C reduced production of every other pro-inflammatory cytokine tested, except for IL-22, which was not affected in any treatment group. Though we did not specifically analyze macrophages, others have shown that I3C or DIM suppresses IL-6, TNFα, IL-1β, IL-23 and IFNγ[94-96]. Therefore, I3C most likely inhibits colitis development via effects on both T cells and macrophages.

In summary, our studies suggest that *H. muridarum* increases susceptibility to DSS-induced colitis by inducing macrophage-associated cytokines and creating a mucosal milieu conducive to Th17 polarization. I3C ameliorates colitis via induction of Tregs, suppression of Th17 cells, and suppression of macrophage-associated pro-inflammatory cytokines. While no mouse model perfectly replicates human IBD, the identities of miRNAs altered by *H. muridarum* and DSS were similar to colitis studies in both mice and humans, although the direction of change was not always consistent. I3C is equally effective in the presence and absence of *H. muridarum*. Further research is warranted on the roles of EHH species in human IBD and the use of I3C or similar AhR agonists for the treatment of inflammatory bowel disease.

ARTICLE HIGHLIGHTS

Research background

Enterohepatic *Helicobacter* (EHH) species can infect humans and many animal species. Some of these species are known to cause disease in animals, while others have been described as commensal. In humans, epidemiological evidence suggests that EHH species are associated with inflammatory bowel disease (IBD), but the specific species involved and mechanisms of action are unknown. New treatments being tested for IBD include natural compounds and microRNA (miRNA)-based therapies. MiRNA is also being investigated as a diagnostic tool.

Research motivation

Given the limitations of performing IBD research in humans, an animal model of EHH-mediated pathology is needed. Such a model should reflect the biological changes seen during human IBD. *Helicobacter muridarum* (*H. muridarum*) has been referred to as a commensal in mice, yet we previously determined that *H. muridarum* worsens colitis resulting from dextran sodium sulfate (DSS). This suggested that EHH species could represent environmental factors that cause or worsen IBD in genetically susceptible individuals. It is also important to determine whether phytochemicals being investigated as IBD treatments are influenced by infection with EHH species because there are no commercially available tests for EHH infection in humans.

Research objectives

We sought to determine how the immune and miRNA profiles of *H. muridarum*-infected wild-type mice compared with DSS-treated mice and with published immune and miRNA profiles of IBD patients. We also determined whether efficacy of a broccoli-derived anti-inflammatory compound, indole-3-carbinol (I3C), was reduced by *H. muridarum* infection.

Research methods

We measured changes in body weight, stool consistency, and stool blood following *H. muridarum* infection, DSS treatment, and/or I3C treatment. We then measured cytokine responses in the colon and plasma and histopathological changes in the colon. MiRNA changes and T cell population changes were measured in mesenteric lymph nodes.

Research results

While *H. muridarum* infection alone did not cause clinical symptoms, it did cause colonic inflammation and induced proinflammatory cytokines. As expected, *H. muridarum* worsened colitis caused by DSS treatment, but it did not prevent amelioration of colitis by I3C treatment. Both the miRNA changes and cytokine responses to *H. muridarum* infection were similar to those seen in human IBD and due to DSS treatment. Changes in cytokines and miRNA were consistent with a Th17 response.

Research conclusions

H. muridarum causes subclinical colitis that increases vulnerability to DSS treatment. Since I3C is an aryl hydrocarbon receptor agonist, the efficacy of I3C in the presence of *H. muridarum* suggests that *H. muridarum* does not influence the aryl hydrocarbon receptor agonist pathway. The strong similarities between cytokine and miRNA profiles induced by DSS and those induced by *H. muridarum* suggest that similar mechanisms could be at play and that the mouse model is suitable for studying host interactions with EHH species.

Research perspectives

This research supports the hypothesis that EHH species could contribute to human IBD by exacerbating the response to other inflammatory stimuli. More research is needed on the prevalence of EHH species in humans and the mechanisms underlying EHH-mediated colonic damage.

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