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AhR Expression on Rorc-Expressing Immune Cells is Essential for I3C-Mediated Protection Against Colitis

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AHR EXPRESSION ON RORC-EXPRESSING IMMUNE CELLS IS ESSENTIAL FOR I3C-MEDIATED PROTECTION AGAINST COLITIS

By

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THESIS SUMMARY

Colitis, an inflammatory bowel disorder, is caused by an array of factors, including luminal microbiota alterations and dysregulation of the immune system. Gut microbiota produce indole, a compound thought to protect the colon from inflammatory damage. In this paper, we explore the effect of indole-3-carbinol, a compound found in many cruciferous vegetables, treatment on an ulcerative colitis mouse model with aryl hydrocarbon receptor (AhR) depletion in Rorc-expressing immune cells. Indole-3-carbinol has been proven to be a ligand for AhR receptors and to reduce inflammation in an interleukin-22-dependent manner in female mice. This study explores the same theory but with both female and male mice.

ABSTRACT

Colitis is an inflammatory bowel disorder (IBD) whose etiology is attributed to modification in the luminal microbiota and dysregulation in the immune response. Indole is a signaling molecule which is naturally produced by gut luminal microbiota. Indole-3-carbinol (I3C) is a compound commonly found in vegetables and a ligand for the aryl hydrocarbon receptor (AhR). Previous studies have detected decreased expression and activation on the AhR receptor in colitis patients, thought to possibly alter gut microbiota metabolism, subsequently promoting colitis. [1] AhR, expressed in a variety of immune and epithelial cells, contributes to gut homeostasis by affecting vital mediators such as regulatory T cell (Treg)/T helper 17 (Th17), colonic epithelial cell (CEC)- regenerating interleukin 22 (IL-22) production, and secretion of the protective mucous layer and antimicrobial peptides (AMPs) by CECs. I3C in previous experiments has been shown to help prevent colitis through induction of IL-22 via innate lymphoid type 3 cells (ILC3s). In this study, we investigated how AhR deficiency in Rorc-expressing cells (which we refer to as AR mice) affects anti-inflammatory immune response during I3C treatment of colitis, whether I3C-AhR interaction in CECs increase IL-22 production during colitis, and the effect of AhR deficiency on epigenetic modifications induced by I3C during colitis. A dextran sodium sulphate (DSS) colitis mouse model was used in this study to mimic the effects of ulcerative colitis and was introduced into drinking water and treatments of I3C treatment were given. Results showed compared to wild-type (WT) AR mice induced with colitis were unaffected by 13C treatment. In addition, the protective IL-22 production by ILC3s was lost for the AR mice. Lastly, we show that I3C treatment does induce epigenetic modifications (i.e. DNA methylation) in the promoter region of IL-22. Thus, we conclude AhR knockout in Rorc-expressing immune cells mice resist the production of IL-22 via ILC3s after I3C treatment during DSS-induced colitis, and loss of this potential protective cytokine prevents I3C-mediated beneficial alterations in the gut microbiome.

INTRODUCTION

Colitis is a disease characterized by chronic inflammation in the digestive tract and tissue annihilation within the intestinal tract. Colitis can progress to form colon cancer over a prolonged period, making preventative treatments of colitis vitally important.[2] There are two distinct forms of colitis which are defined by the location of the inflamed areas and by the depth of lesions in the gastrointestinal tissue: ulcerative colitis and Crohn's disease. Ulcerative colitis is known to cause inflammation limited to the large

intestine/colon, only reaching to the superficial epithelial surface.[3] Crohn's disease exhibits inflammation and typically extends deeper into the colonic tissue layers.[4] The development of colitis is still an ambiguous topic but is known to be attributed to genetics, environmental triggers such as diet, and dysregulation of the luminal microbiota and immune response.[2]

There are two different mouse models for inducing colitis, each representing a distinct form of colitis, using dextran sodium sulfate (DSS) or 2,4,6-trinitrobenzenesulfonic acid (TNBS).[5] The DSS induction provides as a model for ulcerative colitis since DSS is toxic to epithelial colon cells.[5] The TNBS induction portrays Crohn's disease since it produces an immune response.[5] Both models are known to exhibit the clinical symptoms of weight loss, colon shortening, colon damage, and diarrhea/bloody stool. This experiment used the DSS colitis model.

Our lab recently published a report using the TNBS model on female mice to explore whether the compound I3C, a naturally occurring plant product, can be effective in preventing colitis and attempted to identify the related mechanisms.[6] The research uncovered that I3C mediates inflammation by promoting the production of the cytokine, interleukin-22 (IL-22). IL-22 production is linked to the activation of the aryl hydrocarbon receptor.[6] These new findings are expanded on in this paper since this paper describes a nearly identical experiment, except using the DSS model and mice with AhR conditionally knocked out in Rorc-expressing immune cells, which are known to produce IL-22.

The aryl hydrocarbon receptor (AhR) is a cytosolic-bound intracellular receptor, meaning that the receptor is located in the cytoplasm until activated by a ligand.[7] Once the ligand has bound to the receptor, the receptor is able to translocate to the nucleus, heterodimerizes with aryl hydrocarbon receptor nuclear translocator (ARNT) and mediate gene transcription of AhR-responsive genes. The gene transcription mediation by the AhR receptor helps regulate liver detoxification and immune cell regulation.[7] As a result, the AhR receptor can be found in a variety of cell types, including immune cells and colonic epithelial cells.[7] Interestingly, previous research has discovered patients suffering from colitis typically have a decrease in expression of AhR in lamina propia (LP) mononuclear cells.[8]

I3C is a likely candidate for treatment of colitis since it is a known ligand for AhR.[9] I3C already has a number of known therapeutic advantages thus far, such as anti-cancer, antioxidant, anti-microbial, and anti-inflammatory effects.[10] I3C's anti-inflammatory effects are thought to be attributed to it driving IL-22 production, which promotes epithelial cell regeneration and maintaining mucosal barrier integrity.[6] IL-22 can be expressed by many cell types, including T helper cells (Th17/Th22) and innate lymphoid Type 3 cells (ILC3).[11] This experiment attempts to determine the mechanism which I3C undergoes when acting as an anti-inflammatory agent by studying how AhR deficiency in Rorc-expressing immune cells (i.e. ILC3s) affects treatment of colitis. There were six experimental groups used in this experiment as a result: LM+Vehicle, AR+Vehicle, LM+I3C, AR+I3C, LM+Colitis, AR+Colitis, LM+Colitis+I3C, and AR+Colitis+I3C.

This experiment is able to quantify the number of IL-22 expressing immune cells by conducting a staining panel for labeling biomarkers distinct to T cells and ILC3 cells; the lineage monoclonal antibodies CD45+Lin-CD90.2+Rorgt are indicative of ILC3 cells and CD4 for Th22.

Since IL-22 production is already known to be increased by I3C treatment, and I3C is known to be related to DNA transcription through AhR, this experiment also examines the methylation patterns of experimental mice. Increased methylation indicates decreased transcription; decreased methylation indicates increased transcription. Thus, an increase in methylated DNA would suggest a decrease in AhR receptors or a decrease in 13C.

There is no definitive cure for colitis and traditional drug treatments induce many side effects. Glucocorticoids, for example, can cause a variety of alterations in the liver and pancreas.[12] These patients are recommended to have their blood checked regularly by their doctor for signs of these possible side effects. This study examined blood samples from each experimental mouse at the end of the trial, in order to check for similar side effects.

METHODS

Animals

Male and female C57BL/6J mice were purchased from Jackson Laboratory, and experimental mice used were between the age of 8 to 10 weeks, at the average weight of 15-18 grams. The breeding pairs included Ahr-flox (Ahrtm3.1Bra/J) bred with a Rorc-cre (B6.FVB-Tg(Rorc-cre)1Litt/J). The mice were housed at the Association for Assessment and Accreditation of Laboratory Animal Care-accredited (AAALAC-accredited) animal facility in the University of South Carolina School of Medicine, Columbia. The facilities offered precise pathogen-free conditions, with 12-hour light/12-hour dark cycles.

This experiment required the use of six distinct experimental groups. The littermate mice (LM) served as controls in comparison to the AR mice. The groups for the experiment were LM+Vehicle, AR+Vehicle, LM+I3C, AR+I3C, LM+DSS, AR+DSS, LM+DSS+I3C, and AR+DSS+I3C.

Generation of mice lacking AhR in Rorc-expressing cells (AR mice)

The cre-flox recombination technique was used in our lab to generate mice lacking AhR in Rorc-expressing immune cells. AhR-floxed ($AhR^{f/f}$) mice were bred with Rorc(γt)-cre mice to produce AhR knockout (KO) mice in T cells/ILC3s, named AR mice. Each mutant transgene mouse was genotyped using PCR to ensure the conditional deletion from DNA, as seen in **Figure 1**. The DNA was isolated from the tissue via tail snips (≤25 mg). The DNA was isolated from the tail snips using the DNeasy Blood & Tissue Kit (cat. nos. 69504 and 69506) from Qiagen. Once the DNA was isolated, gel electrophoresis was then used to validate the mutant DNA using primers specific to detect the mutant strains. TAE (trise-acetate-EDTA) buffer (VWR) was used with agarose (VWR) to create a standard 1.5% agarose gel. Approximately 1.5 g of agarose was missed with 100 mL of TAE in a microwaveable flask and then microwaved for 1-3 minutes, or until the agarose was completely dissolved. Once

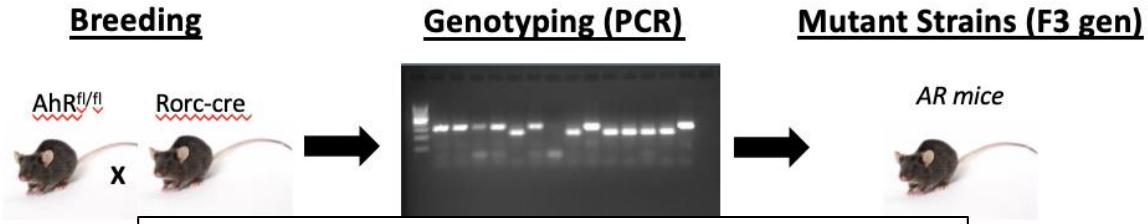

Figure 1. Summary of experimental genotyping.

cooled, approximately 2-3 μl of ethidium bromide (Sigma-Aldrich), EtBr, was added to the mixture. Ethidium bromide would bind to the DNA and was essential for visualizing the DNA under ultraviolet (UV) light. The mixture was then poured into the casting tray of the gel box with the well comb already in place. The poured gel was then left to sit at room temperature for 20-30 minutes, or until it had fully solidified. Once solid, the agarose gel was placed in the gel box, the gel box was filled to TAE until the gel was covered, and a voltage source was then attached to the electrodes. The loading buffer, 1X TAE buffer (Sigma-Alder) was added to each of the DNA samples and the samples were then carefully loaded into the wells of the gel. The gel was run at 80-150 V for approximately 1 hour. The electrodes were removed, and the gel was carefully removed from the gel box. The gel was placed under a UV light (Universal Hood li Molecular Imager with Camera, Bio-Rad) which allowed for the DNA fragments to be visualized. The control counterparts to the mutant mice were controlled, or littermate (LM), mice.

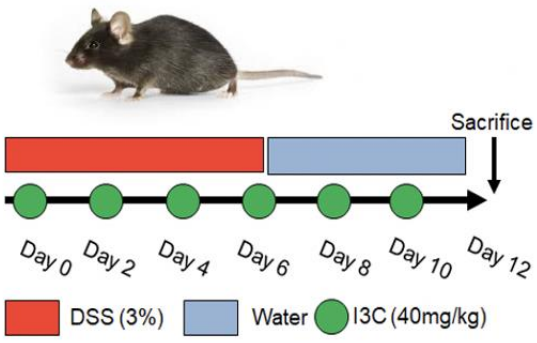

Figure 2. Summary of DSS colitis model with I3C treatments. Colitis was induced using a DSS model and the effectiveness of the I3C treatments were tested with the following experimental groups unless otherwise stated: LM vehicle (n=7), LM I3C (n=5), LM DSS+vehicle (n=7), LM DSS+I3C (n=7), AR DSS+vehicle (n=11), and AR DSS+I3C (n=11).

Induction of colitis and treatment with I3C

The DSS used for this experiment was purchased from MP Biomedical LLC. and was colitis grade. DSS model colitis was induced in 4 of the experimental groups through drinking water (**Figure 2**). The drinking water contained 3% DSS for 6 days, which was followed by 8 days of normal drinking water. Previous experiments have proven 1 week of DSS treatment is sufficient for effectively inducing an ulcerative colitis model.[13] There were 3 experimental groups who received I3C treatment. For the treatment groups with DSS colitis model, mice were given injections of 40 mg/kg in 0.05% DMSO/corn oil starting 1 hour after the administration of the DSS water and continued every other day until the end of the experiment. LM groups were also given 0.05% DMSO/corn oil injections every other day.

Assessment of colitis disease parameters

Clinical parameters for colitis were measured and examined throughout the entirety of the experiment. The mice were weighed daily using a scale (ALC-2100.1, Acculab), in order to determine the percent weight loss over the duration of the trial. Colonoscopies were also performed to assess tissue damage. At experimental endpoints, the mice were euthanized using isoflurane overdose. After the trial completion, the lengths of the colons were measured. Serum (~10 microliters) was analyzed using a VetScan (Allied Analytic) to determine diagnostic blood panel percentages, as outlined in **Figure 3**.

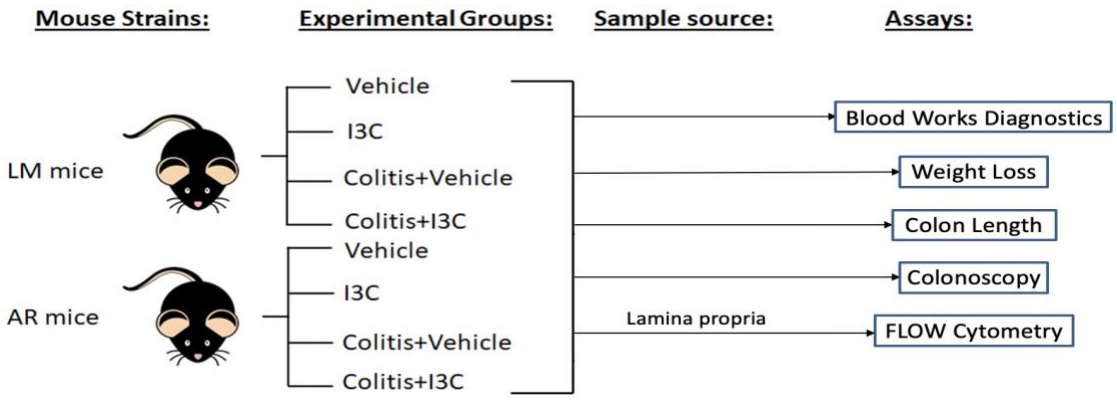

Figure 3. Summary of experimental method.

Lamina propria layer isolation and immune cell validation using flow cytometry

Flow cytometry was used to explore the effects on immune cell response when AhR was depleted in Rorc-expressing cells. Cells from the lamina propria (LP) were isolated by removing and cleaning the intestine of a 6-10-week-old C57BL/6 mouse using PBS (VWR). The intestines were placed into 50 mL conical tubes (VWR) with 15 mL of pre-digestion buffer (VWR) and incubated. The colons were then cut laterally (~0.5 cm pieces) and incubated for 40 minutes at 37°C under a continuous rotation. Once incubated, the samples were vortexed for 10 seconds and applied onto a 100 μm cells strainers (VWR) having been placed on a 50 mL tube. The samples were then transferred into a new 50 mL tube containing 20 mL of HBSS (VWR) and incubated at 37°C for 10-20 minutes under a continuous rotation. The samples were then vortexed for another 10 seconds to ensure they were mixed well and applied onto a MACS SmartStrainer, 100 μm, and placed in a 50 mL tube.

The intestine tissue was then transferred into the gentle MCS C tube which contained the digestion buffer and the tube was sealed tightly. The sample was then incubated again for 30-75 minutes at 37°C under continues rotation. The C tube was the attached nupside down to the sleeve of the gentleMACS Dissociator (Miltenyi Biotec) and the gentleMACS was run. Once the program was complete, the tube was detached from the Dissociator and a short spin (up to 300xg) was completed in order to collect the sample from the bottom of the tube. Another 5 mL of complete RPMI media (VWR) was added, and the cell suspension was applied to a 100 μm cell strainer, inserted in a 50 mL tube. The 100 μm strained was washed with 10 mL of PBS with 3% FBS (FACS Buffer, VWR) and the strainer was then discarded.

The cell suspension was centrifuged at 1300 rpm for 10 minutes at 40°C. We resuspended the cells in 10 mL of FACS buffer and a layer on 5 mL of 90% Percoll, having been diluted using 10x PBS (VWR). The samples were then spun at 2000 rpm for 15 minutes at room temperature and resuspended in 10 mL FACS. We spun the samples again at 1300 rpm for 10 minutes at 40°C and resuspended in 1 mL of RBC lysis buffer on ice for 2 minutes. Once chilled, 10 mL of FACS was added and the samples were spun at 1300 rpm for 10 minutes at 40°C and resuspended in 2 mL of FACS. An automated cell counter (Bio-Rad) was used to record the cell count and viability.

Once the cells were isolated from tissue, they were stained with fluorescent antibodies, indicators of IL-22-producing

cells. The cells were then passed through the FACS Celesta flow cytometer (BD Biosciences). The gating and the analysis of flow plots were performed using FlowJo version 10.5.3 (BD Biosciences). Two specific immune cells were stained in the flow cytometry: ILC3 ($CD45^+Lin^-CD90.2^+Rorgt^+$) and Th22 ($CD3^+CD4^+IL\text{-}17^-$).

Determination of DNA methylation status

The DNA methylation patterns were examined to determine the effects of I3C treatments on transcription in the DSS-model of ulcerative colitis. A genome-wide DNA methylation was conducted using methylated DNA immunoprecipitation sequencing (MeDIP-seq) on cells isolated from CD45+ immune cells from the lamina propria of experimental groups (DSS+Vehicle and DSS+I3C). MeDIP-seq was performed as previously described.[14]

Statistics

All statistical analysis were conducted using GraphPad Prism software unless otherwise indicated. A 1-way ANOVA and Tukey's hoc multiple comparisons tests were used to determine the statistical significance when comparing 3 or more groups. A 2-way ANOVA and Turkey's hoc multiple comparisons tests were used to determine the statistical significance when comparing 3 or more groups with more than one data point. For comparisons between only two 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 conducted involving the use of mice obey NIH guidelines under protocols approved by the IACUC at the University of South Carolina School of Medicine (Columbia).

RESULTS

Treatment with I3C and/or DSS does not effect blood work diagnostics

We conducted a full blood panel on each of the mice at the end of the experiment. The blood panel determined the white blood cell (WBC) count and the percent of lymphocytes, monocytes, and neutrophils, as illustrated in **Figure 4**. The white blood cell count provides information on the immune system since they aid the body to fight infection and disease. Lymphocytes are what determine the specificity of the immune response to infection and disease. Monocytes aid other WBC to remove dead or damaged tissues,

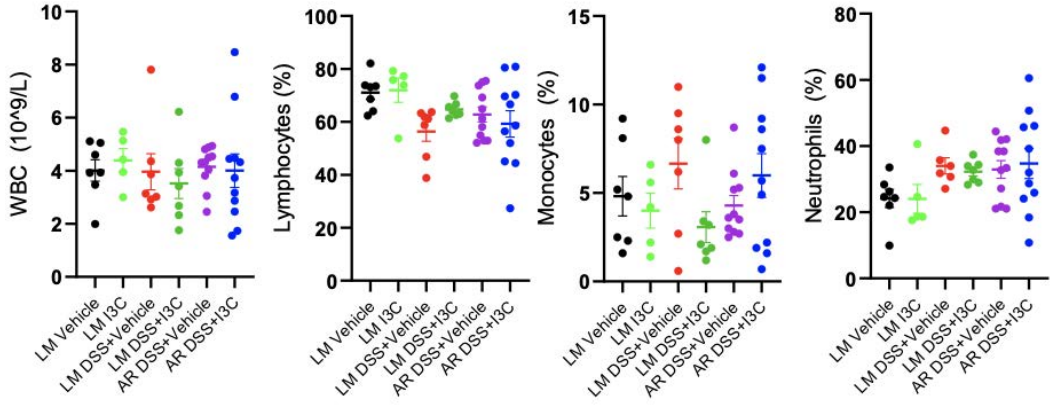

Figure 4. AhR defficiancy in Rorc-expressing immune cells resulted in no effect on blood work. Blood was collected from experimental mice and run on a VetScan to access**,** White blood cell (WBC) count, lymphocyte percent, monocyte percent, and neutrophil percent. Significance was determined using a 1-way ANOVA and Tukey's multiple comparisons tests. There was found to be no significant differences among the experimental groups when examining the blood diagnostics panel.

kill cancer cells, and regulate immunity from infection and disease. Neutrophils are the first immune cells to respond to a foreign pathogen and are responsible for the destruction of the microorganism or molecule.

Previous results from our have proven that neither the I3C treatment does not affect blood-circulated immune cells like the white blood cell count and percent of lymphocytes, monocytes, and neutrophils, which play a role in the immune response. To that end, we studied mice with AhR deficiency in Rorc-expressing immune cell, in comparison to the four control groups, and found no statistically significant differences, suggesting the major immune cell differences are localized in the colon.

AR mice with I3C and DSS treatments loose I3C-mediated beneficial effects

For the purpose of testing the efficacy of I3C treatment in the DSS colitis model, mice with colitis were treated with an I3C regime that has been effective in

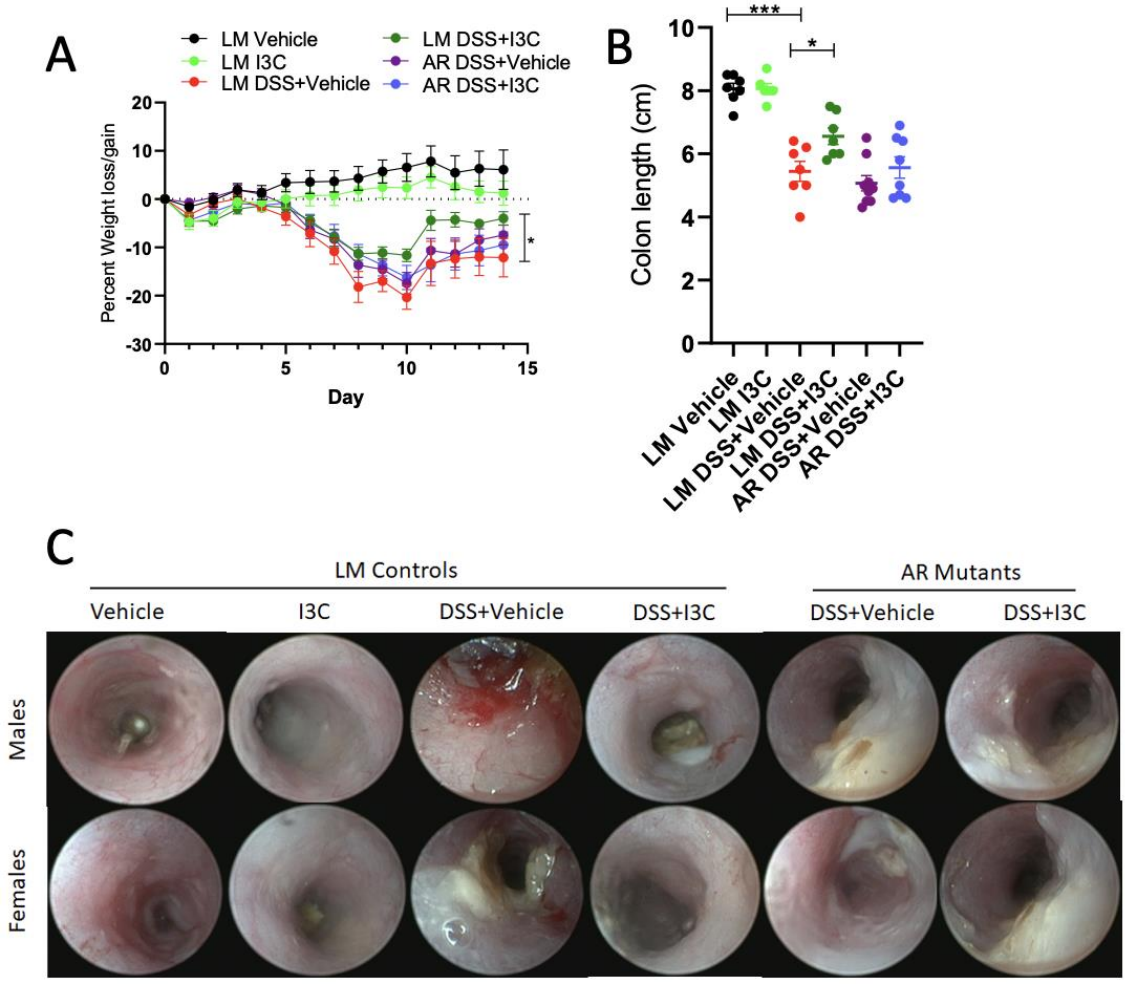

Figure 5. I3C treatment failed to protect against DSS-induced colitis in both AR mice. The parameters assessed for quantifying the disease was **(A)** percent weight loss, **(B)** colon length, and **(C)** Representative colonoscopy images were taken during day 7 of the DSS model (n=5). Scale bars: 100 micrometers (original magnification, x100). Significance was determined using a 1-way ANOVA and Tukey's multiple comparisons tests for the colon lengths and macroscopic colitis score and a 2-way ANOVA and Tukey's multiple comparisons tests for the percent weight loss; *P<0.05; **P<0.01; ***P<0.005; ****P<0.001.

treating other forms of inflammatory diseases.[15] We used 6 experimental groups of mice: vehicle alone (LM vehicle), I3C treatments alone (LM I3C), diseased alone (LM DSS+Vehicle), diseased receiving I3C treatment (LM DSS+I3C), diseased mutant (AR DSS+Vehicle), and diseased mutant receiving I3C treatment (AR DSS+I3C). The percent weight loss was determined each day of the experiment from the start (**Figure 5A**). We observed a significant percent weight loss in the LM DSS+Vehicle, the AR DSS+Vehicle, and the AR DSS+I3C (**Figure 5A**). The percent weight loss was slightly reduced for the LM DSS+I3C (**Figure 5A**). Whereas, the LM Vehicle and the LM I3C experienced no significant weight loss (**Figure 5A**), which suggested AR mice no longer responded to I3C like the LM controls. Next, we measured the colon lengths at the end of the experiment (**Figure 5B**). Mice induced with DSS colitis receiving I3C treatment experienced a reduction in their colon (~1.25 cm) compared to the control groups, LM vehicle and LM I3C (**Figure 5B**). A significant reduction in the colon lengths were found in the LM DSS+Vehicle group (~2.5 cm), the AR DSS+Vehicle group (~3.0 cm), and the AR DSS+I3C group (~2.5 cm). No statistical significant difference was found in the colon lengths between the diseased mutant mice and the diseased mutant mice receiving treatment.

Colonoscopy images revealed that the diseased mutant mice receiving treatment had evidence of ulcerations and tissue annihilation through the lining of the colon, whereas the observations from the diseased LM receiving treatment exhibited far less destruction (**Figure 5C**). The diseased vehicle mice, mutant diseased mice, and the mutant diseased mice receiving I3C treatment displayed

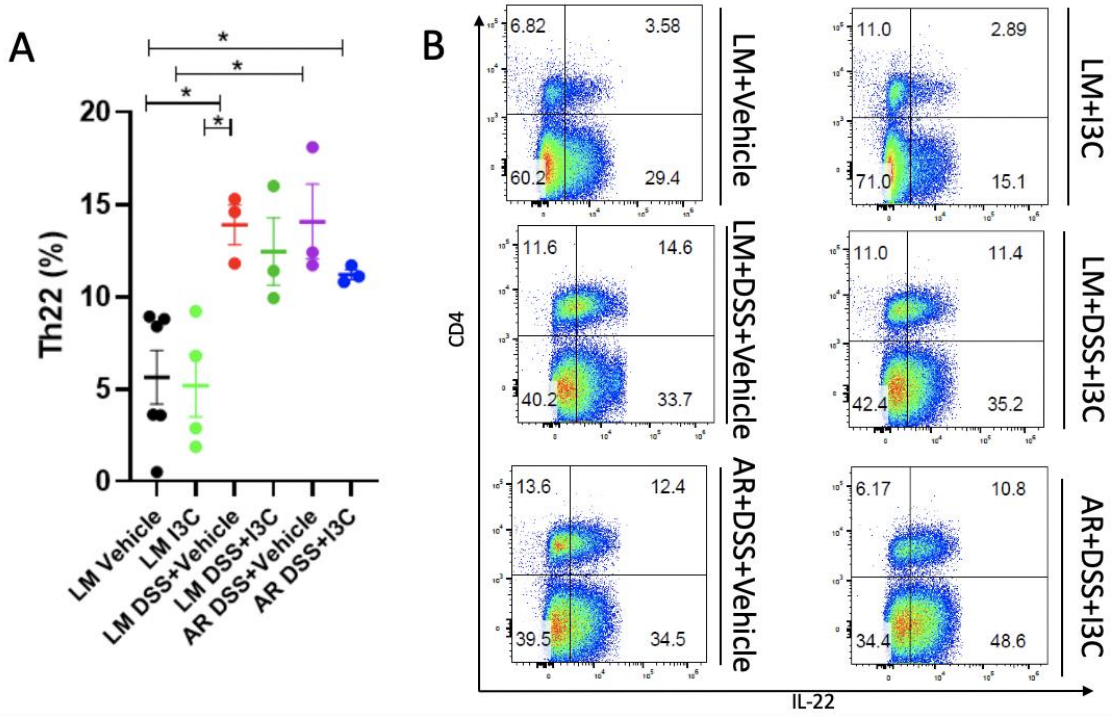

Figure 6. IL-22 secretion by Th22 cells is unaffected in diseased AR mice. (A) Percent of IL-22 producing Th22 cells. **(B)** Representative flow charts for each experimental group. Scale bars: 100 micrometers (original magnification, x100). Significance was determined using a 1-way ANOVA and Tukey's multiple comparisons tests; *P<0.05; **P<0.01; ***P<0.005; ****P<0.001.

significantly increased colon damage in comparison to the controls, LM Vehicle and LM I3C (**Figure 5C**). Taken altogher, it appeared that AR mutants lost the ability to respond to I3C treatment, unlike their LM counterparts.

I3C-mediated increase of IL-22 secreted by T cells is not altered in diseased AR mice

The expression of IL-22 producing Th22 cells in each experimental group was determined using flow cytometry and staining for CD4, a biomarker for T helper cells. The percentages indicated in the bottom left corner of each flow chart provides a numerical value for comparison of experimental groups based on levels of CD4 expressing IL-22. The higher the percentage, the greater the cells expression. The flow charts depict LM+Vehicle mice intensity as 3.58, 2.89 for LM+I3C, 14.6 for LM+DSS+Vehicle, 11.4 for LM+DSS+I3C, 12.4 for AR+DSS+Vehicle, and 10.8 for AR+DSS+I3C mice (**Figure 6B**). The percent of IL-22 producing Th22 cell was also determined for each experimental group based off of the flow cytometry data. We found no statistically significant difference in the percent of Th22 cells between the diseased AR mutant receiving treatment and diseased LM mice receiving I3C treatment (**Figure 6A**), thus suggesting AhR on Rorc-expressing immune cells does not alter IL-22 production by Th22 cells.

I3C-mediated increase of IL-22 secreted by ILC3 cells is prevented in diseased AR mice

Flow cytometry was also used for exploring whether the quantity of ILC3 cells in the lamina propria is affected by AhR depletion in immune cells (AR mice). The flow cytometer determined the percent of IL-22 synthesizing ILC3s (**Figure 7A**)

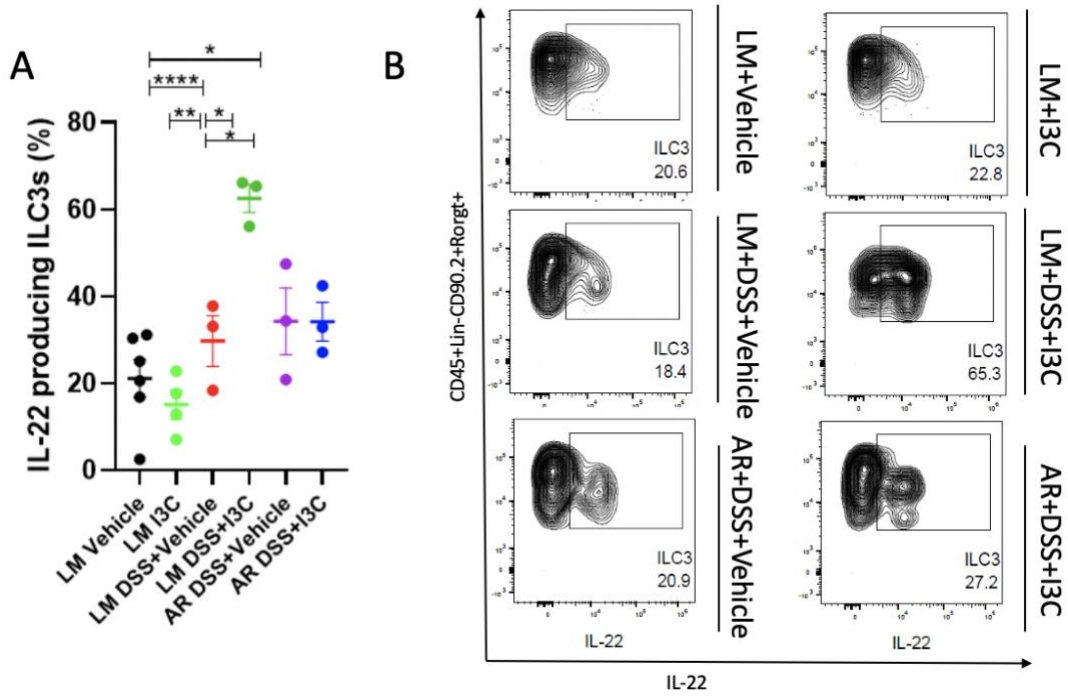

Figure 7. IL-22 secretion by ILC3 cells is prevented in diseased AR mice. (A) Percent of IL-22 producing ILC3 cells. **(B)** Representative flow charts for each experimental group. Scale bars: 100 micrometers (original magnification, x100). Significance was determined using a 1-way ANOVA and Tukey's multiple comparisons tests; *P<0.05; **P<0.01; ***P<0.005; ****P<0.001.

and revealed a representative bar chart (**Figure 7B**). The representative bar charts quantify the area of the shaded region, as seen in the bottom right corner of each flow plot. Thus, larger area values indicate an increased number of IL-22 producing ILC3s identified using the fluorescent markers. The bar charts depict percent quantification of the ILC3s ($CD45^+Lin^-CD90.2^+Rorgt^+$) producing IL-22.

It should be noted that the intensity value indicated by the flow charts for LM+Vehicle can be found as 20.6, 22.8 for LM+I3C, 18.4 for LM+DSS+Vehicle, 65.3 for LM+DSS+I3C, 20.9 for AR+DSS+Vehicle, and 27.2 for AR+DSS+I3C mice (**Figure 7B**). The data shows that in disease LM mice treated with I3C there was a significant increase in IL-22 production, which we had previously shown. However, disease AR mice treated with I3C did not show an increase in IL-22 production in ILC3 cells, which suggests that this increase in protective IL-22 by this immune cell subset was dependent on AhR expression.

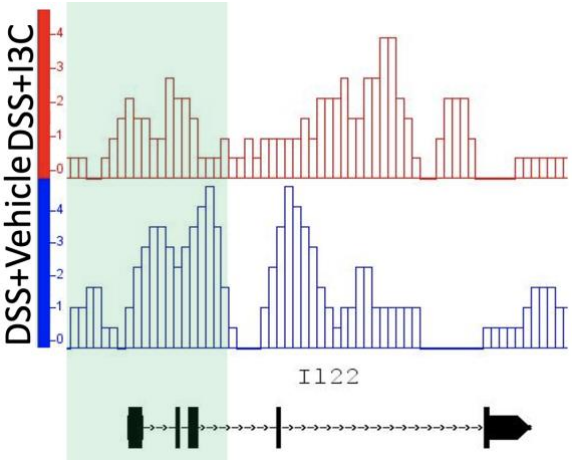

Figure 8. I3C decreases DNA methylation in promoters of IL-22 regulatory genes during DSS-induced colitis. DNA methylation was determined using MeDIP-seq, as described in the Methods. The IL-22 promoter region can be found in the figure highlighted green. The methylation patterns of DSS+Vehicle (bottom) and DSS+I3C (top) samples are displayed in the figure.

Treatment of I3C decreases methylation of IL-22 promoter region in diseased mice

This experiment also sought to begin investigating I3C-mediated epigenetic modifications, DNA methylation specfically, involved in IL-22 regulation. The lab plans on exploring these epigenetic modifications further in future studies with AhR conditional knockout mice. DNA methylation is of great significance since it is relates to the frequency which the gene is being transcribed. Samples from the two diseased wild-type (WT) experimental groups, WT DSS+Vehicle and WT DSS+I3C, were used for MeDIP-seq, and methylation patterns were determined for the IL-22 promoter region of the DNA (**Figure 8**). The DNA methylation of the diseased mice receiving I3C treatment was found to be significantly decreased in comparison to the diseased mice not receiving treatment. This suggests that one of the mechanisms by which I3C increases IL-22 production is by decreasing DNA methylation in the promoter region for this cytokine.

DISCUSSION

Research and new development of safe preventative treatments to inhibit colitis are of great importance. Studies have shown chronic inflammation through the intestine to be linked to alterations in the luminal microbiota and to dysregulation of the immune system.[6] There is an increasing consensus that AhR plays a prominent role in regulating the immune system through the intestine.[16] AhR has been shown in promote the production of interleukin-22 (IL-22) once activated by a ligand.[17] However, the mechanisms which AhR primarily uses to regulate the immune system in the gut microbiome is still

unknown. There appears to be a link with the expression of AhR in Rorc-expressing immune cells that occur during colitis and the expression of IL-22, which is demonstarted further by this study.

The DSS-induced diseased mutant AR mice lost all protective benefits of I3C treatments. The diseased AR mice proved to have no statistically significant difference in their percent weight loss, colon lengths, and macroscopic score when receiving I3C treatment. Whereas, the diseased littermate mice showed a significant improvement and increase in their percent weight loss and colon length when receiving I3C treatment. The representative colonoscopy images display the healing affects of I3C through the colon when comparing the diseased LM mice to the diseased LM mice receiving treatment. However, the colonoscopy images of the diseased mutant AR mice receiving I3C treatment revealed evidence of ulcerations and tissue destruction in the lining of the colon, which was just as severe as those found in the diseased mutant mice not receiving treatment.

As noted before, I3C is a recognized ligand of AhR. Recent reports have shown I3C to have a protective effect by improving the necroptosis and inflammation of intestinal epithelial cells by activating AhR in mice with DSS-induced colitis.[18] In this current study, AhR activation was examined in Rorc-expressing immune cells. There also has become an increasing consensus that upon activation the AhR receptor, there is an increased regulation of IL-22, helping restore the gut homeostasis.[19] Thus, this study has further proven that without an abundance of AhR receptors, I3C has nothing to bind to, which results in no production of IL-22, at least in ILC3s. Similar reports have found ILC3s production of IL-22 to change upon I3C treatment.[6]

Of particular note was the fact that secretion of IL-22 by ILC3 cells was prevented in AR mutant mice receiving I3C treatment. This was a important finding because AhR can be found in many different types of cells; however, it has remained unclear as to which cell type promotes gastrointestinal thereuputic benefits via AhR activation. This study revealed an increase in IL-22 secreting ILC3 cells when diseased LM mice were given I3C treatments. However, the diseased mutant mice with a depletion of AhR in Rorc-expressing immune cells displayed no increase in IL-22 producing ILC3 cells when receiving I3C treatments, proving AhR was essential for this I3C-mediated effect.

In comparison, the T helper type 22 (Th22) cells were unaffected by a depletion of AhR receptors. This would suggest that the AhR receptor primarily use IL3C cells over Th22 cells. We concluded that the AhR receptor primarily translocates in ILC3 cells when producing IL22. Other recent reports have shown collectively that ILC3 cells are responsible for regulating gastrointestinal mucosal homeostasis via controlled generation of IL-22 and IL-17.[20] Interestingly enough, studies have also shown the frequency of ILC3s to be decreased in inflamed tissue.[21]

The methylation patterns of the DSS-induced colitis diseased mice recieving I3C treatment revealed a decrease in methylation, when compared to the diseased mice not receiving treatment. The reduced methylation implies that there is a increase in the transciption of the IL-22 gene. An increase in transciption would result in an increase in creation of the cytokine IL-22, which helps reduce and control inflammation.[22] Other reports have recently suggested a protein coding gene, the G Protein-Coupled Receptor 34, to be a

receptor of ILC3 which triggers tissue repair upon colon damage.[23]

The study's findings are consistent with the theory that DSS-induced diseased mice when treated with I3C are able to manage chronic inflammation and tissue damage through production of IL-22. In conclusion, these studies revealed that I3C mediated protection against colitis was heavily dependent on AhR expressed in Rorc-expressing immune cells, such as ILC3s. Without AhR expression, protective IL-22 was no longer produced after I3C treatment of colitis, thus negating the beneifical effects observed in LM controls.

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