All-trans Retinoic Acid Counteracts Diarrhea and Inhibition of Downregulated in Adenoma Expression in Gut Inflammation

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Background: Intestinal epithelial apical membrane Cl^-/HCO_3^- exchanger DRA (downregulated in adenoma, SLC26A3) has emerged as an important therapeutic target for diarrhea, emphasizing the potential therapeutic role of agents that upregulate DRA. All-trans retinoic acid (ATRA), a key vitamin A metabolite, was earlier shown by us to stimulate DRA expression in intestinal epithelial cells. However, its role in modulating DRA in gut inflammation has not been investigated.

Aims: Our aim was to analyze the efficacy of ATRA in counteracting inflammation-induced decrease in DRA in vitro and in vivo.

Methods: Interferon-γ (IFN-γ)-treated Caco-2 cells and dextran sulfate sodium (DSS)-treated C57BL/6J mice served as in vitro and in vivo models of gut inflammation, respectively. The effect of ATRA on IFN-γ-mediated inhibition of DRA function, expression, and promoter activity were elucidated. In the DSS colitis model, diarrheal phenotype, cytokine response, in vivo imaging, myeloperoxidase activity, and DRA expression were measured in the distal colon.

Results: All-trans retinoic acid (10 μM, 24 h) abrogated IFN-γ (30 ng/mL, 24 h)-induced decrease in DRA function, expression, and promoter activity in Caco-2 cells. All-trans retinoic acid altered IFN-γ signaling via blocking IFN-γ-induced tyrosine phosphorylation of STAT-1. All-trans retinoic acid cotreatment (1 mg/kg BW, i.p. daily) of DSS-treated mice (3% in drinking water for 7 days) alleviated colitis-associated weight loss, diarrheal phenotype, and induction of IL-1β and CXCL1 and a decrease in DRA mRNA and protein levels in the colon.

Conclusion: Our data showing upregulation of DRA under normal and inflammatory conditions by ATRA demonstrate a novel role of this micronutrient in alleviating IBD-associated diarrhea.

Key Words: diarrhea, DRA, DSS-colitis, intestinal inflammation, vitamin A

INTRODUCTION

Diarrhea is one of the most debilitating symptoms in patients with Crohn's disease (CD) and ulcerative colitis (UC).[1] The extent and severity of diarrhea in IBD patients are symptomatic of disease activity and commonly involve dysregulated epithelial electrolyte transport in the intestine. Decreased absorption and/or increased secretion of fluid and electrolytes lead to water retention in the intestinal lumen, resulting in diarrhea. With respect to the mechanism, inflammation-induced decrease in absorption of NaCl has been identified as the predominant cause for IBD-associated diarrhea[1] with minimal or no alterations observed in anion secretion.

In this regard, the predominant route of electroneutral NaCl absorption in the mammalian ileum and colon involves coupled operation of Na^+/H^+ and Cl^-/HCO_3^- exchangers. Key molecular species involved in electroneutral NaCl absorption include[1] Na^+/H^+ exchanger 3 (NHE3, SLC9A3) and Cl^-/HCO_3^- exchanger DRA (downregulated in adenoma, SLC26A3). Disturbances in DRA function and/or expression have been implicated in the pathophysiology of diarrheal disorders. Mutations in DRA gene lead to congenital chloride diarrhea, and DRA knockout mice exhibit diarrheal phenotype.[2] Reduction in the colonic crypt Cl^-/HCO^-_3 exchange activity with a parallel decrease in SLC26A3 mRNA has been reported in patients with ulcerative colitis[3] and in mouse model of dextran sulfate sodium (DSS)-induced colitis.[4–6] A genome-wide association study identified SLC26A3 polymorphism associated with lower DRA expression as a risk factor for the development of ulcerative colitis in Japanese and Chinese cohorts.[7,8] Collectively, DRA has emerged as an important and novel therapeutic target for

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IBD-associated diarrhea, which warrants an in-depth investigation of the mechanisms involved in its regulation in normal physiology and in diarrheal diseases associated with gut inflammation.

Pro-absorptive agents that can upregulate DRA function and expression may serve as potential antidiarrheals for the treatment of IBD-associated diarrhea. In this regard, several lines of evidence suggest that ATRA, the predominant natural metabolite of vitamin A derived from animal and plant food sources, exhibits protective effects in the intestinal mucosa including immunomodulatory and antidiarrheal effects. Studies utilizing 2,4,6 Trinitrobenzene sulfonic acid (TNBS) and DSS-mouse model of colitis showed that ATRA reduced the degree of inflammation and necrosis in the mouse distal colon.[9–11] Vitamin A deficiency has been shown to exacerbate intestinal injury in the rat model of IBD,[12] and lower levels (below normal) of vitamin A are common in patients with IBD and short bowel syndrome.[13] Studies have also reported that repletion of vitamin A in cases of its deficiency reduce the risk of diarrhea and gut barrier dysfunction, indicating an important role for this nutrient in gut mucosal repair.[14] We have previously demonstrated that ATRA treatment upregulates DRA expression via transcriptional mechanisms in Caco-2 cells.[15] However, the direct effects of ATRA on DRA function and expression in intestinal inflammation remain unknown, and understanding such basic mechanisms may lead to better and novel therapies for treatment of IBD-associated diarrhea. Therefore, the current study was undertaken to evaluate the efficacy of ATRA in upregulating DRA expression in both in vitro and in vivo models of gut inflammation. Our studies demonstrated for the first time the counteracting effects of ATRA in attenuating diarrheal phenotype and downregulation of DRA expression in DSS-induced colitis. All-trans retinoic acid also alleviated inhibition of DRA function and expression by Interferon-γ (IFN-γ) in Caco-2 cells.

MATERIALS AND METHODS

Materials

All-trans retinoic acid (ATRA), human recombinant IFN-γ, and 4, 4’-diisothiocyanostilbene-2, 2’ disulfonic acid (DIDS) were purchased from Sigma Aldrich (St. Louis, MO). All-trans retinoic acid stock (10 mM) was made in 100% ethanol and stored in the dark at −80°C. Radionuclide ^{125}I (NaI) was procured from Perkin Elmer (Boston, MA). Downregulated in adenoma (DRA) antibody was raised against the C-terminal amino acid (745–764) sequence: INTNGGLRNRVYEPVETKF of SLC26A3 (accession number: BC025671) at the Research Resource Center (RRC) of the University of Illinois at Chicago. Goat antirabbit antibody conjugated to horseradish peroxidase was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Kits for Luciferase assay and β-galactosidase assay were procured from Promega (Madison, WI) and BD Biosciences (Palo Alto, CA), respectively. All other chemicals were of reagent grade and were obtained from commercial sources.

Cell Culture

Caco-2 cells were obtained from ATCC (American Type Culture Collection, Manassas, VA) and maintained in Eagle’s Minimum Essential medium (EMEM, ATCC) supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, 2 mg/L gentamicin, and 20% fetal bovine serum in 5% CO2-95% air environment at 37°C in T-150 cm^2 plastic flasks. Cells between passages 25 and 45 were used for the present study. Caco-2 cells were plated at a density of 1×10^4 cells/well on 12-well transwell collagen-coated inserts (permeable support). Fully differentiated Caco-2 monolayers (14 days postplating) were treated with ATRA (10 µM) for 24 hours in serum-free cell culture medium in the presence or absence of IFN-γ (30 ng/mL, basolateral treatment) for the assessment of Cl^-/HCO_3^- exchange activity, DRA mRNA, and protein levels.[15, 16]

Measurement of Cl^-/HCO_3^- Exchange Activity

The Cl^-/HCO_3^- exchange activity was determined by measuring DIDS-sensitive $^{125}I^-$ uptake in base loaded cells as previously described in our earlier research.[6, 17] A recent study further validated the use $^{125}I^-$ for chloride transport studies.[18] The Cl^-/HCO_3^- exchange activity was assessed as DIDS-sensitive $^{125}I^-$ uptake, and the specific activity is expressed as nanomoles per milligram protein per 5 minutes. The 5-minute time period was chosen because it was within the linear range of $^{125}I^-$ uptake in this system.

Transient Transfections and Luciferase Assay

For the promoter studies, 1.30×10^7 cells were transiently transfected by electroporation in 100 µL of solution T provided in Amaxa nucleofactor system (Lonza, Allendale, NJ)[15] with full-length DRA promoter (30 µgs) cloned upstream of the luciferase reporter gene and 2.0 µgs of p-CMV-β (β-galactosidase mammalian expression vector, BD Biosciences, Clontech, Palo Alto, CA). The latter plasmid served as an internal control for transfection efficiency. Transfected cells were plated on 12-well Transwell collagen-coated inserts.[16] After 24 hours of transfection, cells were treated with vehicle (100% ethanol) at 0.1% final concentration or ATRA in presence or absence of IFN-γ. After completion of the treatment (24 h), cells were lysed using passive lysis buffer (Promega, Madison, WI). Activities of luciferase and β-galactosidase were measured according to the manufacturer’s instructions in a luminometer (Promega). Promoter activity was calculated as a ratio of luciferase value to β-galactosidase value for each sample and expressed as percentage of control.

Animal Studies and Induction of Colitis

Animal studies were approved by the Animal Care Committee (ACC) of the University of Illinois at Chicago and the Institutional Animal Care and Use Committee (IACUC) at the Jesse Brown Veterans Affairs Medical Center. Eight-week-old male-C57BL6/J mice were obtained from the Jackson Laboratory

(Bar Harbor, ME) and were acclimatized for 7 days with free access to water and standard rodent pellet diet in the animal facility.

Induction of colitis and treatment with All-trans Retinoic Acid

After acclimatization, the animals were randomly divided into 4 groups (n = 5): control, ATRA, DSS, and ATRA+DSS. Acute colitis was induced in 2 of the groups (DSS and ATRA+DSS) through oral administration of 3% (wt/vol) DSS (36,000 to 50,000 Da; MP Biomedicals, Solon, OH) in drinking water for 7 days as previously described.[6] Fresh DSS solution was provided every second day. Mice in control and ATRA group received drinking water without DSS. All-trans retinoic acid (1 mg/kg body weight in corn oil) was administered intraperitoneally (i.p.) daily to the ATRA and ATRA+DSS groups from the commencement of DSS treatment, and the groups were sacrificed on day 8. The animals were observed carefully once daily, for intake of DSS water, weight change, stool consistency, and appearance of gross blood in feces and at the anus. At day 8, the animals were euthanized by exposure to CO_2, and laparotomy was performed. The entire colon was removed, and the lengths and weights were recorded. A small piece from the distal part of the colon was snap frozen in liquid nitrogen for measurement of myeloperoxidase (MPO) activity or embedded in optimal cutting temperature embedding medium (Tissue-Tek O.C.T compound) for immunostaining. Mucosal samples from the distal colon were scraped to study the effect of ATRA on DRA mRNA and protein expression in vivo.

Myeloperoxidase Activity

MPO activity was measured in the distal colon to evaluate the degree of inflammation. Briefly, distal colonic tissue from each experimental group was homogenized in phosphate buffer containing 0.5% w/v hexadecyltrimethylammonium bromide (HTAB), where HTAB acts as a detergent and aids in the release of MPO from primary granules of the neutrophils. The tissue homogenate was subjected to centrifugation at 13,000 rpm for 5 minutes and clear supernatant was collected.[19] Enzyme activity was measured by a colorimetric assay involving hydrogen peroxide dependent oxidation of o-Dianisidine dihydrochloride (Sigma) catalyzed by MPO.[11] Absorbance was read at 450 nm using a microplate reader. MPO activity was expressed as units per milligram of tissue.

Real-time Polymerase Chain Reaction Analysis

Total RNA was extracted from Caco-2 cells or scraped mucosa from mouse colon using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Equal amounts of RNA from treated and control samples were reverse transcribed and amplified in 1-step reaction using Brilliant SYBR Green qRT-PCR Master Mix Kit (Agilent Tech., Santa Clara, CA). The primers for human DRA and glyceraldehyde 3-phosphate dehydrogenesae (GAPDH) are described previously.[15] Specific primers used for quantification of DRA and cytokine levels in mice distal colon are listed in Table 1.

Western Blotting

Whole cell lysates from treated or untreated Caco-2 cells or tissue lysate from distal colon were prepared as previously described.[6, 15, 17] Lysis buffer (Cell Signaling, Danvers, MA) was supplemented with protease inhibitor cocktail from Roche (Indianapolis, IN) and phosphatase inhibitor cocktail 3 (Sigma Aldrich). Protein concentration of the lysates was determined by the Bradford method.[20] To examine the expression levels of DRA, equal amounts (75 µg/sample) of cell or tissue lysates were solubilized in sodium dodecyl sulfate-gel loading buffer and boiled for 5 minutes. Proteins were loaded on a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was probed with anti-DRA (1:100 dilution) or GAPDH antibody (Sigma; 1:3000 dilution) in 1X-PBS and 2.5% nonfat dry milk overnight at 4°C. Expression levels of basal and phosphorylated STAT-1 and STAT-3 were detected using specific antibodies from Cell Signaling (Danvers, MA) at recommended dilutions in 1X-TBST and 1% BSA buffer overnight at 4°C. The membranes were washed 5 times with the wash buffer (1X-PBS or TBS and 0.1% Tween-20) for 5 minutes and probed with HRP-conjugated goat antirabbit antibody (1:2000 dilution) for 1 hour. Bands were visualized with enhanced chemiluminescence detection reagent (Bio-Rad, Hercules, CA).

Hematoxylin and Eosin Staining

To study the histology of the distal colon, hematoxylin and eosin (H&E) staining was performed. Staining was conducted on OCT-embedded distal colonic tissue sections of 5 µm thickness utilizing the H&E staining kit (ScyTek Laboratories, West Logan, UT) as per the manufacturer's protocol.

TABLE 1. Gene-Specific Primers Used for Real-Time PCR Analysis of mRNA Levels

Genes	Primer Sequence (5'-3')
Mouse CXCL1	Forward: AAAGATGCTAAAAGGTGTCCCCA
	Reverse: AATTGTATAGTGTTGTCAGAAGCCA
Mouse IL-1β	Forward: GCAACTGTTCCTGAACTCAACT
	Reverse: ATCTTTTGGGGTCCGTCAACT
Mouse DRA	Forward: TGGTGGGAGTTGTCGTTACA
	Reverse: CCCAGGAGCAACTGAATGAT
Mouse GAPDH	Forward: TGTGTCCGTCGTGGATCTGA
	Reverse: CCTGCTTCACCACCTCTTGAT

Immunofluorescence Studies in Mouse Distal Colon

For immunostaining, 5-µm frozen sections of distal colon were made using a cryostat and were fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature. Fixed sections were permeabilized with 0.5% NP-40 and blocked with 2.5% normal goat serum (NGS) for 60 minutes followed by incubation with rabbit anti-DRA (1:100) and mouse anti-villin (1:100) antibodies in 1% NGS for 2 hours at room temperature. After washes with 1% NGS, the sections were incubated with the secondary antibodies, Alexa Fluor 488-conjugated goat antirabbit IgG (green), and Alexa Fluor 568-conjugated goat antimouse IgG (red) for 60 minutes and then washed and mounted using Slowfade Gold antifade with DAPI reagent (blue, nuclei) (Invitrogen) by using coverslips.[6, 17] Sections were imaged using a Carl Zeiss LSM 510 laser-scanning confocal microscope equipped with 20X water immersion objective.

In Vivo Imaging

In vivo bioluminescent imaging was performed using an in vivo imaging system at Biological Research Laboratories, University of Illinois at Chicago. At day 7, mice from various treatment groups were anesthetized with isoflurane/oxygen and were injected with L-012 (20 mg/kg bwt i.p., Wako Chemical, Neuss, Germany). The L-012 solution was prepared fresh right before the experiment by dissolving 2 mg L-012/mL in sterile phosphate buffer saline (PBS) and kept in the dark at room temperature. After L-012 injection, mice were placed into the light tight chamber of the Xenogen IVIS Spectrum in vivo imaging system (Caliper Life Sciences, Hopkinton, MA), equipped with a cooled CCD camera. Bioluminescence production was measured using the IVIS camera set to obtain images with 1-minute exposure time unless otherwise specified. The Living Image software automatically captured and overlayed the bioluminescence images by registering the photons produced by L-012 during imaging (1 to 5–minute exposure times) depending on signal strength. Bioluminescence signals from the abdominal region of interest (ROI) were used for quantification of total counts and expressed as relative luminescence units (RLU).

Statistical Analysis

Results are expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism (version 6.0). Significance of difference observed between different treatment groups was evaluated by one-way ANOVA followed by the Tukey test. The level of significance was set at $P \leq 0.05$.

RESULTS

All-trans Retinoic Acid Treatment Attenuated IFN-γ-induced Decrease in Cl^-/HCO_3^- Exchange Activity in Caco-2 Cells

We have previously shown that long-term treatment of Caco-2 cells with ATRA increased DRA expression via transcriptional mechanisms.[15] To examine the effects of ATRA on DRA function (Cl^-/HCO_3^- exchange activity), Caco-2 monolayers were treated for 24 hours with different concentrations of ATRA (1, 5, and 10 µM). The Cl^-/HCO_3^-exchange activity was measured as DIDS-sensitive $^{125}I^-$ uptake after base loading the cells, as described in the methods section.[6, 17] As shown in Figure 1A, 10 µM ATRA significantly increased Cl^-/HCO_3^- exchange activity (~2.5-fold) compared with the vehicle-treated control. However, no significant change was observed at 1 and 5 µM ATRA. As the inflammatory cytokine IFN-γ has been shown to inhibit Cl^-/HCO_3^- exchange activity,[6] we sought to determine the efficacy of ATRA

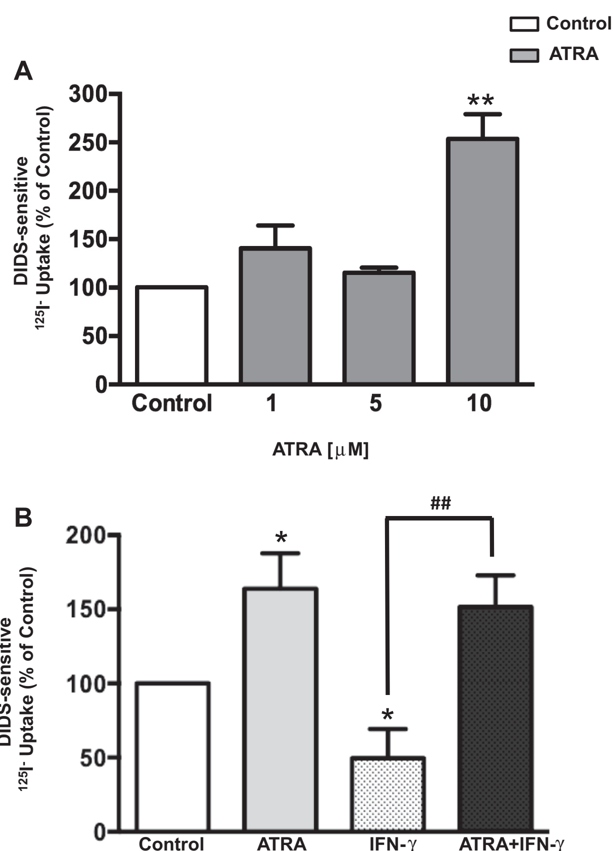

FIGURE 1. All-trans retinoic acid increases apical membrane Cl^-/HCO_3^- exchange activity and attenuates the inhibitory effects of interferon-γ in Caco-2 cells. A, Postconfluent Caco-2 cells were treated with different doses of ATRA (1, 5, and 10 µM) for 24 hours in serum free cell culture medium. Cl^-/HCO_3^- exchange activity was measured as DIDS (600 µM)-sensitive $^{125}I^-$ uptake for 5 min. Results are expressed as % of control and represent mean ± SEM of 3 separate experiments performed in triplicate. $^{**}P < 0.01$ compared with control. B, Caco-2 cells plated on transwells were treated apically with ATRA (10 µM) alone or in combination with interferon- γ (IFN- γ, 30 ng/mL) supplemented in the basolateral chambers of the transwells for 24 hours. Cl^-/HCO_3^- exchange activity was measured as DIDS-sensitive (600 µM) $^{125}I^-$ uptake for 5 minutes. Results are expressed as percentage of control and represent mean ± SEM of 3 separate experiments performed in triplicate. $^{*}P < 0.05$ compared with control and $^{\#\#}P < 0.01$ compared with IFN- γ.

in counteracting the inhibitory effects of IFN-γ (30 ng/mL, 24 h) on DIDS-sensitive $^{125}I^-$ uptake. As expected, IFN-γ treatment resulted in a significant decrease (~50%) in the Cl^-/HCO_3^- exchange activity. However, the decrease in Cl^-/HCO_3^- exchange activity induced by IFN-γ was blocked by cotreatment with ATRA (Fig. 1B). These findings suggest that ATRA exerts anti-inflammatory effects and attenuates IFN-γ-induced decrease in DRA function in intestinal epithelial cells.

All-trans Retinoic Acid Blocked IFN-γ-induced Decrease in DRA mRNA and Protein Levels

Interferon-γ has been shown to play an important role in the pathogenesis of intestinal inflammatory diseases such as ulcerative colitis and Crohn's disease where functional expression of DRA is compromised.[1, 21] Also, our earlier studies showed that IFN-γ decreased DRA mRNA and protein expression.[6, 16] We therefore tested whether ATRA could modulate DRA expression in presence of IFN-γ. Treatment of IFN-γ to Caco-2 cells resulted in decreased DRA mRNA (53%) and protein levels (62%). Similar to our previous report,[15] ATRA alone resulted in a significant upregulation in DRA mRNA (~2-fold) (Fig. 2A) and protein (40%) levels (Fig. 2B–C) compared with the control. Parallel to the results obtained for Cl^-/HCO_3^- exchange activity, ATRA also significantly counteracted the inhibitory effects of IFN-γ on DRA mRNA and protein levels (Figs. 2A–C) ($P \le 0.01$ compared with IFN-γ).

All-trans Retinoic Acid Counteracted the Inhibitory Effects of IFN-γ at the Transcriptional Level

In a previous study, we reported that IFN-γ attenuates DRA expression at the transcriptional level by decreasing DRA promoter activity.[16] To determine the mechanisms of ATRA effects in counteracting IFN-γ-induced inhibition of DRA expression, Caco-2 cells transiently transfected with DRA promoter construct (p-1183/+114) along with pCMV-β-galactosidase expression vector were treated for 24 hours with ATRA (apically) in presence or absence of IFN-γ (basolaterally). DRA promoter activity was determined by firefly luciferase activity normalized to β-galactosidase activity to correct for transfection efficiency. As shown in Figure 3, ATRA treatment significantly stimulated DRA promoter activity (~2.5-fold), whereas IFN-γ decreased the promoter activity by 48.3 ± 1.2%. Cotreatment of cells with ATRA blocked the IFN-γ-induced reduction in DRA promoter activity, indicating that ATRA effects could be via modulation of the IFN-γ-induced signaling cascade that decrease DRA gene transcription.

All-trans Retinoic Acid Abrogated IFN-γ Induced Phosphorylation of STAT1

Interferon-γ primarily functions by employing the JAK/STAT pathway, and the dysregulation of this pathway has

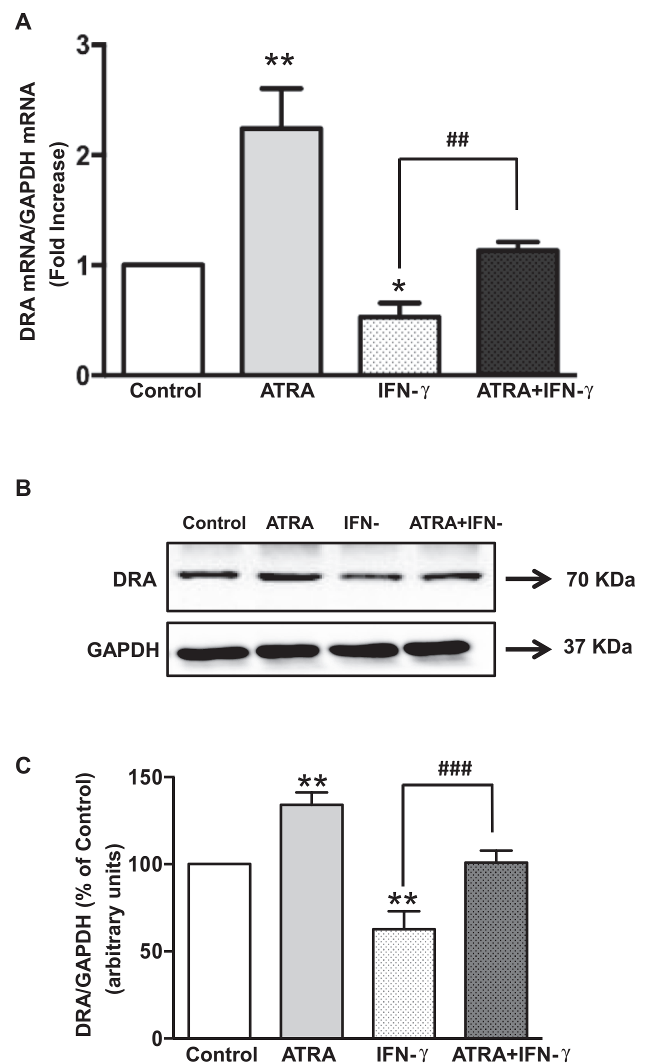

FIGURE 2. All-trans retinoic acid treatment blocks interferon- γ -induced decrease in DRA mRNA and protein levels. Postconfluent Caco-2 cells were treated with ATRA (10 µM, apically) alone or in combination with IFN- γ (30 ng/mL, basolaterally) for 24 hours. A, Total RNA extracted from the control and treated cells was amplified utilizing DRA gene specific primers for real time PCR quantification. Data represent relative DRA mRNA expression normalized to GAPDH mRNA (internal control) levels. Results are expressed as fold changes in mRNA levels compared with control taken as 1.0. B, Protein lysates prepared from different treatment groups were subjected to 7.5% SDS-PAGE, and western blot analysis was performed utilizing rabbit anti-DRA antibody. Blotting for GAPDH was used as a protein loading control. A representative blot of 3 separate experiments is shown. C, Results of densitometric analysis are presented as percentage of control for DRA/GAPDH levels in arbitrary units. Data represents mean ± SEM of 3 separate experiments performed in triplicate. $*P < 0.05$, $**P < 0.01$ compared with control, and $^{##}P < 0.01$, $^{###}P < 0.001$ compared with IFN- γ.

pathological implications.[21] We have previously shown the involvement of STAT1 in the inhibition of DRA gene expression by IFN-γ.[16] The signal for activation and nuclear translocation of STAT1 is the phosphorylation of its tyrosine residue at position 701.[22] As

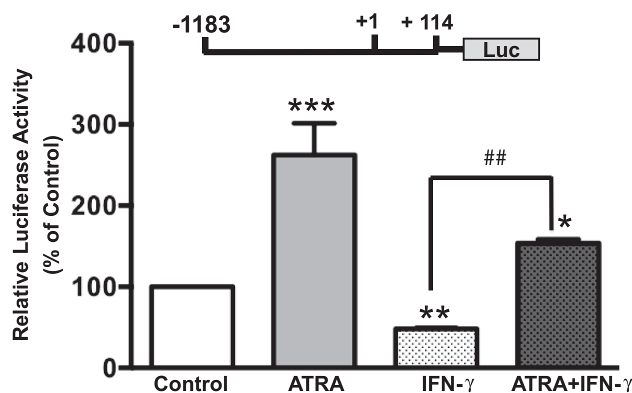

FIGURE 3. All-trans retinoic acid counteracts IFN-γ-induced inhibition of DRA promoter activity: Caco-2 cells transiently transfected with full-length DRA promoter construct (-1183/+114) along with pCMVβ for β–galactosidase to normalize for the transfection efficiency. Twenty-four hours post-transfection, cells were treated with ATRA (10 µM, apically) in absence or presence of IFN- γ(30 ng/mL) supplemented basolaterally. Forty-eight hours post-transfection, cells were harvested and lysed in passive lysis buffer. Promoter activity was assessed by luciferase and β–galactosidase assays. Results are shown as percentage of control and represent the mean ± SEM of values obtained from at least 4 separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control and $^{##}P < 0.01$ compared with IFN-γ.

expected, IFN-γ treatment resulted in STAT1-Y701 phosphorylation as early as 15 minutes and persisted for 24 hours (Fig. 4A: i and ii), whereas phosphorylation of STAT1 in untreated controls was not detected at any of the time points studied. To investigate the mechanisms underlying the protection provided by ATRA in response to IFN-γ, 2 time points (6 h and 24 h) were chosen to test whether ATRA interferes with STAT1-Y701 phosphorylation. Cotreatment of cells with ATRA markedly suppressed IFN-γ induced phosphorylation of STAT1-Y701 both at 6 hours (Fig. 4B: i and ii) and 24 hours (Fig. 4B: iii and iv). However, ATRA alone did not induce STAT1 phosphorylation that was similar to control. These data suggest that ATRA inhibits IFN-γ signaling by attenuating STAT1 phosphorylation and nuclear translocation, thereby preventing the decrease in DRA gene transcription induced by IFN-γ.

All-trans Retinoic Acid Attenuated the Severity of DSS-induced Colitis

To assess the net impact of ATRA treatment on DRA function and expression in the native intestine and its alterations in inflammatory conditions, the DSS-induced mouse model of colitis was utilized. The DSS-colitis model reflects many of the clinical features of ulcerative colitis, and the inflammation is mainly localized to the distal part of the colon.[23] After a 7 day period of acclimatization, the mice were randomly divided into 4 groups (n = 5): control, ATRA, DSS and ATRA+DSS. Mice in the control and ATRA groups were given normal drinking water, whereas the mice in DSS and ATRA+DSS groups received 3% DSS in drinking water for 7 days. All-trans retinoic acid (1 mg/kg bwt) was administered concomitantly by i.p. injections once a day for 7 days to the ATRA and DSS+ATRA groups. The mice in the control and DSS groups were given only the vehicle. A significant body weight loss was recorded in DSS-treated mice from day 3 onward (Fig. 5A) and persisted until day 7. The DSS-treated mice exhibited a higher degree of loose stools as compared with the control, ATRA, and ATRA+DSS treatment groups (visual observation). In addition, the DSS-treated mice also exhibited shortened colon (Fig. 5B) and significantly increased colon weight to length ratio, an indirect measure of diarrheal phenotype (Fig. 5C). All-trans retinoic acid cotreatment partially attenuated the shortening of the colon and significantly decreased the colon weight to length ratio in ATRA+DSS treatment group. However, control and ATRA-treated animals were not significantly different from each other. These data exhibit the protective effect of ATRA in attenuating symptoms of DSS-induced colitis in agreement with an earlier report.[10] Additionally, H&E staining of the distal colon showed that ATRA treatment alleviated the loss of epithelial integrity, immune cell infiltration, and thickening of submucosal layer compared with the DSS-treated mice (Fig. 5D).

All-trans Retinoic Acid Alleviated DSS-induced Increase in MPO Activity and Expression of Proinflammatory Cytokines

Tissue MPO activity serves as an index of neutrophil infiltration at the site of inflammation.[19] As the distal part of the colon is more severely affected in colitis, it was used to determine the MPO activity. MPO activity was significantly higher in the DSS group (~90-fold) as compared with the control animals (Fig. 6A). This increase in MPO activity was significantly attenuated by ATRA treatment to the DSS mice (~85% inhibition as compared with the DSS mice, $P \leq 0.05$). All-trans retinoic acid alone had no effect on the MPO activity. Additionally, mRNA levels of the proinflammatory cytokines (CXCL1 and IL-1β) were also evaluated in the distal colon of mice as these proinflammatory mediators play a key role in pathogenesis of IBD. As shown in Figures 6B and 6C, mRNA levels of the proinflammatory cytokines CXCL1 and IL-1β in colon of DSS-treated mice were significantly elevated (~20-fold and 11-fold, respectively) compared with the control mice. Treatment with ATRA to DSS-colitis mice (ATRA+DSS) significantly attenuated the upregulation of CXCL1 and IL-1β mRNA expression, and the levels were significantly different from the DSS-treated mice ($P \leq 0.005$ compared with DSS) (Figs. 6B and 6C). These results suggest that ATRA alleviates mucosal injury in the DSS colitis model via the suppression of proinflammatory mediators.

All-trans Retinoic Acid Attenuated the Inflammation in Mouse Intestine as Measured by In Vivo Imaging

The protective effect of ATRA in attenuating the severity of DSS-induced inflammation was further determined by in

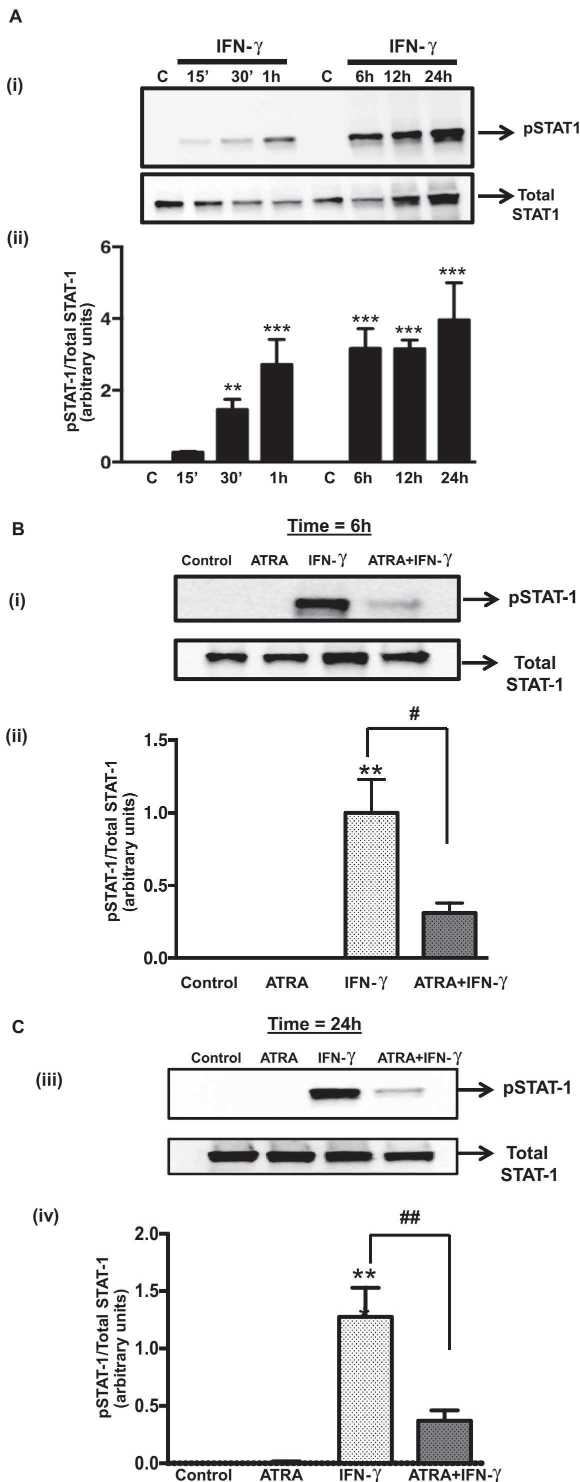

FIGURE 4. All-trans retinoic acid abrogates IFN-γ induced phosphorylation of STAT1 in Caco-2 cells. A, i.) Postconfluent Caco-2 cells were treated with IFN- γ(30 ng/mL) for different time points (15 min, 30 min, 1, 6, 12 and 24 h). Protein lysates prepared from different treatment groups were separated on a 7.5% SDS-PAGE and electrotransferred to nitrocellulose membrane. Blots were probed with rabbit antiphospho-STAT1-Y701 antibody. Phosphorylated STAT1 levels were normalized to total STAT1 to indicate equal loading of protein in each lane. A representative blot of 3 different experiments is shown. A, ii.) The data were quantified by densitometric analysis and expressed as arbitrary units. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control. In separate set of experiments cells were treated with ATRA (10 µM, apically) alone or in combination with IFN- γ(30 ng/mL), basolaterally for (B) i) and ii) 6 hours and (B) iii) and iv) 24 hours. Lysates were prepared and subjected to western blot analysis. A representative immunoblot for phospho-STAT1-Y701 at (B) i.) 6 hours and (C) i) 24 hours is shown. The levels of pSTAT1 were normalized to total STAT1. B, C, ii) Results of densitometric analysis expressed as pSTAT1/total STAT1 levels in arbitrary units. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control and $^{##}P < 0.01$ compared with IFN- γ.

vivo imaging utilizing the luminescent probe L-012. Previous studies have shown that L-012 serves as a sensitive probe for quantifying the generation of reactive oxygen and nitrogen species (RONS) under inflammatory conditions.[24, 25] As shown in Figure 6D (i and ii), L-012-mediated signals were markedly increased in the DSS-treated mice compared with control mice, indicative of severe inflammation. However, consistent with our MPO data, the intensity of the chemiluminescent signals was significantly decreased by ATRA treatment in the DSS-administered mice ($P \leq 0.001$ compared with DSS group). The L-012 signals in mice treated with ATRA alone were similar to the control mice.

All-trans Retinoic Acid Blocked the Activation of STAT3 in Mouse Distal Colon

Activation of STAT3 has been reported in both acute and chronic forms of gut inflammation including IBD.[26, 27] Since the STAT proteins are activated by phosphorylation, we next performed western blotting to determine the levels pSTAT3 in tissue lysates of distal colon from ATRA-treated and untreated DSS-colitis mice. By contrast, pSTAT-3 was markedly upregulated in the colon of DSS-treated mice (>3-fold) as compared with the control ($P < 0.05$). The activation of STAT3 was significantly alleviated by ATRA treatment almost to the level of control or ATRA-treated mice (Fig. 6E, i and ii). The results suggest that suppression of STAT3 signaling may also contribute to the anti-inflammatory effects exerted by ATRA.

All-trans Retinoic Acid Attenuated DSS-induced Decrease in DRA mRNA and Protein Expression

To validate our in vitro data, DRA mRNA and protein expression was determined in distal colon of ATRA-treated mice in the presence and absence of DSS. As shown in Fig. 7A, ATRA treatment resulted in a significant increase in DRA mRNA expression (~3-fold) compared with the vehicle treated control mice. Parallel to the increase in DRA mRNA level, ATRA-treated mice exhibited a marked increase in DRA protein expression (~70%) in comparison with control mice (Fig. 7B i and ii). As reported previously,[4, 6] DRA mRNA and protein expression were significantly reduced in response to DSS

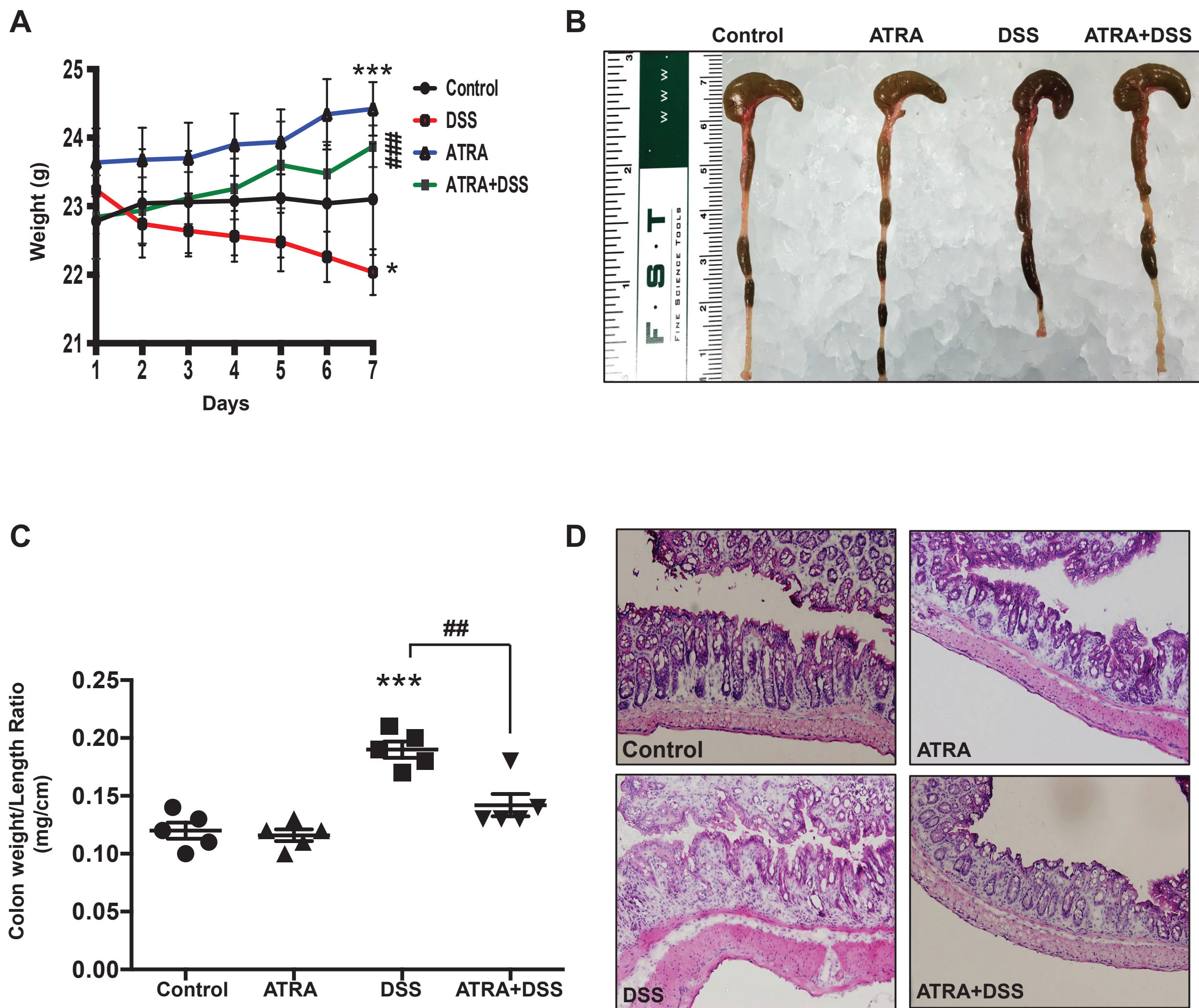

FIGURE 5. All-trans retinoic acid attenuates DSS-induced alterations in body weight and colon weight to length ratio and tissue damage: A, Body weight curves denote the changes in the mean body weight of mice recorded daily for 7 days in different treatment groups (B) representative picture of the colon in control, ATRA-, DSS- and ATRA+DSS-treated mice showing the stool consistency and colon length. C) Colon weight and length (excluding cecum) was recorded for each mice/group on day after harvesting the tissue on day 8. Colon weight to length ratio was calculated for each animal in all the experimental groups. D) Representative image of hematoxylin and eosin staining of colon from control, ATRA-, DSS-, and ATRA+DSS-treated mice. Data are expressed as mean ± SEM for n = 5 mice/group. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with control mice and $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with DSS-treated mice.

treatment (~60%–70%). However, the decrease in both DRA mRNA and protein expression was significantly attenuated in ATRA+DSS mice ($P < 0.001$ for DRA mRNA and $P < 0.01$ for DRA protein vs DSS). The protective effect exerted by ATRA against DSS-induced decrease in DRA expression was also assessed by immunofluorescence studies in mouse colonic sections. All-trans retinoic acid treatment resulted in an increase in DRA expression (stained green) on the apical membrane and abrogated the DSS-induced decrease in apical DRA levels (Fig. 7C). Taken together, the results suggest that ATRA can serve as a potential antidiarrheal agent as it upregulated DRA under normal and inflammatory states, which could be attributed—at least in part—to its ability to inhibit colonic inflammatory cytokines and related signaling pathways.

DISCUSSION

Diarrhea is the one of the most debilitating symptoms manifested in 80% of patients with IBD.[28] The pathogenesis of IBD-associated diarrhea is multifactorial and complex and can be primarily attributed to sustained activation of the mucosal immune system and a failure to abate these responses. Pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, and IL-8, tumor necrosis factor-α, IFN-γ, and prostaglandins are implicated in exacerbating mucosal inflammation[29] in

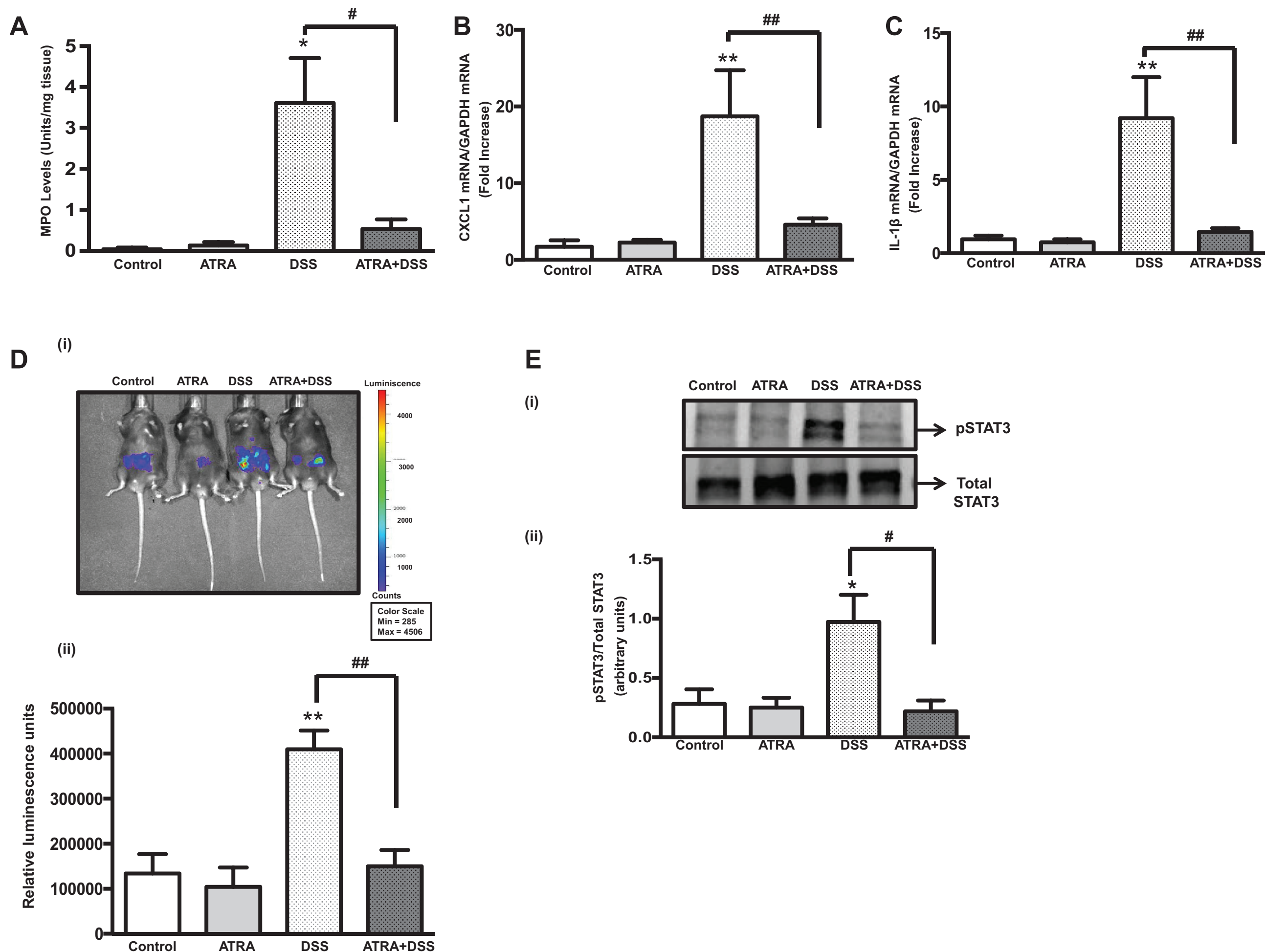

FIGURE 6. All-trans retinoic acid suppresses DSS-induced inflammation in mouse colon: A, Myloperoxidase activity in the distal colonic tissue was measured as described in Materials and Methods section. Data are calculated as MPO units/mg tissue and the mean ± SEM values for n = 5 mice/group are represented as percentage of control. Total RNA isolated from the mucosal scrapings of the distal colon of the mice from different treatment groups was used to measure the mRNA levels of the pro-inflammatory cytokines (B) CXCL1 and (C) IL-1β by RT-PCR using gene specific primers shown in Table 1. GAPDH was used as an internal control. D, In vivo imaging utilizing the bioluminescent probe L-012 was done as described in Materials and Methods section to assess the overall inflammatory status and reactive oxygen and nitrogen species (RONS) production in the experimental mice: i) shows a representative picture of L-012 derived abdominal signals captured by IVIS in mice from control, ATRA, DSS and ATRA+DSS groups; ii) the intensity of signals was quantified by selecting a region of interest (ROI) in the abdominal area and the average photon number was measured. ROI area was kept constant across the groups. E, ATRA inhibits DSS-induced phosphorylation of STAT3 in mice: i) protein lysates prepared from the mucosal scrapings from the distal colon of mice from different treatment groups were separated on a 7.5% SDS-PAGE and electrotransferred to nitrocellulose membrane. Blots were probed with rabbit antiphospho-STAT3-Y705 antibody. Phosphorylated STAT3 levels were normalized to total STAT3 to indicate equal loading of protein in each lane. A representative blot of 3 different experiments is shown; ii) densitometric analysis of band intensities were plotted as mean ± SEM for n = 5 mice/group and was expressed in arbitrary units. Data are expressed as mean ± SEM for n = 5 mice/group. *$P < 0.05$, **$P < 0.01$ compared with control mice and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with DSS-treated mice.

IBD, resulting in loss of epithelial barrier function, reduction in the resorptive area and altered electrolyte homeostasis.[28] Dysregulated absorption of electrolytes in the inflamed mucosa causes a profound decrease in the net absorption of NaCl and water from the lumen of the colon in IBD patients, subsequently leading to diarrhea.[28] In this regard, SLC26A3 or DRA has been identified as the major anion exchanger involved in electroneutral NaCl absorption in the intestine.[1] Several lines of evidence suggest that reduction in DRA function and/or expression is one of the key events in the pathogenesis of diarrhea associated with IBD.[30, 31] Also, DRA expression has been found to be significantly reduced in patients with ulcerative colitis[3, 31] and in animal models of IBD.[4, 6, 32, 33] This warrants a better understanding of the mechanisms of regulation of DRA and identifying novel agents that can upregulate DRA function and expression to rectify reduced chloride absorption in IBD-associated diarrhea. We have previously shown that ATRA may have potential therapeutic value in treating diarrhea due

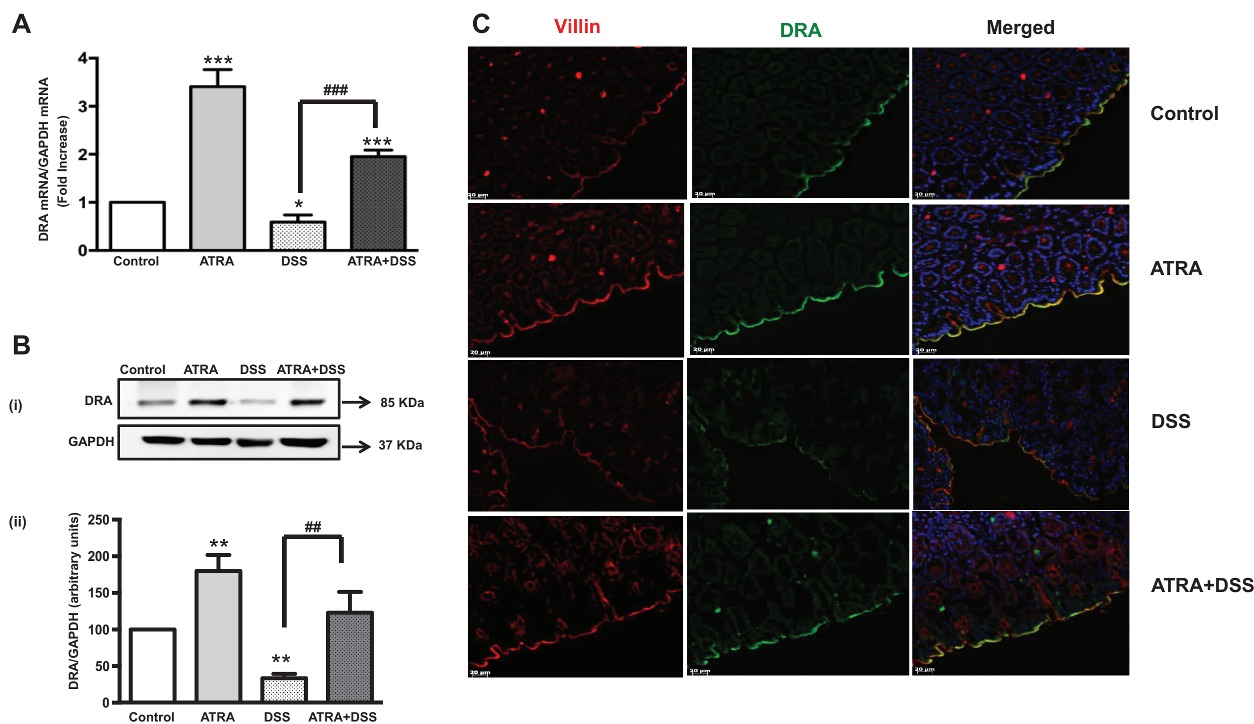

FIGURE 7. All-trans retinoic acid inhibits DSS-induced decrease in DRA mRNA levels and protein expression in distal colon: A, Total RNA was extracted from the mucosal scrapings of distal colon of control, ATRA, DSS and ATRA+DSS-treated mice. Relative expression of DRA mRNA normalized to GAPDH mRNA (internal control) was assessed by real time RT-PCR. Results are expressed as fold change in mRNA levels compared with control taken as 1.0. B, Protein lysates were prepared from the mucosal scrapings from the distal colon of mice from different treatment groups. Equal amount of protein were separated on a 7.5% SDS-PAGE and electrotransferred to nitrocellulose membrane. The blot was probed with anti-DRA or anti-GAPDH antibody and bands were visualized using Enhanced chemiluminiscence (ECL) solution; i) a representative blot is shown; ii) data were quantified by densitometric analysis and expressed as percentage of control in arbitrary units. C) OCT sections from distal colon of control, ATRA-, DSS-, and ATRA+DSS-treated mice were immunostained for DRA (green), the apical membrane marker Villin (red) and nuclei (blue) as described in Materials and Methods section. A representative image is shown. Scale bar, 20 μm. Data are expressed as mean ± SEM for n = 5 mice/group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control mice and $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with DSS-treated mice.

to its efficacy in increasing DRA expression in Caco-2 cells.[15] Furthermore, recent studies have reported the beneficial role of ATRA on intestinal mucosa via attenuation of intestinal inflammation and injury in neonatal rat model of necrotizing enterocolitis[34] and also in mouse models of TNBS[9] and DSS-induced colitis.[11] Whether ATRA exerts its antidiarrheal effects by increasing the Cl^-/HCO_3^- exchange activity and DRA expression under inflammatory states such as colitis, however, is not known.

In the present study, we evaluated the protective effects of ATRA via counteracting decreased DRA function and expression in inflammation utilizing in vitro (in presence of IFN-γ) and in vivo (DSS-colitis) models of inflammation. Interferon-γ is one of the most potent proinflammatory cytokine that has been shown to disrupt the tight junctions[35] and alter intestinal ion transport by reducing the function and expression of transporters involved in sodium absorption (NHE2 and NHE3)[36] and DRA, involved in chloride absorption.[6, 16] All of these are thought to contribute to the pathogenesis of inflammatory diarrhea. In our current studies, cotreatment of Caco-2 cells with ATRA prevented IFN-γ-induced decrease in DIDS-sensitive apical Cl^-/HCO_3^- exchange activity. Consistent with the results obtained for the functional studies, downregulation of DRA mRNA and protein levels induced by IFN-γ were also attenuated by ATRA treatment. These data, for the first time, showed that ATRA, a dietary metabolite, acts as an antidiarrheal agent in regulating expression and function of DRA, a key ion transporter downregulated in inflammatory diarrhea.

Our pervious mechanistic studies have shown that IFN-γ decreased DRA expression at the transcriptional level by activation of JAK/STAT1 pathway, causing nuclear translocation of phosphorylated STAT1 where it binds to the DRA promoter at the GAS (γ-activated sequence) elements, resulting in decreased promoter activity.[16] In the current study, we observed that ATRA cotreatment significantly counteracted IFN-γ-induced decrease in DRA promoter activity. Interferon-γ is known to mediate its effects through the JAK/STAT1 pathway.[16] To assess whether the protective effects of ATRA involve modulation of IFN-γ-induced signaling pathway, we examined the phosphorylation of STAT1. The results clearly indicated the ability of

ATRA to reduce the levels of Tyr-701 phosphorylated STAT1 in response to IFN-γ treatment in Caco-2 cells. Therefore, it can be concluded that ATRA exerted its protective effects on DRA function, expression, and promoter activity by blocking the signaling pathways induced by IFN-γ. Similar results have been reported in other tissues,[22] demonstrating that ATRA induced anti-inflammatory effects occurred via suppressing the activation of JAK/STAT pathway in primary astrocytes in rat brain. Although IFN-γ receptor stimulation is a crucial step necessary for STAT1 activation, the possibility of diminished binding of IFN-γ to its receptor in presence of ATRA seems to be unlikely, as ATRA treatment was given apically, whereas IFN-γ-receptor is expressed basolaterally in the fully differentiated Caco-2 cells.[16] Therefore, a decrease in IFN-γ induced STAT1 phosphorylation is one potential mechanism by which ATRA counteracts the inhibitory effects of IFN-γ on DRA gene transcription.

To validate our in vitro results, we also investigated the effects of ATRA on the expression of DRA in the native intestinal tissue. Consistent with the in vitro results, our in vivo data showed that DRA mRNA and protein expression was significantly increased in distal colon of ATRA-treated mice. However, ATRA treatment to mice showed no effect on the mRNA expression of Na^+/H^+ exchanger-3 (NHE3) (control mice, 1.59 ± 0.33; ATRA mice 1.23 ± 0.27) and another Cl^-/HCO_3^- exchanger, putative anion transporter 1 (PAT1) (control mice, 1.02 ± 0.05; ATRA mice, 1.00 ± 0.17).

There are several studies implicating a decrease in DRA function and expression in diarrhea associated with IBD,[3, 6, 30, 31, 37] and DRA knockout mice have been reported to be more susceptible to DSS damage.[38] In this regard, ATRA has been shown to attenuate inflammation by exerting protective effects by various mechanisms in different models of inflammation. For example, ATRA has been shown to inhibit NF-κB signaling in DSS model of colitis,[10] ameliorate TNBS-induced colitis by modulating Th1/Th2 profile,[9] and downregulate TNBS-induced intestinal fibrosis in mice.[39] The role of ATRA in regulatory T cells (Treg) growth, differentiation, and gut homing and inhibition of Th17 generation and function has also been demonstrated.[40] Since the anti-inflammatory role of ATRA has been demonstrated in various in vivo models of inflammation, we sought to examine the protective role of ATRA via counteracting inhibition of DRA expression utilizing in vivo model of DSS-colitis. Dextran sulfate sodium–induced colitis is a commonly used model of colitis in mice that mimics the clinical and histological features characteristic of UC.[23] Therefore based on our previous studies,[5, 6] we used 3% DSS in drinking water for 7 days to induce colitis in mice. As expected, mice in the DSS group exhibited significant decrease in body weight, shortening of the colon, and loose fecal pellets. Dextran sulfate sodium treatment also resulted in a marked increase in tissue MPO activity, reflective of enhanced neutrophil infiltration and accumulation[19] in the colon of these mice. The DSS-treated mice also showed significantly increased intensity of the L-012 signals, indicative of enhanced production of reactive oxygen and nitrogen species (ROS/RNS) during DSS-induced inflammation. In this regard, L-102 has been shown to be a sensitive luminescent probe for detecting ROS/RNS generated during inflammation in live animals via noninvasive in vivo imaging.[24]

In agreement with previous findings of Hong et al,[10] in the current study ATRA treatment significantly attenuated the typical symptoms of DSS-induced colitis such as weight loss, rectal bleeding, shortening of colon, and increased MPO activity. Further, intensity of L-012 signals was significantly reduced in DSS mice cotreated with ATRA. We also observed a significant reduction in the levels of pro-inflammatory cytokines such as CXCL1 and IL-1β in DSS-treated mice in response to ATRA treatment, reflecting a potent intestinal anti-inflammatory effect of ATRA against tissue injury.

Several recent studies have shown that STAT1 and STAT3 are activated in the colon of human IBD and murine colitis models.[26, 27] We detected a significant increase in phosphorylated levels of STAT3 in DSS mice, which was significantly diminished by ATRA treatment. This provided additional evidence that ATRA suppresses inflammatory responses in colitis mice not only by reducing the expression of inflammatory cytokines but also in part via inhibition of STAT3 phosphorylation. It should be noted that pSTAT1 was not detected in the colonic mucosa of DSS mice in our study (data not shown). Activation of STAT3 has been strongly correlated with the severity and extent of inflammation in various IBD models.[27]

Adverse effects of DSS-induced colitis on intestinal water absorption were evident from the diarrheal phenotype (absence of solid fecal pellets) observed in the colonic lumen of DSS-treated mice. In the intestine, absorption of water is secondary to electrolyte transport. Dextran sulfate sodium–induced downregulation in electrolyte transport, especially the anion exchange process, was indicated from the markedly reduced levels of DRA mRNA and protein expression in the distal colon of DSS-treated mice compared with the control mice. It is important to note that DRA knockout mice exhibit diarrheal phenotype attributed to loss of luminal membrane Cl^-/HCO^-_3 exchange activity. Therefore, DSS-induced decrease in DRA protein could be implicated in the appearance of loose stool consistency and increased colon weight to length ratio (an indirect readout for diarrheal phenotype) observed in the DSS group.[6] As shown here, ATRA administration to DSS-treated mice significantly suppressed the downregulation in DRA levels and exhibited improved stool consistency (solid fecal pellet). Based on these findings, we speculate that ATRA may serve as a potential antidiarrheal agent by reducing overall inflammation associated damage and directly stimulating DRA mRNA and protein expression under inflammatory conditions such as in experimental models of DSS colitis.

CONCLUSION

In summary, our findings demonstrate the efficacy of ATRA in stimulating intestinal DRA function and expression under inflammatory conditions and define novel mechanisms underlying the beneficial effects of ATRA in gut health and disease. Further, our in vivo studies in a mouse model of inflammation clearly suggest that ATRA, a dietary metabolite, could serve as an important and novel antidiarrheal agent and could be exploited for newer and superior treatment modalities for diarrhea associated with IBD.

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