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The transcription factor RXRα in CD11c$^+$ antigen-presenting cells regulates intestinal immune homeostasis and inflammation

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Abstract

Antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages (Mϕs) play a pivotal role in mediating immune tolerance and restoring intestinal immune homeostasis by limiting inflammatory responses against commensal bacteria. However, cell-intrinsic molecular regulators critical for programming intestinal APCs to a regulatory state rather than an inflammatory state are unknown. In this study, we report that the transcription factor retinoid X receptor α (RXRα) signaling in CD11c$^+$ APCs is essential for suppressing intestinal inflammation by imparting anti-inflammatory phenotype. Using a mouse model of ulcerative colitis, we demonstrated that targeted deletion of RXRα in CD11c$^+$ APCs in mice resulted in the loss of T cell homeostasis with enhanced intestinal inflammation and increased histopathological severity of colonic tissue. This was due to the increased production of pro-inflammatory cytokines that drive Th1/Th17 responses and decreased expression of immune regulatory factors that promote Treg differentiation in the colon. Consistent with these findings, pharmacological activation of the RXRα pathway alleviated colitis severity in mice by suppressing the expression of inflammatory cytokines and limiting the Th1/Th17 cell differentiation. These findings identify an essential role for RXRα in APCs in regulating intestinal immune homeostasis and inflammation. Thus, manipulating the RXRα pathway could provide novel opportunities for enhancing regulatory responses and dampening colonic inflammation.

Introduction

Antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages (Mϕs) play a critical role in maintaining a delicate balance between tolerance and immunity in the intestine (1–4). These APCs also play a pivotal role in mediating immune tolerance to oral antigens and restoring mucosal immune homeostasis by limiting inflammatory responses

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against commensal bacteria (1–7). They regulate immune tolerance through the induction of regulatory T cells (Tregs) while restricting the differentiation of pathological Th1/Th17 cells in the gut (8–10). Disruption in immune homeostasis and loss of immune tolerance to gut flora leads to intestinal inflammation and inflammatory bowel disease (IBD) (4, 5, 11). In IBD, these APCs lose their regulatory properties and express high levels of inflammatory factors such as IL-1β, IL-1α, TNF-α, and IL-6, resulting in uncontrolled intestinal inflammation (4, 5, 11). However, molecular pathways that program these APCs towards a regulatory versus an inflammatory state remain fragmentary.

The Retinoid X receptor (RXR) family of nuclear receptors are ligand-activated transcription factors that heterodimerize with several members of the nuclear receptor and regulates fundamental biological processes such as embryogenesis, reproduction, cellular differentiation, homeostasis, metabolism, and hematopoiesis (12, 13). The RXR family includes three isoforms, namely RXRα (NR2B1), RXRβ (NR2B2), and RXRγ (NR2B3), that are differentially expressed in various tissues including immune cells (14–16). Among these isoforms, RXRα is functional and highly expressed in myeloid cells (15, 17). Endogenous ligands of retinoid X receptor α are Vitamin A-derived retinoic acid and fatty acids and play a significant role in retinoic acid (RA) and lipid signaling pathways that are critical for the development of the intestine and gut homeostasis (18, 19). Aberrant retinoic acid (RA) and lipid signaling occur in several inflammatory diseases, including Inflammatory Bowel Diseases (IBD) and IBD-associated colon cancer (1–4). Pharmacological modulators of RXR exist and RXR agonist treatment suppresses inflammation in murine models of sepsis, asthma, atherosclerosis, and liver injury (14, 20–23) Furthermore, past studies have shown the potential involvement of RXRα in mucosal inflammation (24–27). While the focus of most research has been directed toward how the RXRα signaling cascade regulates intestinal stem cell proliferation and epithelial cell maintenance, as well as its effects on cancer initiation and progression (26). However, its role in shaping the functions of intestinal APCs and mucosal immune responses in the gut remains unknown. In addition, the molecular mechanism by which the RXRα signaling pathway in antigen-presenting cells (APCs) regulates intestinal inflammation is still undefined.

In this study, we show that RXRα signaling in $CD11c^+$ APCs plays a vital role in regulating immune tolerance and suppressing intestinal inflammation. Accordingly, our data demonstrate that the $CD11c^+$ APC-specific deletion of RXRα in mice results in loss of immune homeostasis and exacerbated colitis. This was because of decreased expression of immune regulatory factors that are critical for the differentiation of $IL\text{-}10^+$ and $Foxp3^+$ Tregs by intestinal DCs and Mϕs lacking RXRα. Furthermore, our data also show that the RXRα pathway in intestinal APCs is critical for suppressing the expression of inflammatory cytokines that drive Th1/Th17 responses in the intestine. Consistent with these findings, pharmacological activation of the RXRα pathway alleviated colitis severity by suppressing the expression of inflammatory cytokines and limiting the Th1/Th17 cell differentiation in murine models of colitis. Collectively, these findings identify a new and essential role for RXRα in intestinal APCs in regulating intestinal immune homeostasis and inflammation. Thus, manipulating the RXRα pathway could provide novel opportunities for enhancing regulatory responses and treating colonic inflammation.

Materials and Methods

Mice

RXRα floxed (RXRα $^{FL/FL}$ or WT-FL) (28), CD11c^{Cre} (29), C57BL/6, and Rag2$^{-/-}$ mice of 6 to 12 weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME). RXRα^{FL} mice were crossed to transgenic mice expressing Cre recombinase under the control of the CD11c promoter (Jackson Laboratories) to generate mice in which RXRα (RXRα$^{\Delta CD11c}$) was deficient in CD11c^{+} APCs. Successful Cre-mediated deletion was confirmed by polymerase chain reaction (PCR) and protein expression analyses as in our previous studies (30, 31). All experiments were carried out with age-matched littermates unless specified otherwise. All mice were housed under specific pathogen-free conditions at Augusta University, with animal care protocols approved by the Institutional Animal Care and Use Committee.

Antibodies and reagents

Antibodies against mouse CD3 (145–2C11), CD4 (GK1.5), CD8a (53–6.7), CD45 (30-F11), Foxp3 (FJK-16s), IL-10 (JES5–16E3), CD11c (N418), CD11b (M1/70), I-A^{b} (25–9–17), CD64 (X54–5/7.1), F4/80 (BM8), CD90.1 (HIS51), V alpha 2 TCR (B20.1), V beta 5.1/5.2 TCR (MR9–4), IFN-γ (XMG1.2), and IL17A (17B7) were purchased from eBioscience. RXRα antibody was obtained from Cell Signaling Technology. CD11c and CD11b microbeads were purchased from Miltenyi Biotec.

Induction of DSS-induced colonic inflammation

Colonic inflammation was induced, as previously described (32, 33). Briefly, mice were subjected to one cycle of DSS treatment, whereby mice were given 3% DSS (36–50 kDa) in their drinking water (at a dose as indicated in Results) for 7 days, followed by 8 days of normal drinking water. In some experiments, WT mice were treated orally with the RXRα agonist CD3254 (Tocris) at indicated time points. Mice were monitored for weight change, diarrhea, and rectal bleeding as previously described (32, 33). Diarrhea was scored as (0) normal stool; (1) soft but formed pellet; (2) very soft pellet; (3) diarrhea (no pellet); or (4) dysenteric diarrhea. Rectal bleeding was recorded as (0) no bleeding, (2) presence of occult blood in stool, or; (4) gross macroscopic bleeding.

T cell reconstitution of Rag2$^{-/-}$ mice

CD4^{+} T cell reconstitution of Rag2$^{-/-}$ mice was performed as described previously (34, 35). CD4^{+} T cells from WT mouse spleen and lymph nodes (inguinal and axillary) were first enriched using CD4-specific microbeads and MACS column (Miltenyi Biotech; Auburn, CA). CD4^{+} T cell subsets were then further purified by FACS-sorting to collect two different populations of cells, CD4^{+} CD45RB^{high} CD25^{-} cells and CD4^{+} CD25^{+} cells. Approximately 3×10^{5} CD4^{+} CD45RB^{high} CD25^{-} cells were injected i.p. into the indicated recipient Rag2$^{-/-}$mice. Mice were then monitored for body weight twice a week. In some experiments, Rag2$^{-/-}$mice were treated orally with the RXRα agonist CD3254 (Tocris) at indicated time points.

Isolation of intestinal APCs and lymphocytes

APCs and lymphocytes from colons were isolated as described in our previous study (32, 33). Briefly, mice were euthanized, and the colon was washed, cleaned of fat tissue, and longitudinally cut and suspended in 1× HBSS with 20 mM HEPES, 1 mM DTT, and 5 mM EDTA for 30 min at 37°C with shaking to remove epithelial cells. After that, pieces of the colon were digested with collagenase VIII (Sigma) (0.3 mg/ml in RPMI with DNase I (0.1mg/ml) and 2% FCS) for 30 min at 37 °C with shaking (150 r.p.m.). Tissue was processed through a 100-μm-cell strainer, and the resulting suspension was pelleted. Cells derived following collagenase digestion were resuspended for lymphocyte isolation in 7 ml of 40% Percoll and layered on top of 2 ml of 70% Percoll (GE Amersham). After centrifugation for 15 min at 1,500 r.p.m without brakes, the middle layer was removed, washed in 2% FBS in RPMI, and the lymphocytes were obtained. Isolated lymphocytes were cultured with phorbol myristate acetate (50 ng/ml) plus ionomycin (750 ng/ml) in the presence of GolgiStop and Golgiplug for 6 h. Cells were fixed and stained for CD4, IL-10, IFN-γ, and IL-17. For LP DCs and macrophages, the collagenase-digested cells were filtered through a 100-μm strainer and pelleted and stained. For cell sorting, APCs in this preparation were enriched with $CD11c^+$ and $CD11b^+$ magnetic beads according to the manufacturer's instructions (Miltenyi Biotec), and then FACS sorted for DCs ($CD45^+I\text{-}A^{b+}CD11c^+$ $F4/80^-CD64^-$) and macrophage cells ($CD45^+I\text{-}A^{b+}CD11b^+F4/80^+CD64^+$) sorted on FACS Aria at the Augusta University flow cytometer core. After sorting, DCs or Mϕs (10^5) were cultured in 0.2 ml RPMI 1640 complete medium in 96-well round-bottom plates. Cell culture supernatants were analyzed after 48 hr for indicated cytokine production by ELISA.

In vitro lymphocyte co-culture

Colonic DCs or macrophage and OT-II $CD4^+$ T cell co-culture experiments were performed as described previously (33, 35). FACS-sorted colonic dendritic cells ($CD45^+I\text{-}A^{b+}CD11c^+$ $CD64^-$) or macrophage ($CD45^+I\text{-}A^{b+}CD11b^+$ $CD64^+$) cells (1×10^5) were cultured with naïve $CD4^+CD25^-CD62L^+$ OT-II $CD4^+$ transgenic T cells (1×10^5) and OVA peptide (ISQVHAAHAEINEAGR; 1 μg/ml) in a total volume of 200 μl RPMI complete medium. The culture supernatants were analyzed after 96 h and cells were harvested and restimulated for 6 h with plate-bound antibodies against CD3 (5 μg/ml; 145.2C11 from Becton Dickinson) and CD28 (2 μg/ml; 37.51 from Becton Dickinson) in the presence of GolgiStop and Golgiplug for intracellular cytokine detection (IL-17A, IL-10 and IFN-γ).

OT-II $CD4^+$ T cell adoptive transfer

In vivo OT-II $CD4^+$ T cell differentiation assay was performed as described previously (33, 35). Naive $CD4^+CD25^-$ T cells were isolated from the spleen and lymph nodes of $Rag1^{-/-}$ OT-II Thy1.1 transgenic mice and 5×10^6 cells were intravenously transferred into WT-FL and $RXR\alpha^{\Delta CD11c}$ mice. Mice received 5 mg OVA by gavage on 5 consecutive days after transfer.

Ex vivo colon culture and ELISAs

Ex vivo colon culture was performed as described previously (32, 33). Briefly, whole colons were excised and flushed with PBS containing penicillin, streptomycin, and amphotericin B. An approximately 1-cm-long section of the ascending colon was excised, opened longitudinally, and washed three times with sterile HBSS containing penicillin, streptomycin, and amphotericin B. Colon sections were then placed into culture in complete RPMI 1640 media (2% FBS, L-glutamine, penicillin, streptomycin, and amphotericin B) and cultured for 2 d at 37°C with 5% CO_2. Supernatants were then collected, and cytokine concentrations were determined by ELISA. IL-6, IL-10, TNF-α, IL-1α, and IL-1β were quantitated using ELISA kits procured from BioLegend.

Measurement of intestinal permeability

Intestinal permeability was measured on day 10 after DSS treatment as described previously (32, 33). In brief, mice were given fluorescein isothiocyanate (FITC)-dextran by oral gavage at a dose of 0.5 mg/g of body weight. Four hours later, mice were bled, and FITC-dextran was quantified in the serum via a fluorescence spectrophotometer.

Myeloperoxidase (MPO) activity measurement

Myeloperoxidase (MPO) activity measurement was performed as described previously (32, 33). Pieces of colon (100 mg weight) were homogenized in phosphate buffer (20 mM [pH 7.4]) and centrifuged. The pellet was resuspended in phosphate buffer (50 mM [pH 6.0]) containing 0.5% hexadecyltrimethylammonium bromide (Sigma). The sample was freeze-thawed and then sonicated, warming to 60°C for 2 hr and subsequent centrifugation. The redox reaction of 3,3′,5,5′-tetramethylbenzidine (Sigma) by supernatant was used to determine myeloperoxidase (MPO) activity. The reaction was terminated with 2N HCl and absorbance was read at 450 nm.

Real-time PCR

Total mRNA was isolated from the colon or indicated cell type using the Omega Total RNA Kit according to the manufacturer's protocol and as described previously (32, 33). cDNA was generated using the RNA to cDNA Ecodry Premix Kit (Clontech) according to the manufacturer's protocol. cDNA was used as a template for quantitative real-time PCR using SYBR Green Master Mix (Roche), and gene-specific primers (32, 33). PCR analysis was performed using a MyiQ5 ICycler (BioRad). Gene expression was normalized relative to *Gapdh*.

Histopathology and immunohistochemistry

Sections (5 μm thick) from formalin-fixed and paraffin-embedded colons were placed onto glass slides. H&E-stained sections were blindly scored for the severity of colonic inflammation as described previously (32–34). The degree of inflammation was scored as follows: (0) no inflammation, (1) mild inflammation or prominent lymphoid aggregates, (2) moderate inflammation, (3) moderate inflammation associated with crypt loss, and (4) severe inflammation with crypt loss and ulceration. Crypt destruction was graded as follows: (0) no destruction, (1) 1%–33% of crypts destroyed, (2) 34%–66% of crypts destroyed,

and (3) 67%–100% of crypts destroyed. The individual scores from inflammation and crypt damage were summed to derive the histological score for colonic inflammation (maximum score 7).

Statistical analyses

Statistical analyses were performed using GraphPad Prism software. An unpaired one-tailed Student's *t*-test was used to determine statistical significance for mRNA expression levels, Treg percentages, and cytokines released by various cell types between different groups. A *P* value less than 0.05 (*) was considered to be significant, a *P* value less than 0.01 (**) was considered to be very significant, and a *P* value less than 0.001 (***) was considered to be extremely significant.

Results

RXRα-deficiency in CD11c$^+$ APCs enhances susceptibility to DSS-induced colitis in mice.

To understand the role of RXRα in intestinal DCs and Mϕs in regulating immune homeostasis and inflammation in the intestine, first, we analyzed the RXRα expression in colonic DCs and Mϕs. We noted that both colonic CD11c$^+$ DCs (CD45$^+$I-A^{b+}CD11c$^+$ F4/80$^-$CD64$^-$) and CD11c$^+$ Mϕs (CD45$^+$I-A^{b+}CD11b$^+$F4/80$^+$CD64$^+$) express RXRα (Supplementary Fig. 1A, B). Next, to investigate the role of RXRα in CD11c$^+$ APCs, we generated CD11c^{Cre} RXRα$^{FL/FL}$ (RXRα$^{\Delta CD11c}$) mice in which the CD11c promoter/enhancer elements drive the expression of Cre protein (28, 29). The gene of interest is deleted in CD11c$^+$ DCs and Mϕs, as the Cre protein is expressed in both APC subsets (10, 36, 37). To confirm the efficiency of RXRα deletion in CD11c$^+$ APC subsets, we isolated CD11c$^+$ DCs and CD11c$^+$ Mϕs from the intestine of WT-FL (RXRα$^{FL/FL}$) and RXRα$^{\Delta CD11c}$ mice and performed intracellular FACS and mRNA expression for RXRα. We noted a marked decrease in the RXRα expression levels in colonic CD11c$^+$ DCs and CD11c$^+$ Mϕs isolated from RXRα$^{\Delta CD11c}$ mice (Supplementary Fig. 1A, B).

Next, we investigated if signaling via RXRα in APCs would suppress or promote intestinal inflammation. Thus, we challenged WT-FL and RXRα$^{\Delta CD11c}$ mice with 3.0% dextran sulfate sodium (DSS), an experimental model of intestinal injury and inflammation (38, 39). In this murine model of UC, inflammatory cytokines produced by innate immune cells in the colon drive colitis in response to microbiota (38, 39). Upon DSS administration, RXRα$^{\Delta CD11c}$ mice showed more significant weight loss, diarrhea, and rectal bleeding, and a marked reduction in colon length compared to the WT-FL mice in response to DSS (Fig. 1A–E). Myeloperoxidase (MPO) activity, a hallmark of the degree of tissue inflammation and neutrophil infiltration, was markedly increased in the colons of RXRα$^{\Delta CD11c}$ mice after DSS treatment (Fig. 1F). Loss of intestinal permeability and alterations in the expression levels of key tight junction (TJ) proteins such as ZO-1 and occludin are prominent features during DSS-induced colitis. Thus, we tested the gut epithelial barrier integrity by orally feeding the DSS-treated RXRα$^{\Delta CD11c}$ and WT-FL mice with FITC-dextran. We observed an increase in FITC-dextran in the serum of DSS-treated RXRα$^{\Delta CD11c}$ mice after oral gavage (Fig. 1G), indicating severely impaired epithelial barrier integrity. In addition, we also observed a marked decrease in mRNA expression levels of tight junction complex proteins

claudin-1 and occludin in the colons of DSS-treated RXRα$^{\Delta CD11c}$ mice compared to colons of DSS-treated WT-FL mice (Fig. 1H). Consistent with enhanced gut inflammation and delayed recovery, histopathological analysis of colons of DSS-treated RXRα$^{\Delta CD11c}$ mice showed extensive damage to the mucosa with epithelial erosion, loss of crypts, and increased infiltration of immune cells compared to the colons of DSS-treated WT-FL mice (Fig. 1I, J). However, colons from untreated WT-FL and RXRα$^{\Delta CD11c}$ mice showed no morphological sign of damage or inflammation (data not shown). Collectively, our results also show that RXRα deletion in CD11c^{+} APCs in mice results in increased intestinal inflammation with delayed recovery, indicating a possible regulatory role for RXRα in intestinal APCs during ongoing intestinal inflammation.

RXRα-deficiency in APCs leads to an inflammatory environment in the colon during DSS-induced colitis.

The cytokine milieu in the gut microenvironment can suppress or promote intestinal inflammation (1–7). Proinflammatory factors such as IL-6, TNF-α, IL-1β, IL-1α, IL-12p40, and IL-23 promote colitis, whereas the immune regulatory factors such as IL-10, retinoic acid (RA), IDO, and IL-27 suppress colitis (1–7). In DSS-induced intestinal inflammation, inflammatory cytokines produced by innate immune cells in the gut microenvironment drive colitis and augment tissue injury (38, 39). Thus, we analyzed the expression levels of immune regulatory and inflammatory factors that suppress or promote inflammation in the colons of WT-FL and RXRα$^{\Delta CD11c}$ mice treated with or without DSS. Colons of DSS-treated RXRα$^{\Delta CD11c}$ mice expressed significantly higher levels of proinflammatory cytokines IL-6, TNF-α, IL-1β, IL-1α, and IL-12p40 and lower levels of anti-inflammatory cytokine IL-10 compared to colons of DSS-treated WT-FL mice (Fig. 2A). Consistent with these observations, colon explant cultures showed that colons of DSS-treated RXRα$^{\Delta CD11c}$ mice produced higher levels of inflammatory cytokines and lower levels of IL-10 than colons of DSS-treated WT-FL mice (Fig. 2B). Collectively, these results indicate that RXRα-deficiency in CD11c^{+} APCs leads to an imbalance in the expression of proinflammatory versus immune regulatory cytokines in the intestine, resulting in increased expression of cytokines that promote inflammation and tissue injury.

RXRα-deficiency in APCs leads to loss of CD4^{+} T cell homeostasis in the colon during colitis.

The balance between regulatory and effector T cells is critical for gut homeostasis and intestinal APCs play an important role in regulating this balance. Furthermore, the type of cytokine milieu present in the gut microenvironment drives the differentiation and expansion of effector T cells and Tregs (8–10). Although DSS-induced colitis is independent of T and B cell responses, an increased presence of pathological Th1/Th17 cells can accelerate disease severity (40–42). Thus, we next investigated if the increased colitis severity observed in RXRα$^{\Delta CD11c}$ mice was due to changes in Tregs and effector CD4^{+} T cell subsets in the colon. The percentages of IFN-γ^{+} and IL-17A^{+} CD4^{+} T cells were markedly increased in the colon of RXRα$^{\Delta CD11c}$ mice compared with those of WT-FL mice under steady-state and in response to DSS treatment (Fig. 3A, B). In contrast, we observed a marked decrease in the frequency of IL-10^{+} and Foxp3^{+} CD4^{+} regulatory cells in the colon of RXRα$^{\Delta CD11c}$ mice compared with WT-FL mice under steady-state and in response to DSS treatment (Fig. 3A,

C). Consistent with these observations, following *ex vivo* αCD3/αCD28 stimulation, $CD4^+$ T cells isolated from the colons of $RXR\alpha^{\Delta CD11c}$ mice produced markedly higher levels of IFN-γ and IL-17A, and lower levels of IL-10 compared with the $CD4^+$ T cells isolated from the colons of WT-FL mice (Fig. 3D). Collectively, these findings suggest a vital role for RXRα in $CD11c^+$ APCs regulating a delicate balance between Tregs and effector T cell numbers in the colon.

RXRα-deficient intestinal APCs express higher levels of proinflammatory factors and lower levels of anti-inflammatory factors.

As intestinal APCs dictate the fate of naïve $CD4^+$ T cells through differential production of pro-and anti-inflammatory factors (43), we further considered the functional relevance of $CD11c^+$ APC-specific RXRα-mediated signaling in naive $CD4^+$ T cell differentiation. Thus, we evaluated the expression of inflammatory cytokines and immune regulatory factors in $CD11c^+$ DCs and $CD11c^+$ Mϕs isolated from the colon (33, 35). We observed significantly increased mRNA levels for IL-6, TNF-α, IL-1β, IL-1α, IL-23p19, and IL-12p40 in colonic DCs and Mϕs isolated from $RXR\alpha^{\Delta CD11c}$ mice compared with colonic DCs and Mϕs isolated from the WT-FL mice (Fig. 4A, B). In contrast, colonic DCs and Mϕs isolated from the $RXR\alpha^{\Delta CD11c}$ mice expressed markedly lower mRNA levels of Aldh1a1, Aldh1a2, IDO1, IL-10 (Fig. 4C, D). In line with these observations, RXRα-deficient intestinal DCs and Mϕs produced markedly higher levels of IL-6, IL-1β, TNF-α, IL-1α, and IL-23, and lower levels of IL-10 compared with WT intestinal DCs and Mϕs (Fig. 4E). Thus, our data demonstrate that in intestinal DCs and Mϕs, RXRα is critical for limiting inflammatory cytokine expression while inducing anti-inflammatory factors.

RXRα signaling in intestinal APCs limits Th1/Th17 differentiation and promotes Treg differentiation.

As DCs and Mϕs dictate the fate of naive $CD4^+$ T cells through differential the production of pro- and anti-inflammatory cytokines (1, 4, 10), we further considered the functional relevance of DC- and Mϕ-specific RXRα-mediated signaling in naive $CD4^+$ T cell differentiation. Thus, we tested the ability of colonic DCs and Mϕs isolated from $RXR\alpha^{\Delta CD11c}$ mice and WT-FL mice to promote the differentiation of naive OT-II CD4 cells into Treg/Th1/Th17 cells. Intestinal DCs and Mϕs deficient in RXRα are more potent in inducing IFN-γ and IL-17A –producing T cells compared with those of WT DCs and macrophages (Fig. 5A, B). In contrast, RXRα-deficient intestinal DCs and Mϕs were less potent in inducing $IL\text{-}10^+$ and $Foxp3^+$ Tregs than WT DCs and Mϕs (Fig. 5A, B).

To extend these observations *in vivo*, we adoptively transferred naïve OT-II Thy1.1 $CD4^+$ T cells into WT-FL and $RXR\alpha^{\Delta CD11c}$ mice and then challenged these mice orally with OVA. Intracellular cytokine analysis on day 6 post-transfer showed a significant increase in naïve OT-II T cell differentiation towards Th1 and Th17 cells in $RXR\alpha^{\Delta CD11c}$ mice compared to WT-FL mice in the colon (Fig. 5C, D). Further characterization of transferred OT-II T cells showed a marked decrease in the differentiation of $Foxp3^+$ and $IL\text{-}10^+$ Tregs cells in the colon of $RXR\alpha^{\Delta CD11c}$ mice compared to WT-FL mice (Fig. 5C, D). Thus, these data demonstrate that RXRα signaling imparts an anti-inflammatory phenotype on intestinal

APCs by inducing the expression of key immune regulatory genes while suppressing the expression of inflammatory cytokines.

Pharmacological activation of RXRα suppresses colonic inflammation in DSS-induced colitis.

Given that the RXRα pathway in intestinal APCs regulates the expression of pro- and anti-inflammatory factors, we asked if activating RXRα would suppress intestinal inflammation in DSS-induced colitis. Thus, we examined the effects of the pharmacological activation of RXRα in DSS-induced colitis in mice. RXRα agonist treatment mitigated DSS-induced colitis severity in mice as evidenced by lesser weight loss, colon shortening, inflammation, and myeloperoxidase activity (Fig. 6A–C). Histopathology of colons of RXRα agonist-treated mice showed less extensive damage to the mucosa regarding epithelial erosion, loss of crypts, and infiltration of immune cells to DSS (Fig. 6D, E). Consistent with reduced gut inflammation-associated injury, we also observed a decrease in FITC-dextran in the serum of RXRα agonist-treated mice and increased expression of tight junction complex proteins, indicating less damage to the integrity of epithelial barrier (Fig. 6F, G). Consistent with diminished gut inflammation, colons of RXRα agonist-treated mice, expressed and produced lower levels of inflammatory cytokines (IL-6, TNF-α, IL-1β, IL-1α, IL-12) and higher levels of IL-10 than the colons of vehicle-treated mice in response to DSS (Fig. 6H, I). Collectively, these observations suggest that activation of RXRα mice ameliorates DSS-induced colitis and inflammation-associated tissue injury.

Pharmacological activation of RXRα suppresses colonic inflammation in the Rag-deficient T cell–transfer model of colitis.

Given that the RXRα pathway in intestinal APCs limits Th1/Th17 cell differentiation and promotes Treg differentiation, we examined the effects of treating mice with an RXRα-specific agonist (CD3254) in the Rag-deficient T cell–transfer model of colitis. In this murine model of CD, colitis is caused by disruption of T cell homeostasis by uncontrolled Th1 and Th17 responses to commensal microbiota (44, 45). As expected, control $Rag2^{-/-}$ mice adoptively transferred with naive T cells showed rapid body weight loss around 4 wk post–T cell transfer and increased colitis severity (Fig. 7A–C). In contrast, RXRα-agonist treatment significantly delayed disease onset and reduced disease severity (Fig. 7A–C). In line with these observations, the percentages of IFN-γ^+ and IL-$17A^+$ $CD4^+$ T cells were markedly reduced in the colon of RXRα-agonist treated mice compared to control mice (Fig. 7D, E). In contrast, we observed a significant increase in IL-10^+ and $Foxp3^+$ $CD4^+$ Tregs in the colon of RXRα-agonist treated mice (Fig. 7D, E). In line with these observations, T cells isolated from the colon of RXRα-agonist treated mice produced markedly lower levels of IFN-γ and IL-17A and higher levels of IL-10 upon re-stimulation with α-CD3/CD28 *ex vivo* (Fig. 7F). These results suggest that activation of the RXRα pathway suppresses intestinal inflammation by promoting various innate immune regulatory functions of APCs and supporting the induction of Tregs versus Th1/Th17 cell differentiation.

Discussions

The present study defines an essential role for RXRα in intestinal CD11c$^+$ APCs in regulating colonic inflammation and restoring immune homeostasis. A key mechanism contributing to this role of RXRα involves the suppression of proinflammatory cytokines (IL-6, IL-1β, IL-1α, IL12, and TNF-α) with the induction of anti-inflammatory factors IL-10, RA, and IDO in the intestine. Consequently, the absence of RXRα signaling in intestinal DCs and Mϕs suppressed regulatory T cell responses yet promoted Th1 and Th17 cell differentiation. Accordingly, conditional deletion of RXRα in CD11c$^+$ APCs in mice resulted in the loss of immune homeostasis and increased susceptibility to DSS-induced colitis. Conversely, pharmacological activation of RXRα suppressed intestinal inflammation by decreasing inflammatory cytokine expression and limiting Th1/Th17 cell differentiation in murine models of colitis. Collectively, these findings support the hypothesis that RXRα has an immune regulatory role in the colon. Hence, this pathway could be a new target for suppressing intestinal inflammation and restoring immune homeostasis. Several aspects of these findings deserve further comment.

First, the role of intestinal APCs in regulating intestinal tolerance and immune homeostasis has been extensively studied. In IBD, these APC subsets lose their regulatory properties resulting in disruption of immune homeostasis and uncontrolled inflammation against commensal bacteria. However, cell-intrinsic molecular regulators critical for programming intestinal APCs to a regulatory state rather than an inflammatory state are unknown. Our studies demonstrate that RXRα is one of the key transcription factors that programs intestinal APCs to a regulatory state that drives their ability to induce Tregs and suppress intestinal inflammation. The balance between regulatory and effector T cells is critical for gut homeostasis (8, 9). Our study shows that targeted deletion of RXRα in APCs in mice resulted in the loss of balance between T regulatory cells (Tregs) versus pathological Th1/Th17 cells in the colon. Furthermore, conditional deletion of RXRα in APCs exacerbated colitis severity due to a marked increase in Th1/Th17 cells and a decrease in IL-10$^+$ and Foxp3$^+$ Treg cells in the colon. Conversely, pharmacological activation of the RXRα pathway alleviated colitis severity by suppressing the expression of inflammatory cytokines and limiting the Th1/Th17 cell differentiation in the colon, demonstrating a key role for RXRα in suppressing intestinal inflammation and restoring immune homeostasis.

Second, immune regulatory factors such as IL-10, RA, and IDO are critical for driving regulatory T cell differentiation and expansion while limiting the differentiation of Th1/Th17 cells in the gut (5, 10, 11, 46) Importantly, intestinal DCs and Mϕs drive Treg differentiation and expansion, and control intestinal inflammation by expressing these immune regulatory factors (5, 10, 11, 46). Our studies show that the deletion of RXRα in APCs resulted in a significant decrease in the expression of Aldh1a1, Aldh1a2, IDO1, and IL-10, and these APCs were less potent in inducing Tregs. Endogenous ligands of retinoid X receptor α are Vitamin A-derived retinoic acid and fatty acids (19, 47). RXRα regulates gene transcription by heterodimerizing with other nuclear receptors such as retinoic acid receptorsα (RARα), peroxisome proliferator-activated receptors (PPARα, γ), and vitamin D receptor (VDRs) (12, 13). Although our data indicate a significant role for RXRα in regulating the expression of immune regulatory factors in intestinal APCs, it is not clear

whether RXRα regulates these genes directly or indirectly. In the intestine, RARα, PPARα, and PPARγ are associated with retinoic acid (RA) and lipid signaling pathways (18, 19, 35). Other studies have shown that RXRα can modulate β-catenin signaling in the intestinal epithelial cells (26, 27). Transcription factors RARα, PPARα, PPARγ, VDRs and β-catenin signaling regulate the expression of Aldh1a1, Aldh1a2, IDO1, and IL-10 in intestinal APCs (1, 31, 33, 35, 48, 49). It is possible that RXRα might directly regulate the expression of immune regulatory genes through its interaction with RARα, PPARα, PPARγ, and VDRs or indirectly by modulating the Wnt/β-catenin pathway in the intestinal APCs. In the lamina propria (LP), there are three major subsets of DCs: $CD103^+$ $CD11b^-$, $CD103^+$ $CD11b^+$and $CD103^-$ $CD11b^+$ DCs, and these subsets differ in their localization and functions(1–4). However, RXRα expression pattern and how it shapes the functions of these DC subsets is still lacking. Furthermore, the heterogeneity of intestinal DC subsets cannot be addressed with the CD11c-Cre deletion system used in the current study. Further studies are necessary to understand the role of RXRα in regulating the functions of DCs subsets in the intestine.

Lastly, loss of immune tolerance to commensal microflora or commensal dysbiosis results in host susceptibility to colonic inflammation (6, 7). Furthermore, genetic modification of the host leads to microbial dysbiosis resulting in host susceptibility to colonic inflammation (33, 35, 50, 51). Our studies also show that RXRα-deficient intestinal DCs and Mϕs are potent in inducing Th1/Th17 cell differentiation, at least in part due to increased production of inflammatory cytokines. A similar protective role for RXRα was observed in murine models of sepsis, asthma, atherosclerosis, and liver injury (14). So, it is possible that the observed increase in inflammatory cytokine levels and Th1/Th17 cells in the intestine of RXRα mice is due to the loss of immune tolerance to commensal flora or alterations in the composition of gut flora. Additionally, prior studies have shown that RA and IL-10 produced by APCs exert autocrine effects to suppress the expression of inflammatory factors by inducing SOCS1 and SOCS3 genes (10, 31, 33, 52). Thus, it is quite possible that RA and IL-10 signaling could also regulate the expression of proinflammatory factors in intestinal APCs. Further studies are necessary to understand whether RXRα regulates proinflammatory factors in intestinal APCs directly via its interaction with nuclear receptors or indirectly via, RA and IL-10 signaling.

In summary, our study reveals a novel role for RXRα in DCs and Mϕs in regulating intestinal immune homeostasis and inflammation. Additionally, pharmacological activation of RXRα suppressed colitis in mice by inducing regulatory responses and suppressing pathological inflammatory responses in the intestine. These findings have important implications for restoring immune homeostasis and preventing and treating inflammatory bowel disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key points

RXRα in APCs regulates intestinal immune homeostasis and colitis.

RXRα regulates the expression of proinflammatory and anti-inflammatory factors.

Pharmacological activation of the RXRα pathway alleviates colitis severity in mice

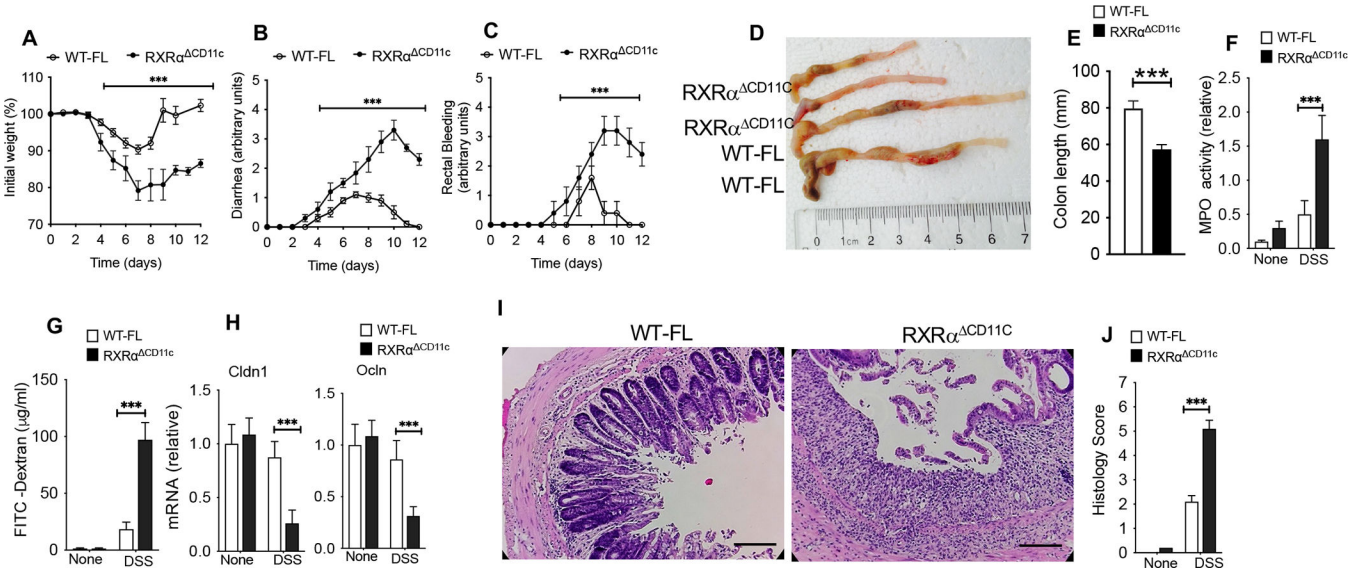

FIGURE 1. RXRα$^{\Delta CD11c}$ mice show increased susceptibility to DSS-induced colitis.

WT-FL and RXRα$^{\Delta CD11c}$ mice were treated with 3% DSS (36–50 kDa) in drinking water for 7 days before returning to normal water and at day 10 the colons of mice were analyzed for inflammation. **(A–E)** Change in body weight, diarrhea, rectal bleeding, and colon length of WT-FL and RXRα$^{\Delta CD11c}$ mice (n ≥ 5). **(F)** Myeloperoxidase (MPO) activity in the colon (n ≥ 5). **(G)** Mice were fed with FITC-dextran on day 10, and 4 hrs later FITC-dextran was quantified in serum (n ≥ 5). **(H)** Quantitative real-time PCR analysis of mRNA expression of tight junction complex proteins claudin-1 (Cldn1) and occludin (Ocln) in the colons of untreated and DSS-treated WT-FL and RXRα$^{\Delta CD11c}$ mice (n ≥ 5). **(I)** Representative images of H&E-stained colonic sections from DSS-treated WT-FL and RXRα$^{\Delta CD11c}$ mice (Scale bars, 100 μm). **(J)** Histopathological score (inflammation + epithelial damage) of colons was graded following analysis of H&E-stained cross sections of colons of DSS-treated WT-FL and RXRα$^{\Delta CD11c}$ mice (n ≥ 5). The error bars indicate mean ± SEM of 5–6 mice/group or representative of at least 2 independent experiments. ***p<0.001.

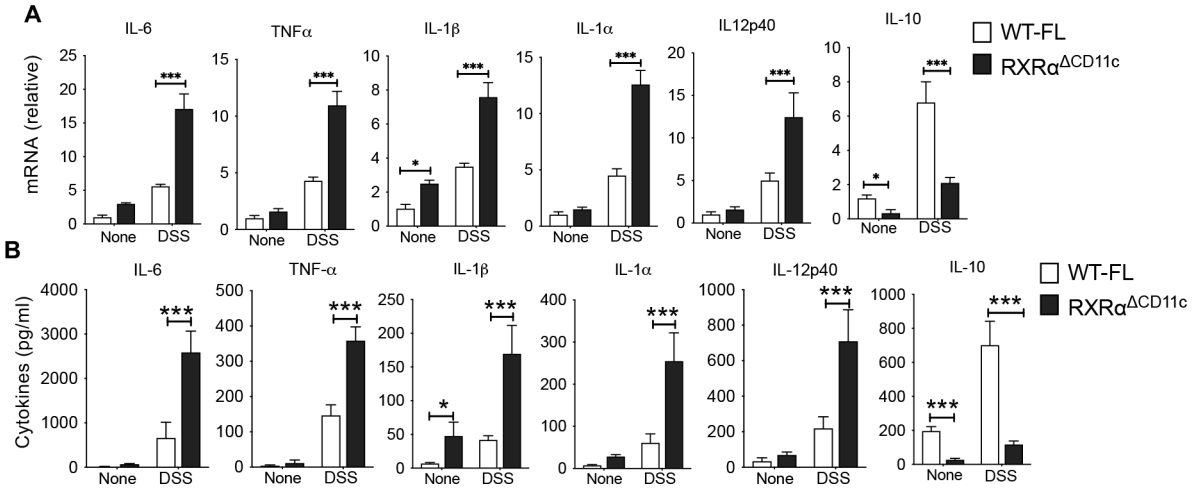

FIGURE 2. RXRα-deficiency in CD11c^{+} APCs augments the expression of inflammatory factors in the colon.

(A) RNA was extracted from colons of untreated and DSS-treated WT-FL and RXRα$^{\Delta CD11c}$ mice. The expression of indicated genes was quantified by qPCR (n>5). **(B)** Excised colon samples of untreated and DSS-treated WT-FL and RXRα$^{\Delta CD11c}$ mice were cultured for 2 days *ex vivo*, and the cytokine levels in the culture supernatants were quantified by ELISA (n>5). The error bars indicate mean ± SEM of 5–6 mice/group or representative of at least 2 independent experiments. *p<0.05; ***p<0.001.

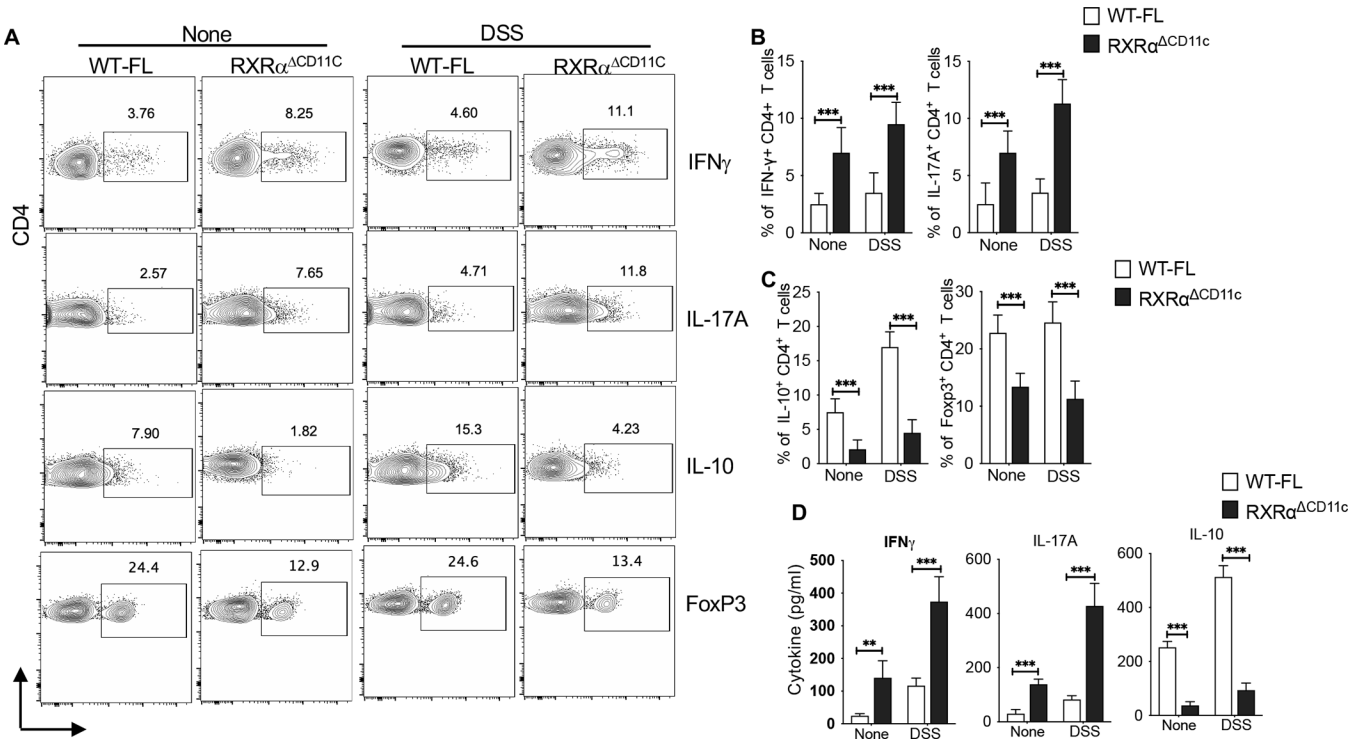

FIGURE 3. RXRα$^{\Delta CD11c}$ mice show loss of CD4^{+} T cell homeostasis in the colon during colitis.

(A) FACS plots representing percentages and **(B, C)** cumulative frequencies of CD4^{+} T cells positive for IL-17A, IFN-γ, IL-10, and Foxp3 isolated from colons of WT-FL and RXRα$^{\Delta CD11c}$ mice treated with or without DSS (n>5). **(D)** Colonic CD4^{+} T cells isolated from untreated and DSS-treated WT-FL and RXRα$^{\Delta CD11c}$ mice were cultured for 48 hrs ex vivo in the presence of anti-CD3 and anti-CD28 antibodies. IL-17A, IFN-γ, and IL-10 cytokine amounts in the culture supernatants were quantified by ELISA (n>4). Error bars show mean values ± SEM of 5–6 mice/group or representative of at least 2 independent experiments. **p<0.01; ***p<0.001.

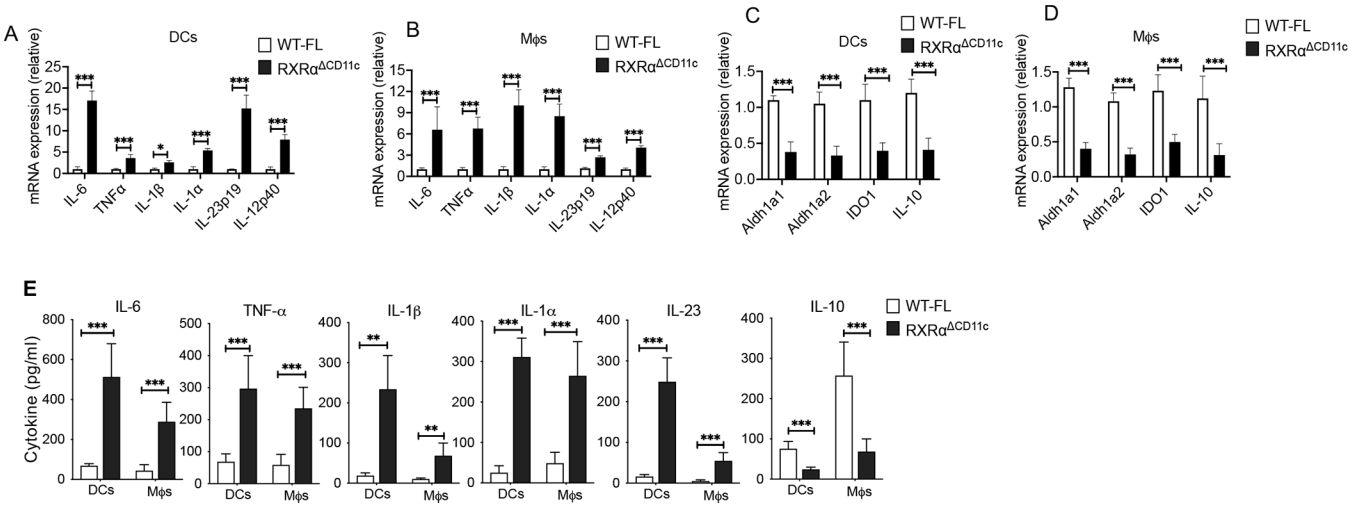

FIGURE 4. RXRα signaling suppresses the expression of inflammatory factors and induces the expression of immune regulatory factors in colonic CD11c$^+$ DCs and Mϕs.

(A-D) Quantitative real-time PCR analysis of the mRNA of indicated genes in colonic CD11c$^+$ DCs and Mϕs sorted from WT-FL and RXRα$^{\Delta CD11c}$ mice. Data are presented as fold change relative to WT (n=5). **(E)** Sorted colonic CD11c$^+$ DCs and Mϕs from WT-FL and RXRα$^{\Delta CD11c}$ mice were cultured for 2 days *ex vivo* and cytokine amounts in the culture supernatants were quantified by ELISA (n=4). Error bars show mean values ± SEM of 5–6 mice/group or representative of at least 2 independent experiments. *p<0.05; **p<0.01; ***p<0.001.

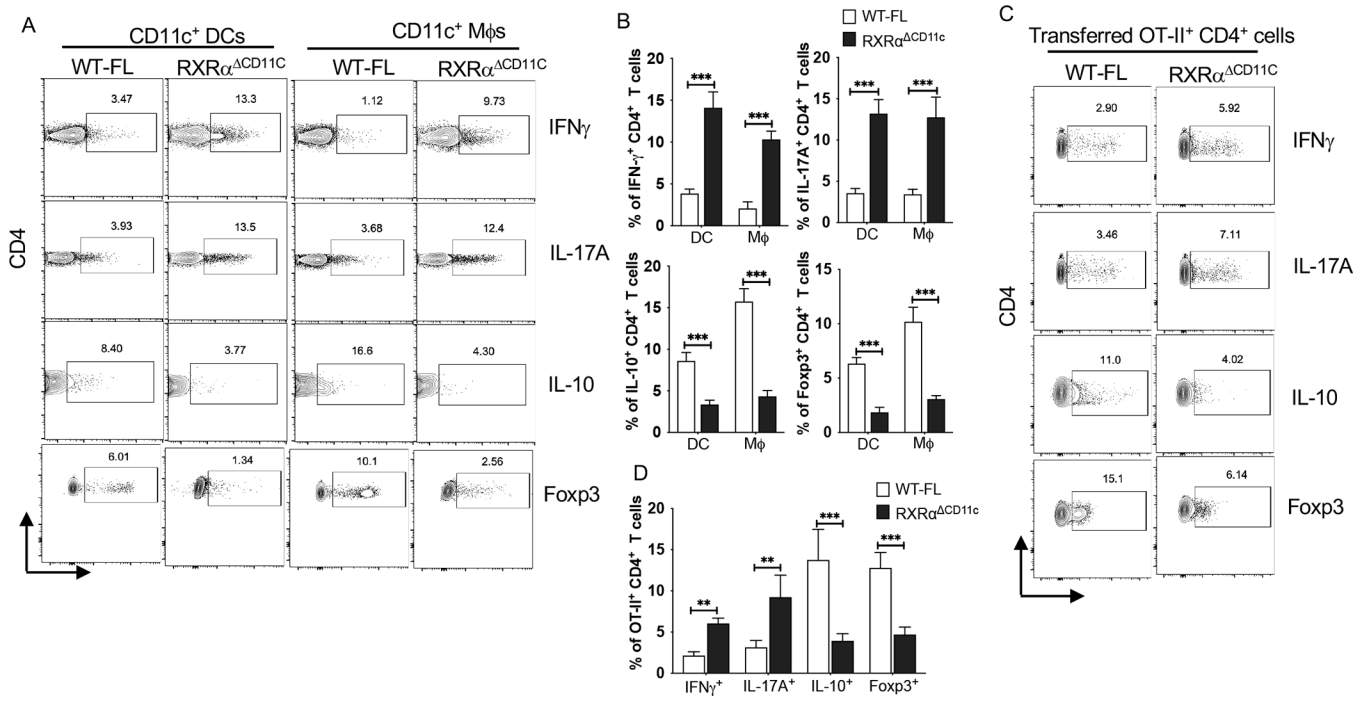

FIGURE 5. RXRα signaling imparts an anti-inflammatory phenotype on colonic DCs and Mϕs.$ $PARABREAKHERE$$

(A, B) Colonic DCs and Mϕs isolated from WT-FL and RXRα$^{\Delta CD11c}$ mice were cultured with naïve OT-II cells in the presence of $OVA_{323–339}$ peptide (OVA). After 5 days, cultured OT-II cells were stimulated *in vitro* for 6 hrs with antibodies to CD3 and CD28 in the presence of brefeldin A and monensin. **(A)** FACS plots representing percentages and **(B)** cumulative frequencies of OT-II T cells positive for IFN-γ, IL-17A, IL-10, and Foxp3 are shown. Data is representative of two experiments (n >5). (**C**, **D**) Representative FACS plot (C) and cumulative frequencies (D) of adoptively transferred naive OT-II CD4$^+$ T cells positive for IFN-γ, IL-17A, IL-10, and Foxp3 isolated from colons of WT-FL and RXRα$^{\Delta CD11c}$ mice treated orally with OVA protein (n >5). Error bars show mean values ± SEM of 5–6 mice/group or representative of at least 2 independent experiments. **p<0.01; ***p<0.001.

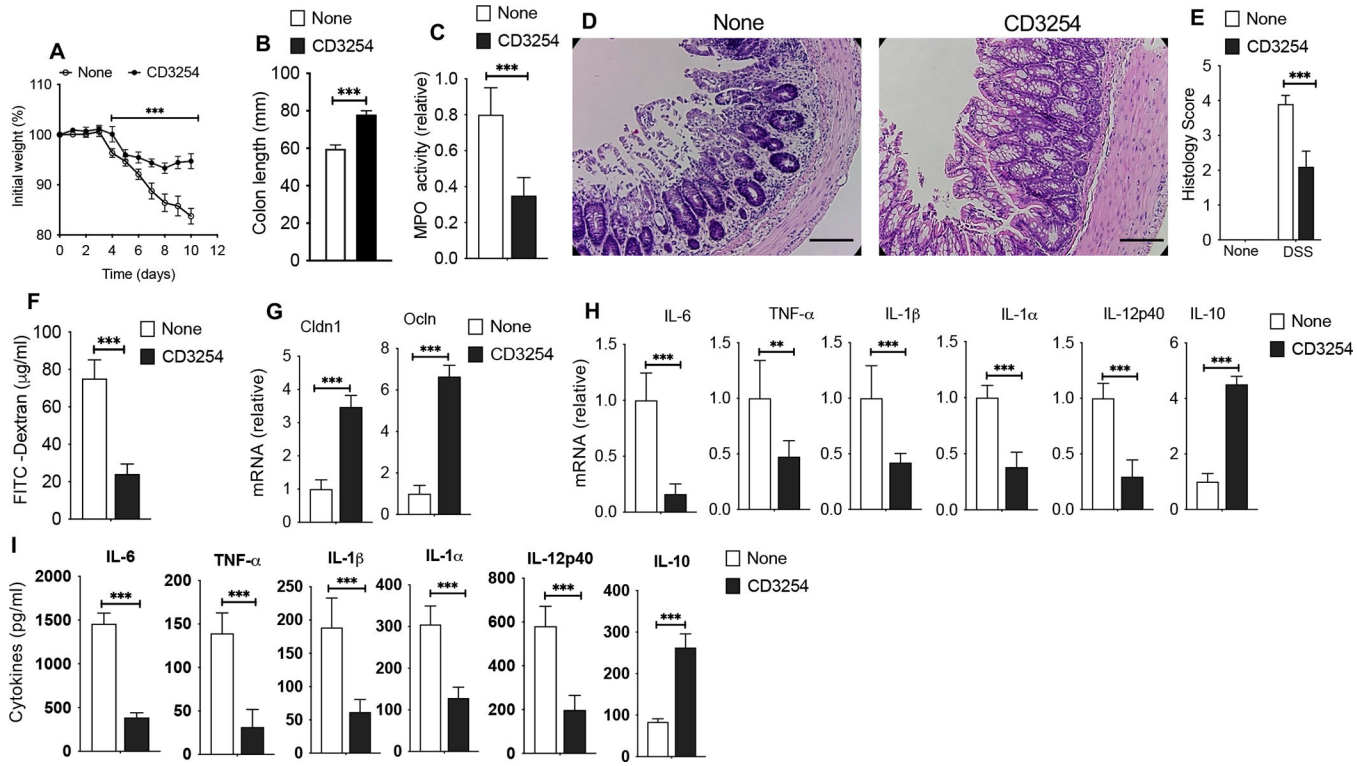

FIGURE 6. RXRα agonist treatment ameliorates DSS-induced colitis.

WT mice were treated with RXRα agonist orally (CD3254; 10 mg/kg) or PBS (None) daily from day 0 to day 7 and received 3% DSS in drinking water from day 1 to day 7 and at day 10 the colons of mice were analyzed for inflammation. **(A)** Change in body weight and **(B)** colon length of mice treated with or without RXRα agonist ($n \geq 5$). **(C)** Myeloperoxidase (MPO) activity in the colon of mice treated with or without CD3254 ($n \geq 5$) **(D, E)** Representative colon histology (H&E staining, (Scale bars, 100 μm)) and histology scores of mice treated with or without CD3254 ($n \geq 5$). **(F)** Mice were fed with FITC-dextran on day 10, and 4 hrs later FITC-dextran was quantified in serum ($n \geq 5$). **(G, H)** Quantitative real-time PCR analysis of mRNA expression of indicated genes in the colons of mice treated with or without CD3254. **(I)** Excised colon samples of mice treated with or without CD3254 were cultured for 2 days *ex vivo*, and the cytokine levels in the culture supernatants were quantified by ELISA ($n \geq 5$). The error bars indicate mean ± SEM of 5–6 mice/group or representative of at least 2 independent experiments. $^{**}p<0.01$; $^{***}p<0.001$

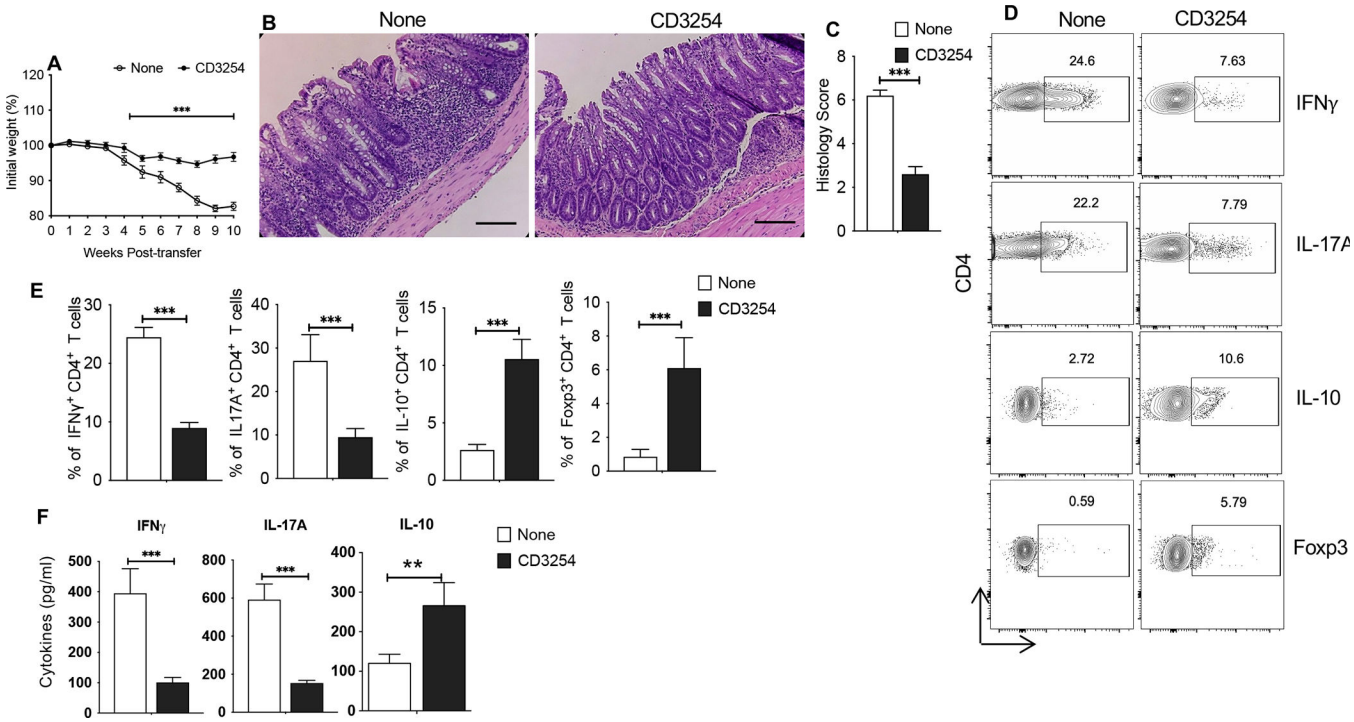

Figure 7. Pharmacological activation of RXRα attenuates intestinal inflammation in the T-cell transfer model of colitis.

(A-F) CD45RB^{hi}CD4^{+} T cells isolated from WT mice were adoptively transferred into Rag2$^{-/-}$ mice. Animals were treated with RXRα agonist orally (CD3254; 10 mg/kg; on Weeks 1, 2, 3, and 4) and monitored over a period of time for percent weight loss compared to the initial weight. **(A)** Percent weight change for Rag2$^{-/-}$ mice treated with CD3254 at various weeks post-naïve CD4^{+} T cell adoptive transfer (n ≥ 5). **(B, C)** Representative colon histology (H&E staining, (Scale bars, 100 μm)) and histology scores of Rag2$^{-/-}$ mice 8 weeks post-naïve CD4^{+} T cell transfer treated with or without CD3254 treatment (n ≥ 5) **(D, E)** Representative FACS plots and cumulative frequencies of colonic IL-17A^{+}, IFN-γ^{+}, IL-10^{+}, and Foxp3^{+} CD4^{+} T cells from Rag2$^{-/-}$ mice treated with CD3254 treatment compared with untreated mice on week 8 post-naïve CD4^{+} T cell adoptive transfer (n ≥ 5). **(F)** Excised colon samples in panel C were cultured for 2 days *ex vivo*, and then the secreted IL-17A, IFN-γ, and IL-10 cytokine amounts in the culture supernatants were quantified by ELISA (n ≥ 5). The error bars indicate mean ± of 5–6 mice/group or representative of at least 2 independent experiments. **p<0.01; ***p<0.001. ±