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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

# Induction of Colonic Regulatory T Cells by Mesalamine by Activating the Aryl Hydrocarbon Receptor

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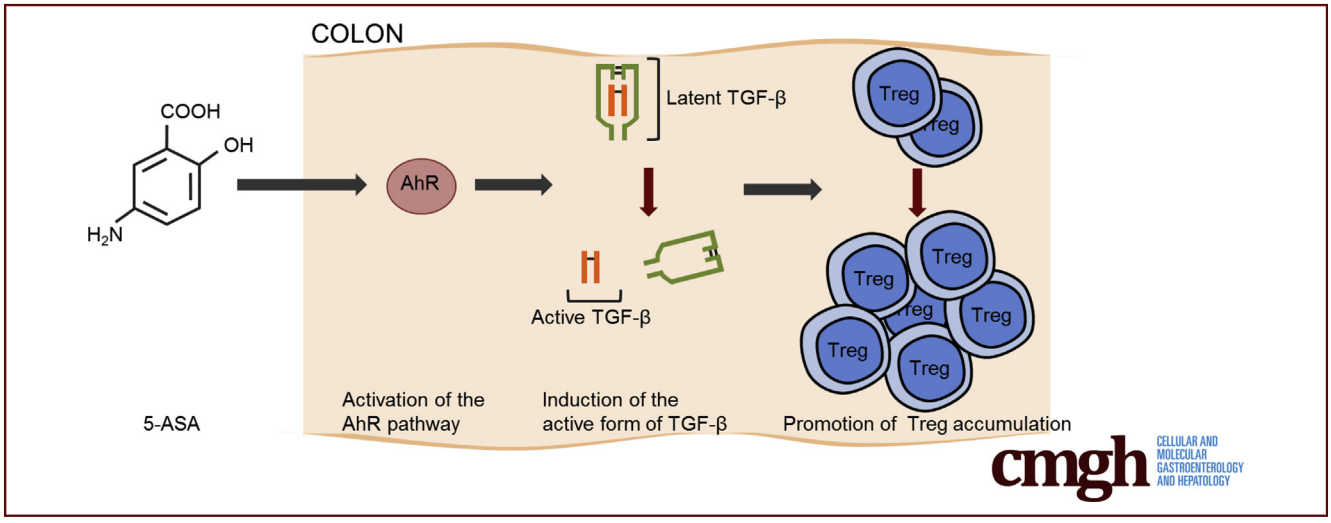


## SUMMARY

We propose a novel anti-inflammatory mechanism of mesalamine for colitis: induction of regulatory T cells in the colon via the aryl hydrocarbon receptor pathway, followed by transforming growth factor-$\beta$ activation.

**BACKGROUND & AIMS:** Mesalamine is a first-line drug for treatment of inflammatory bowel diseases (IBD). However, its mechanisms are not fully understood. $CD4^+$ $Foxp3^+$ regulatory T cells (Tregs) play a potential role in suppressing IBD. This study determined whether the anti-inflammatory activity of mesalamine is related to Treg induction in the colon.

**METHODS:** We examined the frequencies of Tregs in the colons of wild-type mice, mice deficient for aryl hydrocarbon receptor ($AhR^{-/-}$ mice), and bone marrow–chimeric mice lacking AhR in hematopoietic cells (BM-$AhR^{-/-}$ mice), following oral treatment with mesalamine. We also examined the effects of mesalamine on transforming growth factor (TGF)-$\beta$ expression in the colon.

**RESULTS:** Treatment of wild-type mice with mesalamine increased the accumulation of Tregs in the colon and up-regulated the AhR target gene *Cyp1A1*, but this effect was not observed in $AhR^{-/-}$ or BM-$AhR^{-/-}$ mice. In addition, mesalamine promoted *in vitro* differentiation of naive T cells to Tregs, concomitant with AhR activation. Mice treated with mesalamine exhibited increased levels of the active form of TGF-$\beta$ in the colon in an AhR-dependent manner and blockade of TGF-$\beta$ signaling suppressed induction of Tregs by mesalamine in the colon. Furthermore, mice pretreated with mesalamine acquired resistance to dextran sodium sulfate–induced colitis.

**CONCLUSIONS:** We propose a novel anti-inflammatory mechanism of mesalamine for colitis: induction of Tregs in the colon via the AhR pathway, followed by TGF-$\beta$ activation. *(Cell Mol Gastroenterol Hepatol 2017;4:135–151; http://dx.doi.org/10.1016/j.jcmgh.2017.03.010)*

*Keywords:* Mesalamine; Aryl Hydrocarbon Receptor; TGF-$\beta$; Regulatory T Cells.

Mesalamine (or mesalazine or 5-aminosalicylic acid [5-ASA]) is an anti-inflammatory drug indicated for the treatment of inflammatory bowel disease (IBD).[1–3] In particular, mesalamine is the core therapy for both induction and maintenance of remission in patients with mild-to-moderate ulcerative colitis. Mesalamine has pleiotropic effects on various signaling pathways, including the NF-kB, Wnt/$\beta$-catenin, PPAR-$\gamma$, MAPK, and PI3K/Akt axes,[4] which are thought to be responsible for inhibition of colonic inflammation. However, the key mechanisms underlying mesalamine-mediated suppression of colitis remain unclear.

$CD4^+$ $Foxp3^+$ regulatory T cells (Tregs) contribute to the maintenance of immune homeostasis and may play a role in suppressing inflammatory diseases.[5] At steady state, Tregs are abundant in the colonic mucosa in mice, constitute the most dominant $CD4^+$ population in the colon, and suppress excessive immune responses to commensal bacteria.[6] Bacterial colonization immediately after birth, especially by the spore-forming component of the indigenous intestinal microbiota (eg, clusters IV and XIVa of genus *Clostridium*), plays a pivotal role in the colon Treg accumulation by generating a transforming growth factor (TGF)-$\beta$-rich microenvironment.[7] In IBD, the ability of transferred Treg to control inflammatory lesions has been demonstrated in rodent models of IBD.[8–10] In addition, the Treg frequency in the peripheral blood was significantly lower in patients with active IBD than in healthy control subjects, suggesting disrupted Treg homeostasis in IBD.[11,12] Thus, Tregs may play a role in suppression of IBD.[13]

This study tested the hypothesis that mesalamine induces Tregs in the colon, and that these cells mediate the drug's anti-inflammatory effects on colitis. We also investigated how mesalamine affected the accumulation of colon Tregs and whether this mechanism was associated with prevention of colitis.

## Materials and Methods

### Oral Administration of Mesalamine

Male or female 6-to-8-week-old C57BL/6J mice, male mice aged 30 ± 4 weeks (purchased from Japan SLC, Tokyo, Japan), mice bearing a homozygous deletion of the *AhR* gene ($AhR^{-/-}$ mice),[14] and xenobiotic responsive element (XRE)-based sensing via SEAP (DRESSA) mice[15] were bred under specific pathogen-free conditions. Mice were orally treated with phosphate-buffered saline (PBS) or mesalamine (5 or 50 mg/kg in 0.05 N HCl, Sigma-Aldrich Inc, St Louis, MO) once per day for 1 day, 3 days, or 2 weeks. Mice were orally treated with either dimethyl sulfoxide or 5 $\mu$g/kg 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; dioxin, Wako Pure Chemical Co, Osaka, Japan). Mice were orally treated with acetylsalicylic acid (aspirin) added to drinking water (0.6 mg/mL) for 2 weeks. Aspirin was added in drinking water because high doses of 1-shot oral feeding of aspirin induced severe injury of the small intestine as previously described.[16] Based on the amounts of drinking water that the aspirin-treated mice took per day, we estimated that they received ~50 mg/kg of aspirin per day. For some experiments, the TGF-$\beta$ type I receptor kinase inhibitor GW788388 (R&D Systems, Minneapolis, MN) was added in drinking water (8 mg/mL) as previously described.[17] All animal experiments were approved by the institutional review board for animal experiments at the University of Yamanashi and were carried out under the board's guidelines.

### Generation of Bone Marrow Chimeric Mice

Six-week-old recipient wild-type (WT) mice were exposed to X-irradiation (2 doses of 500 rad, 4 hours apart), and then injected intravenously 6 hours later with $1 \times 10^7$ bone marrow (BM) cells derived from the femurs of WT or $AhR^{-/-}$ donor mice. These chimeric mice were used for experiments at 14 weeks old. Two sets of BM chimeric mice were generated: WT mice were reconstituted either with WT mice BM (BM-$AhR^{+/+}$) or with $AhR^{-/-}$ mice BM (BM-$AhR^{-/-}$). The chimerism was confirmed by examining AhR mRNA expression levels in the mesenteric lymph nodes (MLNs) of BM chimeric mice by quantitative polymerase chain reaction (Q-PCR).

### Preparation of Lamina Propria Lymphocytes

Lamina propria lymphocytes (LPLs) were isolated as described previously.[7] Briefly, intestines were opened longitudinally, washed, and shaken for 45 minutes at 37°C in Hank's balanced salt solution containing 5 mM EDTA. After removal of epithelial cells, the intestines were cut into small pieces and shaken for 40 minutes at 37°C in RPMI 1640 supplemented with 2% fetal bovine serum (FBS) and 1 mg/mL collagenase D (Wako Pure Chemical Co). The digested tissues were washed and resuspended in 100% Percoll (GE Healthcare, Little Chalfont, UK) with an underlayer of 40% Percoll followed by centrifugation at 850 $\times$ *g* for 20 minutes. LPLs were collected at the interface of the gradient and washed with RPMI 1640/10% FBS. For some experiments, $CD4^+$ T cells from LPLs were purified by incubating LPLs with antibodies against CD4 coupled to magnetic beads, following manufacturer's instructions (MACS, Miltenyi Biotec, Auburn, CA). $CD4^+$ T-cell population was sorted to a purity of 60%−70%.

***Abbreviations used in this paper:*** **AhR, aryl hydrocarbon receptor; BM, bone marrow; DSS, dextran sodium sulfate; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IBD, inflammatory bowel disease; IFN, interferon; IL, interleukin; LPL, lamina propria lymphocytes; mAb, monoclonal antibody; MLN, mesenteric lymph nodes; PBS, phosphate-buffered saline; Q-PCR, quantitative polymerase chain reaction; RPMI, Roswell Park Memorial Institute; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TGF, transforming growth factor; TNF, tumor necrosis factor; Tregs, regulatory T cells; WT, wild-type; XRE, xenobiotic responsive element.**

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### Preparation of Mesenteric Lymph Nodes and Spleen

Single-cell suspensions were prepared from the MLNs and spleen, by removing fat and connective tissue and smashing between glass slides. Leukocyte suspensions were then washed with RPMI 1640/10% FBS. Additionally, splenic erythrocytes were then lysed with ammonium, chloride solution and the remaining leukocytes washed twice with RPMI 1640/10% FBS.

### Flow Cytometry

For analyses of Tregs, the LPLs were stained with fluorescein isothiocyanate (FITC)-CD3 monoclonal antibody (mAb; clone 145-2C11, eBioscience, San Diego, CA) and APC-CD4 mAb (clone GK1.5, eBioscience), fixed, permeabilized, and stained with PE-Foxp3 mAb (clone FJK-16s, eBioscience) and PerCP-Cy 5.5-Ki67 mAb (clone B56, BD Pharmingen, San Diego, CA) using the Foxp3 staining buffer set (eBioscience). For the analyses of Th1 and Th17 cells, LPLs were stimulated at 37°C for 4 hours with 50 ng/mL phorbol myristate acetate (Sigma-Aldrich Inc), 750 ng/mL ionomycin (Sigma-Aldrich Inc) in the presence of 10 $\mu$g/mL brefeldin A (Sigma-Aldrich Inc). The cells were stained with PE or FITC-CD3 mAb and APC-CD4 mAb, fixed, permeabilized, and stained with FITC-interferon (IFN)-$\gamma$ mAb (clone XMG1.2, eBioscience) or PE-IL-17 mAb (clone Bio17B7, eBioscience) using the fixation and permeabilization (eBioscience). All data were analyzed on a BD ACCURI C6 (BD Biosciences, San Jose, CA). Single-color compensation controls were prepared from cells treated in the same fashion as the test samples.

### Immunofluorescence Study

Colon tissues were isolated from 5 mice treated orally either with PBS or mesalamine, washed, and then embedded in Tissue-Tek OCT compound (Sakura Fintek USA, Inc, Torrance, CA). Cryostat sections of 3 $\mu$m were fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) in 0.1 M phosphate buffer (pH 7.4) for 30 minutes at 4°C, followed by incubation with FITC-conjugated rat antimouse CD4 mAb (Clone RM4-5, BD Pharmingen) or isotype-matched control FITC-conjugated rat IgG2a (Clone R35-95, BD Pharmingen) for 1 hour at 37°C. Endogenous biotin was blocked using avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA), according to the manufacturer's instructions. After blocking, the sections were incubated with biotin-conjugated rat antimouse Foxp3 mAb (Clone FJK-16S, eBioscience) or isotype-matched control biotin-conjugated rat IgG2a (Clone R35-95, BD Pharmingen) for 1 hour at 37°C, and then labeled with Alexa Fluor 594-conjugated streptavidin (Invitrogen, Eugene, OR). The fluorescence images were captured with AxioPlan2 (Zeiss, Jena, Germany). The number of infiltrating $CD4^+$ $Foxp3^+$ cells in mucosa in each section was analyzed using KS400 Image analysis system (Zeiss). A total of 5–7 typical areas were examined in each specimen, and positively stained cell density was calculated per square millimeter.

### Quantitative Real-Time Polymerase Chain Reaction

Q-PCR using cDNA from mouse tissue specimens was performed using the StepOne real-time PCR system (Applied Biosystems, Foster City, CA), using primers and probes for mouse *Foxp3*, *T-bet*, *RorC* (retinoic acid–related orphan receptor C), *Cyp1A1*, *ALDH1A2* (aldehyde dehydrogenase 1A2), *RAR-α* (retinoic acid receptor), *IL33*, *ST2*, *P2X7* (purinergic receptor P2X7), *integrin αV*, *TSP1* (thrombospondin 1), *AhR*, *TGF-β1*, *HO-1* (heme oxigenase-1), *IL1β*, *IL6*, *MPO* (myeloperoxidase), *TNF-α* (tumor necrosis factor), and *18S ribosomal RNA* (rRNA) (Applied Biosystems). Each mRNA level was normalized against *18S rRNA*, and the relative expression levels are shown.

### Enzyme-Linked Immunosorbent Assay

The concentrations of IFN-$\gamma$ and interleukin (IL) 17A in supernatants of cell cultures were measured using specific enzyme-linked immunosorbent assay (ELISA) kits (eBioscience). For quantitation of TGF-$\beta$1, colon tissue samples (0.4 g) were mixed with 1 mL of PBS containing 1% Triton X-100 and protease inhibitor cocktail (CALBIOCHEM, Cambridge, MA) and homogenized using a bead shocker. After centrifugation (15,000 rpm, 15 minutes), supernatants were collected, and the concentrations of TGF-$\beta$1 were measured with or without treatment by 0.2 N HCl using the TGF-$\beta$1 ELISA kit (R&D Systems).

### *T-Cell Differentiation* In Vitro

Naïve $CD4^+$ T cells were isolated from the spleens of WT mice or $AhR^{-/-}$ mice using the Naive $CD4^+$ T-cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany). For Treg cell polarization, naive $CD4^+$ T cells were stimulated with immobilized anti-CD3 mAb (2 $\mu$g/mL, clone145-2G1, eBioscience) and soluble anti-CD28 mAb (1 $\mu$g/mL, clone37.51, eBioscience) supplemented with human TGF-$\beta$1 (2.5 ng/mL, R&D Systems) and mouse IL2 (10 ng/mL, R&D Systems) with the indicated concentration of mesalamine for 3 days. For Th1 or Th17 cell polarization, naive $CD4^+$ T cells were stimulated with immobilized anti-CD3 mAb and soluble anti-CD28 mAb for Th0; with recombinant mouse IL12 (10 ng/mL, R&D Systems) plus anti-IL4 mAb (10 $\mu$g/mL, clone11B11, eBioscience) for Th1; and with human TGF-$\beta$1, mouse IL6 (30 ng/mL, R&D Systems), anti-IL4 mAb, and anti-IFN-$\gamma$ mAb (10 $\mu$g/mL, cloneXMG1.2, eBioscience) for Th17, with the indicated concentration of mesalamine, for 3 days.

### Measurement of Serum SEAP Levels

Activity of SEAP in serum of DRESSA mice was evaluated by a chemiluminescent method using the Great EscAPe SEAP detection kit (BD Biosciences) as previously described.[15]

### Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assay was performed using SimpleChIP Enzymatic Chromatin IP Kit (Agarose

Beads, Cell Signaling, Tokyo, Japan, #9002). Naive $CD4^+$ T cells were isolated from the spleens of WT mice and cultured for 2 hours in Treg differentiation media with or without mesalamine (1 $\mu$M) or TCDD (1 nM), and then cross-linked in 1% formaldehyde, followed by chromatin immunoprecipitation assay. Rabbit polyclonal antibody against AhR was purchased from Biomol (SA-210, Plymouth Meeting, PA). DNA was purified and Q-PCR was performed using the SYBR Green reagent (QIAGEN, Hilden, Germany). Q-PCR reactions were carried out using the following primer pairs: *Cyp1A1*-845, forward 5′-AGGCTCTTCTCACGCAACTC-3′ and reverse 5′-CTGGGGCTACAAAGGGTGAT-3′; *Foxp3* (NCAB-2)-1596, forward 5′-GCCTTGTCAGGAAAAACTCTG-3′ and reverse 5′-GTCCTCGATTTGGCACAGAC-3′; *Foxp3* (CAB) +13343, forward 5′-GCTTTGTGCGAGTGGAGAG-3′ and reverse 5′-AGGGATTGGAGCACTTGTTG-3′.

### Measurement of Transforming Growth Factor-β Activity

TGF-$\beta$ activity in colon samples was measured using clone MFB-F11, embryonic fibroblasts from *TGF-β1*$^{-/-}$ mice stably transfected with a reporter plasmid consisting of TGF-$\beta$-responsive Smad-binding elements coupled to a secreted alkaline phosphatase reporter gene (SBE-SEAP), as previously described.[18]

Briefly, colon tissue samples were prepared as described previously in the Methods section (ELISA) from WT mice or *AhR*$^{-/-}$ mice orally treated with mesalamine for 2 weeks. A total of 2 $\mu$L (1.6 mg) of colon tissue samples were added into the culture of MFB-F11 ($1 \times 10^4$ cells/100 $\mu$L) for 24 hours and activities of SEAP were evaluated. To block TGF-$\beta$ signaling, 10 $\mu$M of TGF-$\beta$ type I receptor kinase inhibitor HTS466284 (Calbiochem, San Diego, CA) was added into the cell culture.

### Fecal IgA Levels

For quantitation of IgA, stool pellets (0.25 g) were mixed with 1 mL of PBS and incubated at 4°C for 2 hours. Then, the pellet was vortexed for 5 minutes. After centrifugation (4,000 $\times$ *g*, 20 minutes), supernatants were collected and IgA concentrations were measured using the IgA ELISA kit (ICL Lab, Portland, OR).

### Induction of Dextran Sodium Sulfate–Induced Colitis

Colitis was induced by feeding 3% dextran sodium sulfate (DSS) dissolved in distilled drinking water (molecular weight 5000; Wako Pure Chemical Co) for 8 days to WT mice or 5 days to *AhR*$^{-/-}$ mice.

### Stool Scores

Stool scores were calculated as the sum of the diarrheal score and the bloody stool score. Briefly, stool consistency was graded as 0 = firm, 1 = loose, 2 = diarrhea, and 3 = severe diarrhea. Blood in the stool was also evaluated as 0 = no blood, 1 = occult blood, and 2 = gross rectal bleeding.

### Histopathologic Scoring

The entire colon was removed and divided into 4 equal segments and fixed with 4% paraformaldehyde. The 2-$\mu$m sectioned colon was stained with hematoxylin and eosin and examined at 40 $\times$ magnification by light microscopy (Olympus BX50, Tokyo, Japan). Histopathologic evaluation was graded using a previously validated scoring system as previously described[19]: (a) inflammation severity: none = 0, slight = 1, moderate = 2, severe = 3; (b) depth of injury: none = 0, mucosal = 1, mucosal and submucosal = 2, transmural = 3; (c) crypt damage: none = 0, basal one-third damaged = 1, basal two-thirds damaged = 2, only surface epithelium intact = 3, entire crypt and epithelium lost = 4; and (d) percentage of area involved: none = 0, 1%–25% = 1, 26%–50% = 2, 51%–75% = 3, 76%–100% = 4. The final scores are the averages of all individual scores of 4 pieces per colon.

### Statistical Analysis

Values represent the mean ± SEM. Statistical analysis was performed using the unpaired Student's *t* test or the 1-way analysis of variance with the Tukey post hoc test. The 1-way analysis of variance with the Tukey post hoc test was used for multiple comparisons of the Q-PCR data (Table 1). A value of $P < .05$ was considered to be significant.

## Results

### Mesalamine Promotes Accumulation of Regulatory T Cells in the Lamina Propria of the Colon

To determine whether mesalamine affects the accumulation of Tregs in the large intestine, we measured

**Table 1.** Changes of mRNA Levels of Genes Related to Treg Differentiation/Proliferation in the Colon of Mice Treated With Mesalamine

| | Genes | Ratio of change ± SEM[a] |
|---|---|---|
| AhR pathway | AhR | 1.55 ± 0.22 |
| Retinoic acid pathway | ALDH1A2 | 0.96 ± 0.14 |
| | RAR-α | 1.40 ± 0.30 |
| IL33/ST2 pathway | IL33 | 1.13 ± 0.13 |
| | ST2 | 0.93 ± 0.29 |
| ATP receptor | P2X7 | 0.96 ± 0.14 |
| Inflammatory cytokine | IL6 | 0.46 ± 0.07 |
| | TNF-α | 0.91 ± 0.22 |

NOTE. Each mRNA level was normalized against 18S rRNA. The relative expression levels (mesalamine-treated mouse colon/PBS-treated mouse colon) are shown. The values are mean ± SEM.
[a]$P > .05$; significance was calculated using 1-way analysis of variance with the Tukey post hoc test.

the proportion of Tregs in the LP of the colon in WT mice treated with or without mesalamine. Patients with IBD generally take 25–50 mg/kg of mesalamine per day (1.5–3.0 g/day).[20] Accordingly, in these experiments, we orally treated male 6-to-8-week-old WT mice with 50 mg/kg mesalamine per day for 2 weeks.

Flow cytometry analysis showed that treatment of mice with mesalamine significantly increased the proportion of $CD4^+$ $Foxp3^+$ Tregs in the LP of the colon, but not in the small intestine, MLNs, or spleen (Figures 1*A* and *B*), although the numbers of colon LPLs were comparable between control mice and mesalamine-treated mice

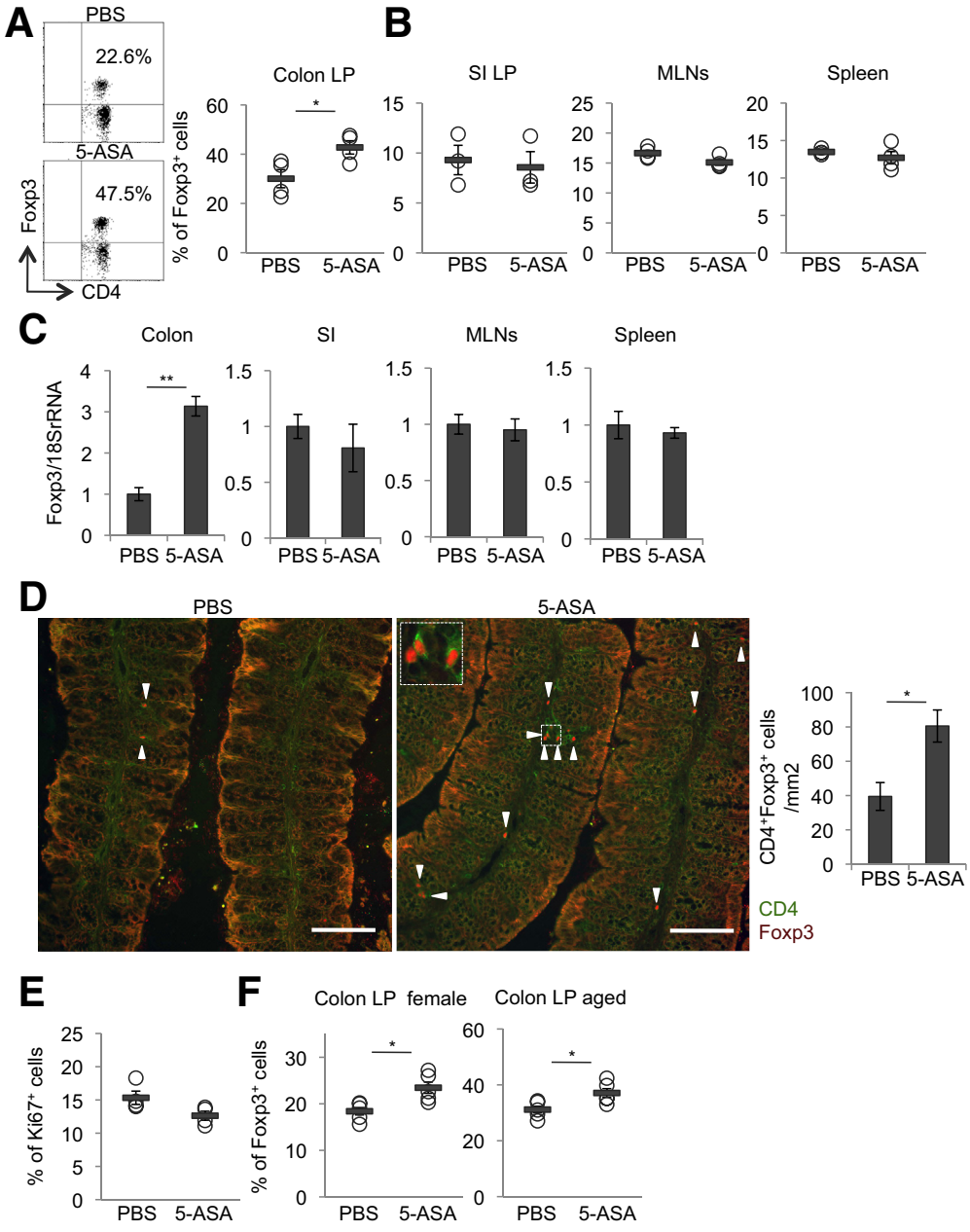



**Figure 1. Mesalamine increases the accumulation of Tregs in the LP of the colon.** (*A–E*) Male 6-week-old WT mice were orally treated with mesalamine for 2 weeks. (*A*) Representative plots (*left*) and quantitative data (*right*) of $Foxp3^+$ cells within the $CD3^+CD4^+$ cell population isolated from the colon LP. n = 4 mice per group. (*B*) Percentages of $Foxp3^+$ cells within the $CD3^+CD4^+$ cell population isolated from the LP of small intestine (SI) (n = 3 mice per group), MLNs (n = 4 mice per group), and spleen (n = 4 mice per group). (*C*) Foxp3 mRNA levels in the colon (n = 8 mice per group), SI (n = 8 mice per group), MLNs (n = 4 mice per group), and spleen (n = 4 mice per group). (*D*) Representative images (*left*) and the numbers of $CD4^+Foxp3^+$ cells in square millimeter in the lamina propria (*right*). Scale bar, 100 $\mu$m. *Arrowheads* indicate $CD4^+Foxp3^+$ cells (n = 5 mice per group). (*E*) The percentages of $Ki67^+$ cells within the $CD3^+CD4^+$ $Foxp3^+$ cell population isolated from the colon LP (n = 4 mice per group). (*F*) Female 6-week-old WT mice (*left*, n = 5 mice per group) or male aged (30 ± 4 week-old) mice (*right*, n = 5 mice per group) were orally treated with mesalamine for 2 weeks. Percentages of $Foxp3^+$ cells within the $CD3^+CD4^+$ cell population isolated from the colon LP. Error bars represent mean ± SEM. $^*P < .05$, $^{**}P < .01$. 5-ASA, mesalamine.

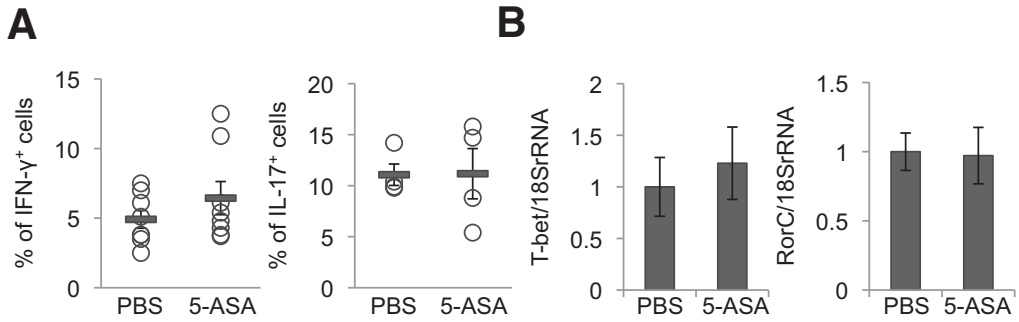



**Figure 2. Mesalamine does not increase the accumulation of Th1 cells and Th17 cells in the LP of the colon.** Wild-type mice were orally treated with 50 mg/kg of mesalamine for 2 weeks. (*A*) Percentages of IFN-$\gamma^+$ cells (n = 8 mice per group) or IL17$^+$ cells (n = 4 mice per group) within the CD3$^+$CD4$^+$ cell population isolated from the colon LP. (*B*) T-bet and RorC mRNA levels in the colon (n = 4 mice per group). Error bars represent mean ± SEM. 5-ASA, mesalamine.

(PBS-treated mice: 1.955 × $10^6$/mouse vs mesalamine-treated mice: 1.86 × $10^6$/mouse; n = 5; $P$ = .8). Consistent with this, *Foxp3* mRNA expression in the colon, but not in the small intestine, MLNs, or spleen, was significantly higher in mesalamine-treated mice than in control mice (Figure 1*C*). Furthermore, immunofluorescence analysis showed that treatment of mice with mesalamine significantly increased the numbers of Tregs in the LP of the colon (Figure 1*D*). In addition, treatment of mice with mesalamine did not affect the proportions of Ki67$^+$ cells of Tregs in the LP of the colon (Figure 1*E*) and there were no differences in *IL6* and *TNF-α* mRNA expression levels between control mice and mesalamine-treated mice (Table 1). We also observed that treatment of female 6-to-8-week-old WT mice or male mice aged 30 ± 4 weeks with 50 mg/kg mesalamine per day for 2 weeks significantly increased the proportion of Tregs in the LP of the colon (Figure 1*F*).

By contrast, 2-week mesalamine treatment of male 6-to-8-week-old WT mice did not affect the numbers of CD4$^+$ IFN-$\gamma^+$ cells (Th1-type cells) or CD4$^+$IL17$^+$ cells (Th17-type cells) in the colon LP or the levels of *T-bet* or *RorC* mRNAs in the colon (Figures 2*A* and *B*).

Collectively, these findings suggest that treatment of WT mice with mesalamine at a dose equivalent to its medical use for IBD treatment can promote the accumulation of Tregs in the colon without affecting other helper T-cell subsets. It seems that the accumulation of colon LP Tregs due to mesalamine treatment is not caused by elevated

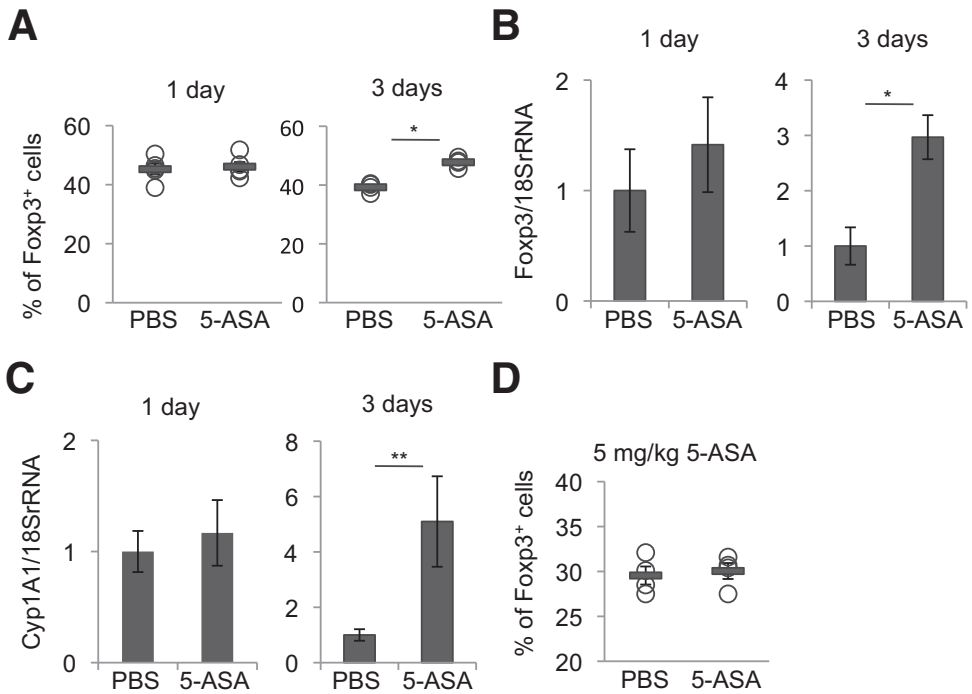



**Figure 3. Treatment of mice with mesalamine for at least 3 consecutive days can promote the accumulation of Tregs in the colon.** (*A–C*) WT mice were orally treated with 50 mg/kg of mesalamine for 1 (n = 5 mice per group) or 3 days (n = 4 mice per group). The percentages of Foxp3$^+$ cells within the CD3$^+$CD4$^+$ cell population isolated from colon LP (*A*) and Foxp3 (*B*) or Cyp1A1 (*C*) mRNA levels in the colons. (*D*) WT mice were orally treated with 5 mg/kg of mesalamine for 2 weeks, and the percentages of Foxp3$^+$ cells within the CD3$^+$CD4$^+$ cell population isolated from the colon LP are shown (n = 4 mice per group). Error bars represent mean ± SEM. **P* < .05, ***P* < .01. 5-ASA, mesalamine.

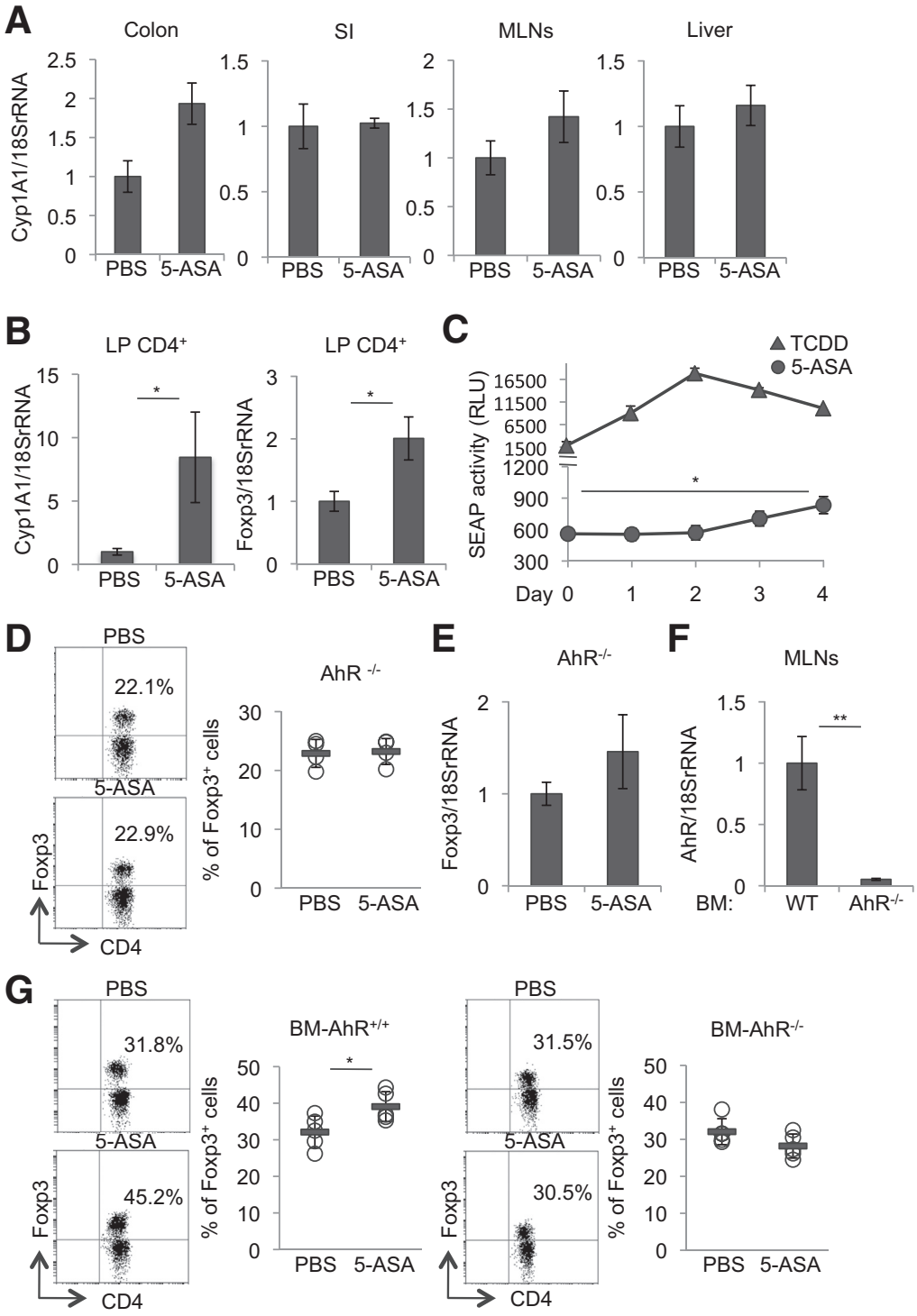



**Figure 4. Mesalamine activates the AhR pathway, thereby inducing colon LP Tregs.** (*A*) Cyp1A1 mRNA levels in the colon, small intestine (SI), MLNs, and liver isolated from WT mice orally treated with 50 mg/kg of mesalamine for 2 weeks (n = 8 mice per group). (*B*) Cyp1A1 and Foxp3 mRNA levels of the $CD4^+$ cells in the LP of the colon isolated from WT mice orally treated with 50 mg/kg of mesalamine for 3 days (n = 5 mice per group). (*C*) Kinetics of serum SEAP in AhR-reporter DRESSA mice following orally treated once with 5 $\mu$g/kg TCDD (n = 3 mice per group) or orally treated with 50 mg/kg mesalamine for 4 days (n = 8 mice per group). (*D*, *E*) Percentages of $Foxp3^+$ cells within the $CD3^+CD4^+$ cell population in the colon LP (*D*) (representative plots [*left*] and quantitative data [*right*]) and Foxp3 mRNA levels in the colon (*E*) isolated from $AhR^{-/-}$ mice orally treated with mesalamine for 2 weeks (n = 4 mice per group). (*F*) AhR mRNA levels in the MLNs from WT mice reconstituted with WT mice BM (BM-$AhR^{+/+}$) or with $AhR^{-/-}$ mice BM (BM-$AhR^{-/-}$) (n = 5 mice per group). (*G*) The percentages of $Foxp3^+$ cells within the $CD3^+CD4^+$ cell populations in the colon LP isolated from WT mice reconstituted with WT mice BM (BM-$AhR^{+/+}$) or WT mice reconstituted with $AhR^{-/-}$ mice BM (BM- $AhR^{-/-}$) orally treated with mesalamine for 2 weeks (n = 5 mice per group). Representative plots (*left*) and quantitative data (*right*). Error bars represent mean ± SEM. *$P < .05$, **$P < .01$. 5-ASA, mesalamine.

proliferation of Tregs or by subclinical inflammation induced by the drug.

### *Treatment of Mice With Mesalamine for Inflammatory Bowel Disease Treatment for at Least 3 Consecutive Days Can Promote the Accumulation of Regulatory T Cells in the Colon*

We next examined the time- and dose-dependent effects of mesalamine on the colonic Treg accumulation in mice. Male 6-to-8-week-old WT mice treated with mesalamine for 3 days, but not 1 day, significantly increased the proportion of colon LP Tregs and *Foxp3* mRNA expression (and *Cyp1A1* mRNA expression; see later) in the colon (Figures 3*A*–*C*). In addition, treatment of mice with 5 mg/kg/day, but not 50 mg/kg/day, of mesalamine for 2 weeks did not increase the proportion of colon LP Tregs (Figure 3*D*). Collectively, these findings suggest that treatment of mice with mesalamine at a dose equivalent to its medical use for IBD treatment (50 mg/kg) for at least 3 consecutive days can promote the accumulation of Tregs in the colon.

### *Mesalamine Activates the Aryl Hydrocarbon Receptor Pathway, Which Is Necessary for Regulatory T Cell Induction*

To obtain mechanistic insight into how mesalamine induces colon LP Tregs, we examined mRNA levels of genes related to Treg differentiation/proliferation in the mouse colon following treatment with 50 mg/kg of mesalamine per day for 2 weeks (Table 1).

Among the genes examined, we observed an increased trend in mRNA levels of cytochrome P-450 family 1A1 (*Cyp1A1*), a major target gene of the aryl hydrocarbon receptor (*AhR*), in the colon, but not the small intestine, MLNs, and liver, of mice treated with mesalamine for 2 weeks (Figure 4*A*). Notably, we found a significant increase in mRNA levels of *Cyp1A1* and *Foxp3* in the colon LP $CD4^+$ T cells of mice treated with mesalamine for 2 weeks (Figure 4*B*). In addition, we found a significant increase in *Cyp1A1* mRNA expression levels in the colon of mice treated with 50 mg/kg of mesalamine for 3 days, but not for 1 day, (Figure 3*C*). Thus, the AhR pathway seemed to be activated by mesalamine in the colon, particularly in the colon LP $CD4^+$ T cells.

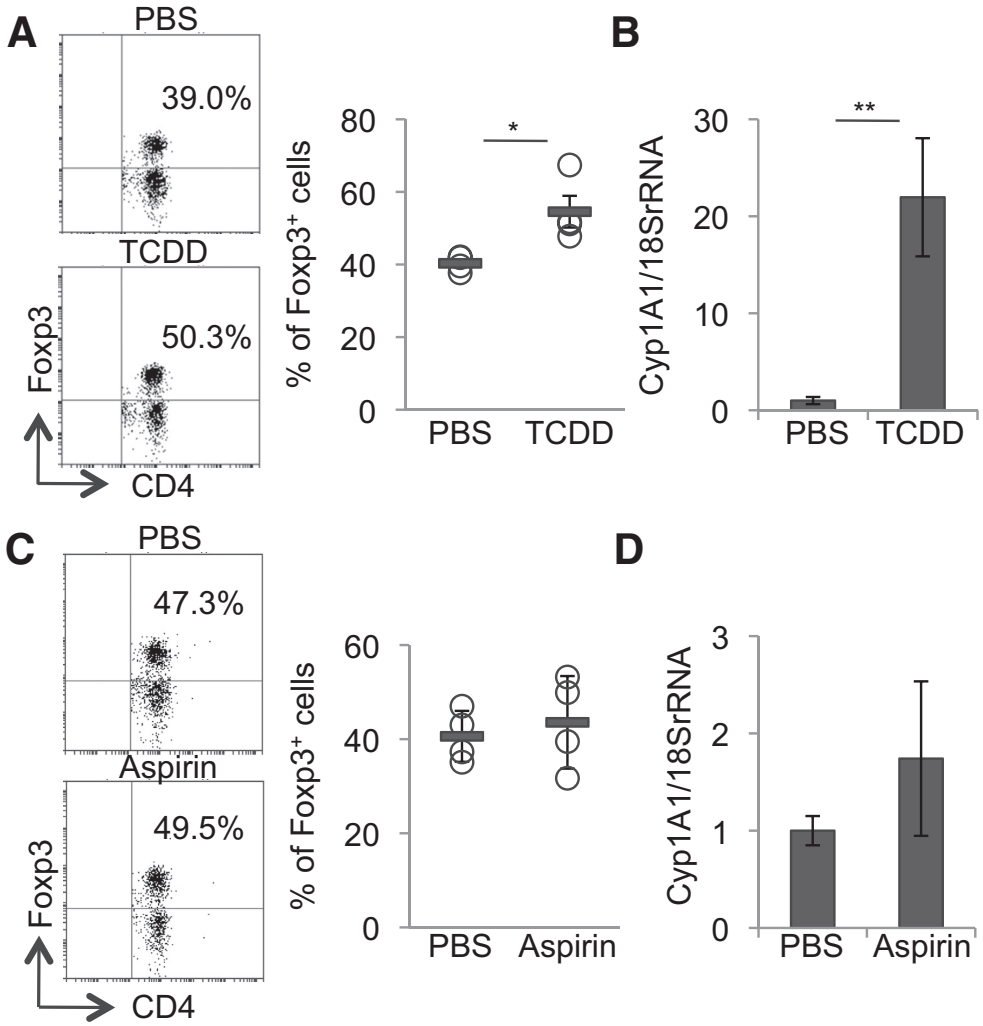



**Figure 5. TCDD, but not aspirin induces Tregs in the LP of the colon, concomitant with AhR activation.** (*A*, *B*) WT mice were orally treated once with 5 $\mu$g/kg of TCDD. Three days later, the percentages of $Foxp3^+$ cells within the $CD3^+CD4^+$ cell population isolated from the colon LP of mice were analyzed by flow cytometry. (*A*) Representative plots (*left*) and quantitative data (*right*). (*B*) Cyp1A1 mRNA levels in the colon. (*C*, *D*) WT mice were orally treated with aspirin added to drinking water for 2 weeks, the percentages of $Foxp3^+$ cells within the $CD3^+CD4^+$ cell population isolated from the colon LP of mice were analyzed by flow cytometry. (*C*) Representative plots (*left*) and quantitative data (*right*). (*D*) Cyp1A1 mRNA levels in the colon. Error bars represent mean ± SEM, n = 4 mice per group. *$P < .05$, **$P < .01$.

The AhR is a ligand-activated transcriptional factor that is ubiquitously expressed in vertebrate cells.[21,22] Upon a ligand binding, AhR translocates to the nucleus and initiates the transcription of the target genes with promoters containing an XRE consensus sequence. Using AhR reporter mice in which the XRE consensus sequence is fused to the serum alkaline phosphatase (SEAP) gene,[15] we detected activation of the AhR pathway after oral treatment with mesalamine for 3 days, although the AhR-activating capacity was much weaker than that of TCDD (dioxin), a strong synthetic AhR ligand (Figure 4*C*).

To determine whether activation of the AhR pathway by mesalamine is required for induction of Tregs in the colon LP, we examined the effects of AhR deficiency on Treg induction by mesalamine. Treatment of mice deficient for AhR ($AhR^{-/-}$ mice)[14] with mesalamine did not affect the proportion of Tregs in the colon LP or *Foxp3* mRNA expression in the colon (Figure 4*D* and *E*). In addition, we generated

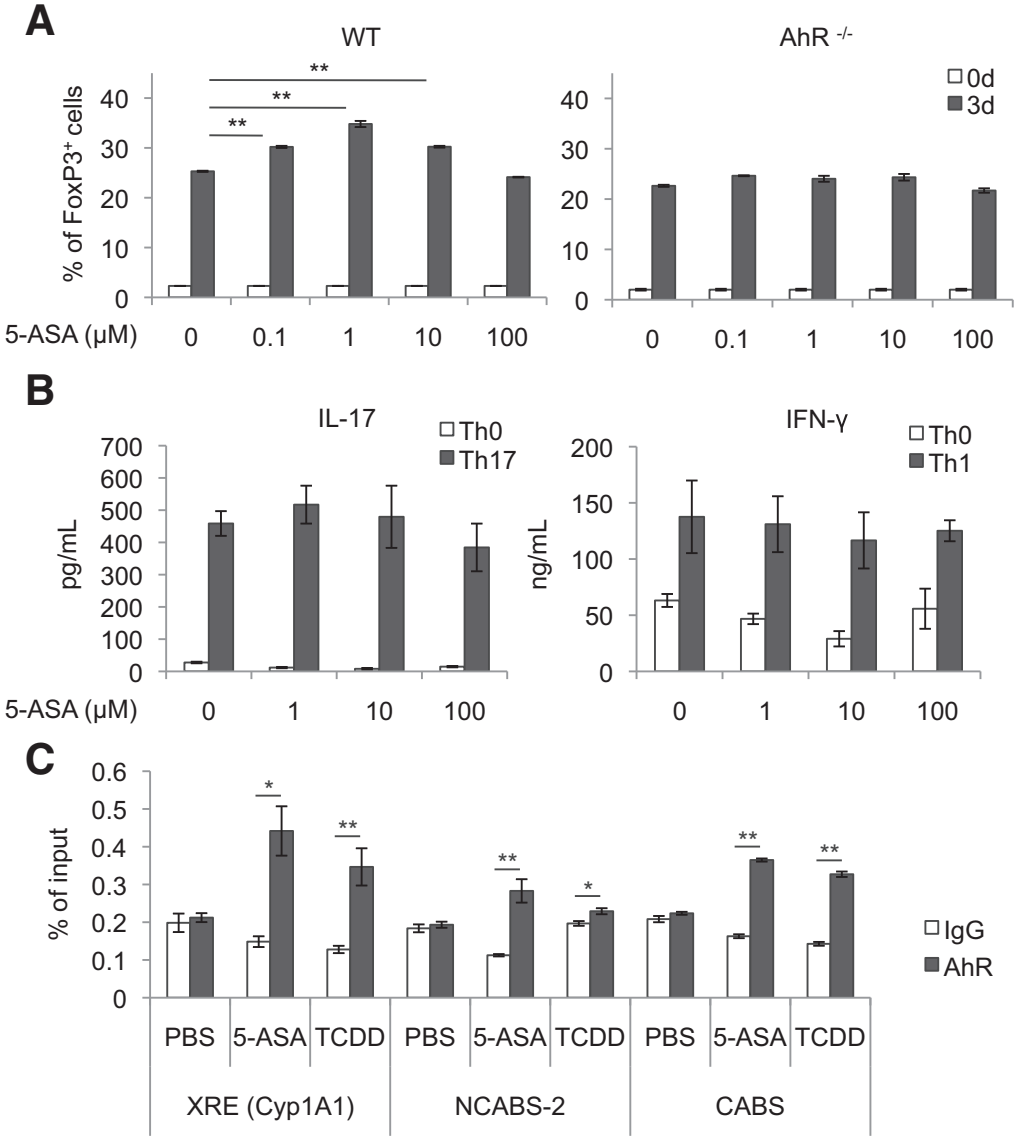
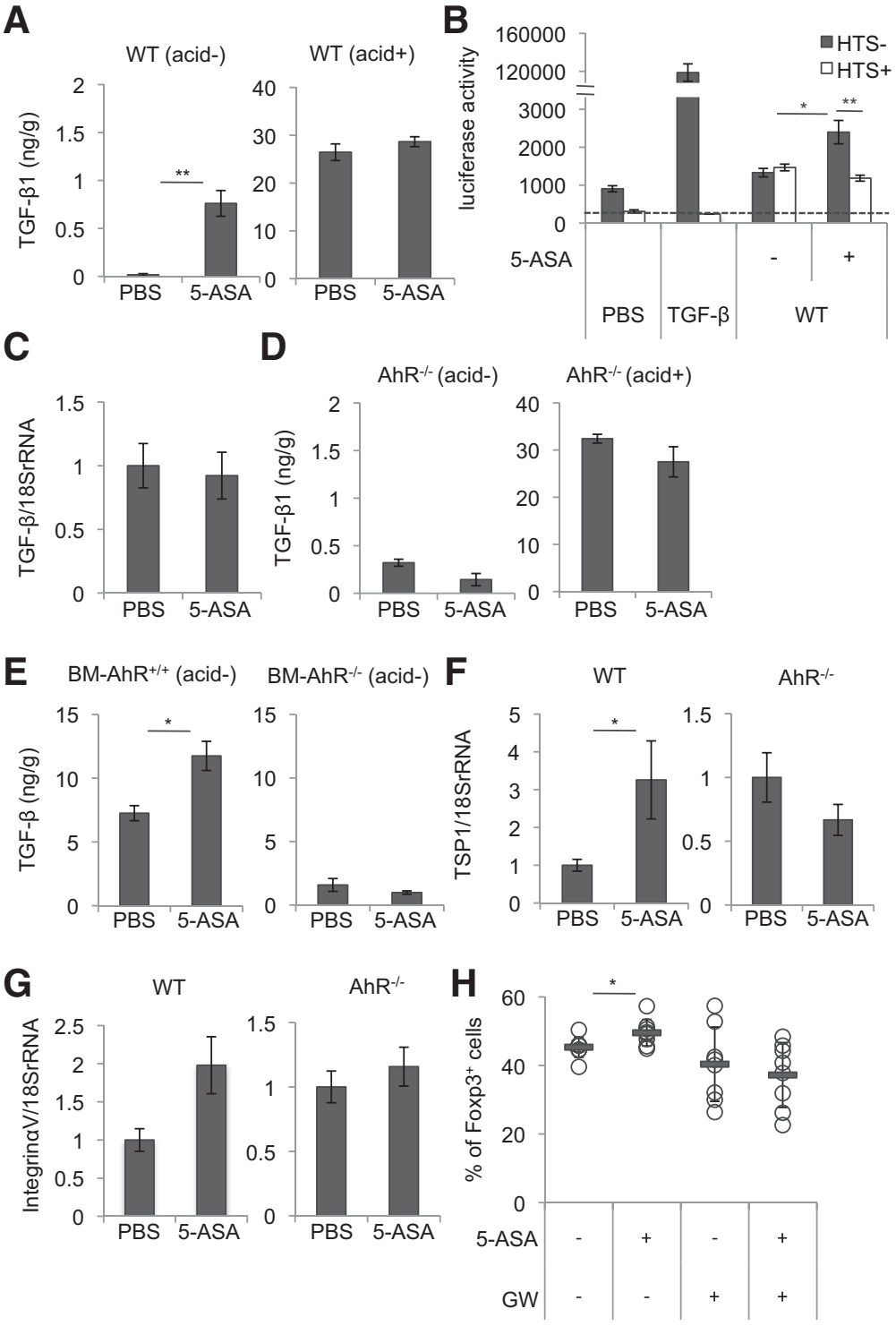



**Figure 6. Mesalamine promotes Treg differentiation, concomitant with AhR activation.** (*A*) Naive $CD4^+$ T cells isolated from the spleen of WT mice or $AhR^{-/-}$ mice were cultured in Treg differentiation conditions with the indicated concentrations of mesalamine for 3 days, and the percentages of $Foxp3^+$ cells within $CD4^+$ cells were analyzed by flow cytometry (n = 3). (*B*) Naive $CD4^+$ T cells isolated from the spleen in WT mice were stimulated in Th0, Th1, or Th17 differentiation conditions with the indicated concentrations of mesalamine for 3 days, and the supernatants of the cultures were collected. The concentrations of IFN-γ or IL17A were measured by ELISA (n = 2). (*C*) Chromatin immunoprecipitation assay analysis of the interaction of AHR with the nonconserved AhR binding site (NCABS) and conserved AhR binding site (CABS) in Foxp3 or XRE sequence of Cyp1A1 in naive $CD4^+$ T cells under Treg differentiation conditions supplemented with PBS, mesalamine (1 μM), or TCDD (1 nM) (n = 3). Error bars represent mean ± SEM. **P* < .05, ***P* < .01. Similar results were obtained from at least 3 independent experiments. 5-ASA, mesalamine.

BM-chimeric mice reconstituted with BM cells derived from *AhR*$^{-/-}$ mice (BM-*AhR*$^{-/-}$ mice) or from WT mice (BM-*AhR*$^{+/+}$ mice). We confirmed the engraftment of the donor cells in the chimeric experiments by analyzing *AhR* mRNA levels in the MLNs (Figure 4*F*). Treatment of BM-*AhR*$^{-/-}$ mice with mesalamine did not affect the frequencies of Tregs in the colon LP, in contrast to BM-*AhR*$^{+/+}$ mice (Figure 4*G*).

As previously described,[23] TCDD increased the proportion of Tregs in the colon LP in association with *Cyp1A1* mRNA induction in the colon (Figures 5*A* and *B*). By contrast, although mesalamine has a structure similar to that of acetylsalicylic acid (aspirin), treatment of mice with ~50 mg/kg of aspirin per day for 2 weeks did not affect the proportion of Tregs in the colon LP and failed to



induce *Cyp1A1* mRNA expression in the colon (Figures 5*C* and *D*).

Collectively, these findings suggest that mesalamine at a dose equivalent to its medical use for IBD treatment for at least 3 consecutive days can activate the AhR pathway in the colon, and that AhR expressed in hematopoietic cells is critical for the induction of colon LP Tregs by mesalamine.

## Mesalamine Promotes Regulatory T-Cell Differentiation In Vitro, Concomitant With Aryl Hydrocarbon Receptor Activation

To obtain mechanistic insight into how mesalamine affects Treg accumulation in the mouse colon, we examined the effects of mesalamine on the *in vitro* differentiation of naive $CD4^+$ T cells to Tregs. Under Treg differentiation conditions, mesalamine promoted differentiation from naive $CD4^+$ T cells to Tregs in a dose-dependent manner, but little effects were observed in naive $CD4^+$ T cells isolated from $AhR^{-/-}$ mice (Figure 6*A*). Mesalamine did not affect differentiation from naive $CD4^+$ T cells into Th1 and Th17 cells under the corresponding differentiation conditions (Figure 6*B*).

There are 4 XRE sequence elements (nonconserved AhR binding sites 1, 2, and 3, and conserved AhR binding site) in the promoter region of the mouse *Foxp3* gene.[23] Chromatin immunoprecipitation assay assays revealed that mesalamine, like TCDD,[24] induced AhR binding to the nonconserved AhR binding site 2 and conserved AhR binding site XRE in T cells under Treg differentiation conditions (Figure 6*C*). We also confirmed that mesalamine induced AhR binding to the XRE in the *Cyp1A1* promoter, similar to TCDD in T cells under Treg differentiation conditions (Figure 6*C*). Collectively, these findings suggest that mesalamine can directly promote Treg differentiation *in vitro*, concomitant with AhR activation.

## Mesalamine Increases the Level of the Active Form of TGF-β1, Which Is Necessary for Regulatory T Cell Induction

TGF-$\beta$ is a critical regulator of Treg differentiation. Therefore, to further investigate how mesalamine induces Tregs via AhR activation, we examined the effects of mesalamine on TGF-$\beta$1 expression and activity in the colon. TGF-$\beta$ is secreted by cells as an inactive precursor (the latent form) that must be activated by several factors including pH change, integrins, and proteases to exert its biologicaeffects.[25] Interestingly, mesalamine significantly increased the amount of the active form of TGF-$\beta$1, but not the total amount of TGF-$\beta$1 (measured after acid treatment of samples), in the colons of WT mice (Figure 7*A*). By using reporter cells (MFB-F11 cells) that can measure as little as 1 pg/mL of active TGF-$\beta$1 *in vitro*,[18] we confirmed that TGF-$\beta$1 activity significantly increased in the colons of mesalamine-treated mice, but not control mice (Figure 7*B*). *TGF-β1* mRNA expression did not increase in the colon following mesalamine treatment (Figure 7*C*). The increase in the amount of the active form of TGF-$\beta$1 in the colon following mesalamine treatment was not observed in $AhR^{-/-}$ or BM-$AhR^{-/-}$ mice, but was observed in BM-$AhR^{+/+}$ mice (Figures 7*D* and E). Consistent with the increase in the level of the active form of TGF-$\beta$1, we observed a significant increase and an increased trend in mRNA levels of thrombspondin-1 (TSP-1)[26] and integrin $\alpha$V,[27] genes related to the conversion of the latent form of TGF-$\beta$ to the active form, respectively, in the colons of mesalamine-treated WT mice, but not $AhR^{-/-}$ mice (Figures 7*F* and *G*).

Importantly, pharmacologic blockade of TGF-$\beta$ signaling in WT mice using GW788388, an inhibitor of TGF-$\beta$ type I receptor kinase,[17] suppressed induction of colonic LP Tregs by mesalamine (Figure 7*H*). These findings suggest that treatment of mice with mesalamine increased the active form of TGF-$\beta$1 in the colon, depending on AhR expressed in hematopoietic cells, which is required for induction of colon Tregs by mesalamine.

## Mesalamine Increases Fecal IgA Production

Tregs and TGF-$\beta$ contribute to IgA production in the mouse intestine.[28,29] If mesalamine indeed increases the numbers of Tregs and the active form of TGF-$\beta$1 in the colon, it should increase IgA production. Accordingly, we examined the production of IgA in the stools of mice treated with 50 mg/kg mesalamine per day for 2 weeks. The amount of IgA in stool samples was significantly higher in mesalamine-treated mice than in control mice and $AhR^{-/-}$ mice (Figure 8*A*). In addition, no increase in the level of stool IgA was observed in BM-$AhR^{-/-}$ mice, but an increase was observed in BM-$AhR^{+/+}$ mice (Figure 8*B*). These

**Figure 7.** (***See previous page***). **Mesalamine increases the level of the active form of TGF-β1, which is necessary for Treg induction.** (*A*) Levels of the active form of TGF-$\beta$1 (acid-) or total TGF-$\beta$1 (acid+) in colons isolated from WT mice orally treated with mesalamine for 2 weeks (n = 5 mice per group). (*B*) Colon tissue samples (1.6 mg) from WT mice orally treated with mesalamine for 2 weeks were added into the culture of TGF-$\beta$ reporter cells (MFB-F11) for 24 hours with or without HTS466284 (a chemical inhibitor of TGF-$\beta$ signaling); then, SEAP activities of the supernatants were measured. 10 ng/mL human TGF-$\beta$1 was used as a positive control (n = 5). (*C*) TGF-$\beta$1 mRNA levels in the colon isolated from WT mice orally treated with mesalamine for 2 weeks (n = 8 mice per group). (*D*) Levels of active TGF-$\beta$1 (acid-) or of total TGF-$\beta$1 (acid+) in the colon isolated from $AhR^{-/-}$ mice treated orally with mesalamine for 2 weeks (n = 4 mice per group). (*E*) Levels of active TGF-$\beta$1 (acid-) or of total TGF-$\beta$1 (acid+) in the colon isolated from bone marrow chimeric mice (BM-$AhR^{+/+}$ mice and BM-$AhR^{-/-}$ mice) treated orally with mesalamine for 2 weeks (n = 5 mice per group). (*F*, *G*) TSP-1 and Integrin $\alpha$V mRNA levels in the colon isolated from WT mice or $AhR^{-/-}$ mice orally treated with mesalamine for 2 weeks (n = 8 mice per group). (*H*) WT mice were orally treated with mesalamine with or without GW788388, an inhibitor of TGF-$\beta$ type I receptor kinase, in drinking water for 2 weeks, and the percentages of $Foxp3^+$ cells within the $CD3^+CD4^+$ cell population isolated from the colon LP were determined (n = 8 mice per group). Error bars represent mean ± SEM. **P* < .05, ***P* < .01. 5-ASA, mesalamine.

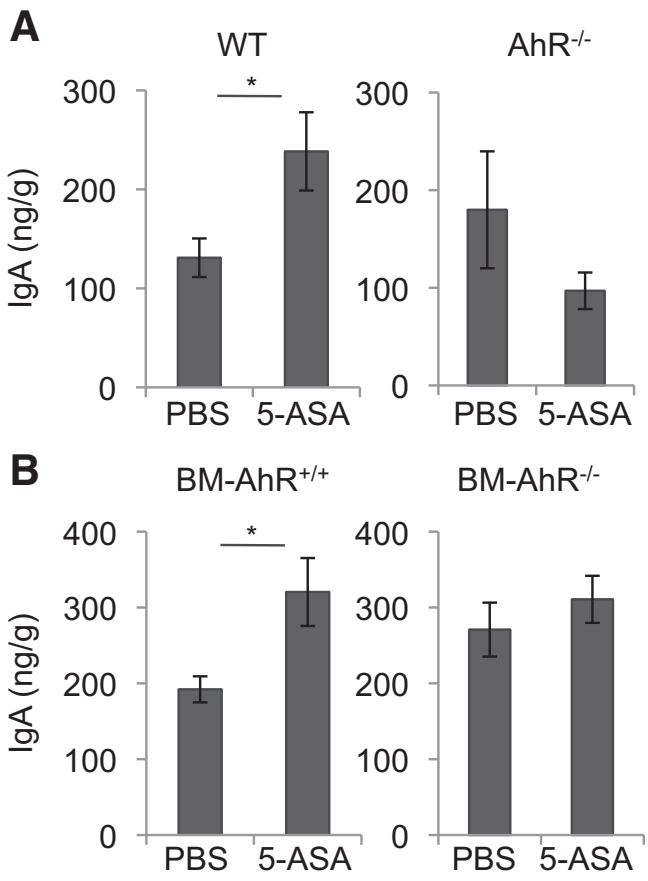



**Figure 8. Mesalamine increases fecal IgA production.** (*A*) WT (n = 6 mice per group) and $AhR^{-/-}$ mice (n = 4 mice per group), or (*B*) bone marrow chimeric mice (BM-$AhR^{+/+}$ mice and BM-$AhR^{-/-}$ mice) (n = 10 mice per group) were orally treated with 50 mg/kg of mesalamine for 2 weeks. Concentrations of IgA of stool samples were measured by ELISA. Error bars represent mean ± SEM. *$P < .05$. 5-ASA, mesalamine.

findings suggest that mesalamine can increase intestinal IgA production in an AhR-dependent manner, which may be associated with increased numbers of Tregs and the active form of TGF-$\beta$1 in the colon.

### *Mice Pretreated With Mesalamine Are Resistant to Colitis*

Colon Tregs induced by a defined mix of *Clostridium* strains confer resistance to DSS-induced colitis, a model of ulcerative colitis.[30] To evaluate the potential benefits of mesalamine-induced colon Tregs, we examined the effects of mesalamine, under colon Treg-inducing conditions, on DSS-induced colitis.

We treated mice orally with 50 mg/kg of mesalamine per day for 2 weeks (Days 1–14) and, in light of the ~5-hour biologic half-life of mesalamine,[31] 24 hours after the last mesalamine administration (Day 15) subjected the mice to DSS treatment to induce colitis (Figure 9*A*). Following the induction of DSS-induced colitis, mesalamine-treated mice exhibited less weight change, stool consistency and bleeding, colon shortening, histologic changes, and colonic expression of inflammatory markers (TNF-$\alpha$, IL1$\beta$, IL6, MPO, and HO-1) than control mice (Figures 9*B–F*). Similarly, $AhR^{-/-}$ mice were treated with 50 mg/kg of mesalamine per day for 2 weeks, and 24 hours later the mice were treated with DSS to induce colitis. However, although $AhR^{-/-}$ mice showed severe phenotypes of DSS-induced colitis compared with WT mice, $AhR^{-/-}$ mice treated with mesalamine exhibited weight changes, stool consistency and bleeding, colon shortening, and expression of inflammatory markers (TNF-$\alpha$, IL1$\beta$, IL6, MPO, and HO-1) comparable with those in $AhR^{-/-}$ mice treated with vehicle following DSS treatment (Figures 10*A–D*). Collectively, although additional mechanisms and cell types could be involved, these findings suggest that mesalamine-mediated induction of colon Tregs via AhR may be responsible for inhibition of DSS-induced colitis by mesalamine.

## Discussion

Mesalamine plays a well-established role in the management of IBD, but its anti-inflammatory mechanisms are not fully understood. The results of this study may reveal a previously unknown anti-inflammatory mechanism of mesalamine: induction of colon Tregs. Mesalamine induced activation of the AhR pathway and increased the level of the active form of TGF-$\beta$1 in the colon, which is critical for the accumulation of colon LP Tregs. In addition, pretreatment of mice with mesalamine inhibited DSS-induced colitis, concomitant with induction of Tregs. Given that mesalamine can induce *Cyp1A1* expression in colon LP $CD4^+$ T cells, AhR expressed in hematopoietic cells is required for the mesalamine-mediated induction of Tregs, mesalamine promotes Treg differentiation via AhR *in vitro*, and mesalamine does not increase the proportion of $Ki67^+$ Tregs in the colon, mesalamine may increase the accumulation of colon Tregs by directly promoting Treg differentiation, rather than proliferation.

Mesalamine promotes the accumulation of Tregs in the colon LP via AhR, without affecting the Th1 and Th17 subsets. AhR is expressed in Tregs and Th17 cells, but not in Th1 or Th2 cells.[24] In contrast to Tregs, we did not observe a significant increase in the proportion of colon Th17 cells following the treatment with mesalamine (Figure 2). Previous studies showed that AhR regulates both Treg and Th17 cell differentiation in a ligand-specific fashion.[24,32] Although TCDD and 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester are potent inducers of Treg differentiation and suppress EAE,[24] 6-formylindolo[3,2-b] carbazole enhances Th17 differentiation and increases severity of the disease.[24,32] These findings pointed out that AHR ligands of different origin and, most likely, different affinities differentially impact the outcome of Treg/Th17 cell differentiation. Thus, it will be an important issue to determine direct binding ability of mesalamine to AhR and, if any, its affinity to AhR for understanding how mesalamine preferentially induces Tregs.

Mesalamine is rapidly adsorbed, extensively acetylated to *N*-acetyl-mesalamine by the *N*-acetyltransferase 1

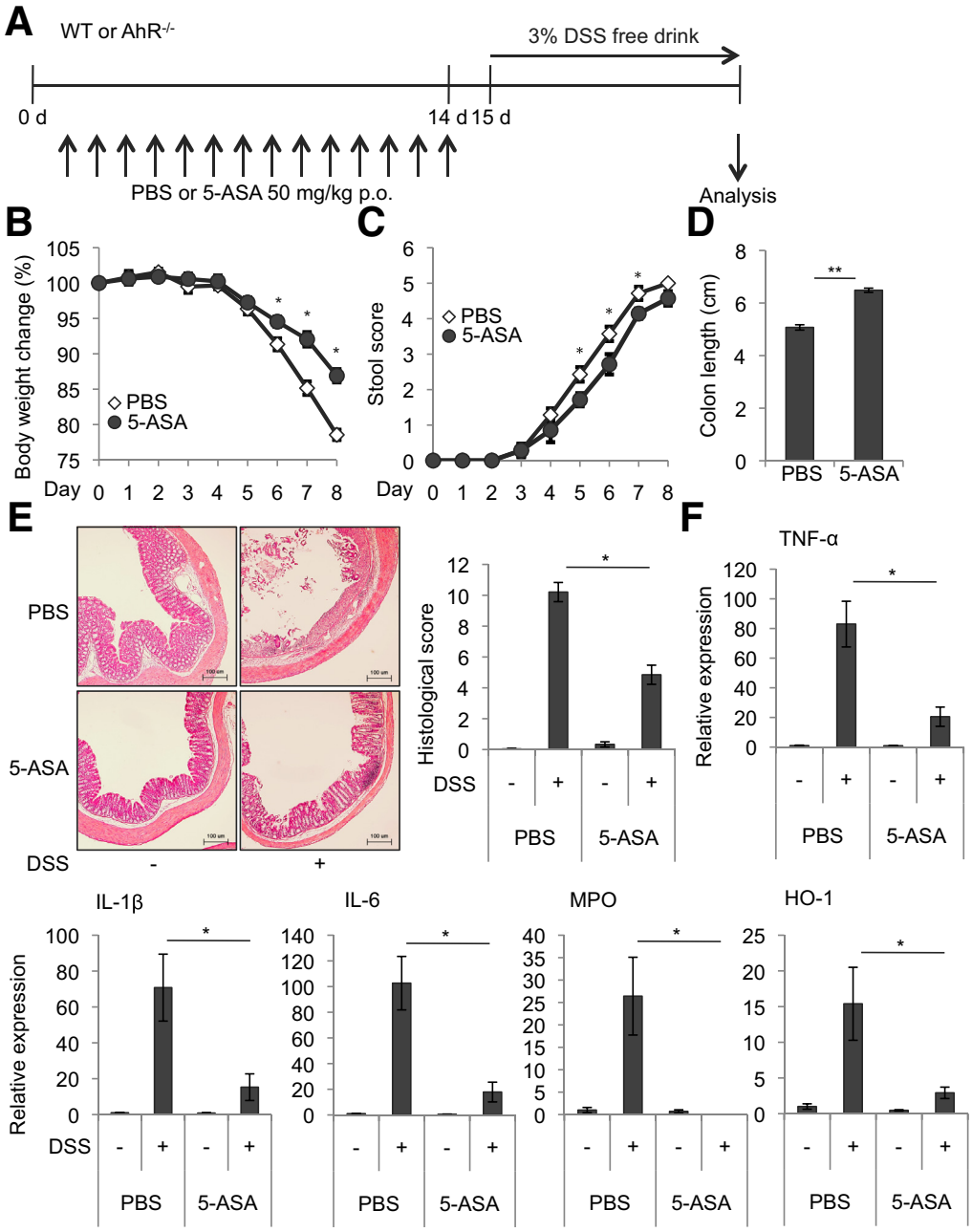



**Figure 9. Mice pretreated with mesalamine acquire the resistance to DSS-induced colitis.** (*A*) Experimental protocol. WT or AhR$^{-/-}$ mice (see Figure 10) were orally treated with mesalamine for 2 weeks; 24 hours after the last administration of mesalamine, the mice were treated with 3% DSS to induce colitis. (*B*) Body weight changes in mice after administration of DSS (n = 7 mice per group). (*C*) Stool score changes in mice after administration of DSS (n = 7 mice per group). (*D*) Colon length in mice after administration of DSS (n = 7 mice per group). (*E*) Colon histopathology. Representative pictures (*left*) and quantitative data (*right*). Scale bar indicates 100 $\mu$m (n = 3 per group). (F) TNF-$\alpha$, IL1$\beta$, IL6, MPO, and HO-1 mRNA levels in the colon after administration of DSS were analyzed by Q-PCR (n = 7 mice per group). Error bars represent mean ± SEM. *$P < .05$, **$P < .01$. 5-ASA, mesalamine.

enzyme in colonic mucosa, and supposed to lose its activity.[33] This metabolic pathway of mesalamine may explain the lack of its systemic effects and also a requirement for a high dose (50 mg/kg) of mesalamine on the colon Tregs accumulation. Given that 5 mg/kg of mesalamine did not affect colon Treg accumulation, we will determine precisely how much dose of mesalamine is a minimum required for the colon Treg induction in our future studies.

It remains to be determined how mesalamine promoted the accumulation of Tregs selectively in the colon LP, concomitant with induction of *Cyp1A1* mRNA in the colon, but not in the small intestine. We speculate that unique microenvironment of the large intestine might play a role in promoting colon Treg accumulation by mesalamine. Interactions between indigenous intestinal miroflora and the host play a critical role in the accumulation of colonic, but not small intestinal, LP Tregs.[7] The commensals stimulate the differentiation, migration, and proliferation of Tregs in the colon LP,[34–36] which may be independent on the MLNs.[37] In addition, colonic Tregs arise by means of commensal bacterial antigen-driven peripheral Treg development, thereby providing tolerance to commensal microbiota.[38] Thus, it is conceivable that colon LP is a unique site where Tregs are being constantly developed under the strong influence of commensal bacteria at steady state.[36,37] This unique Treg-developing microenvironment in the

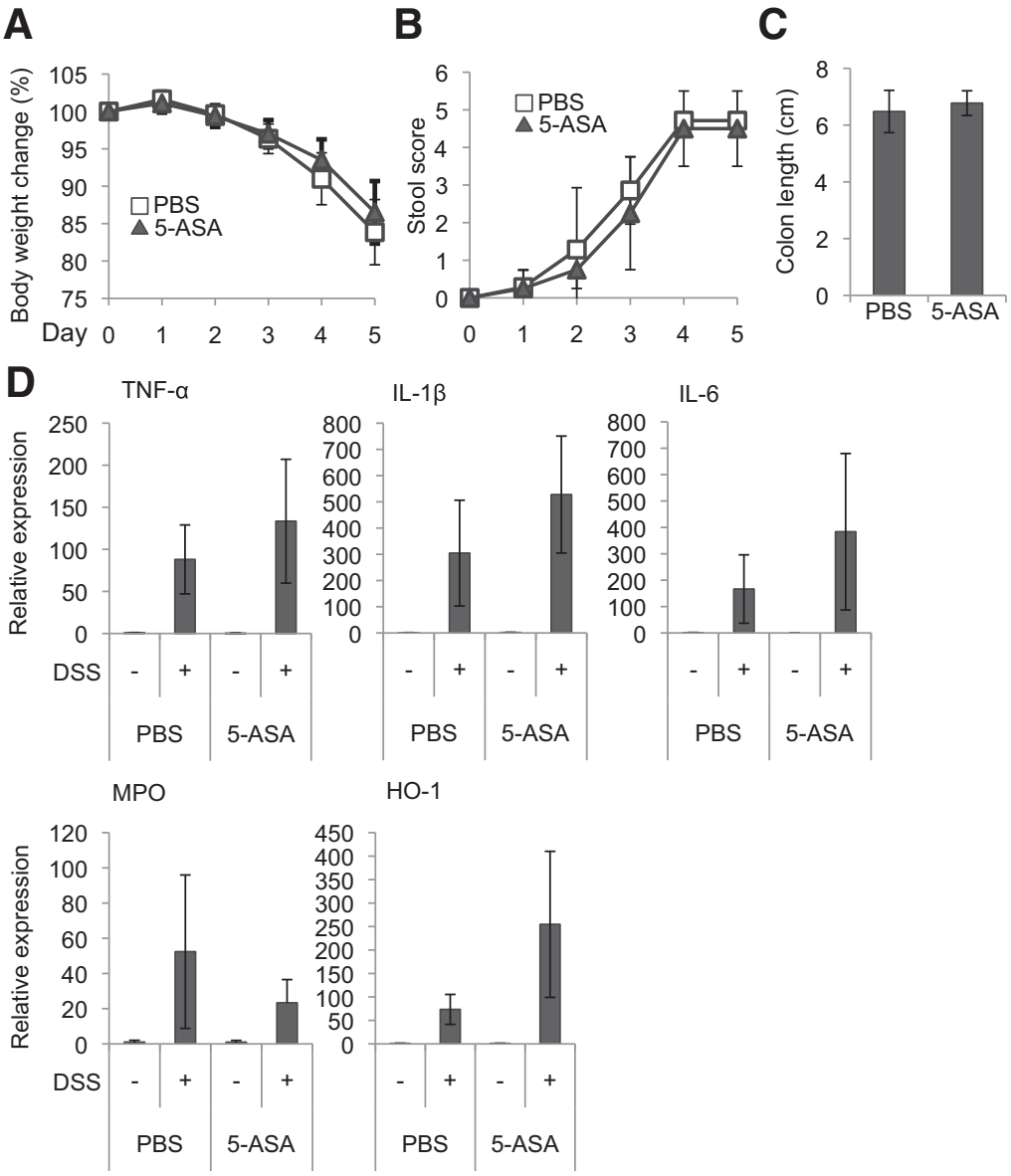



**Figure 10. AhR$^{-/-}$ mice pretreated with mesalamine do not acquire the resistance to DSS-induced colitis.** AhR$^{-/-}$ mice were orally treated with mesalamine for 2 weeks; 24 hours after the last administration of mesalamine, the mice were treated with 3% DSS to induce colitis. (*A*) Body weight changes in mice after administration of DSS (n = 5 mice per group). (*B*) Stool score changes in mice after administration of DSS (n = 5 mice per group). (*C*) Colon length in mice after administration of DSS (n = 5 per group). (*D*) TNF-$\alpha$, IL1$\beta$, IL6, MPO, and HO-1 mRNA levels in the colon after administration of DSS were analyzed by Q-PCR (n = 5 per group). Error bars represent mean ± SEM. 5-ASA, mesalamine.

colon, but not in the small intestine, might somehow facilitate the promotion of colon LP Treg accumulation in response to mesalamine.

Given that mesalamine-induced activation of AhR in hematopoietic cells is required for colon Treg induction and mesalamine can enhance Treg differentiation *in vitro*, concomitant with AhR activation under Treg differentiation conditions, we assume that mesalamine induces the accumulation of colon Tregs by directly promoting Treg differentiation in the colon. The findings that mesalamine can induce *Cyp1A1* expression in the colon LP CD4$^+$ T cells may also support this notion. However, it is also possible that mesalamine induces colon Tregs by acting on AhR expressed in dendritic cells and thereby promoting the development of tolerogenic dendritic cells that support Treg differentiation, as previously described.[39,40] Other possible mechanisms underlying mesalamine-induced colonic Treg accumulation independent of AhR are discussed later.

The precise mechanisms underlying the increase in the level of the active form of TGF-$\beta$1 in the colon by mesalamine via AhR remain to be determined. Given that mesalamine did not affect *TGF-$\beta$1* mRNA levels and total amounts of TGF-$\beta$1 protein, whereas mesalamine increased expression of TSP-1 and integrin $\alpha$V, genes related to conversion of the latent form of TGF-$\beta$1 to the active form, mesalamine might facilitate TGF-$\beta$1 activation in the colon via AhR–mediated control of those genes. Consistent with this notion, previous studies suggest that AhR initiates transcription of *TSP-1* via activation of the promoter[41] and AhR mediates integrin control of TGF-$\beta$ signaling[42]. Similarly, a defined mix of *Clostridium* strains induces colon Tregs, concomitant with increased expression of metalloproteinase 2, 9, and 13,[7]

which have been reported to be involved in the activation of TGF-$\beta$.[25]

We and others showed that activation of the AhR pathway can suppress rodent models of IBD.[43–46] The current findings suggest that the beneficial effect of mesalamine on IBD is linked to its potential to activate AhR in the colon. Because mesalamine is a first-line drug for IBD, our results provide additional strong support for the protective role of AhR against IBD and a rationale for targeting AhR for treatment of IBD. In support of this notion, Chinese herbal medicine Qing-Dai (also known as indigo naturalis), which has been used to treat patients with ulcerative colitis, is recently shown to contain indole compounds that act as AhR ligands.[47] Furthermore, we recently report that 1,4-dihydroxy-2-naphthoic acid, a precursor of menaquinone (vitamin $K_2$) abundantly produced by *Propionibacterium freudenreichii* ET-3 isolated from Swiss-type cheese, acts as an AhR activator and inhibits DSS-induced colitis.[48] Because 1,4-dihydroxy-2-naphthoic acid is commercially available in Japan as a prebiotic supplement without severe adverse effects, 1,4-dihydroxy-2-naphthoic acid might become a promising drug candidate for IBD via AhR activation.

Although we suggest that mesalamine induces colon LP Tregs via AhR, other changes in host responses induced by mesalamine could mediate the colon LP Treg induction independent of AhR. For instance, mesalamine increases PPAR-$\gamma$ expression and activates its target genes,[49] which can lead to the generation of Tregs.[50,51] In addition, salicylate or its derivative, such as acetylsalicylic acid (aspirin), induces AMP-activated protein kinase activation,[52] which can lead to the generation of Tregs.[53] Because mesalamine has a similar structure to salicylate or aspirin, this mechanism may be also involved in mesalamine-mediated colon Treg induction. Therefore, it is highly likely that mesalamine can induce Tregs through multiple mechanisms, including AhR. Because salicylate can also activate AhR,[54] these mechanisms could operate in ways that are not mutually exclusive.

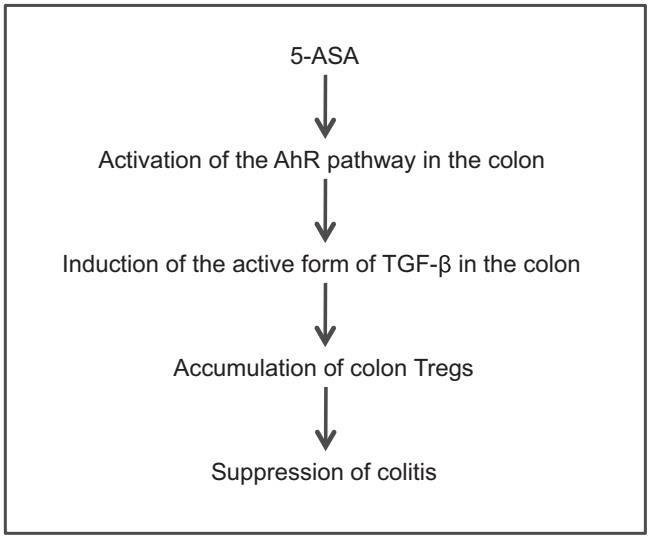



**Figure 11. Proposed model.** 5-ASA, mesalamine.

In summary, we propose a novel anti-inflammatory mechanism of mesalamine for colitis: induction of Tregs in the colon via the AhR pathway, followed by TGF-$\beta$ activation (Figure 11).

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**Author contributions**
Kyoko Oh-oka, Yuko Kojima, Koichiro Uchida, Kimiko Yoda, Shotaro Nakajima, and Jun Hemmi performed the experiments and analyzed the data. Hiroshi Kano, Yoshiaki Fujii-Kuriyama, Ryohei Katoh, and Hiroyuki Ito contributed the reagents. Kyoko Oh-oka, Hiroshi Kano, Hiroyuki Ito, and Atsuhito Nakao conceived the study, designed the experiments, analyzed the data, and wrote the manuscript.

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