Aryl Hydrocarbon Receptor-Induced Signals Up-regulate IL-22 Production and Inhibit Inflammation in the Gastrointestinal Tract

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BACKGROUND & AIMS: The pathogenesis of inflammatory bowel disease (IBD) is believed to involve an altered balance between effector and regulatory T cells. Aryl hydrocarbon receptor (AhR), a ligand-dependent transcription factor that mediates the toxicity of dioxins, controls T-cell responses. We investigated the role of AhR in inflammation and pathogenesis of IBD in humans and mouse models. **METHODS:** AhR expression was evaluated in intestinal tissue samples from patients with IBD and controls by real-time polymerase chain reaction (PCR) and flow cytometry. Intestinal lamina propria mononuclear cells (LPMCs) were activated in the presence or absence of the AhR agonist 6-formylindolo(3, 2-b)carbazole (Ficz). Colitis was induced in mice using trinitrobenzene sulfonic acid (TNBS), dextran sulfate sodium (DSS), or T-cell transfer. Mice were given injections of Ficz or the AhR antagonist 2-metyl-2H-pyrazole-3-carboxylic acid; some mice first received injections of a blocking antibody against interleukin (IL)-22. Cytokines were quantified by real-time PCR and flow cytometry. **RESULTS:** Intestine tissue from patients with IBD expressed significantly less AhR than controls. In LPMCs from patients with IBD, incubation with Ficz reduced levels of interferon gamma (IFN)-γ and up-regulated IL-22. Mice injected with Ficz were protected against TNBS-, DSS-, and T-cell transfer–induced colitis; they had marked down-regulation of inflammatory cytokines and induction of IL-22. Mice given AhR antagonist produced more inflammatory cytokines and less IL-22 and developed a severe colitis. Neutralization of endogenous IL-22 disrupted the protective effect of Ficz on TNBS-induced colitis. **CONCLUSIONS: AhR is down-regulated in intestinal tissue of patients with IBD; AhR signaling, via IL-22, inhibits inflammation and colitis in the gastrointestinal tract of mice. AhR-related compounds might be developed to treat patients with IBDs.**

Keywords: Crohn's disease; Colitis; T-helper cells; Immune Regulation.

The etiology of Crohn's disease (CD) and ulcerative colitis (UC), the major forms of inflammatory bowel diseases (IBD) in humans, remains unknown, but evidence suggests that IBD results from the interaction of genetic and environmental factors that ultimately promote an abnormal immune response leading to organ damage.[1]

Deregulation of various components of the immune system can be seen in the gut of patients with IBD, but hyperactivity of T cells with excessive production of cytokines is perhaps the major immunologic stigmata of these disorders.[1] Such an abnormal T-cell response is in part due to a defective activity of counterregulatory mechanisms and is directed against components of the luminal bacterial flora.[1,2] Consistently, strategies aimed at restoring the balance between inflammatory and anti-inflammatory factors have already been tested with success in both patients with IBD and experimental models of colitis.[3–6]

Aryl hydrocarbon receptor (AhR), a transcription factor ubiquitously expressed in vertebrate cells, mediates a range of cellular events in response to halogenated aromatic hydrocarbons and nonhalogenated polycyclic aromatic hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin.[7] Further ligands are small synthetic compounds and natural chemicals, including derivatives of tryptophan, such as 6-formylindolo(3, 2-b)carbazole (Ficz).[8,9] AhR is present in the cytosol in an inactive form, bound to several cochaperones.[7] After binding with ligands, AhR dissociates from the chaperones and translocates into the nucleus, where it binds to its dimerization partner AhR nuclear translocator, and the AhR/AhR nuclear translocator complex initiates transcription of genes with promoters containing a dioxin-responsive element consensus sequence.[7]

Pioneering studies in AhR-deficient mice have emphasized the role of AhR in the development and functions of various organs. AhR-deficient mice exhibit a spectrum of hepatic and skin defects as well as abnormalities in vascular and hematopoietic development.[10] More recent studies have shown that AhR controls specific immune responses. AhR is highly expressed by T-helper (Th)17 cells, and activation of AhR results in expansion of Th17

Abbreviations used in this paper: AhR, Aryl hydrocarbon receptor; DSS, dextran sulfate sodium; Ficz, 6-formylindolo(3, 2-b)carbazole; IFN, interferon; IL, interleukin; LPMC, lamina propria mononuclear cell; Th, T-helper; TNBS, trinitrobenzene sulfonic acid; TNF, tumor necrosis factor.

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cells and enhanced production of Th17 cytokines.[11–13] Consistently, studies in mice with experimental autoimmune encephalomyelitis, a Th17 cell–driven autoimmune inflammatory disease of the central nervous system that serves as a disease model for multiple sclerosis in humans, showed that AhR activation causes earlier onset of the disease and more severe pathology.[14] AhR also controls Th1/Th2 cell–associated immunity. Mice given a synthetic ligand of AhR, the compound M50367, exhibit reduced Th2 cell responses and enhanced production of interferon (IFN)-γ,[15] whereas AhR-deficient mice seem to have more Th1 and Th2 cells.[16] AhR activation in T cells can also regulate production of interleukin (IL)-22,[17,18] a cytokine that can exert either inflammatory or protective effects in various organs.[18–22] Therefore, depending on the cell context analyzed and type of agonist used, AhR-driven signals could differently modulate Th cell response and act as initiators or attenuators of tissue-damaging T cell–dependent inflammatory processes.

Because the tissue damage in IBD occurs in areas that are massively infiltrated with distinct subsets of Th cells,[23,24] we investigated whether AhR activation controls Th cell–derived cytokine production and pathogenic responses in the gut.

Materials and Methods

Mucosal Samples

Mucosal biopsy samples were obtained from involved colonic and ileum areas of 19 patients with active CD undergoing endoscopy (median age, 39 years; range, 28–59 years). In 5 of these 19 patients with CD, paired biopsy specimens were taken from both involved and uninvolved mucosal areas. Nine patients with CD were receiving corticosteroids, and the remaining patients were treated with mesalazine. Intestinal specimens, taken from 8 patients with moderate to severe CD undergoing intestinal resection for a severe disease poorly responsive to medical treatment, were used to isolate lamina propria mononuclear cells (LPMCs). Colonic mucosal samples were also taken from 16 patients with active UC who were undergoing endoscopy (median age, 37 years; range, 26–52 years). Four of these patients were receiving corticosteroids, and the remaining patients were treated with mesalazine. In 4 of these patients with UC, biopsy specimens were taken from both inflamed and uninflamed mucosa. Healthy controls included colonic mucosal biopsy samples from 17 patients with irritable bowel syndrome, as well as macroscopically and microscopically unaffected colonic specimens from 9 patients undergoing colonic resection for colon cancer (median age, 46 years; range, 35–69 years). Ethical approval was obtained from the local ethics committee.

Protein Extraction and Western Blotting Analysis

All reagents were from Sigma-Aldrich unless specified (Milan, Italy). Total proteins were extracted from biopsy specimens taken from 5 healthy controls, 6 patients with UC, and 6 patients with CD. Total proteins were also prepared from colonic specimens taken from control (ETOH-treated) and colitic (trinitrobenzene sulfonic acid [TNBS]-treated) mice treated or not with Ficz and killed at day 5. For the detection of AhR, proteins were separated on a 10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis gel. A commercially available monoclonal mouse anti-AhR antibody (1 μg/mL; Abcam, Cambridge, England) followed by horseradish peroxidase–conjugated rabbit anti-mouse immunoglobulin (Ig) G antibody (final dilution 1:10,000; Dako, Glostrup, Denmark) was used, and the reaction was detected with a Dura chemiluminescence kit (Pierce, Rockford, IL). After detection of AhR, blots were stripped and incubated with a mouse anti-human β-actin antibody (final dilution, 1:5000) followed by horseradish peroxidase–conjugated goat anti-mouse antibody (final dilution, 1:20,000; Dako). A computer-assisted scanning densitometry was used to analyze the intensity of the immunoreactive bands.

LPMC Isolation and Culture

LPMCs were isolated as described elsewhere[25] and used to characterize AhR or cultured in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 U/mL). Human cells were preincubated with either Ficz (final concentration, 2–200 nmol/L; Alexis, Milan, Italy) or dimethyl sulfoxide for 1 hour and then stimulated with activating CD3/CD28 antibody-coated beads (Miltenyi Biotec, Calderara di Reno, Italy) for 4 to 24 hours.

TNBS-Induced Colitis

TNBS in 50% ethanol was administered to 6- to 8-week-old female Balb/c mice as previously described.[26] Two or 3 mg of TNBS was used for studies with Aryl antagonist or Ficz, respectively. Controls consisted of mice treated with 50% ethanol. AhR antagonist (2-methyl-2H-pyrazole-3-carboxylic acid, CH223191; Calbiochem, Nottingham, England) was dissolved in phosphate-buffered saline and injected intraperitoneally (10 μg/mouse) 1 day before TNBS administration, whereas Ficz (1 μg/mouse) was injected intraperitoneally 1 day after TNBS administration. Ficz was dissolved in dimethyl sulfoxide at a final concentration of 0, 1 μg/μL, and 10 μL of this solution and was then mixed with 140 μL phosphate-buffered saline. Weight changes were recorded daily, mice were killed at day 5, and tissues were collected for histology, RNA analysis, protein analysis, and LPMC isolation. The colitis histologic score was assigned as described elsewhere.[26] In additional studies, mice were killed at different time points (day 0–4) and colonic tissue was used for epithelial cell and LPMC isolation as described elsewhere.[26] For assessing cytokine expression, mice were killed at different time points. To determine whether IL-22 mediates the therapeutic effect of Ficz, a neutralizing murine anti–IL-22 (100 μg/mouse; R&D Systems, Minneapolis, MN) or nonrelevant control antibody (100

μg/mouse) was administered intraperitoneally 2 hours before Ficz treatment. After 5 days, mice were killed and colonic tissues were collected.

Dextran Sulfate Sodium–Induced Colitis

Three cycles of 2.5% dextran sulfate sodium (DSS) were used to induce colitis. Six- to 8-week-old female Balb/c mice received either regular drinking water (control) or DSS in drinking water for 7 days and then allowed to recover by drinking water alone for an additional 7 days. Ficz (1 μg/mouse) was given intraperitoneally 1 day after starting the last DSS cycle. Weight changes were recorded daily, and the day after the last DSS administration, mice were killed and tissues were collected for histology, RNA and protein analysis, and LPMC isolation. Colonic sections were stained with H&E, and histology was scored as described elsewhere.[27]

Adoptive Transfer Colitis

$CD4^+CD25^-CD62L^+$ cells were isolated from the spleens of Bl/6 mice using the mouse naive T-Cell Isolation Kit according to the manufacturer's instructions (purity >98%; Miltenyi Biotec) and injected intraperitoneally to six 8-week-old female Bl/6 recombinase-activating gene 1 (RAG1)-deficient mice. To evaluate the effect of AhR activation on the ongoing colitis, mice were treated with Ficz (1 μg/mouse) or dimethyl sulfoxide 7 days after the appearance of clinical signs of colitis. Ficz was given intraperitoneally every other day, and mice were killed at week 6, 10 days after the first Ficz injection. Colonic sections were stained with H&E, and histology was scored as described elsewhere.[28]

RNA Extraction, Complementary DNA Preparation, and Real-Time Polymerase Chain Reaction

Please see Supplementary Materials and Methods.

Flow Cytometry

Please see Supplementary Materials and Methods.

Statistical Analysis

Differences between groups were compared using either the Mann–Whitney *U* test or Student *t* test.

Results

AhR Expression Is Down-regulated in CD Tissue

A significant decreased AhR RNA and protein expression was seen in patients with CD as compared with healthy controls and patients with UC (Figure 1*A* and *B*). In CD, no significant change in AhR expression was seen in samples taken from patients with ileal or colonic localization of the disease (Supplementary Figure 1). To examine if, in IBD, the expression of AhR correlates with the site of inflammation, AhR transcripts were evaluated in additional biopsy samples taken from inflamed and uninflamed areas of 5 patients with CD and 4 patients with UC. In CD, but not UC, AhR RNA expression was down-regulated in the inflamed mucosa (Figure 1*C*). Flow cytometry analysis of LPMCs confirmed that AhR expression is diminished in CD, particularly in $CD3^+$, $CD4^+$, $CD56^+$, and $CD25^+$ cells (Figure 1*C* and Supplementary Figure 2).

Because CD tissue is infiltrated with Th1/Th17 cells[23,24] and AhR has been involved in the control of Th cell responses,[29] we next determined the effect of AhR signaling on Th1/Th17 cytokine expression in CD and normal LPMCs. To this end, LPMCs were stimulated with activating CD3/CD28 antibody-coated beads in the presence or absence of increasing doses of the AhR ligand Ficz. In both CD and normal LPMCs, Ficz dose-dependently reduced IFN-γ and T-bet and augmented IL-22 RNA expression at 24 hours (Figure 1*D* and *E*), while analysis at early time points (ie, 4 hours) showed that Ficz inhibited IFN-γ and T-bet without affecting IL-22 (not shown). No significant change in AhR and IL-17A RNA was seen after Ficz treatment (Figure 1*D* and not shown).

Ficz Ameliorates TNBS-Induced Colitis

To explore whether AhR-driven signals attenuate experimental colitis, mice were given Ficz 1 day after TNBS administration. TNBS-treated mice exhibited significant weight loss from the first day of TNBS treatment (Figure 2*A*). At day 5, these mice had lost more than 10% of their initial body weight, and there was a 30% rate of mortality (Figure 2*A*). By contrast, TNBS-treated mice rapidly regained weight after Ficz treatment and manifested less mortality (10%) (Figure 2*A*). In addition, histologic examination of colonic tissues as well as blinded histologic scoring of colitis in the different groups showed that mice treated with Ficz developed a less severe colitis (Figure 2*B*; $P = .01$). The expression of IFN-γ, IL-17A, and tumor necrosis factor (TNF)-α was significantly increased in the colons of mice with TNBS-induced colitis in comparison with ethanol-treated mice (Figure 2*C*), while IL-22 RNA expression remained unchanged (Figure 2*C*). Mice treated with Ficz had reduced levels of IFN-γ, IL-17A, and TNF-α and increased expression of IL-22 (Figure 2*C*). Flow cytometry analysis of LPMCs confirmed that Ficz reduced the fractions of IFN-γ or IL-17A–positive cells and enhanced the percentage of IL-22–expressing cells (Figure 2*D*). Inhibition of IFN-γ by Ficz was evident as early as 2 days after induction of TNBS colitis, whereas up-regulation of IL-22 occurred later (ie, day 3) (Supplementary Figure 3). A significant down-regulation of AhR was seen in the colons of mice with TNBS-induced colitis as compared with controls or Ficz-treated mice (Figure 2*C* and Supplementary Figure 4*A*). Time-course studies showed that AhR was reduced in both epithelial cell and LPMC samples and that such a down-regulation occurred at day 3 and day 4 after induction of TNBS colitis (Supplementary Figure 5).

To confirm the anti-inflammatory role of AhR signaling in the gut, we next administered an AhR antagonist to mice with TNBS-induced colitis. In these experiments, a smaller amount of TNBS was used to induce a less intense

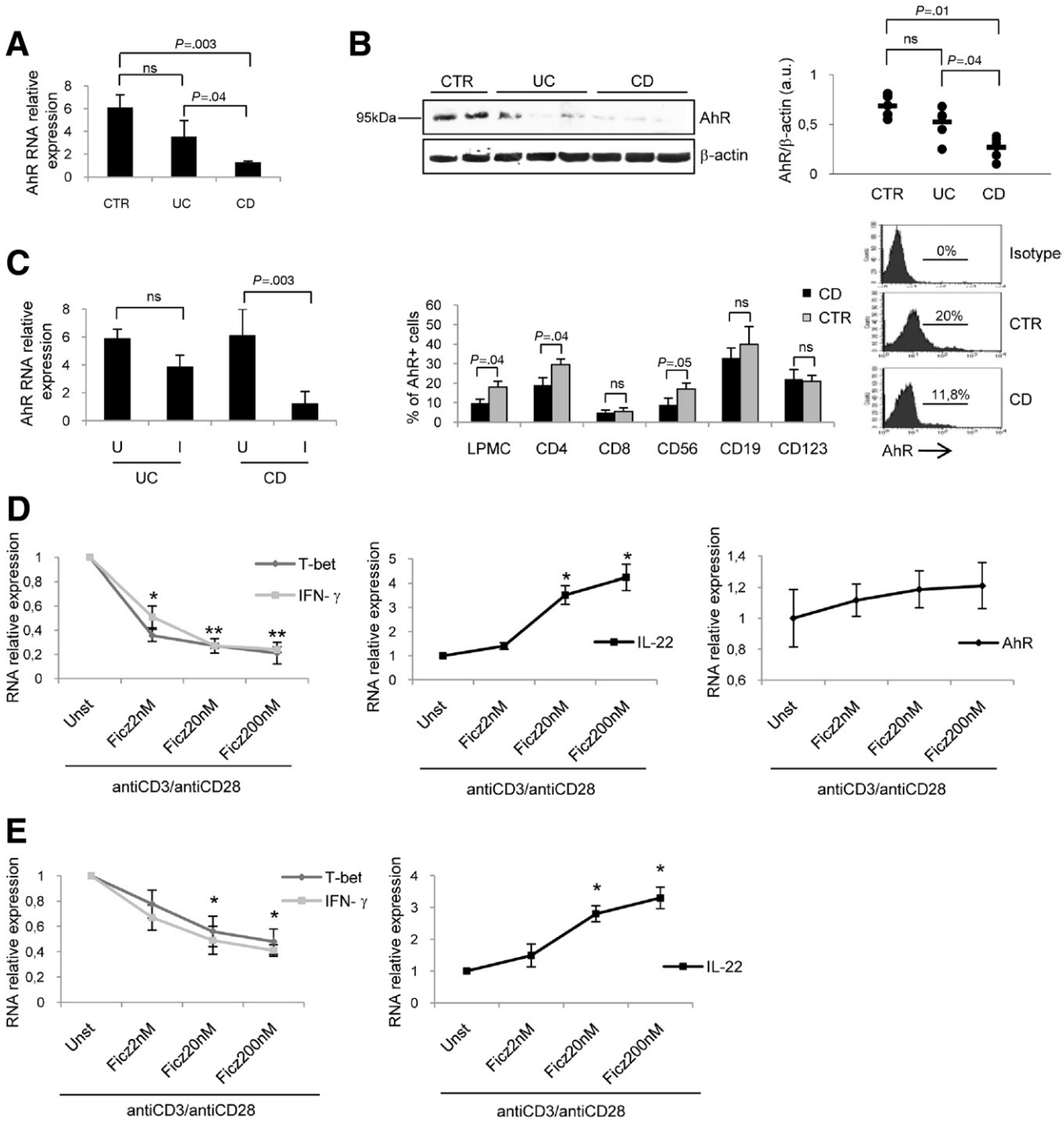

Figure 1. AhR expression is diminished in CD. (*A*) AhR RNA transcripts were evaluated in colonic mucosal samples taken from 6 healthy controls (CTR), inflamed areas of 10 patients with UC, and inflamed areas of 10 patients with CD by real-time polymerase chain reaction and normalized to β-actin. Data indicate mean ± SD of all experiments. (*B, left panel*) Representative Western blots showing AhR and β-actin in proteins extracted from biopsy specimens taken from 2 CTR, inflamed areas of 3 patients with UC, and inflamed areas of 3 patients with CD. One of 2 representative blots analyzing in total samples from 5 controls, 6 patients with UC, and 6 patients with CD is shown. (*B, right panel*) Quantitative analysis of AhR protein in mucosal samples from 5 CTR, 6 patients with UC, and 6 patients with CD, as measured by densitometry scanning of Western blots. Values are expressed in arbitrary units (a.u.). Each point represents the AhR/β-actin ratio in samples taken from a single subject. *Horizontal bars* indicate median values. (*C, left panel*) In CD, down-regulation of AhR occurs in mucosal areas with active inflammation. Transcripts for AhR were evaluated in samples taken from uninflamed (U) and inflamed (I) areas of 4 patients with UC and 5 patients with CD by real-time polymerase chain reaction. Data indicate mean ± SD of all experiments. (*C, right panel*) Flow cytometry analysis of AhR in LPMCs isolated from 5 CTR and 5 patients with CD. Data indicate mean ± SD of all experiments. *Right inset* shows representative histograms of AhR-expressing LPMCs isolated from one patient with CD and one CTR. Staining with an isotype control IgG is also shown. (*D*) Ficz reduces Th1-related markers and enhances IL-22 in CD LPMCs. Cells were incubated with increasing doses of Ficz or dimethyl sulfoxide for 1 hour and then stimulated with anti-CD3/anti-CD28 antibodies for 24 hours. IFN-γ, T-bet, IL-22, and AhR were examined by real-time polymerase chain reaction. Data indicate mean ± SD of 3 experiments in which LPMCs isolated from 3 patients with CD were used (*$P = .03$; **$P = .01$). (*E*) Ficz reduces Th1-related markers and enhances IL-22 in normal LPMCs. Colonic LPMCs were preincubated with increasing doses of Ficz or dimethyl sulfoxide for 1 hour and then stimulated with anti-CD3/anti-CD28 antibodies for 24 hours. IFN-γ, T-bet, and IL-22 were examined by real-time polymerase chain reaction. Data indicate mean ± SD of 4 experiments in which LPMCs isolated from 4 CTR were used (*$P = .03$).

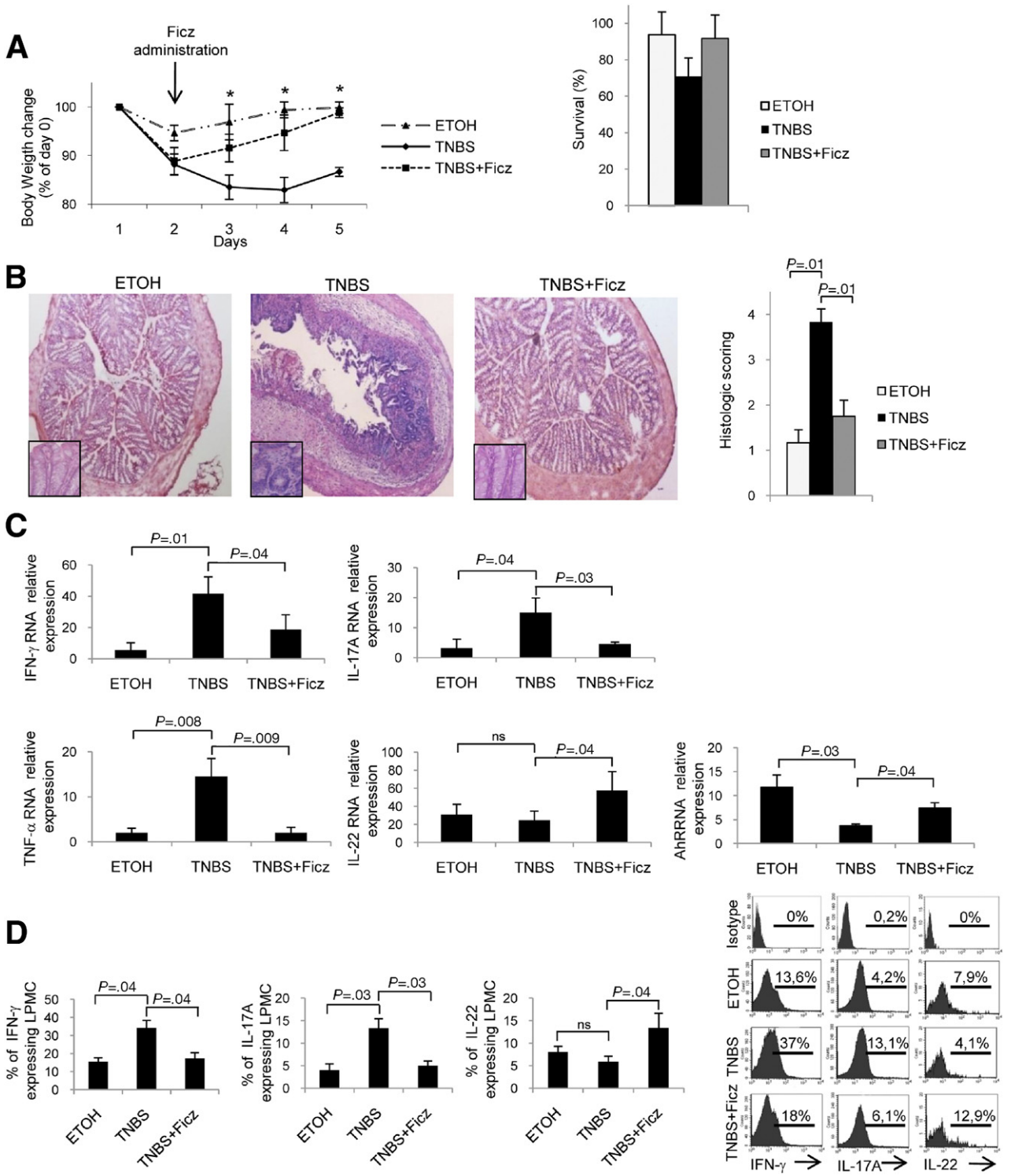

Figure 2. Ficz ameliorates TNBS-induced colitis in mice. (*A*) Ficz was intraperitoneally administered to mice 1 day after the induction of TNBS colitis. Changes in body weight were recorded daily. Data indicate the cumulative mean weight data ± SD of 5 separate experiments. In each experiment, each group consisted of at least 4 mice. TNBS vs TNBS + Ficz, **P* = .03. *Right inset* shows the percentages of survival of control (ETOH-treated) and colitic (TNBS-treated) mice treated or not with Ficz. Data indicate mean ± SD of all experiments. (*B*) Representative H&E-stained colonic sections of control (ETOH-treated) and colitic (TNBS-treated) mice treated or not with Ficz and killed at day 5. Original magnification 40×; *lower left panels*, 100×. *Right inset* shows the histologic score of the colonic sections taken from the 3 groups of mice. Data indicate mean ± SD of all experiments (n = 14 mice per group). (*C*) Transcripts for IFN-γ, IL-17A, TNF-α, IL-22, and AhR in colonic samples of mice treated as indicated in *A* and analyzed by real-time polymerase chain reaction. Data indicate mean ± SD of 5 experiments analyzing in total samples from 14 mice per group. (*D*) Percentages of cytokine-expressing LPMCs isolated from the colons of ETOH- or TNBS-treated mice treated or not with Ficz. Data indicate the mean ± SD of 5 experiments in which LPMCs isolated from 4 mice per group were pooled and analyzed for the intracellular content of IFN-γ, IL-17A, and IL-22 by flow cytometry. *Right insets* show representative histograms of IFN-γ, IL-17A, and IL-22–expressing LPMCs isolated from control (ETOH) and TNBS-treated colitic mice either left untreated or treated with Ficz and analyzed by flow cytometry. Staining with an isotype control IgG is also shown.

colitis. Body weight changes and histologic analysis of colonic sections showed that mice given the AhR antagonist developed a more severe colitis (Figure 3*A* and *B*), which was associated with enhanced expression of IFN-γ, IL-17A, and TNF-α and diminished synthesis of IL-22 (Figure 3*C* and *D*). Moreover, down-regulation of AhR was seen in the colons of mice treated with AhR antagonist as compared with Ficz-treated mice with TNBS-induced colitis (Figure 3*C*).

Ficz Reverses Relapsing DSS-Induced Colitis

In subsequent studies, we evaluated the therapeutic effect of Ficz on a relapsing colitis that was induced in mice by 3 cycles of DSS treatment. In this model, the mice lost weight during each cycle of DSS treatment but then regained the same weight before administration of DSS (Figure 4*A* and not shown). Because the mice receiving oral DSS had persistent, minimal inflammation during the recovery phase, this colitis can be considered as a colitis characterized by persistent inflammation with recurrent relapses of acute inflammation. Histologic examination of the colons obtained from mice at the end of the last cycle of DSS treatment revealed that mice had a marked inflammatory infiltrate of the mucosa with reduction in the number of goblet cells (Figure 4*B*). Interestingly, Ficz-treated mice manifested no significant weight loss during the DSS treatment and a minimal inflammatory infiltrate (Figure 4*A* and *B*). These histologic differences were corroborated by the determination of blinded colitis scores (Figure 4*B*, *right inset*). Administration of Ficz to mice with DSS-induced colitis led to a significant reduction in the expression of inflammatory cytokines and up-regulation of IL-22 (Figure 4*C* and *D*). A significant down-regulation of AhR transcripts and protein was seen in the colons of mice with DSS-induced colitis as compared with controls or Ficz-treated mice (Figure 4*C* and Supplementary Figure 4).

Ficz Suppress T Cell–Mediated Colitis

We next determined whether Ficz had anti-inflammatory effects in a T cell–dependent model of colitis induced by the adoptive transfer of naive T cells into Rag1-deficient mice. As expected, 4 weeks after T-cell reconstitution, mice manifested clinical signs of colitis and weight loss (Figure 5*A* and not shown). Ficz was administered 1 week after the appearance of signs of colitis. No further body weight loss was seen in colitic mice receiving Ficz, and at the end of the experiment the body weight in these mice was significantly greater than that measured in control mice (Figure 5*A*). Histologic examination of colons and histologic scoring of colitis confirmed that Ficz-treated mice had a less severe colitis (Figure 5*B*), and this was associated with diminished production of IFN-γ and IL-17A and up-regulation of IL-22 and AhR (Figure 5*C*).

The Therapeutic Effect of Ficz on TNBS-Induced Colitis Is Partly Dependent on IL-22

Because administration of Ficz to mice with colitis leads to up-regulation of IL-22, a cytokine that is supposed to exert protective effects in the gut,[19,20] we finally evaluated whether the therapeutic effect of AhR on experimental colitis is mediated by IL-22. Mice with TNBS-induced colitis treated with the anti–IL-22 antibody showed a worsening of colitis as compared with mice with TNBS-induced colitis treated with control IgG (Figure 6*A* and *B*). Figure 6*A* and *B* also show that blockade of endogenous IL-22 with the anti–IL-22 largely prevented the anti-inflammatory effect of Ficz on TNBS-induced colitis. Moreover, the anti–IL-22 significantly reduced the inhibitor effect of Ficz on IL-17A and TNF-α (Figure 6*C*). By contrast, in Ficz-treated mice, the anti–IL-22 slightly but not significantly enhanced the expression of IFN-γ (Figure 6*C*). Importantly, Ficz-treated mice given the anti–IL-22 produced significantly less IL-22 as compared with Ficz-treated mice receiving the control IgG (Figure 6*C*).

Discussion

Over the past decades, a considerable amount of data have been accumulated to suggest that CD results from an abnormal immune reaction within the intestinal mucosa that is directed against luminal bacterial antigens and is characterized by excessive production of Th1/Th17 cytokines.[1] Consistently, studies in experimental models of IBD have shown that inhibition of Th1/Th17 cell–driven inflammatory pathways are useful to attenuate tissue-damaging immune responses in the gut.[5,30] In this study, we examined the expression and functional role of AhR in IBD. We initially documented a diminished expression of AhR in the inflamed tissue of patients with CD as compared with uninvolved areas of the same patients and healthy controls. Importantly, the expression of AhR in the uninvolved tissue of patients with CD did not differ from that seen in the colons of controls. These observations, together with the demonstration that patients with CD taking mesalazine had the same AhR expression of those who were receiving corticosteroids (not shown), suggest that down-regulation of AhR in CD is not caused by current therapy. We do not yet know how AhR is regulated in the human gut, and mechanisms involved in the down-regulation of this transcription factor in CD remain to be ascertained. By contrast, we did not find a significant difference in terms of AhR expression between UC and controls. Nonetheless, it is noteworthy that AhR was markedly reduced in some UC samples. The limited number of UC biopsy specimens analyzed in this study does not help clarify in which subset of patients with UC AhR is down-regulated. Studies are now ongoing to clarify this issue.

In subsequent studies, we assessed whether activation of AhR by Ficz reduced inflammatory pathways in the gut. Treatment of CD LPMCs with Ficz for 24 hours led to a significant inhibition of Th1-related markers, which was

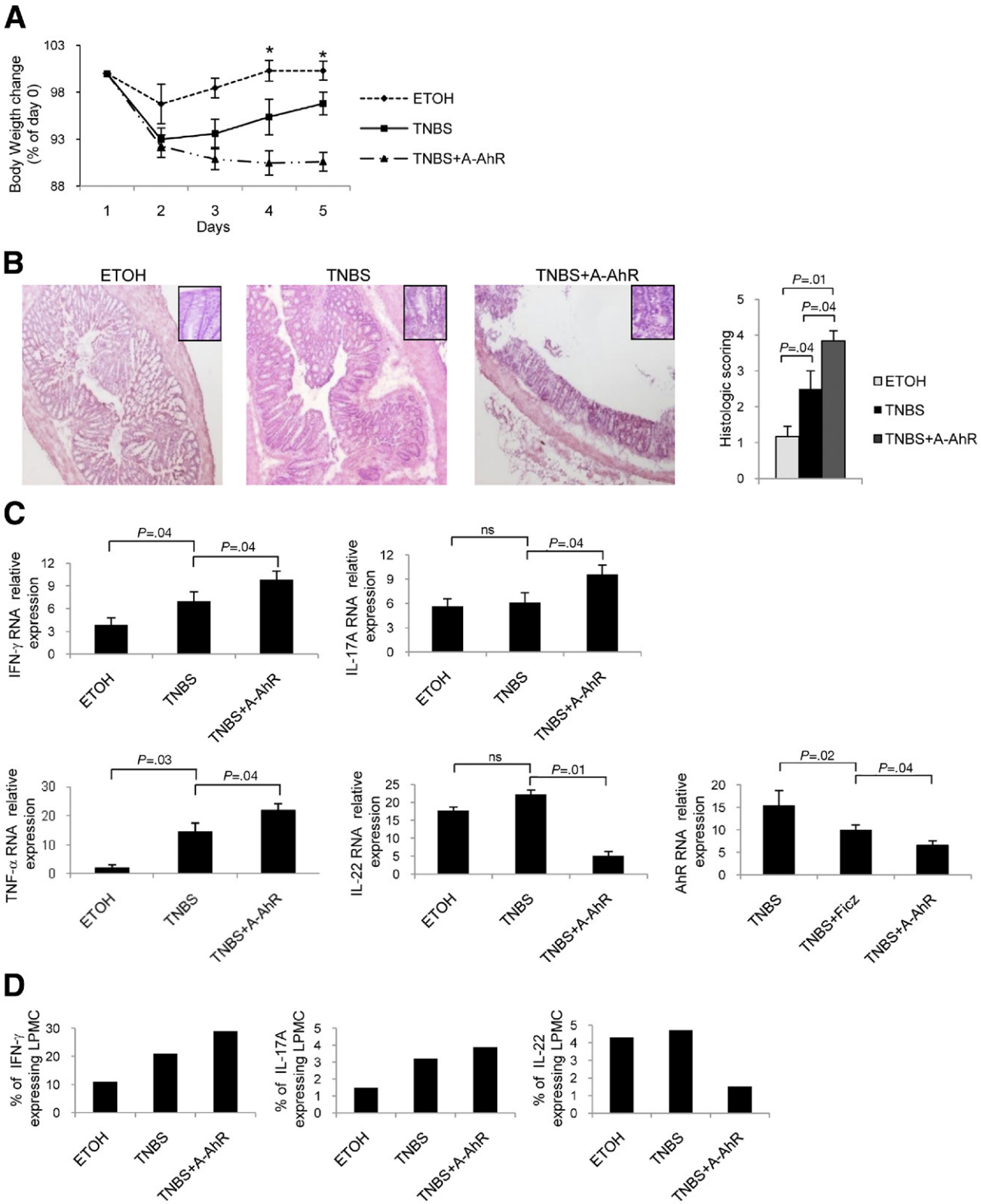

Figure 3. Mice injected with the AhR antagonist (A-AhR) develop a severe TNBS-induced colitis. (*A*) Mice were injected with A-AhR 1 day before the induction of TNBS colitis. Mice treated with ETOH were used as a control for TNBS-induced colitis. Data indicate the cumulative mean body weight data ± SD of 2 separate experiments. In each experiment, each group consisted of at least 4 mice (TNBS vs TNBS+A-AhR, *$P = .04$). (*B*) Representative H&E-stained colonic sections of ETOH- and TNBS-treated mice injected or not with A-AhR and killed at day 5. Original magnification 40×; *top right panels*, 100×. *Right inset* shows the histologic score of the colon sections taken from these mice. Data indicate the mean ± SD of 2 separate experiments in which at least 4 mice per group were considered. (*C*) Mice injected with A-AhR exhibit high levels of inflammatory cytokines and reduced production of IL-22 and AhR in the colon. Colonic samples taken from mice treated as indicated in *A* were analyzed for IFN-γ, IL-17A, TNF-α, IL-22, and AhR by real-time polymerase chain reaction and normalized to β-actin. Data indicate the mean ± SD of 2 separate experiments in which at least 4 mice per group were considered. (*D*) Percentages of cytokine-expressing LPMCs isolated from the colons of control mice (ETOH) and TNBS-treated colitic mice either treated or not with A-AhR. LPMCs isolated from 4 mice per group were pooled and analyzed by flow cytometry.

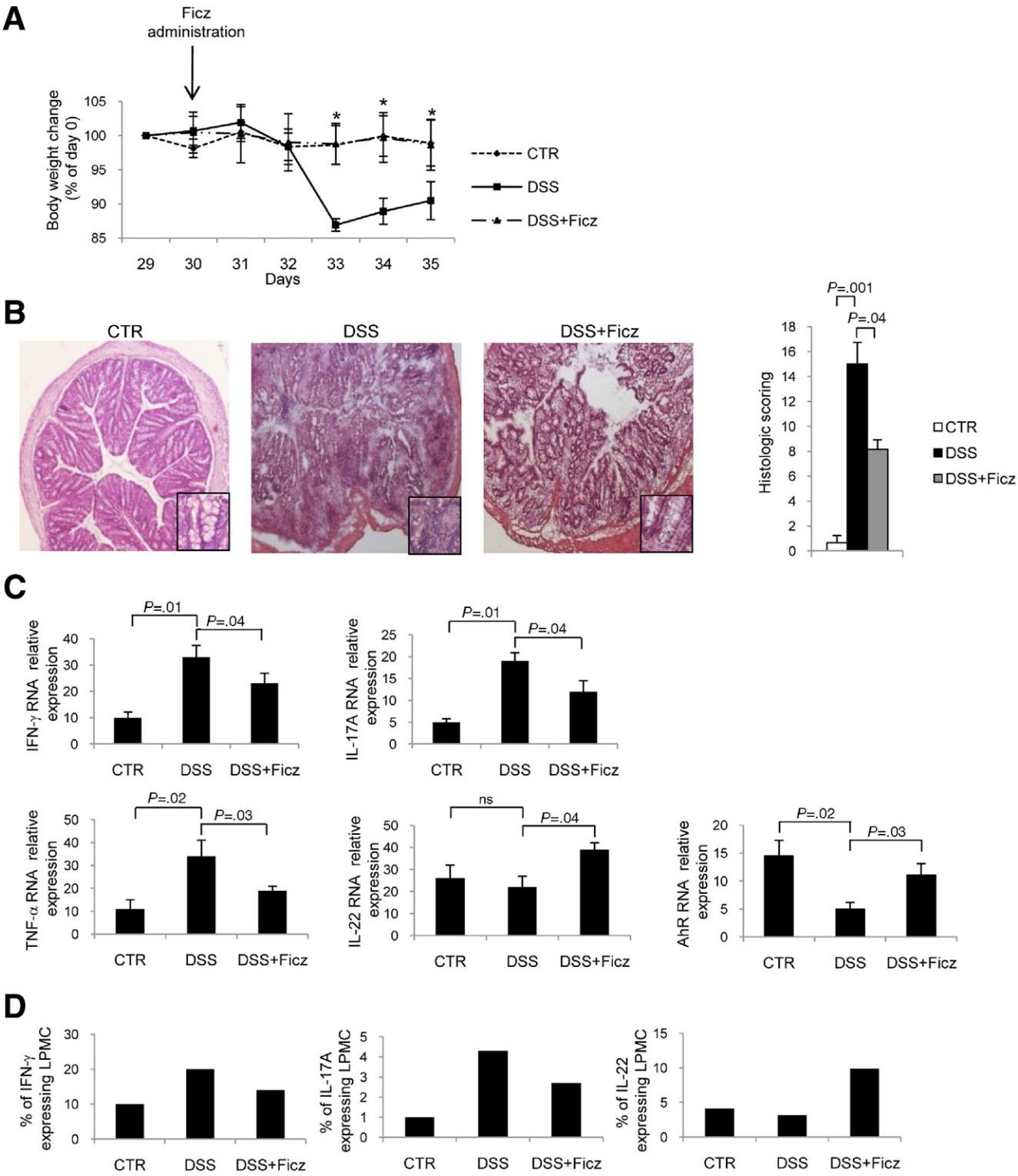

Figure 4. Ficz ameliorates relapsing DSS-induced colitis. (*A*) Three cycles of DSS were used to induce relapsing colitis. Ficz was given intraperitoneally 1 day after starting the last DSS cycle. Weight changes, recorded daily during the last DSS cycle in control mice (CTR), DSS-treated mice, and DSS-treated mice injected with Ficz, are shown. Data indicate mean ± SD (n = 5 mice/group) (DSS + Ficz vs DSS, **P* = .04). (*B*) Representative H&E-stained colonic sections of control mice (CTR), DSS-treated mice, and DSS-treated mice injected with Ficz. Original magnification 40×; *lower right panels*, 100×. *Right inset* shows the histologic scores of the colonic sections of such mice. Data indicate mean ± SD (n = 5 mice/group). (*C*) Ficz reduces inflammatory cytokines and enhances IL-22 in the colon of mice with DSS-induced colitis. Colonic samples taken from control mice (CTR), DSS-treated mice, and DSS-treated mice injected with Ficz were analyzed for IFN-γ, IL-17A, TNF-α, IL-22, and AhR by real-time polymerase chain reaction and normalized to β-actin. Data indicate mean ± SD (n = 5 mice/group). (*D*) Percentages of cytokine-expressing LPMCs isolated from the colons of control and DSS-treated mice either injected or not with Ficz. LPMCs isolated from 5 mice per group were pooled and analyzed by flow cytometry.

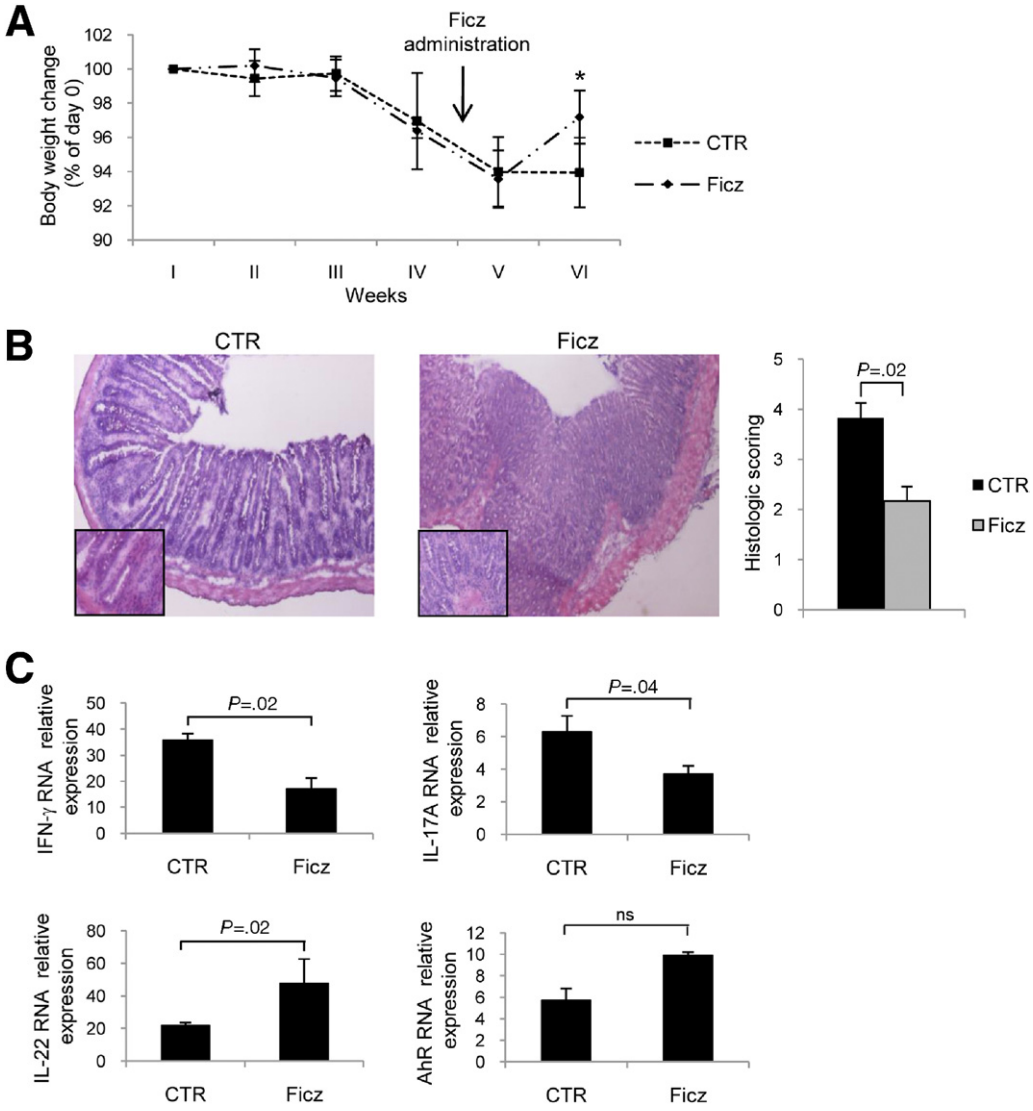

Figure 5. Therapeutic effect of Ficz in T-cell transfer colitis. Rag1-deficient mice were reconstituted with $CD4^+CD25^-CD62L^+$ cells isolated from the spleens of wild-type mice. At week 5, colitic mice were treated with dimethyl sulfoxide (CTR) or Ficz every other day for 1 week. Mice were killed 10 days after the first Ficz injection. *A* shows the body weight changes in reconstituted mice treated with CTR or Ficz. Data indicate mean ± SD (n = 6 mice/group; **P* = .03). (*B*) Representative H&E-stained and histologic scores of the colonic sections of reconstituted mice treated as indicated in *A* and killed 10 days after the first Ficz injection. Original magnification 40×; *lower left panels*, 100×. Data indicate mean ± SD (n = 6 mice/group). (*C*) Colonic samples taken from reconstituted mice treated with dimethyl sulfoxide or Ficz were analyzed for IFN-γ, IL-17A, IL-22, and AhR by real-time polymerase chain reaction and normalized to β-actin. Data indicate mean ± SD (n = 6 mice/group).

paralleled by induction of IL-22. By contrast, in CD LPMCs stimulated with Ficz for 4 hours, there was inhibition of Th1-related markers but no change in IL-22. These findings, together with the demonstration that IL-22 does not directly act on IFN-γ–producing cell types,[22] would seem to indicate that inhibition of Th1 factors and induction of IL-22 in Ficz-treated cells are 2 distinct and unrelated phenomena.

AhR is highly expressed in polarized murine and human Th17 cells, and activation of AhR during Th17 cell development increases the proportion of Th17 cells.[11,12] However, AhR-deficient $CD4^+$ T cells can be induced to differentiate along the Th17 pathway, suggesting that AhR is probably involved in the expansion, rather than induction, of Th17 cells.[11,13] We were not able to see significant changes in IL-17A expression after stimulation of CD LPMCs with Ficz. The reason for this apparent discrepancy remains unknown. A possibility is that, in our cell system, AhR-driven signals did not affect IL-17A expression, because in CD IL-17A is mostly produced by not typical Th17 cells (ie, T cells coexpressing IFN-γ and $CD68^+$ cells).[24,31] In this context, it is however noteworthy that our results are in agreement with data of recent studies showing that stimulation of AhR is crucial to induce, even in differentiated T cells, production of IL-22 without affecting IL-17A.[17,32]

Using well-established models of experimentally induced colitis, we then confirmed the importance of AhR-driven signals in the negative control of pathogenic Th cell responses. Administration of Ficz to mice amelio-

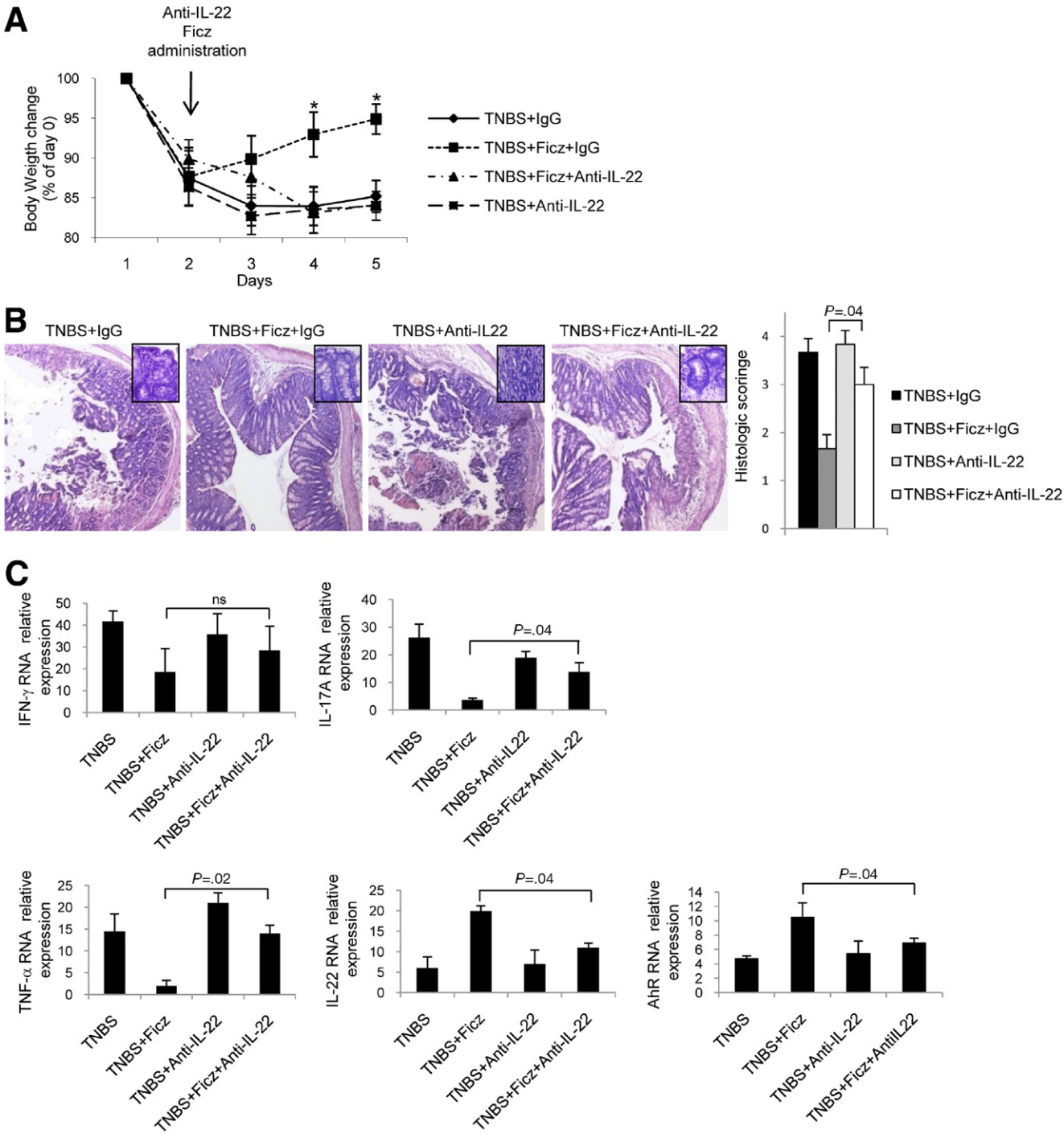

Figure 6. Administration of a blocking IL-22 antibody partially prevents the therapeutic effect of Ficz in mice with TNBS-induced colitis. (*A*) The day after the induction of TNBS colitis, mice were intraperitoneally injected with an anti–IL-22 or control IgG followed 2 hours later by Ficz. Data indicate mean ± SD (n = 5 mice/group) (TNBS + Ficz vs all the other groups, *P = .04). (*B*) Representative H&E-stained colonic sections of mice of the 4 groups. Original magnification 40×; *top right panels*, 100×. *Right inset* shows the histologic score of the colonic sections, and data are expressed as mean ± SD (n = 5 mice/group). (*C*) Blockade of endogenous IL-22 with the anti–IL-22 antibody partly prevents the regulatory effect of Ficz on cytokine expression in mice with TNBS-induced colitis. Mice were treated as indicated in *A* and killed at day 5. Colonic samples were analyzed for IFN-γ, IL-17A, TNF-α, IL-22, and AhR by real-time polymerase chain reaction and normalized to β-actin. Data indicate mean ± SD (n = 5 mice/group).

rated TNBS-, relapsing DSS-, and T-cell transfer–induced colitis. Moreover, treatment of mice with an AhR antagonist enhanced the severity of TNBS-induced colitis. While our study was ongoing, Takamura et al showed that 2,3,7,8-tetrachlorodibenzo-p-dioxin, a chemical ligand of AhR, ameliorated acute DSS-induced colitis by activating a mechanism that seems to be dependent on prostaglandin E_2.[33]

The therapeutic effect of Ficz seen in our models of colitis was paralleled by a marked inhibition of Th1 mol-

ecules and up-regulation of IL-22. Time-course studies performed in mice with TNBS-induced colitis showed that down-regulation of IFN-γ and T-bet following Ficz treatment preceded the induction of IL-22. Induction of IL-22 appeared, however, to be crucial in the anti-inflammatory action of Ficz. Indeed, administration of a neutralizing anti–IL-22 antibody to mice largely prevented the anti-inflammatory effects of Ficz. These findings well fit with previously published data showing that IL-22 promotes intestinal epithelial integrity, mucous secretion, and defensin production, thus reducing gut inflammation.[19,20] Interestingly, the expression of IFN-γ in the colons of Ficz-treated mice with TNBS-induced colitis did not differ from that seen in Ficz-treated mice injected with anti–IL-22. These data suggest that activation of AhR can regulate the ongoing mucosal inflammation by both directly inhibiting Th1 cell responses and up-regulating IL-22. Because AhR is relatively ubiquitous, we cannot however exclude the possibility that the anti-inflammatory effect we have seen in colitic mice treated with Ficz is also partly due to a control of the function of other mucosal cell types (eg, epithelial cells).

In contrast to the human data, administration of Ficz to mice with experimental colitis markedly reduced the synthesis of IL-17A. Although we cannot exclude the possibility that murine and human LPMCs respond differently to AhR activation in terms of IL-17A production, it is conceivable that the reduced synthesis of IL-17A seen in vivo in mice following Ficz treatment is secondary to the IL-22–mediated resolution of colitis. Indeed, the colons of mice with TNBS-induced colitis and treated with Ficz exhibited high levels of IL-17A after anti–IL-22 administration.

In conclusion, our findings delineate a novel mechanism by which production of inflammatory cytokines and pathology can be inhibited in the gut. The demonstration that Ficz ameliorates experimental colitis in mice indicates that AhR-related compounds may have a possible benefit in the treatment of CD.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.04.007.

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Reprint requests

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

RNA Extraction, Complementary DNA Preparation, and Real-Time Polymerase Chain Reaction

RNA was extracted by using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). A constant amount of RNA (1 μg per sample) was reverse transcribed into complementary DNA, and this was amplified using the following conditions: denaturation for 1 minute at 95°C; annealing for 30 seconds at 61°C for human IL-17A, 58°C for human IFN-γ, mouse TNF-α, mouse IFN-γ, and mouse IL-17A, and 60°C for human and mouse β-actin; and followed by 30 seconds of extension at 72°C. Primer sequences were as follows: human IL-17A, forward 5′-ACT ACA ACC GAT CCA CCTCAC-3′, reverse 5′-ACT TTG CCT CCC AGA TCACAG-3′; human IFN-γ, forward 5′-TGGAGACCATCAAGGAAGAC-3′, reverse 5′-GCGTTGGACATTCAAGTCAG-3′; mouse IL-17A, forward 5′-TCAGACTACCTCAACCGTTC-3′, reverse 5′-TTCAGGACCAGGATCTCTTG-3′; mouse IFN-γ, forward 5′-CAATGAACGCTACACACTGC-3′, reverse 3′-GAGTTCACCGTATCTACACC-5′; mouse TNF-α, forward 5′-ACCCTCACACTCAGATCATC-3′, reverse 5′-GAGTAGACAAGGTACAACCC-3′. T-bet and IL-22 were evaluated using commercially available TaqMan probes (Applied Biosystems, Foster City, CA). β-actin (forward 5′-AAGATGACCCAGATCATGTTTGAGACC-′3, reverse 5′-AGCCAGTCCAGACGCAGGAT-′3) was used as a housekeeping gene. Gene expression was calculated using the ΔΔCt algorithm.

Flow Cytometry

Human LPMCs were stained with the following antibodies: anti–CD4-PerCP (1:50, final dilution; BD Biosciences, San Jose, CA), anti–CD8-APC (1:50, final dilution; BD Biosciences), anti–CD56-PE (1:50, final dilution; BD Biosciences), anti–CD19-PE (1:50, final dilution; BD Biosciences), anti–CD123-APC (1:50, final dilution; eBioscience, San Diego, CA), anti–HLA-DR-PE (1:50, final dilution; BD Biosciences), anti–CD3-PerCP (1:50, final dilution; BD Biosciences), anti–CD25-PE (1:50, final dilution; BD Biosciences), and anti–161-APC (1:50, final dilution; BD Biosciences). To assess the intracellular expression of FoxP-3 and AhR, cells were fixed with 1% formaldehyde for 20 minutes and subsequently permeabilized with 0.5% saponin in 1% bovine serum albumin fluorescence-activated cell sorter buffer and stained intracellularly with anti–FoxP3-APC (1:50, final dilution; eBioscience) and anti-AhR (1:50, final dilution; Abcam).

Murine LPMCs were resuspended in RPMI 1640 medium; supplemented with 10% inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL); seeded in 96-well U-bottom culture dishes; and stimulated with phorbol myristate acetate (10 ng/mL), ionomycin (1 μg/mL), and brefeldin A (10 μg/mL; eBioscience). After 5 hours, cells were stained with the following antibodies: anti–IFN-PE (1:50, final dilution; BD Biosciences), anti–IL-17A/allophycocyanin (1:50, final dilution; eBioscience), anti–FoxP3-allophycocyanin (1:50, final dilution; eBioscience), anti–IL-22/allophycocyanin (1:50, final dilution; eBioscience), and anti–CD3-PercP (1:50, final dilution; eBioscience). Appropriate isotype-matched controls (BD Biosciences) were included in all experiments. Cells were analyzed using a FACSCalibur cytometer and Cell QuestPro software (BD Biosciences, San Jose, CA).