

# Aryl hydrocarbon receptor inhibits inflammation in DSS-induced colitis via the MK2/p-MK2/TTP pathway

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**Abstract.** The pathogenesis of inflammatory bowel disease (IBD) is believed to be associated with the abnormal expression of inflammatory factors. The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor, which can suppress the inflammatory response and attenuate experimental colitis. However, the detailed mechanism underlying the effects of AhR remains unclear. The present study investigated the role of AhR in the pathogenesis of IBD. Colitis was induced in mice by administration of 3% dextran sulphate sodium (DSS) for 7 days. The mice were also administered injections of the AhR agonist, 6-formylindolo(3,2-b)carbazole (FICZ), starting 2 days after the first administration of DSS. Furthermore, LoVo cells were treated with lipopolysaccharide (LPS) in the presence or absence of FICZ for 8 h. The protein expression levels of AhR, cytochrome P450 1A1 (CYP1A1) and tristetraprolin (TTP) were assessed by western blotting and immunofluorescence, whereas mRNA expression levels were assessed by reverse transcription-quantitative polymerase chain reaction. The results indicated that injection of mice with FICZ significantly attenuated DSS-induced colitis; in addition, the expression levels of inflammatory cytokines were markedly downregulated. Conversely, the expression levels of AhR and TTP were upregulated. In addition, mice in the AhR-knockout + DSS group exhibited elevated inflammatory cytokine production and developed more severe colitis. In LoVo cells, incubation with FICZ decreased the expression levels of inflammatory cytokines, whereas AhR and TTP expression was increased. In addition, the levels of phosphorylated-mitogen-activated protein kinase-activated protein kinase 2 (p-MK2) were decreased. These results suggested that AhR deficiency resulted in increased susceptibility to colitis, whereas activation of AhR by FICZ could ameliorate DSS-induced colitis via the MK2/p-MK2/TTP pathway.

## Introduction

The pathogenesis of inflammatory bowel disease (IBD) in humans remains incompletely understood; however, considerable evidence indicates that IBD results from an interaction between genetic, immune and environmental factors (1,2). IBD, including ulcerative colitis (UC) and Crohn's disease (CD), is a prominent intestinal disease. The pathogenesis of IBD is predominantly associated with intestinal immunodeficiency; this dysregulated immune response results in chronic gut inflammation and the presence of activated immune cells, which produce copious amounts of interferon (IFN)- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-2 and IL-17, thereby promoting the production of large amounts of inflammatory cytokines, and reactive oxygen and nitrogen metabolites, which ultimately lead to organ damage (3-5). Consistent with these findings, restoration of the balance between inflammatory and anti-inflammatory factors has reported as a useful treatment strategy, which has been successfully tested in patients with IBD and in experimental animal models of colitis (6-9).

Previous studies have reported that the aryl hydrocarbon receptor (AhR) is a transcription factor, which serves a protective role in IBD (10-12). AhR is extensively expressed in vertebrate cells and is a member of the basic helix-loop-helix/Per-Arnt-Sim homology superfamily (13). In the cytosol, AhR is present in an inactive form that binds to numerous co-chaperones (14). Following ligand binding, AhR dissociates from its chaperones and dimerises with AhR nuclear translocator (ARNT). The AhR/ARNT complex then activates the transcription of target genes, including cytochrome P450 1A1 (CYP1A1) (15). AhR has also been revealed to be indirectly activated by endogenous AhR ligands, including 6-formylindolo(3,2-b)carbazole (FICZ) (16). FICZ may activate AhR and significantly inhibit the inflammatory response (17). A previous study in AhR-knockout (KO) mice illustrated the role of AhR in the function and development of various organs; skin defects and a spectrum of hepatic abnormalities, as well as haematopoietic and vascular abnormalities, were observed in AhR-KO mice (18). Therefore, reduced AhR expression may lead to immunodeficiency and cause the immune system to secrete a mass of inflammatory factors. Although numerous studies have demonstrated a role for AhR in the inhibition of inflammation, the detailed underlying mechanism remains unclear.

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It has previously been reported that treatment of tristetraprolin (TTP)-KO mice with dextran sulphate sodium (DSS) leads to increased susceptibility to colitis, decreased colon length and increased production of proinflammatory cytokines (19). These findings suggest that TTP deficiency may result in a defect in the resolution of intestinal inflammation and in exacerbated IBD symptoms. TTP is the prototypic member of the TIS11 family of RNA-binding proteins, which has an important role in regulating the expression of adenylate-uridylate-rich element (ARE)-containing mRNAs (20). RNA-binding proteins, including TTP and human antigen R (HuR), recognise and bind to AREs in the 3'-untranslated regions (3'-UTRs) of their target mRNAs (20,21). Binding of TTP to its target mRNAs promotes the degradation of numerous inflammatory factors, including TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$ , IL-6, IL-8 and cyclooxygenase (COX)-2 (22-24). Furthermore, TTP-KO mice display severe autoimmune dysfunction, inflammatory arthritis and ulcerative colitis, thus suggesting that TTP has an important role in limiting the inflammatory response (25).

A previous study indicated that the expression of TTP is downregulated in AhR<sup>-/-</sup> cells (26). Furthermore, AhR is able to suppress the expression of COX-2, which is mediated by HuR, another RNA-binding protein from the same family as TTP (27). Based on the results of previous studies, the present study hypothesised that AhR may exert protective effects against experimental colitis through the regulation of TTP expression.

## Materials and methods

**Materials.** FICZ was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Foetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from HyClone (GE Healthcare Life Sciences, Logan, UT, USA). Antibodies against AhR (cat. no. ab2770) were purchased from Abcam, Inc. (Cambridge, MA, USA). Antibodies against CYP1A1 (cat. no. 13241-1-AP) and mitogen-activated protein kinase-activated protein kinase 2 (MK2; cat. no. 13949-1-AP) were purchased from Wuhan Sanying Biotechnology (Wuhan, China). Antibodies against TTP (cat. no. sc-14030) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibodies against phosphorylated (p)-MK2 were purchased from Cell Signalling Technology, Inc. (Danvers, MA, USA). Antibodies against GAPDH (cat. no. 10494-1-AP) were purchased from Wuhan Sanying Biotechnology. Goat anti-mouse (cat. no. A0216) and goat anti-rabbit (cat. no. A0208) horseradish peroxidase (HRP)-conjugated secondary antibodies, Cy3-conjugated goat anti-mouse immunoglobulin (Ig)G (cat. no. P0193) and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (cat. no. P0186) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). All primers used in the present study were synthesised by Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

**Cell cultures.** LoVo human intestinal epithelial cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM supplemented with 15% FBS, 100 U/ml streptomycin and 100 U/ml penicillin at 37°C in a 5% CO<sub>2</sub> atmosphere. The medium was refreshed

every 2 days. The cells were subcultured successively when they reached ~80% confluence.

**Cell treatments.** Cells were grown in 6-well plates and were incubated with LPS (10  $\mu$ g/ml) and/or FICZ (100 nM) for 8 h at 37°C. Immunofluorescence and western blotting were used to detect target protein expression, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to determine mRNA expression.

**Small interfering (si)RNA transfection.** For transient knock-down of AhR expression, cells were grown in DMEM with no antibiotics until they reached 80-100% confluence. Transient transfection was performed using Lipofectamine<sup>®</sup> 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After a 48-h transfection period, the cells were harvested. The sequences of the AhR-targeting siRNA were: Sense, 5'-GGAACACCUACAUCUAGAAdTdT-3' and antisense, 3'-dTdT CCUUGUGGAUGUAGAUCUU-5'. The sequences of the negative control siRNA were: Sense, 5'-GGGCAAUCCCCAAGAGGAAdTdT-3' and antisense, 3'-dTdT CCCGUUUAGGGUUCUCCUU-5'.

**DSS-induced colitis.** The present study was approved by the Laboratory Animal Welfare and Ethic Committee of the Third Military Medical University (Chongqing, China). Male C57BL/6J wild-type mice (n=60; age, 6-8 weeks; weight, 18-22 g) and male C57BL/6J AhR-KO mice (n=15; age, 6-8 weeks; weight, 18-22 g) were purchased from the Experimental Animal Center at Daping Hospital of the Third Military Medical University (Chongqing, China). Animals were bred and maintained under specific pathogen-free conditions in a temperature-controlled room (20 $\pm$ 2°C) with circadian light-dark cycles and free access to standard rodent chow and water. Mice were randomly divided into the following three groups: Sham (n=10), DSS (n=10), DSS + FICZ (n=10) group. Colitis was induced by administration of 3% DSS (Sigma-Aldrich, Inc.; Merck KGaA) dissolved in distilled water for 7 days. Mice in Sham group were administered distilled water. In addition, FICZ (1  $\mu$ g/mouse) was administered daily, by intraperitoneal injection, beginning 2 days after the start of DSS administration. Changes in body weight were recorded daily. Following DSS administration for 7 days, the mice were sacrificed and intestinal mucosa samples were collected for histology, and the determination of target mRNA and protein expression levels.

**Histological examination.** Colon segments were washed three times in PBS and were fixed in 4% paraformaldehyde overnight at 4°C. Ethanol was used to dehydrate the tissues, which were embedded in paraffin. The resulting tissue sections (4- $\mu$ m thick) were stained with haematoxylin for 5 min and eosin for 3 min at room temperature. The sections were observed using a confocal microscope (LSM 5 PASCAL; Carl Zeiss, Oberkochen, Germany).

**Immunofluorescence staining.** Cells were fixed with 4% paraformaldehyde for 15 min at room temperature, and permeabilized with 0.2% Triton X-100 for 10 min at room temperature. Cells were then blocked in 5% bovine serum albumin (BSA) (Sigma-Aldrich, Inc.; Merck KGaA) for 2 h at 37°C and incubated with mouse monoclonal anti-AhR (1:50) and rabbit polyclonal anti-TTP

Table I. Sequences of primers used in reverse transcription-quantitative polymerase chain reaction experiments.

Species	Gene name	Forward (5'-3')	Reverse (5'-3')
Human	IL-1 $\beta$	CTTCGACACATGGGATAACG	ATATCCTGTCCCTGGAGGTG
	IL-6	GACAGCCACTCACCTCTTCA	CCTCTTTGCTGCTTTCACAC
	CYP1A1	CCATGTGCGGCCACGGAGTT	ACAGTGCCAGGTGCGGCTT
	TTP	TTTAAGGGAGGCAATGAACC	CAGGAGACACTGGAACCTCA
	$\beta$ -actin	CCACGAAACTACCTTCAACTCC	CGTGATCTCCTTCTGCATCCTG
Mouse	IL-1 $\beta$	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
	IL-6	CTTCCAGCCAGTTGCCTTCTTG	GGTCTGTTGTGGGTGGTATCCTC
	IFN- $\gamma$	GCCACGGCACAGTCATTGA	TGCTGATGGCCTGATTGTCTT
	TNF- $\alpha$	CCACCACGCTCTTCTGTCTACTG	GGGCTACGGGCTTGTCACTC
	CYP1A1	CAATGAGTTTGGGGAGGTTACTG	CCCTTCTCAAATGTCTGTAGTG
	TTP	CTCGGAGGACTTTGGAACAT	TGCAGTAGGCGAAGTAGGTG
	GAPDH	TGAAGGTCGGTGTGAACGGATTTGG	ACGACATACTCAGCACCAGCATCAC

CYP1A1, cytochrome P450 1A1; IFN- $\gamma$ , interferon- $\gamma$ ; IL, interleukin; TNF- $\alpha$ , tumour necrosis factor- $\alpha$ ; TTP, tristetraprolin.

(1:20) antibodies overnight at 4°C. Subsequently, Cy3-conjugated goat anti-mouse immunoglobulin (Ig)G (1:200) and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:200) were used to stain the cells and sections, respectively. Finally, 1 mg/ml DAPI (Invitrogen; Thermo Fisher Scientific, Inc.) was used to stain the nuclei for total cell counting. The fluorescent signals were analysed by confocal laser scanning microscopy (Leica LAS AF Lite, version 2.8.0; Leica, Wetzlar, Germany), after the recording and merging of single-stained images.

**RNA purification and RT-qPCR.** Total RNA from intestinal mucosa and LoVo cells was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Spectrophotometry was used to determine the concentration of total RNA. For RT-qPCR, 1  $\mu$ g total RNA was converted to cDNA using a RT kit (Takara Bio, Inc., Otsu, Japan) in a 20  $\mu$ l volume according to the following temperature protocol: 37°C for 15 min, 85°C for 5 sec, followed by 5 min at 4°C. Subsequently, SYBR-Green-based qPCR was used to measure the mRNA expression levels of CYP1A1, TTP, IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\beta$ , IL-6,  $\beta$ -actin and GAPDH. The average quantification cycle (Cq) value of triplicate wells with each primer set was calculated as the amount of gene product present in the sample. The relative mRNA expression levels were determined according to the ratio between the Cq value of the target gene and  $\beta$ -actin or GAPDH (28). The cycling conditions were as following: 94°C for 10 min, 40 cycles at 94°C for 30 sec, 60°C for 1 min and 68°C for 30 sec, followed by 7 min at 68°C. The primer sequences were designed using online tools (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and are presented in Table I.

**Western blot analysis.** LoVo cells were washed three times with PBS and were lysed in cell lysis buffer (Intron Biotechnology, Inc., Seongnam, Korea) for 20 min on ice. The lysed cells were then centrifuged at 12,000 x g for 10 min at 4°C, and the supernatant was collected. The proteins were extracted

from the intestinal mucosa using lysis buffer, followed by homogenization through sonication and the extraction mixture was centrifuged at 12,000 x g for 15 min at 4°C. The protein concentrations were measured using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology). Protein samples (25  $\mu$ g) were separated by 10% SDS-PAGE and the proteins were transferred to polyvinylidene fluoride membranes (Amersham; GE Healthcare, Chicago, IL, USA). The membranes were blocked in TBS containing 0.05% Tween-20 (TBS-T) and 5% bovine serum albumin (BSA; Sigma-Aldrich, Inc.; Merck KGaA) for 2 h at 37°C and were then incubated with the following primary antibodies diluted in TBS-T containing 5% BSA overnight at 4°C: Mouse anti-AhR (1:500), rabbit anti-CYP1A1 (1:1,000), rabbit anti-TTP (1:200), rabbit anti-MK2 (1:1,000), rabbit anti-p-MK2 (1:1,000) and rabbit anti-GAPDH (1:1,000). The membranes were washed three times in TBS-T, and were then incubated with goat anti-mouse and goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000), after which they were washed a further three times in TBS-T. Enhanced chemiluminescence (Millipore, Billerica, MA, USA) was used to detect the binding of HRP-conjugated antibodies according to the manufacturer's protocol.

**Statistical analysis.** Data are expressed as the mean  $\pm$  standard deviation. Differences among groups were assessed by analysis of variance using SPSS statistical software package (version 13.0; SPSS Inc., Chicago, IL, USA). The groups were compared using one-way analysis of variance (ANOVA) and Q-test. Comparisons between groups were conducted using paired t-tests.  $P \leq 0.05$  was considered to indicate a statistically significant difference.

## Results

**AhR deficiency results in increased colitis severity.** Initially, the present study observed that administration of DSS produces more severe colitis in AhR-KO mice compared with in C57BL/6J



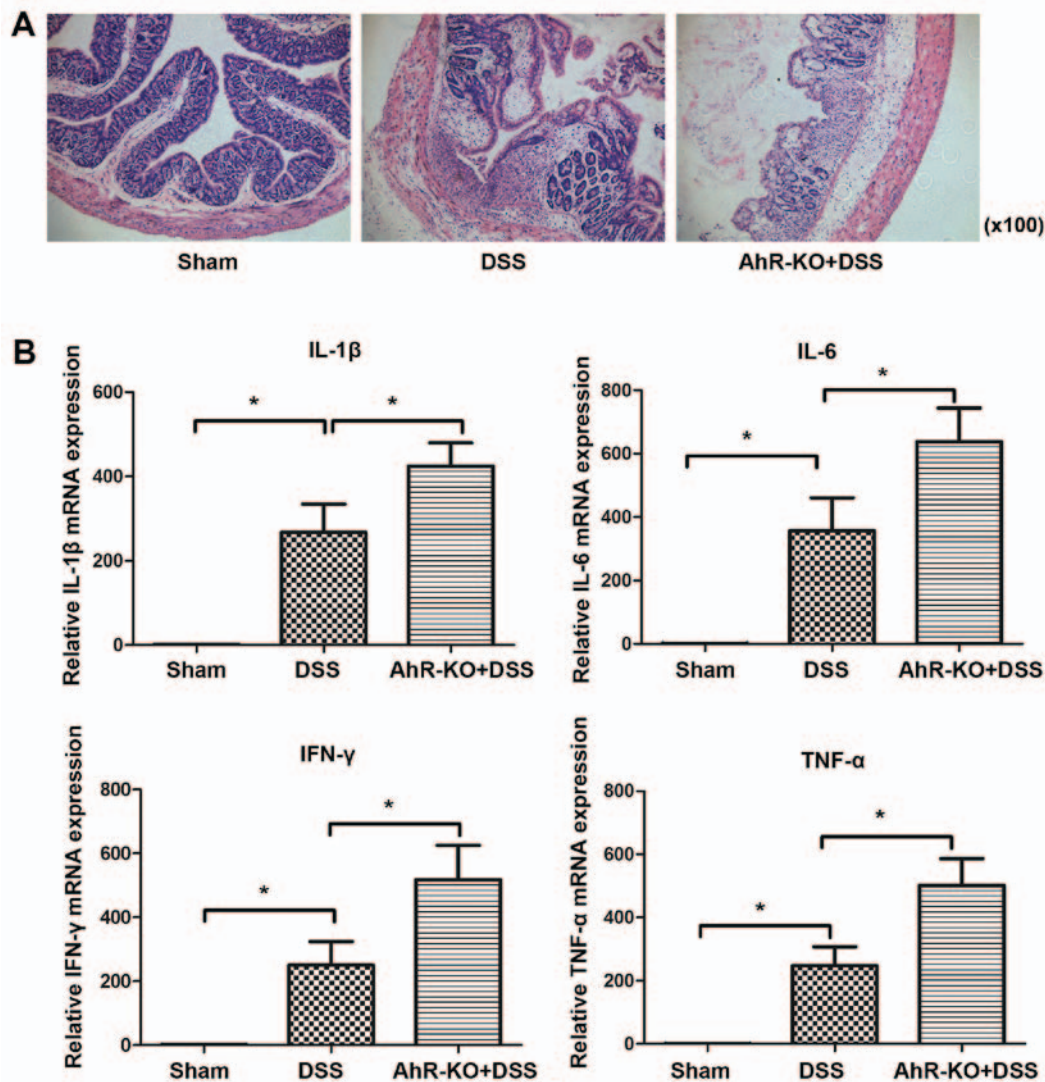


Figure 1. AhR deficiency results in increased colitis severity. AhR-KO mice and C57BL/6J WT mice were treated with 3% DSS for 7 days. (A) Representative haematoxylin and eosin-stained colonic sections from DSS-treated C57BL/6J and AhR-KO mice (magnification, x100). (B) mRNA expression levels of IL-1 $\beta$ , IL-6, IFN- $\gamma$  and TNF- $\alpha$  in colonic samples from mice treated as indicated in (A). mRNA expression was analysed by quantitative polymerase chain reaction. Data are presented as the mean  $\pm$  standard deviation of three experiments, in which samples from 3 mice/group were analysed. \*P<0.05. AhR, aryl hydrocarbon receptor; DSS, dextran sulphate sodium; IFN- $\gamma$ , interferon- $\gamma$ ; IL, interleukin; KO, knockout; TNF- $\alpha$ , tumour necrosis factor- $\alpha$ ; WT, wild-type.

wild-type (WT) mice, as evidenced by histological examination of colonic tissue (Fig. 1A). Furthermore, mice in the AhR-KO group produced more inflammatory factors compared with those in the DSS-treated WT group. The mRNA expression levels of IL-1 $\beta$ , IL-6, IFN- $\gamma$  and TNF- $\alpha$  were significantly increased in the AhR-KO + DSS group compared with in the DSS-treated WT group (Fig. 1B). These findings suggested that a deficiency in AhR may be associated with DSS-induced colitis.

**AhR activation ameliorates DSS-induced colitis.** To investigate whether AhR activation could attenuate experimental colitis, mice were administered FICZ for 5 days, beginning 2 days after the start of DSS administration (Fig. 2). DSS-treated mice exhibited significant weight loss 3–4 days after DSS treatment (Fig. 2B). On day 8, mortality rate in the DSS group was ~30% (Fig. 2C). In addition, colon length was decreased in DSS-treated mice (Fig. 2A). Conversely, DSS + FICZ-treated mice exhibited reduced weight loss and mortality (~16.67%), and colon length was increased compared with in the DSS-treated mice (Fig. 2A–C). In addition,

histological examination of colonic tissues from mice in the various groups revealed that mice treated with FICZ developed less severe colitis (Fig. 2E). The expression levels of IL-1 $\beta$ , IL-6, IFN- $\gamma$  and TNF- $\alpha$  were significantly increased in colon samples from mice in the DSS-induced colitis group compared with in the vehicle-treated group (Fig. 2D). However, in mice treated with FICZ the mRNA expression levels of IFN- $\gamma$ , IL-1 $\beta$ , IL-6 and TNF- $\alpha$  were significantly reduced (Fig. 2D). These findings suggested that administration of FICZ may attenuate inflammation associated with DSS-induced colitis in mice, and that AhR has an important role in this attenuation.

**FICZ upregulates expression of the RNA-binding protein TTP in mice with DSS-induced colitis.** Recent studies have reported that TTP serves an important role in limiting inflammatory responses (29,30). In addition, treatment of TTP-KO mice with DSS results in increased susceptibility to colitis (19). Furthermore, it has been demonstrated that TTP expression is decreased in AhR<sup>-/-</sup> cells (26). To explore whether TTP has

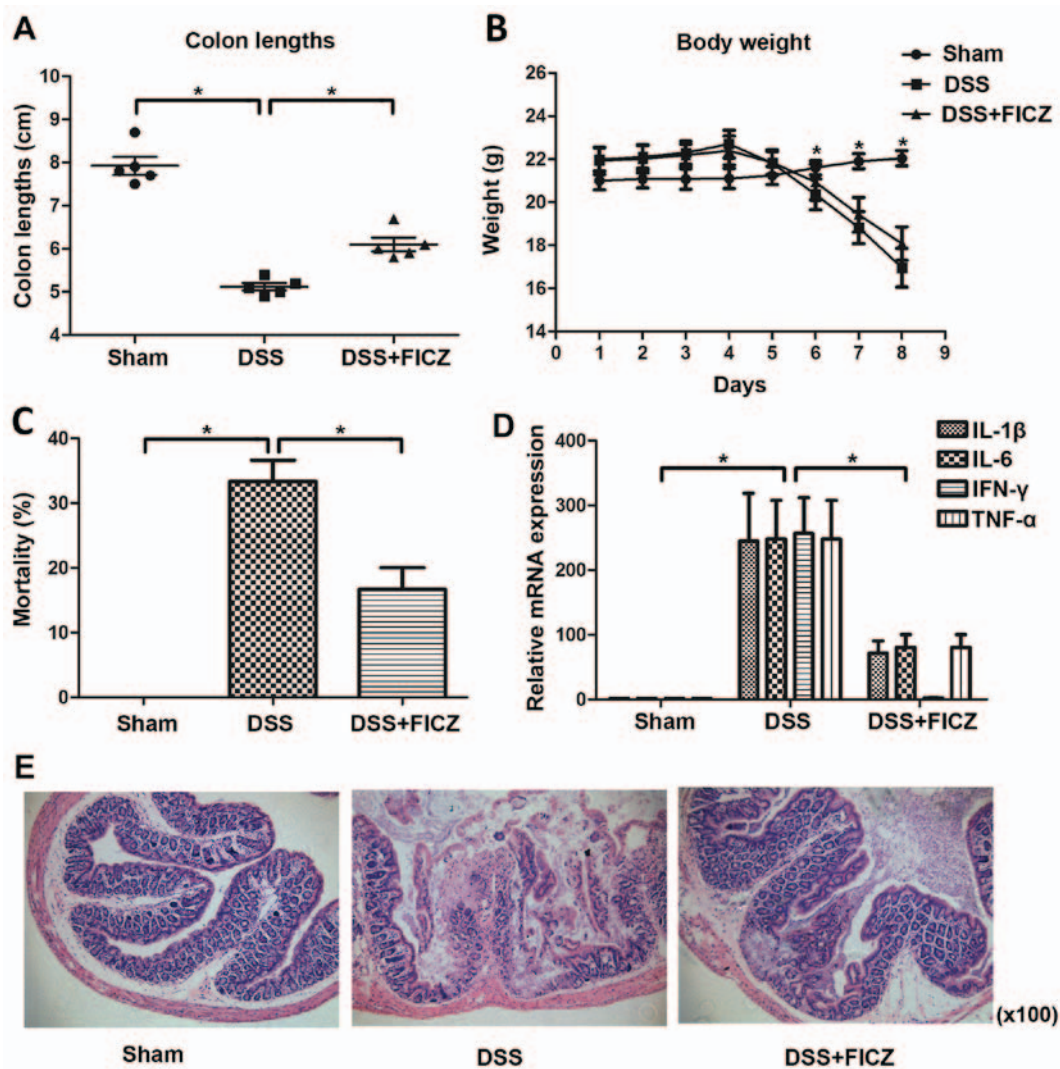


Figure 2. AhR activation ameliorates DSS-induced colitis. (A) FICZ was intraperitoneally administered to mice for 5 days, beginning 2 days after the start of DSS administration. Colon lengths of mice in the sham, DSS and DSS + FICZ groups were measured on day 8. Data are presented as the mean  $\pm$  standard deviation of five experiments in which samples from 10 mice/group were analysed. (B) Changes in body weight were recorded daily. Weight data are presented as the cumulative mean  $\pm$  standard deviation obtained from five separate experiments. In each experiment, each group consisted of  $\geq 10$  mice. (C) Percentage of mortality in the sham, DSS and DSS + FICZ groups was noted on day 8. Data are presented as the mean  $\pm$  standard deviation. (D) mRNA expression levels of IL-1 $\beta$ , IL-6, IFN- $\gamma$  and TNF- $\alpha$  in colonic samples from mice treated as indicated in (A). mRNA expression was analysed by quantitative polymerase chain reaction. Data are presented as the mean  $\pm$  standard deviation of five experiments in which samples from 10 mice/group were analysed. (E) Representative haematoxylin and eosin-stained colonic sections of mice in the sham, DSS and DSS + FICZ groups on day 8 (magnification,  $\times 100$ ). \* $P < 0.05$  ( $n = 10$ /group). AhR, aryl hydrocarbon receptor; DSS, dextran sulphate sodium; FICZ, 6-formylindolo(3,2-b)carbazole; IFN- $\gamma$ , interferon- $\gamma$ ; IL, interleukin; KO, knockout; TNF- $\alpha$ , tumour necrosis factor- $\alpha$ ; WT, wild-type.

a role in alleviating DSS-induced colitis, and to determine the association between TTP and AhR in this process, the expression levels of TTP were detected. The results indicated that the expression levels of AhR, CYP1A1 and TTP were downregulated in AhR-KO mice (Fig. 3A and B). Similarly, the expression levels of AhR and TTP were significantly decreased in mice with DSS-induced colitis (Fig. 3C and D).

The present study demonstrated that TTP expression was downregulated in AhR-KO mice. Therefore, in order to explore whether activation of AhR could upregulate the expression of TTP, mice were intraperitoneally injected with FICZ daily, beginning 2 days after the start of DSS administration. The expression levels of AhR, CYP1A1 and TTP were significantly increased following administration of FICZ to mice with DSS-induced colitis (Fig. 3C and D). Increased AhR, CYP1A1 and TTP expression was confirmed by western blotting (Fig. 3D). Furthermore,

alterations in mRNA expression levels, as quantified by RT-qPCR, correlated with these protein alterations (Fig. 3C).

*FICZ upregulates the RNA-binding protein TTP expression in an in vitro model.* To further investigate the role of AhR and TTP in an *in vitro* model, LoVo human intestinal epithelial cells were treated with LPS (10  $\mu\text{g/ml}$ ) in the presence or absence of FICZ (100 nM) for 8 h. The results demonstrated that IL-1 $\beta$  and IL-6 were significantly increased following treatment of the cells with LPS. However, the mRNA expression levels of IL-1 $\beta$  and IL-6 were significantly downregulated in the LPS + FICZ group compared with in the LPS group (Fig. 4A). This finding suggested that FICZ may inhibit LPS-induced inflammation in an *in vitro* model. Subsequently, the present study aimed to determine whether FICZ modulates the RNA-binding protein TTP. As illustrated in Fig. 4B and C, the protein and mRNA expression levels

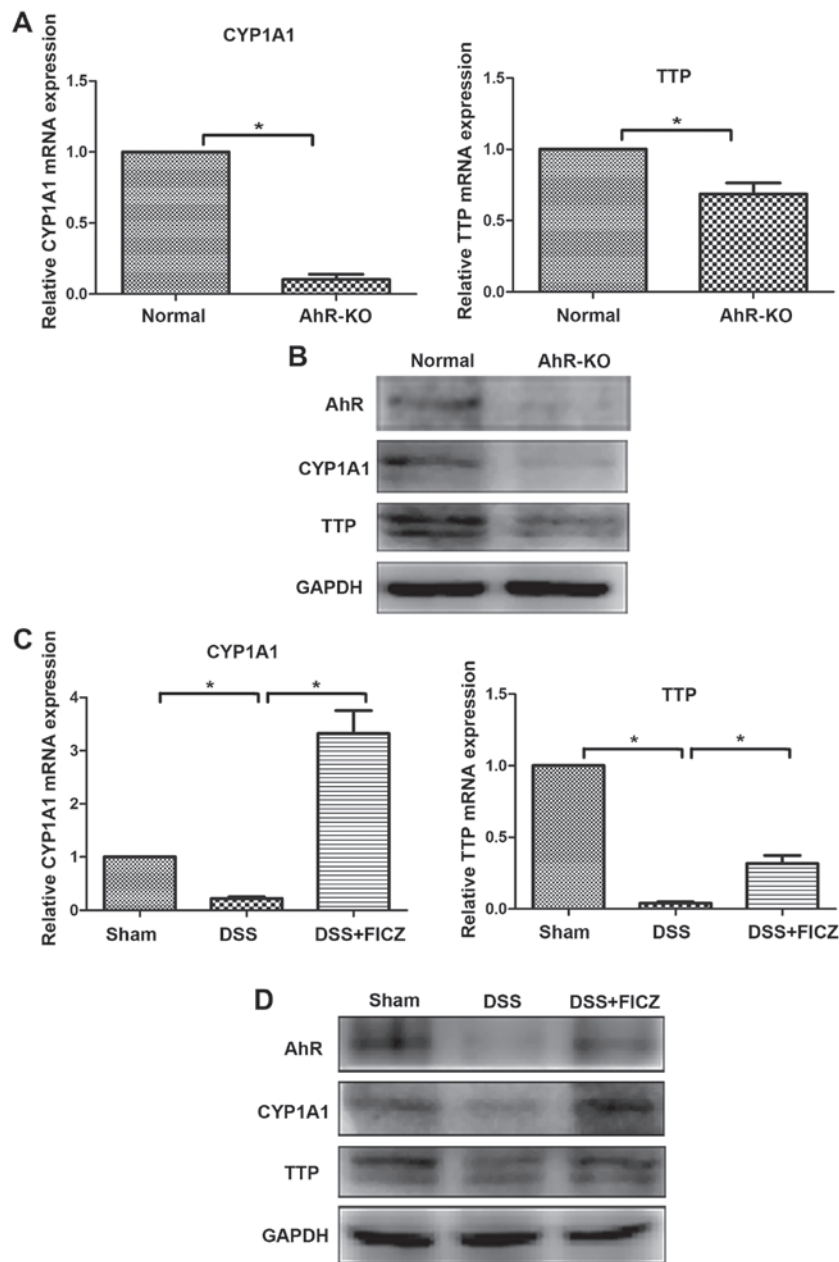


Figure 3. FICZ upregulates expression of the RNA-binding protein TTP in mice with DSS-induced colitis. (A) mRNA expression levels of CYP1A1 and TTP in colonic samples of WT and aryl hydrocarbon receptor (AhR) KO mice were determined by RT-qPCR. (B) Protein expression levels of AhR, CYP1A1 and TTP in colonic samples from WT and AhR-KO mice, as detected by western blotting. (C) FICZ was intraperitoneally administered to mice for 5 days, beginning 2 days after the start of DSS administration. The mRNA expression levels of CYP1A1 and TTP in colonic samples from the sham, DSS and DSS + FICZ groups were determined by RT-qPCR. (D) Mice were treated as indicated in (C), and the protein expression levels of AhR, CYP1A1 and TTP from colonic samples was detected by western blotting. Data are presented as the mean  $\pm$  standard deviation of five experiments in which samples from 10 mice/group were analysed. \* $P < 0.05$ . AhR, aryl hydrocarbon receptor; CYP1A1, cytochrome P450 1A1; DSS, dextran sulphate sodium; FICZ, 6-formylindolo(3,2-b)carbazole; KO, knockout; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TTP, tristetraproline; WT, wild-type.

of TTP were significantly increased in the LPS + FICZ group compared with in the LPS group. These results are in accordance with those obtained in the animal model. Unexpectedly, the protein expression levels of AhR were decreased following treatment with FICZ. This may be due to AhR degradation following FICZ treatment (31,32). Immunofluorescence staining revealed that confluent LoVo cells treated with LPS and FICZ displayed an increased intensity of TTP staining compared with the LPS-treated group. In addition, the intensity of AhR staining was decreased in the LPS and FICZ-treated groups compared to the LPS-treated groups (Fig. 4D).

*AhR regulates TTP expression through the MK2/p-MK2 pathway.* In order to explore the mechanism through which AhR regulates TTP, AhR expression was silenced in LoVo cells, after which the cells were treated with LPS and FICZ for 8 h. As expected, the protein expression levels of AhR and TTP were decreased in cells in which AhR expression was silenced (Fig. 5A). Concurrently, TTP expression in AhR siRNA-transfected cells treated with LPS and FICZ was lower than that in normal cells treated with LPS and FICZ (Fig. 5B). These findings indicated that FICZ may upregulate TTP via AhR. In addition, LoVo cells were treated with LPS and FICZ,



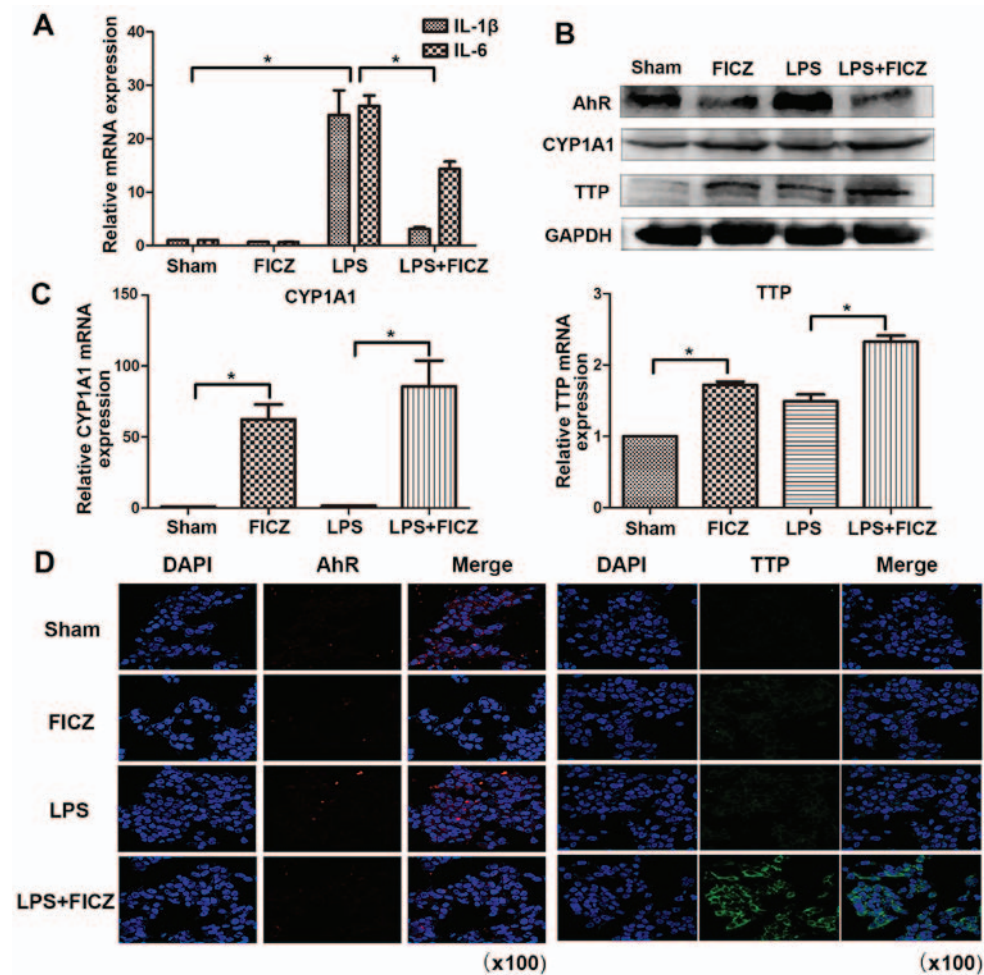


Figure 4. FICZ upregulates the RNA-binding protein TTP expression in an *in vitro* model. (A-D) LoVo human intestinal epithelial cells were treated with or without LPS (10  $\mu$ g/ml) and FICZ (100 nM) for 8 h. The mRNA expression levels of (A) IL-1 $\beta$ , IL-6, (C) CYP1A1 and TTP were determined by reverse transcription-quantitative polymerase chain reaction. (B) Protein expression levels of AhR, CYP1A1 and TTP were semi-quantified by western blotting. (D) Immunofluorescence was used to detect AhR and TTP expression. Red and green signals represent AhR and TTP, respectively (magnification, x100). Data are presented as the mean  $\pm$  standard deviation. \* $P$ <0.05. AhR, aryl hydrocarbon receptor; CYP1A1, cytochrome P450 1A1; FICZ, 6-formylindolo(3,2-*b*) carbazole; IL, interleukin; TTP, tristetraprolin.

and the expression levels of MK2 and p-MK2 were measured, which are the classical upstream kinases of the TTP pathway, in order to determine whether AhR mediates TTP expression through the MK2/p-MK2 pathway. Notably, MK2 expression was not significantly altered; however, p-MK2 expression was reduced in the LPS + FICZ-treated group compared with in the LPS-treated group (Fig. 5C). Consistent with the expected results, these findings indicated that AhR may mediate TTP expression via the MK2/p-MK2 pathway. It should be noted that after AhR expression was silenced, MK2 expression was still not significantly altered; however, FICZ no longer downregulated p-MK2 expression in the LPS + FICZ-treated group (Fig. 5D). These findings suggested that AhR may serve a key role in the FICZ-mediated downregulation of p-MK2 expression.

## Discussion

The present study demonstrated that mice deficient in AhR displayed increased susceptibility to colitis, and indicated that activation of AhR by FICZ could ameliorate DSS-induced colitis. In addition, AhR could inhibit the inflammation that occurs in DSS-induced colitis via the MK2/p-MK2/TTP pathway.

It has previously been reported that AhR serves an important role in IBD. AhR can inhibit inflammation and colitis in mice via the upregulation of IL-22 (11), and AhR activation significantly inhibited the production of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in LPS-treated dendritic cells (17), and attenuated LPS-induced inflammatory responses in SW480 human colon carcinoma cells (10). Furthermore, AhR can be activated by 2,3,7,8-tetrachlorodibenzo-p-dioxin and attenuates inflammation associated with CD (33). In addition,  $\beta$ -naphthoflavone, a nontoxic agonist of AhR, has been reported to suppress the pathogenesis of DSS-induced colitis and attenuate DSS-induced colitis (10). These findings suggested that AhR expression in intestinal epithelial cells is involved in the prevention of colitis.

The present study examined the expression and functional role of AhR in a mouse model of DSS-induced colitis. The results indicated that DSS induces more severe colitis in AhR-KO mice compared with in wild-type mice. This finding suggested that a deficiency in AhR may result in a decrease in resistance to inflammation. In addition, the present study assessed whether activation of AhR by FICZ could attenuate DSS-induced colitis. Treatment of mice with FICZ for 5 days, starting 2 days after the initiation of DSS administration,

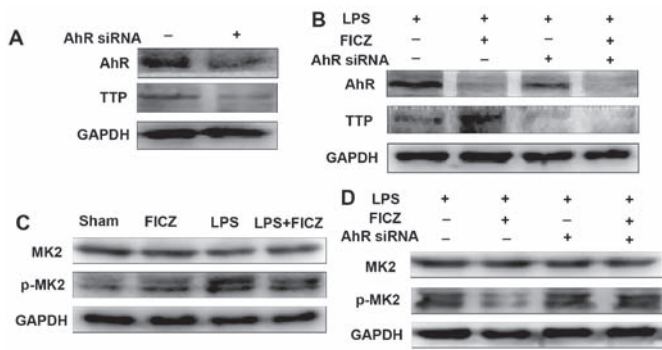


Figure 5. AhR regulates TTP expression via the MK2/p-MK2 pathway. (A) Confluent LoVo cells were transfected with AhR siRNA for 48 h. Protein expression levels of AhR and TTP were detected by western blotting. (B) Cells were transfected with AhR siRNA, and were treated with or without LPS (10  $\mu$ g/ml) and FICZ (100 nM) for 8 h; western blotting was used to detect the protein expression levels of AhR and TTP. (C) LoVo human intestinal epithelial cells were treated with or without LPS (10  $\mu$ g/ml) and FICZ (100 nM) for 8 h. Protein expression levels of MK2 and p-MK2 were detected by western blotting. (D) Cells were treated as indicated in (C) and were transfected with AhR siRNA; western blotting was used to detect the protein expression levels of MK2 and p-MK2. AhR, aryl hydrocarbon receptor; FICZ, 6-formylindolo(3,2-b)carbazole; LPS, lipopolysaccharide; MK2, mitogen-activated protein kinase-activated protein kinase 2; p-MK2, phosphorylated-MK2; siRNA, small interfering RNA; TTP, tristetraprolin.

significantly inhibited the expression of inflammatory factors. Based on these findings, it may be suggested that AhR activation attenuates DSS-induced colitis.

The main aetiology of IBD is thought to be associated with disordered immune function, in which the organism releases a mass of inflammatory factors, resulting in an imbalance between pro- and anti-inflammatory factors, which in turn disrupts intestinal barrier function. In recent years, the mechanisms through which factors that mediate the stability of mRNAs coding inflammatory factors exert their anti-inflammatory effects have attracted more attention. RNA-binding proteins, which may bind to AREs located in the 3'-UTRs of target mRNAs and direct them to exosomes for rapid degradation (34,35), can reduce the expression of various inflammatory factors. RNA-binding proteins, including TTP and HuR, are found in numerous protein families. It has previously been reported that TTP-KO mice display more severe autoimmune dysfunction, inflammatory arthritis and UC compared with their WT counterparts (20). Furthermore, in AhR<sup>-/-</sup> cells, the expression of TTP is downregulated (26). AhR also inhibits inflammation via the downregulation of another RNA-binding protein, HuR (27). These findings suggested that AhR activation may regulate activity of the RNA-binding protein TTP.

The present study assessed whether AhR could regulate TTP expression in the gut and investigated the role of TTP in IBD. Initially, the results demonstrated that TTP expression was decreased in DSS-treated mice compared with in vehicle-treated animals. In addition, consistent with results reported in the literature (26), the expression levels of AhR and TTP were downregulated in AhR-KO mice. When DSS-treated mice were treated with FICZ, TTP expression was upregulated. These findings suggested that TTP expression may be associated with IBD and that FICZ, which activates the AhR pathway, may exert its anti-inflammatory effects by upregulating the RNA-binding protein TTP.

Although AhR mediates the expression of TTP, the mechanism underlying this effect remains unknown. It has been suggested that phosphorylation of MK2, which occurs upstream of TTP, modulates the activity and expression of TTP. MK2 can phosphorylate the TTP protein (36,37). In addition, the stability, expression and function of TTP are reported to be regulated by the MK2/p-MK2 pathway (36,38,39). Activation of the MK2/p-MK2 pathway has been reported to abolish TTP-mediated suppression of IL-6 3'-UTR reporter activity (40). In addition, it has been demonstrated that the MK2/p-MK2 cascade is involved in regulating the stability of ARE-containing mRNAs (41). Recent studies have clearly indicated that the MK2/p-MK2 signalling cascade can regulate TTP-mediated mRNA stability of IL-6 and TNF- $\alpha$  (42).

In the present study, following treatment with FICZ, MK2 expression was not significantly altered; however, the expression of p-MK2 was downregulated. Conversely, when AhR expression was silenced, FICZ no longer downregulated p-MK2 expression. These findings suggested that FICZ upregulates the expression of TTP by downregulating p-MK2 expression.

In conclusion, the present study demonstrated that AhR serves an important role in attenuating DSS-induced colitis. AhR-mediated protection operates in part by inducing TTP via the MK2/p-MK2 pathway. The present study is the first, to the best of our knowledge, to demonstrate a dependence on TTP for AhR-mediated protection against DSS-induced colitis. Since AhR may serve important roles in protection against DSS-induced colitis, the AhR-TTP pathway may be considered an attractive candidate for the treatment of UC.

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