MicroRNA-124 Promotes Intestinal Inflammation by Targeting Aryl Hydrocarbon Receptor in Crohn’s Disease

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Abstract

**Background and Aims:** Dysregulation of microRNAs (miRNAs) is associated with a variety of diseases, including Crohn’s disease (CD), but the essential biological functions and crucial targets of miRNAs remain largely unknown. The present study investigated the aberrant colonic mucosal miRNAs in active CD patients.

**Methods:** miRNA levels were assayed in inflamed colon of active CD patients by quantitative real-time polymerase chain reaction. The influence of differential expressed miR-124 on its putative target, the aryl hydrocarbon receptor (AHR), was investigated in CD patients, intestinal epithelial cells (IECs) and 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis mice. The role of miR-124 was further studied in experimental colitis mice by intracolonic administration of miR-124 inhibitors or precursors.

**Results:** We found an inverse correlation between miR-124 and AHR protein levels in colon tissues and IECs of active CD patients. Further results demonstrated that miR-124 suppressed AHR expression by directly targeting the AHR 3′-untranslated region (3′-UTR) in Caco-2 cells and HT-29 cells. MiR-124 mediated the inflammatory response in lipopolysaccharide-stimulated cells through retroregulation of AHR in vitro. Downregulation or upregulation of miR-124 in TNBS-induced colitic colon alleviated or aggravated experimental colitis, respectively.

**Conclusions:** These findings suggest that miR-124 induces intestinal inflammation by inhibiting AHR to modulate pro-inflammatory cytokine production and thereby promotes the pathogenesis of CD.

**Key Words:** Crohn’s disease; miR-124, aryl hydrocarbon receptor; intestinal epithelial cell
1. Introduction

Inflammatory bowel disease (IBD) is a chronic relapsing inflammatory disease, including Crohn’s disease (CD) and ulcerative colitis (UC). Crohn’s disease can affect any part of the gastrointestinal tract and frequently presents with diarrhoea, fever, abdominal pain, clinical signs of bowel obstruction, as well as extra-intestinal manifestations and associated immune disorders. Crohn’s disease is thought to result from an abnormal and continuing immune response to environmental factors such as commensal bacteria in a genetically susceptible host. Many environmental risk factors have been investigated and shown to have regulatory effects on the development and clinical course of CD.

The aryl hydrocarbon receptor (AHR) is a ligand-inducible transcription factor that is highly conserved in evolution and present in many cell types. In its inactive state, AHR resides in the cytosol bound to a molecular chaperone complex (Hsp90/XAP2/p23). Following ligand binding, AHR dissociates from the chaperones and translocates to the nucleus, where it heterodimerizes with aryl hydrocarbon nuclear translocator. This heterodimer then activates target genes with promoters containing consensus regulatory sequences. The AHR is exported to the cytosol and degraded by the proteasome after transcriptional regulation has occurred. There is a variety of ligands of AHR, including xenobiotic ligands, endogenous activator, dietary compounds. The AHR, which is involved as a component of the host response to environmental stimuli, is essential in the regulation of immune responses and helps to control immune homeostasis. While low to normal activation of AHR can protect the host from environmental insults, excessive activation or deficiency of AHR indicates dysregulation of immune homeostasis and the development of inflammation. Many studies have indicated that AHR regulates the differentiation of Th17 and Treg cells. Abnormal expression of AHR shifts the balance between Th17 and Treg cells and thus contributes to the pathogenesis of several animal models of autoimmune disease, including experimental autoimmune encephalomyelitis, collagen-induced arthritis and colitis. Recent research shows that AHR is downregulated in intestinal tissue of IBD patients, and AHR signalling inhibits experimental colitis in the gastrointestinal tract of mice via interleukin (IL)-22. The AHR can ameliorate dextran sodium sulphate (DSS)-induced colitis in mice by influencing differentiation of Th17 and Treg cells as well. These novel data indicate that AHR delivers protective signals in the gastrointestinal tract and its function in mucosal cell types, e.g. intestinal epithelial cells (IECs).

miRNAs are a recently discovered class of small, non-coding, single-stranded RNAs that are involved in post-transcriptional gene regulation. They function by base-pairing with complementary sites on target messenger RNAs (miRNAs), thus causing either translational inhibition or cleavage of the mRNA complex. miRNA has been recognized as an important element in the regulation of innate and adaptive immune responses. Changes in miRNA expression are described in many autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis, psoriasis and IBD. Although altered miRNA profiles in both tissue and serum of CD patients have been reported, the precise mechanistic links between miRNA alterations and target genes remain unknown.

miR-124, a brain-specific miRNA, has been reported to target AHR in neuroblastoma. Recent studies have suggested that miR-124 is downregulated in lung, gastric and colorectal cancer and is also involved in an inflammatory feedback loop in hepatocellular carcinoma. In the present study we investigated the role of AHR expression-associated miRNAs in modulating the pathogenesis of CD. By assaying miRNA levels, we first identified high expression of miR-124 in CD and further demonstrated the potential pro-inflammatory role of miR-124 on its target, AHR, in IECs, CD patients and 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis mice.

2. Methods

2.1. Tissue samples

Tissue biopsy samples were obtained from inflamed colonic and ileal areas of patients with active CD (n = 20) and normal control subjects (n = 20) undergoing screening colonoscopies or surgery in Jiangsu Province Hospital (Jiangsu, China). All tissues were frozen in liquid nitrogen immediately and then store at −80°C until use. Characteristics of CD patients and controls are summarized in Table 1.

2.2. Cell culture

Caco-2 and HT-29 human colon carcinoma cell lines were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Caco-2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, CA, USA) supplemented with 10% foetal bovine serum (FBS; Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco). HT-29 cells were cultured in RPMI-1640 medium (Gibco) containing 10% FBS and 100 U/ml penicillin and 100 µg/ml streptomycin. All cells were incubated at 37°C in a 5% CO₂, water-saturated atmosphere.

2.3. Rapid isolation and purification of primary IECs

Primary IECs were isolated from human/mouse intestine at low temperature using chelating agents according to previous methods with slight modification. Briefly, the ileum and colon were separated, washed and dissected with forceps and scissors in precooled Hanks balanced salt solution (HBSS; Gibco) containing 1 mM dithiothreitol (DTT; Fisher Biotech, Fair Lawn, NJ). Then the mucosal strips were stirred in the chelating agent solution (1.5 mM KCl, 96 mM NaCl, 8 mM KH₂PO₄, 5 mM Na₂HPO₄, 44 mM sucrose, 55 mM d-sorbitol, 5 mM EDTA, 5 mM EGTA) at 4°C for 1 h. Detached IECs were harvested in the suspension after 10 vigorous shakes of the vessel. For further purification, the suspension was immediately passed through a 80-µm nylon mesh, which allowed single cells, such as fibroblasts, lymphocytes and monocytes, to pass through while retaining the crypts. The filter was then immediately inverted and the highly purified crypts were backwashed and resuspended in DMEM in the centrifuge tube. To increase the yield of purified IECs, the mucosal strips were again incubated in the chelating agent solution, at 4°C for another 1 h. The isolated epithelium was collected by centrifugation (200g for 5min) for short-term function studies, including quantitative reverse transcription–polymerase chain reaction (qRT-PCR) and Western blotting. Detailed protocols describing the methods of primary intestinal immune cell isolation are shown in Supplementary Methods.

2.4. RNA isolation and qRT-PCR

Total RNA from the cultured cells and human tissues was extracted using TriZol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. We performed qRT-PCR of miRNAs using TaqMan microRNA probes (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Briefly, 1 µg of total...
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RNA was reverse-transcribed to cDNA using a stem-loop RT primer (Applied Biosystems) and AMV reverse transcriptase (TaKaRa, Dalian, China). Real-time PCR was conducted using a TaqMan PCR kit on an Applied Biosystems 7500 Sequence Detection System. After the reaction, the cycle threshold (C<sub>T</sub>) values were determined using fixed threshold settings, and the mean C<sub>T</sub> was determined from the duplicate PCRs. A comparative threshold cycle (ΔC<sub>T</sub>) method was used to compare each condition with controls, and values are expressed as 2<sup>−ΔΔC_T</sup>. The relative amount of miRNA was normalized to U6.

To quantify AHR mRNA, 1 µg of total RNA was reverse-transcribed to cDNA using oligo dT and Thermoscript (TaKaRa). Next, real-time PCR was conducted with SYBR Green (Invitrogen), the RT product and specific primers for AHR and β-actin. The sequences of the primers were as follows: human AHR (sense), 5'-CAAATCTTCCAAGCGGCATA-3'; human AHR (antisense), 5'-CGCTCATTGCGATAGTG-3'. After the reactions, the C<sub>T</sub> values were determined by setting a fixed threshold. The relative level of AHR mRNA was normalized to β-actin.

2.5. Overexpression or knockdown of miR-124

Synthetic pre-miR-124, anti-miR-124 and scrambled negative control RNA (pre-scramble and anti-scramble) were purchased from GenePharma (Shanghai, China). Caco-2 cells and HT-29 cells were seeded in 6-well plates and transfected with Lipofectamine 2000 (Invitrogen) the following day, when the cells were approximately 70% confluent. In each well, equal amounts of these RNA oligonucleotides were used. Twenty-four hours after transfection, the cells were harvested for qRT-PCR and Western blotting or stimulated with lipopolysaccharide (LPS, Sigma).

2.6. Small interfering RNA assay

A small interfering RNA (siRNA) sequence targeting the human AHR gene was designed and synthesized by GenePharma. The

| Gender/age (y) | Diagnosis | Tissue | Duration (y) | Treatment |
|---------------|-----------|--------|--------------|-----------|
| M/28          | CD/active | Ileum  | 6            | 5-ASA + prednisone |
| M/37          | CD/active | Ileum  | 3            | Prednisone |
| F/43          | CD/active | Colon  | 18           | Infliximab + azathioprine |
| M/38          | CD/active | Ileum  | 5            | Infliximab + prednisone |
| F/47          | CD/active | Colon  | 15           | Infliximab + prednisone |
| M/22          | CD/active | Ileocolon | 6          | 5-ASA + prednisone |
| F/30          | CD/active | Ileum  | 9            | 5-ASA + prednisone |
| M/31          | CD/active | Ileum  | 10           | Infliximab + azathioprine |
| M/25          | CD/active | Ileum  | 4            | 5-ASA + prednisone |
| F/26          | CD/active | Colon  | 8            | Infliximab + prednisone |
| M/31          | CD/active | Colon  | 2            | Prednisone |
| M/57          | CD/active | Ileum  | 12           | Infliximab + prednisone |
| F/42          | CD/active | Ileum  | 18           | Infliximab + azathioprine |
| M/20          | CD/active | Ileocolon | 5          | 5-ASA + prednisone |
| F/15          | CD/active | Ileum  | 2            | 5-ASA |
| M/22          | CD/active | Colon  | 5            | 5-ASA + prednisone |
| M/29          | CD/active | Ileum  | 8            | Prednisone |
| F/44          | CD/active | Ileocolon | 12         | 5-ASA + prednisone |
| M/33          | CD/active | Ileum  | 6            | 5-ASA + prednisone |
| M/22          | NC        | Colon  | 0            | NA |
| M/25          | NC        | Ileum  | 0            | NA |
| M/69          | NC        | Colon  | 0            | NA |
| F/18          | NC        | Ileum  | 0            | NA |
| F/61          | NC        | Colon  | 0            | NA |
| M/47          | NC        | Ileum  | 0            | NA |
| M/52          | NC        | Colon  | 0            | NA |
| M/45          | NC        | Colon  | 0            | NA |
| M/21          | NC        | Ileocolon | 0         | NA |
| M/48          | NC        | Colon  | 0            | NA |
| F/19          | NC        | Ileum  | 0            | NA |
| M/54          | NC        | Colon  | 0            | NA |
| M/16          | NC        | Colon  | 0            | NA |
| F/39          | NC        | Colon  | 0            | NA |
| M/23          | NC        | Ileocolon | 0         | NA |
| M/25          | NC        | Colon  | 0            | NA |
| F/27          | NC        | Ileocolon | 0         | NA |
| M/42          | NC        | Colon  | 0            | NA |
| M/30          | NC        | Colon  | 0            | NA |
| M/19          | NC        | Colon  | 0            | NA |

M, male; F, female; CD, Crohn's disease; NC, normal control; 5-ASA, 5-aminosalicylic acid; NA, not applicable.

Table 1. Clinical characteristics of patients
siRNA sequence was 5'-GCCUGUAUUACCACACUTF-3'. A scrambled siRNA was used as a negative control. AHR siRNA and scrambled control RNA were transfected into Caco-2 cells or HT-29 cells using Lipofectamine 2000 according to the manufacturer's instructions. Total protein and RNA were extracted 24 h after transfection. The AHR protein and mRNA expression levels were assessed by Western blotting and qRT-PCR.

2.7. Luciferase reporter assay
To test the direct binding of miR-124 to the target gene, AHR, we performed a luciferase reporter assay. The entire 3'-untranslated region (3'-UTR) of human AHR, containing a presumed miR-124 complementary site (seed sequence, GUGCCUU), was amplified with PCR using human genomic DNA as a template. The PCR products were inserted into the p-MIR-reporter plasmid (Ambion). Efficient insertion was confirmed by sequencing. To test the binding specificity, we mutated the miR-124 seed sequence from GUGCCUU to CACGGAA, and the mutant AHR 3'-UTR was inserted into an equivalent luciferase reporter. For luciferase reporter assays, Caco-2 or HT-29 cells were cultured in 24-well plates, and 1 µg of firefly luciferase reporter plasmid, 1 µg of a β-galactosidase (β-gal) expression plasmid (Ambion), and equal amounts (100 pmol) of pre-miR-124, anti-miR-124 or the scrambled negative control RNA were transfected into each well using Lipofectamine 2000. The β-gal plasmid was used as a transfection control. Twenty-four hours after transfection, the cells were assayed using a luciferase assay kit (Promega, Madison, WI, USA).

2.8. Protein extraction and Western blotting
Total cell lysates or tissue homogenates were prepared by using a RIPA lysis buffer (Beyotime, China). The protein concentration was calculated with a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein were separated by electrophoresis in 10% sodium dodecyl sulphate–polyacrylamide gels before transfer onto polyvinylidene difluoride (PVDF) membranes (Millipore). The level of AHR protein was analysed by Western blotting with polyclonal anti-human AHR antibody (Abcam, Cambridge, MA, USA). The protein levels were normalized by probing the same blots with a tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Alpha Innotech (San Leandro, CA) imaging software was used to quantify Western blot data.

2.9. Cytokine enzyme-linked immunosorbent assays
Caco-2 cells or HT-29 cells were pretreated with pre-miR-124, anti-miR-124, scrambled negative control RNA and AHR siRNA for 24 h. Pretreated cells were then stimulated with LPS (1 µg/ml) or left untreated. The cytokines tumour necrosis factor-α (TNF-α), interleukin (IL) 6 (IL-6) and IL-1β were measured in culture supernatants using enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems, Minneapolis, MN, USA.

For the expression of TNF-α, IL-6 and IL-1β in the TNBS-induced colitis model, full-thickness colonic tissue specimens of mice were homogenized in PBS containing a cocktail of protease inhibitors (KeyGen Biotech, Nanjing, China) supplemented with 1 mM phenylmethylsulphonyl fluoride (PMSF) and then subjected to centrifugation to obtain the supernatants, which were finally tested for protein level with the BCA protein assay (Thermo Scientific). To measure these inflammatory cytokines, mouse double-antibody sandwich ELISA kits (R&D Systems) were developed. Absorbance was measured at 450 nm and compared with the respective standard curve of the cytokines.

2.10. Immunohistochemistry
Paraffin-embedded intestinal mucosal biopsy samples from 5 controls and 6 active CD patients were boiled in 10 mM sodium citrate buffer with 0.05% Tween 20 for 20 minutes to perform antigen retrieval. Endogenous peroxidase activity was blocked with peroxidase blocking solution for 15 minutes. Next, sections were blocked with 10% goat serum for 1.5 hours at room temperature and then incubated with primary antibody for AHR (Abcam) at 4°C overnight. Isotype-matched monoclonal antibody (Iso, anti-rabbit IgG mAb, Cell Signaling Technology, USA) with no specific affinity to proteins of interest was used as the negative control. The slides were subsequently incubated with horseradish peroxidase-labelled secondary antibody and developed with diaminobenzidine. Counterstaining was performed with haematoxylin.

2.11. Establishment of colitis model and design of treatment
Female BALB/c mice aged 7 weeks were purchased from the Laboratory Animal Center of Nanjing Medical University (Nanjing, China). All animals received appropriate care according to the requirements of the Animal Care and Use Committee of Nanjing Medical University.

Experimental colitis was induced with TNBS (Sigma) as published previously.21 Briefly, 3 mg of TNBS in 100 µl of 50% ethanol was slowly administered via a transrectal polyethylene catheter inserted 4 cm from the anus (n = 8). Mice were then kept in a vertical position for 30 s. An equivalent volume of 50% ethanol was instilled into control mice (n = 8). Polyetherimide (PEI, 25 kDa; Sigma)/miRNA precursors or inhibitor complexes were prepared by mixing equal volume of solution of 4 mg/ml PEI and 2 mg/ml miRNA precursors/inhibitors. To determine the effect of anti-miR-124 in TNBS colitis, mice were treated with PEI/anti-miR-124, PEI/scrambled miRNA, PEI alone or saline at a dose of 5 mg miRNA inhibitor/kg body weight 12 h after a 3-mg TNBS injection (n = 8 per group). When investigating the effect of miR-124 precursor (pre-miR-124) in TNBS colitis, 2 mg of TNBS was used to induce moderate colitis. After 12 h, the mice were given pre-miR-124 (5 mg miRNA precursors/kg body weight) (n = 8). The total volume of the solution intracolonically injected into mice was 100 µl. Mice were sacrificed on day 3 after induction of colitis.

2.12. Cell localization of miRNA inhibitors
Frozen colon sections from mice, harvested 24 h after Cy3-labelled miRNA inhibitor (GenePharma)/PEI complex administration, were first stained with a mouse antiamouse IEC marker (pan-cytokeratin [PCK]) antibody (Abcam) at 4°C overnight. The corresponding fluorescein isothiocyanate (FITC)-labelled goat antimouse (KPL, Gaithersburg, MD, USA) secondary antibody was applied at room temperature for 45 minutes, followed by nuclear 4,6-diamidino-2-phenylindole (DAPI; Sigma) staining.

2.13. Assessment of colonic inflammatory changes
The mice were observed daily for behaviour and body weight. On day 3, the colon was excised and cut into sections for macroscopic observation, histopathological study and immunofluorescence examination. The disease activity index (DAI) was used to evaluate the severity of intestinal inflammation based on weight loss, stool bleeding and stool consistency in accordance with a previously reported method.24 The DAI scores (range 0–4) for each parameter were summed for each mouse and each group. Histopathological studies were performed on paraffin-embedded colon sections stained with haematoxylin and
eosin. Histological evaluation was graded from 0 to 4 as described in a previous report. Additionally, intestinal tissues samples were evaluated by performing miRNA quantification, mRNA expression analysis, Western blotting and ELISA, as described above.

2.14. Statistical analysis

All the Western blotting images are representative of at least three independent experiments. The luciferase reporter assay and qRT-PCR were performed in triplicate, and each experiment was repeated several times. The data shown are the mean ± SD of at least three independent experiments. The differences were considered statistically significant at \( p < 0.05 \) using Student’s \( t \)-test.

2.15. Ethical considerations

This study was approved by the Institutional Review Board of the Affiliated Zhongda Hospital of Southeast University. All patients and normal control individuals signed a written informed consent form.

3. Results

3.1. Downregulation of AHR and upregulation of miR-124 in active CD tissues and IECs

We assessed AHR protein expression by Western blotting and found that expression was downregulated in active CD tissue samples compared with the normal controls (Figure 1A). However, unlike AHR protein expression, which was consistently downregulated in active CD tissues, AHR mRNA varied randomly between active CD tissues and normal controls (Figure 1B). Given the disparity between AHR mRNA and protein in CD intestinal tissues, it is quite likely that a post-transcriptional regulatory mechanism exists. Because miRNA is an important post-transcriptional regulator of gene expression, we hypothesized that miRNAs may be involved in the regulation of AHR expression. Based on the present understanding of miRNA expression in CD, we investigated whether a suite of inflammation-related miRNAs, including miR-146a, miR-146b, miR-145, miR-155, miR-124, miR-191, miR-106a, miR-200c, miR-143, miR-9, miR-21 and miR-150, were differentially expressed in active CD compared with normal controls by qRT-PCR. As shown in Figure 1C, miR-124 and miR-9 were upregulated in CD tissues relative to healthy subjects. The inverse correlation between miR-124 and AHR protein expression and the disparity between miR-124 and AHR mRNA levels was subsequently illustrated using Pearson correlation scatter plots (Figure 1D), whereas correlation between miR-9 and AHR was not detected (Supplementary Figure 1). To further quantify the cellular level of miR-124 and its differential expression in inflamed sites, epithelia of ileum and colon were isolated separately for qRT-PCR and Western blotting analysis (Figure 1E, F). As expected, the change in expression of miR-124 and AHR in sorted IECs from CD patients had the same trend as those in intestinal CD samples of CD patients compared with the controls. MiR-124 was expressed intensely in the inflamed colonic and ileac epithelial cells while AHR protein was barely detected when compared with the normal colonic and ileac epithelial cells. Furthermore, there was no significant difference between inflamed colon and ileum in either RNA or protein expression (Figure 1E, F). Immunohistochemistry analysis also revealed the downregulation of AHR protein in the inflamed colon and ileum mucosa of active CD patients compared with the normal controls, especially in the epithelial layer (Figure 1G).

Taken together, these results imply an miR-124 mediated post-transcriptional regulation of AHR expression in active CD.

3.2. Validation of human colonic AHR as a direct target of miR-124

Given the inverse correlation between miR-124 and AHR, we next used three algorithms (TargetScan, miRanda and PicTar) in combination to calculate whether human AHR is a putative target of miR-124. As shown in Figure 2A, a hybrid between AHR 3′-UTR and miR-124 was observed. The free energy value of this hybrid was −22.2 kcal/mol, which was well within the range of genuine miRNA–target pairs. Moreover, there was perfect base-pairing between the ‘seed’ (the core sequence that encompasses the first 2–8 bases of the mature miRNA) and cognate targets, and the seeds were exactly conserved across species. Thus, it is quite possible that human AHR is a direct target of miR-124.

We then determined whether the overexpression or knockdown of miR-124 had an impact on AHR expression. Overexpression of miR-124 was achieved by transfecting Caco-2 cells and HT-29 cells with pre-miR-124 (synthetic RNA oligonucleotides mimicking miR-124 precursors), while knockdown of miR-124 was performed by transfecting cells with anti-miR-124 (chemically modified antisense oligonucleotides designed to specifically target mature miR-124). As shown in Figure 2B, C, overexpression of miR-124 in Caco-2 cells strongly increased cellular miR-124 expression but decreased AHR protein levels in comparison with the pre-scramble control group. In contrast, compared with anti-scramble control, inhibition of miR-124 in Caco-2 cells decreased cellular miR-124 expression but increased AHR levels. In HT-29 cells miR-124 and AHR protein showed similar changes (Figure 2B, D). Although the miR-124 intracellular level was altered significantly after pre-miR-124 or anti-miR-124 treatment, overexpression or knockdown of miR-124 did not affect AHR mRNA stability in either cell line (Figure 2E). siRNA against AHR was transfected into the two cell lines to serve as a positive control. Efficient silencing of AHR was confirmed by Western blotting (Supplementary Figure 2).

To examine whether miR-124 directly targets the 3′-UTR of human AHR mRNA, a full-length segment of the AHR 3′-UTR containing a possible miR-124 complementary site was cloned into a luciferase reporter plasmid. The resulting plasmid was transfected into Caco-2 and HT-29 cells along with a control plasmid (β-gal) and pre-miR-124, pre-scramble control, anti-miR-124 and anti-scramble control. As expected, in both cell lines overexpression of miR-124 resulted in a reduction of approximately 50% in luciferase reporter activity compared with cells treated with the pre-scramble control, whereas in Caco-2 cells inhibition of miR-124 did not result in a significant increase in reporter activity compared with cells transfected with the anti-scramble control (Figure 2F). Furthermore, after mutating the nucleotides of the seeding sequence in the human AHR 3′-UTR, the inhibitory effect of pre-miR-124 and the promoting effect of anti-miR-124 on luciferase reporter activity were largely abolished (Figure 2F).

These results unequivocally demonstrate that miR-124 directly recognizes the 3′-UTR of AHR transcripts and regulates its protein expression at the post-transcriptional level, which fully conforms to the canonical mechanism of miRNA-mediated regulation.

3.3. MiR-124 promoted LPS-induced inflammatory response by suppressing AHR in vitro

We next focused on studying the role of miR-124 in regulating AHR. According to previous reports, mice injected with AHR ligand were protected against colitis induced by TNBS, DSS and T-cell transfer. This suggests that AHR has an anti-inflammation effect during the progression of inflammation.
Figure 1. Downregulation of aryl hydrocarbon receptor (AHR) and upregulation of miR-124 in active Crohn’s disease (CD) tissues and intestinal epithelial cells (IECs). (A) Representative Western blots (left panel) and quantitative analysis (right panel) of AHR protein levels in intestinal mucosal samples of active CD patients and normal controls (NCs). **p < 0.01 versus NC. (B) Expression of AHR mRNA in active CD and NC tissues was analysed by qRT-PCR and normalized to β-actin. (C) Expression of inflammation-related miRNAs in active CD and NC tissues was analysed by quantitative reverse transcription–polymerase chain reaction (qRT-PCR) and normalized to U6. ***p < 0.001 versus NCs. (D) Pearson’s correlation scatter plots of fold changes in miR-124 and AHR mRNA and protein. (E) Expression of miR-124 (left panel) and AHR mRNA (right panel) in IECs of colon and ileum were analysed by qRT-PCR. **p < 0.01. (F) Representative Western blots (left panel) and quantitative analysis (right panel) of AHR expression in IECs of colon and ileum. ***p < 0.001. All data are expressed as mean ± SEM. (G) Representative immunohistochemical analysis (left panel) and quantitative data (right panel) showing AHR protein expression in colon and ileum mucosa of active CD tissues and controls. No positive expression was detectable in sites labelled with isotype-matched control monoclonal antibody. Original magnification x400. Scale bars = 50 μm. ***p < 0.001 versus NCs.

Figure 2. Identification of aryl hydrocarbon receptor (AHR) as a target of miR-124. (A) Schematic description of conserved binding site for miR-124 and human AHR. (B) quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis of miR-124 expression after transfection with pre-miR-124, pre-scramble, anti-miR-124, and anti-scramble in Caco-2 cells and HT-29 cells. ***p < 0.001. (C) Western blotting analysis of AHR protein expression level after transfection with pre-miR-124 or pre-scramble (left panel), and anti-miR-124 or anti-scramble (right panel) in Caco-2 cells. **p < 0.01. (D) Western blotting analysis of AHR protein expression level after transfection with pre-miR-124 or pre-scramble (left panel), and anti-miR-124 or anti-scramble (right panel) in HT-29 cells. **p < 0.001. (E) qRT-PCR analysis of AHR mRNA expression after transfection in Caco-2 cells and HT-29 cells. (F) Luciferase reporter activity in Caco-2 cells (left panel) and HT-29 cells (right panel). Luciferase reporters carrying wild-type (WT) or mutant (Mut) AHR 3’-UTR were cotransfected into both cell lines along with the indicated oligonucleotides. ***p < 0.001. All values are expressed as mean ± SEM.

First, we stimulated Caco-2 cells and HT-29 cells with LPS to induce inflammation, and assessed AHR and miR-124 expression at 6, 12, 24, 48 h (Figure 3A–C). The influence of LPS stimulation differed between AHR expression and miR-124 level. While the mRNA and protein expression of AHR were decreased in both cell lines after LPS stimulation, the miR-124 level was increased at the
same time, which suggested that LPS stimulation could reduce AHR RNA expression in an miR-124 independent way. The changes in AHR and miR-124 were not only negatively correlated but also time-varying.

We then sought to investigate whether miR-124 can modulate inflammatory process by targeting AHR in LPS-stimulated cells. First, cells pre-transfected with pre-miR-124, pre-scramble control, anti-miR-124, anti-scramble control and AHR siRNA were stimulated with LPS for the indicated times. MiR-124 overexpression and AHR knockdown significantly inhibited AHR protein expression in both cell lines (Figure 3D). In contrast, miR-124 knockdown prominently upregulated AHR expression in HT-29 cells, whereas a slight but non-significant elevation of AHR protein level was detected in Caco-2 cells (Figure 3E). To further determine the function of miR-124 in IECs during intestinal inflammation, we examined the production of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in human intestinal enterocyte-like Caco-2 cells. As shown in Figure 3F, pretreatment with pre-miR-124 and AHR siRNA significantly increased the expression of the 4 pro-inflammatory cytokines (TNF-α, IL-1β and IL-6). Pretreatment with anti-miR-124 decreased the release of TNF-α and IL-6, while the level of IL-1β did not change apparently.

These results demonstrate that overexpression of miR-124 upregulates the expression of pro-inflammatory cytokines by suppressing AHR protein level, which consequently aggravates the inflammation induced by LPS.

3.4. Anti-miR-124 treatment ameliorates TNBS-induced colitis by suppressing AHR

We first established TNBS-induced colitis as a murine model of CD to determine whether miR-124 and AHR are differentially expressed in colitis. The time-course analysis found that the expression of miR-124 in was strikingly elevated in mouse colon following TNBS treatment, and the colonic AHR protein level was significantly decreased accordingly (Supplementary Figure 3A, B). To confirm whether anti-miR-124 has an anti-inflammatory effect on CD, we then administered anti-miR-124 to mice intracolonically. To identify the cellular localization of miR-124 inhibitors after anti-miR-124 administration, we performed immunofluorescence staining with an IEC marker (PCK) on frozen sections prepared from colons of Cy3 miRNA inhibitor-treated mice. As shown in Figure 4A, anti-miR-124 was predominantly expressed in IECs (PCK cells). Anti-miR-124 treatment resulted in prominent protection from colitis as assessed by DAI values, macroscopy, colon length and histopathological damage of the colon. Mice treated with anti-miR-124 experienced a dramatic decrease in DAI (Figure 4B). After anti-miR-124 treatment, a striking improvement of macroscopic inflammation and an appreciable increase in colon length became apparent (Figure 4C). Histopathological examination showed marked crypt architecture damage, inflammatory cell infiltration and ulceration in TNBS-induced colitis colon, especially in the epithelial layer. However, anti-miR-124 treatment significantly improved this damage (Figure 4D). The histological scores confirmed the anti-inflammatory effect of anti-miR-124 (Supplementary Figure 3C). These results suggest that anti-miR-124 expression is protective against colitis induced by TNBS.

To study the possible relationship between miR-124 and AHR in the pathogenesis of colitis, we examined AHR expression after intra-colonic administration of anti-miR-124, anti-scramble or 30% ethanol. As shown in Figure 4E, F, miR-124 was significantly increased and AHR protein was reduced in epithelial cells of murine colitis colon. After intra-colonic administration of anti-miR-124, AHR protein expression was increased dramatically in TNBS-induced colitis colon and epithelial cells but was barely detected in immune cells (Figure 4F, Supplementary Figure 3D), suggesting that miR-124 and AHR are predominantly expressed in epithelial cells of mouse colon rather than colonic immune cells, but in different ways.
there was no significant alteration in AHR mRNA before and after treatment with anti-miR-124 in murine colon and IECs, which accords with the results observed in CD patients and in vitro experiments (Supplementary Figure 3E). Furthermore, the expression of TNF-α, IL-1β, IL-6 and IL-22 in TNBS-induced colitis decreased significantly after intra-colonic administration of anti-miR-124 (Supplementary Figure 3F).

Taken together, these results demonstrate that anti-miR-124 enhances AHR protein expression, diminishes pro-inflammatory cytokine production in TNBS-induced colitis, and generates a therapeutic effect in intestinal inflammation.

3.5. Pre-miR-124 aggravates TNBS-induced colitis

To confirm the role of anti-miR-124 in TNBS-induced colitis, we next administered pre-miR-124 to mice that had been given a low dose of TNBS to induce moderate colitis. Colon pictures and histopathological and DAI examinations showed that mice given pre-miR-124 developed more severe colitis (Figure 5A–D), which was associated with enhanced expression of pro-inflammatory cytokines (Supplementary Figure 4). Pre-miR-124 further increased colonic and epithelial miR-124 levels and reduced AHR protein expression (Figure 5E, F).

Together, these results confirm that pre-miR-124 induces a reduction in AHR in IECs and consequently a severe colitis.

4. Discussion

During recent decades, studies in experimental models of IBD have shown that activation of the intestinal AHR pathway can attenuate inflammatory responses in the gut, but there is little knowledge about the regulation of AHR in colonic inflammatory conditions. Since the investigation of miRNAs in both the innate and the adaptive immune system, significant effort has been devoted to analysing the expression of miRNAs in autoimmune diseases and understanding their potential role in their pathogenesis, diagnosis and treatment.

2 In the present study, we examined the expression of AHR and the functional role of AHR in CD. While there was a diminished AHR protein level in active CD patients compared with normal controls, miR-124 expression was significantly increased in CD patients. There was an inverse correlation between miR-124 and AHR protein expression. Furthermore, by using computational bioinformatics we identified a conserved miR-124 binding site in the AHR 3’-UTR. We evaluated the expression of the AHR after knocking down or overexpressing miR-124, and there was a clear inverse correlation between miR-124 level and AHR protein expression in both cell lines. We further substantiated that the AHR was a target of miR-124 by detecting the low activity of a luciferase reporter containing the AHR 3’-UTR in cells with high miR-124 expression.

3 miR-124 was first thought to be a brain-specific miRNA. This conserved miRNA can regulate neuron differentiation, neuron activation, neuronal development and neural plasticity. It is also involved in many inflammatory and autoimmune diseases, miR-124 is a key regulator of microglia quiescence in the central nervous system and suppresses experimental autoimmune encephalomyelitis by deactivating macrophages via the C/EBP-α-PU.1 pathway. Another study showed that miR-124 plays a key role in regulating the proliferation and chemokine production of fibroblast-like synoviocytes in rheumatoid arthritis patients. Recently, miR-124 has been found to be downregulated in colon tissue of paediatric patients with UC. It appears that a reduced level of miR-124 could promote inflammation in UC in children by regulating the expression of signal transducer and activator of transcription 3 (STAT3).
Therefore, it is reasonable to hypothesize that miR-124 is a potential post-transcription regulator of AHR expression during the colonic inflammatory process in CD patients.

The gastrointestinal tract is continuously exposed to a variety of antigens, including enteric bacteria. To maintain the homeostasis of the gut without development of intestinal inflammation, the intestine possesses an immune system that suppresses excessive immune responses against antigen stimulation. Gut immunity is composed of acquired and innate immunity, mediated by IECs, for example. These cells not only create a physical barrier to prevent invasion of pathogens and the influx of antigens, but also facilitate interactions between commensals and immune cells. They act as sensors for antigens through some immune receptors, such as toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors. TLR signalling pathways can induce the production of pro-inflammatory cytokines and chemokines in IECs to recruit immune cells, which is important in the pathogenesis of IBD. LPS is an important structural component of the outer membrane of Gram-negative bacteria, and has been hypothesized to be an important risk factor for IBD. It interacts with TLR4 and subsequently activates nuclear factor κB (NF-κB) through a myeloid differentiation factor 88 (MyD88)-dependent pathway, leading to the production of various pro-inflammatory cytokines, including TNF-α and IL-6. However, AHR has been found to negatively regulate inflammatory responses mediated by LPS in macrophages and IECs. Masuda et al. showed that LPS-induced production of pro-inflammatory cytokines was significantly increased in AHR-deficient macrophages compared with that in wild-type (WT) cells. They found that AHR formed a complex with STAT1, and the complex may control LPS-induced inflammatory responses by inhibiting transcriptional activity of NF-κB. A study by Kimura et al. demonstrated that AHR also suppressed the expression of pro-inflammatory cytokine IL-6 by inhibiting histamine production in LPS-stimulated macrophages. Furthermore, Furumatsu and colleagues found that AHR down-regulation in SW480 cells enhanced LPS-evoked inflammatory responses and that AHR activation attenuated the inflammatory responses induced by LPS.

To determine the role and molecular mechanism of miR-124 in CD by targeting AHR, we used LPS-stimulated cells as an in vitro inflammatory model of the human intestinal epithelium. The changes in AHR and miR-124 were negatively correlated in LPS-stimulated cells. The mRNA and protein expression levels of AHR were decreased while the level of miR-124 was increased, which suggested that the anti-inflammatory effect of AHR might be inhibited by miR-124 and that LPS stimulation might reduce AHR mRNA expression in an miR-124-independent way. In the present study, we further demonstrated that miR-124 overexpression enhanced the secretion of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in LPS-stimulated Caco-2 cells by suppressing AHR expression. On the contrary, knockdown of miR-124 inhibited inflammatory responses triggered by LPS. Our data indicated that miR-124 played a pro-inflammatory role in LPS-induced inflammatory responses by suppressing AHR. In other words, inhibiting miR-124 provides a promising candidate for anti-inflammatory therapies. Experiments on the murine model of TNBS-induced colitis further validated this conclusion. In the current study in vivo, we demonstrated the inhibitory effects of anti-miR-124 and promoting effects of pre-miR-124 in IECs but not in the intestinal immune cells during the inflammatory process in TNBS-induced colitis.

In summary, our study shows that upregulation of miR-124 expression and downregulation of AHR protein level may serve as a molecular signature of CD. The pathway of miR-124 targeting AHR plays an important role in the development of intestinal inflammation. Knockdown of miR-124 suppresses inflammation in LPS-stimulated cells and TNBS-induced colitis by downregulating AHR. Therefore, inhibiting colonic miR-124 expression provides a
new and bright prospect for a promising therapeutic target in the clinical treatment of CD.

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Conflict of Interest
The authors have declared that no competing interests exist.

Author Contributions
YZ, TM and WC performed the experiments, analysed the data and wrote the manuscript; YC, ML, LR and JC participated in some of the experiments; RC and YF contributed reagents/materials/analysis tools; HZ provided vital guidance to the study; RS conceived the study and designed and supervised the experiments. YZ, TM and WC contributed equally to this study and share first authorship. All the authors read and approved the final manuscript.

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