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ARTICLE

Baicalein ameliorates ulcerative colitis by improving intestinal epithelial barrier via AhR/IL-22 pathway in ILC3s

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Ulcerative colitis (UC) is a chronic inflammatory disease of the gastrointestinal tract, which is closely related to gut barrier dysfunction. Emerging evidence shows that interleukin-22 (IL-22) derived from group 3 innate lymphoid cells (ILC3s) confers benefits on intestinal barrier, and IL-22 expression is controlled by aryl hydrocarbon receptor (AhR). Previous studies show that baicalein protects the colon from inflammatory damage. In this study we elucidated the molecular mechanisms underlying the protective effect of baicalein on intestinal barrier function in colitis mice. Mice were administered baicalein (10, 20, 40 mg·kg^{-1}·d^{-1}, i.g.) for 10 days; the mice freely drank 3% dextran sulfate sodium (DSS) on D1–D7 to induce colitis. We showed that baicalein administration simultaneously ameliorated gut inflammation, decreased intestinal permeability, restored tight junctions of colons possibly via promoting AhR/IL-22 pathway. Co-administration of AhR antagonist CH223191 (10 mg/kg, i.p.) partially blocked the therapeutic effects of baicalein in colitis mice, whereas AhR agonist FICZ (1 μg, i.p.) ameliorated symptoms and gut barrier function in colitis mice. In a murine lymphocyte line MNK-3, baicalein (5–20 μM) dose-dependently increased the expression of AhR downstream target protein CYP1A1, and enhanced IL-22 production through facilitating AhR nuclear translocation, these effects were greatly diminished in shAhR-MNK3 cells, suggesting that baicalein induced IL-22 production in AhR-dependent manner. To further clarify that, we constructed an in vitro system consisting of MNK-3 and Caco-2 cells, in which MNK-3 cell supernatant treated with baicalein could decrease FITC-dextran permeability and promoted the expression of tight junction proteins ZO-1 and occluding in Caco-2 cells. In conclusion, this study demonstrates that baicalein ameliorates colitis by improving intestinal epithelial barrier via AhR/IL-22 pathway in ILC3s, thus providing a potential therapy for UC.

Keywords: ulcerative colitis; baicalein; epithelial barrier; group 3 innate lymphoid cells; interleukin-22; aryl hydrocarbon receptor; CH223191; FICZ

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INTRODUCTION

Ulcerative colitis (UC) is characterized by mucosal inflammation starting from the rectum and rapidly spreading to the proximal colon, which usually appears in the mucosal layer and damages the intestinal wall [1, 2]. Intestinal barrier function is a sensitive prognostic indicator of UC patients [3]. When the intestinal barrier is disrupted, microbes will cross from the lumen into the lamina propria, inducing an excessive immune response and aggravating inflammation [4]. Hence, in the treatment of UC, it is essential to repair the compromised intestinal mucosa in addition to administering antibiotics and anti-inflammatory treatment [5].

The regeneration and reconstruction of the intestinal epithelium mainly depend on interleukin-22 (IL-22). IL-22 has two main functions: (1) strengthen the intestinal barrier by promoting intestinal tight junctions [6]; and (2) increase antimicrobial peptides and mucins to reinforce mucosal defense [7]. Therefore, promotion of IL-22 production is essential to epithelial repair.

IL-22 is mainly produced by helper T cells (Th1, Th17 and Th22 cells), group 3 innate lymphoid cells (ILC3s), etc., of which ILC3s are the primary source and regulated by aryl hydrocarbon receptor (AhR) [8, 9]. As a ligand-dependent transcription factor, AhR remains inactive, combined with several chaperone complexes in the cytoplasm. Once an agonist binds to AhR, the AhR–ligand complex shuttles to the nucleus, interacts with xenobiotic response elements (XRE) and initiates the transcriptional regulation of downstream target genes. Moreover, AhR may synergize with RORγt to induce IL-22 transcription [10]. The relevance of AhR to inflammatory bowel disease (IBD) has been extensively described elsewhere. AhR expression was decreased in the ILC3s of inflammatory intestinal tissues [11]. It was reported that the AhR agonist 6-formylindolo[3,2-b] carbazole (FICZ) directly activated AhR in intestinal epithelial cells (IECs), thereby reducing intestinal permeability and preserving the epithelium [12]. Although AhR is critical in regulating the intestinal barrier, most acknowledged AhR ligands have various toxicities [13, 14], limiting their druggability. There is an urgent need to find safe AhR ligands for the treatment of UC.

Baicalein, derived from *Scutellaria baicalensis* Georgi, is metabolized from baicalin by gut microbiota. The oral bioavailability of baicalein (27.8%) is much higher than that of baicalin (2.2%) owing

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to its fat solubility and hydrophobicity. The absorption rates of baicalein in stomach, small intestine and colon of rats were respectively 34.53%, 30.61% and 4.89% [15]. Additionally, baicalein is widely distributed in multiple organs after being absorbed into the blood [16, 17].

Various pharmacological activities of baicalein such as anti-inflammatory and the repair of the intestinal epithelium have been proved [18]. Baicalein also restores the intestinal epithelial barrier of mice induced by ovalbumin (OVA) sensitization [19]. Simultaneously, baicalein is a potential ligand of AhR [20]. In the present study, we worked to investigate anti-colitis efficacy of baicalein, and to explore whether and how AhR/IL-22 mediate the protective effects of baicalein on intestinal barrier.

MATERIALS AND METHODS

Drugs and reagents

Baicalein (98% purity, S25956) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). FICZ (HY-12451) as an AhR agonist was purchased from Medchem Express Co., Ltd (Monmouth Junction, USA). CH223191 (A8609) as an AhR inhibitor was purchased from APExBIO (Houston, USA). DSS (160110) was purchased from MP Biomedicals (Irvine, USA). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS, 42F2362K) were purchased from Gibco (Grand Island, USA). Percoll (BS909-100mL) was purchased from Biosharp (Hefei, China). Nuclear protein extraction kit (R0050) and Luria–Bertani agar (L1015) were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). AG RNAex Pro Reagent (AG21102), SYBR® Green Premix Pro Taq HS qPCR kit (AG11701) and Evo M-MLV RT Kit with gDNA Clean (AG11705) for quantitative polymerase chain reaction (qPCR) were purchased from Accurate Biotechnology Co., Ltd. (Changsha, China). Mouse IL-22 ELISA Kit (EMC119) was purchased from Neobioscience Technology Company (Shenzhen, China). Anti-β-actin antibody (BM0627) was purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). APC anti-mouse IL-22 antibody (516409) and Alexa Fluor® 700 anti-mouse CD45 antibody (103127) were purchased from Biolegend (San Diego, USA). Foxp3/transcription factor staining buffer set (00-5523-00) and PE anti-mouse RORγt antibody (2054914) were purchased from Thermo Fisher Scientific (Waltham, USA). Purified rat anti-mouse CD16/CD32 (553141) was purchased from BD Pharmingen (Santiago, Chile). Anti-mouse IgG (Alexa Fluor® 488 Conjugate, 4408) was purchased from Cell Signaling Technology (Beverly, USA). Cy3-labeled goat anti-rat IgG (A0507) was purchased from Beyotime Biotechnology (Shanghai, China). Antibodies against Zona occludens (ZO-1, sc-33725), occludin (sc-133256), AhR (sc-133088), and cytochrome P450 (CYP) 1A1 (sc-393979) were purchased from Santa Cruz Biotechnology (Dallas, USA).

Induction of colitis murine model and administration of drugs

Male C57BL/6 mice (6–8 weeks), weighing 20–22 g, were purchased from SPF Biotechnology Co., Ltd. (Beijing, China). All mice were housed in a specific pathogen-free facility in the Guangzhou University of Chinese Medicine (Guangzhou, China), at standard temperature (22 ± 2 °C), and humidity (55% ± 2%) and maintained under a 12 h light/dark cycle. All animal experiments were conducted according to the International Ethics Guidelines and the National Institutes of Health Guidelines Concerning the Care and Use of Laboratory Animals. The experiments were approved by the Experimental Animal Ethics Committee, School of Pharmaceutical Sciences, Guangzhou University of Chinese Medicine.

In this study, C57BL/6 mice were randomly divided into the following groups: control group, model group, baicalein group (BL-10 mg/kg, BZ-20 mg/kg and BH-40 mg/kg), FICZ group (AhR agonist, 1 μg per mouse), and CH223191 (AhR inhibitor group, 10 mg/kg) + baicalein (40 mg/kg) group (CH + B). Except for the control group, the mice in the other groups drank 3% DSS freely for 7 days. Meanwhile, mice in the control and model groups were orally administered deionized water. Mice in the baicalein group were orally administered baicalein dissolved in deionized water (10, 20 and 40 mg/kg). Mice in the FICZ group were intraperitoneally injected FICZ. Mice in the CH + B group were intraperitoneally injected CH22319 and orally administered baicalein for 10 days. On the 11th day, all the mice were euthanized (Fig. 1a).

Pharmacodynamic study of baicalein on colitis mice

Peripheral blood was collected for detecting the proportion of blood cells using an auto hematology analyzer (BC-2800 Vet) on day 11. The length of the colons was measured. A segment of colon was fixed in 4% paraformaldehyde for at least 48 h, then embedded with paraffin, sliced into 4 μm slices, stained with haematoxylin-eosin (H&E), and finally observed using a microscope. Spleens and thymus were removed to calculate organ indexes. The following formula was implemented: index of spleen = wet weight of spleen (mg) × 10/bodyweight (g); Index of thymus = wet weight of thymus (mg) × 10/bodyweight (g).

Bacterial culture

Spleens and mesenteric lymph nodes (MLNs) of mice were separated on day 11, and then were ground mechanically at 4 °C for 1 min. The tissue homogenate was added into Luria–Bertani agar and cultured at 37 °C for 12 h. In the end, the number of bacterial colonies in each group was calculated.

Western blot

The colons were ground with a homogenizer and cleaved by RIPA lysate to obtain the whole protein. Cells were cleaved with the nuclear protein extraction kit to obtain cytoplasmic protein and nucleoprotein. Next, proteins were denatured and then separated by SDS-PAGE gel electrophoresis. After that, protein samples in the polyacrylamide gel were transferred to a polyvinylidene fluoride (PVDF) membrane at 300 mA for 2 h. Afterwards, PVDF membrane was sealed with 5% non-fat powdered milk for 2 h and incubated with anti-AhR antibody and anti-CYP1A1 antibody at 4 °C overnight. The next day, PVDF membrane was incubated with goat anti-mouse IgG/HRP at room temperature for 1.5 h. Finally, the PVDF membrane was washed three times and imaged using a chemiluminescence imaging apparatus (Tanon 5200, Shanghai, China).

Fluorescence in situ hybridization (FISH)

Paraffin sections of colons were collected for performing FISH targeting the bacterial 16S rDNA. The tissues were then sealed with 200 μL blocking buffer at 55 °C for 2 h. Subsequently, the tissues were hybridized with the denatured probe. After hybridization, paraffin sections were washed with washing buffer and dehydrated with 70%, 85%, and 100% ethanol for 2 min, in turn. Finally, sections of colons were dyed with DAPI and observed using a confocal laser scanning microscope.

Small animal imaging

All mice were fasted for 12 h prior to dissection, and they were orally given 50 mg/kg 4 kDa fluorescein isothiocyanate dextran (FITC-dextran, FD4). After 4 h, the distribution of FITC-dextran in mice was observed using a small animal imaging system (NIGHT OWL LB 983, Germany).

The peripheral blood of mice was collected and centrifuged (3000 r/min, 4 °C, 10 min). Then, 100 μL serum was obtained from the supernatant and added to a 96-well plate. Finally, the fluorescence intensity of serum was measured using a fluorescence microplate reader (excitation wavelength: 480 nm, emission wavelength: 520 nm).

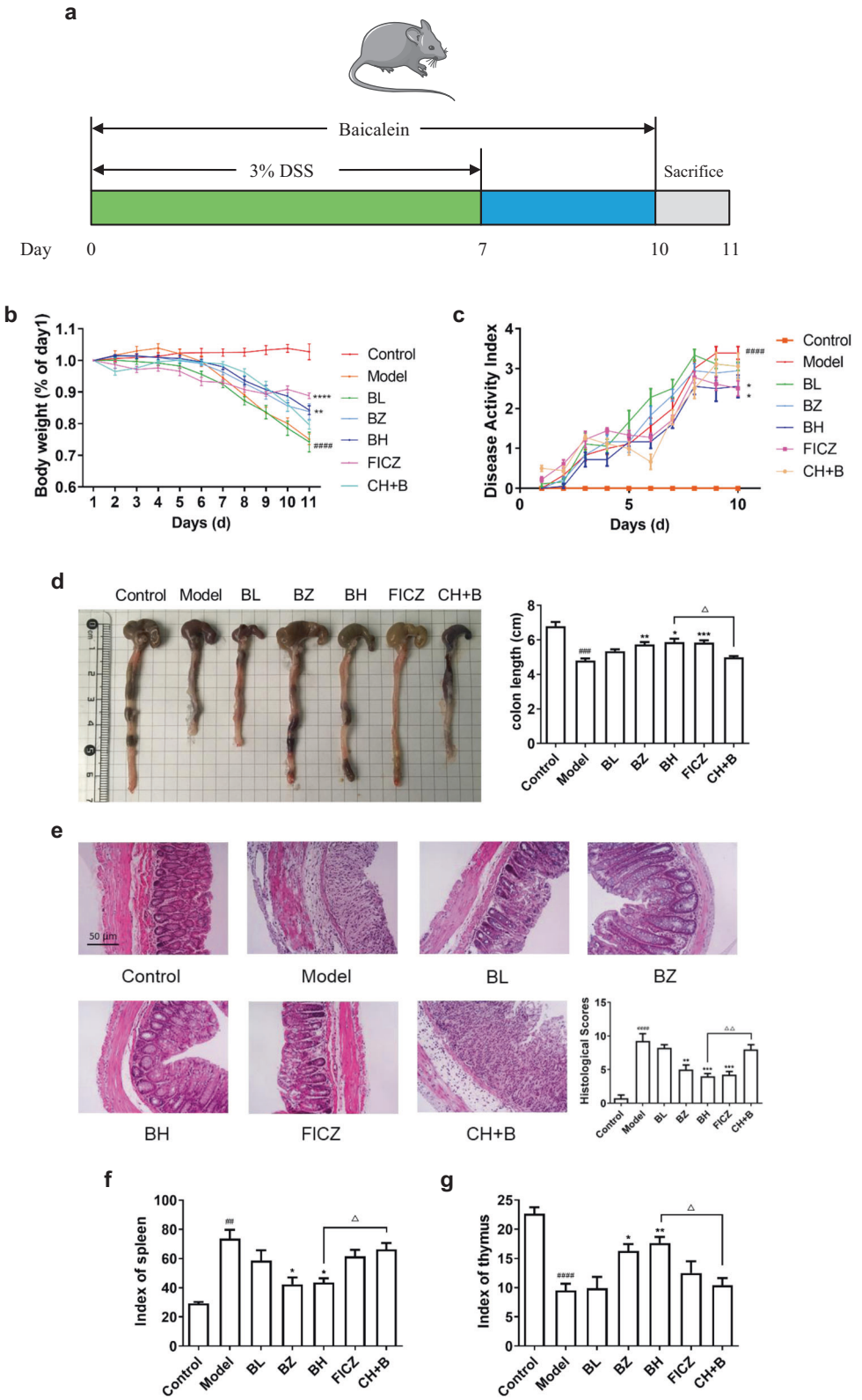

Fig. 1 Baicalein ameliorated DSS-induced mice and the effect was dependent on AhR. a Establishment of murine colitis model and administration of baicalein. **b** Body weight change. **c** DAI scores. **d** Length of colons. **e** Haematoxylin and eosin staining of colon (200×). **f** Index of spleen. **g** Index of thymus, $n = 6$ in each group. BL, low dose of baicalein (10 mg/kg); BZ, medium dose of baicalein (20 mg/kg); BH, high dose of baicalein (40 mg/kg); FICZ, AhR agonist; CH + B, CH223191 (AhR antagonist) + baicalein (40 mg/kg). $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.001$ vs. the control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the model. $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$ vs. the BH.

Immunofluorescence

Frozen sections of colons were fixed in acetone for 20 min. Then, 10% goat serum was used for sealing the sections for 1.5 h. After that, the tissues were incubated with anti-ZO-1 antibody (1:100) and anti-occludin antibody (1:250) overnight at 4 °C. Subsequently, frozen sections were incubated with anti-mouse IgG (Alexa Fluor® 488 Conjugate) and anti-rat IgG (Cy3 Conjugate) in the dark at room temperature for 60 min. After incubation, the tissues were incubated with 30 μL DAPI in the dark at room temperature for 10 min. In the end, frozen sections were observed using a confocal laser scanning microscope.

Extraction of colonic lamina propria lymphocytes and flow cytometry analysis

The Lineage Cocktail contains the following FITC-conjugated antibodies: anti-mouse CD3, anti-mouse CD11b, anti-mouse B220, anti-mouse Ly-6G, and anti-mouse TER-119. Lineage$^-$ and CD45$^+$ cells in the lamina propria are usually labeled as ILCs, of which RORγt$^+$ cells are classified as ILC3s [21]. In our study, Lineage$^-$ CD45$^+$ RORγt$^+$ cells were represented as ILC3s, and the ratio of IL-22$^+$ ILC3s was evaluated.

Colonic tissues were cut into small pieces and digested with 5 mL digestive solution at 37 °C for 2 × 20 min, followed by digestion with 3 mL collagenase IV solution (1 mg/mL) at 37 °C for 60 min. Then, the tissue suspension was passed through a 100 μm and 70 μm screen. The filtrate was washed and resuspended with 3 mL RPMI-1640 complete medium. After that, 3 mL 80% percoll, 3 mL 40% percoll, and 3 mL cell suspension were slowly added to a tube in turn. Finally, lamina propria lymphocytes were isolated using density gradient centrifugation (800 × *g*, 20 min, 20 °C).

Colonic lamina propria lymphocytes were sealed by CD16/CD32 (1:100) at 4 °C for 5 min. After that, lymphocytes were incubated with Lineage-FITC and CD45-Alexa Fluor® 700 antibody at 4 °C for 30 min. Then, lymphocytes were fixed and permeabilized with fixation/permeabilization working solution at room temperature in the dark for 40 min. Afterwards, lymphocytes were incubated with RORγt-PE at room temperature in the dark for 30 min. Finally, colonic lamina propria lymphocytes were detected using a flow cytometer (BD LSRFortessa, USA).

Cell culture

We used an MNK-3 cell line, which expressed the same signaling transcription factors, cytokines, adhesion molecules, and chemokine receptors as ILC3. They can proliferate and passage in vitro for the study of physiological characteristics. Thus, it is an effective cell model for studying ILC3s [22].

MNK-3 cells, which were purchased from Bluefbio Biology Technology Development Co., Ltd. (Shanghai, China), were cultured with DMEM containing 10% FBS and 1% penicillin/streptomycin solution in 5% CO_2 at 37 °C. Caco-2 cells, which were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), were cultured with DMEM containing 10% FBS, 1% penicillin/streptomycin solution, and 1% non-essential amino acids solution in 5% CO_2 at 37 °C.

MNK-3 cells were divided into 5 groups including control group, baicalein groups at three doses (5, 10 and 20 μM) and FICZ group (400 nM). All drugs were incubated with MNK-3 cells for 24 h.

Cell transfection

MNK-3 cells were seeded in a 48-well plate. Different concentrations of lentivirus that carried green fluorescent protein and two types of assistant transfection reagents (Histrans G A/P) were mixed to transfect cells for 12 h, followed by culturing in DMEM for 72 h. The infection condition was optimized according to the maximum fluorescence intensity detected by flow cytometry (BD Accuri C5, USA). After lentivirus transfection, the transfected cells were cultured in DMEM containing a suitable concentration of puromycin (the minimum concentration to kill normal MNK-3 cells) for purification.

Table 1. Primer sequences.

Gene	Primer sequences
AHR-F (mouse)	5′-ATGGAGAGGTGCTTCAGGTGCCG-3′
AHR-R (mouse)	5′-ATGGAGGGGTGGCTGAAGTGGAGT-3′
CYP1A1-F (mouse)	5′-TCTGTGCCATTTGCTTTGGC-3′
CYP1A1-R (mouse)	5′-AGGCATTCAGGGAAGGGTTG-3′
IL-22-F (mouse)	5′-CATGCAGGAGGTGGTACCTT-3′
IL-22-R (mouse)	5′-CAGACGCAAGCATTTCTCAG-3′
GAPDH-F (mouse)	5′-GGTTGTCTCCTGCGACTTCA-3′
GAPDH-R (mouse)	5′-TGGTCCAGGGTTTCTTACTCC-3′

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from MNK-3 cells was extracted by Ag RNAEX pro reagent. Then, DNA intermingled with total RNA was removed and the RNA was reverse-transcribed to cDNA by EVO M-MLV RT Kit. In the end, cDNA was mixed with primer and 2× SYBR® Green Pro Taq HS and amplified using PCR amplifier (Bio-Rad T100). The primer sequences used were listed in Table 1.

ELISA

The content of IL-22 derived from MNK-3 cell supernatant or homogenate of murine colonic tissues was detected using the mouse IL-22 ELISA kit according to the product manual.

Construction of an in vitro culture system consisting of MNK3 and Caco2 cells

Caco-2 cells were seeded in a transwell chamber (6.5 mm) with 0.4 μm polyester film and cultured for 13 days to allow the formation of tight junctions [23]. Caco-2 cells were stimulated with lipopolysaccharide (LPS) for 24 h to establish an epithelial barrier injury model. MNK-3 cells were treated with 5, 10, and 20 μM baicalein for 24 h. Then, MNK-3 cells were cultured in the fresh medium without baicalein for another 24 h. Afterwards, the supernatant of MNK-3 cells was collected to treat the Caco-2 cells stimulated by LPS. Finally, FITC-dextran flux and tight junction protein expression were observed to evaluate the epithelial barrier function of Caco-2 cells.

FITC-dextran (0.1 mg/mL) was added to the upper side of the transwell chamber and incubated for 2 h. The FITC-dextran content in the lower side was measured using fluorescence spectroscopy.

Molecular docking

The molecular structures of baicalein and AhR (PDB ID: 5V0L) were constructed and preprocessed using Autodock Tools 1.5.6. Then, the central location, length, width and height of the Grid Box were determined according to the interaction between baicalein and AhR. Afterwards, molecular docking was performed by Autodock 1.5.6, and the docking results were visualized using PyMOL 2.1 to analyze the interaction between baicalein and the active site in AhR. Finally, Lamarckian was used to calculate the binding free energy.

Statistical analysis

All data were statistically analyzed using GraphPad Prism 8.3 software (San Diego, USA). Firstly, all data were tested for normality. If the data did not conform to normal distribution, non-parametric test was used. If the data conformed to normal distribution, all data were tested for equal standard deviations (SDs). Then, if the SDs were not equal, Dunnett's T3 test was used for comparison

among the groups. Finally, if the SDs were equal, Tukey's test was used for comparison among the groups.

RESULTS

Baicalein ameliorated DSS-induced colitis mice

To determine the protective effect of baicalein against UC, several conventional indications were examined. Basically, mice treated with baicalein exhibited less weight loss (Fig. 1b), decreased disease activity index (DAI) (Fig. 1c), with downregulated spleen index (Fig. 1f) and upregulated thymus index (Fig. 1g). Baicalein alleviated the decrease of colon length (Fig. 1d) and restored abnormal peripheral blood cell proportion induced by DSS (Supplementary Fig. S1a–d). In addition, administration of baicalein remarkably relieved pathological changes in colons including colonic epithelial cells desquamating, inflammatory cells infiltration, extensive edema in the muscle layer, and increased pathological tissue score (Fig. 1e). Results above-described indicated baicalein's protective effects on intestinal epithelium against UC.

Baicalein improved intestinal barrier structure and function in colitis mice

As intestinal barrier dysfunction has been widely accepted as distinctive feature of UC, we then evaluated intestinal permeability and gut barrier function in colitis mice. In DSS-induced colitis mice, a large number of bacteria were cultivated from spleens and MLNs (Fig. 2a, b). Many bacteria infiltrated the intestinal epithelium, lamina propria, and even the muscular layer (Fig. 2c). After treatment with baicalein, the above indices were mitigated. In addition, compared with the untreated ones, the baicalein-treated colitis mice showed increased expression of tight junction proteins ZO-1 and occludin (Fig. 2d). Baicalein also prevented the FITC-dextran leakage (Fig. 2e) and elevated levels of serum FITC-dextran challenged with DSS (Fig. 2f). Collectively, those above indicated that baicalein could reduce intestinal permeability and protect the gut barrier in colitis.

Baicalein regulated AhR/IL-22 pathway in ILC3s of colitis mice

To explore the underlying mechanisms of baicalein's protective effects on intestinal epithelium, we checked the AhR/IL-22 pathway in ILC3s of colitis mice. The expression of AhR downstream target protein CYP1A1, proportion of IL-22$^+$ ILC3s in colonic lamina propria, and IL-22 content in colon dramatically decreased in colitis mice (Fig. 3a–c). These changes above were restored after baicalein treatment, which suggested that baicalein might attenuate colitis by upregulating AhR/IL-22 pathway in ILC3s.

Protective effects of baicalein on colitis were dependent on AhR

To further examine whether the protective effects of baicalein on the gut barrier integrity are mediated by AhR, we treated colitis mice with the AhR agonist FICZ, or AhR antagonist CH223191, based on an examination of the pharmacodynamic indicators, gut barrier and AhR/IL-22 pathway (Figs. 1–3). We determined that CH223191 partially blocked the therapeutic effects of baicalein in colitis mice. Nevertheless, FICZ ameliorated symptoms and gut barrier function in colitis mice (Figs. 1–3). Together, these studies suggested that baicalein conferred positive effects on intestinal epithelial barrier function in DSS-induced colitis mice, which depended on AhR.

Baicalein activated AhR/IL-22 pathway in MNK-3 cells in vitro

The MNK-3 cell line, a murine lymphocyte line that expresses the same signaling transcription factors and cytokines as ILC3, was used to evaluate the effect of baicalein on AhR in ILC3s for in vitro studies. Baicalein showed no cytotoxicity in MNK-3 cells at concentrations of 1–160 μM (Supplementary Fig. S2). As mentioned in the Introduction, activated AhR by agonist manifests as nuclear translocation, promoting the expression of downstream proteins such as CYP1A1 and IL-22. The results here showed that baicalein had no significant effects on the expression of AhR mRNA (Fig. 4a), but substantially increased the expression of CYP1A1 (Fig. 4b) and IL-22 mRNA (Fig. 4c). Furthermore, the expression of AhR protein increased in the nucleus but decreased in the cytoplasm after administration of baicalein, which indicated that baicalein promoted the translocation of AhR to the nucleus (Fig. 4d). Simultaneously, the expression levels of CYP1A1 protein and IL-22 were enhanced in a dose-dependent manner by baicalein (Fig. 4e, f). In addition, molecular docking models also predicted that baicalein was bound to AhR with three combination types including hydrophobic interaction (Arg 78, Gln 249, Gln 262 and His 155), π-stacking (His 155) and hydrogen bond (Gln 249). The calculated binding energy for baicalein and FICZ was −6.76 kcal/mol and −7.26 kcal/mol respectively (Fig. 4g, Supplementary Table S1). These findings demonstrated that baicalein might be a potent AhR agonist and upregulated AhR/IL-22 pathway in MNK-3 cells.

Baicalein induced IL-22 production by MNK-3 cells in an AhR-dependent manner

In this study, a pLV shRNA vector system was used to efficiently deliver shAhR into cells via lentivirus transfection. After screening the transfection conditions and purification with puromycin, the transfection efficiency reached 94.1% (Fig. 5a–c, Supplementary Fig. S3). Subsequently, lentiviruses with AhR sequence 1 remarkably inhibited AhR mRNA expression (Fig. 5d) and AhR protein expression (Fig. 5e). Therefore, we used it to construct a shAhR-MNK3 stable transfection cell line.

To identify whether baicalein-activated AhR largely contributed to IL-22 production, MNK-3 cells and shAhR-MNK-3 cells were treated with baicalein. As expected, in MNK-3 cells, baicalein and FICZ noticeably boosted the nuclear translocation of AhR (Fig. 6a, b), remarkably increased the expression of CYP1A1 protein (Fig. 6c), and promoted IL-22 secretion (Fig. 6d). In contrast, in shAhR-MNK3 cells, the action of baicalein on AhR activation and IL-22 production disappeared. These results confirmed that baicalein induced IL-22 production in an AhR-dependent manner.

Protective effect of baicalein on Caco-2 cells was mediated by IL-22 that MNK-3 cells secreted

To understand how ILC3s affect intestinal barrier in the presence of baicalein, we constructed an in vitro system consisting of MNK-3 and Caco-2 cells to simulate the intestinal microenvironment. In this system, MNK-3 cells were used to replace ILC3s owing to their similarity [22]. It was found that the FITC-dextran flux of Caco-2 cells markedly increased when exposed to 40 μg/mL LPS (Fig. 7a), which was markedly reserved by 25 ng/mL IL-22. (Fig. 7b). Next, we observed that the supernatant of MNK-3 cells treated with baicalein prevented the increased FITC-dextran flux of Caco-2 cells induced by LPS (Fig. 7c). MNK-3 cells' supernatant additionally promoted the expression of the barrier-sealing proteins ZO-1 and occludin to facilitate tight junction assembly in Caco-2 cells, whose effect was consistent with IL-22 (Fig. 7d). Combined with the above results, we found that baicalein induced MNK-3 cells to secret IL-22, thus supporting the intestinal barrier of Caco-2 cells under LPS stimulation.

DISCUSSION

Currently, UC is mainly endorsed as a "barrier organ disease", and clinical therapies of UC focusing on anti-inflammation with 5-ASA or glucocorticoids show a high recurrence rate in patients. Therefore, new therapeutic agents aiming at improving the intestinal barrier are needed. In this study, we demonstrated that baicalein, which is derived from *Scutellaria baicalensis* Georgi, had

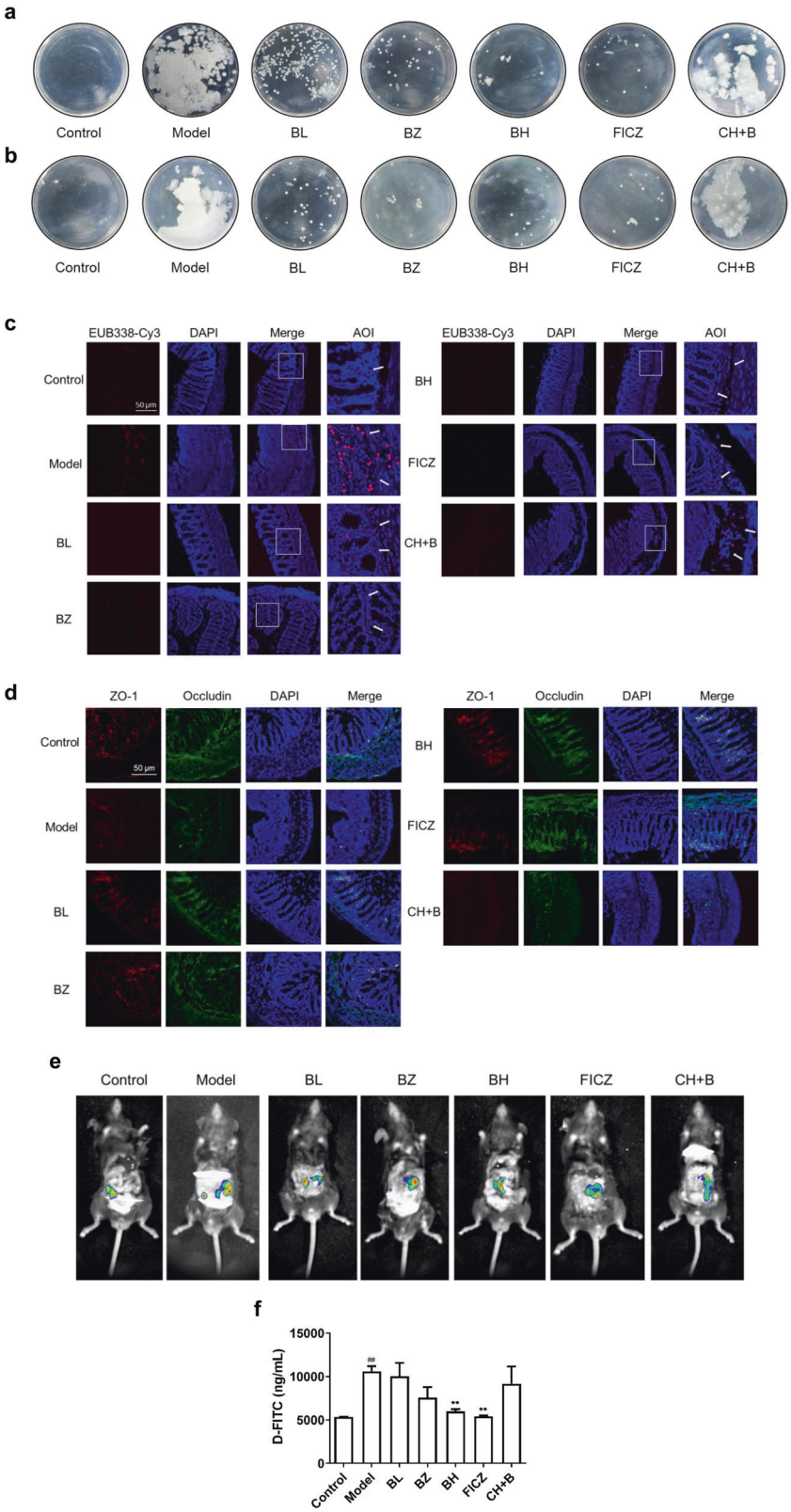

Fig. 2 Baicalein improved the intestinal barrier structure and function in colitis mice. a Bacterial loads in spleen were detected by bacteria culture. **b** Bacterial loads in MLNs were detected by bacteria culture. **c** Bacterial infiltration in intestine was observed using FISH (200×). **d** The expression levels of tight junction proteins ZO-1 and occludin were observed by immunofluorescence (200×). **e** FITC-dextran distribution in colitis mice was observed by small animal imaging. **f** Content of FITC-dextran in serum ($n = 5$). BL, low dose of baicalein (10 mg/kg); BZ, medium dose of baicalein (20 mg/kg); BH, high dose of baicalein (40 mg/kg); FICZ, AhR agonist; CH + B, CH223191 (AhR antagonist) + baicalein (40 mg/kg). $^{\#\#}P < 0.01$ vs. the control. $^{**}P < 0.01'$ vs. the model.

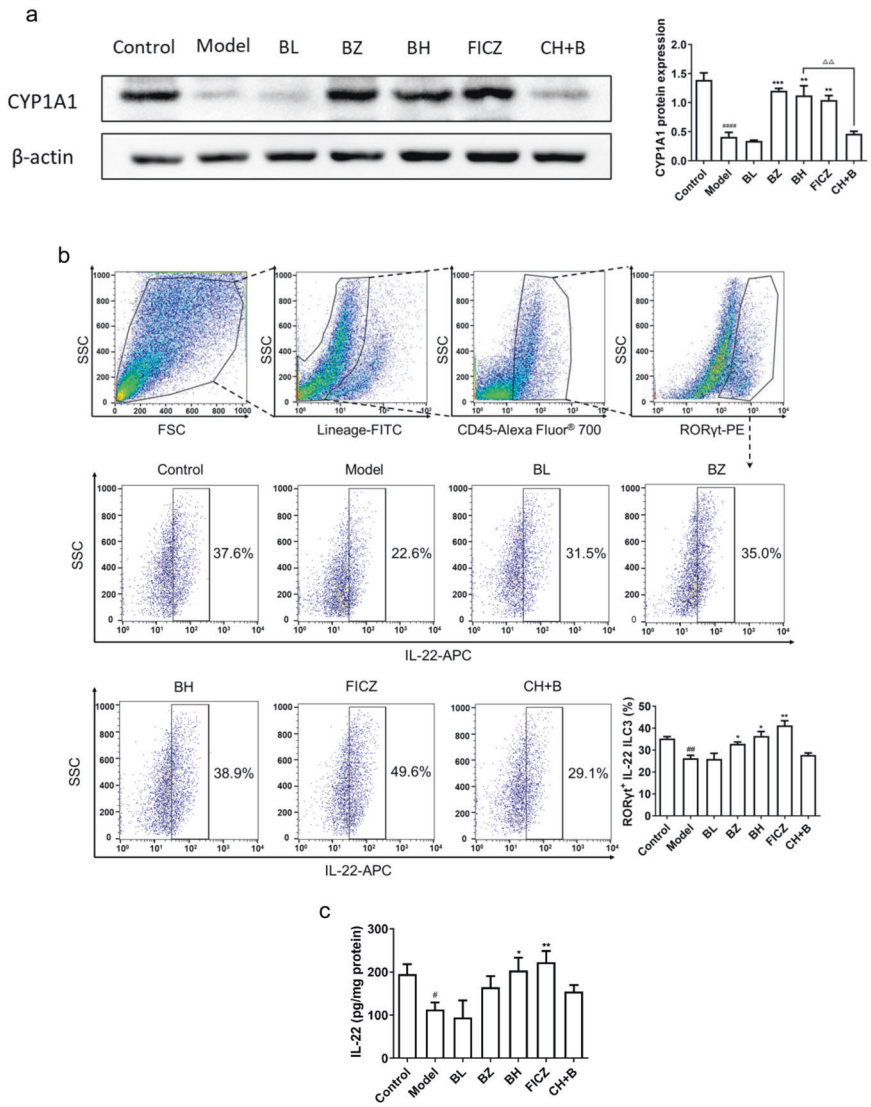

Fig. 3 Baicalein regulated AhR/IL-22 pathway in ILC3s of colitis mice. a CYP1A1 protein expression of colon was evaluated using Western blot ($n = 3$). **b** The proportion of ILC3s was evaluated using flow cytometry ($n = 6$). **c** IL-22 content in colon was evaluated using ELISA ($n = 5$). BL, low dose of baicalein (10 mg/kg); BZ, medium dose of baicalein (20 mg/kg); BH, high dose of baicalein (40 mg/kg); FICZ, AhR agonist; CH + B, CH223191 (AhR antagonist) + baicalein (40 mg/kg). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#\#}P < 0.001$ vs. the control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the model. $^{\triangle\triangle}P < 0.01$ vs. the BH.

protective effect on intestinal epithelia by activating AhR/IL-22 pathway of ILC3s in UC.

At the beginning of this study, we confirmed the therapeutic effect of baicalein on UC. Then, we mainly focused on the restoring gut barrier function of baicalein. Berg et al. [24] first described bacterial translocation as a phenomenon in which intestinal bacteria crossed the epithelial layer to the lamina propria, and then migrated to other tissues. In IBD patients, bacterial translocation is more likely to result from impaired intestinal barrier [25, 26]. Of note, the loss of the mucus layer leads to the adherence of microbiota to the epithelium [27], accelerating the process of bacterial translocation. Consequently, baicalein may alleviate bacterial infiltration and translocation by improving the intestinal barrier. Actually, in line with the previous findings, bacterial infiltration in the colon and translocation to the MLNs and spleens of colitis mice increased, and baicalein reduced this pathological change. Generally, the altered tight junction comprised of occludin and claudins weakened the barrier function and epithelial integrity [28, 29]. Our results also showed that baicalein modulated the tight junctions of the intestinal epithelium. Here, we showed that baicalein, used as a curative treatment, was able to reverse DSS-induced disease outcomes and repair the dysfunctional gut barrier.

Up to now, little is known regarding the mechanisms by which baicalein regulate gut barrier. Emerging evidence indicates that interaction between ILC3s and IL-22 is crucial in the regulation of intestinal barrier function and structure [30, 31] since ILC3s are the main source of IL-22 in the intestine [32]. We further hypothesized that the protective effect of baicalein on the epithelial barrier might be associated with ILC3s-IL-22. We confirmed that baicalein could recover the proportion of IL-22^{+} ILC3s and IL-22 content in colitis mice. Consistent with in vitro data, the supernatant of MNK-3 cells treated with baicalein, which may contain high levels of IL-22 repaired the compromised intestinal barrier function of Caco-2 cells. However, this begged the question on how baicalein improved the ability of ILC3s to secrete IL-22.

Qiu et al. reported that AhR might cooperate with RORγt to promote IL-22 gene transcription [33]. It is assumed that AhR

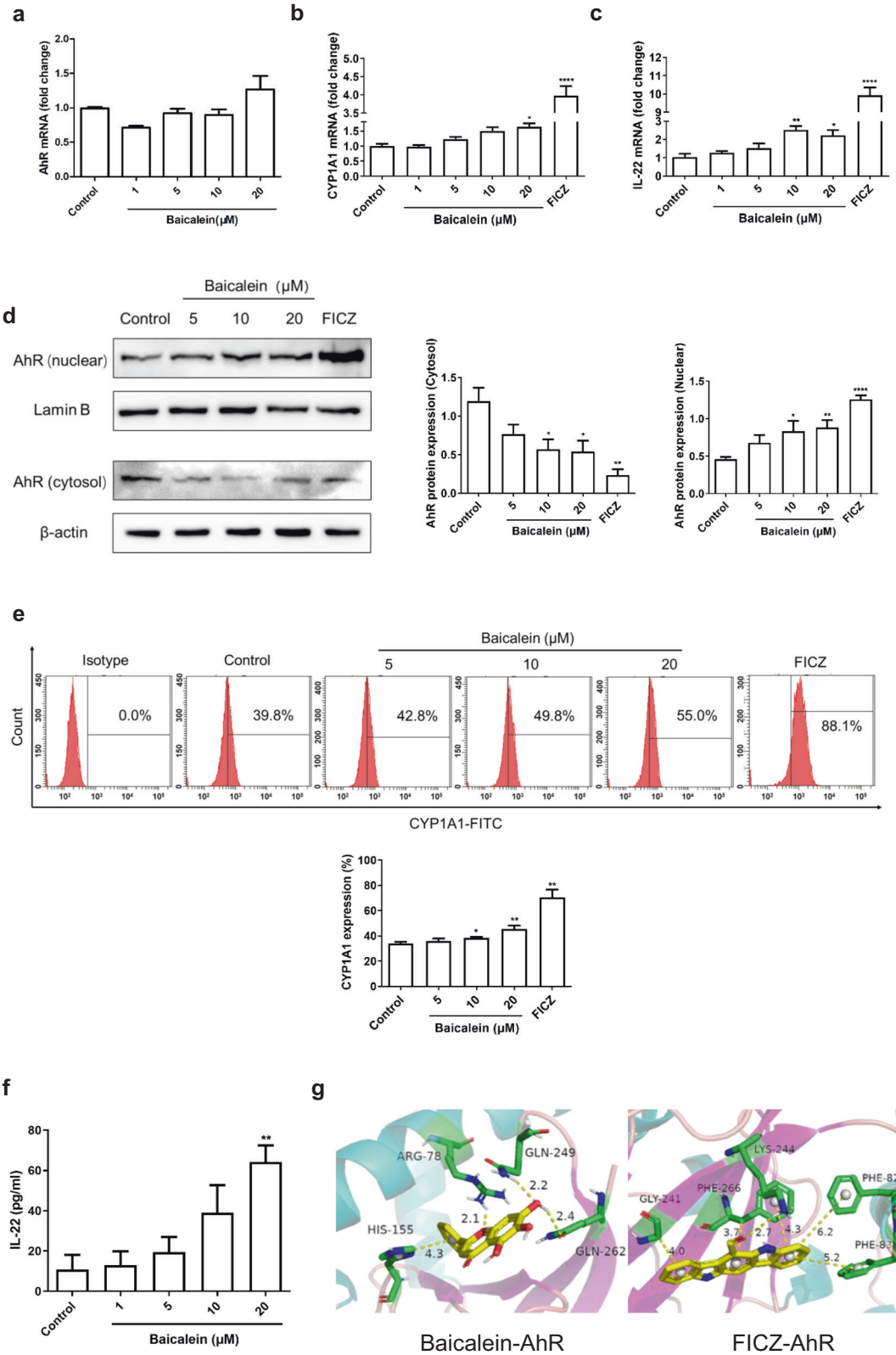

Fig. 4 Baicalein activated AhR/IL-22 pathway in MNK-3 cells in vitro. a AhR mRNA expression. **b** CYP1A1 mRNA expression. **c** IL-22 mRNA expression. **d** AhR protein expression in the cytoplasm and nucleus was detected using Western blot. **e** CYP1A1 protein expression was detected using flow cytometry; **f** The content of IL-22 secreted by MNK-3 cells was evaluated using ELISA. **g** Molecular docking models of baicalein-AhR and FICZ-AhR were constructed using AutoDock 1.5.6. $n = 3$ in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.001$ vs. the control.

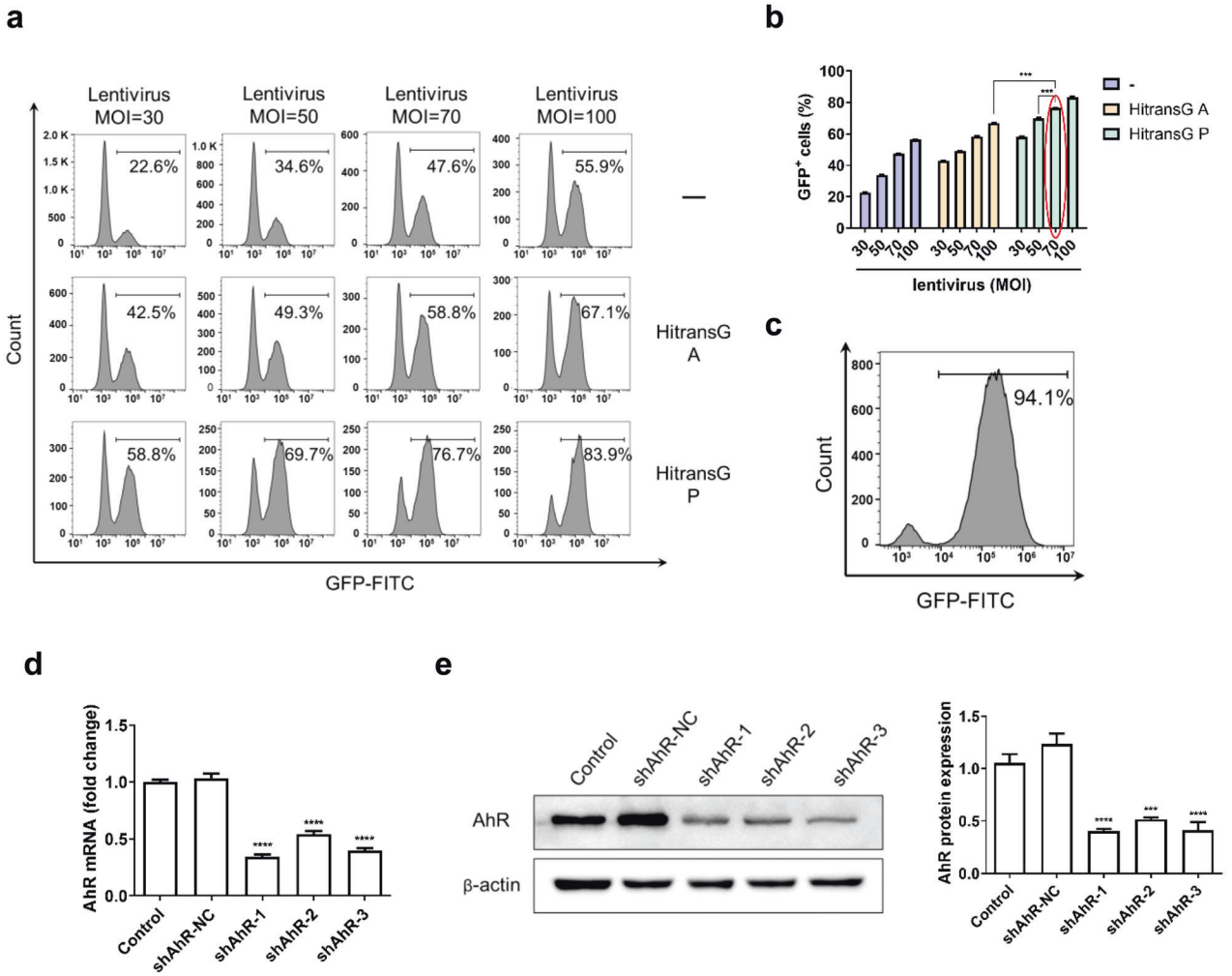

Fig. 5 Establishment of shAhR-MNK3 stable transfected cell line. a Transfection conditions for MNK-3 cells were optimized using flow cytometry. **b** Bar chart of the multiple transfection conditions for MNK-3 cells. **c** Fluorescence intensity corresponding to the optimal transfection condition was detected through flow cytometry. **d**, **e** The transfection effect of lentivirus in MNK-3 cells was evaluated using RT-PCR and Western blot. $n = 3$ in each group. $^{***}P < 0.001$, $^{****}P < 0.001$ vs. the control.

controlled IL-22 transcription by interacting with RORγt and binding to RORES, or alternatively acquiring activity to enhance DNA-binding under the promotion of RORγt [9, 34]. Therefore, we speculated that baicalein could directly augment IL-22 production by activating AhR in ILC3s.

AhR nuclear translocation and CYP1A1 upregulation directly reflect the activation effect of baicalein on AhR. Our in vivo results showed that CYP1A1 protein expression was markedly decreased in colitis mice. One explanation for it is that various microbially derived tryptophan metabolites acting as ligands of AhR, such as indole-3-acetic acid, indoleacrylic acid, indole-3-propionic acid, and tryptophan, were impaired in the context of colitis [35–37]. These metabolites have been shown to improve colitis via various pathways. Whereas, these studies did not address the effects of exogenous AhR ligands in this disease model. Consistent with previous studies [12], AhR agonist FICZ protected mice against colitis while AhR antagonist CH223191 reserved positive effects of baicalein, suggesting that anti-colitis effects of baicalein were AhR-dependent.

In line with in vivo data, baicalein effectively facilitated the nuclear translocation of AhR, increased CYP1A1 expression and promoted IL-22 production in MNK-3 cells. Conversely, the above effects were not observed in shAhR-MNK3 cells, in which AhR expression was substantially knocked down. This implied that baicalein-induced IL-22 production was AhR-dependent. Additionally, molecular docking analysis predicted that baicalein was a potent agonist of AhR. We determined that baicalein boosted AhR nuclear translocation by binding to AhR and forming a molecular complex, followed by binding with the IL-22 promoter and regulating the transcription and translation of IL-22.

Interestingly, FICZ could remarkably reinforce the secretion of IL-22 by shAhR-MNK3 cells, suggesting that the effects of FICZ on MNK-3 cells were not solely mediated by AhR. Of note, in ILC3, IL-22 expression is co-regulated by transcription factors including RORγt [38], AhR [33], signal transducer and activator of transcription (STAT) 3 [39], and STAT5 [40]. STAT3 and STAT5 preferentially bind to the STAT binding site at the IL-22 locus, initiating IL-22 gene transcription independently of AhR [41, 42]. Recent studies uncovered that FICZ reinforced phosphorylation of STAT3 and STAT5 in Naïve $CD4^+$ T cells [43]. Therefore, we speculated that FICZ enhanced the transcription and translation of IL-22 by boosting STAT3 and STAT5 phosphorylation and promoting their binding to the IL-22 promoter, independent of the AhR pathway.

AhR is a ligand-activated transcription factor that mediates different effects in various immune cells. Our previous study found that baicalein could regulate T regulatory cell differentiation

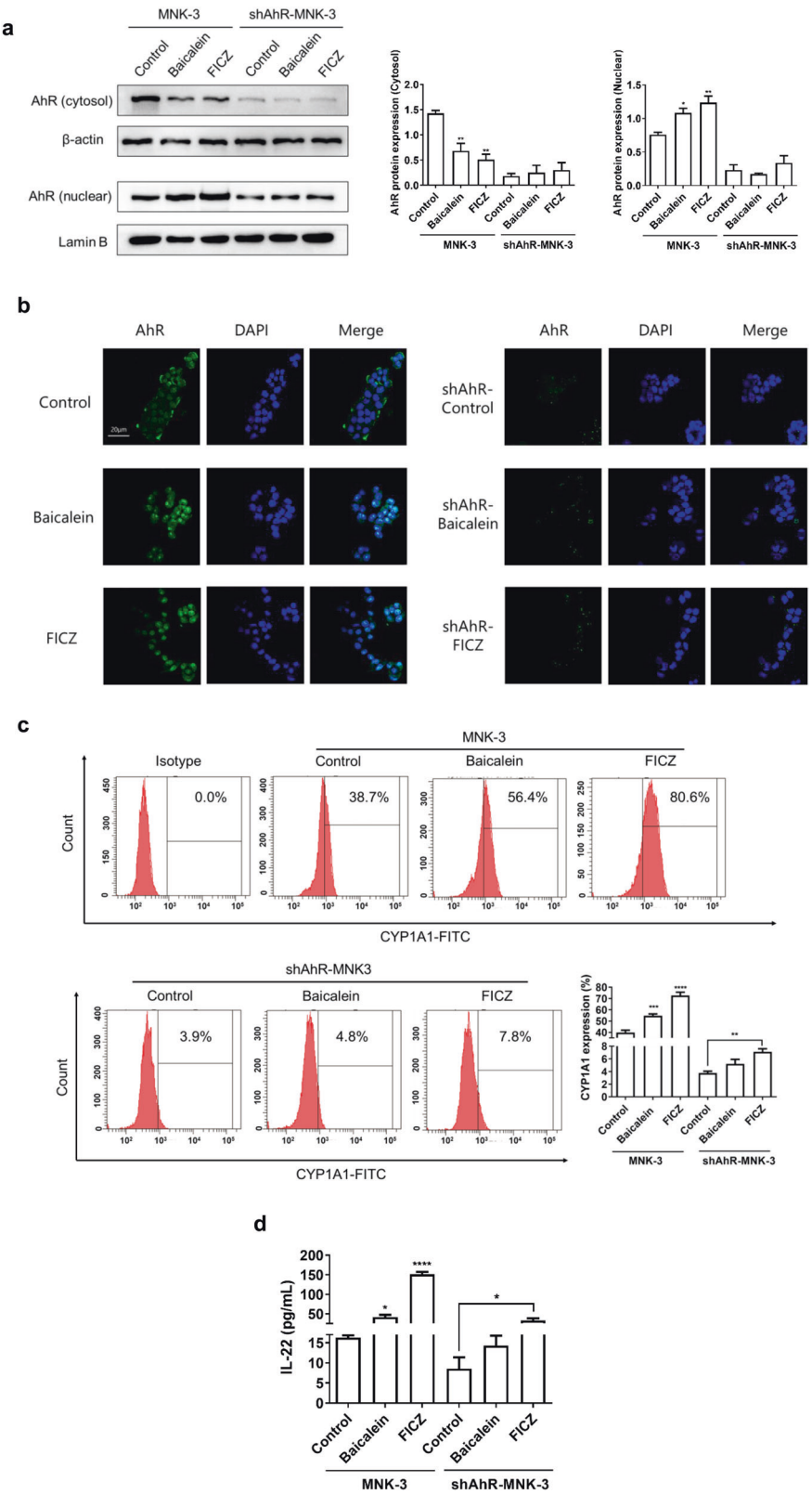

Fig. 6 Baicalein had little effect on the AhR/IL-22 pathway in shAhR-MNK3 cells in vitro. a AhR protein expression in the cytoplasm and nucleus of MNK-3 cells and shAhR-MNK3 cells was detected using Western blot. **b** Nuclear translocation of AhR in MNK-3 cells and shAhR-MNK3 cells was observed using immunofluorescence (400×). **c** CYP1A1 protein expression in MNK-3 cells and shAhR-MNK3 cells was detected using flow cytometry. **d** The effect of baicalein on IL-22 secreted by MNK-3 cells and shAhR-MNK3 cells was evaluated using ELISA. $n = 3$ in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.001$ vs. the control.

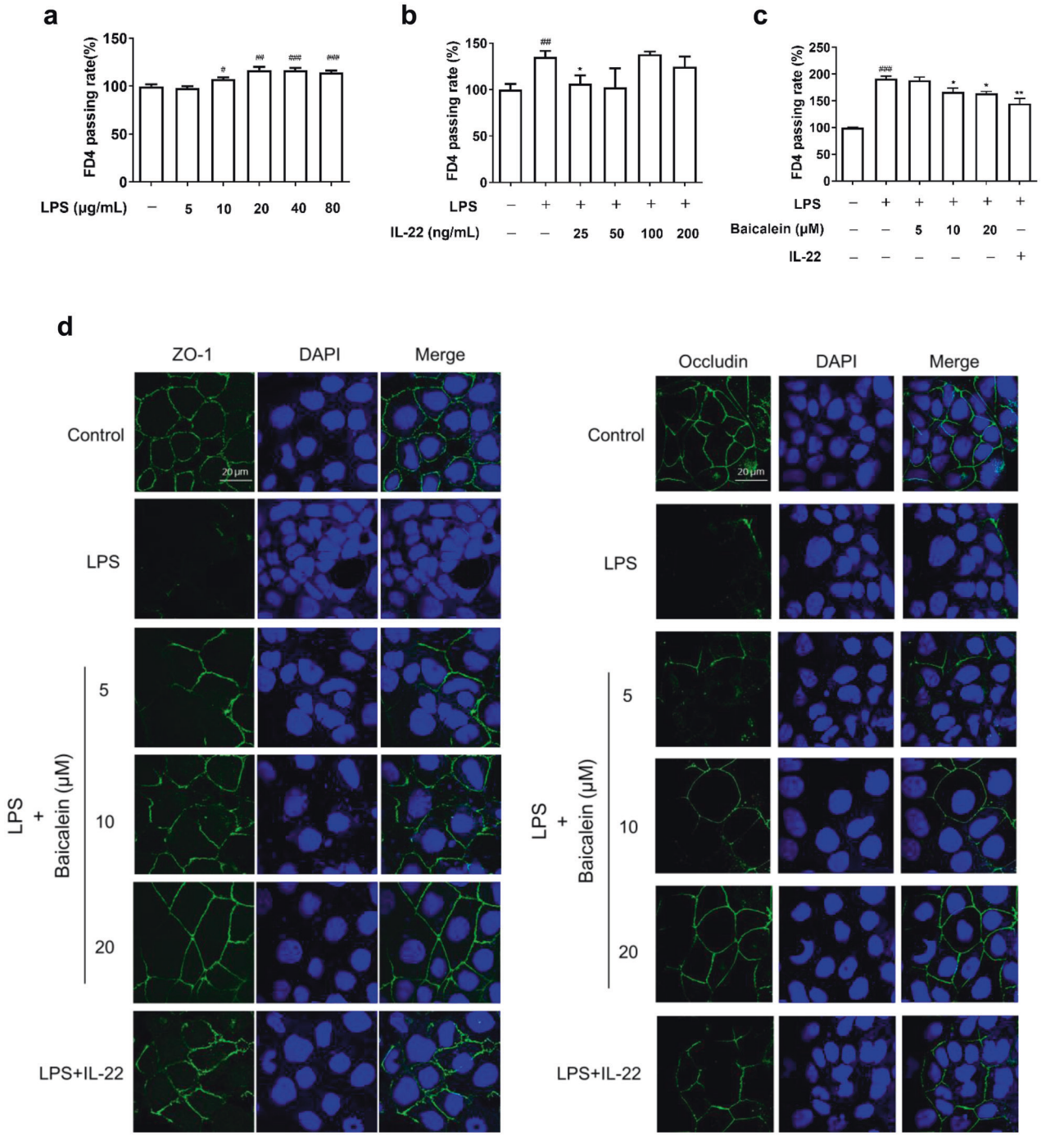

Fig. 7 Protective effect of baicalein on Caco-2 cells was mediated by IL-22 that MNK-3 cells secreted. a FITC-dextran flux of Caco-2 cells treated with LPS was detected. **b** FITC-dextran flux of Caco-2 cells treated with LPS and IL-22 was detected. **c** Caco-2 cells were challenged with LPS and cured with MNK-3 cells' supernatant treated with baicalein, and then the FITC-dextran flux was detected. **d** The expression of ZO-1 and occludin was observed using immunofluorescence (400×); $n = 3$ in each group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. the control. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the model.

through AhR to reduce intestinal inflammation [44]. Meanwhile, during the experiment, we accidentally noticed that baicalein also restored IL-22 balance and repaired the gut barrier following AhR activation in ILC3s. Besides, Zhong et al. demonstrated that baicalein exerted anti-inflammatory effects by suppressing NF-κB and STAT3 signaling pathways in ICR colitis mice model [18]. An interesting future direction will be to understand if these pathways synergize with baicalein-activated pathway identified in this study to ameliorate colitis mice.

Taken together, our study illustrated that baicalein alleviated DSS-induced disease outcomes and improved intestinal barrier structure and function. Moreover, we demonstrated the underlying mechanism by which baicalein activated AhR and initiated AhR translocation to the nucleus, enhancing IL-22 production in ILC3s, and consequently, maintaining the tight junctions to improve the gut barrier (Fig. 8). Our studies provided novel insights on how baicalein affected gut barrier function and laid the groundwork for clinical application of baicalein.

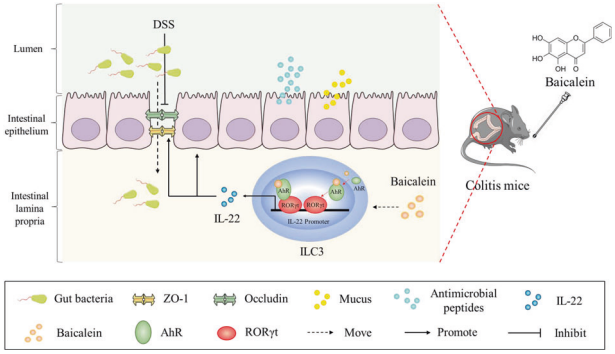

Fig. 8 Mechanisms of protection against colitis by baicalein via activation of the AhR/IL-22 pathway in ILC3s. Intestinal epithelial barrier in DSS-induced colitis mice was disrupted, resulting in intestinal inflammation and tissue damage. Baicalein promoted the translocation of AhR to the nucleus and enhanced IL-22 production by ILC3s, which maintained the tight junctions and regulated the intestinal barrier.

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AUTHOR CONTRIBUTIONS

YYL performed experiments and wrote the article. XJW, YLS, QW, SWH, ZFP contributed to establishment and treatment of colitis mice model. YPC, JJL, MLZ, XQX, ZYW performed some cell studies. JYC analyzed and organized the data. LZ and XL designed ideas and edited the paper.

ADDITIONAL INFORMATION

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