

CD73 blockade alleviates intestinal inflammatory responses by regulating macrophage differentiation in ulcerative colitis

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Abstract. Ulcerative colitis (UC) is a type of inflammatory bowel disease characterized by excessive and persistent inflammation. Intestinal macrophages play a considerable role in regulating inflammatory immune reactions in the gut mucosa. It has previously been reported that CD73 is related to the pathogenesis of inflammatory or immune-related diseases; however, the roles of CD73 in UC remain unclear. In this study, CD73 expression in the inflamed mucosa of patients with UC was examined using reverse transcription-quantitative PCR (RT-qPCR), western blotting, and immunohistochemistry. Adenosine 5'-(α , β -methylene) diphosphate (APCP) was used to block the expression of CD73. Furthermore, the mRNA levels of proinflammatory mediators associated with macrophages following the blocking of CD73 were examined using RT-qPCR. Finally, the regulatory function of CD73 in intestinal inflammation was assessed by administering APCP in a mouse model of dextran sulfate sodium salt (DSS)-induced colitis. Notably, it was found that CD73 expression was significantly increased in the colonic mucosal tissues of patients with UC. Blockade of CD73 inhibited the expression of pro-inflammatory cytokines but promoted the production of anti-inflammatory cytokines in macrophages, while its promotion of M2 macrophage polarization was also verified. *In vivo*, CD73 blockade markedly alleviated DSS-induced colitis in mice, as characterized by reduced weight loss, reduction in the incidence of diarrhea, and reduced amount of bloody stool. Mechanistically, it was shown that CD73 regulated macrophage differentiation via the NF- κ B and ERK signaling pathways. In conclusion, the findings of the present study indicate that CD73 may have a potential impact on the

pathogenesis of UC by modulating the immune response of macrophage differentiation; thus, providing a novel pathway for modulating mucosal inflammation in UC.

Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory and immune-related disease with unknown pathogenesis, and it is typically categorized into two subtypes: Crohn's disease (CD) and ulcerative colitis (UC). In UC, mucosal inflammation can affect the colon and rectum, which comprises the mucosa and submucosa of the large intestine (1). Although the pathogenesis of IBD remains unclear, studies have shown that an imbalance in the intestinal microenvironment, immune dysfunction, environmental changes, and genetic factors may lead to UC. Under normal conditions, the process of the inflammatory response can be self-limiting, allowing for the complete remission of inflammation and internal homeostasis (2). At present, however, the excessive and persistent inflammation that occurs in UC suggests an immunological pathogenesis (3).

More recently, an increasing number of studies have shown that macrophages play crucial roles in preventing an excessive immune response. Intestinal macrophages, as innate immune cells, play a significant role in modulating the inflammatory response in the gut mucosa (4). Intestinal macrophages, composed of newly recruited circulating monocytes and resident macrophages, can regulate tissue repair and promote wound healing during infection and autoimmunity (5). Concurrently, intestinal macrophages have also been reported to produce various cytokines and inflammatory mediators that participate in gut homeostasis (6-8). For example, macrophages induce the production of IL-1 β in the microbiota and this promotes the release of colony-stimulating factor-2 (CSF2) by type 3 innate lymphoid cells, and this subsequently stimulates macrophages to secrete IL-10, thereby maintaining intestinal homeostasis via antigen-specific CD4+CD25+ regulatory T(Treg) cell expansion (6). In addition, macrophages can polarize into M1-like macrophages that produce various proinflammatory cytokines, including TNF- α , IL-6, and iNOS, thus increasing inflammatory damage to the intestinal mucosa. Conversely, M2-like macrophages express mannose receptors (MRC1/CD206) and produced several anti-inflammatory

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factors, such as IL-10, TGF- β , and Fizz-1, further giving rise to the abatement of intestinal inflammation (7,9). Thus, when macrophages are dysregulated, it contributes to a cascade of inflammatory immune responses in IBD, indicating that modulating macrophage differentiation could be a promising therapeutic strategy for treating patients with UC.

CD73 is an extracellular-5'-nucleotidase encoded by the ecto-5'-nucleotidase CD73(NT5E) gene (10). The upstream CD39 catalyzes ATP to produce adenosine monophosphate (AMP), which is converted into adenosine by CD73, and adenosine binds to the downstream adenosine receptor (A2AR) (11). It has been shown that CD73-positive extracellular vesicles (EVs) contribute to suppressing antitumor immune responses in the tumor microenvironment, and that EVs derived from murine regulatory T cells display AMP activity. Moreover, APCP can specifically block the activity of AMP (12). Previous studies have also demonstrated that APCP can block the expression of CD73 (13,14). Additionally, APCP promotes tumor immune escape, development, and metastasis by regulating the cell cycle, apoptosis, and several signaling pathways, including EGFR, NF- κ B, VEGF, and AKT/ERK, which are key mediators of inflammatory responses in the regulation of innate and adaptive immune responses (15). Nevertheless, the understanding of the effect of CD73 in UC remains to be elucidated.

In the present study, the expression of CD73 in colonic mucosal tissues from patients with UC and its correlation with the endoscopic index was assessed. CD73 activity was also blocked in macrophages to assess its effect on the production of proinflammatory and anti-inflammatory cytokines, and to determine the role of CD73 in the differentiation of macrophages. It was found that CD73 inhibited M2 macrophage polarization in both mouse and human-derived macrophages. In addition, blockade of CD73 in mice reduced dextran sulfate sodium salt (DSS)-induced colitis. Moreover, the results showed that the function of CD73 in the modulation of inflammation induced by lipopolysaccharide (LPS) involved regulation of the NF- κ B and ERK signaling pathways. Together, these findings show that CD73 inhibits M2 macrophage polarization and that it may regulate inflammation via the NF- κ B and ERK signaling pathways to reduce UC.

Materials and methods

Patients. All colonic biopsy samples were collected from the Department of Gastroenterology of the Affiliated Hospital of Jining Medical College (UC: 8 men and 7 women; healthy controls: 9 men and 8 women). The median age of the patients with UC was 56 years and the age range was 42-73 years. The median age of the healthy controls was 41 years and the age range was 26-50 years. The diagnostic criteria of UC were based on the clinical examination, radiological examination, endoscopic examination, and histological criteria (16). Written informed consent was also obtained from all participants prior to the study. Additionally, this study was approved by the Institutional Review Board for Clinical Research. The severity of the disease and intestinal mucosal lesions were graded using the Ulcerative Colitis Endoscopic Index of Severity (UCEIS) criteria (17).

Mice. C57BL/6J mice were purchased from the Peng Yue Experimental Animal Breeding Company and kept under specific pathogen-free conditions in the animal house of Jining Medical University. Mice were used for experiments at 8-10 weeks of age, weighing 20-25 g. The number of mice used for the experiment was 14. All animal studies were approved by and conducted according to the permission of the Ethical Committee of Jining Medical University.

DSS-induced colitis model. Age- and sex-matched mice were divided into four groups: The negative control-DSS group (intraperitoneal injection of PBS), and the CD73 inhibitor-DSS group (intraperitoneal injection of APCP) (4 mg/kg). There were four mice in each of these two groups. Mice exposed to DSS received 2.5% DSS (MP Biomedicals, M.W. 36,000-50,000 kDa) for 7 days, and then received sterile water for a further 3 days from day 8. The other two groups of mice were given sterile water for 9 continuous days as the negative controls. There were three mice in each of these two groups. Subsequent disease progression in the development of colitis was assessed by monitoring indicators, including daily weight changes, fecal blood, and diarrhea, which are common clinical indicators of colitis (18). Colitis severity was calculated using the disease activity index (DAI) based on a composite score of weight loss, stool consistency, and bleeding, ranging from 0 to 12, as described previously (19). All parameters were scored from days 0-9, and mice were sacrificed on day 9. The mice were executed by asphyxiation with a 60% CO₂ volume displacement rate followed by cervical dislocation when the mice did not respond to painful stimuli. Mice were euthanized when DSS mice reached ~85% body weight loss. In the DSS group, 2 mice died due to colitis, and the remaining 12 mice were euthanized as above. After ensuring that the animals were free of vital signs (cardiac arrest, dilated pupils, whitening of the eyelids, and no visual response), the dead animals were placed in yellow waste bags and left for 30 min before reconfirming whether there were any surviving animals.

Cell culture. A human monocyte leukemia cell line (THP-1) was provided by the Medical Research Center of the Affiliated Hospital of Jinan Medical University. THP1 cells were grown to a density of 2.5×10^5 cells/ml in 10% FBS-RPMI (Gibco; Thermo Fisher Scientific, Inc.) medium at 37°C in a humidified incubator supplied with 5% CO₂. In all experiments, THP-1 cells were transformed into adherent macrophages in complete medium treated with 200 ng/ml phorbol12-myristate 13-acetate (PMA) (MilliporeSigma) for 24 h.

Isolation of peritoneal macrophages (PMs). PMs were harvested from mice injected intraperitoneally with 3% Brewer's thioglycollate medium (Kingmore Biotech Co., Ltd.). After 3 days, mice were sacrificed and their abdominal cavities were flushed 2-3 times with DMEM (Gibco; Thermo Fisher Scientific, Inc.) to obtain primary murine PMs, which were cultured in complete DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (100 U/ml).

Isolation of bone marrow-derived macrophages (BMDMs). BMDMs were obtained from the tibia and femur of C57BL/6 mice and cultured in complete DMEM supplemented with granulocyte-macrophage (GM)-CSF (10 ng/ml; PeproTech,

Table I. Sequences of the primers used for quantitative PCR.

Gene	Species	Forward, 5'-3'	Reverse, 5'-3'
GAPDH	Human	GGAGCCAAAAGGGTCATCATCT	GAGGAGCCATCCACAGTCTTCT
CD73	Human	GCCTGGGAGCTTACGATTTTG	TAGTGCCCTGGTACTGGTCG
GAPDH	Mouse	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
TGF- β	Mouse	CTCCCGTGGCTTCTAGTGTC	GCCTTAGTTTGGACAGGATCTG
IL-10	Mouse	CTTACTGACTGGCATGAGGATCA	GCAGCTCTAGGAGCATGTGG
CD206	Mouse	CTCTGTTCAGCTATTGGACGC	CGGAATTTCTGGGATTCAGCTTC
Fizz-1	Mouse	CCAATCCAGCTAACTATCCCTCC	CCAGTCAACGAGTAAGCACAG
IL-6	Mouse	TAGTCCTTCCACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
IL-1 β	Mouse	GCAACTGTTCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
INOS	Mouse	GTTCTCAGCCCAACAATAACAAGA	GTGGACGGGTTCGATGTAC
TNF- α	Mouse	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG

Inc.). On day 4, half of the medium was replaced with supplemented DMEM containing GM-CSF (10 ng/ml). On day 7, BMDMs were harvested and plated at a density of 2×10^6 cells/ml, and when the cells had adhered and grown confluent, they were used for subsequent experiments.

Cell stimulation. For inflammatory response analysis, cells were stimulated with LPS (500 ng/ml) to induce an inflammatory state with or without APCP (20 nm/ml) treatment after 6 h. For macrophage polarization, cells were induced with LPS (200 ng/ml; PeproTech, Inc.) and IFN- γ (10 ng/ml; PeproTech, Inc.) treatment to induce M1 polarization or with IL-13 (20 ng/ml; PeproTech, Inc.) and IL-4 (20 ng/ml; PeproTech, Inc.) treatment to induce M2 polarization in the medium for the indicated lengths of times.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells or tissues using TRIzol[®] reagent, according to the manufacturer's protocol (Ambion; Thermo Fisher Scientific, Inc.). RNA quantity and quality were assessed using a NanoVue spectrophotometer (GE Healthcare Life Sciences), with a 260/280 ratio of 1.8 to 2.0 considered suitable. All primers were synthesized by ShengGong BioTeck, and the sequences are shown in Table I. Total RNA was reverse transcribed into cDNA using a 5x All-In-One RT MasterMix kit (ABM) according to the manufacturer's protocol. mRNA expression was measured using a fluorescence qPCR kit (UltraSYBR Mixture; CW Biosciences), according to the manufacturer's instructions, on an iQ SYBR Green on a CFX96 Real-Time System (Bio-Rad). The following cycling conditions were used: 95°C for 10 min, 95°C for sec, and 60°C for 30 sec, at which point, the plate was read. This was followed by 40 cycles of 95°C for 1 min, 55°C for 1 min, 55.0 to 95.0°C in increments of 0.5°C for 10 sec which included a plate read to obtain the melt curve. Data were analyzed using the $2^{-\Delta\Delta C_t}$ method (20). GAPDH was used as the housekeeping gene.

Immunohistochemistry (IHC). Colonic biopsy samples from patients with UC and healthy donors were fixed and embedded to determine CD73 expression. The sections were incubated

with Envision flex peroxidase-blocking reagent for 10 min at room temperature, followed by incubation with a rabbit anti-human CD73 antibody (1:200; cat. no. ab133582; Abcam) at 4°C overnight. After washing with PBS, the sections were incubated with conjugated anti-rabbit antibodies (1:150; cat. no. ab170902; Abcam) for 60 min at 4°C. The color was developed using 3,3'-diaminobenzidine, and the sections were counterstained with hematoxylin (1:20) at room temperature. Observation of CD73⁺ cells was performed using a light microscope (magnification, x5 and x200).

Western blot analysis. Cells were lysed using RIPA lysis buffer containing protease inhibitors (cat. no. P0013B; Beyotime Institute of Biotechnology). Cell lysates were quantified using a BCA assay, followed by separation using 10% SDS-PAGE. The amount of protein loaded per lane was 10 μ l. Subsequently, proteins were transferred to PVDF membranes (MilliporeSigma) for 1 h at 100 V. To block non-specific binding sites, the membranes were placed in 3% BSA for at least 1 h at room temperature and then incubated with primary antibodies against p65 (1:2,000; cat. no. 8242T; Cell Signaling Technology, Inc.), p-p65 (1:2,000; cat. no. 3033S; Cell Signaling Technology, Inc.), ERK (1:2,000; cat. no. 4695T; Cell Signaling Technology, Inc.), p-ERK (1:2,000; cat. no. 4370T; Cell Signaling Technology, Inc.) or GAPDH (1:5,000; cat. no. D16H11; Cell Signaling Technology, Inc.) at 4°C overnight. After the incubation with HRP-labeled goat anti-rabbit/mouse secondary antibodies (1:3,000; cat. no. AS014/ZB-2305; Beyotime Institute of Biotechnology) for at least 1 h at 4°C, blots were analyzed using an ECL kit (Thermo Fisher Scientific, Inc.) to detect the target proteins. GAPDH was used as the internal control. Densitometry analysis was performed using ImageJ (1.x; National Institutes of Health).

Statistical Analyses. Data are presented as the mean \pm SEM and were analyzed using an unpaired Student's t-test or one-way ANOVA with a Tukey's post hoc test. Spearman's correlation analysis was performed to determine the correlation between CD73 expression and UCEIS. Statistical analyses were performed using SPSS software v21.0 (IBM Corp.).

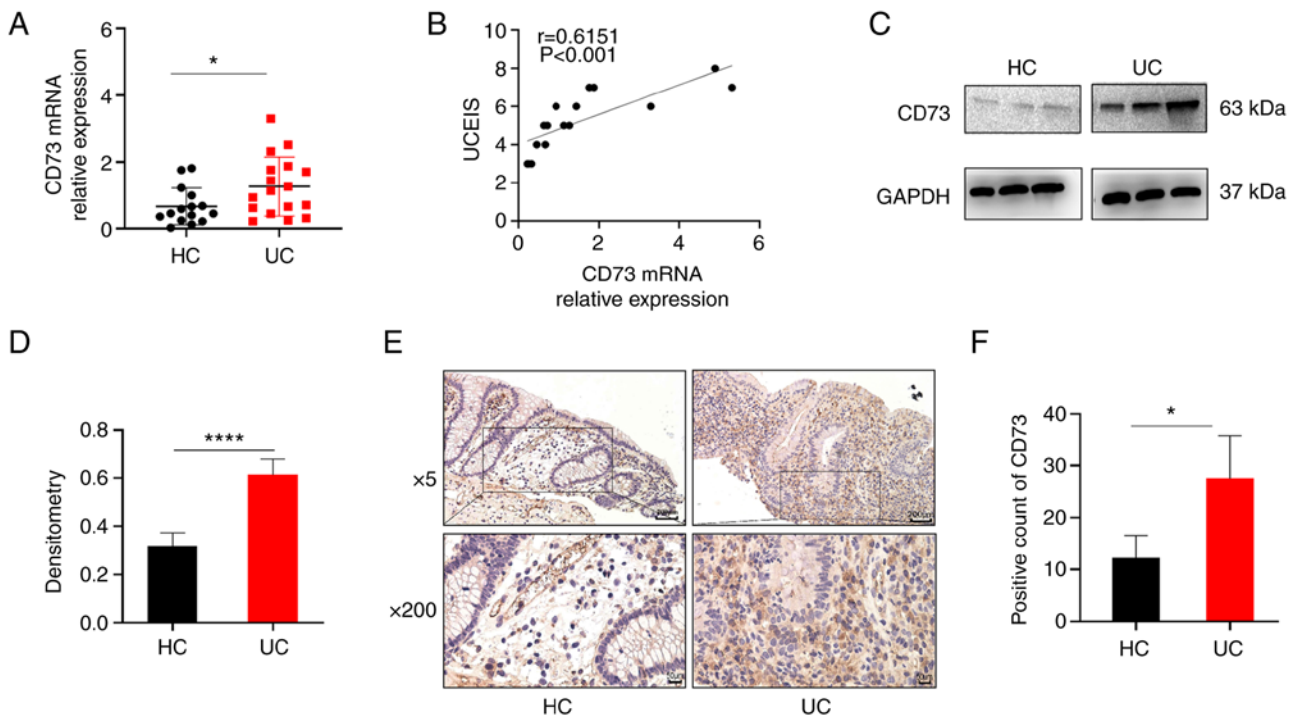


Figure 1. CD73 expression is increased in colonic mucosa from patients with UC and its expression is correlated with disease activity. (A) CD73 gene expression in colonic mucosa in patients with UC (n=15) and healthy controls (n=17). (B) Correlation analysis was performed between the UCEIS and mRNA expression of CD73 in the inflamed mucosa from UC patients. (C and D) CD73 expression in colonic mucosa from patients with UC (n=3) and HC (n=3) was examined by western blotting. The histogram shows the integrated density of the western blots. (E) Immunohistochemistry staining of CD73 in the colon tissue and (F) the count of CD73⁺ cells in the colonic mucosa. Magnification, x5 and x200; scale bar, 200 and 50 μ m). Data are presented as the mean \pm SEM. Data were compared using a Student's paired t-test. *P<0.05 and ****P<0.0001. UC, ulcerative colitis; HC, healthy control; UCEIS, Ulcerative Colitis Endoscopic Index of Severity.

P<0.05 was considered to indicate a statistically significant difference.

Results

CD73 expression is increased in the inflamed mucosa of patients with UC. Based on previous studies, CD73 has been reported to participate in the pathogenesis of several immune diseases (10,15). However, the role of CD73 in UC remains unclear. Hence, inflamed mucosa samples from patients with UC and healthy controls were obtained, and it was found that CD73 expression was notably increased in the inflamed mucosa of UC patients compared with that in the healthy controls using RT-qPCR (Fig. 1A). Furthermore, it was hypothesized that CD73 expression may be associated with UC disease activity. Thus, the correlation between CD73 expression and UCEIS, the international standard criteria for assessing endoscopic disease activity in patients with UC, was determined. As shown in Fig. 1B, CD73 expression was positively correlated with UCEIS in UC ($r=0.6151$, $P<0.001$).

Western blot analysis further indicated that CD73 protein expression was significantly increased in the mucosa of patients with UC compared with that in healthy controls and this increase was statistically significant (Fig. 1C and D). In addition, IHC staining showed that the inflamed mucosa of patients with UC contained a significantly higher number of CD73-positive cells than that in the healthy controls (Fig. 1E and F). Together, these data indicate that CD73 expression is upregulated in the inflamed tissues of patients

with UC and may therefore play a crucial role in UC pathogenesis.

CD73 blockade reduces proinflammatory cytokines levels and increases anti-inflammatory cytokine levels in mouse macrophages. The differentiation of inflammatory monocytes into pro-degradative macrophages during chronic intestinal inflammation induces UC and the secretion of large amounts of pro-inflammatory factors (21). To determine whether CD73 affects colitis by promoting the pro-inflammatory response induced by LPS in macrophages, mouse PMs were isolated and cultured from wild-type mice (Fig. 2A).

Firstly, the expression of CD73 was determined in M0 macrophages stimulated with different concentrations of APCP for different lengths of time to confirm the optimum concentration and stimulation time of APCP to inhibit the expression of CD73. The expression of CD73 in PMs was significantly inhibited by APCP (Fig. 2B). As shown in the results, the optimal time was 6 h and the optimal concentration was 20 nm/ml. Next, PMs were treated with LPS to induce an inflammatory state in the cells. The mRNA expression levels of macrophage-related inflammatory cytokines obtained from PMs treated with or without APCP after LPS administration were determined. As shown in Fig. 3A, RT-qPCR analysis showed that APCP significantly reduced IL-6, iNOS, and IL-1 β levels in LPS-stimulated PMs, but increased the expression of CD206, a characteristic anti-inflammatory cytokine in M2 macrophages (Fig. 3B). Thus, CD73 appears to affect the expression of the inflammatory mediators related with macrophages to promote an inflammatory reaction.

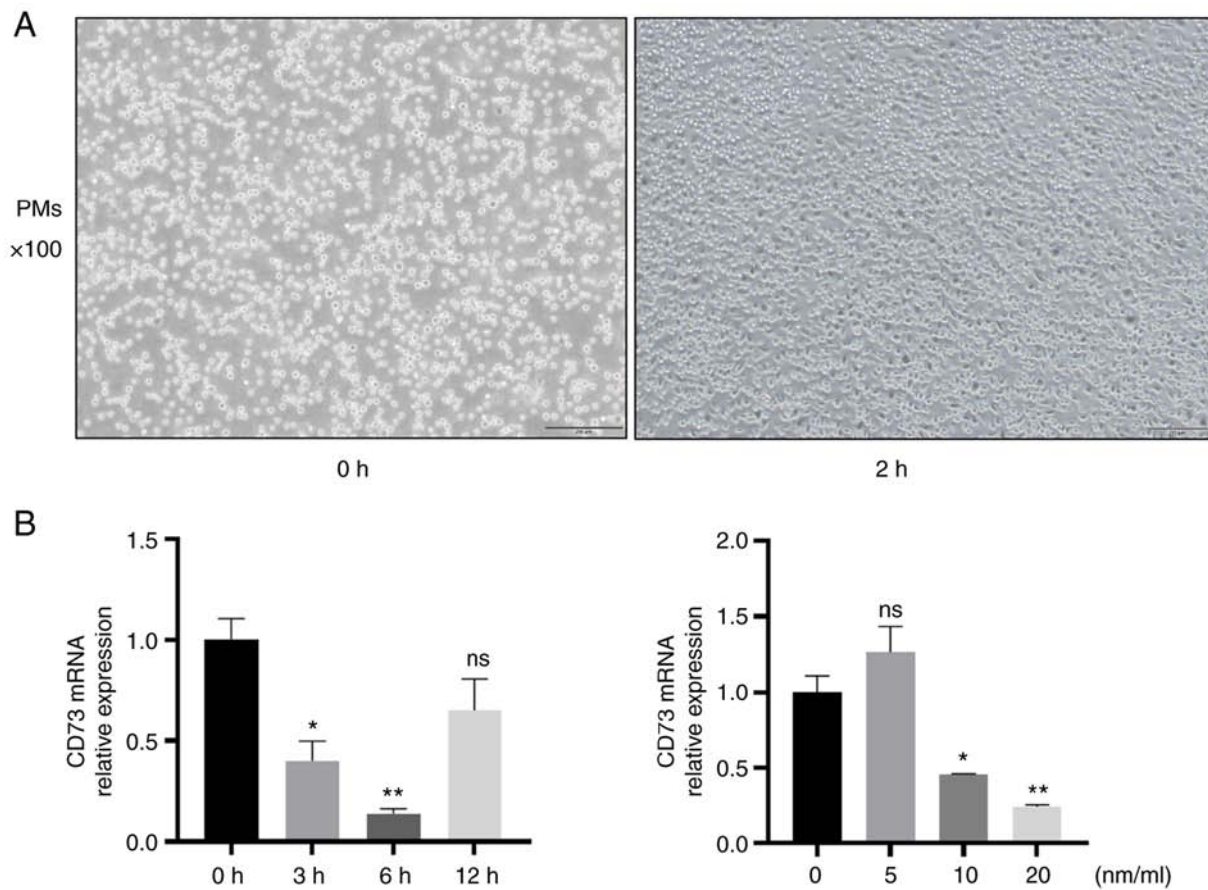


Figure 2. Determination of the optimum concentration and time of APCP treatment. (A) Images of PMs. Magnification, $\times 100$; scale bar, $200 \mu\text{m}$. (B) PMs were treated with APCP for 0, 3, 6, or 12 h with 0, 5, 10, or 20 nm/ml. The expression of mRNA CD73 was assessed ($n=3$). * $P<0.05$ and ** $P<0.01$. ns, not significant; PM, peritoneal macrophage; APCP, adenosine 5'-(α , β -methylene) diphosphate.

It has been suggested that PMs have distinct heterogeneities, and yet BMDMs are the most homogeneous due to macrophage CSF (M-CSF) induction (22). To avoid the variation in polarization caused by the induction of macrophages from different sources, our experimental hypothesis was verified using BMDMs (Fig. 3C). The expression of related inflammatory mediators in BMDMs was consistent with that in PMs. The mRNA levels of proinflammatory factors, such as IL-6, IL-1 β , and iNOS, were lower in the APCP-treated groups than those in the control groups; however, the expression of anti-inflammatory factors such as CD206, IL-10, and TGF- β was higher in the APCP-treated groups (Fig. 3D-E). Interestingly, the effect of CD73 on BMDMs towards M2 macrophage polarization was greater than that on PMs in an inflammatory state. Taken together, these data suggested that CD73 blockade by APCP significantly inhibited the pro-inflammatory responses induced by LPS in macrophages.

CD73 blockade promotes M2 macrophage polarization. To explore the effect of CD73 on the polarization of intestinal macrophages, the expression of several genes related to M1 and M2 macrophages was detected by RT-qPCR. M0 macrophages were induced towards M1 polarization with LPS and IFN- γ treatment, or towards M2 polarization using IL-4 and IL-13 treatment. The expression of M1 macrophage markers, such as IL-1 β and iNOS, was decreased in the groups treated with LPS and IFN- γ . However, this was not statistically significant (Fig. 4A). In contrast,

the inhibition of CD73 measurably increased the percentage of CD206, TGF- β , Fizz-1, and IL-10 in mouse PMs treated with IL-4 and IL-13 (Fig. 4B), which are the characteristic markers of M2 macrophages. Furthermore, these results additionally suggested that the blockade of CD73 could enhance polarization towards M2 macrophages in regulating inflammatory responses.

To further confirm the findings, analogous polarization experiments were conducted using BMDMs. Inhibition of CD73 increased the expression of the characteristic markers of M2 macrophages and decreased the expression of M1 macrophage genes (Fig. 4C and D). Interestingly, the suppression of M2 macrophage polarization induced by CD73 on BMDMs was not significant compared to that in PMs. Therefore, these data demonstrate that CD73 regulates LPS-induced pro-inflammatory responses in macrophages, while suppressing M2-like macrophage polarization.

CD73 blockade promotes anti-inflammatory cytokine production and enhances M2 macrophage polarization in THP1 cells. Next, whether CD73 had a similar effect on M2 macrophage polarization on human THP1 cells. As shown in Fig. 5A, THP1 cells were pulsed with PMA to induce macrophage differentiation. Consistently, THP1 macrophages expressed high levels of the M2 markers such as CD206 upon LPS treatment (Fig. 5B). Moreover, THP1 macrophages expressed upregulated levels of the M2 marker Fizz-1 upon culture with IL-4 and IL-13 (Fig. 5C). Collectively, these

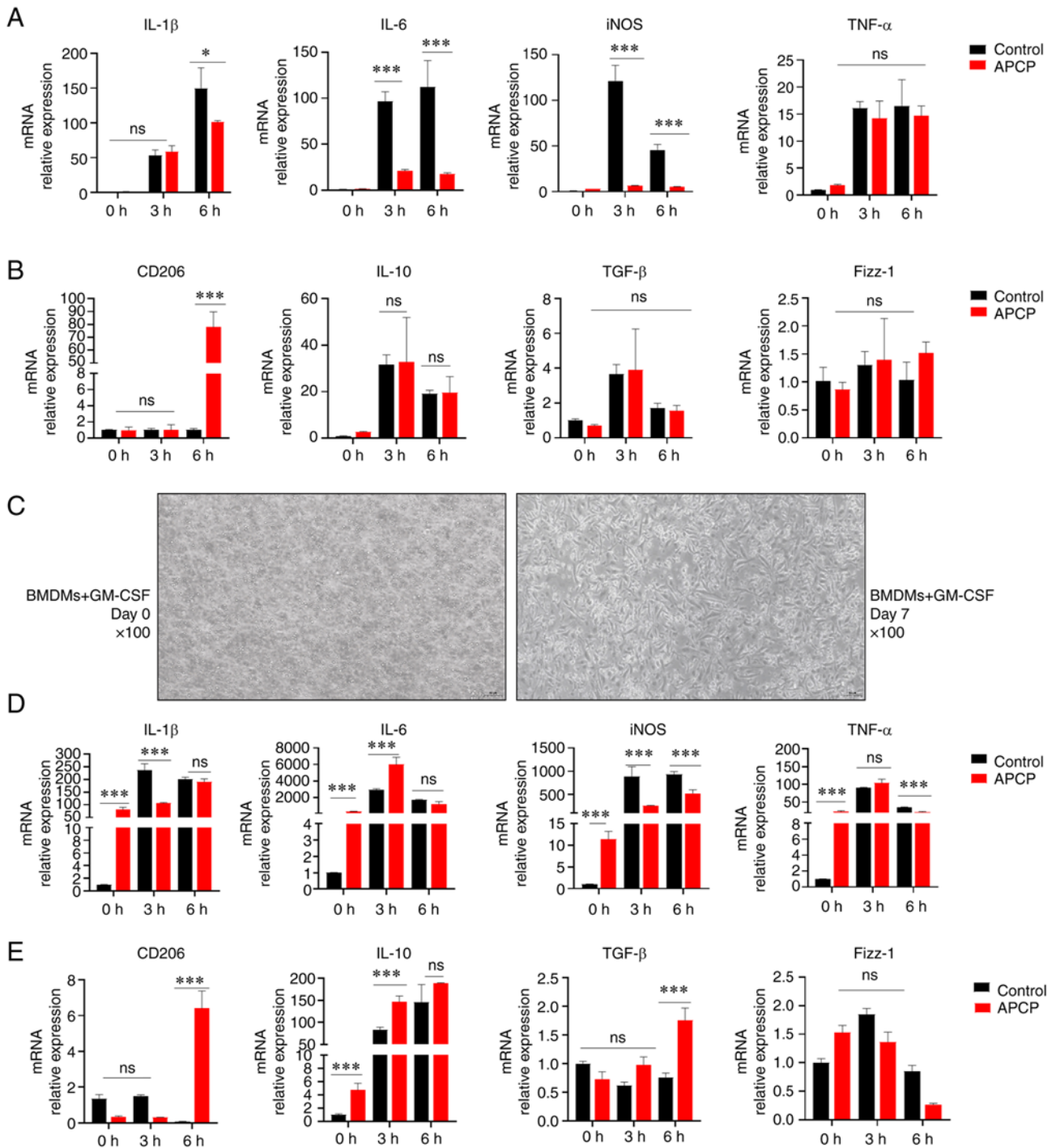


Figure 3. Blocking of CD73 suppresses the production of proinflammatory cytokines and promotes the production of anti-inflammatory cytokines. (A) mRNA expression of IL-1 β , IL-6, iNOS, and TNF- α in PMs treated with or without APCP after LPS (500 ng/ml) stimulation after 0, 3, and 6 h (n=3). (B) Gene expression of CD206, IL-10, TGF- β , and Fizz-1 in PMs treated with or without APCP after LPS (500 ng/ml) stimulation after 0, 3, and 6 h (n=3). (C) Images of treated BMDMs (magnification, $\times 100$; scale bar, 50 μ m). (D) Gene expression of IL-1 β , IL-6, iNOS, and TNF- α in BMDMs treated with or without APCP after LPS (500 ng/ml) stimulation after 0, 3, and 6 h (n=3). (E) Gene expression of CD206, IL-10, TGF- β , and Fizz-1 in BMDMs treated with or without APCP after LPS (500 ng/ml) stimulation after 0, 3, and 6 h (n=3). Gene expression was normalized to GAPDH in each group. * $P < 0.05$ and *** $P < 0.001$. ns, not significant; PM, peritoneal macrophage; APCP, adenosine 5'-(α , β -methylene) diphosphate; BMDM, bone marrow-derived macrophage; iNOS, inducible nitric oxide synthase; GM-CSF, granulocyte-macrophage-colony stimulating factor.

results further indicated that the blockade of CD73 increased M2 macrophage polarization in human macrophage cells, thereby exerting anti-inflammatory effects.

CD73 blockade alleviates DSS-induced colitis in mice. To verify the effect of CD73 on mucosal inflammation, the

changes in intestinal inflammation after blocking of CD73 in an animal model of DSS-induced colitis was assessed (Fig. 6A). DSS-treated and non-DSS-treated mice were injected intraperitoneally with APCP daily; PBS was additionally used as a negative control. Clinical disease severity was scored based on weight loss, diarrhea, and bleeding. Mice

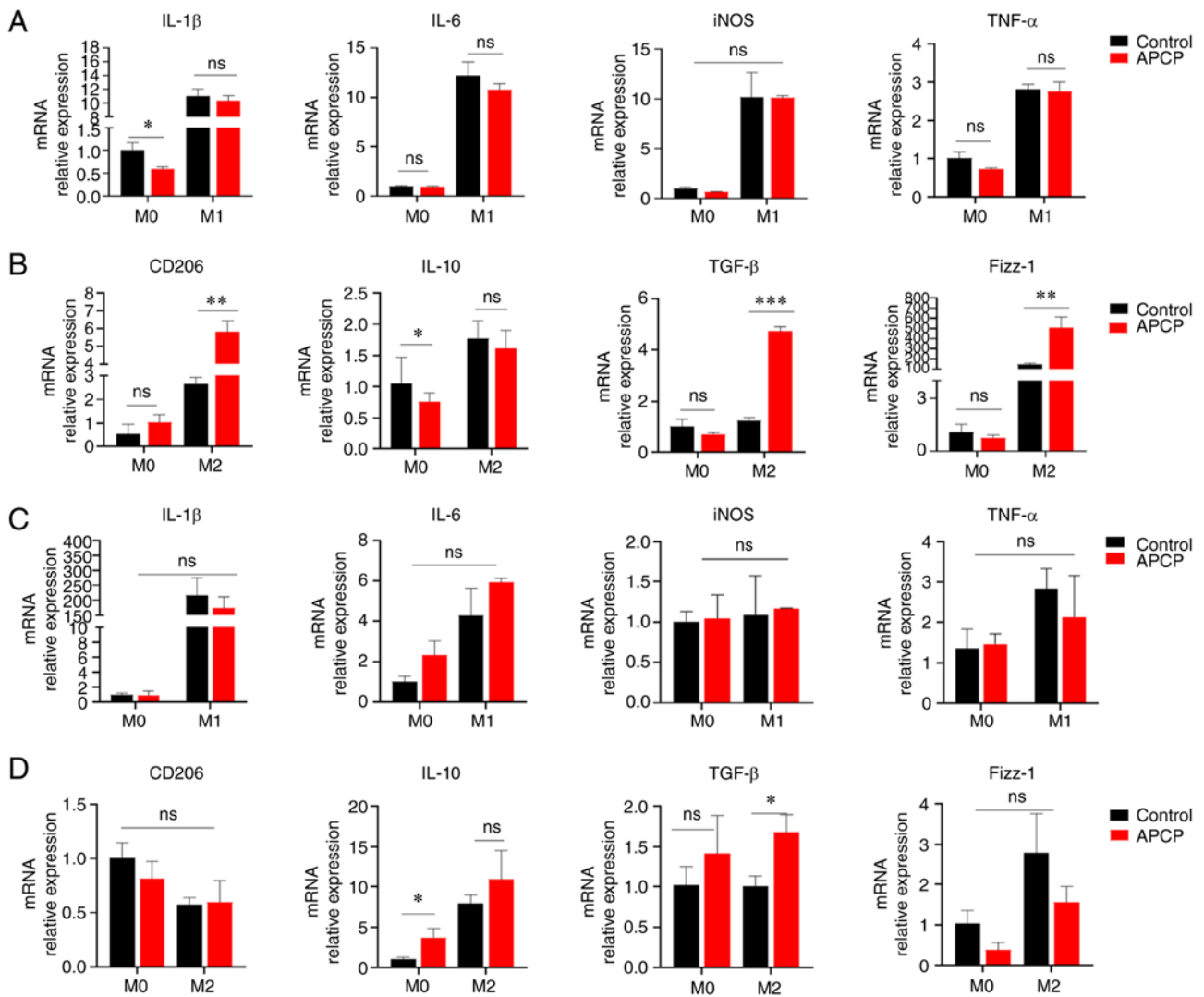


Figure 4. Blocking of CD73 increases M2 macrophage polarization. (A) The mRNA expression levels of IL-6, TNF- α , IL-1 β , and iNOS in PMs were determined after inducing differentiation into M0, M1, or M2 macrophages for 72 h (n=3). (B) The mRNA levels of TGF- β , Fizz-1, IL-10, and CD206 in PMs were determined (n=3). (C) The mRNA levels of IL-6, TNF- α , IL-1 β , and iNOS in BMDMs were determined (n=3). (D) The mRNA levels of TGF- β , Fizz-1, IL-10, and CD206 in BMDMs were determined (n=3). *P<0.05, **P<0.01 and ***P<0.001. ns, not significant; BMDM, bone marrow-derived macrophage; PM, peritoneal macrophage.

that were treated with APCP exhibited a significant increase in body weight compared with the negative control-DSS group (Fig. 6B). Treatment with DSS resulted in severe diarrhea and bleeding, and mice in the negative control DSS group had a shortened colonic length, severe diarrhea, and more bleeding than those treated with APCP (Fig. 6C). It has been previously that histological inflammation in UC patients is correlated with endoscopic inflammation of the ileal pouch, reflecting the severity of colonic inflammation to some extent (23). Following DSS treatment, the intestine produced remarkable inflammatory responses, primarily manifesting as inflammatory cell infiltration, loss of cup cells, crypt loss, crypt abscess formation, and reactive epithelial hyperplasia. Importantly, mice treated with APCP exhibited a significantly alleviated inflammatory reaction, compared with those treated with PBS, as demonstrated by hematoxylin and eosin histopathology analysis (Fig. 6D). These data demonstrate that the blocking of CD73 reduces inflammation in the DSS-induced colitis model.

Furthermore, the production of macrophage-associated inflammatory cytokines was also examined in the BMDMs using RT-qPCR. The levels of IL-1 β , TNF- α , iNOS, and IL-6 were significantly decreased in M1 macrophages of mice that received APCP treatment compared with the mice that did not receive APCP. Importantly, there was no significant difference in M0 macrophages that did undergo LPS-induced polarization (Fig. 6E). Interestingly, the expression of M2-associated inflammatory factors was elevated in both M0 and M2 macrophages in the APCP-treated mice. However, only the elevated levels of IL-10 were statistically significant, while CD206, TGF- β , and Fizz-1 were not significantly differentially expressed. Thus, CD73 affected the differentiation of macrophages *in vivo*, and also promoted intestinal inflammation by promoting M1 macrophage polarization and inhibiting M2 polarization.

CD73 regulates macrophage polarization via inhibition of p-p65 and ERK phosphorylation. To determine the mechanism

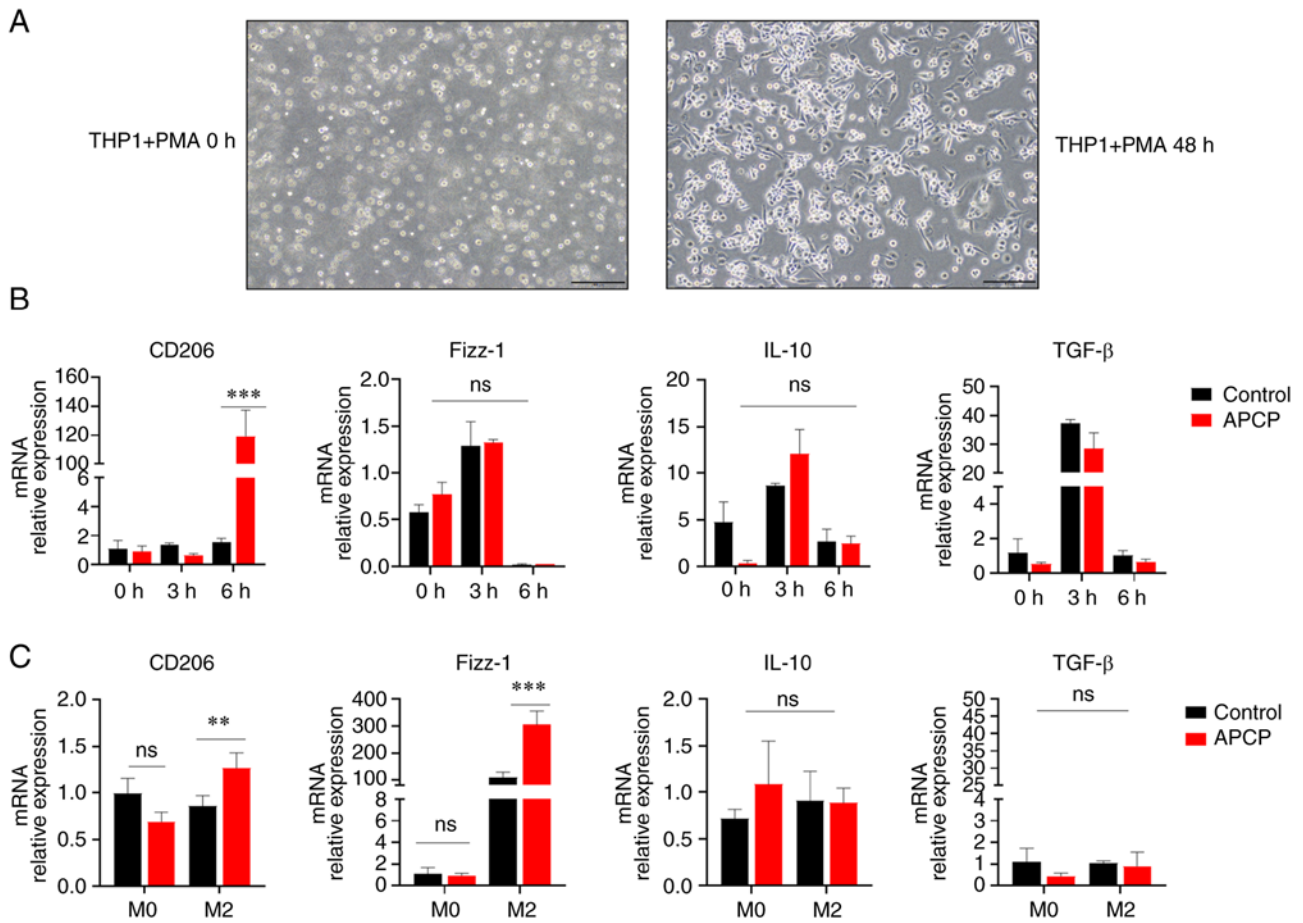


Figure 5. Blocking of CD73 promotes the production of anti-inflammatory cytokines and enhances M2 macrophage polarization in THP1 cells. (A) Images of THP1 cells treated with PMA. Magnification, x100; scale bar, 200 μ m. (B) mRNA expression of CD206, IL-10, TGF- β , and Fizz-1 in THP1 following stimulation with LPS (500 ng/ml) for 0, 3, or 6 h following treatment with APCP (20 nmol/ml) (n=3). (C) The mRNA levels of TGF- β , Fizz-1, IL-10, and CD206 in BMDMs were determined after IL-4 and IL-13-induced differentiation into M2 macrophages for 72 h (n=3). *P<0.01 and ***P<0.001. ns, not significant; BMDM, bone marrow-derived macrophage; PMA, phorbol12-myristate 13-acetate.

underlying the effects of CD73 in colitis, the signaling pathways closely related to macrophage polarization related to CD73 were assessed. Mechanistically, previous studies have reported that CD73 promotes optimal TLR-mediated response in tumor cells (24). LPS activates TLR4 to activate the NF- κ B and ERK signaling pathways (25). Thus, PMs were treated with LPS for lengths of time and the levels of p-p65 were assessed. The results showed that there were significantly detectable differences between the control and CD73-inhibited macrophages. Additionally, inhibition of CD73 increased the expression levels of several proteins in The ERK signaling pathway, including p-p65 and ERK (Fig. 7A). Subsequently, the ratio of phosphorylated proteins to the respective non-phosphorylated proteins in these pathways was assessed to further confirm that the p-p65 and ERK pathways were inhibited after CD73 blockade (Fig. 7B and C). Taken together, these data indicate that CD73 regulates LPS-induced pro-inflammatory responses in macrophages by modulating the activation of the NF- κ B and ERK signaling pathways.

Discussion

UC is a multifactorial chronic disease prone to relapse that is characterized by abnormal systemic and local dysregulation

of the mucosal immune response (26). Immune modulation is necessary to reduce the immune response and limit immunopathology following an intestinal infection or inflammation, which would otherwise cause the accumulation of large quantities of immune cells (12). Our understanding of the etiology and complexity of immune regulation in UC is still relatively limited (3,27). Although it has been shown that CD73 acts as an immunological factor that dampens the anti-tumor T-cell-mediated immune response, the investigation of CD73 function in UC is limited. The expression in CD73 on immune cells is species-specific (12). It is expressed on most immune cells including B cells, T cell subsets such as Tregs, and NK cells (28,29). Given the immunoregulatory abilities of CD73 in the immune response, it was hypothesized that CD73 may play an important part in IBD-associated macrophages by regulating pathways related to inflammation and immunity. In the present study, it was found that CD73 expression was upregulated in the inflamed mucosa of patients with UC, and that increased CD73 expression was positively correlated with disease activity and UCEIS. Importantly, colitis in mice was significantly alleviated after inhibition of CD73 with APCP. These data indicate that CD73 may play an important role in UC pathogenesis by regulating the immune response.

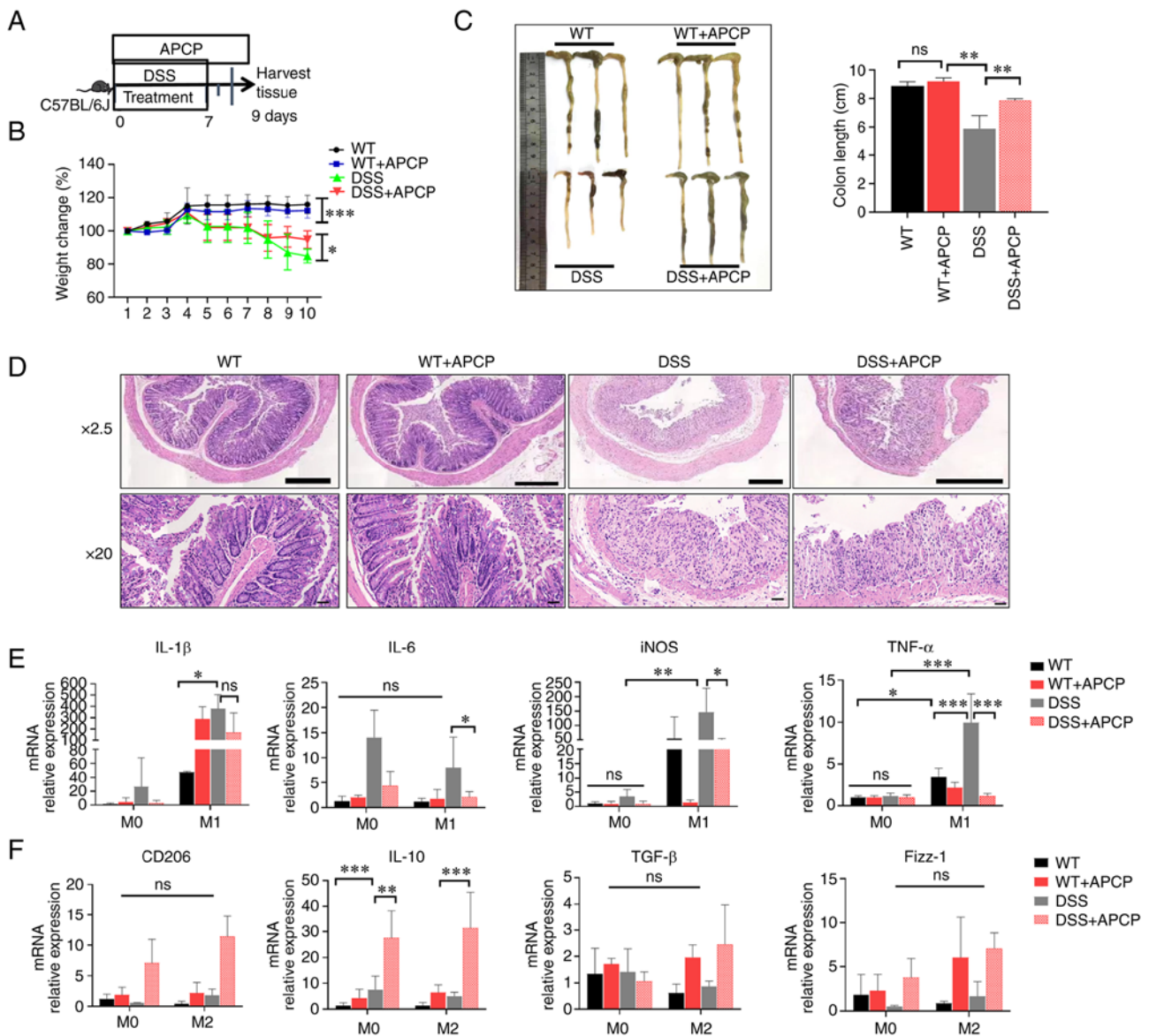


Figure 6. Blocking of CD73 alleviates DSS-induced colitis in mice. (A) Flow diagram of the establishment of the mouse model. (B) Changes in body weight were observed and are presented as a percentage of the initial body weight at the start of experiments. (C) Extracted colons and statistical analysis of the length of colons 9 days after DSS induction. (D) H&E biopsies of colon tissues in the different groups. Magnification, $\times 2.5$ and $\times 20$; scale bar, $50 \mu\text{m}$. (E) The mRNA levels of IL-6, TNF- α , IL-1 β , and iNOS in BMDMs from DSS-induced colitis mice. (F) The mRNA levels of TGF- β , Fizz-1, IL-10, and CD206 in BMDMs from DSS-induced colitis mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ns, not significant; H&E, hematoxylin and eosin; DSS, dextran sulfate sodium; BMDM, bone marrow-derived macrophage.

Macrophages, the key innate immune system cells, play important roles in several diseases, including IBD (2,30). Previous studies have demonstrated that LPS enhances M1 polarization, whereas IL13 and IL4 are crucial for polarization towards M2-associated genes (30). However, the mechanism underlying M1/M2-switch regulation associated with UC development remains to be elucidated. Classically activated M1-like macrophages are involved in initiating and maintaining inflammation, whereas alternatively activated M2-like macrophages are associated with inflammation regression (31). M1-like macrophages produce a wide range of proinflammatory cytokines, including TNF- α , IL-6, and iNOS (32), while M2-like macrophages produce large quantities of anti-inflammatory cytokines, including IL-10, TGF- β , and CD206, which can suppress intestinal inflammation (33). Macrophages with

increased M1 polarization and decreased M2 polarization are typically observed in the colon tissues of UC patients, and an M1/M2 imbalance is associated with the pathogenesis of UC (34). In the present study, the production of M1 and M2-related cytokines during inflammation was assessed, and the results showed that CD73 blockade reduced LPS-induced proinflammatory cytokine expression and increased the levels of markers of M2 macrophages, confirming that CD73 inhibited M2 macrophage activation while promoting M1 macrophage activation in intestinal mucosal inflammation of UC.

It has been shown that CD73 expression on tumor cells sufficiently facilitates tumor growth and metastasis by generating extracellular adenosine to mediate immune evasion and promote colitis-associated tumorigenesis in mice (15). The

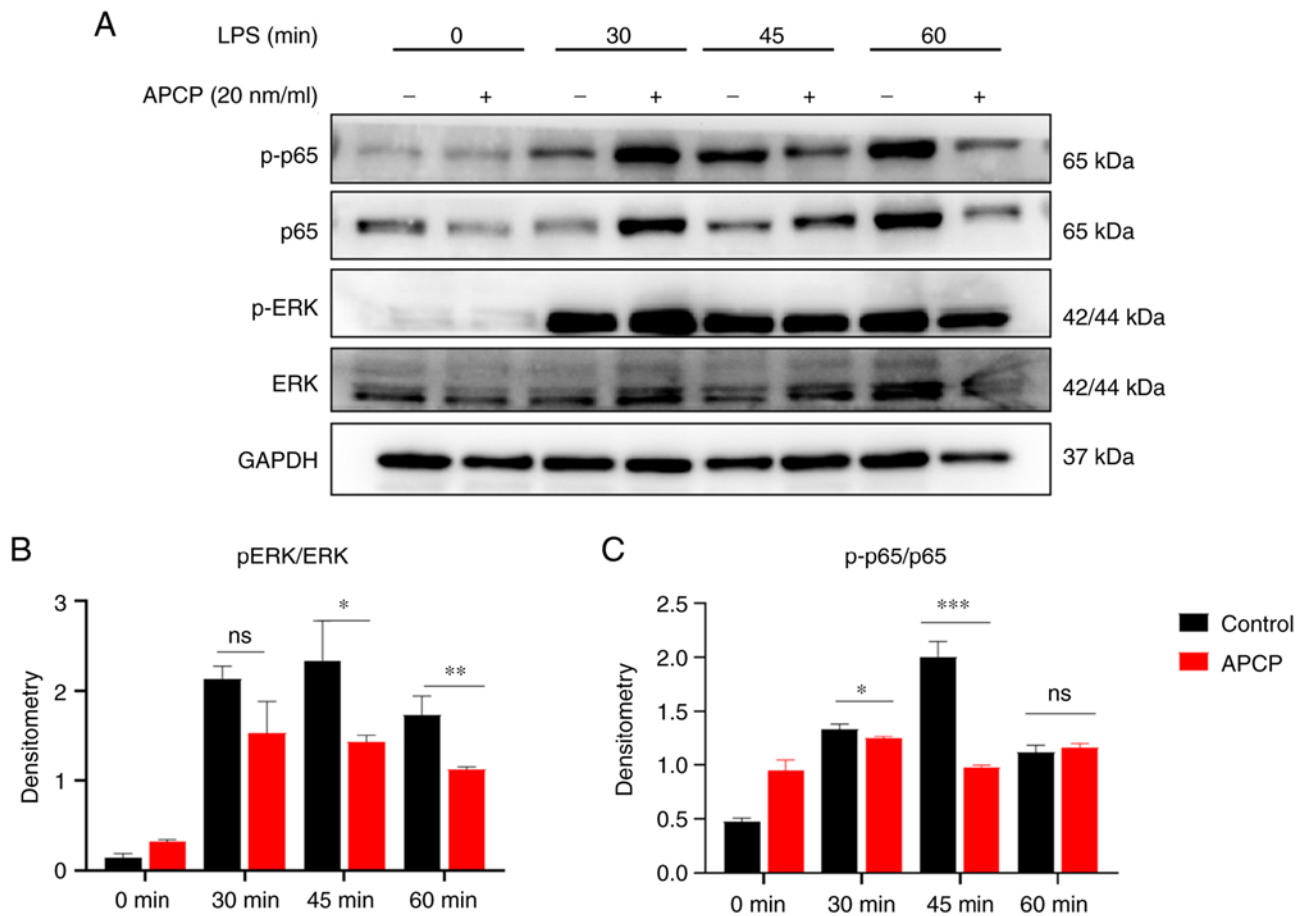


Figure 7. NF- κ B and ERK signaling is impaired in APCP-treated macrophages. (A) PMs were pretreated with APCP for 6 h, after which cells were stimulated with LPS (200 ng/ml) for 30, 45, or 60 min. Cell lysates were prepared and subjected to immunoblotting with the indicated antibodies. The histograms show the ratio of (B) p-ERK/ERK and (C) p-p65/p65 signaling. Data are shown as the mean \pm SEM. Data were compared using a Student's t-test. * P <0.05, ** P <0.01, *** P <0.001. LPS, lipopolysaccharide; APCP, adenosine 5'-(α , β -methylene) diphosphate.

activity of CD73 regulates macrophage function by converting M2 to M1 phenotypes. A growing body of clinical evidence and research data supports the involvement of purinergic signaling in the pathogenesis of IBD through inflammatory responses and modulation of bacterial alterations (35,36). Thus, how CD73 regulated intestinal inflammation *in vivo* was assessed. It was found that CD73 blockade by APCP could markedly alleviate DSS-induced colitis in mice. Moreover, CD73 blockade significantly reduced the quantities of inflammatory cytokines in BMDMs from DSS-induced colitis mice, which was consistent with the results *in vitro*. However, M1-related pro-inflammatory factors were significantly suppressed in mice injected intraperitoneally with APCP, while M2-related anti-inflammatory factors were not significantly elevated. It is hypothesized that the reason for this condition is the inflammatory state developed in mice by DSS.

One limitation of the present study was that a selective inhibitor rather than CD73 knockout mice was used. CD73-knockout mice are being established to further validate the results of the present study. Additionally, this study only investigated the inflammatory effect of CD73 on DSS-induced colitis in mice, and the study of other types of colitis should be assessed. It is hypothesized that CD73 knockout mice could be used to investigate the effects of CD73 on colonic inflammation and the bacterial flora in the Trinitro-benzene-sulfonic acid colitis model and the *Citrobacter*

Rodentium colitis model (37,38). A previous study showed that the efficacy of organoid transplantation on the treatment of intestinal inflammation in experimental colitis models is relatively mature (39); thus organoid transplantation technology may be useful for further investigation of the mechanism of CD73 action on the intestinal epithelium in UC at a later stage.

Excessive NF- κ B activation exacerbates the severity of intestinal inflammation in UC patients (40). As such, NF- κ B blockade has become a promising therapeutic strategy for the management of UC (41). The phosphorylation of p65, which initiates the activation of NF- κ B, is a key regulator of the NF- κ B pathway (42), which regulates multiple aspects of innate and adaptive immunity. Canonical NF- κ B induces the production of proinflammatory cytokines, such as TNF- α and IL-1 β in the innate immune system, resulting in an inflammatory response (43). Interestingly, it was shown that CD73 blockade significantly inhibited p-p65 and p65 expression, suggesting that CD73 may exert immune effects in inflammation via NF- κ B activation. ERK is a serine/threonine protein kinase that mediates fundamental biological processes and cellular responses to external stress signals, and also regulates the synthesis of inflammation and apoptosis mediators (44). Accumulating evidence has revealed that ERK also regulates the transcriptional activity of NF- κ B-p65 (45). Thus, the protein expression levels of p-ERK and ERK were assessed following CD73

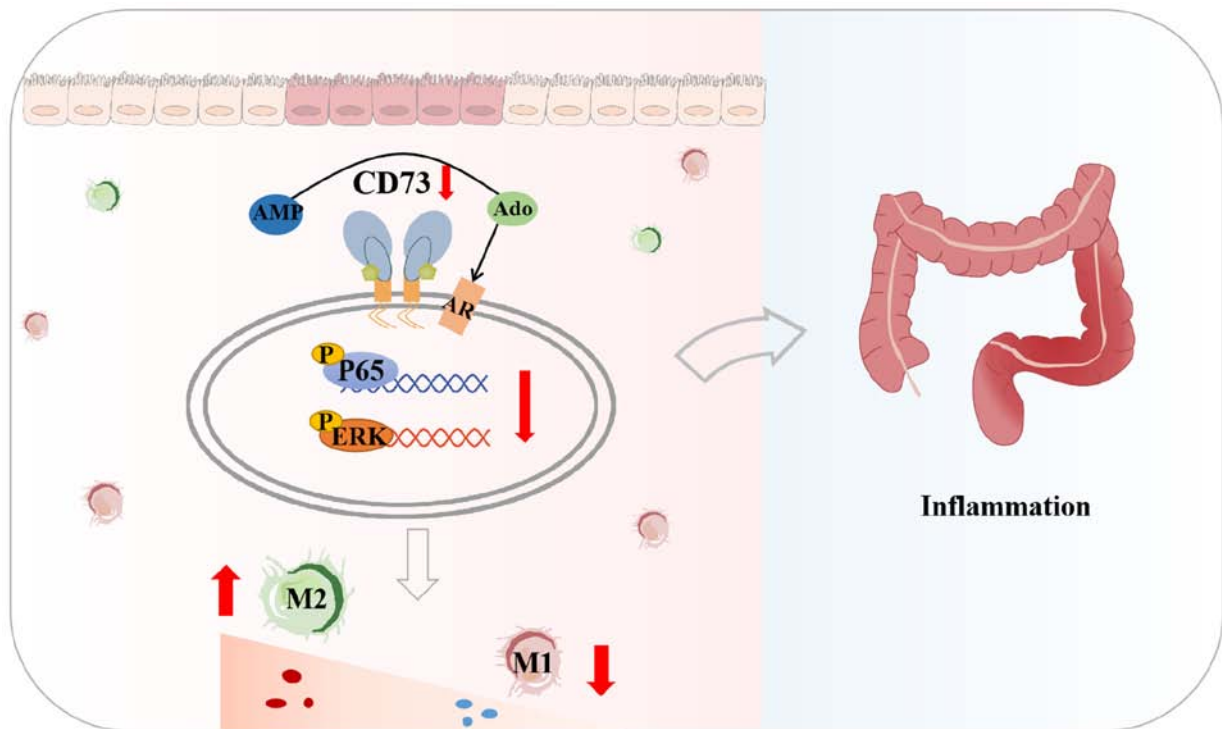


Figure 8. CD73 modulates intestinal macrophage polarization via the NF- κ B and ERK signaling pathways to maintain intestinal mucosal homeostasis. CD73 in macrophages increase the release of proinflammatory cytokines (e.g., IL-10, TNF- α and IL-6) to aggravate intestinal inflammation. In addition, the blockade of CD73 contributed to an increase in polarization towards an M2-like phenotype and a decrease in polarization towards an M1-like phenotype. Thus, CD73 played a critical role in regulating macrophage polarization and in the maintenance of intestinal mucosal homeostasis via the NF- κ B and ERK axis. Ado, adenosine; AR, adenosine receptors; AMP, adenosine monophosphate.

inhibition. In agreement with the p-p65 results, the blockade of CD73 also decreased the expression of the p-ERK and ERK. These data suggested that CD73 exhibited pro-inflammatory effects via activation of NF- κ B p65 and ERK. Together, it was shown that CD73 expression regulates the intestinal inflammatory response through the regulation of the clinical phenotype and inflammatory mediator mechanisms of macrophages.

In conclusion, the results of the present study showed that CD73 regulates the intestinal inflammatory immune response by switching M1/M2 macrophage polarization as well as the expression of related inflammatory factors via NF- κ B and ERK signaling, thereby exerting proinflammatory effects (Fig. 8). Therefore, CD73 may be considered as a diagnostic biomarker and drug target for the treatment of inflammation-related diseases. Targeting CD73 in the appropriate cell types should be considered as a potential treatment of inflammatory diseases such as UC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

RW and GZ conceived and designed the study. CW and RW performed the experiments. YiW, YaW contributed to acquisition of the clinical data and specimens. FZ, RW, YaW and YY analyzed the data. GJ, YY, YiW and RW helped to interpret the data, and write and review the manuscript. GJ, GZ and YY confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was reviewed and approved by the Ethics Committee of Jining Medical University (approval no. 2021-09-C002; Jining, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Larabi A, Barnich N and Nguyen HTT: New insights into the interplay between autophagy, gut microbiota and inflammatory responses in IBD. *Autophagy* 16: 38-51, 2020.
- Na YR, Stakenborg M, Seok SH and Matteoli G: Macrophages in intestinal inflammation and resolution: A potential therapeutic target in IBD. *Nat Rev Gastroenterol Hepatol* 16: 531-543, 2019.
- Schett G and Neurath MF: Resolution of chronic inflammatory disease: Universal and tissue-specific concepts. *Nat Commun* 9: 3261, 2018.
- Matheis F, Muller PA, Graves CL, Gabanyi I, Kerner ZJ, Costa-Borges D, Ahrends T, Rosenstiel P and Mucida D: Adrenergic signaling in muscularis macrophages limits infection-induced neuronal loss. *Cell* 180: 64-78 e16, 2020.
- Lu H, Zhang C, Wu W, *et al*: MCP1 restrains mucosal inflammation by orchestrating the intestinal monocyte to macrophage maturation via an ATF3-AP1S2 axis. *Gut* 72: 882-895, 2023.
- Mortha A, Chudnovskiy A, Hashimoto D, Bogunovic M, Spencer SP, Belkaid Y and Merad M: Microbiota-dependent crosstalk between macrophages and ILC3 promotes intestinal homeostasis. *Science* 343: 1249288, 2014.
- Chistiakov DA, Myasoedova VA, Revin VV, Orekhov AN and Bobryshev YV: The impact of interferon-regulatory factors to macrophage differentiation and polarization into M1 and M2. *Immunobiology* 223: 101-111, 2018.
- Lissner D, Schumann M, Batra A, Kredel LI, Kühl AA, Erben U, May C, Schulzke JD and Siegmund B: Monocyte and M1 macrophage-induced barrier defect contributes to chronic intestinal inflammation in IBD. *Inflamm Bowel Dis* 21: 1297-1305, 2015.
- Biswas A, Shouval DS, Griffith A, Goettel JA, Field M, Kang YH, Konnikova L, Janssen E and Redhu NS: WASP-mediated regulation of anti-inflammatory macrophages is IL-10 dependent and is critical for intestinal homeostasis. *Nat Commun* 9: 1779, 2018.
- Liu XH, Wu XR, Lan N, Zheng XB, Zhou C, Hu T, Chen YF, Cai ZR, Chen ZX, Lan P and Wu XJ: CD73 promotes colitis-associated tumorigenesis in mice. *Oncol Lett* 20: 1221-1230, 2020.
- Zarek PE, Huang CT, Lutz ER, Kowalski J, Horton MR, Linden J, Drake CG and Powell JD: A2A receptor signaling promotes peripheral tolerance by inducing T-cell anergy and the generation of adaptive regulatory T cells. *Blood* 111: 251-259, 2008.
- Schneider E, Rissiek A, Winzer R, Puig B, Rissiek B, Haag F, Mittrücker HW, Magnus T and Tolosa E: Generation and function of non-cell-bound CD73 in inflammation. *Front Immunol* 10: 1729, 2019.
- Petruk N, Tuominen S, Akerfelt M, Mattsson J, Sandholm J, Nees M, Yegutkin GG, Jukkola A, Tuomela J and Selander KS: CD73 facilitates EMT progression and promotes lung metastases in triple-negative breast cancer. *Sci Rep* 11: 6035, 2021.
- Lopes DV, de Fraga Dias A, Silva LFL, Scholl JN, Sévigny J, Battastini AMO and Figueiró F: Influence of NSAIDs and methotrexate on CD73 expression and glioma cell growth. *Purinergic Signal* 17: 273-284, 2021.
- Röh M, Wainwright DA, Wu JD, Wan Y and Zhang B: Targeting CD73 to augment cancer immunotherapy. *Curr Opin Pharmacol* 53: 66-76, 2020.
- Núñez FP, Krugliak Cleveland N, Quera R and Rubin DT: Evolving role of endoscopy in inflammatory bowel disease: Going beyond diagnosis. *World J Gastroenterol* 27: 2521-2530, 2021.
- Zhang XF, Li P, Ding XL, Chen H, Wang SJ, Jin SB, Guo J and Tian ZB: Comparing the clinical application values of the Degree of Ulcerative Colitis Burden of Luminal Inflammation (DUBLIN) score and Ulcerative Colitis Endoscopic Index of Severity (UCEIS) in patients with ulcerative colitis. *Gastroenterol Rep (Oxf)* 9: 533-542, 2021.
- Chassaing B, Aitken JD, Malleshappa M and Vijay-Kumar M: Dextran sulfate sodium (DSS)-induced colitis in mice. *Curr Protoc Immunol* 104: 15.25.1-15.25.14, 2019.
- Yang R, Liao Y, Wang L, He P, Hu Y, Yuan D, Wu Z and Sun X: Exosomes Derived From M2b Macrophages Attenuate DSS-Induced Colitis. *Front Immunol* 10: 2346, 2019.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.
- Huang B, Chen Z, Geng L, Wang J, Liang H, Cao Y, Chen H, Huang W, Su M, Wang H, *et al*: Mucosal profiling of pediatric-onset colitis and IBD reveals common pathogenesis and therapeutic pathways. *Cell* 179: 1160-1176 e24, 2019.
- Zhao YL, Tian PX, Han F, Zheng J, Xia XX, Xue WJ, Ding XM and Ding CG: Comparison of the characteristics of macrophages derived from murine spleen, peritoneal cavity, and bone marrow. *J Zhejiang Univ Sci B* 18: 1055-1063, 2017.
- Toritani K, Kimura H, Otani M, Fukuoka H, Kunisaki R, Watanabe J, Ishibe A, Misumi T, Inayama Y and Endo I: Inflammatory bowel disease-specific findings are common morphological changes in the ileal pouch with ulcerative colitis. *Sci Rep* 12: 20361, 2022.
- Liu B, Zheng Y, Yin F, Yu J, Silverman N and Pan D: Toll receptor-mediated hippo signaling controls innate immunity in *Drosophila*. *Cell* 164: 406-419, 2016.
- Ren Q, Guo F, Tao S, Huang R, Ma L and Fu P: Flavonoid fisetin alleviates kidney inflammation and apoptosis via inhibiting Src-mediated NF- κ B p65 and MAPK signaling pathways in septic AKI mice. *Biomed Pharmacother* 122: 109772, 2020.
- Maloy KJ and Powrie F: Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature* 474: 298-306, 2011.
- Liu TC and Stappenbeck TS: Genetics and pathogenesis of inflammatory bowel disease. *Annu Rev Pathol* 11: 127-148, 2016.
- Rackowski F, Rissiek A, Ricklefs I, Heiss K, Schumacher V, Wundenberg K, Haag F, Koch-Nolte F, Tolosa E and Mittrücker HW: CD39 is upregulated during activation of mouse and human T cells and attenuates the immune response to *Listeria monocytogenes*. *PLoS One* 13: e0197151, 2018.
- Liang D, Zuo A, Zhao R, Shao H, Born WK, O'Brien RL, Kaplan HJ and Sun D: CD73 Expressed on $\gamma\delta$ T cells shapes their regulatory effect in experimental autoimmune uveitis. *PLoS One* 11: e0150078, 2016.
- Lawrence T and Natoli G: Transcriptional regulation of macrophage polarization: Enabling diversity with identity. *Nat Rev Immunol* 11: 750-761, 2011.
- Liu X, Ren X, Zhou L, Liu K, Deng L, Qing Q, Li J, Zhi F and Li M: Tollip orchestrates macrophage polarization to alleviate intestinal mucosal inflammation. *J Crohns Colitis* 16: 1151-1167, 2022.
- Yao X, Dong G, Zhu Y, Yan F, Zhang H, Ma Q, Fu X, Li X, Zhang Q, Zhang J, *et al*: Leukadherin-1-Mediated Activation of CD11b Inhibits LPS-Induced pro-inflammatory response in macrophages and protects mice against endotoxic shock by blocking LPS-TLR4 interaction. *Front Immunol* 10: 215, 2019.
- Shouval DS, Biswas A, Goettel JA, McCann K, Conaway E, Redhu NS, Mascanfroni ID, Al Adham Z, Lavoie S, Ibourk M, *et al*: Interleukin-10 receptor signaling in innate immune cells regulates mucosal immune tolerance and anti-inflammatory macrophage function. *Immunity* 40: 706-719, 2014.
- Guilliams M, Thierry GR, Bonnardel J and Bajenoff M: Establishment and maintenance of the macrophage niche. *Immunity* 52: 434-451, 2020.
- Longhi MS, Moss A, Jiang ZG and Robson SC: Purinergic signaling during intestinal inflammation. *J Mol Med (Berl)* 95: 915-925, 2017.
- Costales MG, Alam MS, Cavanaugh C and Williams KM: Extracellular adenosine produced by ecto-5'-nucleotidase (CD73) regulates macrophage pro-inflammatory responses, nitric oxide production, and favors *Salmonella* persistence. *Nitric Oxide* 72: 7-15, 2018.
- Wirtz S, Popp V, Kindermann M, Gerlach K, Weigmann B, Fichtner-Feigl S and Neurath MF: Chemically induced mouse models of acute and chronic intestinal inflammation. *Nat Protoc* 12: 1295-1309, 2017.
- Yang W, Liu H, Xu L, Yu T, Zhao X, Yao S, Zhao Q, Barnes S, Cohn SM, Dann SM, *et al*: GPR120 inhibits colitis through regulation of CD4(+) T Cell Interleukin 10. *Gastroenterology* 162: 150-165, 2022.
- Watanabe S, Kobayashi S, Ogasawara N, Okamoto R, Nakamura T, Watanabe M, Jensen KB and Yui S: Transplantation of intestinal organoids into a mouse model of colitis. *Nat Protoc* 17: 649-671, 2022.
- Andresen L, Jorgensen VL, Perner A, Hansen A, Eugen-Olsen J and Rask-Madsen J: Activation of nuclear factor kappaB in colonic mucosa from patients with collagenous and ulcerative colitis. *Gut* 54: 503-509, 2005.
- Zhu Y, Yang S, Zhao N, Liu C, Zhang F, Guo Y and Liu H: CXCL8 chemokine in ulcerative colitis. *Biomed Pharmacother* 138: 111427, 2021.
- Barnabei L, Laplantine E, Mbongo W, Rieux-Laucat F and Weil R: NF- κ B: At the borders of autoimmunity and inflammation. *Front Immunol* 12: 716469, 2021.

43. Yu H, Lin L, Zhang Z, Zhang H and Hu H: Targeting NF- κ B pathway for the therapy of diseases: Mechanism and clinical study. *Signal Transduct Target Ther* 5: 209, 2020.
44. Zhang Y, Zhou S, Zhou J, Wang D and Zhou T: Regulation of NF- κ B/MAPK signaling pathway attenuates the acute lung inflammation in *Klebsiella pneumoniae* rats by mollugin treatment. *Microb Pathog* 132: 369-373, 2019.
45. Zhuo Y, Li D, Cui L, Li C, Zhang S, Zhang Q, Zhang L, Wang X and Yang L: Treatment with 3,4-dihydroxyphenylethyl alcohol glycoside ameliorates sepsis-induced ALI in mice by reducing inflammation and regulating M1 polarization. *Biomed Pharmacother* 116: 109012, 2019.



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