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ORIGINAL ARTICLE

Human umbilical cord mesenchymal stem cells alleviate inflammatory bowel disease by inhibiting ERK phosphorylation in neutrophils

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Abstract

Inflammatory bowel disease (IBD) can be caused by a variety of factors, including hereditary and environmental influences, that lead to dysfunction of the intestinal immune system. Mesenchymal stem cells (MSCs) exhibit important regulatory roles in relieving inflammation and repairing damaged tissues. Although neutrophils are important participants in the development of inflammatory reactions, they are also essential for maintaining intestinal balance during the process of mitigation of IBD by MSCs. Here, we constructed a dextran sulfate sodium (DSS)-induced mouse IBD model and evaluated the effects of treatment with human umbilical cord MSCs. Mouse body weight, faecal traits, colon/spleen gross morphology, tissue histology and immunohistochemical staining, and inflammatory factors were analysed. Magnetic beads were used to sort infiltrating neutrophils from intestinal tissues, and their phenotypes were identified. The neutrophil inflammatory environment was also simulated in vitro, and signalling pathways involved in MSC regulation of neutrophil phenotype were analysed. Human umbilical cord MSCs effectively alleviated DSS-induced weight loss, colon shortening, and intestinal mucosal injury, and reduced clinical disease activity index. The number of neutrophils that infiltrated the intestines of mice treated with human umbilical cord MSCs were decreased and polarised toward the N2 phenotype; at the same time, ERK phosphorylation was inhibited. In vitro experiments showed that addition of the ERK phosphorylation inhibitor, PD98059, down-regulated the expression of N1 neutrophils, while up-regulating that of N2 neutrophils. The colon tissues from patients with IBD were infiltrated with neutrophils. Further, relative to healthy controls, the markers of N1 neutrophils (ICAM-1, FAS, and CCL3) were highly expressed in colon tissues from patients with IBD, whereas the markers of N2 neutrophils (VEGF, CCL2, and CXCR4) were almost undetectable. In conclusion, during alleviation of IBD, human umbilical cord MSCs polarise neutrophils toward the "N2" phenotype by inhibiting activation of ERK signalling.

Keywords Mesenchymal stem cells · Inflammatory bowel disease · Neutrophils · ERK · N2 polarisation

Abbreviations

CD	Crohn's disease
CXCR4	CXC chemokine receptor 4
DAI	Disease activity index
DSS	Dextran sodium sulfate
FAS	Factor-associated suicide
FCM	Flow cytometry
HE	Haematoxylin–eosin
IBD	Inflammatory bowel disease
ICAM-1	Intercellular cell adhesion molecule-1
IHC	Immunohistochemistry analysis
IL	Interleukin
LPS	Lipopolysaccharide
MACS	Magnetic-activated cell sorting
MCP1/CCL2	Low expression of monocyte chemotactic protein 1
MIP-1α/CCL3	Giant phage cell inflammatory protein-1α
MSC	Mesenchymal stem cell
QRT-PCR	Quantitative real-time polymerase chain reaction
TNF	Tumour necrosis factor

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UC	Ulcerative colitis
VEGF	Vascular endothelial growth factor

Introduction

Inflammatory bowel disease (IBD) describes a group of conditions caused by interactions between environmental, genetic, infection, and immune factors, which mainly affect the intestinal mucosal immune system. IBD includes the chronic inflammatory gastrointestinal diseases, ulcerative colitis (UC) and Crohn's disease (CD). Imbalances between pro-inflammatory and anti-inflammatory cytokines, as well as activation of immune cells, such as macrophages and neutrophils, can aggravate the inflammatory response in IBD. The incidence of IBD has increased rapidly in recent years (Andrews et al. 2016; Owczarek et al. 2016). Clinically, chronic gastrointestinal inflammation is a major risk factor for gastrointestinal tumours in patients with IBD (Axelrad et al. 2016). It is documented that interventions in IBD can effectively prevent the occurrence and development of gastrointestinal cancer; however, traditional therapies for IBD are unsatisfactory (Uranga et al. 2016), and hence there is an urgent need to find new alternatives for clinical treatment of this condition.

Mesenchymal stem cells (MSCs) have self-renewal and tissue repair abilities, and can differentiate into bone, cartilage, and fat. In addition, they can influence tissue repair through paracrine mechanisms or via direct contact between cells. MSCs relieve inflammation and promote the amelioration of IBD (Lin et al. 2015), while neutrophils have a significant role in the inflammatory process. Neutrophils are short-lived terminally differentiated cells that can be activated to improve their viability under infectious or inflammatory conditions (Zhang et al. 2018). Long-term studies have shown that neutrophils may have dual roles in that they are both beneficial for the resolution of inflammation and harmful when over-activated, leading to damage to accessory tissues. Hence, neutrophil dysfunction and high reactivity can cause intestinal inflammation, while functional neutrophils are also essential for maintaining intestinal balance.

Neutrophils have two phenotypes, N1 and N2 (Fridlender et al. 2009). Generally, N1- and N2-polarised neutrophils can be distinguished according to their phenotype and function (Zhang et al. 2016). N1-polarised neutrophils have a mature phenotype and short lifespan, and express high levels of factor-associated suicide (FAS), intercellular cell adhesion molecule-1 (ICAM-1), and giant phage cell inflammatory protein-1α (MIP-1α, also known as CCL3). Further, they express low levels of monocyte chemotactic protein 1 (MCP1, also known as CCL2), CXC chemokine receptor 4 (CXCR-4), vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8). N1 neutrophils mediate cytotoxic effects, as well as mediating tumour rejection and anti-tumour immunity. In contrast, N2-polarised neutrophils have a long lifespan and an immature cell phenotype, expressing high levels of CCL2, CXCR4, VEGF, and IL-8, and low levels of FAS, ICAM-1, and CCL3. N2 cells show low levels of cytotoxicity, but high pro-angiogenic, pro-metastatic, and immunosuppressive activity. The phenomenon of N2 neutrophil generation is referred to as polarisation (Piccard et al. 2012; Zhang et al. 2018).

To study the mechanism by which human umbilical cord MSCs regulate neutrophil phenotype polarisation and reduce IBD inflammatory responses, we established a mouse IBD model, induced using dextran sulfate sodium (DSS). Our results show that human umbilical cord MSCs induced neutrophils toward N2 phenotype polarisation by inhibiting ERK phosphorylation during repair of IBD.

Materials and methods

Experiments involving animals and humans were approved by the Ethical Committee of Jiangsu University (2012258).

Cell culture

MSCs were derived from human umbilical cord (Zhenjiang Fourth People's Hospital, Jiangsu, China) using previously described isolation and culture methods (Qiao et al. 2008). MSCs were cultured in α-MEM medium (Invitrogen, Carlsbad, CA, USA), while HL-60 cells (Institute of Biological Sciences at the Chinese Academy of Science, Shanghai, China) were cultured in RPMI 1640 medium (Invitrogen). Cells were maintained in medium supplemented with 10% fetal bovine serum (FBS; Invitrogen) at 37 °C in humidified air with 5% CO_2. HL-60 cells were stimulated and induced by adding DMSO (final concentration 2.5%) during logarithmic growth phase; after 48 h, cells were collected for subsequent experiments.

Neutrophil isolation

C57BL/6 mice were sacrificed, and femurs and tibias removed immediately. Bone marrow was washed out using physiological saline solution, and red blood cells lysed using a hypotonic lysing procedure. The cell suspension was then filtered through a 200-mesh cell screen and physically purified neutrophils obtained using Percoll separation solution (Percoll; Absin, China). Neutrophils were cultured in RPMI 1640 medium supplemented with 10% FBS at a density of 1×10^6 per well.

Animal model establishment and treatment

C57BL/6 mice (8 weeks old) were purchased from the Laboratory Animal Research Center of Jiangsu University (Jiangsu, China). All experimental procedures were conducted in accordance with the Animal Use and Care Committee of Jiangsu University.

Mice were divided into the following groups ($n = 6$ per group): control group (Neg), IBD group (DSS), and MSC group (MSC). Control group mice were provided with autoclaved purified water, while the IBD and MSC groups were provided with 3% DSS in autoclaved purified water. The MSC group were intraperitoneally injected with 3×10^6 human umbilical cord MSCs on the 3rd, 6th, and 9th days after drinking 3% DSS water. When mice in the IBD group were observed to have clear symptoms, such as blood in the stool and weight loss, all mice were sacrificed, and colon and spleen tissues collected for subsequent experiments.

For in vivo cell localisation assay, 3×10^6 MSCs were incubated with 10 μg DIR (Ouhe Technology, Beijing, China) for 30 min at 37 °C after incubation, then the labelled MSCs were collected and intraperitoneally injected into the mice. The presence of labelled MSCs in colon tissues was detected in a live animal imaging system.

Magnetic-activated cell sorting (MACS)

The mice were sacrificed, then soaked in 75% ethanol for 5 min. The colon was removed, the intestinal contents washed with PBS, and the colon was minced and digested with collagenase lysate at 37 °C for 20 min, with constant shaking. The cell suspension was then filtered through a 200-mesh cell screen and incubated with anti-Ly-6B (7/4) MicroBeads (10 μl/10^7 cells) (Miltenyi, Germany) for 30 min at 4 °C. Finally, labelled cells were collected using LS columns (Miltenyi) and washed with PBS before use in experiments.

Flow cytometry (FCM)

MSCs were resuspended in PBS to 10^5/200 μl, then incubated with CD105 + flow antibody (PE; 1:200; Biosciences), CD73 + flow antibody (FITC; 1:200; Biosciences), CD90 + flow antibody (FITC; 1:200; Biosciences), CD11b + flow antibody (FITC; 1:200; Biosciences), and CD34 + flow antibody (FITC; 1:200; Biosciences), respectively, with shaking at 4 °C for 30 min. Flow cytometry was performed to characterise the MSCs.

Cells isolated from colon tissues were resuspended in PBS to 10^5/200 μl, then incubated with CD11b + flow antibody (FITC; 1:200; Biosciences) with shaking at 4 °C for 30 min. We verified the availability of neutrophils by flow cytometry analysis to determine the proportion of CD11b-positive cells.

Co-culture of HL-60/neutrophils with MSCs

HL-60/neutrophils (10^6) were cultured in 6-well culture plates and stimulated with lipopolysaccharide (LPS) (2 μl, 100 ng/ml; Sigma) to induce an inflammatory environment, treated with ERK phosphorylation inhibitor PD98059 (1 μl, 50 μM; CST), and co-cultured with MSCs (1×10^5) in a trans-well system (0.4 μm; Corning) for 72 h.

Haematoxylin–eosin (HE) staining

To clearly observe structural damage to the colonic mucosa and spleen tissue, HE staining was performed according to standard procedures. Tissue samples were immersed in 4% paraformaldehyde, embedded in paraffin, cut into 4-μM sections and stained with HE stain.

Immunohistochemistry analysis (IHC)

Paraffin-embedded mouse colon and spleen tissues were dewaxed after high-temperature fixation, and then incubated in 3% hydrogen peroxide for 30 min to inhibit endogenous peroxidase activity, and boiled in citrate buffer for 30 min for antigen retrieval. Sections were then blocked with 5% BSA and incubated with CD11b (1:200; Bioworld), ICAM-1 (1:200; Abcam), FAS (1:200; Absin), CCL3 (1:200; Absin), VEGF (1:200; Abcam), CXCR4 (1:200; Abcam), and CCL2 (1:200; ABclonal) primary antibodies overnight at 4 °C. Then, the samples were incubated with secondary antibodies for 30 min at room temperature and colour development carried out using diaminobenzidine, with counterstaining using haematoxylin, before observation under a microscope.

RNA extraction and quantitative real-time PCR

RNA was extracted from the colon mucosa, neutrophils, and HL-60 cells using Trizol Reagent (Life Technologies, Carlsbad, CA, USA) and cDNA synthesized using the HiScript 1st Strand cDNA Synthesis Kit (Vazyme Biotech, Shanghai, China). Quantitative real-time polymerase chain reaction (QRT-PCR) was conducted in a Step One Plus Real-Time PCR System (Applied Biosystems, Life Technologies, USA) to detect gene expression. The sequences of primers are listed in Tables 1 and 2.

Western blotting analysis

Colon mucosa, neutrophils, and HL-60 cells were lysed in RIPA lysis buffer, supplemented by proteinase inhibitors (Vazyme Biotech, Shanghai, China). Protein samples were then subjected to gradient separation by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and then the separated proteins transferred to polyvinylidene

Table 1 Primer sequences for RT-PCR (species: mouse)

Genes	Primer sequence	Annealing temp. (°C)	Amplicon size (bp)
β-Actin	For: CTCAGGAGGAGCAATGATCT	58	129
	Rev: GACCTGTACGCCAACACAGT		
CD11b	For: CCCCAAGAAAGTAGCAAGG	60	202
	Rev: TGTCTCATCAAAGAAGGCAC		
IL-1β	For: AGCTTCAGGCAGGCAGTATC	61	215
	Rev: TCATCTCGGAGCCTGTAGTG		
IL-6	For: AAGTCCGGAGAGGAGACTTC	58	487
	Rev: TGGATGGTCTTGGTCCTTAG		
IL-8	For: CATCTTCGCTGTCGTCCTT	62	326
	Rev: AGAGGGTAGTAGAGGTGTTTGC		
IL-10	For: CCTGGCTCAGCACTGCTATG	58	151
	Rev: TCACCTGGCTGAAGGCAGTC		
TNF-α	For: AACTCCAGGCGGTGCCTATG	63	242
	Rev: TCCAGCTGCTCCTCCACTTG		
ICAM-1	For: CGTGCTGTATGGTCCTCG	60	422
	Rev: GGGCTTGTCCCTTGAGTT		
FAS	For: GCACAGAAGGGAAGGAGT	60	163
	Rev: CCAGGAGAATCGCAGTAG		
CCL3	For: ACTGACCTGGAACTGAATG	60	171
	Rev: GAAGAGTCCCTCGATGTG		
VEGF	For: TGGATGTCTACCAGCGAAGC	58	497
	Rev: GGCAGTGAAGGCAAGCAG		
CCL2	For: CACAACCACCTCAAGCAC	58	122
	Rev: AAGGGAATACCATAACATCA		

fluoride membranes (IPVH00010; Millipore, USA). Membranes were blocked with 5% milk in Tris-buffered saline-Tween 20 and incubated with the following corresponding primary antibodies: anti-β-actin (1:800; Bioworld), anti-NF-κB (1:500; ABclonal), and anti-p-NF-κB (1:500; ABclonal), anti-ICAM-1 (1:1000; Abcam), anti-FAS (1:200; Absin), anti-CCL3 (1:200; Absin), anti-VEGF (1:1000; Abcam), anti-CXCR4 (1:1000; Abcam), anti-CCL2 (1:200; ABclonal), anti-ERK (1:500; CST), and anti-p-ERK (1:500; CST). After incubation with primary antibody for at least 6 h at 4 °C, western blots were incubated with secondary antibody for 30 min at 37 °C, then visualised using chemiluminescence (Millipore) and detected using gel imaging software (GE Healthcare Life Sciences, USA).

Statistical analysis

All data are presented as mean ± SEM. Statistical analysis tests (Student's *t* test or analysis of variance; ANOVA) were conducted using Prism software (GraphPad, San Diego, CA, USA), with $P < 0.05$ considered statistically significant.

Results

Human umbilical cord MSCs alleviate DSS-induced IBD

First, flow cytometry analysis revealed expression of CD105, CD73 and CD90 in MSCs were positive; however, CD11b and CD34 were negative (Fig. S1a). To study the location of MSCs in damaged colon tissue of IBD mice, we labelled hucMSCs with DIR (Fig. S1b). 12 h after the intraperitoneal injection, we observed that mouse treated with DIR-MSCs showed red fluorescence in the abdomen (Fig. S1c). The colon tissue was removed after the mice were sacrificed, and it was observed that the IBD mice treated with DIR-MSCs showed strong red fluorescence (Fig. S1d). These results suggested that MSCs can target damaged colon tissue.

To determine whether MSCs could cause remission of DSS-induced inflammation, we first designed a mouse model of inflammation. The weight of mice in the normal control group increased with time; however, mice in the DSS group showed weight loss on the 6th day, as well as bloody stools. The weight of mice in the MSC group did not change in the first 6 days, after which their body

Table 2 Primer sequences for RT-PCR (species: human)

Genes	Primer sequence	Annealing temp. (°C)	Amplicon size (bp)
CD11b	For: GGCCTCAGAGAATACCAGT	60	195
	Rev: GGGTGAACACGGAATCGTT		
IL-1β	For: TACGAATCTCCGACCACCA	60	256
	Rev: GGACCAGACATCACCAAGC		
IL-6	For: TACATCCTCGACGGCATCTC	61	252
	Rev: AGCTCTGGCTTGTTCCTCAC		
IL-10	For: GGAGGACTTTAAGGGTTA	60	266
	Rev: CACTCATGGCTTTGTAGAT		
ICAM-1	For: GTCACCTATGGCAACGACTC	64	172
	Rev: TCTGGCTTCGTCAGAATCAC		
FAS	For: TCGGAGGATTGCTCAACAAC	62	183
	Rev: ATGATGCAGGCCTTCCAAGT		
CCL3	For: TTCCGTCACCTGCTCAGAAT	62	204
	Rev: GCAGCAAGTGATGCAGAGAA		
VEGF	For: CCATTGTGGAGGCAGAGAAA	60	237
	Rev: GATCAGGGAGAGAGAGATTGGA		
CCL2	For: GAACCGAGAGGCTGAGACTA	52	151
	Rev: GCCTCTGCACTGAGATCTTC		
CXCR4	For: TCATCCTCATCCTGGCTTTC	60	200
	Rev: CAAACTCACACCCTTGCTTG		

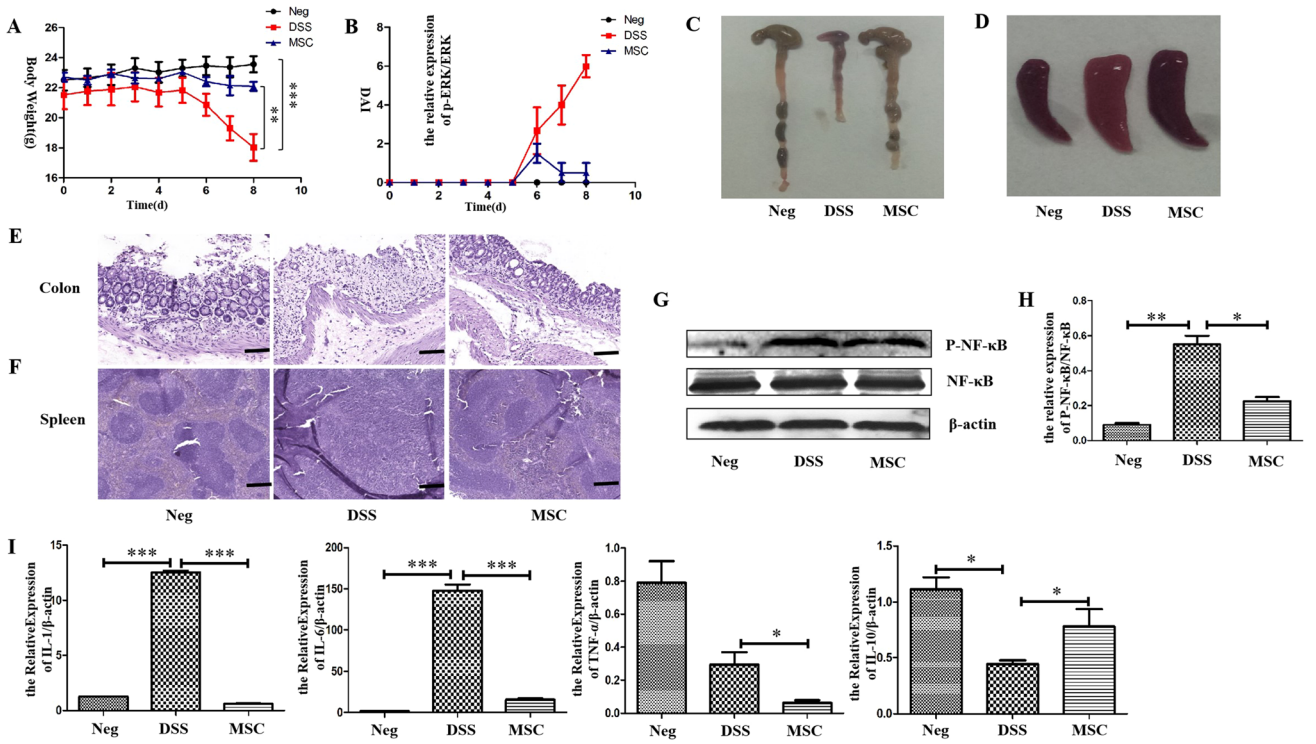


Fig. 1 Umbilical cord MSCs attenuate DSS-induced IBD. *Neg* normal control group; *DSS* DSS-induced mouse IBD group; *MSC* human umbilical cord MSC-treated IBD group. **a** Curves showing changes in mouse body weight. **b** Mouse clinical disease activity index (*DAI*). **c** Mouse colon (gross view). **d** Mouse spleen (gross view). **e** Mouse colon tissue stained with HE (*scale bar* 50 μm). **f** HE staining of mouse spleen tissue (*scale bar* 50 μm). **g** Western blotting analysis of NF-κB phosphorylation expression levels in mouse colon tissue; **h** Greyscale scanning analysis of (**g**). **i** QRT-PCR analysis of expression levels of cellular inflammatory factors (*IL-1β*, *IL-6*, *TNF-α*, *IL-10*) in mouse colon tissue. $n \geq 6$ for each group. Data shown are representative of three independent experiments and presented as mean ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by ANOVA

Table 3 The clinic disease activity index (DAI) value

Values	Weight loss (%)	Stool consistency	Rectal bleeding
0	None	Normal	Normal
1	1–5		±
2	5–10	Loose stool	+
3	10–15		++
4	More than 15	Watery diarrhea	+++

DAI = (weight loss + stool consistency + rectal bleeding)

weights decreased slightly, but not obviously (Fig. 1a). Disease activity index (DAI) values was used to describe the severity of IBD (Table 3). The DAI for the three groups showed no changes in the first 5 days; however, the DAI of mice in the DSS group increased sharply on the 6th day and continued to rise. Although the DAI of the mice in the MSC group also started to rise on the 6th day, it quickly returned to a downward trend (Fig. 1b). Mice were then sacrificed, and their colonic mucosa and spleens harvested for further analysis. Colon tissues in the normal group were longer and the spleens were completely intact. Conversely, the colons of mice in the DSS group were significantly shortened, and the spleens enlarged and congested. After treatment with MSCs, the length of the colon was recovered, and blood flow of the spleen restored (Fig. 1c, d). DSS-induced inflammation also caused severe damage to the intestinal villi structures, whereas, after treatment with MSCs, these pathological changes to the colon were alleviated (Fig. 1e, f). In colon tissues, western blotting showed that NF-κB phosphorylation was up-regulated in the DSS group, while phosphorylation was decreased in the MSC group and the differences were statistically significant (Fig. 1g, h; $P < 0.05$). QRT-PCR showed that levels of the pro-inflammatory cell factors, *IL-1β*, *IL-6*, and *TNF-α*, increased in the colonic mucosa of IBD mice, while they were significantly lower in colon tissues from mice in the MSC group. In contrast, levels of the anti-inflammatory factor, *IL-10*, were decreased in the colonic mucosa of mice from the DSS group, with higher levels in colonic mucosa from MSC group mice (Fig. 1i; $P < 0.05$). These data indicate that human umbilical cord MSCs can effectively alleviate DSS-induced inflammatory bowel disease in mice.

Sorting and identification of infiltrating neutrophils in mouse colon tissue

Neutrophils express high levels of CD11b and IL-8, which are important chemokines that mediate neutrophil aggregation. After the induction of inflammation, colon tissues expressed high levels of CD11b and IL-8, which were decreased in the MSC-treated group, although expression

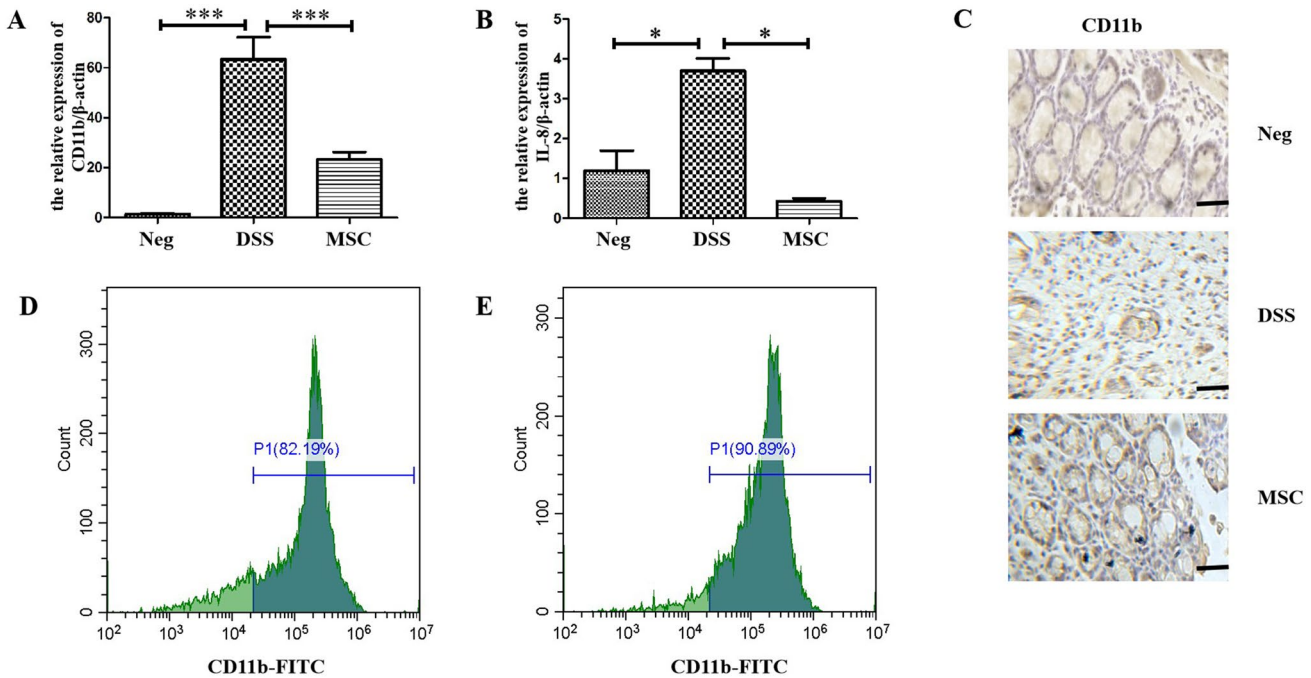


Fig. 2 Sorting and identification of infiltrating neutrophils in mouse colon tissue. *Neg* normal control group; *DSS* DSS-induced mouse IBD group; *MSC* human umbilical cord MSC treated IBD group. QRT-PCR analysis of neutrophil-related gene expression levels in mouse colon tissues; **a** *CD11b*, **b** *IL-8*. **c** IHC analysis of CD11b expression levels in mouse colon tissues (*scale bar* 50 μm). Neutrophils identified by flow cytometry sorted from **d** IBD and **e** MSC group colon tissue. $n \geq 6$ for each group. Data are representative of three independent experiments and presented as mean ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by ANOVA

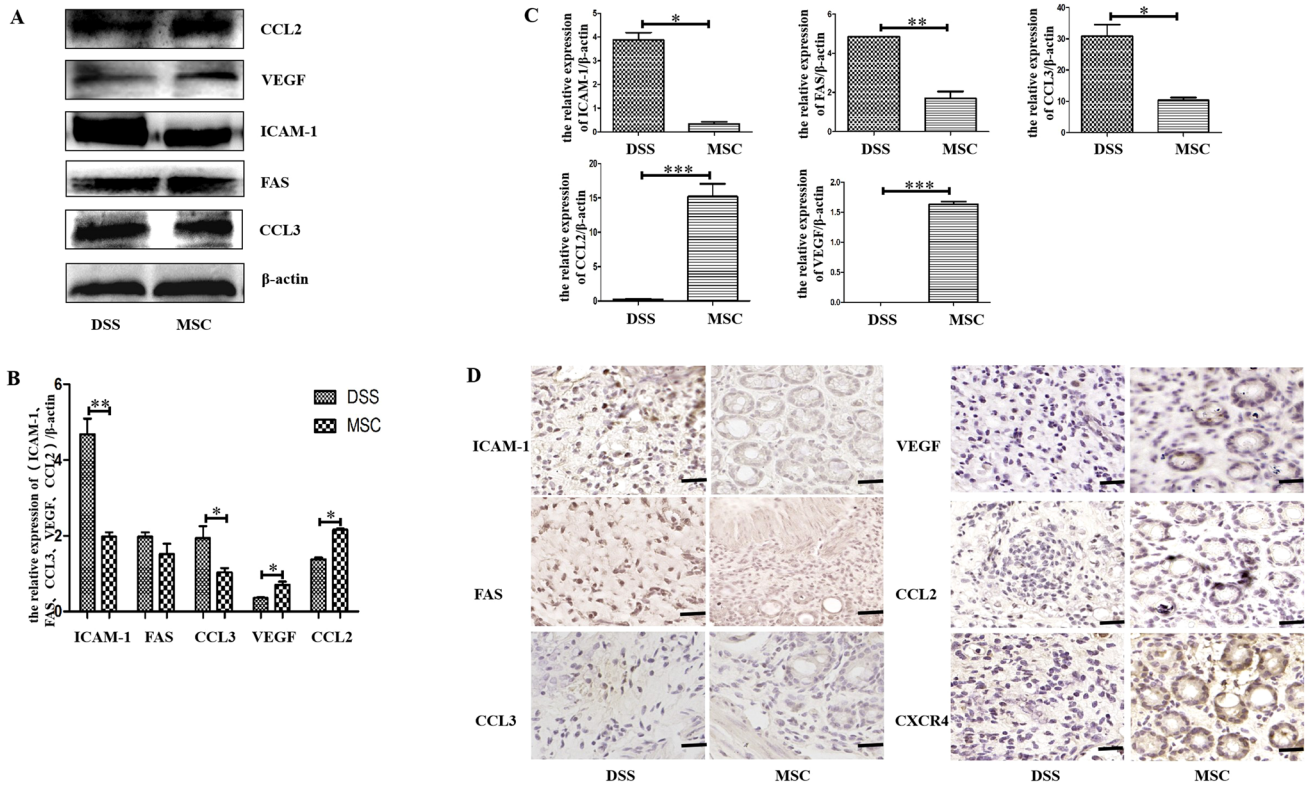


Fig. 3 Human umbilical cord MSCs induce neutrophils toward N2 phenotype polarisation during IBD repair. *DSS* DSS-induced mouse IBD group; *MSC* human umbilical cord MSC-treated IBD group. **a** Western blotting analysis of neutrophil surface molecules expression in colon tissue. **b** Greyscale scanning analysis of (**a**). **c** RT-PCR analysis of expression levels of mouse neutrophil surface molecules. **d** IHC analysis of expression levels of neutrophil surface molecule in mouse colon tissues (*scale bar* 50 μm). $n \geq 6$ for each group. Data shown are representative of three independent experiments and presented as mean ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by ANOVA

levels in both groups were significantly higher than those in controls (Fig. 2a, b; $P < 0.05$), indicating substantial neutrophil infiltration in the intestinal tract of DSS-treated mice. Whilst IHC revealed no neutrophil infiltration in the intestinal tissues of the control group, large numbers of infiltrating neutrophils were detected in the DSS group, with slightly fewer in the MSC group (Fig. 2c). After filtration of cells from intestinal tissue by MACS, the neutrophil yields from the DSS and MSC groups were 82.19% and 90.89%, respectively (Fig. 2d, e).

Human umbilical cord MSCs induce neutrophils toward N2 phenotype polarisation during repair of IBD

Western blotting showed that neutrophils extracted from colon tissue from the DSS group expressed high levels of ICAM-1, FAS, and CCL3, which are surface molecules typical of N1-polarised neutrophils, with low levels of CCL2 and VEGF, which are surface molecules indicating N2-polarised neutrophils. After treatment with MSCs, expression levels of ICAM-1, FAS, and CCL3 in neutrophils decreased, while those of CCL2 and VEGF increased (Fig. 3a). Greyscale scanning analysis indicated that these modulations were statistically significant (Fig. 3b; $P < 0.05$). QRT-PCR also demonstrated similar results, with decreased *ICAM-1*, *FAS*, and *CCL3*, and increased *CCL2* and *VEGF* expression levels in the MSC group (Fig. 3c; $P < 0.05$). Moreover, IHC revealed similar results (Fig. 3d). Together, these results indicate that intestinal inflammation is alleviated in mice after treatment with MSCs, and that neutrophils infiltrating colon tissue are polarised from an N1 to an N2 phenotype.

We also obtained intestinal tissue specimens from healthy individuals and patients with IBD from Nanjing Jiangning People's Hospital. The results of IHC analysis showed that colon tissues from patients with IBD were strongly positive for CD11b expression (Fig. 4a). Further, relative to healthy controls, ICAM-1, FAS, and CCL3 were highly expressed in colon tissues from patients with IBD, whereas VEGF, CCL2, and CXCR4 were almost undetectable (Fig. 4b).

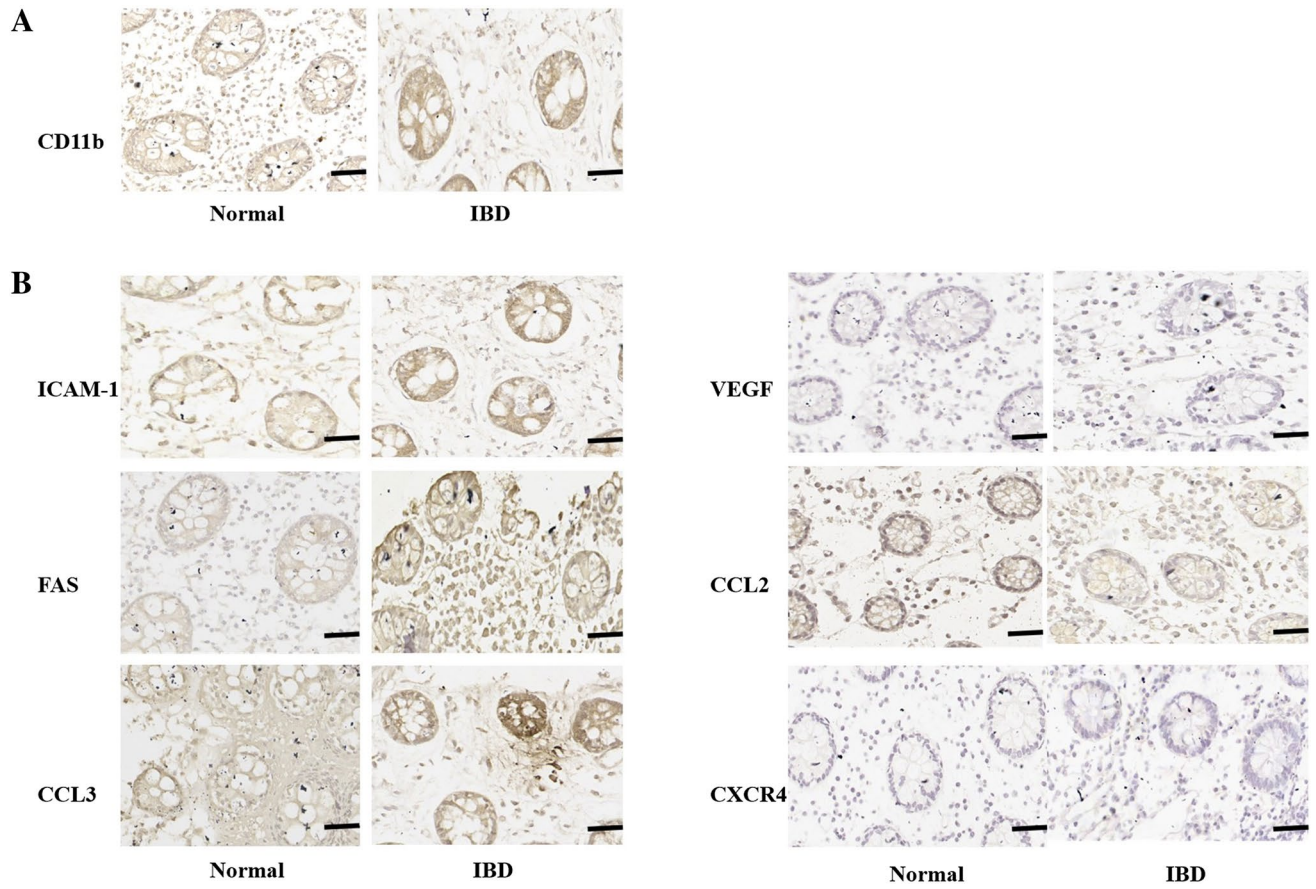


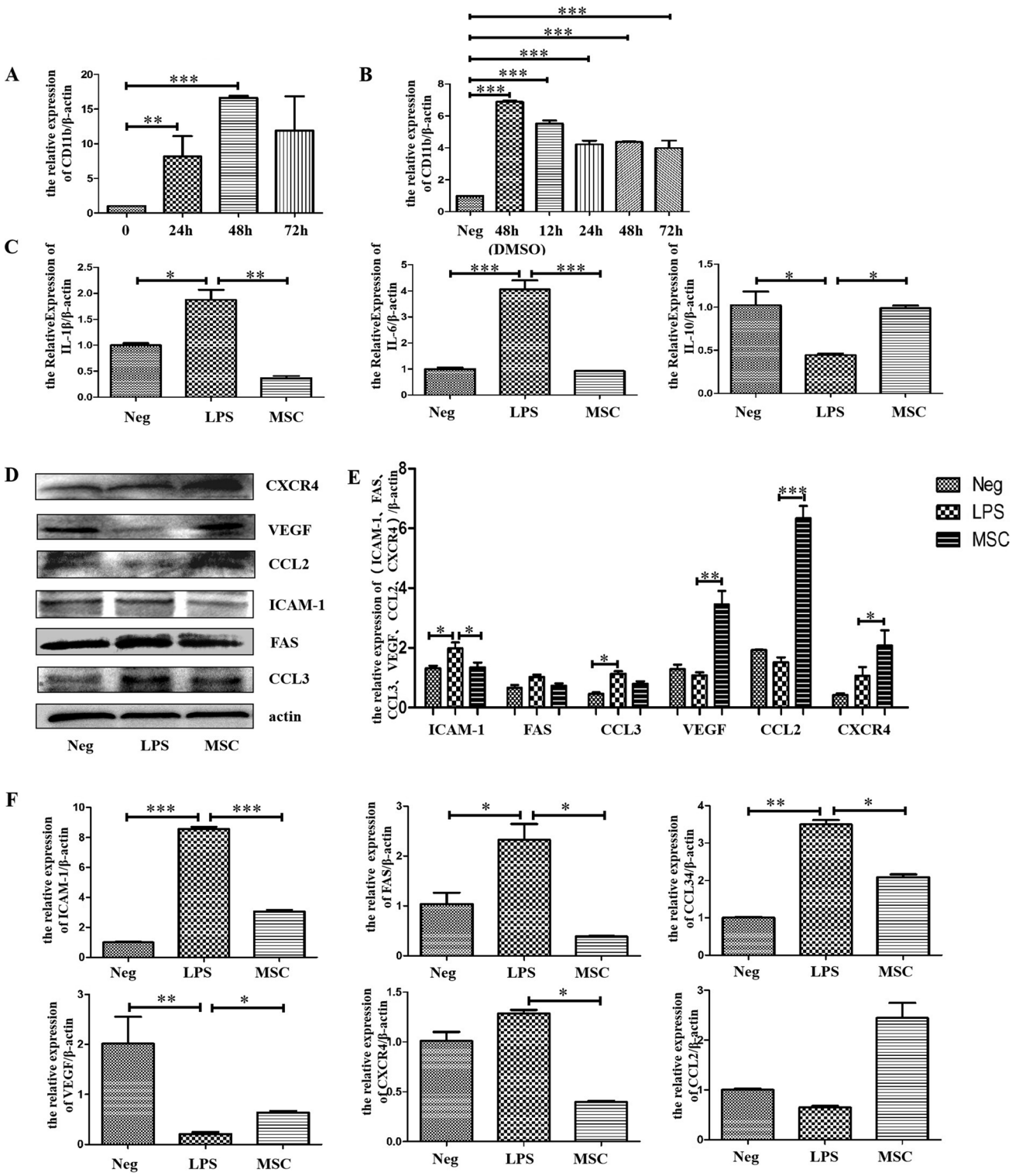
Fig. 4 Neutrophils are inhibited from N2-type polarisation in colon tissue from patients with IBD. *Normal* healthy control individual; *IBD* patient with IBD. **a** IHC analysis of CD11b expression levels in human colon tissues (*scale bar* 50 μm). **b** IHC analysis of neutrophil surface molecule expression levels in human tissues (*scale bar* 50 μm). $n \geq 6$ for each group. Data shown are representative of three independent experiments

Human umbilical cord MSCs induce neutrophil-like N2-type polarisation in an inflammatory environment

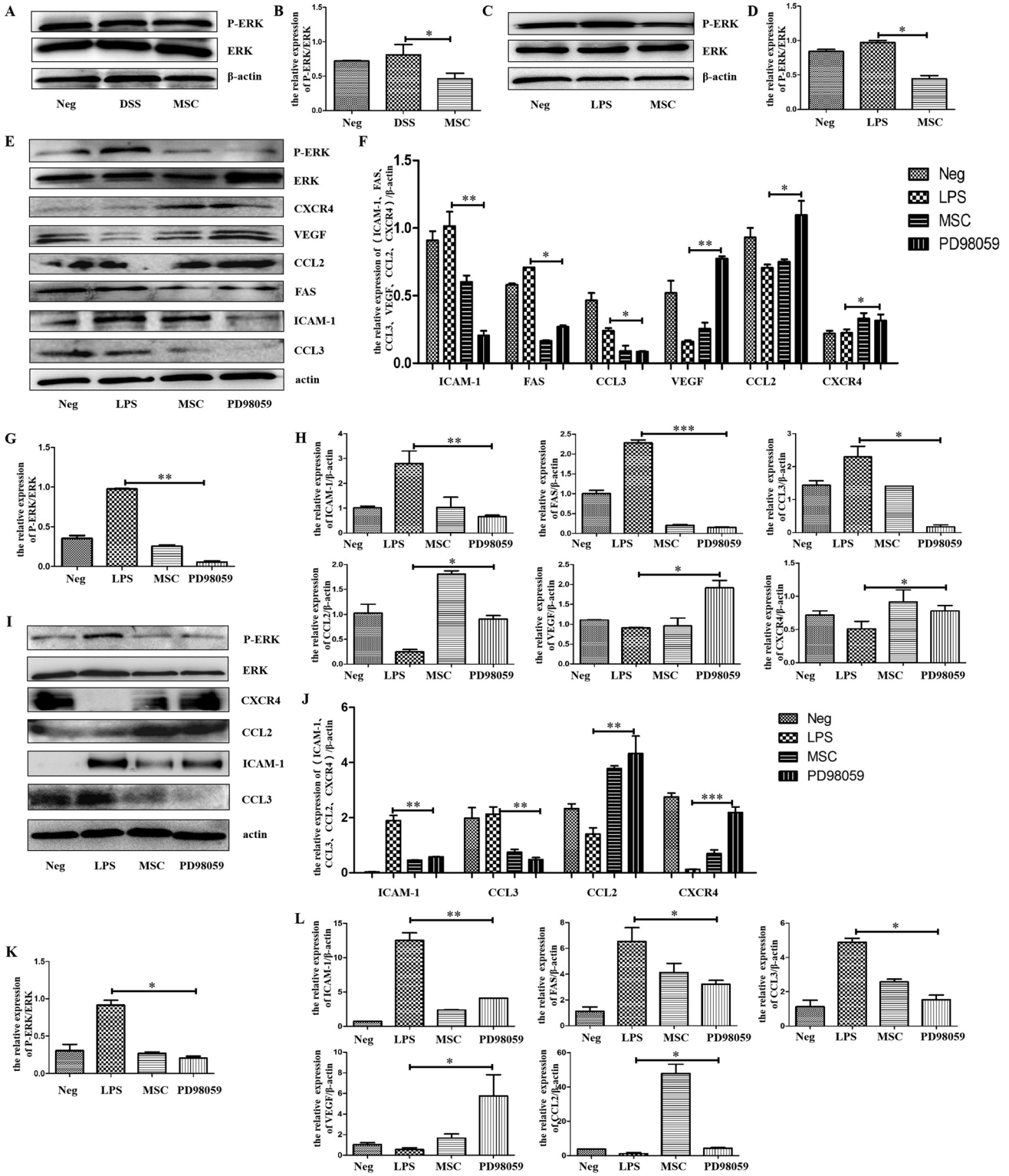
Previous reports have shown that HL-60 cells can differentiate into neutrophils after induction with DMSO, as determined by neutrophil cell morphology, chemical activation, and chemotaxis (Teimourian and Moghanloo 2016). Expression of CD11b was significantly increased after induction of HL-60 cells with DMSO for 48 h (Fig. 5a, $P < 0.05$). After induction, HL-60 cells were cultured in normal nutrient solution and still expressed high levels of CD11b (Fig. 5b, $P < 0.05$). In our next experiment, we conducted in vitro culture of neutrophils (from HL-60 cells induced with DMSO), and added LPS to simulate an inflammatory environment, and MSCs to simulate the inflammatory remission process. QRT-PCR showed that expression of the pro-inflammatory factors, *IL-1β* and *IL-6*, was increased in the LPS-treated group, while it was significantly lower in the MSC-treated group. In contrast, the anti-inflammatory factor, *IL-10*, was decreased in the LPS-treated group, while it was increased in the MSC-treated group (Fig. 5c, $P < 0.05$).

Western blotting showed that neutrophils (from HL-60 cells induced with DMSO) in the LPS group expressed

Fig. 5 Human umbilical cord MSCs induce neutrophil-like N2 phenotype polarisation in an inflammatory environment. *Neg* normal control group; *LPS* LPS-induced inflammation group; *MSC* LPS inflammation group treated with human umbilical cord MSCs. **a** HL-60 cells were cultured in 2.5% DMSO nutrient solution for 0, 24, 48, and 72 h, followed by RT-PCR analysis of *CD11b* expression levels. **b** HL-60 cells were cultured in 2.5% DMSO nutrient solution for 48 h, or in normal nutrient solution for 0, 12, 24, 48, and 72 h, followed by RT-PCR analysis of *CD11b* expression levels. **c** QRT-PCR analysis of inflammation-related cell inflammatory factors (*IL-1β*, *IL-6*, *TNF-α*, and *IL-10*) expression levels. **d** Western blotting analysis of neutrophil surface molecule expression levels in an inflammatory environment; **e** Greyscale scanning analysis of (**d**). **f** RT-PCR analysis of surface molecule expression levels on neutrophils cultured in an inflammatory environment. $n \geq 6$ for each group. Data shown are representative of three independent experiments and presented as mean ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by ANOVA



high levels of ICAM-1, FAS, and CCL3, and low levels of CCL2, VEGF, and CXCR4 (Fig. 5d); however, treatment with MSCs led to decreased expression of ICAM-1, FAS, and CCL3, while CCL2, VEGF, and CXCR4 were significantly increased in the LPS-treated group (Fig. 5e; $P < 0.05$). Furthermore, QRT-PCR also showed similar trend of alterations in expression levels, where those of *ICAM-1*, *FAS*, and *CCL3* were decreased, while *CCL2*, *VEGF*, and *CXCR4* were increased in the MSC group compared with the LPS group (Fig. 5f; $P < 0.05$). We also demonstrated, via in vitro



analysis, that neutrophils expressed high levels of molecules typical of an N1-polarised phenotype in the inflammatory environment, while, after treatment with MSCs, they began to express high levels of molecules associated with an N2-polarised phenotype, indicating that MSCs induced N2 polarisation.

◂**Fig. 6** Human umbilical cord MSCs induce neutrophils toward N2 phenotype polarisation by inhibiting ERK phosphorylation. *Neg* normal control group; *DSS* DSS-induced mouse IBD group; *MSC* human umbilical cord MSC-treated IBD group. **a** Western blotting analysis of p-ERK and ERK expression levels in mouse colon tissues; **b** Greyscale scanning of (**a**); Neg, normal control group; LPS, LPS-induced inflammation group; MSC, human umbilical cord MSC-treated LPS inflammation group; PD98059, LPS inflammation group treated with ERK phosphorylation inhibitor. **c** Western blotting analysis of p-ERK and ERK expression levels in mouse colon tissues; **d** Greyscale scanning of (**c**). **e** Western blotting analysis of neutrophil surface molecule expression levels in an inflammatory environment; **f**, **g** Greyscale scanning analysis of (**e**). **h** RT-PCR analysis of neutrophil surface molecule expression levels in an inflammatory environment. **i** Western blotting analysis of mouse bone marrow-derived neutrophil surface molecule expression levels in an inflammatory environment; **j**, **k** Greyscale scanning analysis of **i**. **l** RT-PCR analysis of mouse bone marrow-derived neutrophil surface molecule expression levels in the inflammatory environment. $n \geq 6$ for each group. Data are representative of three independent experiments and presented as mean ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by ANOVA

Human umbilical cord MSCs induce neutrophils toward N2 phenotype polarisation by inhibiting ERK phosphorylation

In colon tissues, up-regulation of ERK phosphorylation was detected in the DSS group, while phosphorylation was decreased in the MSC group (Fig. 6a). Similarly, in vitro cell assays indicated increased p-ERK expression in neutrophils under LPS stimulation; however, levels decreased after MSCs treatment (Fig. 6c). Greyscale analysis of western blotting data indicated that the differences were statistically significant (Fig. 6b, d, $P < 0.05$).

We speculated that neutrophil polarisation from the N1 toward the N2 phenotype may be related to activation of the ERK signalling pathway. As PD98059 has a significant inhibitory effect on ERK phosphorylation, we assessed neutrophil polarisation and ERK phosphorylation on addition of PD98059. Western blotting showed that ERK phosphorylation was significantly inhibited in neutrophils (from HL-60 cells induced with DMSO) after addition of PD98059, with low levels of ICAM-1, FAS, and CCL3 and high levels of CCL2, VEGF, and CXCR4, relative to the LPS group (Fig. 6e); these changes were statistically significant (Fig. 6f, g, $P < 0.05$). QRT-PCR also demonstrated that *ICAM-1*, *FAS*, and *CCL3* expression in neutrophils decreased, whilst levels of *CCL2*, *VEGF*, and *CXCR4* increased in the PD98059 group compared with the LPS group (Fig. 6h, $P < 0.05$).

To further verify these observations, we isolated neutrophils from the bone marrow of C57BL/6 mice and treated them to assess whether similar results were obtained. Again, the PD98059-treated group exhibited low expression levels of ICAM-1 and CCL3, with high levels of CCL2 and CXCR4 (Fig. 6i), and the differences were statistically significant (Fig. 6j, k; $P < 0.05$). QRT-PCR also demonstrated that levels of *ICAM-1*, *FAS*, and *CCL3* were decreased in bone marrow neutrophils, while those of *CCL2* and *VEGF* increased in the PD98059 group relative to the LPS group (Fig. 6l; $P < 0.05$). Together, these observations indicate that, in the inflammatory environment, ERK phosphorylation is inhibited by MSCs, consequently enhancing the polarisation of neutrophils from an N1 to an N2 phenotype.

Discussion

MSC transplantation can be used to treat many diseases; for example, myocardial infarction, acute liver injury, and collagen-induced arthritis (Qian et al. 2008; Mao et al. 2010; Fu et al. 2014; He et al. 2016; Kim et al. 2016). The therapeutic value of MSCs in various diseases has attracted widespread attention, with the number of clinical trials involving MSCs increasing almost exponentially in recent years. Cell therapy may also have great potential for the relief of enteritis (Okamoto and Watanabe 2016).

In this study, we show that IBD mice exhibit significant clinical features (weight loss, colon shortening, bloody stools, and secretion of pro-inflammatory factors in intestinal tissue), and that intestinal inflammation can be relieved by MSC treatment in this model. During the process of alleviation, the expression levels of anti-inflammatory factors are increased; however, the mechanisms by which MSCs alleviate IBD were unclear. It is established that MSCs exert various regulatory functions on immune cells, including: inhibition of effector T cell differentiation, accompanied by induction of their apoptosis (Ren et al. 2008; Akiyama et al. 2012); enhancing the production of regulatory T cells (Selmani et al. 2008; Kavanagh and Mahon 2011); inhibiting B cell differentiation (Corcione et al. 2006); inhibiting differentiation, maturation, and antigen presentation by dendritic cells (Nauta et al. 2006); inhibiting the proliferation and cytotoxicity of NK cells (Sotiropoulou et al. 2006); and inducing transition from pro-inflammatory (M1) to anti-inflammatory (M2) macrophages (Dayan et al. 2011; Jiang et al. 2013; Qi et al. 2014).

Neutrophils, as effector cells of acute inflammation, have a role in maintaining intestinal homeostasis and in IBD pathogenesis. In the initial stages of inflammation in patients with IBD, neutrophils enter the intestinal mucosa and aggregate to conduct phagocytosis, thereby promoting healing of the mucosa and alleviating inflammation; however, large numbers of neutrophils infiltrating the inflammatory mucosa and accumulating in the epithelial cells causes damage to the mucosal structure, impairing the epithelial barrier and stimulating production of mediators of inflammation (Wera et al. 2016). We suspected that this process involves not only a change in the number of neutrophils but also a degree of functional alteration of these cells. The

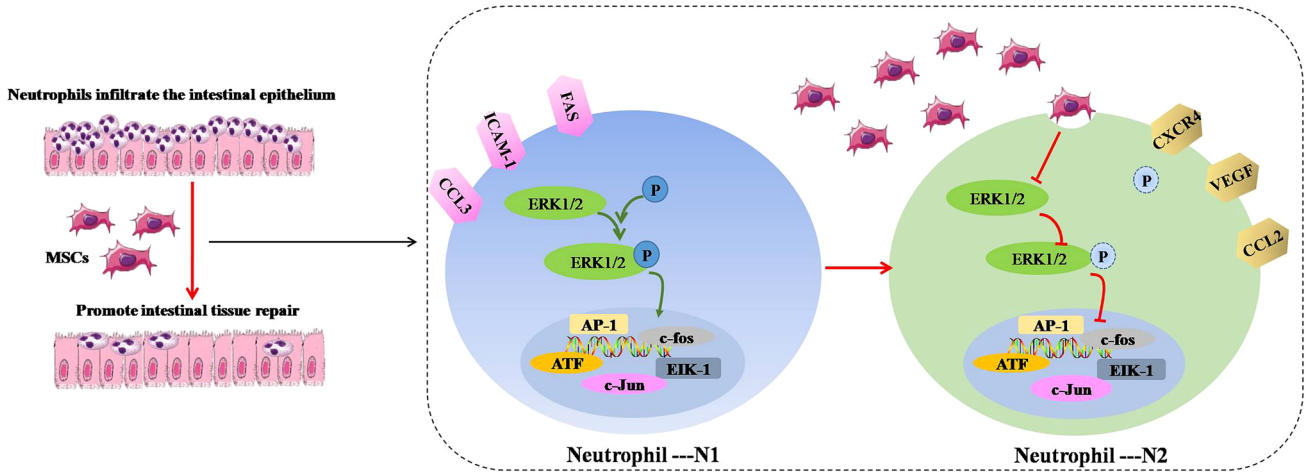


Fig. 7 Mechanism of MSC regulation of neutrophils to alleviate inflammatory bowel disease. During intestinal inflammation, neutrophils infiltrate the intestinal epithelium, MSCs inhibit ERK phosphorylation, and the neutrophil phenotype changes toward N2-type polarisation, thereby repairing intestinal damage. ERK1/2 is activated by phosphorylation in neutrophils and translocated into the nucleus from the cytoplasm, where it mediates transcriptional activation of Elk-1, ATF, Ap-1, c-fos, and c-Jun, and participates in numerous biological reactions, such as cell proliferation and differentiation, and maintenance of cell morphology

expression of IL-8 is significantly increased in patients with IBD, and promotes the accumulation of neutrophils in the inflammatory mucosa (Mumy and McCormick 2009). IL-8 not only recruits granulocytes but also leads to neutrophil activation, increased neutrophil CD11b expression, and reactive oxygen species (ROS) production (Wera et al. 2016). Neutrophils can be polarised into two phenotypes, N1 and N2. We found that many infiltrating neutrophils in colon tissues from IBD mice had significant N1 characteristics; however, on MSC treatment, although the neutrophils still infiltrated in the tissue, they moved toward high levels of expression of N2 phenotype markers. In in vitro experiments, we simulated an inflammatory environment and subsequently added MSCs and found that, when the neutrophils were analysed, their surface molecule expression levels had changed significantly. These data clearly demonstrate that MSCs have an important regulatory influence on neutrophil phenotype polarisation. Among several studies that have shown the immunomodulatory effects of MSCs on neutrophils is the report of Jiang et al. (2016) who showed that MSCs can inhibit tissue damage caused by excessively activated neutrophils. Further, Zhang et al. (2018) demonstrated that tumour-derived exosomes promote gastric cancer cell migration by inducing N2 polarisation of neutrophils, while Zhou and Liu (2017) reported that $CD177^{+}$ neutrophils act as a new neutrophil subtype, increasing bactericidal activity, enhancing IL-22 expression, reducing the production of pro-inflammatory factors, and thus effectively alleviating IBD.

Based on this phenomenon, we speculated that specific signalling pathways would play important roles in the association between MSCs and neutrophils. It has recently been reported that L-fucose can alleviate colitis by preventing macrophage M1 polarisation, suppressing the NLRP3 inflammasome and NF-κB activation, and down-regulating pro-inflammatory cell cytokines (He et al. 2019). Scavenger receptor A stimulation can also cause selective ERK phosphorylation, which up-regulates ROS levels in neutrophil medium and induces the formation of classical neutrophil extracellular traps by activating NADPH oxidase 2 (Zhu et al. 2019). Further, Liang Mianzhen extract attenuates complete Freund adjuvant-induced inflammatory pain and reduces neutrophil infiltration by inhibiting peripheral and central levels of ERK1/2 and NF-κB signalling pathways (Qin et al. 2019).

Our results showed that the phenotype of neutrophils infiltrating colonic tissue was altered in mice with IBD remission when ERK phosphorylation was inhibited. To test whether these changes in neutrophil phenotype were influenced by ERK phosphorylation, we simulated an inflammatory environment in vitro and added the ERK phosphorylation inhibitor, PD98059. When ERK phosphorylation was inhibited, neutrophils were polarised from the N1 to the N2 phenotype.

Conclusion

Our study is the first to demonstrate that MSCs can relieve the intestinal inflammation process in IBD mice and slow the progress of inflammation. During this process, neutrophils infiltrate the colonic tissue, and change phenotype from N1 to N2 following treatment with MSCs. Further, the expression of pro- and anti-inflammatory factors were balanced to alleviate the development and progression of inflammation.

Moreover, we found that the ERK signalling pathway has a specific regulatory role in this process. Inhibition of ERK phosphorylation led to neutrophil polarisation from N1 to N2, which in turn played a significant role in alleviating the occurrence and development of IBD (Fig. 7). These results suggest that neutrophils of different phenotypes have significant roles in inflammation during IBD and provide new avenues for exploration, including potential MSC-mediated treatment of this condition.

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Author contributions GW, conception and design, collection and/or assembly of data, data analysis and interpretation, and manuscript writing; MDMJ, collection and/or assembly of data and data analysis; JY, conception and design, collection of data, data analysis and interpretation; JW, collection and/or assembly of data and data analysis; XC, data analysis and interpretation; DKWO, collection and/or assembly of data and data analysis; YY, provision of study material and interpretation; HQ, data analysis and interpretation; XZ, collection and/or assembly of data; WX, data analysis and interpretation; FM, study design, data analysis and interpretation, manuscript writing, and final approval of manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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