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Original Article

Blockade of CXCR2 suppresses proinflammatory activities of neutrophils in ulcerative colitis

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Abstract: Ulcerative colitis (UC) is one chronically remittent and progressive inflammatory disorder. Chemokine receptor CXCR2 is reported to be involved in the pathogenesis of several inflammatory diseases. However, how CXCR2 modulate mucosal inflammation in UC is still obscure. In this study, CXCR2 expression was determined in inflamed mucosa and peripheral blood cells from patients with UC by qRT-PCR. Neutrophils isolated from peripheral blood were pretreated with CXCR2 inhibitor (SB225002), and proinflammatory mediators were examined by qRT-PCR, ELISA and IF. The migratory capacity of neutrophils after SB225002 treatment was examined by using Transwell plate. Furthermore, SB225002 was administrated daily in DSS-induced colitis mice. We found that CXCR2 expression was significantly increased in colonic mucosal tissues and peripheral blood cells from patients with active UC. Besides, CXCR2 was highly expressed in neutrophils, and was positively correlated with disease activity. Inhibition of CXCR2 in neutrophils decreased the production of proinflammatory mediators, such as reactive oxygen species (ROS), MPO, S100a8, S100a9, TNF-α, IL-1β, IL-8 and IL-6, and the migratory capacity of neutrophils was markedly impaired after SB225002 treatment. Moreover, blockade of CXCR2 with SB225002 could markedly ameliorate DSS-induced colitis in mice. In summary, CXCR2 plays a critical role in the pathogenesis of UC through modulating immune responses of neutrophils. Blockade of CXCR2 may serve as a new therapeutic approach for treatment of UC.

Keywords: CXCR2, neutrophil, ulcerative colitis, mucosal inflammation

Introduction

Ulcerative colitis (UC), belonging to one subtype of inflammatory bowel diseases (IBD), is a chronic, relapsing and remitting inflammatory disease, mainly affecting the colon, with symptoms of diarrhea with mucus or bloody purulent stool, tenesmus and lower abdominal pain [1]. It is reported that UC is caused by the dysregulated immune response against intestinal microflora combined with the imbalance of anti-inflammatory or proinflammatory pathways [2]. The aberrant immunological responses that take place in gut can affect the epithelial barrier, increase intestinal permeability for novel antigens and further lead to a persistent chronic inflammation [3]. Both innate and adaptive immunity have been suggested in maintaining intestinal homeostasis and various inflammatory immune cells are involved in this process, such as neutrophils, $CD4^+$ T cells, and macrophages [4].

Neutrophils, also known as polymorphonuclear leukocytes (PMN), are short-lived effector cells in innate immune system, which are the most abundant leukocyte population in the blood, and are the typically first leukocytes to be recruited to the inflammatory sites. When contacting with invading microbes, neutrophils are adapted to perform a wide variety of antimicrobial functions, such as phagocytosis, release of reactive oxygen species (ROS), degranulation and formation of neutrophil extracellular traps (NET) to eliminate intestinal microbial infection [5, 6]. In UC, neutrophil migration into the co-

lonic mucosa is a hallmark of inflammation. In addition, the extent of neutrophil infiltration correlates with disease severity [7].

CXCR2, a chemokine receptor expressed in neutrophils, has been reported to play a vital role in the recruitment of neutrophils. CXCR2 has been proved to be a potent mediator of PMN recruitment in preclinical models of arthritis, allergy, respiratory disease, and ulcerative colitis [8]. Decreased expression of CXCR2 in neutrophils is found in patients with bacterial infection and associated with impaired migration into sites of inflammation [9]. The expression of CXCR2 in neutrophils has been widely described for its roles in immunity, cancer and inflammation [10-13], however, the exact role of CXCR2 in neutrophils in regulating colonic mucosal inflammation in UC is still obscure.

In this study, we found that CXCR2 expression was increased in colonic mucosal tissues and peripheral blood cells from patients with active UC. Besides, CXCR2 was highly expressed in neutrophils, and positively correlated with disease activity. Inhibition of CXCR2 in neutrophils decreased the production of proinflammatory mediators, and the migratory capacity of neutrophils was markedly impaired after SB225002 treatment. Moreover, blockade of CXCR2 with SB225002 could markedly ameliorate DSS-induced colitis in mice.

Materials and methods

Patients

All patients with UC were recruited from the Department of Gastroenterology, the Affiliated Hospital of Jining Medical University (Jining, Shandong, China) from July 2017 to December 2019. EDTA anti-coagulated blood samples (10 mL) were obtained from patients with active UC (A-UC, n=14), patients with UC in remission (R-UC, n=14) and healthy controls (HC, n=10) after overnight fasting. Colon biopsy samples were obtained from A-UC (n=21), R-UC (n=16) and HC (n=18) who underwent endoscopy. The diagnoses of UC were based on clinical characteristics, radiological and endoscopic examination and histological findings. The clinical characteristics of these patients are shown in Supplementary Table 1. International standard criteria Mayo scores were used to assess the severity of disease in UC patients [14]. Mayo scores were evaluated as: stool frequency: normal stools (0), 1-2 stools (1), 3-4 stools (2), ≥ 5 stools (3); rectal bleeding: no blood seen (0), streaks of blood with stool less than half the time (1), obvious blood with stool most of the time (2), obvious blood alone passed (3); physician Global assessment: normal (0), mild disease (1), moderate disease (2), severe disease (3). Intestinal mucosal lesions of UC patients were graded by ulcerative colitis endoscopic index of severity (UCEIS) by ileocolonoscopy. UCEIS was evaluated as: vascular pattern: normal (0), patchy obliteration (1), obliterated (2); bleeding: none (0), mucosal (1), luminal mild (2), luminal moderate or severe (3); erosions and ulcers: none (0), erosions (1), superficial ulcer (2), deep ulcer (3). This study was approved by the Institutional Review Board for Clinical Research of the Affiliated Hospital of Jining Medical University. Written informed consent was also obtained from all subjects before study.

Isolation of neutrophils

Peripheral neutrophils from patients with UC were isolated with Ficoll-Hypaque (GE Healthcare, Piscataway, NJ, USA) density gradient centrifugation as described previously [15]. Briefly, peripheral blood was collected in EDTA anti-coagulated tubes and slowly laid on the surface of Ficoll, followed by gradient centrifuged at 2000 rpm for 20 min at 20°C. The lowest layer was collected and neutrophils were obtained after incubating with red blood cell lysis buffer (BD Biosciences, San Diego, CA, USA).

Isolation of immune cell subsets

EDTA anti-coagulated peripheral blood samples were collected from HC, and peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation using Ficoll-Paque™, and then incubated by anti-human $CD4^+$ T cells Particles (BD; San Diego, CA, USA) at room temperature for 30 minutes. After the isolation on cell separation magnet, $CD4^+$ T cells were obtained. The purity of these cell population was > 90% as assessed by flow cytometry. $CD14^+$ monocytes, $CD8^+$ T cells and B cells were isolated using their corresponding magnetic bead by the same method.

Culture of neutrophils in vitro and cytokine analysis

2×10^6 peripheral neutrophils were cultured in vitro in 1 ml RPMI 1640-10% fetal bovine serum (FBS) and CXCR2 inhibitor SB225002

(500 nM, Selleckchem) was added to stimulate the cells in the presence of LPS (200 ng/ml) at 37°C in 5% CO_2 humidified air for 3 h. Cells were then collected for qRT-PCR analysis. For the detection of protein levels of cytokines produced by neutrophils, 2×10^6 neutrophils were stimulated with SB225002 (500 nM) in the presence of LPS (200 ng/ml) for 24 h. Supernatants were collected for ELISA.

ROS and MPO examination from neutrophils

For the detection of ROS and MPO produced by neutrophils, 2×10^6 neutrophils were stimulated with CXCR2 inhibitor SB225002 (500 nM) in the presence of LPS (200 ng/ml) for 3 h. 1×10^4 cells were then collected and incubated in HBSS in the presence of peroxidase and Amplex@ Red reagent in 96-well plates according to the manufacturer's instruction illustrated in the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Life Invitrogen).

Mice

Specific pathogen-free C57BL/6 N mice were raised under specific pathogen-free conditions with filtered air, and allowed free access to sterile water and autoclaved food. Mice used in experiment were at 8-10 weeks of age and 20-25 g of weight. Animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of Jining Medical University.

DSS-induced colitis model in mice

DSS-induced colitis model was established as described previously [15, 16]. Briefly, two groups of C57BL/6 N (10 mice per group) were given 2.5% DSS (molecular mass, 36000-50000, MP Biomedicals, Solon, Ohio, USA) in the drinking water for continuous 7 days, and at 8th day, all mice were given sterile water for another 3 days. One group of mice were administered with CXCR2 selective inhibitor (SB225002, 1 mg/kg) daily intraperitoneally (i.p.), and another group of mice were administered with PBS as controls. Other two groups of mice (10 mice per group) were given sterile water for ten continuous days as negative controls. During the observation of 10 days, characteristics of acute colitis were observed daily, including diarrhea, bloody stools, body weight and survival rates. At the 10th day, all mice were sacrificed, and colonic tissues were obtained. A small part of colon (0.5 cm) was fixed in 10% paraformaldehyde overnight used for H&E staining, and another small part of colon (0.5-1.0 cm) was used for RNA extraction and qRT-PCR analysis. Furthermore, bone marrow cells of mice were also isolated after red blood cell lysis. Neutrophils were then isolated from bone marrow of mice using neutrophil isolation kit (Miltenyi Biotec, order: 130097658), and used for the migration capacity analysis.

Isolation of lamina propria mononuclear cells

As described previously [15], the colon was removed from mice, sliced into 0.5-1.0 cm pieces and washed with cold PBS to remove faecal contents. After the digestion by EDTA, colon tissues were then digested by collagenase A (Sigma-Aldrich, St. Louis, Missouri, USA), and single cell suspension was collected and further purified via density gradient centrifugation with 40% and 70% Percoll-RPMI solution. Lamina propria mononuclear cells (LPMCs) were collected from the interface and suspended in 10% FBS-RPMI medium.

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

Total RNA was extracted from the fresh-frozen biopsies or mouse colonic tissues, and the quantity and quality were assessed using a NanoVue spectrophotometer (GE Healthcare, Piscataway, NJ, USA), with a 260/280 ratio between 1.8 to 2.0. The complementary DNA (cDNA) was synthesized with 5 × All-in-one RT mastermix (abm) according to the manufacturer's instructions. The synthesized cDNA was stored at -20°C. qRT-PCR was performed using SYBR green methodology according to the following conditions: 95°C for 1 min, followed by 40 cycles at 95°C for 15 s and 60°C for 30 s with 40 cycles. All samples for qRT-PCR analysis were performed in triplicate wells. All the primers were synthesized from ShengGong BioTeck (Shanghai, China) and GAPDH was used as the endogenous reference gene (Supplementary Table 2). The relative levels of target gene expression were calculated as a ratio relative to the GAPDH reference. qRT-PCR analysis was carried out using the 2-ΔΔCt method.

ELISA

The supernatants were harvested from neutrophils culture or biopsies culture, and used to

detect the concentration of TNF-α, IL-1β, IL-8 and IL-6 by ELISA according to the manufacturer's protocols (BioLegend, San Diego, CA, USA). Briefly, captured antibodies were incubated in 96-well plates at 4°C overnight. Nonspecific antigens were blocked with assay diluents. The standard and samples were added and incubated at 37°C for 2 h. After thoroughly washing with 0.05% Tween-PBS, the plates were incubated with detection antibodies for 1 h and HRP for 30 min. Finally, the color was developed with TMB and the value of OD was detected with a microplate spectrophotometer (BioTek).

Analysis of NET formation by fluorescence microscopy

Neutrophils were isolated from peripheral blood and stimulated with LPS (200 ng/mL) for 2 hours. They (2×10^5) were seeded on the poly-l-lysine-coated glass slides, fixed with 4% paraformaldehyde, and then stained with Hoechst 33342 (1:1000). NETs were visualized on a confocal microscopy (LSM 710, ZEISS; Oberkochen, Germany).

Migration assay of neutrophils in vitro

Neutrophils (5×10^5) suspended in 100 μL medium were placed into the top well of the Transwell chamber and medium containing human CXCL1 or PBS was added into the bottom well. After 1 h of incubation at 37°C, neutrophils that migrated were counted under a light microscope on at least 5 randomly high-power field (HPF).

Statistical analysis

Data were expressed as mean ± SEM, and analyzed using SPSS V.20.0 (SPSS; Chicago, IL, USA). All experiments were conducted at least in triplicate. All the data were normally distributed. Data were using unpaired Student's T-test, one-way analysis of variance (ANOVA) *post-hoc* Tukey Multiple Comparison Test or two-way ANOVA *post-hoc* Bonferroni Multiple Comparison Test. Spearman correlation was performed to analyze the correlation of CXCR2 expression with simple ulcerative colitis endoscopic index of severity (UCEIS), Mayo index, and erythrocyte sedimentation rate (ESR). The statistical significance level was set at $P < 0.05$.

Results

CXCR2 expression is highly increased in inflamed mucosa and peripheral blood cells in patients with active UC, and positively correlated with disease activity

CXCR2 has been proved to be involved in the pathogenesis of arthritis, allergy, and respiratory inflammation, then we hypothesized that CXCR2 may also involve in the induction and development of UC. Thus, inflamed mucosa were collected from patients with UC and HC, and CXCR2 expression was found to be significantly increased in inflamed mucosa patients with A-UC compared with R-UC or HC. However, there was no significant difference between patients with R-UC and HC (**Figure 1A**). Furthermore, we compared CXCR2 expression in inflamed and unaffected mucosa from the same UC patients and found that CXCR2 expression was markedly increased in inflamed mucosa than that in unaffected controls (**Figure 1B**). Peripheral blood cells were isolated from patients with A-UC and HC, and CXCR2 expression was detected by qRT-PCR. We found that CXCR2 expression was also highly increased in peripheral blood cells from A-UC patients, which was consistent with the results in colonic inflamed mucosa (**Figure 1C**).

We then analyzed the correlation of CXCR2 expression in colonic mucosa with Mayo index and UCEIS. Interestingly, CXCR2 expression in colonic mucosa from UC patients was positively correlated with Mayo index (**Figure 1D**) and UCEIS (**Figure 1E**). Furthermore, we also observed a positive correlation between the CXCR2 expression in peripheral blood cells from UC patients and ESR (**Figure 1F**). These data indicate that CXCR2 expression in inflamed mucosa and peripheral blood cells in patients with active UC is positively correlated with disease activity.

CXCR2 is highly expressed in neutrophils

To determine the phenotypic expression of CXCR2 in different subsets of cells, we isolated neutrophils, $CD14^+$ monocytes, $CD4^+$ T cells, $CD8^+$ T cell, and $CD20^+$ B cells from healthy donors. The purity of these cell population was > 90% as assessed by flow cytometry (Supplementary Figure 1). Expression of CXCR2 was analyzed by qRT-PCR. As shown in **Figure 2A**,

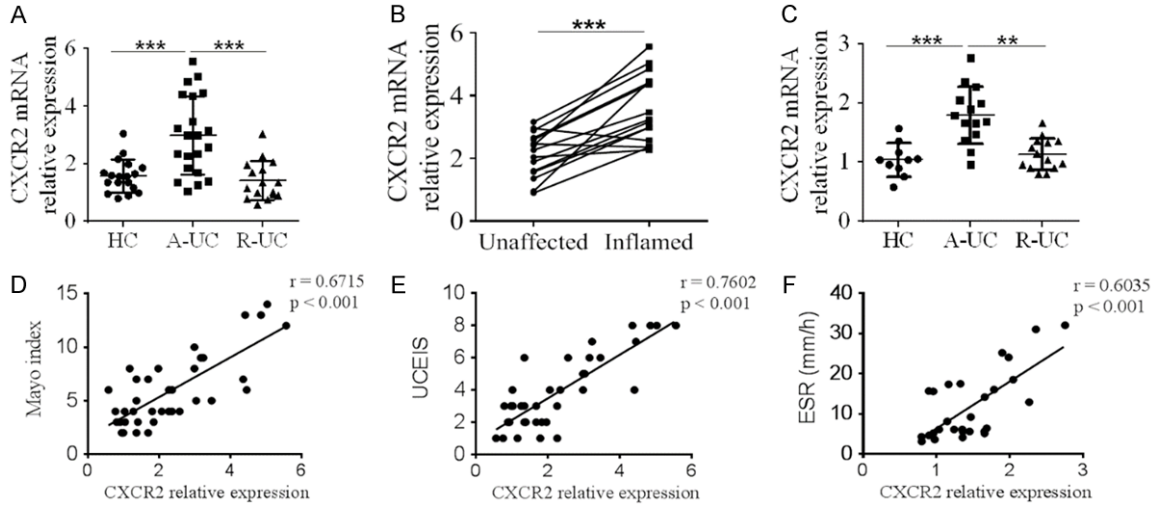

Figure 1. CXCR2 expression is highly increased in patients with active UC and correlated with disease activity. A. Colonic biopsies were collected from patients with A-UC (n=21), patients with R-UC (n=16), and HC (n=18). Expression of CXCR2 mRNA was examined by qRT-PCR. B. CXCR2 mRNA expression in inflamed and unaffected intestinal mucosa from the same patients with A-UC (n=15) was examined by qRT-PCR. GAPDH was used as a housekeeping gene. C. Peripheral blood samples were collected from patients with A-UC (n=14), patients with R-UC (n=14), and HC (n=10). Expression of CXCR2 mRNA was detected by qRT-PCR. $^{**}P < 0.01$, $^{***}P < 0.001$. D. Correlation analysis was performed between Mayo index and CXCR2 mRNA expression in inflamed mucosa from patients with UC ($^{***}P < 0.001$). E. Correlation analysis was performed between UCEIS and CXCR2 mRNA expression in inflamed mucosa from patients with UC ($^{***}P < 0.001$). F. Correlation analysis was performed between ESR and CXCR2 mRNA expression in peripheral blood samples from patients with UC ($^{***}P < 0.001$).

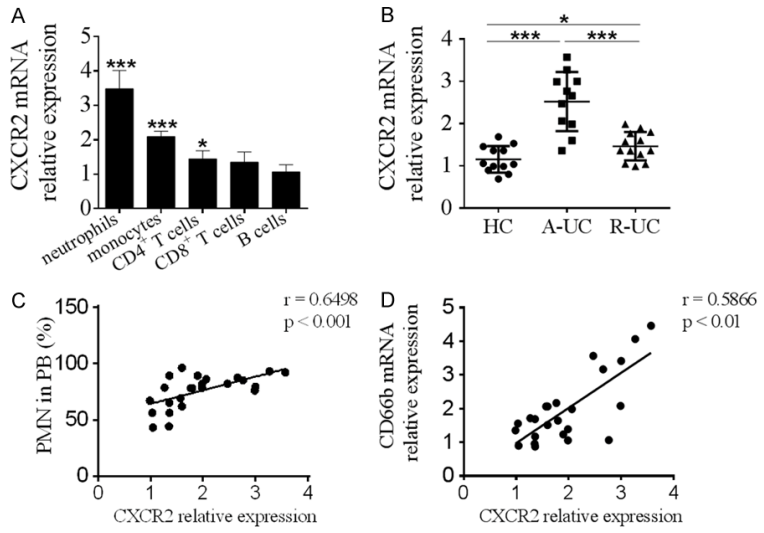

Figure 2. CXCR2 is highly expressed in neutrophils. A. Expression of CXCR2 in different subsets of immune cells was examined by qRT-PCR. Peripheral blood PMN, $CD14^+$ monocytes, $CD4^+$ T cells, $CD8^+$ T cells, and $CD20^+$ B cells (1×10^6) were isolated from healthy donors (n=10), and expression of CXCR2 was detected by qRT-PCR. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus B cells. B. Peripheral blood neutrophils were collected from patients with A-UC (n=11), patients with R-UC (n=13), and HC (n=12). Expression of CXCR2 mRNA was detected by qRT-PCR. $^{*}P < 0.05$, $^{***}P < 0.001$. C. Correlation analysis was performed between the percentage of PMN in peripheral blood and CXCR2 mRNA expression in neutrophils from patients with UC ($^{***}P < 0.001$). D. Correlation analysis was performed between CD66b mRNA expression and CXCR2 mRNA expression in neutrophils from patients with UC ($^{**}P < 0.01$).

CXCR2 is highly expressed in neutrophils. Thus, peripheral blood neutrophils were isolated from patients with A-UC, and we found that CXCR2 mRNA expression was highly increased in peripheral neutrophils from A-UC compared with HC (**Figure 2B**). We then analyzed the correlation of CXCR2 expression in peripheral blood neutrophils with percentage of PMN in peripheral blood. Interestingly, we found that CXCR2 expression was positively correlated with the percentage of PMN in peripheral blood (**Figure 2C**). CD66b is reported to be a specific marker expressed in neutrophils [15]. As shown in Supplementary Figure 2, CD66b expression was markedly increased in peripheral neutrophils from A-UC compared with HC. And CXCR2 expression in peripheral neutrophils was positively corre-

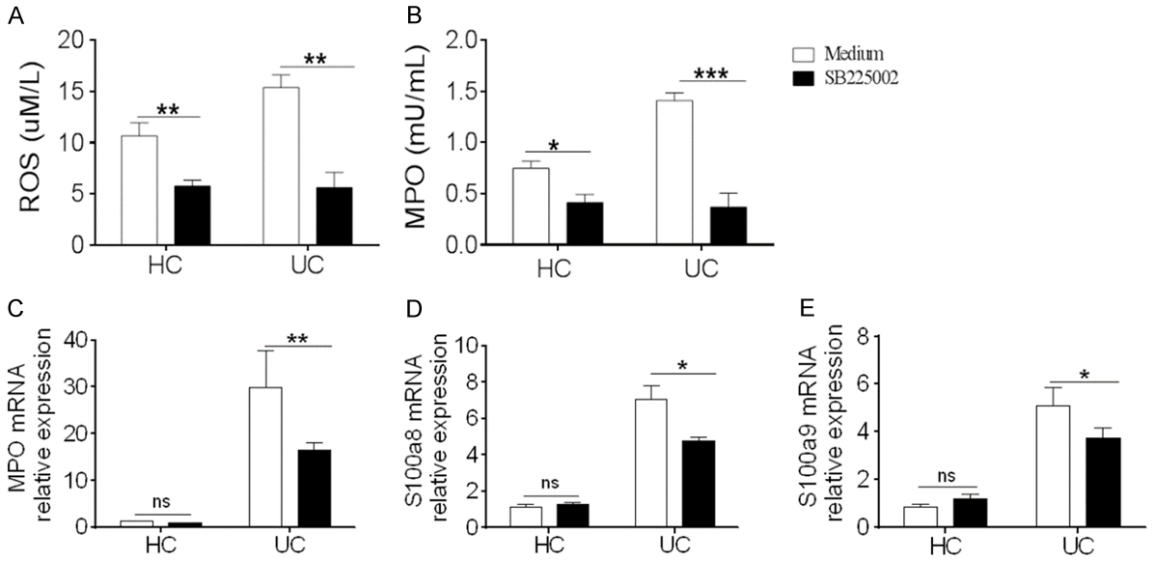

Figure 3. Blockade of CXCR2 in neutrophils suppresses the release of reactive oxygen species (ROS), MPO, and antimicrobial peptides. Peripheral neutrophils were isolated from patients with A-UC (n=6) and HC (n=6), and pretreated with SB225002 (500 nM) and LPS (200 ng/mL) for 3 hours. The levels of ROS (A) and MPO (B) produced by peripheral neutrophils (1×10^4) were measured using Amplex Red Hydrogen Peroxide Assay Kit. (C-E) Expression of MPO (C), S100a8 (D) and S100a9 (E) produced by peripheral neutrophils was analyzed by qRT-PCR. GAPDH was used as the housekeeping gene. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

lated with CD66b expression (**Figure 2D**). Therefore, these data suggest that CXCR2 is highly expressed in peripheral neutrophils and it may play an important role in the pathogenesis of UC.

Blockade of CXCR2 in neutrophils suppresses the release of ROS, MPO, and antimicrobial peptides

Neutrophils, as the effector cells in innate immunity, could release antibacterial proteins from their granules, and produce ROS when encountering the invading pathogens [6]. Neutrophils were isolated from the peripheral blood from patients with UC and HC, and then stimulated with LPS and SB225002. Production of ROS and MPO was detected using the Amplex Red assay. We observed that neutrophils isolated from HC produced relatively low levels of ROS and MPO compared with that from patients with A-UC (**Figure 3A** and **3B**). Moreover, treatment with SB225002 could significantly reduce the levels of ROS and MPO in neutrophils from patients with UC or HC (**Figure 3A** and **3B**). We then examined the expression of MPO, S100a8 and S100a9 in neutrophils by qRT-PCR. As shown in **Figure 3C-E**, SB225002 treatment markedly downregulated the expression of MPO, S100a8 and S100a9 in neutrophils isolated from patients with A-UC. However, it did not show any significant difference in neutrophils isolated from HC.

Blockade of CXCR2 in neutrophils suppresses production of proinflammatory cytokines

The production of proinflammatory cytokines was a typical characteristic in UC, then we hypothesized that whether CXCR2 was involved in the modulation of proinflammatory cytokines production in neutrophils. To this end, peripheral neutrophils were isolated from patients with A-UC and HC, and pretreated with SB225002 and LPS for 3 hours. Culture media were replenished and incubated for another 24 h. Supernatants were collected for ELISA analysis. As shown in **Figure 4A-D**, proinflammatory cytokins (TNF-α, IL-1β, IL-8 and IL-6) were significantly suppressed by SB225002 treatment. And the same results were observed in mRNA expression of TNF-α, IL-1β, IL-8 and IL-6 by qRT-PCR (**Figure 4E-H**). However, SB225002 treatment had no effect on the expression of IFN-γ, IL-4 and IL-17A (Supplementary Figure 3). Taken together, our data reveal that blockade of CXCR2 in neutrophils suppressed production of proinflammatory cytokines.

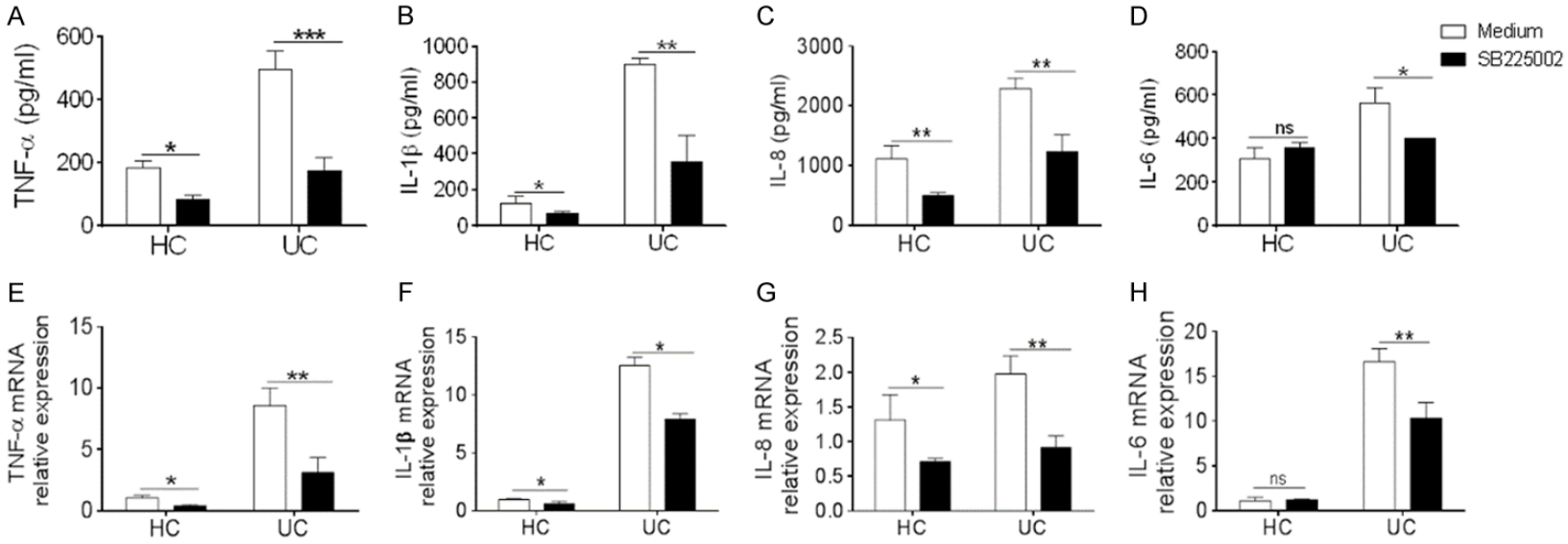

Figure 4. Blockade of CXCR2 in neutrophils suppresses production of proinflammatory cytokines. Peripheral neutrophils were isolated from patients with A-UC (n=6) and HC (n=6), and pretreated with SB225002 (500 nM) and LPS (200 ng/mL) for 3 hours. Culture media were replenished and incubated for another 24 h. Supernatants were collected and protein production of TNF-α (A), IL-1β (B), IL-8 (C) and IL-6 (D) were measured by ELISA. Peripheral neutrophils were isolated from patients with A-UC (n=6) and HC (n=6), and pretreated with SB225002 (500 nM) and LPS (200 ng/mL) for 3 hours. Expression of TNF-α (E), IL-1β (F), IL-8 (G) and IL-6 (H) was analyzed by qRT-PCR. GAPDH was used as the housekeeping gene. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

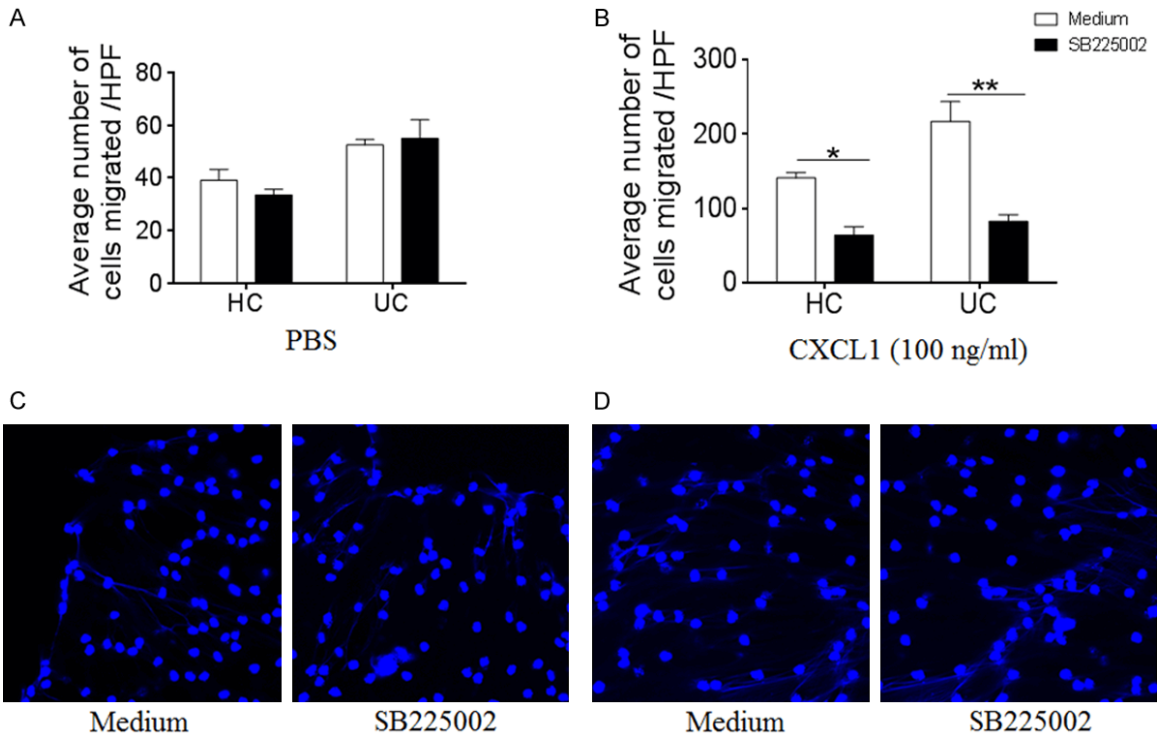

Figure 5. CXCR2 promotes the migratory capacity of neutrophils *ex vivo*, and doesn't affect NET formation. Peripheral neutrophils were isolated from patients with A-UC (n=6) and HC (n=6), and pretreated with SB225002 (500 nM) and LPS (200 ng/mL) for 3 hours. Neutrophil migration was then analyzed by a Transwell plate (8 μm) under the stimulation with PBS (A) or CXCL1 (100 ng/mL, B) for 1 h. **P* < 0.05, ***P* < 0.01. (C, D) Peripheral neutrophils were isolated from patients with A-UC (n=6) and HC (n=6), and pretreated with SB225002 (500 nM) and LPS (200 ng/mL) for 3 hours. Neutrophils were stained with Hoechst 33342, and the formation of NET by neutrophils from HC (C) and patients with A-UC (D) was examined by immunofluorescence microscopy (× 200).

CXCR2 promotes the migratory capacity of neutrophils ex vivo, and doesn't affect NET formation

Large amounts of neutrophils infiltrated in inflamed mucosa, and leaded to the formation of crypt abscess during active inflammation, which was considered as the typical pathological features of UC. Therefore, we sought to determine whether CXCR2 affected the migratory capacity of neutrophils. To this end, peripheral neutrophils were isolated from patients with A-UC and HC, and pretreated with SB-225002 and LPS for 3 hours. The migratory capacity of neutrophils was examined by the Transwell plate. We observed that there were no significant differences of neutrophil migration between UC and HC, with SB225002 treatment or the control under the stimulation of PBS (**Figure 5A**). Interestingly, we found that under the stimulation of CXCL1, SB225002 treatment significantly suppressed the migratory capacity of neutrophils compared with controls under the stimulation of CXCL1 (**Figure 5B**).

Studies have reported that neutrophils can eliminate microorganisms by releasing NET, which can trap the invading microorganisms [5]. We then determined the NET formation in neutrophils. Peripheral neutrophils were isolated from patients with A-UC and HC, pretreated with SB225002 and LPS for 3 hours, and stained with Hoechst 33342. We observed that there was no significant difference of NET formation between neutrophils with or without SB225002 treatment in patients with A-UC or HC (**Figure 5C** and **5D**).

Blockade of CXCR2 alleviates DSS-induced colitis in mice

To determine the role of CXCR2 in the pathogenesis of colonic inflammation, DSS-induced colitis model was induced as described in Materials and Methods. We observed that

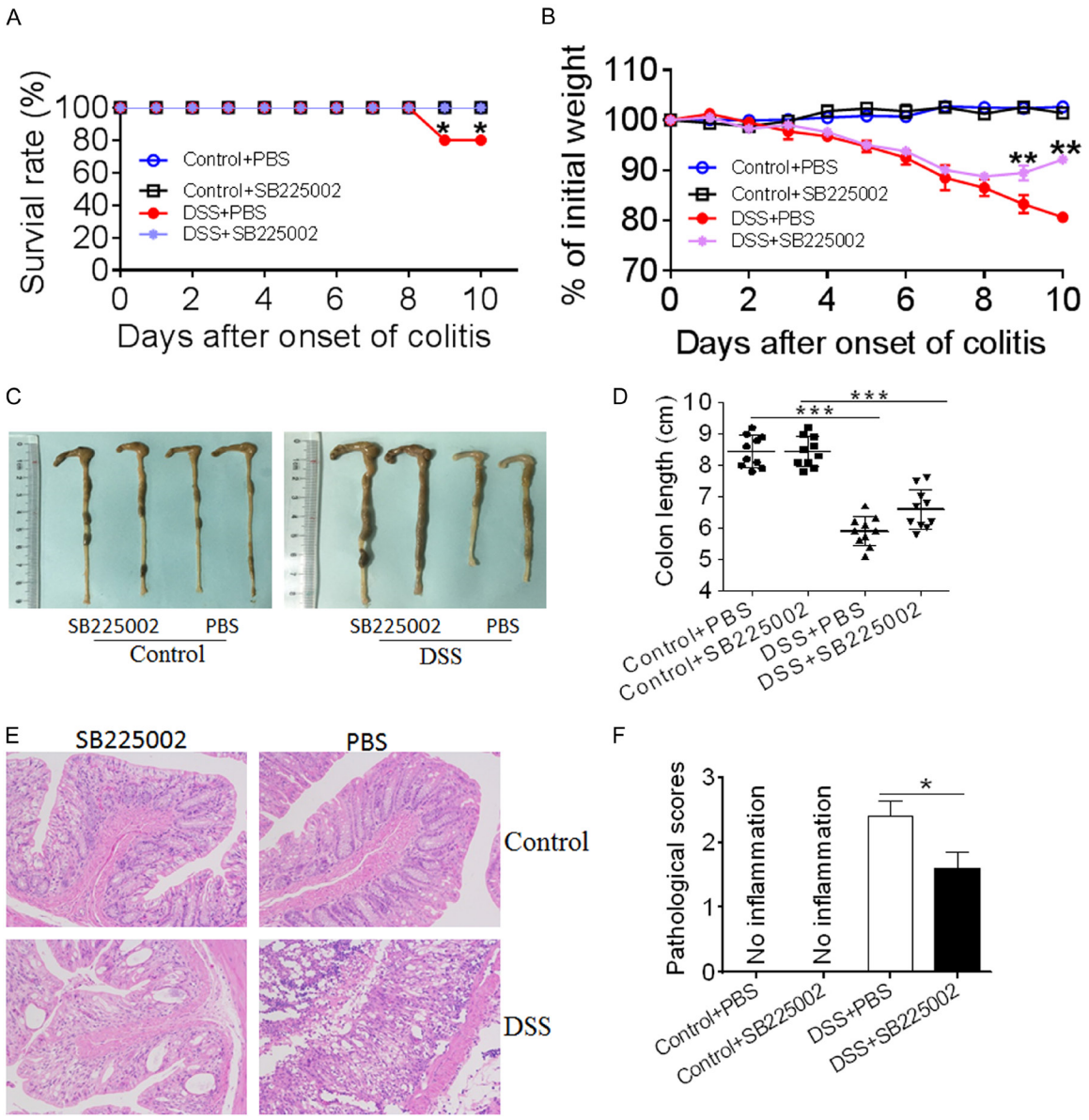

Figure 6. Blockade of CXCR2 alleviates DSS-induced colitis in mice. DSS-induced colitis in C57BL/6 mice was induced as indicated. Two groups of DSS-exposed mice (n=10) were treated with SB225002 (1 mg/kg) or PBS as controls daily intraperitoneally. Two groups of none DSS-exposed mice (n=10) were also treated with SB225002 or PBS as negative controls. A. The survival rates of mice over 10 days. B. The changes of body weight were observed and expressed as a percentage of initial body weight at the start of experiments. C. Gross morphology of colonic tissues on day 10. D. The statistical length of colons in different groups. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus controls. E. Colonic sections were stained with H&E (× 200). F. The changes in pathological scores from colonic sections were calculated as indicated. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus controls.

expression of CXCR2 was markedly increased in inflamed colon of DSS-treated WT mice compared with controls (Supplementary Figure 4). CXCR2 selective inhibitor SB225002 (1 mg/kg) was then administrated daily intraperitoneally as indicated. As shown in **Figure 6A**, SB225002 treatment could significantly enhanced the survival rate of mice exposed to DSS during a period of 10-day observation. And mice with the treatment of SB225002 developed much milder colitis, characterized by a slighter loss of body weight, less shorted colon length, lower levels of pathological scores compared with those in mice without SB225002 treatment (**Figure 6B-F**). Fresh colonic samples were also obtained and cultured *in vitro* for 24 h, and the supernatants were collected to detect cytokines expression by ELISA. We observed

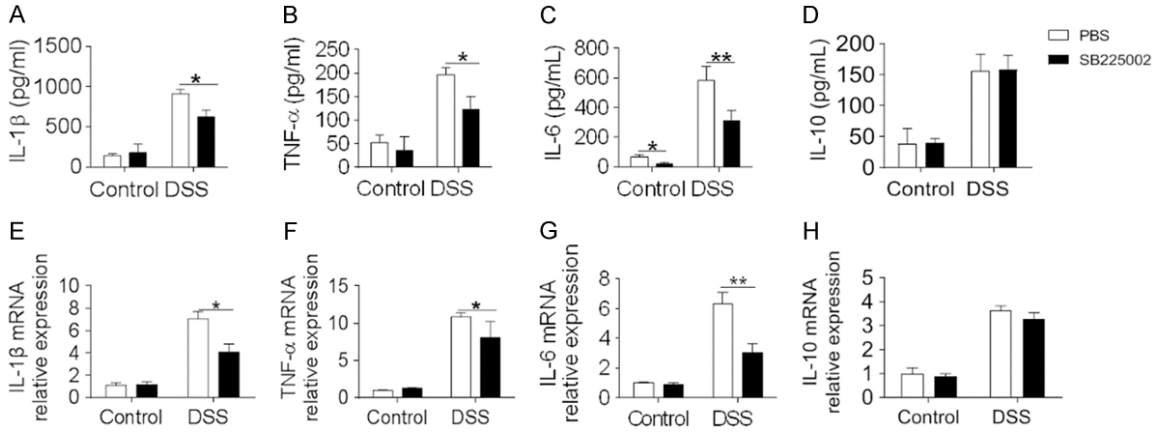

Figure 7. Cytokines profiles in colonic tissues from DSS-induced colitis mice. A-D. Colonic tissues from mice on day 10 after DSS-induced colitis were cultured ex vivo at 37 °C for 24 h, the supernatants were then collected for detection of inflammatory cytokines by ELISA. E-H. Colonic tissues were obtained from mice on day 10 after DSS-induced colitis, and total RNA was extracted to detect mRNA levels of various cytokines by qRT-PCR. **P* < 0.05, ***P* < 0.01 versus controls.

that levels of IL-1β, TNF-α and IL-6 were significantly decreased in mice with treatment of SB225002 (**Figure 7A-C**). However, the IL-10 level was not altered in SB225002-treated mice (**Figure 7D**). Furthermore, total RNA was extracted to detect inflammatory cytokines expression by qRT-PCR. As shown in **Figure 7E-H**, IL-1β, TNF-α and IL-6 expression were significantly decreased in mice with the treatment of SB225002, and IL-10 expression was not changed, which were consistent with the protein levels of ELISA.

CXCR2 promotes the migratory capacity of neutrophils in vitro

To determine how CXCR2 regulates intestinal mucosal inflammation, we isolated neutrophils from LPMCs from the mice. We observed that the percentage of neutrophils was markedly increased from DSS-induced colitis mice compared with that from the control mice (**Figure 8A** and **8B**). Moreover, SB225002 treatment could significantly reduce the percentage of neutrophils in LPMCs from DSS-induced colitis mice (**Figure 8A** and **8B**), which suggested that CXCR2 might be involved in the regulation of neutrophils migration. To further confirm our hypothesis, neutrophils were isolated from the bone marrow of mice on day 10 after DSS-induced colitis, and the migration was then analyzed by a Transwell plate under the stimulation with CXCL1 for 30 min. As shown in **Figure 8C**, SB225002 treatment significantly suppressed the migratory capacity of neutrophils compared with controls. Taken together, our data reveals that CXCR2 promotes the migratory capacity of neutrophils.

Discussion

UC, one of the chronic inflammatory diseases, is reported to be caused by abnormal immune response against the normal microbiota [17]. The typical pathological feature of UC is the formation of crypt abscesses, which is caused by the accumulation of large amounts of neutrophils. Neutrophils are one of major immune cells involved in the pathogenesis of UC [7]. In this study, we found that CXCR2 was highly expressed in neutrophils, and increased CXCR2 expression in colonic mucosal tissues and peripheral blood cells was positively correlated with disease activities. Inhibition of CXCR2 in neutrophils decreased the production of proinflammatory mediators, and the migratory capacity of neutrophils was markedly suppressed after SB225002 treatment. Moreover, blockade of CXCR2 with SB225002 could significantly ameliorate DSS-induced colitis in mice.

CXCR2, belonging to G protein coupled receptors (GPCR), is critically involved in the process and regulation of immune-mediated inflammatory diseases in different models [9, 18, 19]. CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8 are the known ligands of CXCR2 [20]. CXCLs/CXCR2 signal plays a vital role in both cancer and various inflammatory or im-

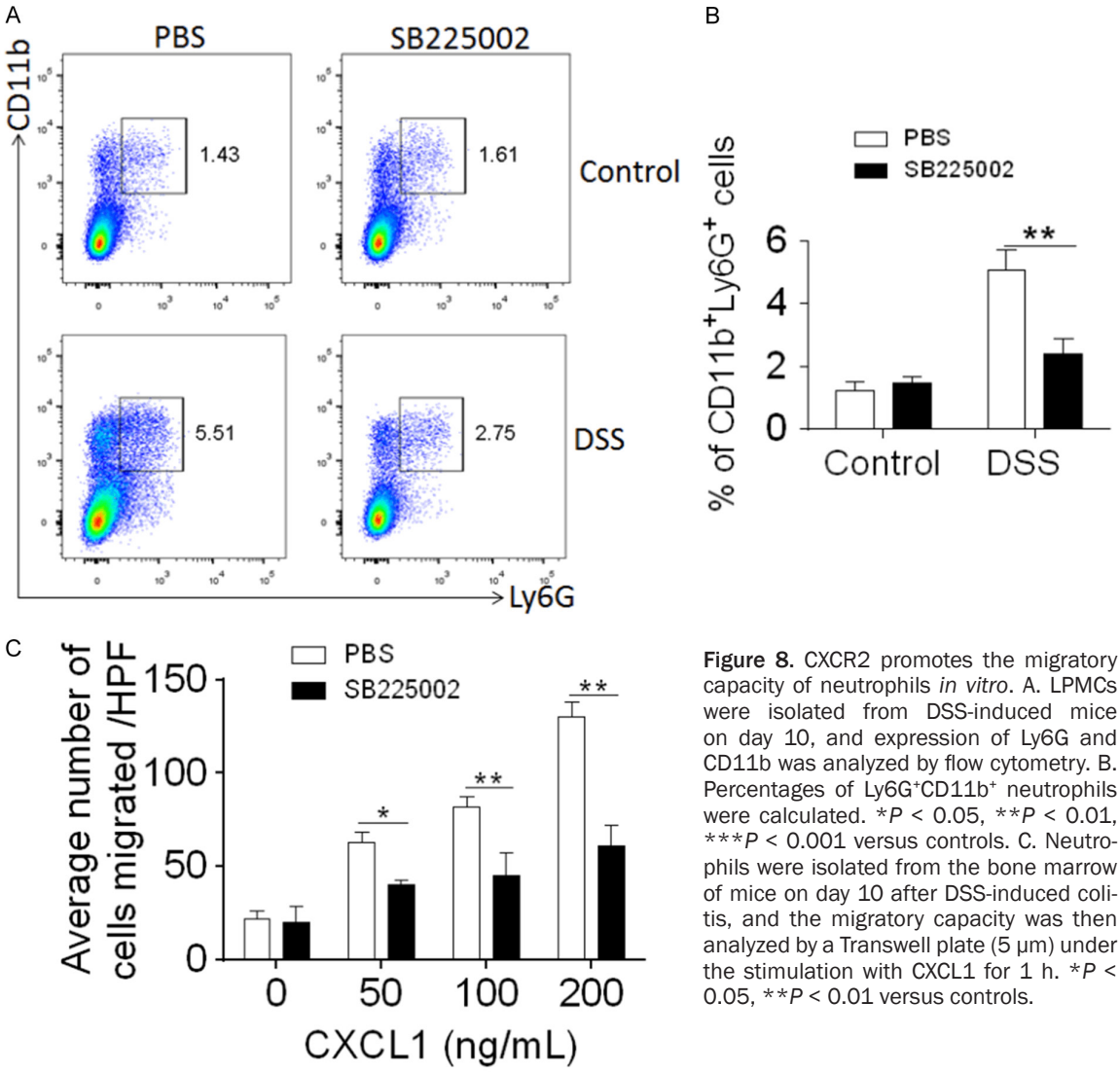

Figure 8. CXCR2 promotes the migratory capacity of neutrophils *in vitro*. A. LPMCs were isolated from DSS-induced mice on day 10, and expression of Ly6G and CD11b was analyzed by flow cytometry. B. Percentages of $Ly6G^{+}CD11b^{+}$ neutrophils were calculated. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus controls. C. Neutrophils were isolated from the bone marrow of mice on day 10 after DSS-induced colitis, and the migratory capacity was then analyzed by a Transwell plate (5 μm) under the stimulation with CXCL1 for 1 h. $^{*}P < 0.05$, $^{**}P < 0.01$ versus controls.

mune disease, such as pancreatic inflammation, allergic airway inflammation, antiviral immune response, experimental hypertension and Vascular Dysfunction, stress-induced depression, cardiac remodeling and dysfunction, acute lung injury [10, 21-26]. CXCR2 deficiency in a murine model further demonstrates increased necrosis and reduced vascular density [27]. Further, CXCR2 expression in tumor cells indicates a poor prognosis and promotes tumor invasion and metastasis [28]. In our study, we observed that CXCR2 expression was remarkably increased in colonic mucosal tissues and peripheral blood cells from patients with active UC. Moreover, increased CXCR2 expression was positively correlated with Mayo index, UCEIS and ESR, which were important markers to evaluate disease activity. Previous studies have revealed that CXCR2 was expressed in various cell types, such as neutrophils, monocytes, eosinophils, endothelial cells, mast cells and oligodendrocytes [29]. Our studies found that CXCR2 was expressed in neutrophils with the highest level, which was consistent with previous study [30]. We then observed that CXCR2 mRNA expression was highly increased in peripheral neutrophils from A-UC compared with HC, and it was positively correlated with the percentage of PMN in peripheral blood and CD66b expression. These data indicate that CXCR2 might play an important role in the pathogenesis of UC via regulating the immune response of neutrophils.

Extensive infiltration of neutrophils during chronic inflammation is one of the most impor-

tant pathogenic factors in various inflammatory diseases. UC is characterized by recurring episodes of inflammation and tissue injury [31, 32]. It is reported that such relapsing inflammation is regarded as a pathological feature and a hallmark of UC, and is associated with dysregulated neutrophil infiltration of the intestinal mucosa [3, 33], which could trigger an uncontrolled positive feedback amplification loop leading to tissue damage and resolution delay. Neutrophils play a beneficial and detrimental role in intestinal inflammation [34]. When epithelium is injured, neutrophils are crucial to protect from invading pathogens. Neutrophils can be recruited to sites of infection to attack invasive pathogens through phagocytosis and degranulation, and engulf invasive pathogens by releasing antimicrobial peptides, producing ROS [3, 35]. However, when not properly eliminated, neutrophils can contribute to significant tissue damages during acute and chronic diseases. The tissue-damaging effects of neutrophils are primarily attributed to their capacity to generate high levels of ROS. Accumulation of ROS in tissues gradually increases intracellular levels of hydrogen peroxide and superoxides, which oxidize and generate modified DNA bases [36]. In our study, we revealed that blockade of CXCR2 inhibited neutrophils to produce ROS, MPO and calprotectin (S100a8, S100a8). Abnormal production of inflammatory cytokines (e.g, TNF-α, IL-1β, IL-6, and IL-10) are the hallmark of UC. It is reported that under spontaneous or specific circumstances, neutrophils are capable of producing several inflammatory cytokines, which are further involved in the regulation of innate and adaptive immune responses to some extent [37]. Neutrophil-derived cytokines have been studied to be highly associated with intestinal mucosal inflammation. As shown in our study, we observed that CXCR2 promoted neutrophils to produce the inflammatory levels of TNF-α, IL-1β and IL-6. However, IL-10 levels were not altered in neutrophils after blockade of CXCR2. Collectively, our data indicate that CXCR2 blockade could regulate the detrimental role of neutrophils in intestinal mucosal inflammation of UC.

Evidence has demonstrated that successful elimination of microbial infection depends on efficient neutrophil migration into sites of inflammation [38, 39]. A defect of neutrophil migration has also been correlated with acute inflammation in human [40]. Massive infiltration of neutrophil in gut mucosa is thought to be involved in the pathogenesis of IBD, and neutrophil migration into intestinal crypts is associated with patient symptoms and mucosal injury [41]. CXCR2, as a chemokine receptor for CXCL1, CXCL2, and CXCL8, has been reported to play a vital role in the recruitment of neutrophils during inflammation [42]. Thus, we hypothesized that CXCR2 might regulate the migration of neutrophils in UC. We observed that under the stimulation of CXCL1, SB225002 treatment significantly suppressed the migratory capacity of neutrophils compared with controls under the stimulation of CXCL1. Evidences have demonstrated that highly activated neutrophils can eliminate microorganisms by releasing NET, which composed of decondensed chromatin decorated with granular proteins that together form extracellular fibres [43]. NETs could trap and kill microbes, activate dendritic and T cells, and are implicated in autoimmune and vascular diseases [44]. However, other studies reported that NETs enhanced procoagulant activity and induced intestinal damage and thrombotic tendency in patients with IBD [45, 46]. Thus, we investigated whether CXCR2 affected NETs formation in neutrophils. Interestingly, our data revealed that there was no significant difference of NET formation between neutrophils with or without SB225002 treatment in patients with A-UC or HC.

Studies have increasingly focused on using CXCR2 antagonists in therapeutic strategies for cancer and related diseases. CXCR2 has been considered as a potential pharmacological target in controlling inflammatory damage in the pathogenesis of chronic obstructive pulmonary disease, asthma, arthritis, and hepatitis [10, 22, 26, 47-49]. Moreover, several CXCR2 blockade strategies have been used in registered clinical trials [50]. Thus, we investigated that how CXCR2 regulated intestinal mucosal inflammation *in vivo*. We found that blockade of CXCR2 by its selective inhibitor SB225002 could markedly alleviate DSS-induced colitis in mice, characterized by a slighter loss of body weight, less shorted colon length, lower levels of pathological scores and inflammatory cytokines. Furthermore, CXCR2 blockade significantly reduced the percentage of neutrophils in LPMCs from DSS-induced colitis mice, indicating the suppressed migratory ca-

pacity of neutrophils after CXCR2 blockade, which was consistent with the results in neutrophils from UC patients.

In summary, our data indicate that CXCR2 is a novel indispensable regulator in the pathogenesis of UC by promoting the production of inflammatory mediators, and the migratory capacity of neutrophils. Blockade of CXCR2 could ameliorate intestinal mucosal inflammation induced by DSS in mice. Our study has shed a new light on elucidating the role of CXCR2 in the pathogenesis of UC, and targeting CXCR2 could be a promising pharmacological target in the treatment of UC.

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Disclosure of conflict of interest

None.

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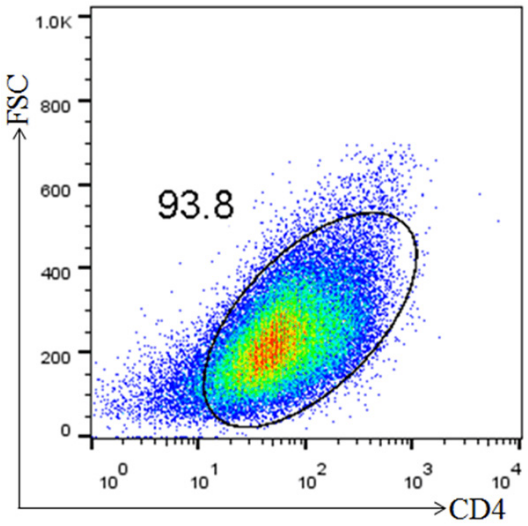
Supplementary Table 1. Clinical characteristics of patients with UC

	Blood samples		Biopsy samples	
	HC	UC (A/R)	HC	UC (A/R)
Number of patients	10	28 (14/14)	18	37 (21/16)
Age (y)	36.15±5.56	38.23±17.26	35.16±6.16	39.16±10.41
Gender				
Male	6	12	10	20
Female	4	16	8	17
Disease duration (month)		36.15±14.16		29.41±9.26
Current therapy				
5-aminosalicylates		18		28
Biologics		0		0
Azathioprine		0		0
methotrexate		0		0
prednisolone acetate		1		4
methylprednisolone		0		2
Disease extent (UC)				
E1		10		14
E2		5		13
E3		3		10
CRP (mg/L)		34.15±15.16		30.16±16.16
ESR (mm/h)		26.45±15.15		30.15±14.15

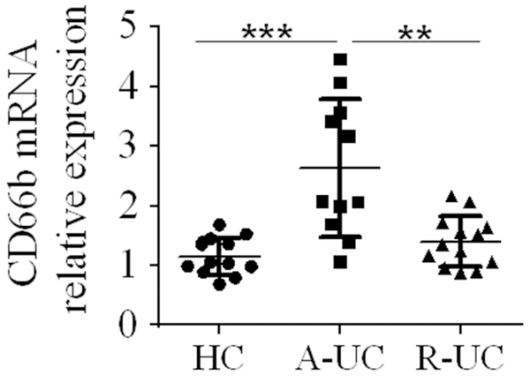
According to the Montreal classification system. A/R: Active/Remission.

Supplementary Table 2. The primers using in qRT-PCR analysis

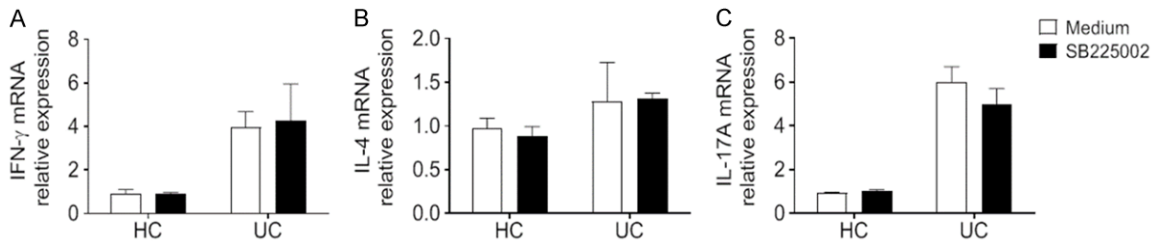
Gene	Species	DNA sequence (sense 5'-3')	DNA sequence (anti-sense 5'-3')
CRCX2	Human	CCTGTCTTACTTTTCCGAAGGAC	TTGCTGTATTGTTGCCCATGT
MPO	Human	CCAGATCATCACTTACCGGGA	CACTGAGTCATTGTAGGAACGG
S100a8	Human	ATGCCGTCTACAGGGATGAC	ACTGAGGACACTCGGTCTCTA
S100a9	Human	GGTCATAGAACACATCATGGAGG	GGCCTGGCTTATGGTGGTG
TNF-α	Human	GAGGCCAAGCCCTGGTATG	CGGGCCGATTGATCTCAGC
IL-8	Human	ACTGAGAGTGATTGAGAGTGGAC	AACCCTCTGCACCCAGTTTTC
IL-1β	Human	TTCGACACATGGGATAACGAGG	TTTTTGCTGTGAGTCCCGGAG
IL-6	Human	CCTGAACCTTCCAAAGATGGC	TTCACCAGGCAAGTCTCCTCA
CD66b	Human	CCATGCAGGGGTAAATCTCAAC	TGGAATGTGCCATTGACAGAC
CXCR2	Mouse	TGTCTGGGCTGCATCTAAAGT	AGGTAACCTCCTTCACGTATGAG
IL-1β	Mouse	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
TNF	Mouse	CTGAACTTCGGGGTGATCGG	GGCTTGTCACTCGAATTTTGAGA
IL-6	Mouse	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
IL-10	Mouse	CTTACTGACTGGCATGAGGATCA	GCAGCTCTAGGAGCATGTGG
GAPDH	Human	CTGGGCTACACTGAGCACC	AAGTGGTCGTTGAGGGCAATG
GAPDH	Mouse	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA



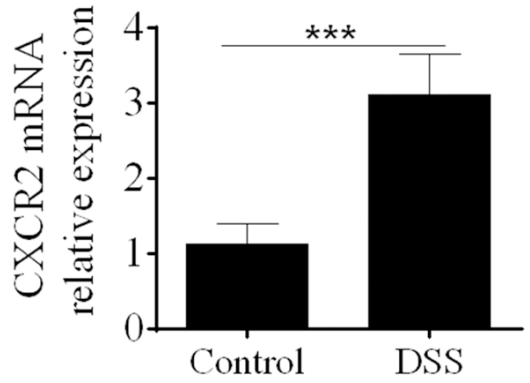
Supplementary Figure 1. The purity of $CD4^+$ T cells isolated by magnetic bead.



Supplementary Figure 2. CD66b expression is highly increased in peripheral neutrophils from patients with active UC. Peripheral blood neutrophils were collected from patients with A-UC (n=11), patients with R-UC (n=13), and HC (n=12). Expression of CD66b mRNA was detected by qRT-PCR. $^{**}P < 0.01$, $^{***}P < 0.001$.



Supplementary Figure 3. Inflammatory cytokines expression in neutrophils pretreated with SB225002, as described in **Figure 4**.



Supplementary Figure 4. CXCR2 expression is markedly increased in colonic tissues from DSS induced colitis mice. Colonic tissues were obtained from mice on day 10 after DSS-induced colitis and the control mice, and CXCR2 mRNA expression was detected by qRT-PCR. ***$P < 0.001$.