IgG plasma cells initiate changes in the protein C system in mouse ulcerative colitis through $CD14^+CD64^+$ macrophage activation

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Conflict of interest
None declared

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Abstract

Background. Inhibition of the protein C system (PCS) might be one of the mechanisms of ulcerative colitis (UC).

Objectives. The aim of the study was to explore the role of IgG plasma cells in changes in the PCS in UC.

Material and methods. Dextran sulfate sodium (DSS) was chosen to induce mouse UC. Inflammation was assessed using hematoxylin & eosin (H&E) staining and immunofluorescence. The profiling of colonic plasma cells and macrophages from colitis mice was analyzed with flow cytometry. After stimulation of macrophages with IgG type immune complex (IgG-IC), western blot was used to determine tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) protein levels. After co-incubation of colonic mucosa microvascular endothelial cells (MVECs) with TNF-α or IL-6, mitogen-activated protein kinase (MAPK) expression was detected.

Results. The DSS-colitis mice showed higher inflammatory indexes ($p < 0.05$ or $p < 0.01$), accompanied by greater infiltration of $CD38^+IgG^+$ plasma cells ($p < 0.01$), $CD14^+CD64^+$ macrophages ($p < 0.01$) and IgG-IC than healthy mice. Enhancement of TNF-α and IL-6 protein expression was demonstrated in this subset of macrophages when stimulated by IgG-IC ($p < 0.01$). After MVECs were incubated with TNF-α or IL-6, the expression of β-arrestin1, pP38 MAPK and pJNK MAPK exhibited an increase ($p < 0.05$ or $p < 0.01$), but downregulation of endothelial protein C receptor (EPCR) expression was observed ($p < 0.05$ or $p < 0.01$); this inhibition of EPCR expression was reversed by SB203580, SP600125 or U0126 ($p < 0.05$ or $p < 0.01$). In addition, changes in activated protein C (APC) presented results similar to those for EPCR expression ($p < 0.05$ or $p < 0.01$).

Conclusions. These results reveal that the PCS is inhibited during UC processing. There is a possibility that the interaction between IgG plasma cells and $CD14^+CD64^+$ macrophages, as well as further secretion of cytokines from $CD14^+CD64^+$ macrophages by the formation and stimulation of IgG-IC, subsequently influence MVECs through the β-arrestin-MAPK pathway. Enhancement of PCS activity may represent a novel approach for treating UC.

Key words: macrophages, ulcerative colitis, MAPK, protein C system, plasma cells

Ulcerative colitis (UC), the primary type of inflammatory bowel disease (IBD), is a recurrent, chronic intestinal inflammation of unknown cause, and a high risk of cancer is known to be associated with this disease.[1] Recently, the incidence of UC in China has rapidly increased, and as a consequence has gained more attention from researchers. Because of its unknown etiology, long course and the variety in the presentation of the lesions, it is often difficult to cure, and has become a very common treatment-resistant digestive disease.

The pathogenesis of UC is not fully understood. Recent research has revealed that IBD-induced pathogenesis extends beyond typical immune cells; however, non-immune cells, for example colonic mucosal microvascular endothelial cells (MVECs), are also involved in the pathological process.[2] Because the development of UC entails an interaction between the coagulation system and inflammation, hypercoagulation is a complication often found in patients. This complication manifests as microthrombus formation and microcirculation disturbance,[3] which develop into the primary disease. Damage to endothelial cells plays a vital role in the process of coagulation activation in UC patients, while the protein C system (PCS) is a major mediator of endothelial cell function. Recent studies have shown that the PCS plays a regulative role both in human MVECs and murine colitis.[4,5] Therefore, an imbalance of the PCS may induce the mediators of pathogenesis in UC patients. Our previous research showed that colon mucosal macrophages acted on mucosal MVECs by secreting pro-inflammatory cytokines and further inhibiting the PCS.[6]

At present, it is unclear what precisely is the initiator of PCS inhibition by macrophages. Studies have shown that redundant activation of humoral immunity is closely related to the pathological process of UC,[7] and acute UC is accompanied by activation of the humoral immune response. Lamina propria lymphocytes (mainly activated B cell subtypes) have been found to be an important source of immunoglobulins and autoantibodies. Plasma cells are the terminal effector cells derived from the development and differentiation of B lymphocytes in the connective tissue. Plasma cells have the important function of synthesizing and secreting antibodies, which they distribute mainly to the mucosa. Our previous research found that a large number of plasma cells accumulated in the colonic mucosa of DSS-colitis mice,[6] while neither the profiling of plasma cells involved in UC nor the interaction between plasma cells and macrophages in inhibiting the PCS have been elucidated. Therefore, exploring the changes and mechanisms of the PCS in UC pathology, as well as finding new drug targets, are of great importance.

In order to investigate the relationship and mechanisms between the PCS and UC, we used a DSS-induced model of colitis for this study. Topics covered in the discussion section primarily include the role of regulative signals of inflammation–coagulation, further elucidation of the pathological mechanism of UC in vivo and in vitro, the pathway of plasma cells-IgG-IC-macrophages-pro-inflammatory cytokines-MVECs-PCS during UC, as well as theoretical and experimental evidence for elucidating the mechanism and exploring new drugs for UC treatment.

Material and methods

The animals

C57BL/6J mice, 6–8 weeks old, half male and female (22–24 g, n = 70), were provided by the Laboratory Animal Center of Henan Province, Zhengzhou, China (Certificate No. SCXK (YU) 2015-0004). The mice were housed in a standard environment for 1 week (24 ±1°C, natural light-dark cycle) and were granted free access to normal tap water and standard rodent chow. The mice were fasted for 12 h before the experiment, and were randomly assigned to their respective groups. In the present study, 5 experiments were performed: 1) for histological evaluation and plasma cells (both the control group (n = 8) and the UC group (n = 8)); 2) for macrophages both at the histological level and at the cellular level (both the control group (n = 8) and the UC group (n = 8)); 3) for immune complex (both groups; (n = 8 each)); 4) for macrophage isolation and stimulation (only non-UC mice were used (n = 8), and were divided into control group and IgG-IC group); 5) for MVEC isolation and stimulation (only non-UC mice were used (n = 8), and were divided into control group, tumor necrosis factor α/interleukin 6 (TNF-α/IL-6) group, SB203580 group, SP600125 group, and U0126 group). All the animal experimental procedures complied with international principles for the care and use of laboratory animals. The procedures were conducted in accordance with guidelines from the Animal Ethics Committee of the Henan University School of Medicine, Kaifeng, China.

Induction of experimental murine colitis

Experimental ulcerative colitis was induced in 30 mice by providing them with 4% DSS (Sigma-Aldrich, St. Louis, USA) as the sole source of drinking water for 7 consecutive days, according to the method described by Schicho et al.[8] During these 7 days, the weight, general physical condition, stool characteristics, and gross and occult blood of the mice were measured daily. The death rate in the DSS-induced UC group left 8 mice in each experiment (1, 2 and 3) by the end of this period; at the end, all the surviving mice were sacrificed by cervical dislocation. Plasma was prepared by centrifugation of blood samples at 12,000 g, 4°C, for 10 min, then frozen at –80°C. A colon segment was cut longitudinally 1 cm above the anus, carefully rinsed with normal saline, and then weighed and measured for length and macroscopic scoring. The detached colon was separated into several parts. Some tissues were used for

the isolation of plasma cells and macrophages, some tissues were immediately fixed in 10% neutral-buffered formalin for histological analysis and immunofluorescence, while others were preserved at –80°C for F4/80 staining.

Macroscopic scoring

The macroscore was documented based on the evaluation system devised by Hartmann et al.,[9] namely: 1) body weight loss from baseline (0 points: none, 1 points: 1–5%, 2 points: 5–10%, 3 points: 10–20%, 4 points: more than 20%); 2) stool consistency (0 points for normal stools, 2 points for loose and pasty stools that failed to stick to the anus, 4 points for liquid stools that remained adhesive to the anus); 3) bleeding (0 points for a negative hemoccult test, 2 points for a positive one, 4 points for any gross bleeding from the rectum). A total clinical score ranging from 0 (healthy) to 4 (the maximum score for DSS-induced colitis) was obtained by dividing the sum of the above scores by 3.

Histology evaluation

Colon segments were fixed as described above and then dehydrated and embedded in paraffin. The tissue was sliced into 5-μm slices, stained with hematoxylin and eosin (H&E), and observed under a microscope to evaluate the degree of tissue damage. An experienced blinded researcher evaluated 5 sections for each colon sample and scored them as described by Kihara et al.[10] Each score was assessed as follows: 1) inflammation severity (0 points: none; 1 points: mild; 2 points: moderate; 3 points: severe); 2) inflammation extent (0 points: none; 1 points: mucosa; 2 points: submucosa; 3 points: transmural); 3) crypt damage (0 points: none; 1 points: basal 1/3 damage; 2 points: basal 2/3 damage; 3 points: crypt lost, surface epithelium present; 4 points: crypt and surface epithelium lost); 4) percent involvement (0 points: 0%; 1 points: 1–25%; 2 points: 26–50%; 3 points: 51–75%; 4 points: 76–100%). The total histologic score, ranging from 0 to 14, was obtained by adding the 4 subscores.

Isolation and identification of the phenotype of mouse colonic plasma cells

The immunomagnetic bead technique was used to purify $CD54^+$ cells (BD Pharmingen, Heidelberg, Germany) as described by Medina et al.[11] Using this method, lamina propria cells were obtained and stained with anti-$CD54^+$ antibody for 10 min, followed by adjustment to a concentration of 1×10^7/mL. Dynabeads™ Goat Anti-Mouse IgG (Invitrogen, Thermo Fisher Scientific, Waltham, USA) were applied to isolate plasma cells through a magnetic separator. The phenotype of the plasma cells was tested using flow cytometry with anti-IgA, IgG, IgM, and CD38 antibody, respectively.

Macrophage evaluation in colonic smooth muscle

Macrophages were visualized using F4/80 staining according to the method described by Li et al.[12] Briefly, the staining was performed by pre-incubation of whole-mount preparations of colonic smooth muscle in 10% normal goat serum (NGS) in phosphate-buffered saline (PBS) for 1 h at room temperature, followed by incubation with the primary antibody (F4/80, rat anti-mouse MCA 497, 1:1 in PBS) overnight at 4°C. The specimens were incubated with a secondary antibody – Alexa Fluor 488 donkey anti-rat, 1:100 in PBS (Invitrogen) – for 1 h at room temperature, and were then mounted in Eukitt® quick-hardening mounting medium (Sigma-Aldrich). Phosphate-buffered saline was used as negative control, and the macrophages in the colonic smooth muscle strips were counted.

Isolation and identification of mouse colonic macrophages

Mouse colonic macrophages were isolated and cultured in accordance with the method described by Chen et al.[13] In brief, the mouse colon was cut longitudinally, quickly placed into Hanks solution containing 1 g/L ethylenediaminetetraacetic acid (EDTA) at 37°C for 60 min, and then subjected to further digestion with 1 g/L collagenase for 2 h. The cell suspensions were resuspended in ISO osmotic fluid and the precipitation was collected. After staining with CD14 monoclonal fluorescent antibody (BD Pharmingen), intestinal macrophages were identified with flow cytometry.

Determination of macrophage type using flow cytometry

To determine the properties of the macrophages that participate in the pathogenesis of UC, the macrophage receptors were tested as previously described.[14] In brief, the cell concentration was adjusted to 1×10^6/mL, and labeled antibodies CD16/32 (eBioscience, Thermo Fisher Scientific) or CD64 (BioLegend, San Diego, USA) were added and incubated with the samples. The cells were resuspended in PBS and centrifuged; after resuspension with PBS, the samples were subjected to flow cytometry.

IgA, IgM or IgG type immune complex in colonic tissue using immunofluorescence

After routine dewaxing and hydration, antigens were retrieved through the use of immunohistochemistry. Next, 5% bovine serum albumen (BSA) was used to block the specimens, after which primary antibodies anti-IgA, anti-IgM and anti-IgG (Southern Biotech, Birmingham, USA) were added and then incubated for 1 night at 4°C. Next, the slides were incubated for 1 h with

fluorescent-labeled secondary antibodies protected from light, followed by 4′,6-diamidino-2-phenylindole (DAPI) staining and glycerol mounting.

TNF-α and IL-6 protein expression in the macrophages

IgG type immune complex (IgG-IC) was mimicked with the plate-immobilized IgG method as described by Uo et al.[14] Isolated macrophages from normal mice, stimulated with IgG-IC or soluble human IgG (hIgG), were collected and the protein concentration in the supernatant was tested. Colonic macrophages were collected and prepared in lysis buffer for western blotting in accordance with the method described previously.[15] Primary antibodies – rabbit anti-TNF-α and rabbit anti-IL-6 (both from Abcam, Cambridge, UK) – were used at a concentration of 1:500, and the β-actin antibody (Abcam) was used at a concentration of 1:1,000. Then the secondary antibody, Pierce® Goat Anti-Rabbit IgG (H+L) (Thermo Fisher Scientific), was added at a concentration of 1:10,000 and incubated for 1.5 h at room temperature. Photos were taken with the Bio-Rad ChemiDoc XRS+ imaging system (Bio-Rad Laboratories, Hercules, USA) and analyzed using an image analysis system in order to determine the optical density of the bands.

Isolation and culture of colonic mucosa MVECs

Colonic mucosa MVECs were isolated using the method described by Scaldaferri et al.[16] Murine colonic tissues were digested enzymatically (37°C, 0.25% trypsin, 1 mL) and then cultured for 96 h in MCDB 131 medium containing 20% serum, 1×10^5 IU/L penicillin, heparin, 0.1 g/L streptomycin, endothelial cell growth factor, and 0.584 g/L glutamine. After digestion, the cell suspension was collected and cultured in fresh medium for 72 h. The purified cells were digested using cell dissociation buffer and cultured in fresh medium to determine factor VIII activity. The 3rd generation cells were used for further investigation.

Activated protein C activity change induced by IL-6 or TNF-α in MVECs by chromogenic substrate

Following the method described by Zhang et al.,[17] colonic mucosa MVECs were incubated with IL-6 or TNF-α for 24 h in the presence of SB203580, SP600125 or U0126, and washed 3 times in Tris-buffered saline (TBS) containing 50 mM Tris-HCl, 120 mM NaCl, 2.7 mM KCl, and 3 mg/mL BSA. Then 200 nM of protein C (Enzyme Research Laboratories, Swansea, UK) and 0.6 U/mL thrombomodulin (TM) were incubated for 1 h at 37°C, and a chromogenic substrate (Chromogenix; Diapharma Group, West Chester, USA) was used to test APC activity.

Effects of IL-6 or TNF-α on the expression of EPCR, TM, β-arrestin1, pP38 MAPK, pJNK MAPK, and pERK MAPK of colonic mucosa MVECs measured using western blot

Next, to determine the signal pathway involved in the inhibition of the PCS in UC, MVECs were used to test the expression of endothelial protein C receptor (EPCR), TM, β-arrestin1, pP38 mitogen-activated protein kinase (MAPK), pJNK MAPK, and pERK MAPK with western blot, using their respective antibodies. Primary antibodies goat anti-EPCR, rabbit anti-TM and goat anti-pJNK MAPK (all from Santa Cruz Biotechnology, Santa Cruz, USA) were used here at a concentration of 1:500; rabbit anti-β-arrestin1, rabbit anti-pP38 MAPK, and rabbit anti-pERK MAPK (all from Abcam) were used here at a concentration of 1:500; and the β-actin antibody (Abcam) was used at a concentration of 1:1,000.

Statistical analysis

The data was compiled and expressed as means ± standard error of the mean (SEM). To determine statistical significance, all the data was analyzed using the independent-samples t-test or one-way analysis of variance (ANOVA) followed by the post hoc test of least significant difference (LSD) for multiple comparison test using SPSS v. 13.0 software (SPSS Inc., Chicago, USA). P-values <0.05 were regarded as statistically significant.

Results

Macroscopic scores elevated in DSS-induced colitis

The mice that consumed 4% DSS as the sole source of drinking water for 7 days (n = 8) developed severe colitis, manifested as colon length shortening ($p < 0.01$), increased spleen weight ($p < 0.05$) and bloody stools, which typically appeared after 3–4 days and continued until the end of the experimental period (Table 1). The macroscopic scores ($p < 0.01$) are presented in Table 1.

Table 1. Pathological impairment parameters, macroscopic scores and microscopic scores of DSS-induced ulcerative colitis (UC) in mice (n = 8 per group)

Variable	Control	UC
Body weight (% of 0 day)	103.1 ±5.6	88.3 ±3.6
Colon length [cm]	7.2 ±0.4	2.9 ±0.4**
Spleen weight (% of control)	100.2 ±5.4	121.3 ±6.9*
Macroscopic scores	0	3.5 ±0.3**
Microscopic scores	0	8.9 ±0.6**

*$p < 0.05$ and **$p < 0.01$ vs the control group.

Microscopic score increased in DSS-induced colitis

Histological examination of the control group animals (n = 8) revealed no signs of colitis. By contrast, the group treated with DSS presented obvious colitis, manifested as multiple erosive lesions, loss of complete crypts in the colon (Table 1), with evident infiltration of inflammatory cells into the colonic submucosa, leading to a higher histological score compared to the controls ($p < 0.01$, Table 1).

IgG type plasma cells and CD14$^+$CD64$^+$ macrophages infiltrate the inflamed mucosa of DSS-induced colitis

Our previous research confirmed that CD38 and CD54 staining showed positive expression in the submucosa of DSS-induced colitis, and a significantly increased number of CD38$^+$CD54$^+$ cells.[18] In the present study, our further analysis was consistent with those results: CD38 and IgG expression were upregulated in isolated plasma cells from the DSS mouse colons (Fig. 1A–C, $p < 0.01$), while the number of CD38$^+$IgM$^+$ and CD38$^+$IgA$^+$ plasma cells from the DSS mouse colons had not changed in comparison with the controls (Fig. 1A–C, $p > 0.05$).

F4/80 staining showed that the quantity of macrophages was increased in the DSS mouse colons (Fig. 2A). In order to further determine the profile of the macrophages, the macrophages were separated and identified. The results revealed that the heavily infiltrated macrophages in UC have a unique phenotype: CD14$^+$CD64$^+$ (Fig. 2B–E, $p < 0.01$); the quantity of CD14$^+$CD16/32$^+$ macrophages was not affected (Fig. 2B–E, $p > 0.05$).

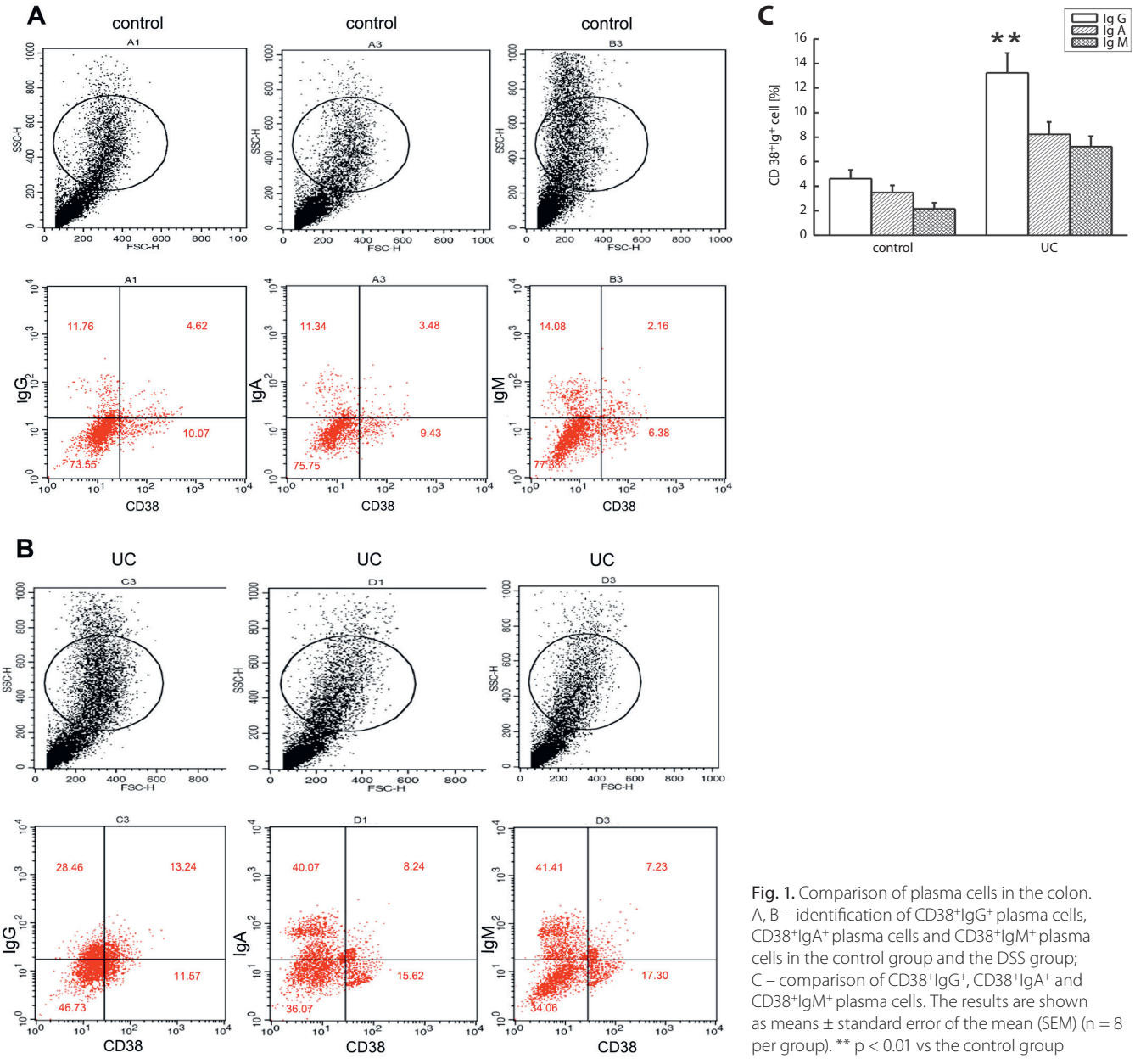

Fig. 1. Comparison of plasma cells in the colon. A, B – identification of CD38$^+$IgG$^+$ plasma cells, CD38$^+$IgA$^+$ plasma cells and CD38$^+$IgM$^+$ plasma cells in the control group and the DSS group; C – comparison of CD38$^+$IgG$^+$, CD38$^+$IgA$^+$ and CD38$^+$IgM$^+$ plasma cells. The results are shown as means ± standard error of the mean (SEM) (n = 8 per group). ** $p < 0.01$ vs the control group

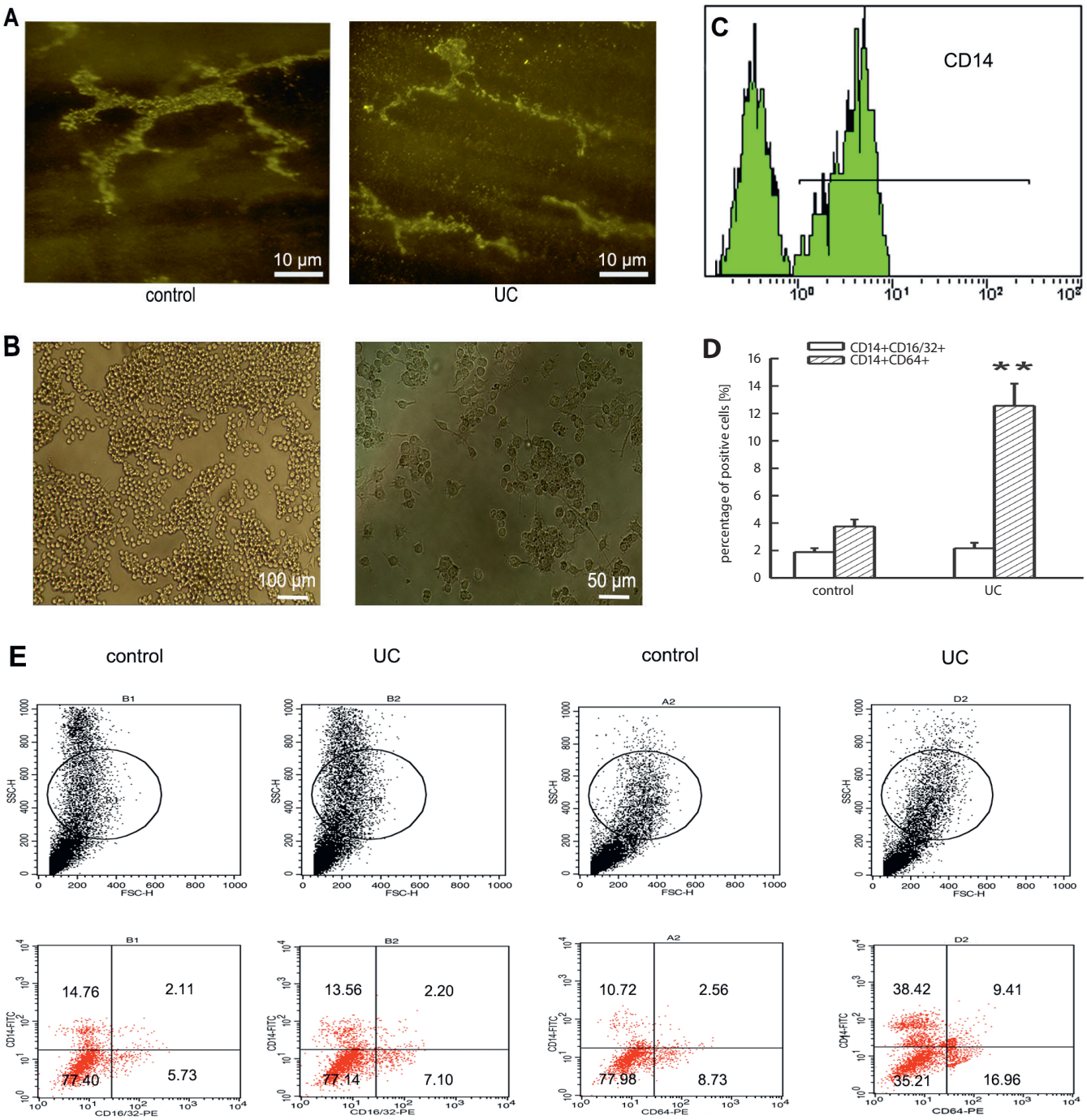

Fig. 2. Comparison of macrophages in the colon. A – F4/80 staining; B, C – isolation and identification of macrophages; D – statistical profiling of CD16/32 or CD64 expression in the control group and UC group; E – CD16/32 and CD64 expression in the control group and UC group. The results are shown as means ± standard error of the mean (SEM) (n = 8 per group). **p < 0.01 vs the control group

IgG type immune complex deposited in colon with DSS-induced colitis

As shown in Fig. 3, IgG-IC was more strongly expressed in the colonic mucosa of the DSS mice when compared to the control group. Vessel walls, especially small vessel walls, and the mesenchymal and glandular epithelia were the main locations of IgG-IC deposition. Low expression of IgA and IgM was found in both the control and the DSS mice.

Elevated release of pro-inflammatory cytokines from macrophages induced by IgG-IC

The mechanism through which IgG plasma cells contributed to the pathogenesis of UC and the inhibition of PCS remains uncertain. Based on the large deposition of IgG-IC and the increased macrophages in the colonic tissues of DSS colitis, we assessed the potential involvement of IgG-IC-macrophages-pro-inflammatory cytokine

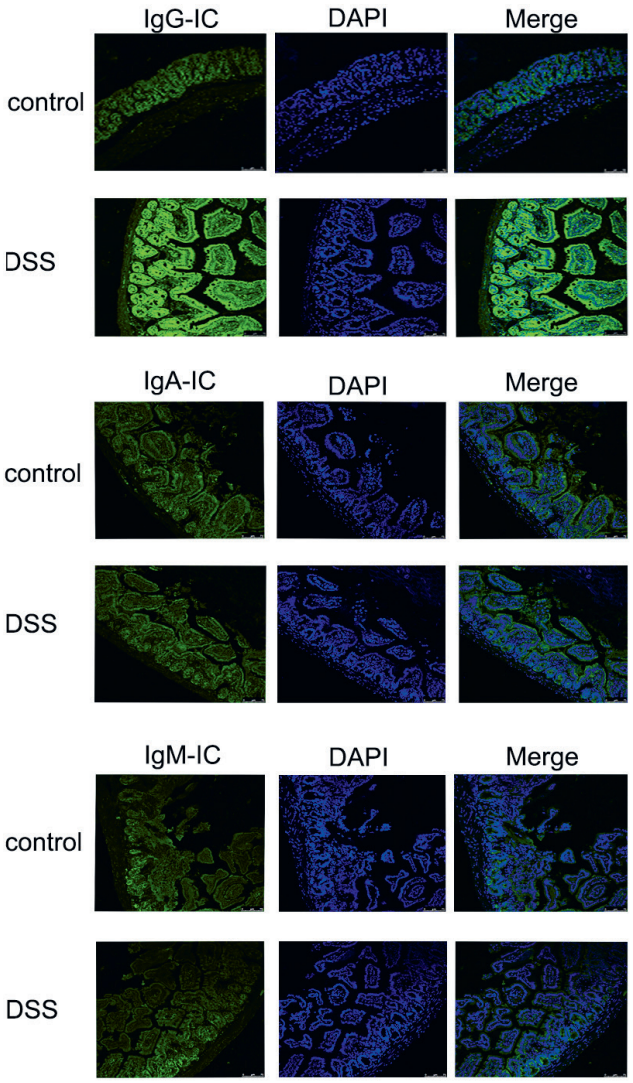

Fig. 3. The type of immune complex in colon tissue. The control group and UC group marked by anti-IgA, anti-IgG and anti-IgM antibody (scale bar: 75 μm)

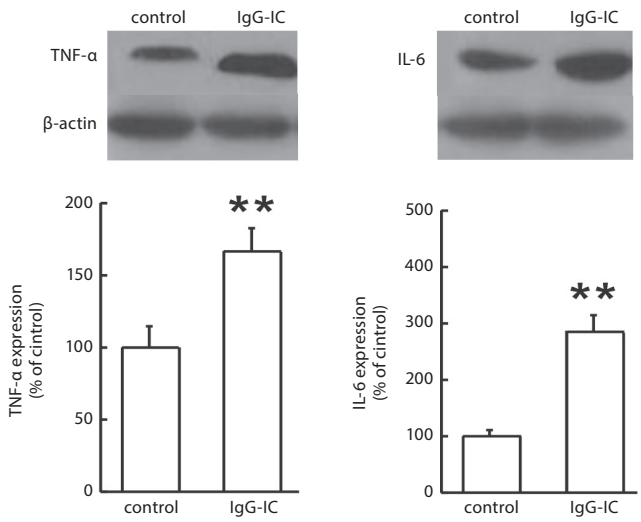

Fig. 4. The effect of IgG-IC on TNF-α and IL-6 protein levels in macrophages. The results are shown as means ± standard error of the mean (SEM) (n = 8 each group). ** $p < 0.01$ vs the control group

signaling in UC. To confirm the hypothesis, we examined cytokine secretion by macrophages when stimulated with IgG-IC mimicked by the plate-immobilized IgG method. As expected, we found that the TNF-α and IL-6 protein levels were elevated in the macrophages (Fig. 4, $p < 0.01$).

Inhibited PCS activity of microvascular cells induced by TNF-α or IL-6

To further explore the detailed mechanism involved in the change in the PCS, we separated and cultured MVECs from colonic mucosa. To identify the cells, we used fluorescence microscopy to detect factor VIII. After incubation with TNF-α or IL-6, APC activity was decreased ($p < 0.01$, Table 2). The reduction due to TNF-α was reversed by the p38 MAPK inhibitor SB203580 or JNK MAPK inhibitor SP600125; in addition, SB203580 and SP600125 reversed the effect of IL-6 on microvascular cells ($p < 0.05$ or $p < 0.01$; Table 2). Meanwhile, after MVECs were incubated with TNF-α or IL-6, the expression of β-arrestin1, pP38 MAPK and pJNK MAPK exhibited an increase (Fig. 5A, $p < 0.05$ or $p < 0.01$). The EPCR expression was reduced by TNF-α or IL-6 administration (Fig. 5B, $p < 0.05$ or $p < 0.01$). However, TM expression was not affected (Fig. 5B, $p > 0.05$). Furthermore, the inhibited expression of EPCR was reversed when the TNF-α-stimulated cells were pre-treated with SB203580, SP600125 or U0126 (Fig. 5B, $p < 0.05$ or $p < 0.01$), but only SB203580 reversed the effect of IL-6 on microvascular cells (Fig. 5B, $p < 0.05$); meanwhile, the expression of TM was not influenced by SB203580, SP600125 or U0126 in TNF-α- or IL-6-stimulated MVECs (Fig. 5B, $p > 0.05$).

Table 2. Change in activated protein C (APC) activity induced by tumor necrosis factor α (TNF-α) or interleukin (IL-6) in microvascular endothelial cells (MVECs) (n = 8 per group)

Group	APC activity
Control	1.0 ±0.1
TNF-α	0.6 ±0.07**
IL-6	0.4 ±0.06**
SB203580+TNF-α	0.9 ±0.08#
SP600125+TNF-α	0.9 ±0.07#
U0126+TNF-α	0.8 ±0.1
SB203580+IL-6	0.7 ±0.1&
SP600125+IL-6	0.8 ±0.1&&
U0126+IL-6	0.5 ±0.07

**$p < 0.01$ vs the control group; #$p < 0.05$ vs the TNF-α group; &$p < 0.05$ and &&$p < 0.01$ vs the IL-6 group.

Discussion

Several studies have demonstrated that a large number of IBD patients present with blood hypercoagulability, which includes venous thrombosis and pulmonary

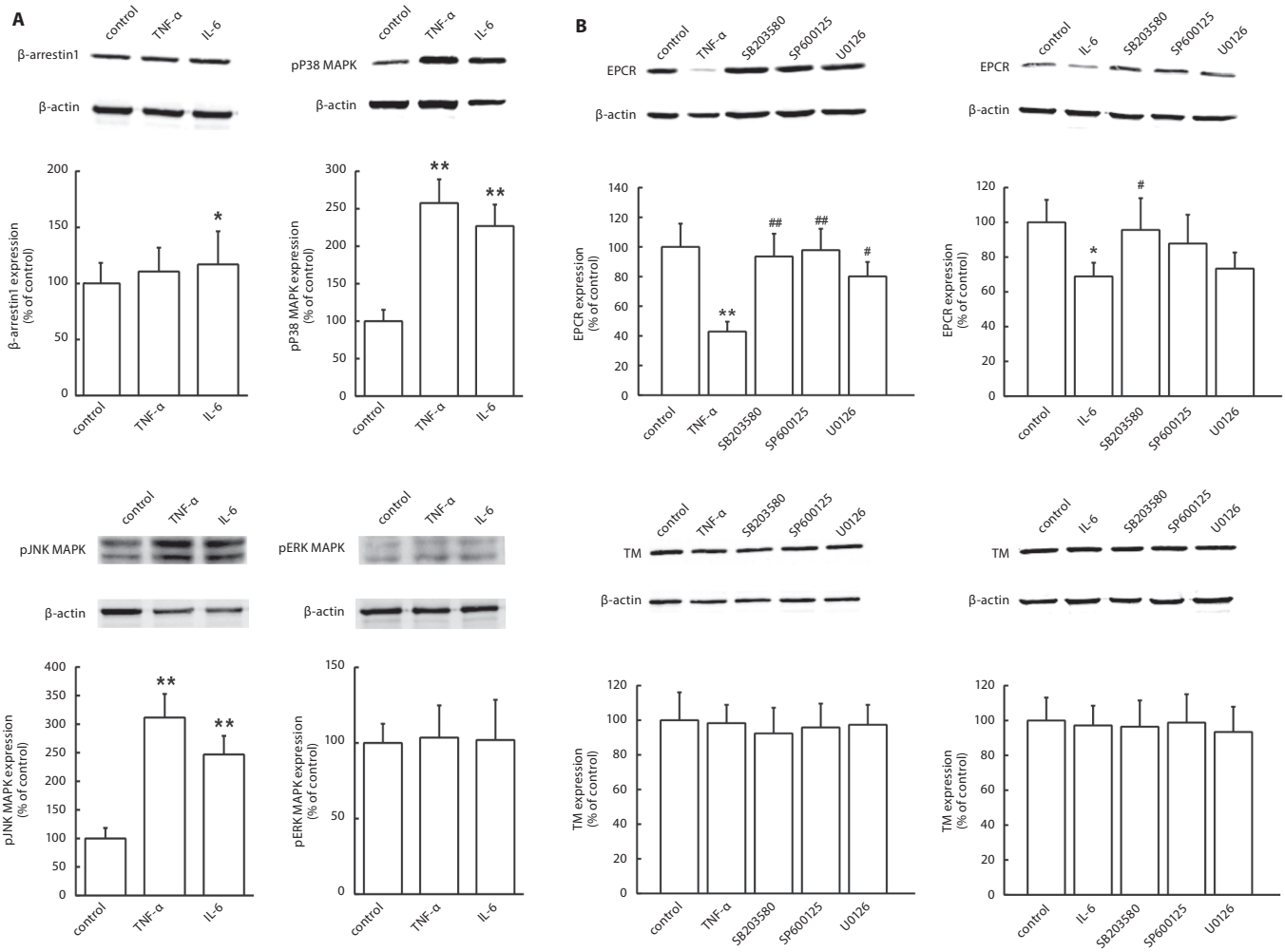

Fig. 5. Changes of the PCS in MVECs. A: changes in β-arrestin1, pP38 MAPK, pJNK MAPK, and pERK MAPK protein expression induced by TNF-α or IL-6 in MVECs by western blot; B: changes in EPCR and TM protein expression induced by TNF-α or IL-6 in MVECs by western blot. The results are shown as the means ± standard error of the mean (SEM) (n = 8 each group). *p < 0.05, and **p < 0.01 vs the control group; #p < 0.05, and ##p < 0.01 vs TNF-α or IL-6 group

embolism.[19–21] Hypercoagulability is accompanied by inflammation and remits when the inflammation disappears. Murthy et al.[22] found that congenital genetic factors do not play a major role in the formation of hypercoagulability in IBD patients. Thrombosis leads to intestinal mucosal ischemia or necrosis and severe ulceration, adding to the intestinal mucosal damage of UC patients. Therefore, thrombosis has been identified as a major contributing factor to the deterioration and death of UC patients.

Until now, no consensus has been reached on the mechanisms involved in coagulation system abnormalities in the pathogenesis of UC,[23,24] while the anticoagulation system is recognized as playing a key role.

The PCS, the most complex and most effective physiological mechanism of anticoagulation, comprises EPCR, protein S (PS), protein C (PC), protein C inhibitor, and TM. It plays an essential role in the regulation of blood coagulation and has a direct effect on maintaining homeostasis of the coagulation and anticoagulation systems.[25] There are few reports about the PCS in UC patients. Ulcerative colitis is closely related to injury of MVECs, and partial constituents of the PCS are the offspring of endothelial cells. We therefore speculate that the PCS plays a vital role in UC pathogenesis. In our previous research, we observed decreased activity of both PC and PS in the plasma of DSS-induced UC mice, and downregulated expression of EPCR.[6] These findings support the theory that the activity of the PCS is inhibited in DSS-induced mouse colitis, while the detailed mechanism of PCS inhibition remains unclear.

In view of the elevated plasma cells in DSS-induced colitis observed in our previous study,[18] we speculated that plasma cells may play a vital role in UC. It has not been determined whether the infiltrated plasma cells represent the mechanism of PCS inhibition, nor have the definite type and characteristics of the plasma cells involved been identified.[26,27] In order to investigate the changes in the number and type of plasma cells in UC, we observed the number of plasma cells from the inflamed mucosa of UC colons and normal colons using immunofluorescence, and found that the number of plasma cells increased in vivo.[18] In addition, flow cytometry was utilized in the present study to test

the number and type of plasma cells from separated lamina propria cells of mouse colonic mucosa. We found that the proportion of $CD38^+IgM^+$ plasma cells and $CD38^+IgA^+$ plasma cells in the UC mice had not changed when compared to the controls, but $CD38^+IgG^+$ plasma cells had increased significantly. These findings provided further confirmation that elevated IgG type plasma cells locally invade the colonic mucosa in UC, and these results were consistent with a recent report on enhanced IgG type plasma cells in UC and other autoimmune diseases.[14] These data suggested that the change in IgG type plasma cells is related to disease activity and plays a vital role in UC.

So far, the detailed mechanism of IgG type plasma cells in UC pathogenesis has not been elucidated. Halstensen et al. reported that IgG and activated immune complexes gather in colonic epithelial cells.[28] Under normal circumstances, tiny amounts of circulatory immune complexes in the body result from the immune response. However, in certain pathophysiological states, decreased phagocytic function of mononuclear macrophages leads to the elevation of immune complexes, furthering the deposition of large amounts of immune complexes on the injured organs. In the present study, DSS-induced colitis showed obvious deposition of immune complexes, mainly of the IgG type. These results were consistent with increased IgG type plasma cells in DSS-induced colitis.

Recent evidence has suggested that macrophages play a key role in homeostasis by inhibiting the dampening of inflammation.[29] In the present study, we found that positive F4/80 staining was higher in the UC mice than in the control group. Considering the increased levels of intestinal mucosa macrophages in UC, we hypothesize that macrophages contribute to inflammation or exacerbate it. Intestinal macrophages were isolated from colonic mucosa of both the UC and control colons, and as expected, the quantity of macrophages in DSS-induced colitis was higher than in the control mice. In addition, no significant differences were observed between DSS-induced colitis mice and normal mice in terms of the number of $CD14^+CD16/32^+$ macrophages, and $CD14^+CD64^+$ macrophages were significantly increased in DSS-induced colitis. CD64, a member of IgG Fc receptor family, which is mainly expressed on the mononuclear macrophage surface, participates in antibody-dependent cellular cytotoxicity (ADCC) and enhances inflammatory cytokine production by phagocytic cells. When cultured with IgG-IC, macrophages from the control mice produced larger amounts of TNF-α and IL-6 than when they were cultured with hIgG. Therefore, we speculate that IgG-IC induced secretion of inflammatory cytokines such as TNF-α and IL-6 by combining with CD64 on the macrophage surface.

Several studies have found that the immune system and coagulation system are linked in the pathogenesis of IBD.[19–21] Cytokines, including TNF-α and IL-6, have the ability to activate the coagulation pathway[30,31] and induce tissue factor (TF) expression in monocytes and endothelial cells.[32] However, the role of the inflammatory cytokines produced by macrophages in the changes in the PCS and in the pathogenesis of UC is unclear. In order to elucidate the mechanism of these cytokines in the PC pathway, we isolated and cultured colonic mucosal MVECs. After the MVECs were incubated with TNF-α or IL-6, we found decreased activity of APC and downregulated expression of EPCR. Thus, these results indicated that these inflammatory cytokines resulted in changes in the PC signal by stimulating colonic MVECs.

The MAPK pathway regulates inflammation through several proteins and kinases upstream and downstream, and it further influences gene transcription. Studies have shown that overactivation of MAPK is closely related with UC progression, and may represent a new target for anti-inflammatory therapy.[33–37] Our study found compatible results: pP38 MAPK and pJNK MAPK expression were upregulated at the protein levels, while pERK MAPK expression was not changed, indicating that the change in EPCR was mediated by p38 MAPK and JNK MAPK.

β-arrestin serves as a scaffolding protein. It is connected to G protein-coupled receptors and the kinase-mediated signal transduction pathway, and it is involved in the regulation of GPCR-activated ERK, JNK, and p38 MAPK pathways.[38] Recent studies have shown that the regulation of β-arrestin varies on different MAPK signaling pathways.[39–41] Since the MAPK pathway is the key mediator of UC inflammatory signals, and β-arrestin regulates MAPK, we speculate that the β-arrestin-MAPK pathway is involved in the regulation of PCS in UC. Our study showed that the inhibition of EPCR expression induced by TNF-α or IL-6 was reversed by SB203580 and SP600125. In addition, after stimulation with TNF-α or IL-6, β-arrestin1 expression was upregulated, which was accompanied by upregulation of p38 MAPK and pJNK MAPK. These results further confirm that the inhibition of EPCR is mediated by the p38 MAPK and JNK MAPK pathways.

Conclusions

In summary, we found that IgG plasma cells interacted with colonic $CD14^+CD64^+$ macrophages by forming IgG-IC during DSS-induced colitis in mice, and that macrophages produced inflammatory cytokines in response to IgG-IC stimulation, which resulted in inhibition of the PCS via colonic mucosal MVECs. Therefore, enhancement of PCS activity provides a theoretical feasible and clinically significant candidate for identifying new drug targets for the treatment of UC.

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