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PATHWAYS OF COMPLEMENT ACTIVATION FOLLOWING INTESTINAL ISCHEMIA-REPERFUSION IN MACAQUE

PUTEVI AKTIVACIJE KOMPLEMENATA POSLE INTESTINALNE ISHEMIJE-REPERFUZIJE KOD MAKAKE MAJMUNA

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Summary: Complement activation is a key component in the inflammation cascade. In the present study, intestinal ischemia-reperfusion (IIR) was introduced to macaques, and the pathways of complement activation in the multiple organ dysfunction syndrome (MODS) following IIR were investigated, which may provide evidence on the mechanisms underlying the endogenous protection in systemic inflammatory response. IIR was performed by clamping superior mesenteric artery and releasing clamp in 5 macaques. Immunization rate nephelometry and CH50 total complement detection were employed to measure the serum concentration of C3, C4, C-reactive protein (CRP) and total complements. Immunocytochemistry was carried out to detect the contents of IL-1 and NF-κB in polymorphonuclear cells (PMN). Flow cytometry was done to measure the apoptosis rate of PMN. At 24 h after IIR, the amount of total complement (106.6± 18.07 U/mL) was reduced to 62.1±9.52 U/mL ($P<0.05$). In addition, the C3 was reduced by 30% ($P<0.05$) but C4 remained unchanged after IIR (0.1342±0.07 vs 0.1420± 0.06, $P>0.05$). The apoptosis rate (15.4%±1.14%) of PMN was markedly reduced (3.5%±0.53%) following IIR ($P<0.05$) accompanied by increased contents of IL-1 and NF-κB. Moreover, CRP was also significantly elevated after IIR (4.33±1.13 mg/L vs 17.73±0.86 mg/L; $P<0.01$). Following IIR, complements are activated through the alternative pathway. Complement activation fragments can inhibit the apoptosis of PMN and elevate the expressions of acute phase inflammatory proteins including CRP and IL-1, which promotes the inflammation cascade and facilitates the occurrence of MODS.

Keywords: intestinal ischemia-reperfusion, macaque, polymorphonuclear cells, multiple organ dysfunction syndrome

Kratak sadržaj: Aktivacija komplemenata predstavlja ključnu komponentu inflamacijske kaskade. U ovoj studiji, kod makake majmuna izazvana je intestinalna ishemija-reperfuzija (IIR) a zatim su posle IIR istraživani putevi aktivacije komplemenata u okviru sindroma višestruke disfunkcije organa, što može doneti dokaze o mehanizmima endogene zaštite u sistemskom inflamacijskom odgovoru. IIR je izazvana stezanjem gornje mezenterične arterije i prekidanjem stiska kod pet makake majmuna. *Immunization rate* nefelometrija i detekcija totalnog komplementa CH50 primenjene su za merenje serumske koncentracije C3, C4, C-reaktivnog proteina (CRP) i totalnih komplemenata. Sprovedena je imunocitohemija radi utvrđivanja sadržaja IL-1 i NF-κB u polimorfonuklearnim ćelijama (PMN). Stopa apoptoze PMN izmerena je pomoću protočne citometrije. Posle 24 časa od IIR, sadržaj totalnog komplementa (106,6±18,07 U/mL) bio je snižen na 62,1±9,52 U/mL ($P<0,05$). Pored toga, C3 je bio snižen za 30% ($p<0,05$), ali je C4 ostao nepromenjen posle IIR (0,1342±0,07 prema 0,1420±0,06, $P>0,05$). Stopa apoptoze PMN (15,4%±1,14%) bila je značajno niža (3,5%±0,53%) posle IIR ($P<0,05$) uz povećan sadržaj IL-1 i NF-κB. štaviše, CRP je bio značajno povišen posle IIR (4,33±1,13 mg/L prema 17,73±0,86 mg/L; $P<0,01$). Posle IIR, komplementi su aktivirani preko alternativnog puta. Fragmenti aktivacije komplemenata mogu sprečiti apoptozu PMN i pojačati ekspresiju inflamacijskih proteina akutne faze, uključujući CRP i IL-1, što podstiče inflamacijsku kaskadu i olakšava nastanak sindroma višestruke disfunkcije organa.

Ključne reči: intestinalna ishemija-reperfuzija, makake, polimorfonuklearne ćelije, sindrom višestruke disfunkcije organa

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Introduction

Local trauma and infection may result in systemic inflammatory response which has the possibility to progress into multiple organ dysfunction syndrome (MODS). Except for polymorphonuclear neutrophils (PMN), the activation of the complement

system is also involved in the spread of local inflammation to the whole body. Complements are important for the defense against pathogen invasion, and critical components of natural immunity and acquired immunity. Studies have confirmed that complements participate in numerous defense reactions and immune regulation, and also mediate the immune pathological damage. They have been regarded as a biologically important immune response system and a response amplification system. However, overactivation of complements may result in MODS (1–2).

Complements can be activated in three pathways: the classic pathway, alternative pathway and mannose-binding lectin pathway (3). When inflammation of any cause spreads to the whole body, complements may be activated in different pathways. Intestinal ischemia-reperfusion (IIR) has been regarded as a critical pathophysiological process causing MODS following trauma, stress and infection (4). In this process, the pathways in which complements are activated and the correlation between complement activation and PMN are largely unknown. In the present study, IIR was introduced to macaques, and the serum concentrations of complements, circulating immune complexes (CIC) and related cytokines were determined, which may elucidate the correlation between the complement activation pathway and PMN and provide evidence for the regulation of the inflammation cascade.

Materials and Methods

Reagents

Percoll separation buffer (Pharmacia, USA), RPMI 1640 (GIBCO, USA), Annexin V FITC/PI (Jingmei China), IL-1 and NF-κB immunohistochemistry kit (Boster China), and the compound dimethylaniline thiazide (Veterinary Research Institute, Quartermaster University of PLA, China) were used in the present study.

Animal experiment

Healthy adult macaques weighing 7.2±0.6 kg were purchased from Chengdu Wildlife World. All animals were quarantined in Wildlife World and the investigators received training at Sichuan University and were qualified to perform animal experiments.

Animal processing

Animals underwent food deprivation for 12 h and water deprivation for 2 h. The macaques were housed at 20–22 °C under a 12 h:12 h light/dark cycle. Five healthy macaques were anesthetized intramuscularly with the compound dimethylaniline thiazide (0.2±0.1 mL/kg) and anesthesia was maintained with intravenous diazepam (0.16±0.09 mg/kg/h). Following skin sterilization, a middle line incision was made in the abdomen and the abdominal cavity was exposed. Superior mesenteric artery was exposed and clamped for 1 h, followed by the release of clamp for reperfusion. At 1 h after reperfusion, 0.9% saline was intravenously transfused at 0.1–0.2 mL/kg/min, and 20 g of glucose were administered within 24 h after reperfusion. The vital signs were monitored. At 24 h after IIR, the small intestine, heart, liver, kidney, lung and brain were collected for histological examination.

Detection of complements by the kinetic rate nephelometry method

Peripheral venous blood (2.5 mL) was drawn before IIR and at 2, 6, and 24 h after IIR and the serum collected. The C3 and C4 contents were determined with a kinetic rate nephelometer (Beckman Coulter, USA) and corresponding reagents.

Detection of total complements by the CH_{50} method

The alsever's solution anti-coagulated sheep red blood cells were rinsed in normal saline thrice and centrifuged at 400×g for 20 min. The condensed cell (0.2 mL) was mixed in barbital buffer (9.8 mL) to prepare 2% sheep red blood cell suspension. Once 1:2000 hemolysin of equal volume was added, the mixture was incubated at 37 °C for 30 min. The fresh serum was diluted to different concentrations and then used to react with sensitized sheep red blood cells. A minimal serum which produced 50% hemolysis served as an endpoint, and the total hemolytic activity of complements was measured. Standard tubes for hemolysis detection: 1% sheep red blood cell suspension was mixed in 4 mL of distilled water, which was regarded as complete hemolysis mixture; complete hemolysis mixture (2 mL) was mixed in 2 mL of buffer, which was regarded as 50% hemolysis mixture. The mixture was diluted to 1:4000 when the content of hemolysin was 1 U/mL. Complement unit (U/mL) = 1/serum volume × dilution (5).

Detection of CIC concentration

The CIC was determined using the polyethylene glycol (PEG) precipitation method in a Beckman Coulter DU 8 UV spectrophotometer (Beckman Coulter, USA) (6). In brief, 0.2 mL of serum was mixed in 1.8 mL of PEG (37.5 g/L) followed by incubation at 4 °C for 1 h and subsequent centrifugation at 600 ×g for 15 min. Supernatant was removed and sediments were washed in 37.5 g/L PEG once followed by centrifugation. The supernatant was removed and sediments were mixed in 3 mL of 0.1 mol/L NaOH. Then, the optical density was measured at 340 nm.

Detection of C reactive protein (CRP) concentration by kinetic rate nephelometry

Peripheral blood (2.5 mL) was collected before IIR and at 24 h after IIR and serum was collected by centrifugation. Kinetic rate nephelometer (Beckman Coulter, USA) and corresponding reagents were employed to detect the C-reactive protein.

Detection of IL-1 and NF-κB by immunocytochemistry

PMN was separated from anti-coagulated blood by using Percoll separation solution and smeared followed by fixation in 4% paraformaldehyde for 30 min. Then, cells were treated with H_2O_2 at room temperature for 10 min to inactivate endogenous peroxidase. After washing in distilled water thrice, cells were blocked in 5% BSA at room temperature for 20 min. Then, these PMNs were treated with rabbit anti-human IL-1 or NF-κB at 37 °C for 1 h. After washing in PBS (pH7.4) thrice (2 min each), cells were treated with biotin conjugated goat anti-rabbit IgG at 37 °C for 20 min. After washing in PBS (pH 7.4) four times (5 min each), visualization was done with a DAB kit. One drop of solution A, B and C was added to 1 mL of distilled water followed by mixing. Then, this mixture was added to cells, followed by incubation at room temperature for about 20 min. After washing in distilled water, counterstaining was done with hematoxylin, followed by dehydration and mounting. Cells were observed under a light microscope. Positive cells had yellowish brown granules in the cytoplasm or nucleus.

Isolation and purification of PMNs by Percoll discontinuous density gradient centrifugation

The separation solution consists of 5 mL of 72% Percoll (low) and 5 mL of 63% Percoll (up). In brief, peripheral blood (5 mL) was collected before IIR and at 2, 6 and 24 h after IIR and anti-coagulated with heparin. Then, the anti-coagulated blood was gently added to Percoll solution followed by centrifugation at 600×g for 20 min. The residual red blood cells were lysed in isotonic ammonium chloride solution (155 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$ and 0.1 mmol/L EDTA). The PMNs were washed in Hanks solution twice and then suspended in RPMI 1640 medium containing 10% fetal calf serum. Cells were observed under a light microscope. Trypan blue staining was performed to detect cell viability. The cell density was adjusted to 5×10^5/mL.

Detection of PMN apoptosis rate by flow cytometry

Blood was collected before IIR and at 2, 6 and 24 h after IIR, followed by PMN separation. Then, these PMNs were washed in PBS twice, followed by centrifugation and removal of supernatant. PMNs were mixed in 100 μL of buffer, followed by addition of 5 μL of Annexin V and 10 μL of PI and subsequent incubation at room temperature in the dark for 15 min. Cells were then mixed in 400 μL of PBS and subjected to flow cytometry (ELITE ESP; Coulter, USA).

Statistical analysis

Data were expressed as mean ± standard deviation ($\bar{x}\pm SD$). Apoptosis rate before and after IIR was compared with the analysis of variance and the remaining parameters were compared with the t test. Statistical analysis was performed with SPSS version 13.0.

Results

Contents of C3 and C4

At 2 h after IIR, the serum content of C3 was markedly reduced ($P<0.05$) and still remained at a low concentration even after reperfusion had been performed. At 2 h after IIR, the content of C4 was slightly reduced when compared with that before IIR ($P>0.05$). In addition, the C4 content gradually returned to the concentration before IIR over time. The amount of total complements reduced progressively after IIR, and was markedly lower at 6 h after IIR than before IIR ($P<0.05$) (*Table I*).

Measurement of CIC and CRP

The CIC remained unchanged after IIR ($P>0.05$), but the CRP concentration was significantly increased

Table I Serum complement concentrations before and after IIR.

Time point	C_3 (g/L)	C_4 (g/L)	Total complement (U/mL)
Before IIR	0.9992±0.14	0.1342±0.07	106.6±18.07
After IIR			
2 h	0.8662±0.09*	0.1250±0.05**	93.32±9.14
6 h	0.7984±0.16*	0.1494±0.06**	76.76±9.49*
24 h	0.7024±0.18*	0.1420±0.06**	62.16±9.52*

*P<0.05 and **P>0.05 vs before IIR

Table II CIC and CRP before and after IIR.

Time point	CIC (O.D)	CRP (mg/L)
Before IIR	0.013±0.005	4.33±1.31
After IIR	0.030±0.01**	17.73±0.86*

*P<0.05 and **P>0.05 vs before IIR

after IIR when compared with that before IIR ($P<0.01$) (*Table II*).

NF-κB expression in PMNs

Cells positive for NF-κB were yellowish brown and the positive granules were located in the cytoplasm. Before IIR, PMNs were negative for NF-κB, and NF-κB expression was significantly increased after IIR (*Figure 1*).

IL-1 expression in PMN

Immunocytochemistry showed a few PMN were positive for IL-1 before IIR (brown granules in the cytoplasm). After IIR, the number of PMNs positive for IL-1 was significantly increased (*Figure 2*).

Apoptosis rate of PMN

Flow cytometry showed the apoptosis rate of PMN in peripheral blood was dramatically reduced at 2, 6, and 24 h after IIR when compared with that before IIR (*Table III*). The apoptosis rate before IIR ($15.4\pm1.41\%$) was reduced to $3.5\pm0.53\%$ ($P<0.05$).

Complement and CRP in isolated hepatocytes

The hepatocytes were isolated and cultured. The concentrations of complements and CRP in the hepatocytes collected from macaques undergoing IIR were compared with those from healthy animals. Following IIR, the concentrations of C3, C4 and CRP were 0.0712 ± 0.028 g/L, 0.0173 ± 0.0013 g/L and

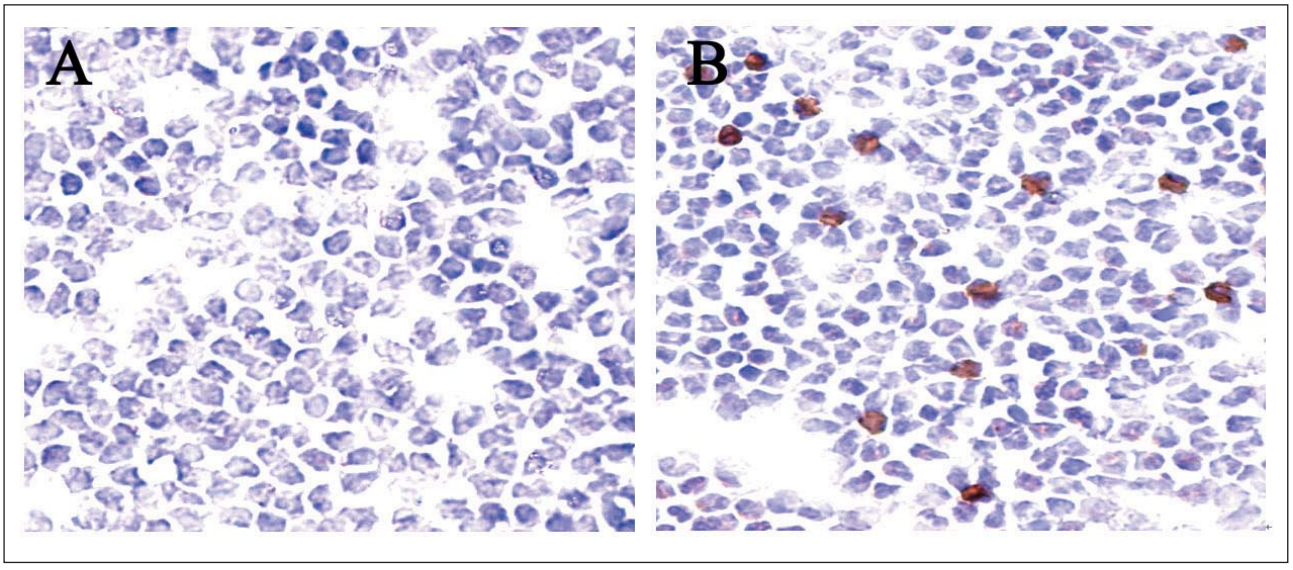

Figure 1 NF-κB expression in PMNs (immunocytochemistry, 100×). A, Before IIR; B, after IIR.

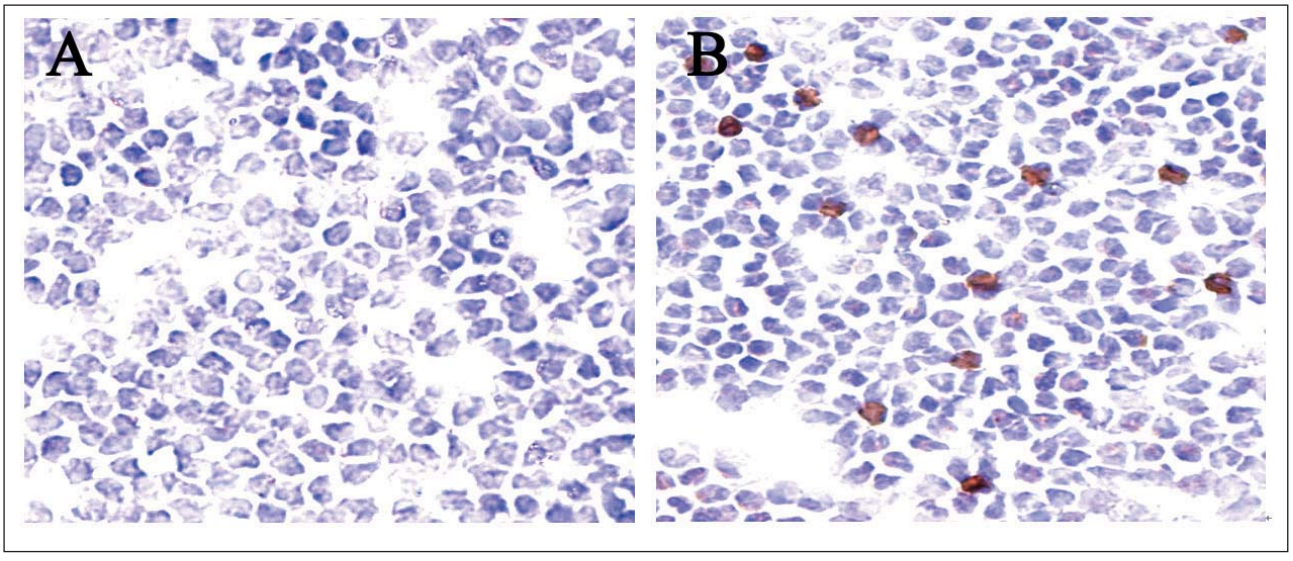

Figure 2 IL-1 expression in PMN (immunocytochemistry, 200×). A, Before IIR; B, after IIR.

Table III Apoptosis rate of PMN during MODS.

Group	Apoptosis rate of PMN (%)
Before ischemia	15.4±1.41
2 h after reperfusion	11.0±1.02*
6 h after reperfusion	7.1±0.54*
24 h after reperfusion	3.5±0.53**

*P<0.05 and **P<0.01 vs before IIR

Table IV Effect of IIR on the generation of complements and CRP in hepatocytes.

Group	C_3 (g/L)	C_4 (g/L)	CRP (mg/L)
Normal hepatocytes	0.0972±0.067	0.02157±0.0084	3.32±2.19
Hepatocytes from IIR animals	0.0712±0.028*	0.0173±0.0013*	2.8640±1.89*

*P>0.05 vs normal hepatocytes

2.8640±1.89 g/L, respectively. In the hepatocytes from healthy animals, the concentrations of C3, C4 and CRP were 0.0972±0.067 g/L, 0.02157± 0.0084 g/L and 3.32±2.19 g/L, respectively. There were no marked differences in the concentrations of C3, C4 and CRP between the hepatocytes collected from IIR animals and healthy animals (*Table IV*).

Histological examinations of major organs at 24 h after IIR

At 24 h after IIR, MODS developed in all macaques undergoing IIR. Inflammatory injury was present in the intestine, lung, liver and kidney at histological examination.

Discussion

Following stimulation by numerous pathogenic factors, inflammatory response and immune response may be present. Under normal conditions, these responses are moderate and protective. Once over-responses are present or the responses last for a long time, these responses may finally result in MODS. A lot of studies have demonstrated complement activation as one of the mechanisms of excessive inflammatory response (7–9). Some biologically active fragments produced in complement activation (C_{3a}, C_{5a}, $C_{5b\text{-}9}$) have been considered as risk factors of MODS, and complement inhibitors can block the enzymatic reaction of C_3 and C_5, reducing the injury during MODS (10).

Complements are a large restrictive protein cleavage system and consist of more than 30 proteins which exist in the form of an inactive zymogen in peripheral blood. The complement activation may induce a cascade reaction to form a membrane attack complex leading to cell lysis. Complements are not only involved in non-specific immune response but closely related to specific immune response. Complement activation is initiated once the antigen-antibody complex binds to C_{1q}, and this pathway is the first one identified in complement activation and known as the classic pathway. Except for the C_1, C and C_2 dependent pathway, C_3, B factor, D factor and P factor can lead to the activation of the C_5–C_9 membrane attack complex, which is also known as the alternative pathway of complement activation. In the complement system, the amount of C_3 and C_4 is the highest. Both complements are the intersections between two pathways and the key of biological functions of the complement system. C_4 is involved in the classic pathway, but C_3 acts in both the classic and alternative pathways (9). Following IIR, the intestinal barrier is damaged. However, the specific pathway in which complements are activated during MODS following IIR is still unknown. Our findings suggested C_3 was obviously consumed following IIR, but C_4 remained unchanged. In addition, CIC was also maintained at a stable concentration. This indicates that the alternative pathway, but not the classic pathway, is involved in the complement activation and inflammation cascade. It has been found that IIR can cause inflammatory injury to the liver. In our study, C_3 and C_4 were also measured from the hepatocytes collected from animals undergoing IIR. Results revealed no marked difference in both complements between the normal hepatocytes and those from IIR animals. This suggests the complement synthesis in the hepatocytes is intact, and the reduction of complements is attributed to their consumption following IIR.

In addition, there is a positive feedback loop in the alternative pathway of complement activation, and this loop is also an important mechanism of the complement activation cascade. The activated complements (C_{3a} and C_{5a}) can inhibit the apoptosis of neutrophils (11–13), prolong the survival of PMN and promote the release of elastase, collagenase, cathepsin and inflammatory products from neutrophils. Our previous study showed the survival of PMN of macaques undergoing IIR was prolonged, which was related to the reduction of circulating somatostatin. Results of the present study further demonstrated that the prolongation of PMN survival was also correlated with complement activation in an alternative pathway dependent manner. In the PMN with delayed apoptosis, the activity of NF-κB, a critical transcription factor, was significantly increased, which increased the expressions of acute phase inflammatory proteins including CRP and IL-1. IL-1 can stimulate adjacent endothelial cells and activate NF-κB, resulting in the

production of more IL-1 (14). IL-1 can inhibit the apoptosis of PMNs and recruit and activate neutropils as a chemokine leading to degranulation of PMNs. The ROS produced by PMNs also activate complements (12). There is a mutual activation of complements and PMNs, in which ROS play an important role, which form a positive feedback loop leading to the spread of inflammation. In addition, the activated complements (C3a and C5a) can also induce respiratory burst in PMNs to produce ROS, resulting in the activation of complements (15, 16), which increases tissue damage. Following IIR, complements were activated in the alternative pathway, which was characterized by an inflammation cascade, resulting in MODS in all animals undergoing IIR in the present study.

It has been regarded that the occurrence and development of MODS are closely related to both the natural and acquired immunity (17). The present study investigated the pathway in which complements were activated in IIR. Our findings indicated the occurrence was mainly related to the natural immunity. In the acquired immunity, complements are activated in the classic pathway in which there is no characteristic inflammation amplification and the tissue injury is also focal. Our findings further demonstrated that, after IIR, the CIC remained unchanged, suggesting the classic pathway of complement activation is not activated and the humoral immunity is also stable. These findings indicate short term deficiency of the acquired immunity may result in subsequent severe and refractory infection. In the present study, macaques were recruited because their physiology is similar to that of humans. Thus, our findings may provide evidence for the clinical treatment of MODS.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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