ORIGINAL RESEARCH

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Role of spleen-derived CD11b+Gr-1+ cells in sepsis-induced acute kidney injury

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Abstract

Purpose: CD11b+Gr-1+ cells play a key role in inflammation and the purpose of this study was to determine whether splenic CD11b+Gr-1+ cells are mobilized to the kidney and lead to acute kidney injury during sepsis.

Methods: The sepsis model was generated via cecum ligation and puncture (CLP). The mice were randomly distributed into control, sham operated, CLP and CLP+splenectomy (CLPS) groups (n=5–10/group). The percentage of CD11b+Gr-1+ cells in circulating, bone marrow and spleen were determined. Plasma concentrations of interleukin-6, interleukin-1 β , creatinine (Cr) and neutrophil gelatinase-associated lipocalin were measured. CD11b+Gr-1+ cells were detected by immunofluorescence and qRT-PCR. Hematoxylin-eosin (HE) and periodic acid-Schiff (PAS) staining and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) were performed. Expression of mammalian target of rapamycin (mTOR), hypoxia-inducible factor-1 α (HIF-1 α) and cleaved caspase-3 was measured.

Results: The percentage of CD11b+Gr-1+ cells in blood was significantly higher in the CLP group and lower in CLPS group. CD11b+Gr-1+ cells in the spleen were significantly lower in the CLP group. In the CLP group, the plasma concentrations of interleukin-6, interleukin-1 β , Cr and neutrophil gelatinase-associated lipocalin were higher. The expression of Gr-1 and CD11b were higher in CLP. The CD11b+Gr-1+ cells were detected in the kidneys of the CLP group. HE, PAS and TUNEL showed inflammatory cell infiltration and cell apoptosis in CLP. Western blot indicated dephosphorylation of mTOR, down-expression of HIF-1 α and increased expression of cleaved caspase-3 in sepsis kidney.

Conclusion: Splenic CD11b+Gr-1+ cells migrated to the kidney in sepsis, which led to acute kidney injury via the inhibition of mTOR/HIF-1 α .

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Introduction

Sepsis is an important public health problem that causes high morbidity around the world. The most common reason for acute kidney injury (AKI) is sepsis [1]. Studies revealed that sepsis is a key risk factor of AKI [2-4]; however, the mechanism of sepsis-induced AKI is still largely unknown.

Numerous pieces of evidence have shown the progression of sepsis-induced AKI, including identification of a variety of factors involved in this process. Inflammation and cell apoptosis play key roles in the progression of sepsis-induced AKI [5]. Unlike in the ischemia/reperfusion model of AKI, cell apoptosis was a major pathological change involved in sepsis-induced AKI. CD11b+Gr-1⁺ myeloid-derived suppressor cells (MDSCs) were found to be key in AKI [6]. Inflammatory cell infiltration and release of pro-inflammation cytokines were the main pathways in sepsis-induced AKI [7,8]. However, the detailed mechanism of sepsis-induced AKI is still not clear. Bone marrow-derived CD11b+Gr-1⁺ cells were considered as a major cause in tissue damage and inflammatory reaction; however, the role of spleen-derived CD11b+Gr-1⁺ cells in sepsis-induced AKI remains unknown.

Accordingly, we designed this study to determine if the spleen-derived CD11b+Gr-1⁺ MDSCs act on sepsis-induced AKI. Furthermore, the molecular mechanism of sepsis-induced AKI was studied.

Methods

Animals and sepsis model

Sepsis model was generated in male C57BL/6J mice (8–10 weeks old, weight: 20–25 g, purchased from Qing Long Shan animal breeding farm, Nanjing, China) via cecum ligation and puncture (CLP) in the specific pathogen-free animal house in Key Laboratory of Non-coding RNA Transformation Research of Anhui Higher Education Institution (Wann Nan Medical College). This animal study was approved by the Yi Ji Shan Hospital Animal Care Committee (Y201803).

The mice were randomly distributed into four groups: control non-operated (control, n=5), control operated (Sham, n=5), CLP (n=10) and CLP+splenectomy (CLPS, n=10). Sepsis was induced by CLP as described previously [9]. Briefly, fed mice were anesthetized intraperitoneally (i.p.) with 1% sodium pentobarbital (50 mg/kg). Under aseptic conditions, a 1 cm midline laparotomy was performed to allow the exposure of the cecum. The cecum was ligated with a 5-0 silk suture at its base, below the ileocecal valve, without causing bowel obstruction. The cecum was then

punctured one single time (crossing both intestine walls) with a 26-gauge needle. The cecum was ligated and placed back in the abdominal cavity, and the peritoneal wall and skin incision were sutured closed. Sham-operated mice underwent a similar procedure, but the cecum was not ligated or punctured. The CLPS mice underwent the same procedure as for CLP after splenectomy. Immediately after surgery, all mice received 1 ml of sterile saline subcutaneously. Chow pellets were kept inside the cage after the CLP procedure for the entire period of the experiment. No antibiotics or anti-inflammatory was administered. After 24 h of sepsis model established, all mice were sacrificed in deep anesthesia with overdose sodium pentobarbital (Sigma, USA) (130 mg/kg intraperitoneal injection).

Determination of plasma concentration of creatinine, neutrophil gelatinase-associated lipocalin, interleukin-6 and interleukin-1 β of animal model

After executing, mouse blood was collected with an ethylenediaminetetraacetic acid-coated tube. After centrifuged 500 g for 20 minutes, plasma was collected. Plasma concentration of creatinine (Cr), neutrophil gelatinase-associated lipocalin (NGAL), interleukin-6 (IL-6) and interleukin-1 β (IL-1 β) were analyzed by Mouse Cr ELISA Kit, Mouse NGAL ELISA Kit, Mouse IL-6 ELISA Kit and Mouse IL-1 β ELISA Kit, respectively (Enzyme-linked Biotechnology, China).

Flow cytometry

Bone marrow-derived cells from the femurs of euthanized mice were flushed with ice-cold PBS with 1% BSA and depleted of red blood cells (RBCs) using RBC lysing buffer (BD Biosciences, USA). Spleen was cut into small pieces, ground into a cell suspension and depleted of RBCs using RBC-lysing buffer (BD Biosciences) (except for CLPS). Total nucleated cells in peripheral blood were isolated after erythrocyte lysis. For FACS analysis, cell suspensions were stained with PE-conjugated anti-Mouse CD11b antibodies and FITC-conjugated anti-Mouse Gr-1 (BD Biosciences) for 1 h on ice. After washed with 1% BSA contained PBS. The percentage of CD11b+Gr-1⁺ cells was evaluated by multicolor flow cytometry using a flow cytometer (EPICS® ALTRA, Beckman Coulter, USA).

Morphological analysis

Two-micrometer thick sections were made for hematoxylin-eosin (HE) and periodic acid-Schiff (PAS) staining, using HE staining kit (Beyotime, China) and PAS staining kit (Nanjing KeyGen Biotech, China). Stained sections were

subsequently examined by light microscopy in a blinded manner.

TUNEL immunohistochemistry staining

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) was performed to detect apoptotic nuclei by using terminal deoxynucleotidyl transferase-mediated *in situ* fluorescein-conjugated, dUTP nick end-labelling technique according to the manufacturer's protocol (Roche, USA). The sections were incubated with mouse monoclonal antibody (Abcam, USA) to recognizing cardiac myosin heavy chain to specifically recognize apoptotic cardiomyocytes. The fluorescence staining was viewed with a confocal laser-scanning microscope. The number of apoptotic cells with TUNEL-positive nuclei was counted by two independent observers blinded to the treatment group and expressed as a percentage of total myocyte population.

Immunofluorescence staining

The frozen section of the kidney was made. The tissue section (2 μ m thick) was stained with PE-conjugated anti-mouse CD11b antibodies and FITC-conjugated anti-Mouse Gr-1 (BD Biosciences) for 1 h at room temperature to measure the expression of CD11b and Gr-1. The tissue was further stained with 4,6-diamidino-2-phenylindole (DAPI, Roche, Switzerland). After washing with phosphate-buffered saline three times, fluorescence microscopy (Olympus, Japan) was used to detect fluorescence.

mRNA expression of CD11b, Gr-1, IL-6 and IL-1 β in the myocardium of mice

mRNA levels of mouse kidney of various factors, including CD11b, Gr-1, IL-6 and IL-1 β , were detected by qRT-PCR. The mouse kidney was stored in Trizol (Axygen, USA) at -80°C. Total RNA was extracted as previously described and cDNA was synthesized using the PrimeScript™ RT reagent kit with gDNA Eraser (TAKARA, Japan). qRT-PCR was performed using CD11b primers (forward: CCATGACCTTCCAAGAGAATGC, Reverse: ACCGGCTTGTGCTGTAGTC), Gr-1 primers (Forward: TCATCCTTCTTGTGGTCCCTA, Reverse: AAGGGGCAGGTAGTTGTG), IL-6 (Forward: TAGTCCTTCTACCCCAATTTCC, Reverse: TTGGTCCTTAGCCACTCCTTC), IL-1 β (Forward: GCAACTGTTCTGAACTCAACT, Reverse: ATCTTTTGGGGTCCGTCAACT) and GAPDH (Forward: GCCTCAAGATCATCAGCAAT, Reverse: GGACTGTGGTCATGAGTCCT). After room temperature incubation (22°C, 30 min), hot start (94°C, 15 min), and 40–

42 cycles of PCR (94°C, 1 min; 52.5°C, 1 min; 72°C, 1 min), CD11b, Gr-1, IL-6 and IL-1 β mRNA expression was normalized to GAPDH and calculated as $2^{-\Delta\Delta C_t}$.

Western blot

For western blot analysis, the protein was extracted from mice kidney tissue using Tissue Protein Extraction Kit (Beyotime, China) according to the protocol provided by the manufacturer. Protein concentrations were measured using the BCA assay kit (Pierce, USA). Western blots of the protein samples were performed to detect the mammalian target of rapamycin (mTOR) and phosphorylated Ser²⁴⁴⁸-mTOR (P-mTOR) using rabbit monoclonal anti-mouse mTOR antibodies and rabbit monoclonal anti-mouse P-mTOR antibody (Cell Signaling Technology, USA). Cleaved caspase-3 and hypoxia-inducible factor-1 α (HIF-1 α) expression were detected by rabbit monoclonal anti-mouse cleaved caspase-3 (Abcam, UK) and rabbit anti-mouse HIF-1 α (Santa Cruz, UK). GAPDH was used as a loading control (KangCheng Bio-tech, China). After primary antibody incubation, the blots were incubated with the appropriate secondary horseradish peroxidase-conjugated mouse monoclonal anti-rabbit antibody (Boster, China). Each membrane was washed and then developed using the Super Signal chemiluminescent substrate (Pierce, USA).

Statistical analyses

For all experiments, the data were analyzed using one way ANOVA, and values are expressed as the means $\pm$ SEM. All statistical analyses were performed using SPSS software (SAS Institute Inc., USA); *p* values < 0.05 were considered to indicate significance.

Results

Percentage of CD11b⁺Gr-1⁺ cells in blood, spleen and bone marrow

All mice survived the surgery and post-surgical period. Compared with control, sham and CLPS, the percentage of CD11b⁺/Gr-1⁺ cells in blood was significantly higher in CLP group than in other groups (mean: 34.8 $\pm$ 1.1% vs 3.2 $\pm$ 0.2%, 2.8 $\pm$ 0.2%, and 21.7 $\pm$ 0.9%, respectively; *p* < 0.05). In the spleen, the percentage of CD11b⁺/Gr-1⁺ cells was significantly lower in CLP than in control and sham (2.0 $\pm$ 0.1% vs 4.1 $\pm$ 0.1% and 4.1 $\pm$ 0.2%, respectively; *p* < 0.05). In the bone marrow, the percentage of CD11b⁺/Gr-1⁺ cells in CLP was similar with the percentage of CD11b⁺/Gr-1⁺ cells in CLPS, control and sham (mean: 31.2 $\pm$ 1.0% vs 26.4 $\pm$ 1.8%, 31.3 $\pm$ 1.0% and 31.6 $\pm$ 0.7%, respectively; *p* > 0.05, Figure 1A-D).

FIGURE 1. Percentage of CD11b⁺Gr-1⁺ cells in blood, spleen and bone marrow.

A: Representative flow cytometry analyses of CD11b⁺Gr-1⁺ cells expression in blood, spleen and bone marrow in the four groups. The percentage of circulating CD11b⁺Gr-1⁺ cells in the sepsis group was higher than in the control, sham and CLPS groups; however, the percentage of splenic CD11b⁺Gr-1⁺ cells in the sepsis group was lower than in the control and sham groups. In the bone marrow, the percentage of CD11b⁺Gr-1⁺ cells in the four groups was similar.

B: Histogram showing the percentage of CD11b⁺Gr-1⁺ in blood.

C: Histogram showing the percentage of CD11b⁺Gr-1⁺ in the spleen.

D: Histogram showing the percentage of CD11b⁺Gr-1⁺ in bone marrow (n=5 for control and sham groups, n=10 for CLP and CLPS groups; **p*<0.05 vs control, sham and CLPS, # *p*>0.05 vs control, sham and CLPS).

Concentration of plasma of creatinine, NGAL, IL-6 and IL-1 β

The plasma concentration of Cr was significantly higher in CLP than in control, sham and CLPS ($85.0 \pm 5.2 \mu\text{mol/L}$ vs $41.6 \pm 3.4 \mu\text{mol/L}$, $41.4 \pm 3.7 \mu\text{mol/L}$ and $61.0 \pm 5.8 \mu\text{mol/L}$, respectively; $p < 0.05$). Plasma NGAL concentration was significantly higher in CLP than in control, sham and CLPS ($181.5 \pm 13.3 \text{ ng/mL}$ vs $30.0 \pm 1.1 \text{ ng/mL}$, $34.4 \pm 8.2 \text{ ng/mL}$ and $105.3 \pm 11.2 \text{ ng/mL}$, respectively; $p < 0.05$, Figure 2A-B).

Further, plasma concentration of IL-6 in CLP was higher than in control, sham and CLPS ($21.5 \pm 0.6 \text{ pg/mL}$ vs $4.7 \pm 0.3 \text{ pg/mL}$, $4.6 \pm 0.5 \text{ pg/mL}$ and $10.0 \pm 0.5 \text{ pg/mL}$, respectively; $p < 0.05$). As well, plasma concentration of IL-1 β in CLP was higher than in control, sham and CLPS ($27.3 \pm 1.5 \text{ pg/mL}$ vs $7.1 \pm 0.6 \text{ pg/mL}$, $7.3 \pm 1.2 \text{ pg/mL}$ and $15.2 \pm 1.0 \text{ pg/mL}$, respectively; $p < 0.05$, Figure 2C-D).

mRNA expression of CD11b, Gr-1, IL-6, and IL-1 β in kidney tissue

qRT-PCR indicated that in kidney tissue, the mRNA levels of CD11b and Gr-1 were higher in CLP than in control, sham and CLPS. As similar, in kidney tissue, the expression of IL-6 and IL-1 β was higher in CLP than in control, sham and CLPS ($p < 0.05$, Figure 2E-H).

Expression of CD11b and Gr-1 in kidney tissue and cell apoptosis analysis

The HE staining showed that more inflammatory cells accumulated in tubular interstitium in the CLP group compared with control and sham. A decreased number of inflammatory cells was observed in the CLPS group than in CLP. In the CLP group, PAS showed marked tubular interstitial damage, such as extracellular matrix overproduction, increased inflammatory cell infiltration, protein cast formation and tubular dilatation. In CLPS, the inflammatory cell infiltration and tubular interstitial damage were alleviated (Figure 3A). Inflammatory cell count in each group is shown in Figure 3B. TUNEL staining in kidney tissue showed that cell apoptosis was notably increased in the CLP group compared with control, sham ($67.8 \pm 4.0/\text{field}$ vs $2.0 \pm 1.4/\text{field}$ and $2.2 \pm 1.0/\text{field}$, respectively; $p < 0.05$). However, the number of apoptotic cells was decreased in CLPS compared with CLP ($22.3 \pm 2.8/\text{field}$ vs $67.8 \pm 4.0/\text{field}$, $p < 0.05$). No obvious change was detected between control and sham (Figure 3C and 3D). Immunofluorescence indicated that in the kidney tissue, CD11b+Gr-1+ cells tended to be higher in CLP than in control and sham. In the CLPS group, the number of CD11b+Gr-1+ cells was lower than in the CLP group (Figure 3E).

mTOR/HIF-1 α signal pathway participated in sepsis-induced acute kidney injury

In the CLP group, western blot showed that mTOR was dephosphorylated and the protein expression of HIF-1 α was down-regulated compared with control and sham groups. However, the expression of cleaved caspase-3 was up-regulated. In CLPS, the phosphorylation of mTOR was higher than in CLP. The expression of cleaved caspase-3 was down-regulated and compared to the CLP group, the expression of HIF-1 α was up-regulated in CLPS (Figure 4).

Discussion

In this study, we showed that splenic CD11b+Gr-1+ cells, rapidly mobilized to the circulation and to the kidney, were further associated with an inflammatory reaction that inhibited mTOR/HIF-1 α signal pathway and promoted cell apoptosis.

Previous studies have identified a population of immature myeloid cells, termed myeloid-derived suppressor cells (MDSCs). In mice, CD11b and Gr-1 were considered as markers of MDSCs. In humans, MDSCs are most commonly defined as CD14-CD11b+ cells [10]. The research on the biological behavior of MDSCs has focused on immunoregulation function, especially in inflammatory disease and cancer [11,12]. Inflammatory reaction was strongly correlated with CD11b+Gr-1+ cells. It had been shown that CD11b+Gr-1+ cells participated in sepsis. The MDSCs strongly contributed to T-cell dysfunction in patients with sepsis and were associated with the incidence of infections [13]. However, the role of MDSCs in sepsis and trauma was paradoxical [14]. A dramatic increase of MDSCs in sepsis suggested that MDSCs promote the progress of sepsis [15]. In contrast, some research suggested that the expansion of MDSCs during sepsis may actually be protective [16,17]. Our data showed that the plasma concentration of IL-6 and IL-1 β and the percentage of CD11b+Gr-1+ cells in circulating increased equally in the CLP group. Furthermore, the apoptotic cell number in the kidney in the CLP group was significantly higher than the other groups. This indicated that the inflammatory reaction caused by CD11b+Gr-1+ cells may play a key role in sepsis-induced acute kidney injury. However, further study in cell depletion mouse models are needed to confirm this effect of CD11b+Gr-1+ cells.

Various studies showed that bone marrow was rich in mononuclear cells, including CD11b+Gr-1+ cells [18,19]. In the mice AKI model, two types of bone marrow-derived CD11b+Gr-1+ cells were found to have regulated the progress of AKI [6]. In recent studies, the role of spleen-

FIGURE 2. Plasma concentration of Cr, NGAL, IL-6 and IL-1 β and mRNA expression of Gr-1, CD11b, IL-6 and IL-1 β in kidney tissue.

FIGURE 3. CD11b⁺Gr-1⁺ cells mobilized to the kidney in sepsis and were associated with tubular interstitial damage.

FIGURE 3 - E:

E) Representative immunofluorescence staining showed that in the CLP group, both CD11b and Gr-1 expressed in kidney tissue. In control and sham groups, CD11b and Gr-1 were negatively expressed in kidney tissue. In CLPS, the expression of CD11b and Gr-1 was lower than CLP (n=5 for control and sham, n=10 for CLP and CLPS; * $p < 0.05$ vs control, sham and CLPS).

derived CD11b⁺Gr-1⁺ cells was considered to be the primary factor involved in organ damage and inflammation [20,21]. However, whether spleen-derived CD11b⁺Gr-1⁺ cells played a part in sepsis-induced AKI is uncertain. In the present study, we suggest that in sepsis-induced AKI, spleen-derived CD11b⁺Gr-1⁺ cells are rapidly mobilized to circulation and kidney, where they activate inflammatory reaction and lead to organ damage. Furthermore, experiments in this CLPS mouse model confirmed that splenic CD11b⁺Gr-1⁺ cells were the main source of circulating CD11b⁺Gr-1⁺ cells in sepsis.

The mTOR signal pathway has been shown to have played a key role in cell proliferation, tissue injury and immunoregulation [22]. Activating mTOR promotes cell replication and repair of injury, which indicated that mTOR promoted cell replicative senescence [23,24]. However, in premature cell senescence, mTOR inhibited the oxidative stress-induced senescence [25-27]. Further, mTOR is a key molecular in the regulation of the differentiation of the CD11b⁺Gr-1⁺ cells and immunomodulatory in inflammatory

reaction [28]. The previous study showed that in AKI, mTOR signal inhibition regulated the induction of MDSCs and strengthening their immunosuppressive activity [6]. Activation of mTOR was associated with vasodilator and pro-inflammatory factor formation in LPS-induced hypotension and inflammation [29]. These results indicated that inhibition of mTOR protected tissue from injury. Nevertheless, like the controversial effect of mTOR in cell replicative senescence and premature senescence, the role of mTOR in organ damage is in doubt. The key role of mTOR may be to modulate the balance of pro-inflammatory and anti-inflammatory activities [28]. Our data suggest that in sepsis-induced AKI, the expression of phosphorylated mTOR was significantly down-regulated and further associated with the down-regulation of HIF-1 α and up-regulation of cleaved caspase-3, which indicated that the inhibition of mTOR was associated with cell apoptosis in sepsis-induced AKI. HIF-1 α was a component of the mTOR signal pathway and it has been shown that mTOR/HIF-1 α regulated the progress of glycolysis [30,31]. HIF-1 α

FIGURE 4. mTOR/HIF-1 α pathway in septic kidney tissue.

has been widely studied as a protective molecule, especially during myocardial ischemic injury [32,33]. Similarly, HIF-1 α induction during kidney reperfusion is a protective mechanism that has been implicated in normal renal tissue repair after ischemia/reperfusion [34]. HIF-1 α -modified adipose-derived stem cell implantation could provide advanced benefits in the protection against AKI [35].

In this study, we found that, in the sepsis state, after CD11b⁺Gr-1⁺ migration to the kidney the expression of HIF-1 α was inhibited; causing cell apoptosis. These data suggest that mTOR/HIF-1 α pathway may participate in sepsis-induced AKI. Further studies with the mTOR or HIF-1 α knock out mice may demonstrate the mechanism by which CD11b⁺Gr-1⁺ cells cause kidney injury in sepsis.

Limitations

There are two important limitations to this study. Firstly, the CD11b+Gr-1⁺ cells depletion mice model or mTOR and HIF-1 α knock out mice were not used; this reduced the reliability of the study. Secondly, further study is needed to determine if the changed phosphorylation mTOR protein expression affected the differentiation of CD11b+Gr-1⁺ cells.

Conclusion

In this sepsis-induced AKI mice model, spleen-derived CD11b+Gr-1⁺ cells rapidly migrated to the circulation and the kidney, which may be associated with sepsis-induced AKI. Furthermore, the mTOR/HIF-1 α signal pathway may play a key role in the progression of AKI.

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