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Nucleolin Mediates LPS-induced Expression of Inflammatory Mediators and Activation of Signaling Pathways*

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Summary: In this study, we investigated the effects of nucleolin on lipopolysaccharide (LPS)-induced activation of MAPK and NF-KappaB (NF-κB) signaling pathways and secretion of TNF-α, IL-1β and HMGB1 in THP-1 monocytes. Immunofluorescence assay and Western blotting were used to identify the nucleolin expression in cell membrane, cytoplasm and nucleus of THP-1 monocytes. Inactivation of nucleolin was induced by neutralizing antibody against nucleolin. THP-1 monocytes were pretreated with anti-nucleolin antibody for 1 h prior to LPS challenge. The irrelevant IgG group was used as control. Secretion of inflammatory mediators (TNF-α, IL-1β and HMGB1) and activation of MAPK and NF-κB/I-κB signaling pathways were examined to assess the effects of nucleolin on LPS-mediated inflammatory response. Nucleolin existed in cell membrane, cytoplasm and nucleus of THP-1 monocytes. Pretreatment of anti-nucleolin antibody significantly inhibited the LPS-induced secretion of TNF-α, IL-1β and HMGB1. P38, JNK, ERK and NF-κB subunit p65 inhibitors could significantly inhibit the secretion of IL-1β, TNF-α and HMGB1 induced by LPS. Moreover, the phosphorylation of p38, JNK, ERK and p65 (or nuclear translocation of p65) was significantly increased after LPS challenge. In contrast, pretreatment of anti-nucleolin antibody could significantly inhibit the LPS-induced phosphorylation of p38, JNK, ERK and p65 (or nuclear translocation of p65). However, the irrelevant IgG, as a negative control, had no effect on LPS-induced secretion of TNF-α and IL-1β and phosphorylation of p38, JNK, ERK and p65 (or nuclear translocation of p65). We demonstrated that nucleolin mediated the LPS-induced activation of MAPK and NF-κB signaling pathways, and regulated the secretion of inflammatory mediators (TNF-α, IL-1β and HMGB1).
Key words: nucleolin; THP-1 monocytes; lipopolysaccharide; MAPK; NF-κB signaling pathway; inflammatory mediators

As a type of phosphoric acid protein in eukaryotic cells, nucleolin (rRNA) is mainly involved in the ribosomal transcription, ribosome subunit assembly, maturation and so on[1–4]. In most cells, nucleolin is mainly expressed in the nucleus, but it can also exist in the cytoplasm in glycosylated or phosphorylated form. Recent studies have found that nucleolin is expressed on the surface of a variety of immune cells, tumor cells and vascular endothelial cells, it acts as receptors for many proteins, viruses and bacteria or co-receptors, and it is involved in the pathogenic microbial infection, inflammation, angiogenesis and other biological processes[5–8]. Other studies have confirmed that nucleolin expressed on the cell membrane of THP-1 monocytic cells can recognize polysaccharide in CD43 of early apoptotic cells and mediate phagocytosis of early apoptotic Jurkat cells[9].

Lipopolysaccharide (LPS), also known as an endotoxin, plays a dominant role in Gram-negative bacteria-induced systemic inflammatory response syndrome (SIRS). After binding to LPS binding protein (LBP) in serum, LPS/LBP complex interacts with several membrane receptors, such as CD14, toll-like receptor 2, 4 (TLR2, 4), triggering receptor expressed on myeloid cells (TREM-1) and macrophage scavenger receptor (MSR) on monocytes/macrophages and polymorphonuclear leukocytes. Such interactions subsequently activate MAPK and/or NF-KappaB (NF-κB) signaling pathways as well as their corresponding transcription factors, triggering the up-regulation of inflammatory mediators, such as tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), high mobility group box 1 (HMGB1) and so on[10–12]. However, it remains unclear whether nucleolin can affect the

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expression of LPS-induced inflammatory mediators and the activation of intracellular signaling pathways. Based on the general structures (polysaccharide fraction) of CD43 molecule and LPS, we inferred that nucleolin on the cell surface might recognize LPS. All of these findings indicated that nucleolin might be involved in mediating the expression of the inflammatory mediators induced by LPS and the activation of intracellular signaling pathways.

The present study aimed to investigate the roles of nucleolin in LPS-induced activation of MAPK and NF-κB signaling pathways and secretion of inflammatory mediators (TNF-α, IL-1β and HMGB1) in THP-1 monocytes. Human THP-1 cells were pretreated with anti-nucleolin antibody for 1 h prior to LPS challenge. Moreover, we also examined the secretion of inflammatory mediators (TNF-α, IL-1β and HMGB1) and activation of MAPK (p38, JNK and ERK) and NF-κB signaling (NF-κB subunit p65) pathways in order to assess the role of nucleolin in LPS-mediated inflammatory response.

1 MATERIALS AND METHODS

All experiments were performed under conditions specified by law and reviewed and approved by the ethics committee of the First Hospital of Changsha and carried out in accordance with the approved protocol.

1.1 Culture of THP-1 Cells and Experimental Groups

THP-1 cells at a density of 2×10^6 per bottle (T75) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL ampicillin and 100 μg/mL streptomycin at 37°C in an incubator containing 5% CO_2.

Cells were divided into three groups as follows: (1) LPS group, in which THP-1 cells were stimulated with LPS (1 mg/L) for various durations (no serum). The secretion of inflammatory mediators was determined at 0, 4, 12, 24 and 48 h, while the phosphorylation of p38, JNK, ERK and NF-κB subunit p65 was detected at 0, 30 and 60 min; (2) LPS+C23 Abs group, in which THP-1 cells were pretreated with 1 mg/L C23 Abs[13, 14] (mouse anti-C23 monoclonal antibody was purchased from Santa Cruz1, USA) for 1 h and then stimulated with 1 mg/L LPS for different durations (no serum); (3) LPS+IgG group (negative control group), in which THP-1 cells were pretreated with 1 mg/L IgG for 1 h and then stimulated with 1 mg/L LPS for different durations (no serum).

1.2 Immunocytochemistry

Cells were cultured on glass coverslips, fixed with 4% formaldehyde and permeabilized with 0.1% Triton X-100 at 4°C for 10 min. Coverslips were blocked with 2% BSA at room temperature for 1 h and incubated with mouse anti-C23, Fas (Santa Cruz Biotechnology, USA) or rabbit anti-NF-κB subunit p65 mAbs (Santa Cruz Biotechnology, USA), followed by incubation with Cy3 or FITC-conjugated sheep anti-mouse IgG or FITC-conjugated sheep anti-rabbit IgG (BosterBiotech, USA), respectively. Nuclear morphology was analyzed with Hoechst 33258 staining. Cells were washed with PBS containing 0.2% BSA three times (3 min each wash) between all incubation steps. Images were acquired with a fluorescence microscope (ECLIPSE 80i, Nikon, Japan).

1.3 Extraction of Cell Membrane and Nuclear Protein Fractions

Extraction of cell membrane and nuclear proteins was conducted according to the Pierce eukaryotic cell membrane and nuclear protein extraction kits (Pierce, USA), respectively. The components of cell membrane, cytoplasm and nuclear proteins were collected and stored at –70°C.

1.4 Western Blotting Analysis

Protein concentrations were determined by Bradford assay. Albumin was used to establish the standard curve. Equal amounts of whole-cell lysates were subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PVDF membranes. Blots were incubated with primary Abs specific for phospho-p38 (T180/Y182, R&D Cell Signaling Technology, USA), phospho-ERK1/2 (Thr202/Tyr204, Cell Signaling Technology, USA), phospho-JNK1/2 (Thr183/Tyr185, Cell Signaling Technology, USA), NF-κB subunit p65 (Santa Cruz Biotechnology, USA), GAPDH (Jingmei Biotechnology, China) or proliferating cell nuclear antigen (PCNA, BD Biosciences, USA) at 25°C for 2 h. Subsequently, the bolts were incubated with peroxidase-conjugated secondary Abs (Boster Biotech) at 25°C for 1 h. The immunoreactive signals were visualized with chemiluminescence (Beyotime Institute of Biotechnology, China) according to the manufacturer's instructions and quantitated by Scion image software.

1.5 Enzyme Linked Immunosorbent Assay

Levels of IL-1β, TNF-α and HMGB1 in the culture medium were determined by commercially available ELISA kits for TNF-α, IL-1β (Cat. No: EK0392, ab181421, Wuhan Boster Company, China) and HMGB1 (Cat. No: F1062, Shanghai Xitang Biotechnology Co., Ltd., China) according to the manufacturer's instructions. Cell culture supernatant was collected 4, 12, 24 and 48 h after LPS challenge, and 200 μL supernatant was added into a 96-well plate coated with the corresponding mAbs of inflammatory factors and incubated at 37°C for 2 h. Subsequently, liquid was removed, and 200 μL enzyme-labeled secondary antibody was added into each well, followed by incubation at 37°C for 2 h. Wells were washed with TBS (0.01 mmol/L) three times (3 min each wash) and then incubated with substrate solution (200 μL) at

25°C for 30 min. Next, the stop solution was added to terminate the reaction. The absorbance (*A*) value was measured by an enzyme-labeled instrument (Startfax 2100, USA) at a wavelength of 450 nm. The sample concentration was calculated by linear equation.

1.6 Statistical analysis

Data were expressed as means±SEM based on at least three independent experiments. Statistical analysis was performed by one-way ANOVA (LSD test) for multiple testing. The differences were considered as statistically significant if $P<0.05$.

2 RESULTS

2.1 Nucleolin in Cell Membrane, Cytoplasm and Nucleus of THP-1 Monocytes

Immunofluorescence analysis showed that nucleolin was localized on the cell surface of THP-1 monocytes. Similarly, Fas was localized on the surface of the cell membrane in the positive control group (fig. 1). Western blotting analysis showed that nucleolin existed in cell membrane, cytoplasm and nucleus, while Fas was only expressed in cell membrane, and GAPDH was used as the internal control of cytoplasmic protein, indicating no obvious contamination between the components (fig. 2). These findings suggested that nucleolin could be expressed on the cell membrane and in the cytoplasm or nucleus of THP-1 monocytes.

2.2 Role of Nucleolin in LPS-induced Expression of Inflammatory Mediators in THP-1 Monocytes

Under normal culture conditions, THP-1 cells secreted a small amount of TNF-α, IL-1β and HMGB1. After THP-1 cells were stimulated with LPS for 0, 4, 8 and 12 h, the LPS-induced secretion of TNF-α and IL-1β was increased in a time-dependent manner. Similarly, after THP-1 cells were stimulated with LPS for 0, 12, 24 and 48 h, the LPS-induced secretion of HMGB1 was increased in a time-dependent manner. Pretreatment of anti-nucleolin antibody significantly inhibited such LPS-induced secretion of TNF-α, IL-1β and HMGB1. However, the irrelevant IgG, which was used as a negative control, had no effect on LPS-induced secretion of TNF-α, IL-1β and HMGB1 (fig. 3).

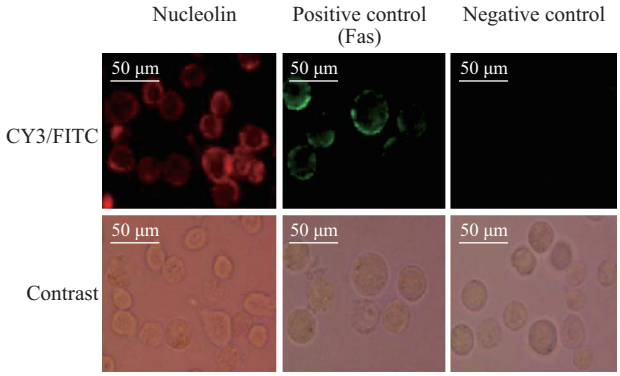

Fig. 1 Localization of nucleolin in THP-1 monocytes
Fas: transmembrane receptor that belongs to TNF family as positive control. Contrast: same visual field under contrast phase; CY3: expression of nucleolin with CY3-labeled Abs (red); FITC: expression of fas with FITC-labeled Abs (green)

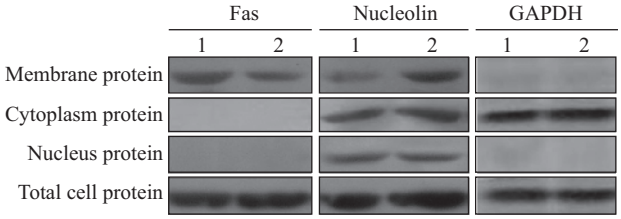

Fig. 2 Subcellular distributions of nucleolin using Western blotting analysis
samples 1 and 2: two samples of cells derived from the different culture flasks; membrane protein: membrane fractions of THP-1; cytoplasm protein: cytoplasmic fractions of THP-1; nucleus protein: nucleus fractions of THP-1; total cell protein: whole cell lysates of THP-1

2.3 Role of MAPK and NF-κB Signaling Pathways in LPS-induced Inflammatory Mediator Secretion with Specific Inhibitors

We observed the causative role of MAPK and NF-κB signaling pathways in LPS-induced IL-1β, TNF-α and HMGB1 secretion with specific inhibitors. After

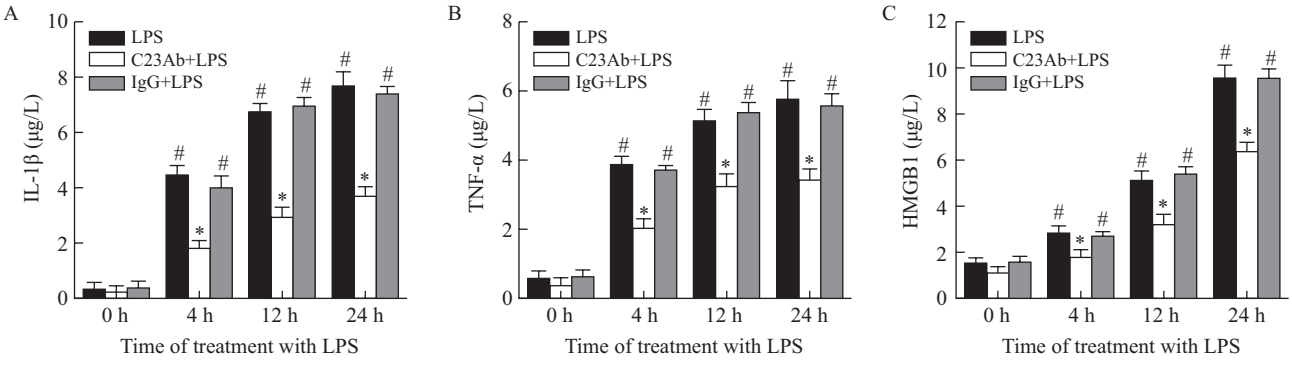

Fig. 3 ELISA showed the inhibition of the effect of pretreatment of anti-nucleolin antibody on the secretion of TNF-α, IL-1β and HMGB1 in THP-1 monocytes
A: secretion of IL-1β; B: secretion of TNF-α; C: secretion of HMGB1. Data were expressed as mean±SD, $n=5$; $^{*}P<0.05$ *vs.* the LPS group and IgG+LPS group, $^{\#}P<0.05$ *vs.* the 0 h group

THP-1 cells were stimulated with LPS for 48 h, the LPS-induced secretion of IL-1β, TNF-α and HMGB1 was significantly increased. However, p38, JNK, ERK and p65 inhibitors could significantly inhibit the secretion of IL-1β, TNF-α and HMGB1 induced by LPS (fig. 4).

2.4 Role of Nucleolin in LPS-induced Activation of Signaling Pathways in THP-1 Monocytes

Results showed that the phosphorylation of p38, JNK and ERK was significantly increased at 30 min and 60 min after LPS challenge. Pretreatment of anti-nucleolin antibody could significantly inhibit the phosphorylation of p38, JNK and ERK. However, the irrelevant IgG had no effect on LPS-induced phosphorylation of p38, JNK and ERK. In addition to MAPK activation, the NF-κB p65 signal transduction pathways are also involved in signaling pathways of inflammatory response. The results showed that little p65 was distributed in the nucleus of THP-1 cells without LPS stimulation. After LPS challenge for 30 min, the distribution of p65 in the nucleus was increased. After LPS challenge for 60 min, the distribution of p65 in the nucleus was significantly increased. Pretreatment of anti-nucleolin antibody had significantly inhibitory effect on LPS-induced phosphorylation of NF-κB p65 (fig. 5). Meanwhile, immunofluorescence analysis showed that nuclear translocation of p65, the transcriptionally active component of the NF-κB complex, induced by LPS was strongly inhibited by pretreatment of anti-nucleolin antibody (fig. 6).

3 DISCUSSION

A large number of studies have shown that nucleolin is expressed on the cell surface of a variety of cells, and it participates in bacterial and viral invasions of host cells, inflammatory immune response, angiogenesis, inhibition of tumor cell proliferation, and other physiological processes[15–19]. However, many investigators have demonstrated that LPS-induced inflammatory response can't be completely abolished by functional deficiency of CD14, TLR2, 4 and LBP. Therefore, LPS/LBP-CD14-TLR2, 4 is not the only pathway during LPS-induced inflammation[20, 21]. These results suggest that there must be other LPS receptors on the membrane of inflammatory cells. According to the current knowledge of cell-surface nucleolin, we postulated that cell-surface nucleolin might serve as a novel receptor of LPS, and it was involved in the LPS-mediated inflammation. In the present study, we found that nucleolin existed on the surface of THP-1 cell membrane by indirect immunofluorescence and immunoblotting. This finding laid a foundation for studying the role of nucleolin in the expression and secretion of inflammatory mediators and activation of signaling pathways in THP-1 cells.

At present, it is generally believed that TNF-α and IL-1β, the "early" pro-inflammatory mediators, are the key inflammatory factors causing uncontrolled inflammatory response and tissue damage[22, 23]. Recent studies have shown that HMGB1 is a key "late" proinflammatory mediator, which plays an important role in sepsis. Moreover, HMGB1 can further induce the expression and release of various proinflammatory mediators. In addition, it is also involved in the activation of MAPK signal transduction pathway and the nuclear translocation of NF-κB (p65) signaling pathway[24, 25]. Our previous RT-PCR results have shown that the expression of TNF-α and IL-1β mRNA was significantly increased at 2 and 3 h after the treatment with 1000 μg/L LPS, respectively. After 1 h of pretreatment with anti-nucleolin antibody, the expression levels of

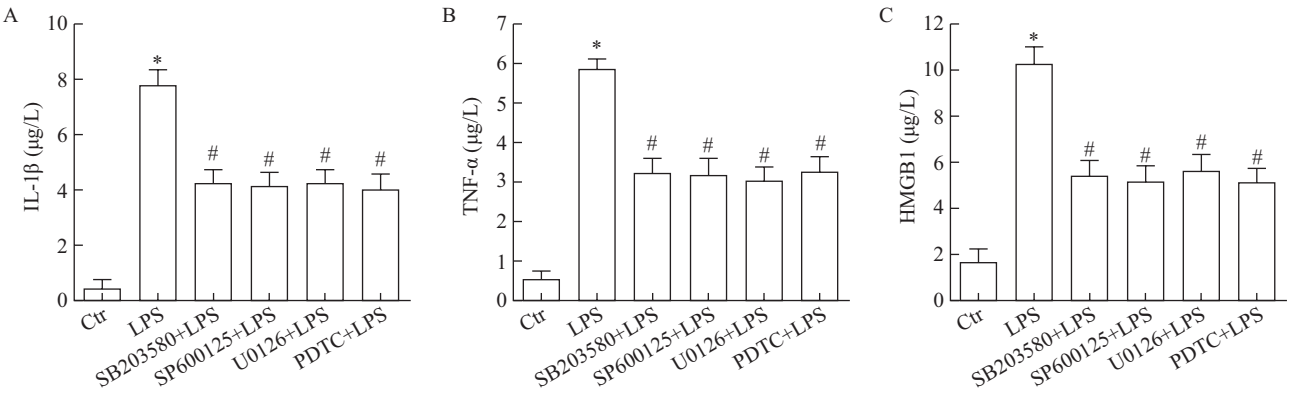

Fig. 4 ELISA showed the causative role of MAPK and NF-κB signaling pathways in LPS-induced IL-1β, TNF-α and HMGB1 secretion with specific inhibitors (SB203580 preincubated with 20 μmol/L for 1 h and cultured for 24 h in the presence of 1 mg/L LPS; SP600125 preincubated with 40 nmol/L for 1 h and cultured for 24 h in the presence of 1 mg/L LPS; U0126 preincubated with 60 nmol/L for 1 h and cultured for 24 h in the presence of 1 mg/L LPS; PDTC preincubated with 100 μmol/L for 1 h and cultured for 48 h in the presence of 1 mg/L LPS)

A: effects of p38, JNK, ERK and p65 inhibitors on LPS-induced IL-1β secretion; B: effects of p38, JNK, ERK and p65 inhibitors on LPS-induced TNF-α secretion; C: effects of p38, JNK, ERK and p65 inhibitors on LPS-induced HMGB1 secretion.

Ctr: control group; SB203580: P38 inhibitor; SP600125: JNK inhibitor; U0126: ERK inhibitor; PDTC: p65 inhibitor. Data were expressed as mean±SD, n=5. $^{*}P$<0.05 *vs.* control group, $^{\#}P$<0.05 *vs.* LPS group and control group. Ctr: control group

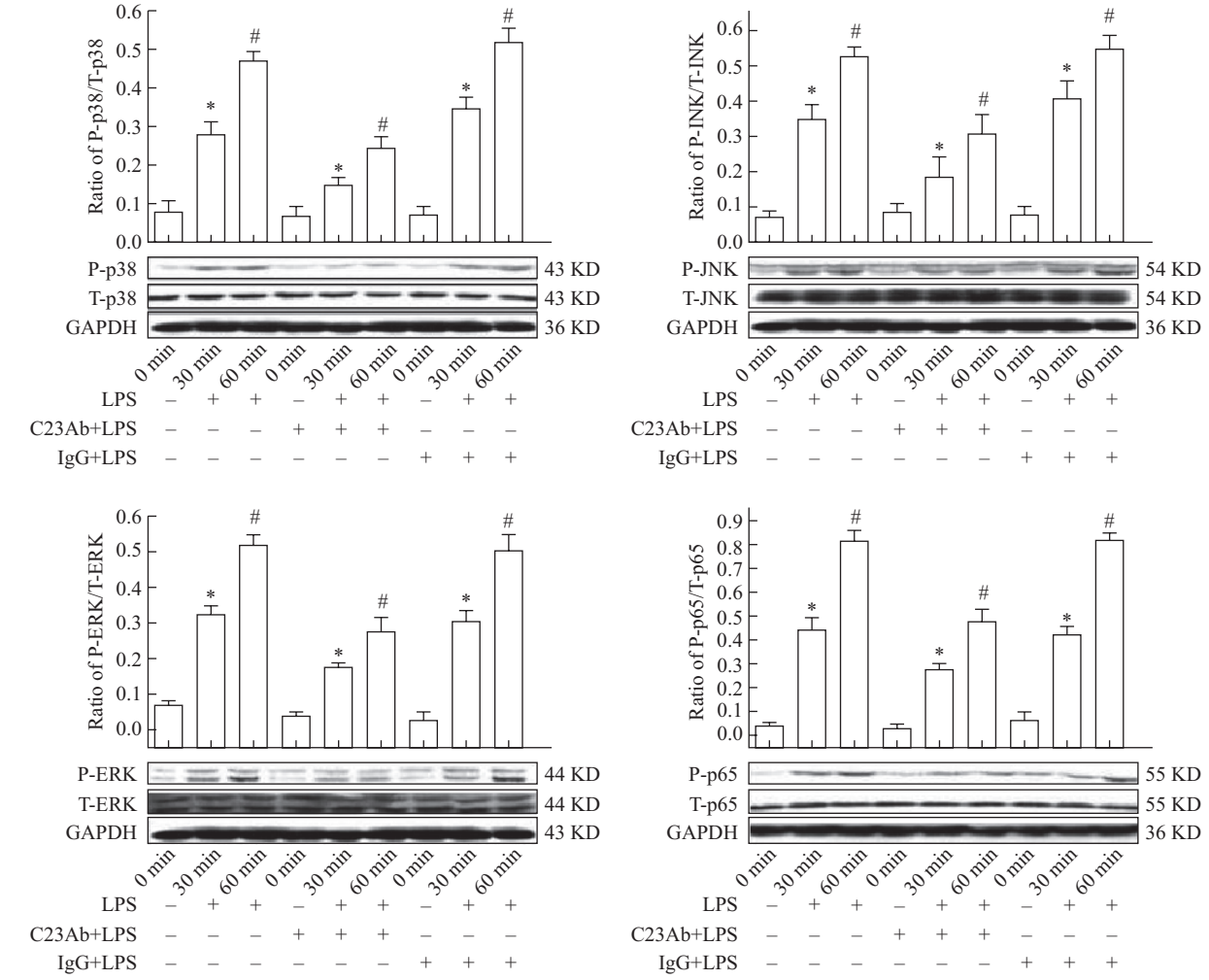

Fig. 5 Western blotting analysis showed the effect of pretreatment of anti-nucleolin antibody on the phosphorylation of p38, JNK, ERK and p65

Relative densitometric assay for the ratio of p38, JNK, ERK and p65 protein bands to T-p38, T-JNK, T-ERK and T-p65 protein bands respectively. Data were expressed as mean±SD, n=5. $^{*}P$<0.05 *vs.* LPS group, $^{\#}P$<0.05 *vs.* C23Ab+LPS group and LPS group

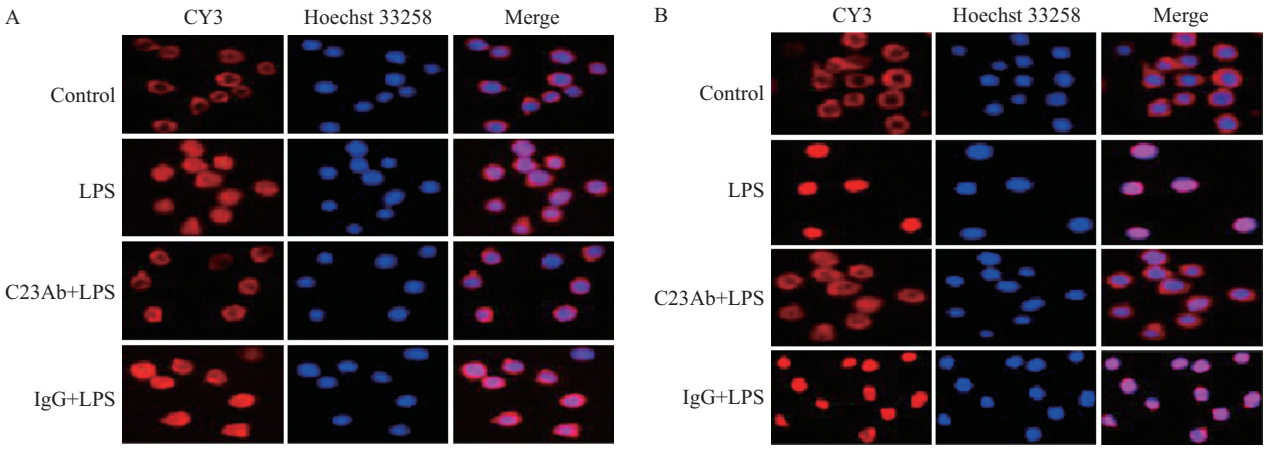

Fig. 6 Indirect immunofluorescence showed the effect of pretreatment of anti-nucleolin antibody on the nuclear translocation of NF-κB (p65) induced by LPS

A: LPS for 30 min. B: LPS for 60 min

TNF-α and IL-1β mRNA were decreased as compared with the untreated group and irrelevant IgG+LPS group[26]. It preliminarily explained that cell-surface nucleolin was involved in the expression and secretion of inflammatory mediators (IL-1β and TNF-α) of THP-1 cells induced by LPS. However, whether the

cell surface nucleolin mediated the expression and secretion of advanced inflammatory mediators caused by LPS remained unclear. The signaling pathways through which nucleolin on the surface of THP-1 cells mediates the inflammatory response of LPS need to be further explored. In the present study, we attempted to clarify the roles of nucleolin in LPS-induced secretion of TNF-α, IL-1β and HMGB1 in monocytes. THP-1 monocytes were pretreated with anti-nucleolin antibody or IgG for 1 h prior to LPS challenge. Secretion of inflammatory mediators (TNF-α, IL-1β and HMGB1) was examined to assess the cell-surface nucleolin in LPS-mediated inflammatory response. The unrelated antibody treatment (IgG group) was used as a control. ELISA results showed that the levels of TNF-α and IL-1β were significantly increased at 4, 12 and 24 h after LPS challenge, and the HMGB1 level was also significantly increased at 12, 24 and 48 h after LPS challenge. Pretreatment of anti-nucleolin antibody significantly inhibited the LPS-induced secretion of TNF-α, IL-1β and HMGB1 as compared with the LPS-stimulated cells. However, the irrelevant IgG had no effect on LPS-induced secretion of TNF-α, IL-1β and HMGB1. These results suggested that nucleolin existed in cell membrane, cytoplasm and nucleus of THP-1 monocytes, and it was involved in the LPS-mediated expression and secretion of TNF-α, IL-1β and HMGB1. The action mechanism might be that nucleolin existing on the cell surface of THP-1 monocytes functioned as LPS receptor, or the interaction of LPS with other LPS receptors resulted in the transformation of inflammatory signals into the cells. Previous studies have shown that cell-surface nucleolin can act as a receptor for a variety of protein molecules, bacteria and viruses, many ligands can be transported into the cell via binding to cell-surface nucleolin, and molecular chaperone plays a fundamental role in the intracellular translocation of extracellular ligands[13, 27, 28]. However, it remains largely unexplored whether nucleolin is involved in the regulation of LPS-induced inflammatory signals. We observed the causative role of MAPK and NF-κB signaling pathways in LPS-induced IL-1β, TNF-α and HMGB1 secretion with specific inhibitors. The results confirmed that p38, JNK, ERK and p65 inhibitors could significantly inhibit the secretion of IL-1β, TNF-α and HMGB1 induced by LPS. It suggested that LPS induced IL-1β, TNF-α and HMGB1 secretion through MAPK and NF-κB signaling pathways. In order to further reveal the mechanism underlying the inflammatory response mediated by the nucleolin, THP-1 cells were pretreated with anti-nucleolin antibody for 1 h prior to LPS challenge. Activation of MAPK and NF-κB signaling pathways was detected to assess the role of cell-surface nucleolin in LPS-mediated inflammatory response. Following pretreatment of anti-nucleolin antibody for 1 h, THP-1 monocytes were challenged with LPS for 30 min and 60 min, and the LPS-stimulated group and irrelevant IgG group were used as controls. Total cell protein and nuclear protein were extracted, and activation of JNK, ERK, p38 and p65 in the MAPK and NF-κB signaling pathways was detected by Western blotting analysis. Results showed that the phosphorylation of p38, JNK, ERK and p65 was significantly increased at 30 min and 60 min after LPS challenge. Pretreatment of anti-nucleolin antibody could significantly inhibit the phosphorylation of p38, JNK, ERK and p65 as compared with the LPS group. However, the irrelevant IgG had no effect on LPS-induced phosphorylation of p38, JNK, ERK and p65. Similar to this finding, pretreatment of anti-nucleolin antibody could significantly inhibit the nuclear translocation of NF-κB (p65). However, the irrelevant IgG had no effect on LPS-induced phosphorylation (the nuclear translocation) of p65, suggesting that nucleolin mediated LPS-induced activation of MAPK and NF-κB signaling pathways as well as the expressions of inflammatory mediators (TNF-α, IL-1β and HMGB1).

A large number of studies have shown that cell-surface nucleolin can act as a receptor for a variety of protein molecules, bacteria and viruses, and it plays a role in the transmission of extracellular signals[7, 9, 29]. Hirano *et al* have found that nucleolin on cell membrane of THP-1 cells can recognize polysaccharide in CD43 of early apoptotic Jurkat cell membrane and mediate phagocytosis of early apoptotic Jurkat cells. Anti-CD43 antibody, anti-nucleolin antibody and pretreatment of oligosaccharide can inhibit the recognition and phagocytosis of THP-1 cells to the early apoptotic cells[9]. Based on above-mentioned data, we hypothesized that nucleolin on the cell membrane might be involved in the inflammatory response induced by LPS. In summary, in this study, our results demonstrated that pretreatment of anti-nucleolin antibody inhibited the LPS-induced expression and secretion of inflammatory mediators (TNF-α, IL-1β and HMGB1) by inactivating MAPK and NF-κB pathways. However, we also found that the LPS-induced expression and secretion of inflammatory mediators (TNF-α, IL-1β and HMGB1) and activation of MAPK and NF-κB signaling pathways could not be completely eliminated after blocking the effect of nucleolin. This finding suggested that other receptors were involved in the activation of the above-mentioned inflammatory mediators and inflammatory signaling pathways induced by LPS, such as scavenger receptors, CD14, CD11/CD18, toll like receptors, loren protein and LBP and so on[30–32]. These receptors also play an important role in LPS-induced inflammatory response. Nucleolin exists on various eukaryotic cell membranes and acts as a receptor for various physiological ligands, which has been confirmed by many studies. After binding to nucleolin, various ligands can be actively transported into the cells, suggesting that nucleolin

enters the cells through an unclear signal pathway after interacting with LPS to play its biological function. For example, does the direct binding of LPS to cell-surface nucleolin play an important role in the proinflammatory response? What are the downstream signaling molecules and transcription activators of nucleolin-induced inflammatory response? In addition to TNF-α, IL-1β and HMGB1, can the blockade of nucleolin can also affect the expression of other inflammatory mediators? Besides the MAPK and NF-κB signaling pathways, is the inflammatory response mediated by other signaling pathways? In addition, the effects of nucleolin on the mortality, the expression of plasma inflammatory mediators and organ damage in mice need to be further clarified. Solving above-mentioned questions will enrich the LPS-induced proinflammatory mechanisms, providing more valuable insights into the prevention and treatment of Gram-negative bacterial infection-induced sepsis and other inflammatory diseases.

Conflict of Interest Statement

All authors declare that there is no conflict of interest and financial interest.

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