Original Article
Protective role of JNK inhibitor SP600125 in sepsis-induced acute lung injury

Liming Lou, Dandan Hu, Suzhen Chen, Shiqiang Wang, Yikai Xu, Yenuo Huang, Yali Shi, Hong Zhang

Department of Respiratory Medicine, The Third Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, Zhejiang, China

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Abstract: Background: Whether the c-Jun N-terminal kinase (JNK) pathway mediates apoptosis in sepsis-induced acute lung injury is not known. Here we investigated the effect of JNK inhibition in a rat model of sepsis-induced lung injury, and assessed expression of JNK and endoplasmic reticulum stress-related proteins. Methods: Sepsis was established by cecal ligation and puncture (CLP) in 48 male Sprague-Dawley rats. 32 additional rats underwent sham surgery. 24 CLP rats and 24 sham rats received tail vein injection of 30 mg/kg SP600125. The rest received saline injection. At 6, 12 and 24 h after surgery, blood, bronchoalveolar lavage fluid (BALF) and lung tissue were collected. p-JNK, XBP-1, ATF-4 and CHOP levels of the lung tissue were measured by western blot; and the JNK mRNA levels were measured by qPCR. Results: The W/D ratios of lungs in the CLP group were significantly higher than those in the sham group, but lower those in the CLP+JNK inhibitor group (P<0.05). TUNEL staining revealed significantly more apoptotic cells in the lungs of the CLP group than the sham group, and in the CLP+JNK inhibitor group the apoptotic index was significantly reduced (P<0.05). XBP-1, ATF-4, CHOP and p-JNK protein levels and JNK mRNA levels were significantly elevated in the CLP group (P<0.05), but significantly ameliorated in the CLP+JNK inhibitor group (P<0.05). Conclusions: Inhibition of the JNK signaling pathway mitigates sepsis-induced lung injury. Our results suggest that JNK signaling promotes endoplasmic reticulum stress and thus apoptosis in sepsis-induced lung injury.

Keywords: Apoptosis, cecal ligation and puncture, endoplasmic reticulum stress, JNK pathway, sepsis

Introduction

Sepsis is a systemic inflammatory response syndrome (SIRS) caused by infection. Epidemiological survey data suggest that sepsis affects 750,000 patients in the US every year, of which about 9% develop severe sepsis and 3% develop septic shock [1]. The incidence of sepsis in Chinese intensive care units (ICU) is 8.68% and the mortality rate can be as high as 50% [2]. Sepsis can damage multiple organs, and the lungs are often the first target organ. Sepsis-induced multiple organ dysfunction syndrome (MODS) is the major cause of death in critically ill patients [3]. Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) can occur early during sepsis. The incidence of ALI and ARDS in critically ill patients can reach 7% with a mortality rate as high as 40%. Thus ALI and ARDS have become the primary cause of death following sepsis [4, 5]. However, ALI remains difficult to treat as the pathophysiology of the disease remains unclear [6].

Recent studies have shown that apoptosis of pulmonary vascular endothelial cells (PVEC) plays an important role in sepsis-induced lung injury [7]. The role of endoplasmic reticulum stress (ERS) and c-Jun NH2-terminal kinase (JNK) signaling in apoptosis are currently hot research topics. ERS can be induced by ischemia and hypoxia, glucose/nutrient deprivation, ATP exhaustion, oxidative stress and destabilization of the Ca^{2+} steady state. Moderate ERS plays a protective role in the body, for the active reconstruction of intracellular homeostasis. However, sustained excessive ERS would lead to apoptosis or even death [8]. The JNK pathway is an important pathway in the mitogen-activated protein kinase (MAPK) family, which promotes cell growth, differentia-
JNK pathway, sepsis and acute lung injury

In addition, the JNK pathway can be activated by a variety of extracellular stress factors to mediate apoptosis [9]. The JNK signaling pathway has been implicated in ERS apoptosis.

Ischemic reperfusion injury is reported to cause excessive ERS in lung tissue. Silencing of JNK mRNA by siRNA can reduce the apoptosis and tissue injury induced by ERS [10], suggesting JNK pathway activation could also mediate apoptosis via ERS. The JNK pathway is reported to be involved in the regulation of inflammatory mediators and epithelial cell connections in sepsis [11]. However, whether the JNK pathway further mediates apoptosis in lung tissue by ERS remains to be determined. The rat CLP model mimics the clinical features of acute peritonitis caused by organ perforation, and is the most widely used animal model for the study of sepsis [12]. In this study, this model was used to investigate the role of JNK signaling and ERS in occurrence and development of lung injury during sepsis.

Materials and methods

Reagents

RIPA lysis buffer (Applygen Technologies Inc.), BCA protein quantification kit (Applygen Technologies, Inc.) PVDF membrane (pore size: 0.22 μm, Millipore, USA); primary anti-p-JNK and anti-β-actin antibodies (Santa Cruz Biotechnology, Inc. USA); SDS-PAGE electrophoretic buffer (Beyotime Biotechnology); membrane transfer buffer for western (Beyotime Biotechnology); Horseradish peroxidase labeled rabbit anti-goat secondary antibody (Maibio Co., Ltd. Shanghai); pre-stained protein marker (Fermentas, Canada); chloroform and isopropanol alcohol (Zhenxing No.1 Chemical Plant, Shanghai); dithiothreitol (DTT, 0.1 M, Invitrogen, USA); RNasin (40 U/μl, Promega, USA); dNTP (10 mM), random primers, and DNasel (5 U/μl) (Takara, Japan); reverse transcriptase (Invitrogen, USA); SYBR I and 50× Calibration (Bio-Rad, USA), HS ExTaq enzyme and 10× Buffer (TakaRa, Japan).

Animals and grouping

This study was approved by the experimental animal management committee of Zhejiang Chinese Medical University. Male Sprague-Dawley rats weighing 200±20 g were purchased from the experimental animal center of Zhejiang Chinese Medical University, License Number: SYXK (Zhe) 2013-0184. The rats were housed in the experimental animal center of Zhejiang Chinese Medical University at 20-24°C with humidity of 60%-80% and a 12 h light/dark cycle. Animals were provided food and water ad libitum by the experimental animal center of Zhejiang Chinese Medical University in accordance with rodent feeding standards. Animals were housed for one week to adapt to the environment before being divided, randomly, into four groups: sham group (8), CLP group (24), Sham+JNK inhibitor group (24), and CLP+JNK inhibitor group (24).

Modeling and drug administration

All rats were fasted for 24 hours before surgery. The sepsis model was established by cecal ligation and puncture (CLP) as previously described [13]. CLP group and CLP+JNK inhibitor group accepted surgery. Sham and sham+JNK inhibitor group had the same surgery as the CLP group, but without ligation and puncture. Sham+JNK inhibitor group and the CLP+JNK inhibitor group received tail vein injection of 30 mg/kg SP600125 (MCE, USA) dissolved in PBS with 2% DMSO once after surgery [14]. The Sham surgery group and CLP group were administered the same volume of PBS containing 2% DMSO by tail vein injection. Eight rats were selected randomly after 6, 12 and 24 h from each of the CLP group, sham+JNK inhibitor group and CLP+JNK inhibitor groups, and anaesthetized by intraperitoneal injection of 2% pentobarbital sodium (50 mg/kg). Blood samples were collected from the abdominal aorta and rats were sacrificed. Samples were collected from the sham group after 24 h.

Sample collection

Rat chests were opened immediately after rats were sacrificed to observe pathologic changes of lung tissues. The left lung was taken for alveolar wash. The trachea was sealed by 5 mL syringe, and 2 mL of PBS was injected twice. Lungs were then gently shaken and aspirated repeatedly. Bronchoalveolar lavage fluid (BALF) was collected, with a guaranteed recovery rate >80%. BALF was transferred to test tubes, and centrifuged at 2500 r/min for 10 min at room temperature. Then the supernatant was col-
lected and preserved at -80°C. The right upper lobe of the lung was fixed in 4% neutral formaldehyde solution, then embedded in paraffin and sliced for pathologic examination. The surface moisture of the middle lobe tissue of the right lung was dried by filter paper, and the tissue was put in a dry clean glass tube, to measure the tissue wet/dry (W/D) ratio. The lower lobe of the right lung was rapidly divided into two parts and preserved in EP tubes at -80°C.

**Index detection**

**HE staining of the lung tissues:** The upper lobe of the right lung was fixed in 4% neutral formaldehyde solution for 48 h, then embedded in paraffin and sliced into 5 μm slices. The slices were stained with H&E as follows. Slices were dehydrated in gradient alcohol dewatering with 70% (4 h), 90% (4-12 h), 95% (2 h×twice) and 100% (2 h×twice) alcohol. Vitrification was performed by immersion in cedar oil overnight twice; then dipped twice in soft wax and twice in hard wax. Slices were embedded by immersion in paraffin; wax repairmen; slicing; slice mounting; and baking at 37°C for 24 h. Slices were dewaxed by xylene three times, and dehydrated with alcohol gradient (100%, 90% and 70%). Then the slices were air dried and stained with HE for 10-20 min. Staining was observed by microscope.

**Wet/dry (W/D) ratio**

The middle lobe of the right lung was collected and surface fluid and blood infiltration was dried by filter paper. After weighing, the samples were dehydrated for 72 h at 80°C until reaching a constant weight. Dry tissue weight was measured, and W/D ratios reflect the degree of tissue edema.

**Detection of total protein, white blood cell and polymorphonuclear neutrophil (PMN) count in BALF**

The protein content of BALF was assessed as follows. After centrifugation, the BALF supernatant was collected and BCA content was measured using a BCA protein quantification kit (Applygen Technologies Inc.) according to the manufacturer’s protocol.

The total white blood cell count and classification in BALF was assessed as follows. After centrifugation, the pellet was washed with cold PBS, and re-centrifuged cells were resuspended in PBS. Total white blood cell was assessed using a hemocytometer and light microscope. The remaining samples were smeared and dried naturally for Wright-Giemsa staining. Cells were classified under light microscope. 5 smears were selected from each group, and the number of cells in 5 fields of view were counted and averaged.

**Measurement of BALF TNF-α and IL-6 content**

BALF supernatant (200 μl) was collected for measurement of TNF-α and IL-6 levels by ELISA (Amersham Inc. USA) according to the manufacturer’s protocols. The data were collected from two independent experiments.

**Measurement of lung tissue myeloperoxidase (MPO) content**

A Rat MPO kit (Nanjing Jiansheng Bioengineering Institute) was used according to the manufacturers’ protocols.

**Apoptosis in lung tissues**

Lung tissues were collected and paraffin sections were prepared. Apoptosis was detected in situ in lung tissue sections using a TUNEL apoptosis detection kit (Roche Inc., USA). DAB was detected using a ZSGB-Bio kit (Beijing) as follows: paraffin embedded slices were pretreated according to conventional dewaxing and dehydration procedures. The samples were incubated at room temperature for 15-30 min with protease K (20 μg/ml dissolved in Tris/HCl, pH 7.4~8.0), followed by incubation at 37°C for 15 min; then washed in PBS twice. Samples were then dried and incubated with 50 μl TUNEL reaction mixture for 60 mins at 37°C in a wet box to prevent evaporation and ensure uniform distribution of TUNEL reaction mixture. Cover slides were used for the entire incubation. The slides were then washed with PBS three times and staining was assessed under fluorescent microscope. For signal transformation and analysis, samples were dried, and 50 μl of transforming reagent-POD was added. The samples were incubated at 37°C in a wet box for 30 min to prevent evaporation and ensure uniform distribution of POD, and cover slides were used during the entire incubation. After three washes with PBS, 50~100 μl of DAB sub-
strate solution was added for 10 mins at room temperature. Then the samples were washed another three times with PBS. The nucleus was stained by hematoxylin. After slice mounting, the images were analyzed by light microscope. Meaningful organs were selected for analysis and graded according to the number of positive cells (0~1% positive cells, grade 0; 1~10%, grade 1; 10~50%, grade 2; 50~80%, grade 3; 80~100%, grade 4) and staining intensity (0, negative; 1, weakly positive; 2, positive, 3, strong positive). IHS=A×B.

Detection of XBP-1, ATF-4, CHOP and phosphorylated JNK protein expression in lung tissues

The levels of XBP-1, ATF-4, CHOP and phosphorylated JNK in lung tissues was assessed by immunoblotting using a BCA protein quantification kit (Applygen Technologies Inc.), PVDF membrane (pore size of 0.22 μm, Millipore, USA), primary anti-p-JNK and anti-β-actin proteins (Santa Cruz Biotechnology, Inc. USA), SDS-PAGE electrophoretic buffer (Beyotime Biotechnology), membrane transfer buffer for western (Beyotime Biotechnology), Horseradish peroxidase labeled rabbit anti-goat secondary antibody (Maibio Co., Ltd. Shanghai), pre-stained protein marker (Fermentas, Canada), ECL chemiluminescence reagent kit (Applygen Technologies Inc.), FluorChem M (multicolor fluorescence and chemiluminescence gel imaging system, ProteinSimple Inc. USA), and 3 MM filter paper (Whatman, UK). The main steps were as follows: protein extraction and quantification by BCA kit. According to the protein quantification result, 50 μg of total protein was boiled in water bath at 100°C for 3 min. SDS-PAGE electrophoresis was performed, then proteins were transferred to PVDF membranes by wet transfer. 3% BSA solution was used for blocking and then membranes were stained with anti-XBP-1 (Santa Cruz, 1:800), anti-ATF-4 (Abcam, 1:1000), anti-CHOP (Santa Cruz, 1:800), anti-p-JNK (Santa Cruz, 1:800) and anti-β-actin (Santa Cruz, 1:1000) antibodies on a rocker at 4°C overnight. Membranes were then washed with TBST and HRP-labeled secondary antibodies (1:10000) were applied for one hour at room temperature. After washing with TBST, ECL reagent mixture was evenly distributed on the PVDF membrane at room temperature for 4 mins. FluorChem M detection was used for direct imaging. Image J image processing software was used for gray level analysis.

Detection of JNK mRNA expression levels in lung tissues

Quantitative real-time PCR was used for mRNA detection. Myocardial tissue RNA was extracted by a conventional Trizol method (Invitrogen Inc.) according to the manufacture’s protocol. Primers were designed, and synthesized by Takara Inc. as follows: JNK-F: 5’-AGTGTAGAAGTGGATGCACTA-3’, JNK-R: 5’-AGTGCTCTCGTGATGTTAC-3’ (182 bp); GAPDH-F: 5’-TGCACTCTGACGGTCGGTCT-3’, GAPDH-R: 5’-GGCAGGACCTGTGGCTGGG-3’ (130 bp). RT-PCR kit (Lot: DRR047A). Real time PCR detection kit (Takara Inc. Lot: DRR420A) was used following PCR using a Step one plus PCR machine (ABI Biosystems, USA). The reaction mix contained 1 μl total cDNA, 0.5 μl forward and 0.5 μl of reverse primers and other kit components. The cycling conditions for JNK and GAPDH were pre-denature at 94°C for 5 min; followed by 45 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 40 seconds; final extension at 72°C for 5 min. GAPDH was used as an internal reference for analysis of JNK expression. Relative quantification was used to analyze ΔCt values in each sample (ΔCt = Ct value of target genes-Ct value of reference gene), ΔΔCt=ΔCt value of the experimental group-ΔCt value of the control group. 2-ΔΔCt was then calculated.

Statistical analysis

All data were analyzed by using SPSS 20.0 statistical software. The measurement data were represented as Mean±SD. Analysis of variance was used for comparison among groups. For the post hoc test, LSD test was used for the data with homogeneity of variance, while Dunnett’s T3 test was used for the data with heterogeneity of variance. P value <0.05 was considered significant.

Results

General conditions of rats

After CLP surgery, the general conditions of rats were recorded. Rats in the sham group
regained consciousness 30 min after the surgery, exhibiting smooth respiration and sensitive responses. Their hair was smooth and glossy, and appetite and defecation were normal. Postoperatively the condition of the

sham+JNK inhibitor group was similar to that of the sham group. Postoperative recovery was delayed in rats in the CLP group, exhibiting accelerated respiration, decreased activity, closed eyes and slow responses. CLP group rats excreted watery, yellow stool with a fishy odor, and had visible peritoneal effusion with odor. Rats in the CLP+JNK inhibitor group had mild postoperative reactions. Their activities and response to the environment were better than that of the CLP group.

Before sacrifice, one of the experimental animals in the CLP group died at 12 h (1/8), and two in the experimental group died at 24 h (2/8); one in the CLP+JNK inhibitor group died at 24 h (1/8), while no animals in other groups died. Mortality did not differ significantly between groups.

Pathological observation of lung tissues

Compared to Sham and Sham+JNK inhibitor group, more PMN and inflammatory cells infiltrate lung surfaces in CLP group, damaging alveolar epithelial cells and capillary endothelial cells. As a result, blood vessel permeability increases, and alveolar surfactants are released. However, administration of JNK inhibitor partially restored the abnormal morphological phenotypes (Figure 1A). The W/D ratio reflects the water content in the lung and thus the degree of edema. We observed that the W/D ratios of the lung tissues in the CLP group were significantly increased at 6 h, 12 h as well as 24 h after CLP modeling when compared with the sham group (P<0.05, P<0.05, and P<0.05, respectively). In addition, although
Figure 2. Eight rats were selected randomly from each group 6 h after surgery or sham surgery. Eight further rats were selected randomly from each CLP 12 and 24 h after surgery. Total protein content of BALF (A), BALF leukocyte content (B), PMN content (C) were measured. Lung tissue levels of TNF-α and IL-6 were also measured (D and E). *P<0.05 when compared with the sham group; **P<0.05 when compared with the CLP group at the same time points. N=6 per group.
the W/D ratio of the lung tissues in the CLP+JNK inhibitor group were still higher than that in the sham group, they were significantly lower than that in the CLP group at all time points (P<0.05, P<0.05, and P<0.05, respectively), see Figure 1B. Lung tissue MPO content in the CLP group was also higher than in the sham group, but significantly reduced when compared with that in the CLP+JNK inhibitor group at each time point (P<0.05, Figure 2B and 2C). Additionally, TNF-α and IL-6 levels were elevated in the CLP group (P<0.05), but significantly reduced at each time point in the CLP+JNK inhibitor group (P<0.05, Figure 2D and 2E).

Figure 3. Eight rats were selected randomly from each group 6 h after surgery or sham surgery. Eight further rats were selected randomly from each CLP 12 and 24 h after surgery. Lung tissue apoptosis was assessed using TUNEL staining (A) and apoptosis index was calculated (B). *P<0.05 when compared with the sham group; #P<0.05 when compared with the CLP group at the same time points. N=6 per group.

Apoptosis in lung tissues

TUNEL staining showed no obvious apoptosis in the lung tissues of rats in the sham group. However, TUNEL-positive cells were observed in the lung tissues of rats in the CLP group and the nuclei of apoptotic cells were brownish yellow. Over time, the number of apoptotic cells increased significantly; the number of apoptotic cells in lung tissues of CLP+JNK inhibitor group animals was significantly lower than in the CLP group (Figure 3A). The apoptosis index was significantly increased at 6, 12 and 24 h post surgery in the CLP group (P<0.05), but was significantly ameliorated in the CLP+JNK inhibitor group at each time point (P<0.05, Figure 3B).

XBP-1, ATF-4, CHOP and P-JNK expression in lung tissues

Expression of JNK mRNA in lung tissue was significantly elevated 6, 12 and 24 h post surgery in the CLP group (P<0.05), but was significantly ameliorated in the CLP+JNK inhibitor group at each time point (P<0.05, Figure 4A). The levels of XBP-1, ATF-4, CHOP and P-JNK protein were also significantly elevated in the CLP group (P<0.05, Figure 4A-F). In the CLP+JNK inhibitor
Figure 4. Eight rats were selected randomly from each group 6 h after surgery or sham surgery. Eight further rats were selected randomly from each CLP 12 and 24 h after surgery. Lung tissue JNK mRNA levels were measured by qPCR (A) and p-JNK, XBP-1, ATF-4 and CHOP protein levels were measured by western blot (B-F).

*P<0.05 when compared with the sham group; **P<0.05 when compared with the CLP group at the same time points. N=6 per group.
group, levels of these proteins were significantly ameliorated at each time point (all \( P<0.05 \), Figure 4A-F).

**Discussion**

In this study, CLP was used to establish a rat model of sepsis, and the role of JNK signaling and ERS in development of lung injury was assessed. We observed that CLP rats had delayed recovery after surgery, manifested in accelerated respiration, reduced activity, closed eyes, slow responses; excretion of watery stool with visible peritoneal effusion. These observations suggest successful establishment of a rat sepsis model.

During sepsis, PMN and inflammatory cells infiltrate lung surfaces, damaging alveolar epithelial cells and capillary endothelial cells. As a result, blood vessel permeability increases, and alveolar surfactants are released. These factors cause thickening of the alveolar wall, edema, and pulmonary capillary and alveolar hemorrhage, which precipitates severe pulmonary edema and severe ventilation/blood flow disorders, manifested as refractory hypoxemia and eventually development of ALI/ARDS [15]. When sepsis occurs, intravascular fluids and macromolecular proteins that could not pass through the blood vessel wall extravasate to the alveolar space, increasing protein levels in BALF and aggravating pulmonary edema. In our CLP-induced sepsis model we observed increased BALF protein levels and elevated W/D ratios, indicating permeability of the pulmonary capillary, and pulmonary edema, damage to the pulmonary interstitium and the permeability of pulmonary alveolar vessels. Histological analysis of lung tissues revealed damage to the pulmonary alveolar structure, severe edema in the pulmonary alveolar and the interstitium, thickening of the alveolar interval, infiltration of inflammatory cells, pulmonary capillary hemorrhage, suggesting successful establishment of the rat sepsis model.

ERS is a hot topic in sepsis research. The endoplasmic reticulum signaling proteins PEAK-ATF4-CHOP and IRE-XBP1-CHOP are two pathways that determine cell fates under stress. Activating transcription factor 4 (ATF4) and X-box binding protein 1 (XBP1) are protective; however, extensive ERS causes ATF4 and XBP1 to induce expression of CHOP and CCAAT enhancer binding protein (C/EBP) homologous protein [16], promoting apoptosis [17]. CHOP is an ERS-specific protein, with only low level expression under normal conditions. Sustained and excessive stress extensively activates CHOP by the unfolded protein response (UPR), regulating downstream pro-apoptotic genes to promote apoptosis [17]. Previous studies have shown that CHOP knockdown could enhance cellular resistance to ERS-induced apoptosis [18]. Blocking CHOP could improve survival of sepsis patients [19]. In this study we demonstrated that in a rat model of sepsis, expression of ATF4, XBP1, and CHOP was significantly upregulated in lung tissues 6 h after CLP (in comparison to sham group animals), and increased over the following 24 h. These data suggested that during sepsis, expression of ATF4, XBP1 and CHOP proteins in lung tissues increased progressively, and likely promoted the apoptosis and thus lung injury observed over the same time period in model animals.

The JNK signaling pathway is an important target for the regulation of cellular physiology and pathology. JNK can be activated to its phosphorylated form, and acts to regulate cell functions [20]. JNK plays important roles in activation of transcription factors, mRNA stability, stress response and apoptosis promotion [21]. During excessive ERS, activated IRE1 binds to TRAF2, forming a complex with apoptotic signal regulating kinase 1, which further activates JNK. Phosphorylated JNK activates apoptotic protein Bim, inhibiting anti-apoptotic protein Bcl-2, and thereby inducing apoptosis [22]. Apoptosis-induced by ERS was correlated with upregulated JNK and CHOP expression [23]. Thus, sustained ERS during sepsis may lead to endoplasmic reticulum dysfunction, and promote apoptosis leading to lung injury.

In this study we explored the impact of administration of the JNK inhibitor SP600125 on CLP-induced sepsis. Administration of JNK inhibitor significantly improved histological features of the lung, the W/D ratio and apoptosis index, significantly ameliorating the symptoms of sepsis in these animals. XBP-1, ATF-4, CHOP and p-JNK protein levels and JNK mRNA levels were also significantly ameliorated in animals administered JNK inhibitor, further confirming the crucial role of the JNK pathway in sepsis-induced lung injury. JNK signaling and ERS are thus
likely to be crucial mechanisms in sepsis-induced lung injury in sepsis. Further study will be required to elucidate the specific molecular mechanisms involved, and to validate these findings in humans.

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Disclosure of conflict of interest
None.

Address correspondence to: Hong Zhang, Department of Respiratory Medicine, The Third Affiliated Hospital of Zhejiang Chinese Medical University, 219 Moganshan Road, Xihu District, Hangzhou 310005, Zhejiang, China. Tel: +86-13588420208; Fax: +86-21-57643271; E-mail: Doctorzhang0208@163.com

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JNK pathway, sepsis and acute lung injury


