

# Circulating level of high mobility group box-1 predicts the severity of community-acquired pneumonia: Regulation of inflammatory responses via the c-Jun N-terminal signaling pathway in macrophages

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**Abstract.** High mobility group box-1 (HMGB-1) has been reported to serve significant roles in various inflammatory diseases. However, the correlation between the circulating level of HMGB-1 and severity of community-acquired pneumonia (CAP) remains unclear. The present study investigated differential alterations in plasma HMGB-1 levels of patients with CAP prior to and following antibiotic treatment, and further analyzed the association between CAP severity and HMGB-1 levels. Furthermore, lipopolysaccharide (LPS)-induced HMGB-1 expression in RAW264.7 macrophages and the relevant signaling pathways were examined. Plasma HMGB-1 levels of 90 patients with CAP and 52 healthy controls were measured using a commercial ELISA. The levels of plasma HMGB-1 were significantly elevated in CAP patients compared with the controls, and antibiotic treatment was effective in reducing HMGB-1 levels. Plasma HMGB-1 correlated with the pneumonia severity index score ( $r=0.566$ ,  $P<0.001$ ). Furthermore, LPS-stimulation significantly upregulated HMGB-1 secretion via the c-Jun N-terminal kinase (JNK) signaling pathway in RAW264.7 macrophages, whereas pretreatment with the JNK inhibitor SP600125 markedly downregulated LPS-induced HMGB-1 levels. In conclusion, plasma HMGB-1 levels may serve a role in the diagnosis and clinical assessment of CAP severity. These findings may provide information on novel targets for the treatment of CAP.

## Introduction

High mobility group box-1 (HMGB-1) is a type of nuclear protein named for its high mobility in PAGE, and serves as a classic non-histone protein within nuclei (1). Previous studies largely focused on the intranuclear functions of HMGB-1, including its involvement in nucleosome stability, regulation of gene transcription and reconstruction and restoration of DNA (2-5). In addition, HMGB-1 is highly correlated to formation, invasion and metastasis of cancer cells (6-8).

Previously, HMGB-1 has been classified as a type of cytokine (1,9). Extracellular HMGB-1 induces inflammatory responses and may activate cells of the immune system, including activating the immune response of monocytes (9). Wang *et al* (10) first revealed that extracellular HMGB-1 serves as a mediator for inflammatory responses, and is a key inflammatory mediator for sepsis. Thereafter, the inflammatory effects of extracellular HMGB-1 attracted considerable interest. Previous studies have demonstrated that extracellular HMGB-1 is a key inflammatory mediator and proinflammatory cytokine present in the pathogenesis of sepsis, arthritis, acute pancreatitis, systemic lupus erythematosus, meningitis, kidney diseases, cardiovascular diseases and other diseases (11-15). In addition, HMGB-1 serves key functions in pulmonary fibrosis and pulmonary inflammation (16-19). Tseng *et al* (16) revealed that HMGB-1 content in the serum samples of patients in intensive care diagnosed with severe pneumonia serves as a predictor for mortality. Ito *et al* (17) assessed HMGB-1 content in the serum samples of H1N1 influenza-infected children, and identified a significant increase in HMGB-1 content in the samples of patients diagnosed with severe pneumonia complications. It was hypothesized that HMGB-1 is important in the recovery process from pneumonia. In addition, a previous study has indicated that HMGB-1 content in pneumonia patients who subsequently develop sepsis is significantly increased compared with pneumonia patients without complications (20). However, few studies have examined the correlation and severity of community-acquired pneumonia (CAP) and HMGB-1.

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CAP is defined as an acute lung infection occurring in individuals who are not hospitalized (or have been hospitalized for <48 h), and is a common and life-threatening lower respiratory tract infection (21,22). Despite numerous treatment options, the CAP mortality rate remains high. Therefore, identifying biomarkers to assist the early diagnosis of pneumonia is crucial (22). Although previous studies of the authors have aimed to identify the biomarkers of pneumonia (21-24), the correlation of HMGB-1 levels with the severity of pneumonia remains undefined. The present study aimed to examine alterations in HMGB-1 expression and severity of pneumonia in patients prior to and following treatment. Furthermore, the present study examined lipopolysaccharide (LPS)-induced RAW 264.7 macrophages to simulate the pathogenesis of pneumonia and investigate the performance of HMGB-1 and the relevant signaling pathways.

## Materials and methods

**Subjects.** The present study enrolled 144 people (90 CAP patients and 54 healthy controls) from February 2009 to December 2013 from Chung Shan Medical University Hospital (Taichung, Taiwan). Individuals that visited the Department of Family and Community Medicine for a health examination in Chung Shan Medical University Hospital were selected as healthy controls. All CAP patients were empirically treated with antimicrobial agents. All CAP patients were given antibiotics intravenously within the first 48 h. The pretreatment blood samples were obtained prior to patients with CAP receiving treatment protocols, and posttreatment blood samples were obtained within 3 days when the pneumonia had resolved. Blood samples were used to determine white blood cell (WBC) counts, neutrophils, and c-reactive protein (CRP) and plasma concentrations of HMGB-1 prior to and following antibiotic treatment of CAP patients. Blood samples of control subjects were additionally collected and tested. Blood samples were placed in tubes containing EDTA, immediately centrifuged at  $2,500 \times g$ , and stored at  $-80^{\circ}\text{C}$ . The present study was approved by the Chung Shan Medical University Hospital Institutional Review Board (Taichung, Taiwan) and all participants gave their informed consent. Pneumonia severity was assessed using the Pneumonia Severity Index (PSI) (25).

**Measurement of plasma HMGB-1.** An ELISA kit (YHB1552Hu; Shanghai YH Biosearch Laboratory, Shanghai, China) was used to measure concentrations of HMGB-1 in blood samples or in the conditional medium of RAW264.7 macrophage. For each plasma sample,  $40 \mu\text{l}$  was directly transferred to microtest strip wells of the ELISA plate combined with a detection antibody and subsequently incubated for 1 h at  $37^{\circ}\text{C}$ . Following 4 washing steps, antibody binding was detected with a horseradish peroxidase (HRP)-conjugated streptavidin secondary antibody for 1 h at  $37^{\circ}\text{C}$  supplied with the kit and developed with a substrate solution. Next, the reaction was stopped, and the optical density was determined at a wavelength of 450 nm using a microplate reader (STNERGY/H4, BioTek Instruments, Inc., Winooski, VT, USA). Sample results were calculated from a standard curve generated by dilutions of a known amount of recombinant HMGB-1 protein. Each standard or sample was assayed in duplicate.

**Cells and cell culture.** RAW264.7 macrophages were purchased from the American Type Culture Collection (Manassas, VA, USA) and were cultured in Dulbecco's Modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 2 mM glutamine, 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin and 400 ng/ml hydrocortisone (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). All cell cultures were maintained at  $37^{\circ}\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$ . For LPS treatment, the conditioned medium was removed from RAW264.7 macrophages and replaced with fresh medium prior to the addition of LPS, following which cells were incubated for the indicated time periods *in vitro*.

**Western blot analysis.** Cellular lysates were prepared by suspending cells in a 10 cm dish (density,  $2 \times 10^6$  cells) with 200  $\mu\text{l}$  radioimmunoprecipitation assay buffer (Sigma-Aldrich, Merck KGaA) containing protease inhibitors cocktail as described previously (26). The nitrocellulose blot was subsequently incubated with 5% non-fat milk in Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) for 1 h to block non-specific binding. Following this, the membrane was incubated with the following rabbit primary antibodies overnight at  $4^{\circ}\text{C}$ : Polyclonal anti-HMGB-1 (6893; 1:500), anti-extracellular signal regulated kinase (ERK) 1/2 (9102; 1:1,000), anti-c-Jun N-terminal kinase (JNK) 1/2 (9258; 1:1,000), p38 (612168; 1:1,000), anti-phosphorylated (p)-ERK (4370; 1:1,000), anti-p-JNK 1/2 (9251; 1:1,000) and anti-p-p38 (4511; 1:1,000), all purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Blots were subsequently incubated with a HRP-conjugated goat anti-rabbit (sc-2004; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or anti-mouse IgG (sc-2005; Santa Cruz Biotechnology, Inc.) secondary antibody for 1 h at  $4^{\circ}\text{C}$ .  $\beta$ -actin served as an internal control. Proteins were detected using an Enhanced Chemiluminescence commercial kit (RPN 2132; GE Healthcare Life Sciences, Chalfont, UK) and relative optical density was quantified using an AlphaImager<sup>®</sup> HP system (Alpha Innotech Corporation, San Leandro, CA, USA).

**Statistical analysis.** Statistical analyses were performed using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). All data are expressed as the mean  $\pm$  standard deviation. N is expressed as percentages for categorical variables. To compare between untreated patients and healthy individuals, the Mann-Whitney *U*-test and Wilcoxon signed-ranks test were performed for continuous variables. A linear regression analysis was applied for correlations of HMGB-1 with PSI scores of CAP patients.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Antibiotic treatment reduces CRP levels in CAP patients.** Demographic data and clinical characteristics of the participants are presented in Table I. In total, 142 age and gender matched CAP and control participants were included in the analysis. Patients with CAP exhibited significantly increased CRP levels (12.99 vs. 0.47 mg/dl,  $P < 0.001$ ), WBC counts ( $13,316$  vs.  $6,301$  cells/ $\text{mm}^3$ ,  $P < 0.001$ ) and neutrophil counts

Table I. Laboratory data of controls and patients with CAP prior to and following treatment.

Clinical variable	C (n=52)	Before antibiotic treatment (n=90)	After antibiotic treatment (n=90)	P UT/C <sup>a</sup>	P UT/T <sup>b</sup>
Age	60.25±10.69 <sup>c</sup>	65.02±18.10 <sup>c</sup>		=0.085	
Gender					
Male	35 (67.3%)	65 (72.2%)		=0.536	
Female	17 (32.7%)	25 (27.8%)			
CRP (mg/dl)	0.47±0.27	12.99±6.50	2.82±3.16	<0.001	<0.001
WBCs (cells/mm <sup>3</sup> )	6,301.2±1,827.2	13,316.3±4,797.26	9,413.7±3,791.4	<0.001	<0.001
Neutrophils (cells/mm <sup>3</sup> )	3,683.2±1,353.2	10,657.3±4,261.7	6,930.2±3,718.1	<0.001	<0.001
PSI score					
I		12 (13.3%)			
II		14 (15.5%)			
III		24 (26.7%)			
IV		33 (36.7%)			
V		7 (7.8%)			

CAP, community-acquired pneumonia; CRP, C-reactive protein; WBCs, white blood cells; C, controls; UT, patients with community-acquired pneumonia prior to receiving antibiotic treatment; T, patients with community-acquired pneumonia following antibiotic treatment. <sup>a</sup>The statistical difference was analyzed by the Mann-Whitney U-test. <sup>b</sup>The statistical difference was analyzed by the Wilcoxon signed-ranks test. <sup>c</sup>Data are expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

(10,657 vs. 3,683 cells/mm<sup>3</sup>, P<0.001) compared with the controls. In these patients, antibiotic treatment significantly reduced median CRP levels (prior to antibiotic treatment, 12.99 mg/dl; following antibiotic treatment, 2.82 mg/dl; P<0.001), WBC counts (prior to antibiotic treatment, 13,316 cells/mm<sup>3</sup>; following antibiotic treatment, 9,413 cells/mm<sup>3</sup>; P<0.001), and neutrophil counts (prior to antibiotic treatment, 10,657 cells/mm<sup>3</sup>; following antibiotic treatment, 6,930 cells/mm<sup>3</sup>; P<0.001).

*Antibiotic treatment significantly reduces HMGB-1 expression in CAP patients.* CAP patients exhibited significantly higher plasma HMGB-1 levels than the controls (11.29±8.44 and 6.00±3.11 ng/ml, respectively, P<0.001). However, antibiotic treatment significantly reduced the expression of HMGB-1 (prior to antibiotic treatment, 11.29±8.44 ng/ml; following treatment, 7.38±5.19 ng/ml; P<0.001; Fig. 1).

*HMGB-1 levels and CAP severity correlations.* The correlation between HMGB-1 concentration and CAP severity in patients prior to antibiotic treatment was assessed using the PSI scoring system. As presented in Fig. 2A, significant differences in HMGB-1 levels were observed between Class I and Class IV (P<0.001), Class I and V (P<0.001), II and IV (P=0.003), II and V (P<0.002), III and IV (P=0.001), and III and V (P<0.001) patients. Furthermore, significantly different HMGB-1 levels were observed in patients who were classified as low risk compared with those classified as medium risk (P<0.001), and between low risk and high risk patients (P<0.0001) according to PSI scores (Fig. 2B). Furthermore, significant correlations were observed between HMGB-1 levels and PSI scores (Spearman's correlation coefficient: r=0.566; P<0.001; Fig. 2C).

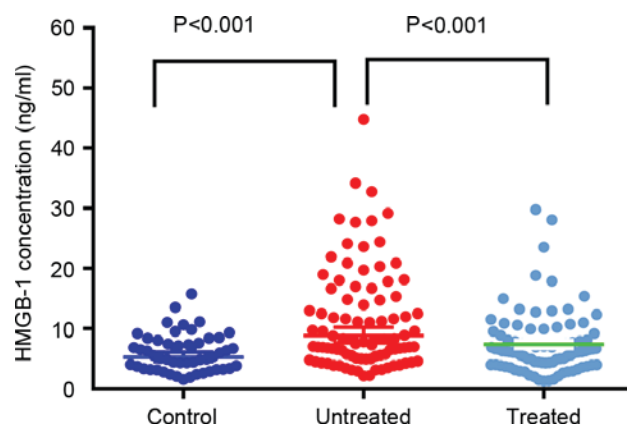


Figure 1. Levels of plasma HMGB-1 in control subjects and patients with CAP prior to and following antibiotic treatment. HMGB-1 levels were significantly elevated in patients with CAP prior to treatment compared with the controls, and significantly decreased in CAP patients following treatment. HMGB-1, high mobility group box-1; CAP, community-acquired pneumonia.

*LPS treatment in macrophages.* LPS-induced inflammatory responses in RAW264.7 macrophages is a reliable model to study bacterial pneumonia *in vitro* (27,28). Western blotting and an ELISA assay were used to investigate the expression and secretion of HMGB-1 following LPS treatment. As detected by western blotting, LPS treatment significantly increased HMGB-1 protein expression levels in RAW264.7 macrophages at 3, 6 and 12 h (Fig. 3A). In addition, the ELISA assay revealed that HMGB-1 secretion elevated to 4.18 ng/ml 6 h after LPS induction (Fig. 3B).

Mitogen-activated protein kinase (MAPK) serves a crucial role in LPS-induced signal transduction pathways that lead to cytokine synthesis in macrophages (29,30). Thus, the effect of

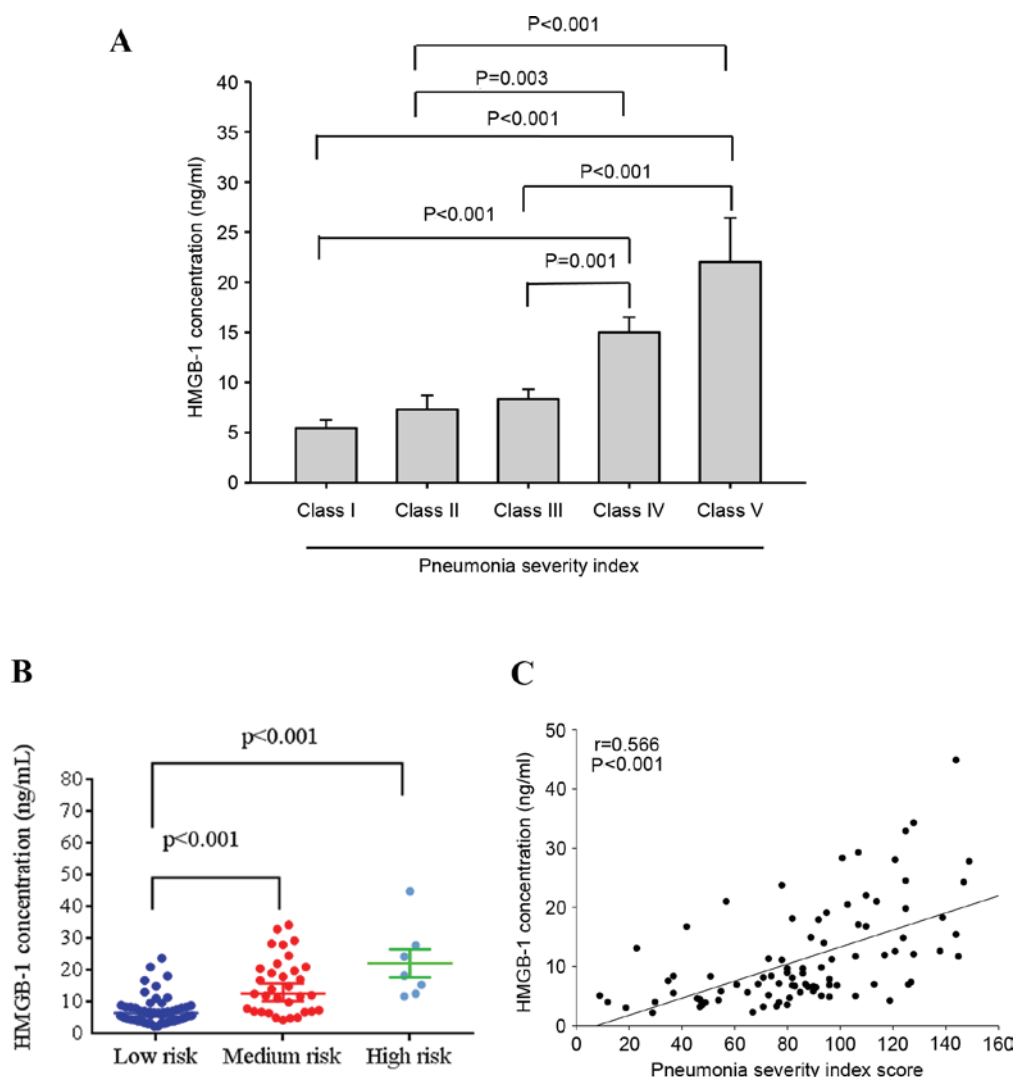


Figure 2. Levels of HMGB-1 and PSI scores in 90 patients with community acquired pneumonia. (A) Significant differences were observed between Class IV, V, I and II PSI scores. Data are expressed as the mean  $\pm$  standard deviation. (B) Significant differences were observed between low and medium risk, and low and high risk patients. (C) A significantly positive correlation was observed between plasma HMGB-1 levels and PSI scores. HMGB-1, high mobility group box-1; PSI, pneumonia severity index.

LPS treatment on the expression levels of MAPK signaling pathway proteins were investigated by western blotting to elucidate their underlying mechanisms. LPS treatment significantly increased phosphorylation levels ERK 1/2, p38 and JNK 1/2 in RAW264.7 macrophages ( $P < 0.05$ ; Fig. 3C). To further determine whether LPS-induced HMGB-1 overexpression was caused primarily by the MAPK signaling pathway, its effects on specific inhibitors of ERK 1/2 (U0126), p38 (SB203580) and JNK 1/2 (SP600125) in RAW264.7 macrophages were investigated. As assessed by western blotting, LPS-induced HMGB-1 overexpression was significantly reduced by the JNK 1/2 inhibitor (SP600125; Fig. 3D). In addition, similar results were obtained by ELISA assay (Fig. 3E). Therefore, induction of the JNK 1/2 signaling pathway may induce expression of HMGB-1.

## Discussion

In terms of the biological function of HMGB-1, previous studies initially focused on its intranuclear functions (9,31,32).

Following identification of the extracellular functions of HMGB-1, a number of studies revealed that HMGB-1 expression levels in plasma may serve as a biomarker for pneumonia and other diseases (13,15,20). Similarly, the present study demonstrated that HMGB-1 is associated with the pathogenesis of CAP. To the best of our knowledge, this is the first report describing the correlation between plasma HMGB-1 expression and CAP severity.

The present study demonstrated a correlation between the severity of CAP and HMGB-1 expression. The PSI and CURB-65 criteria have been used to evaluate hospital length of stay and mortality rates of CAP patients for numerous years (33-35). However, these indicators require large amount of clinical data and the collection of excessive data. Subsequently, numerous traditional and novel biomarkers successfully emerged to serve as a predictor for the severity of CAP, including WBCs, CRP and procalcitonin (23,24). CRP and WBCs are widely employed in clinical tests. However, they are inadequate for evaluating the severity and mortality risks of CAP.

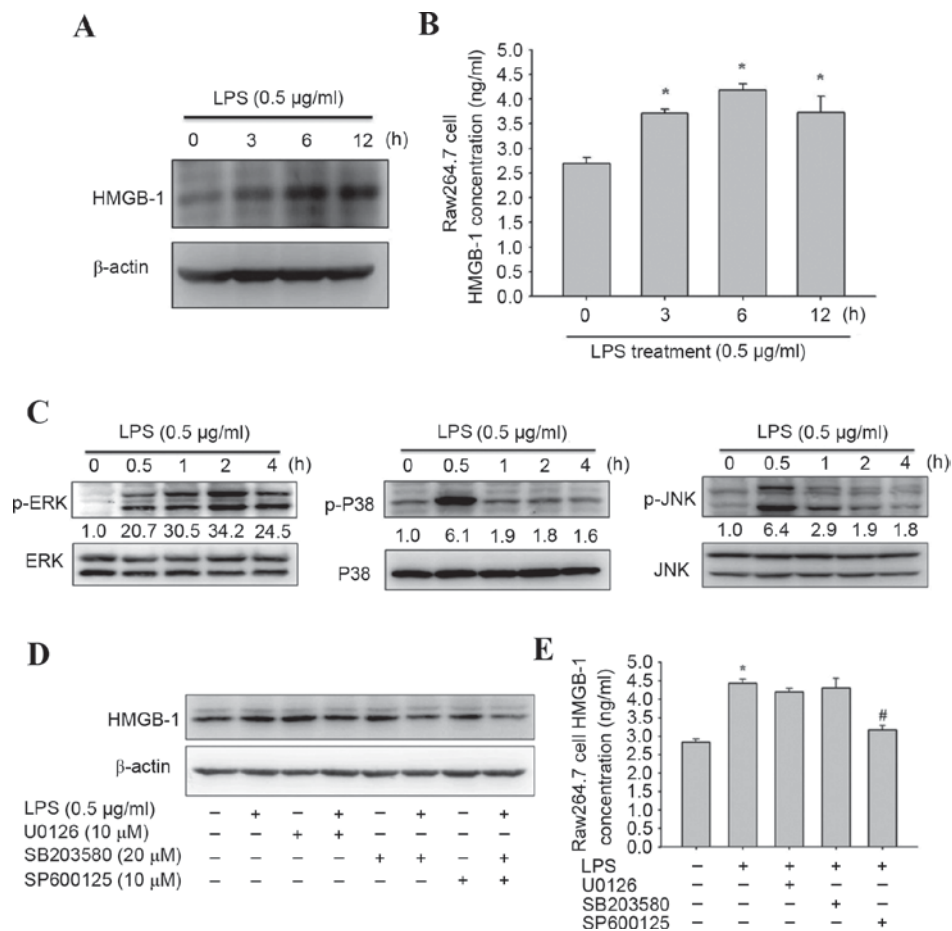


Figure 3. Effects of LPS on HMGB-1 expression and mitogen activated protein kinase signaling pathway proteins. RAW264.7 macrophages were treated with 0.5  $\mu$ g/ml LPS for 0, 3, 6 or 12 h. (A) Representative western blot images of HMGB-1 protein expression levels.  $\beta$ -actin served as an internal control. (B) Quantification of ELISA assay to determine HMGB-1 secretion. (C) RAW264.7 macrophages were treated with 0.5  $\mu$ g/ml LPS for 0, 0.5, 1, 2 or 4 h. Representative western blot images of ERK 1/2, JNK, p38, p-ERK, p-JNK and p-p38 protein expression levels. Total Erk, total p38 and total JNK were the loading controls. (D) Representative western blot images and (E) ELISA assay results of HGMB-1 levels in macrophages following pretreatment with 10  $\mu$ M U0126, 20  $\mu$ M SB203580 or 10  $\mu$ M SP600125 for 30 min, and incubation with 0.5  $\mu$ g/ml LPS for 6 h. Data are expressed as the mean  $\pm$  standard deviation (n=3). \*P<0.05 vs. untreated group; #P<0.05 vs. LPS-treated group. LPS, lipopolysaccharide; p, phosphorylated; ERK, extracellular signal regulated kinase; JNK, c-Jun N-terminal kinase; HMGB-1, high mobility group box-1.

In the past decade, HMGB-1 has been classified as a type of cytokine (1,9). Wang *et al* (10) demonstrated that, following the peak release of early inflammatory cytokines, macrophages begin to release HMGB-1 (10). Another study has indicated that HMGB-1 is a type of late-phase inflammatory cytokine that has greater clinical significance than early inflammatory cytokines such as tumor necrosis factor (TNF) or interleukin-1 (9). Although the association of TNF with the pathogenesis of CAP has been confirmed, its specificity and correlation with CAP severity are relatively poor (36). Furthermore, anti-HMGB-1 antibody treatment significantly reduces the mortality of LPS-induced mice (37). This indicates that HMGB-1 is a late inflammatory mediator of endotoxin lethality. In addition to clinical specimens, the present study examined LPS-induced macrophages to simulate the pathogenesis of pneumonia. These results revealed significant increases in protein expression levels of HMGB1, which are observed in the JNK 1/2 signaling pathway. RAW264.7 cells are widely used as macrophages for *in vitro* experiments. Once these cells are stimulated using LPS, they express and release HMGB-1 and numerous other cytokines (38-40). This method was used in the present study to verify the importance of HMGB-1 in

pneumonia. In addition to cell models, previous studies have indicated in animal models that processing LPS causes lung damage and increases the concentration of HMGB-1 (41,42), which is consistent with the data from the present study. Thus, in addition to serving as an auxiliary factor for the pneumonia severity, HMGB-1 may become an auxiliary tool for pneumonia treatment in the future. One of the limitations of the present study is the lack of microbial data. Thus, future studies are required to detect the association between different microbial pathogens and HMGB-1.

In conclusion, plasma HMGB-1 levels may be used for predicting CAP severity in Taiwanese populations. Plasma HMGB-1 may additionally be applied to distinguish patients with CAP from healthy participants and evaluate the effects of antibiotic treatment on patients with CAP. These results suggested HMGB-1 as a predictive marker for the clinical diagnosis and severity assessment of CAP.

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