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## Original Article

# Protective Effect of an Anti-HMGB-1 Neutralizing Antibody on Hemozoin-Induced Alveolar Epithelial Cell in a Model of Malaria Associated ALI/ARDS

Tachpon Techarang<sup>1,2</sup>, Pitchanee Jariyapong<sup>1</sup>, Parnpen Viriyavejakul<sup>3</sup>, Supattra Glaharn<sup>3</sup>, Charit Srisook<sup>3</sup>, \*Chuchard Punsawad<sup>1,2</sup>

1. Department of Medical Sciences, School of Medicine, Walailak University, Nakhon Si Thammarat, Thailand
2. Tropical Medicine Research Unit, Research Institute for Health Sciences, Walailak University, Nakhon Si Thammarat, Thailand
3. Department of Tropical Pathology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

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**\*Correspondence  
Email:**  
[chuchard.pu@wu.ac.th](mailto:chuchard.pu@wu.ac.th)

### Abstract

**Background:** We aimed to determine whether neutralizing high mobility group box-1 (HMGB-1) prevents the release of HMGB-1 and proinflammatory cytokines on hemozoin (Hz)-induced alveolar epithelial cell in a model of malaria associated ALI/ARDS.

**Methods:** This study was conducted in the Department of Tropical Pathology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand in 2020. Human pulmonary alveolar epithelial cells (HPAEPiCs) were exposed to medium alone or 20  $\mu$ M Hz for 24 h and incubated with different concentrations (1, 5, and 10  $\mu$ g/ml) of anti-HMGB-1 monoclonal antibody (mAb) for various times (0, 4, 12, 24, and 48 h). The levels of HMGB-1, TNF- $\alpha$  and IFN- $\gamma$  in the supernatants were measured by ELISA. The mRNA expression of RAGE, TLR-2 and TLR-4 were analyzed by real-time PCR.

**Results:** The HPAEPiCs treated with 10  $\mu$ g/ml anti-HMGB-1 mAb showed a significant reduction in HMGB-1 release into the supernatant compared with those treated with 1 and 5  $\mu$ g/ml anti-HMGB-1 mAb. The levels of TNF- $\alpha$  and IFN- $\gamma$  were significantly decreased in the supernatant of HPAEPiCs treated with 1, 5, and 10  $\mu$ g/ml anti-HMGB-1 mAb for 4, 12, 24, and 48 h compared with those stimulated with Hz alone. The mRNA expression levels of RAGE, TLR-2, and TLR-4 were significantly decreased after 24 h of anti-HMGB-1 antibody treatment at all concentrations.

**Conclusion:** An anti-HMGB-1 antibody could be an effective agent for inhibiting the release of HMGB-1, TNF- $\alpha$  and IFN- $\gamma$ . Furthermore, a neutralizing anti-HMGB-1 antibody could be applicable for the treatment of malaria-associated ALI/ARDS.



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## Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are major complications of severe malaria and are characterized by the abrupt onset of clinically significant hypoxemia and bilateral diffuse infiltrates (1). The accumulation of hemozoin (Hz), a waste product of hemoglobin digestion produced by the malaria parasite, in the lungs might be an important inflammatory stimulus, leading subsequent malaria-associated ALI/ARDS. The level of pulmonary Hz was significantly correlated with inflammation, lung weight and alveolar edema in malaria-infected mice (2), and high levels of Hz were present in lung autopsies of malaria patients with increased disease severity (3). Furthermore, Hz induced alveolar epithelial cell apoptosis with released proinflammatory cytokines, including tumor necrosis factor alpha (TNF- $\alpha$ ), interferon gamma (IFN- $\gamma$ ), transforming growth factor beta (TGF- $\beta$ ), interleukin 1 (IL-1) and IL-6 (2, 4, 5).

High mobility group box-1 (HMGB-1) is crucially implicated in lung diseases and acts as an independent biomarker and therapeutic target for related lung diseases (6, 7). Levels of circulating HMGB-1 were elevated in *Plasmodium falciparum* malaria patients (8-10) and were related to severe malaria and fatal outcomes (10). In addition, an in vitro study showed HMGB-1 release from human peripheral blood mononuclear cells after stimulation with *P. falciparum*-infected erythrocytes, which was consistent with the results obtained from an analysis of malaria patients with *P. falciparum* infection (10), suggesting that HMGB-1 may play an important role in the pathogenesis of malaria. Importantly, the role of HMGB-1 in malaria-associated ALI/ARDS and the mechanisms that mediate the induction of alveolar epithelial cell injury remain

largely unexplained and represent a major gap in the research to be explored.

HMGB-1 was first described as nuclear DNA binding protein that functions as a pro-inflammatory mediator (6, 11-13) and is involved in several cellular processes, such as maturation, proliferation, motility, survival and death (11). HMGB-1 is expressed in the nuclei of almost all eukaryotic cells and is encoded by the human HMGB1 gene (13q12) (14). HMGB-1 is actively released by inflammatory cells or secreted passively by necrotic and apoptotic cells (6, 11-13). After HMGB-1 is released into the extracellular space, the protein binds to specific receptors on the cell surface, including receptor for advanced glycation end products (RAGE), toll-like receptor-2 (TLR-2) and TLR-4 (10, 12, 15). The interaction between extracellular HMGB-1 and its receptors mediates the release of proinflammatory cytokines and chemokines such as TNF- $\alpha$ , IFN- $\gamma$ , IL-1 and IL-6, contributing to the progression of sepsis (13, 16-18). After administration of anti-HMGB-1 antibody, decreased levels of HMGB-1 and proinflammatory cytokines were found in plasma and pulmonary epithelial lining fluid of patients with ALI/ARDS (18, 19). A previous study on the experimental *P. berghei* ANKA rodent parasite model of severe malaria demonstrated that treatment of malaria-infected mice with HMGB-1 monoclonal antibody did not affect disease outcome, parasitemia or cytokine levels (10).

However, there is little information available about neutralizing strategies using anti-HMGB-1 antibody, which presumably blocks the ability of HMGB-1 to bind to cell surface receptors in malaria-associated ALI/ARDS. Therefore, we aimed to determine whether neutralizing HMGB-1 prevents the release of HMGB-1 and proinflammatory cytokines, as well as the gene expression of receptors for

HMGB-1 in an in vitro model of Hz-induced pulmonary alveolar epithelial cell injury.

## **Materials and Methods**

### ***Cell Culture***

This study was conducted in the Department of Tropical Pathology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand in 2020. Primary human pulmonary alveolar epithelial cells (HPAEpiCs, type II alveolar epithelial cells) were obtained from ScienCell Research Laboratories (Catalog no. 3200, ScienCell Research Laboratories, USA). Cells were cultured in alveolar epithelial cell medium (Catalog no. 3201) containing 10% inactivated fetal bovine serum (Catalog no. 0010), 1% epithelial cell growth supplement (Catalog no. 4152), and 1% penicillin/streptomycin solution (100 U/100 mg/ml, Catalog no. 0503). Cells between passages 4 and 8 were used for the experiments, and the cells were maintained at 37 °C in a 5% CO<sub>2</sub> humidified incubator until use.

### ***Stimulation of Alveolar Epithelial Cell Injury with Hemozoin***

HPAEpiCs were seeded at a density of  $1 \times 10^5$  cell/ml into 24-well plates and grown until they reached 80% confluence. Then, the cells were exposed to Hz (Catalog no. t1rl-hz, InvivoGen, USA) at different concentrations (5, 20, and 50 µM) or complete alveolar epithelial cell medium and incubated in a humidified atmosphere at 37 °C with 5% CO<sub>2</sub> for various times (0.5, 1, 1.5, 2, 3, 4, 12, 24, and 48 h). After stimulation, cell viability was determined, and the number of cells was counted under a light microscope with a hemocytometer after the cells were stained with trypan blue solution (Thermo Fisher Scientific, USA) to calculate the percentage of cell viability.

### ***Hemozoin-Induced Model of Alveolar Epithelial Cell Injury and Treatment with the Anti-HMGB-1 Antibody***

HPAEpiCs at 80% confluence were stimulated with 20 µM Hz or medium alone for 24 h. After 24 h of incubation, the medium was discarded and fresh complete medium with or without various concentrations (1, 5, and 10 µg/ml) of human HMGB-1 monoclonal antibody (IgG clone 951420; Catalog no. MAB16901, R&D Systems, USA) was added and incubated for different times (0, 4, 12, 24, and 48 h). At the end of the incubation time, the supernatant of each culture was collected and stored at -80 °C until use. The cultured cells were fixed in GENEzol™ reagent (Catalog no. GZR100, Geneaid, Taiwan) for RNA extraction.

### ***Enzyme-Linked Immunosorbent Assay***

The levels of HMGB-1, TNF-α and IFN-γ in the supernatant were measured by using commercial ELISA kits (Catalog no. E-EL-H1554, Elabscience, USA; Catalog no. 900-TM25 and 900-TM27, PeproTech, USA) according to the manufacturer's instructions. Briefly, the supernatant was added to the coated plate and incubated for 90 min at 37 °C. After incubation, the contents of each well were discarded, and the detection antibody was added and incubated for 60 min at 37 °C. After the plate was washed with phosphate-buffered saline, horseradish peroxidase-conjugated antibody was added to each well and incubated for 30 min at 37 °C. The substrate solution was added and incubated for 20 min. Finally, the stop solution was added, and the optical density (OD) value was immediately measured at 450 nm. Each assay was performed in triplicate. The detection limits were 25.31 pg/ml for HMGB-1 and 0.16 pg/ml for both TNF-α and IFN-γ. The results are expressed as the mean ± SEM.

### **RNA Extraction and Real-Time Polymerase Chain Reaction (Real-Time PCR)**

Total RNA was extracted by using GENEzol™ reagent (Catalog no. GZR100, Geneaid, Taiwan). The iScript™ Reverse Transcription Supermix for real-time PCR (Catalog no. 1708840, Bio-Rad, USA) was used to construct the first-strand cDNA from the total RNA. Then, the cDNAs were analyzed by using 5× HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Catalog no. 08-24-00001, Solis BioDyne, Estonia) on an ABI 7300 system (Applied Biosystems, CA). The following primers (Bio Basic Inc, Canada) were used in this experiment: RAGE, forward 5'-GACAGAAGCTTGGAAGGTC-3', reverse 5'-GAATCTTACGGTAGACACGG-3'; TLR-2, forward 5'-GGGCAGTCTTGAACATTTAG-3', reverse 5'-TGTCATATTTCCACTCTC-3'; TLR-4, forward 5'-GAAAGTGACAAAACCTGAGC-3', reverse 5'-CTTCAACTGAGAAACCTTG-3'; and β-actin, forward 5'-CAGCACAATGAAGATCAAGA-3', reverse 5'-AAAGGGTGTAAACGCAACTAA-3'. The qPCR conditions were set at 95 °C for 15 min, followed by 40 cycles of 20 sec at 95 °C, 20 sec at 55-58 °C, and 30 sec at 72 °C. Expression of β-actin, the internal control, was used for normalization. The mRNA expression was determined using the  $2^{-\Delta\Delta C_t}$  method (20). Each analysis was performed in triplicate.

### **Statistical Analysis**

The results are presented as the mean ± SEM. The Kolmogorov-Smirnov goodness of fit test was used to test the normality of the data. One-way analysis of variance (ANOVA),

followed by Fisher's least significant difference (LSD) test, was used to determine differences among treatments. The statistical analyses were determined by using IBM SPSS statistics version 23.0 software (SPSS, IL, USA). A value of  $P < 0.05$  was considered to indicate a statistically significant difference.

## **Results**

### **Optimization of the dose and time required for Hz-induced alveolar epithelial cell injury**

The direct effects of synthetic Hz on HPAEpiCs were observed after stimulation with various concentrations of synthetic Hz for different times. Cell viability was evaluated by using trypan blue staining, and the cells were counted to calculate the percentage of cell viability. Table 1 shows the percentages of viable cells after exposure to synthetic Hz. No differences in cell viability were observed after treatment with 5 μM synthetic Hz. After 48 h of incubation, the lowest cell viability was  $44.33 \pm 25.59\%$  after treatment with 20 μM synthetic Hz and  $31.67 \pm 18.28\%$  after treatment with 50 μM synthetic Hz, indicating that these concentrations and time points induced severe damage to cells and were not suitable for pretreatment conditions to induce cell injury. At 24 h after treatment, there was no difference in the percentage of viable cells between the groups treated with 20 μM ( $60.33 \pm 34.83\%$ ) and 50 μM ( $51.00 \pm 29.44\%$ ) Hz. Therefore, 20 μM synthetic Hz for 24 h was selected as the pretreatment condition to induce cell injury.

**Table 1:** Percentage of viable cells after exposure to Hz at different concentrations and times

Times )h(	Percentage of viable cells )mean $\pm$ SEM(			
	Control	5 $\mu$ M Hz	20 $\mu$ M Hz	50 $\mu$ M Hz
0	100.00 $\pm$ 57.73	100.00 $\pm$ 57.73	100.00 $\pm$ 57.73	100.00 $\pm$ 57.73
0.5	100.00 $\pm$ 57.73	98.33 $\pm$ 56.77	94.33 $\pm$ 54.46	92.67 $\pm$ 53.50
1	100.00 $\pm$ 57.73	98.33 $\pm$ 56.77	90.33 $\pm$ 52.15	85.60 $\pm$ 49.46
1.5	100.00 $\pm$ 57.73	97.33 $\pm$ 56.19	84.67 $\pm$ 48.88	78.33 $\pm$ 45.22
2	100.00 $\pm$ 57.73	96.67 $\pm$ 55.81	81.33 $\pm$ 46.95	73.67 $\pm$ 42.53
3	100.00 $\pm$ 57.73	95.33 $\pm$ 55.04	76.67 $\pm$ 44.26	70.33 $\pm$ 40.60
4	100.00 $\pm$ 57.73	95.00 $\pm$ 54.85	76.00 $\pm$ 43.88	65.67 $\pm$ 37.91
12	100.00 $\pm$ 57.73	93.33 $\pm$ 53.88	65.67 $\pm$ 37.91	57.33 $\pm$ 33.10
24	100.00 $\pm$ 57.73	90.67 $\pm$ 52.35	60.33 $\pm$ 34.83	51.00 $\pm$ 29.44
48	99.33 $\pm$ 57.35	73.33 $\pm$ 42.34	44.33 $\pm$ 25.59	31.67 $\pm$ 18.28

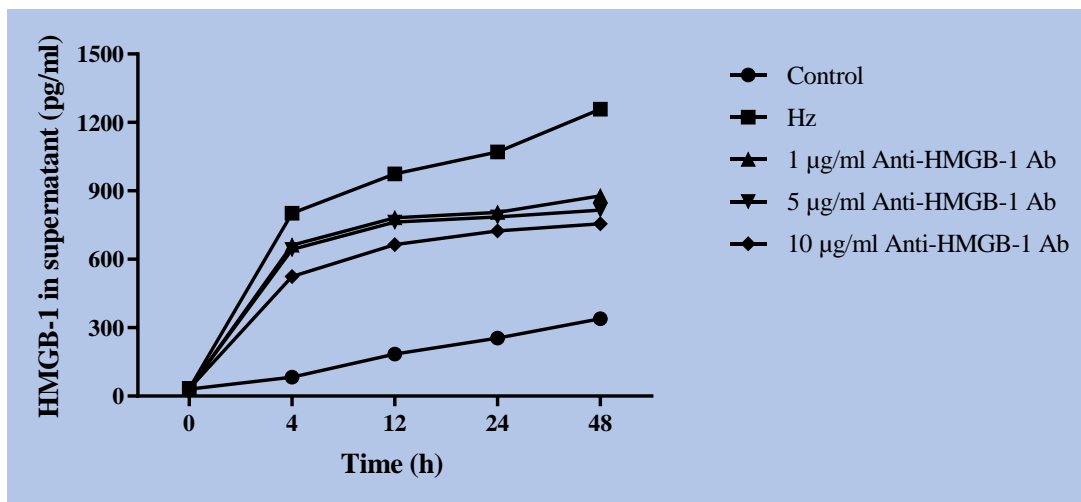
#### *Effect of anti-HMGB-1 neutralizing antibody on the release of HMGB-1*

To examine whether the anti-HMGB-1 neutralizing antibody inhibits the release of HMGB-1, different concentrations (1, 5, and 10  $\mu$ g/ml) of anti-HMGB-1 monoclonal antibody were used for different times (0, 4, 12, 24, and 48 h) after stimulation with 20  $\mu$ M synthetic Hz for 24 h. The culture supernatant was collected, and HMGB-1 levels were measured by using ELISA. The results showed that the levels of HMGB-1 after treatment with the anti-HMGB-1 monoclonal antibody were reduced in a dose-dependent manner, whereas the supernatant of HPAEpiCs exposed to synthetic Hz had increased HMGB-1 levels in a time-dependent manner. After treatment with at all concentrations of the anti-HMGB-1 monoclonal antibody, the levels of HMGB-1 in the supernatant were significantly decreased compared with those of HPAEpiCs exposed to synthetic Hz alone ( $P < 0.05$ ) (Fig. 1). At all doses of the antibody, the levels of HMGB-1 at 4 h after treatment were lower than those at 12, 24 and 48 h after treatment. HPAEpiCs treated with 10  $\mu$ g/ml anti-HMGB-1 monoclonal antibody showed a significant reduction in HMGB-1 release into the supernatant compared with those treated

with 1  $\mu$ g/ml and 5  $\mu$ g/ml anti-HMGB-1 monoclonal antibody (Fig. 1). The lowest level of HMGB-1 in the supernatant was observed after treatment with 10  $\mu$ g/ml anti-HMGB-1 monoclonal antibody for 4 h (Fig. 1). These findings indicate that HMGB-1 neutralization using an anti-HMGB-1 monoclonal antibody could reduce the release of HMGB-1 from HPAEpiCs after stimulation with synthetic Hz.

#### *Effect of the anti-HMGB-1 neutralizing antibody on the release of proinflammatory cytokines*

Hz induced cell apoptosis and proinflammatory cytokine secretion in an in vitro culture model (4, 5). To examine the effects of the anti-HMGB-1 neutralizing antibody on the release of proinflammatory cytokines, the levels of TNF- $\alpha$  and IFN- $\gamma$  in the culture supernatants were analyzed by ELISA after anti-HMGB-1 monoclonal antibody treatment. As expected, the levels of TNF- $\alpha$  and IFN- $\gamma$  were substantially increased in the culture supernatants of HPAEpiCs stimulated with synthetic Hz as a positive control. As shown in Fig. 2a and Fig. 2b, neutralization of HMGB-1 using the anti-HMGB-1 monoclonal antibody inhibited the release of TNF- $\alpha$  and IFN- $\gamma$  in a dose-dependent manner.

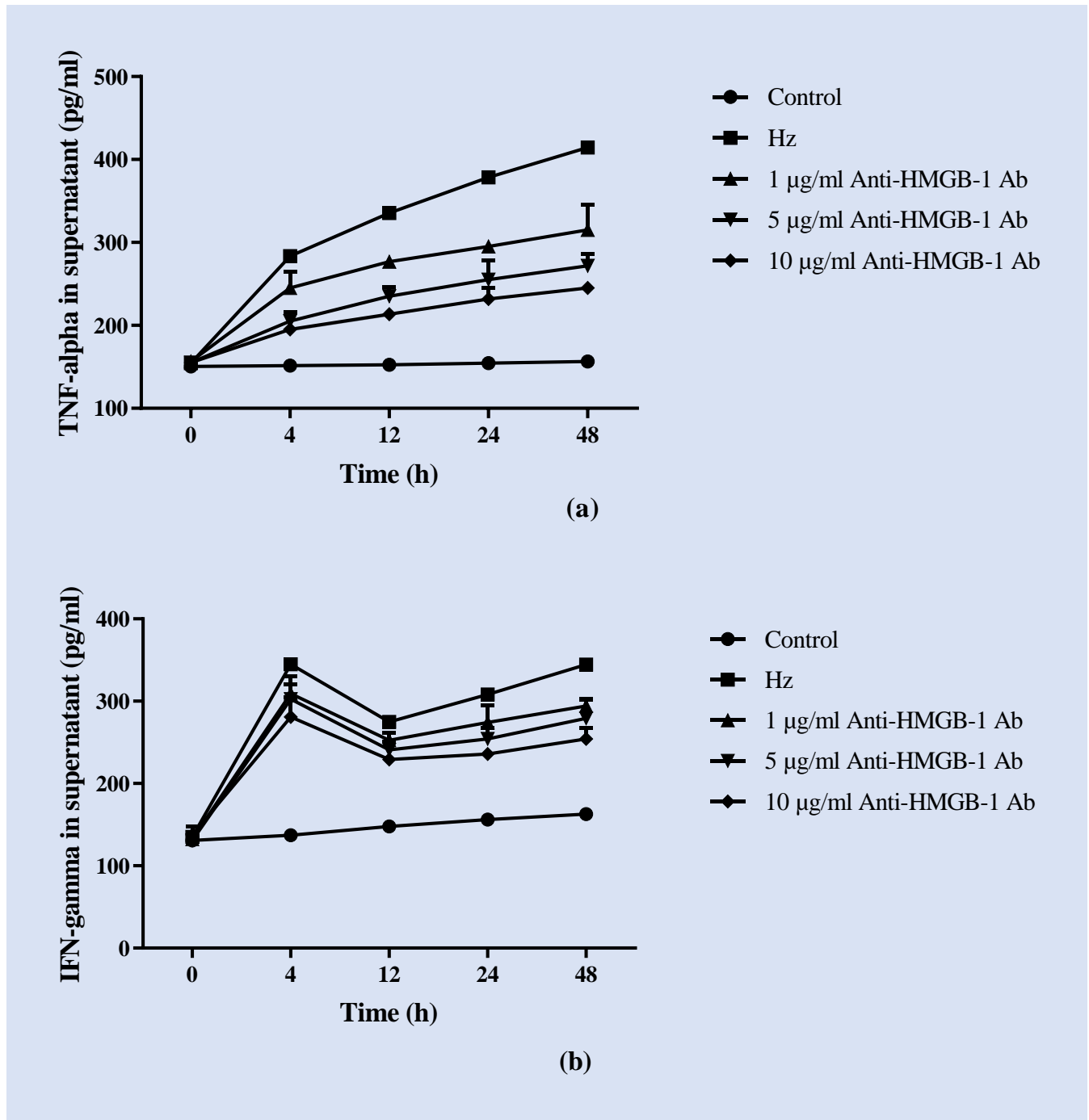


**Fig. 1:** Levels of HMGB-1 in the culture supernatant of human pulmonary alveolar epithelial cells (HPAEpiCs) after stimulation with 20  $\mu$ M synthetic Hz and treatment with different concentrations (1, 5, and 10  $\mu$ g/ml) of anti-HMGB-1 monoclonal antibody for different times (0, 4, 12, 24, and 48 h). The levels of HMGB-1 were measured by ELISA. The data are expressed as the mean  $\pm$  SEM of 3 independent experiments

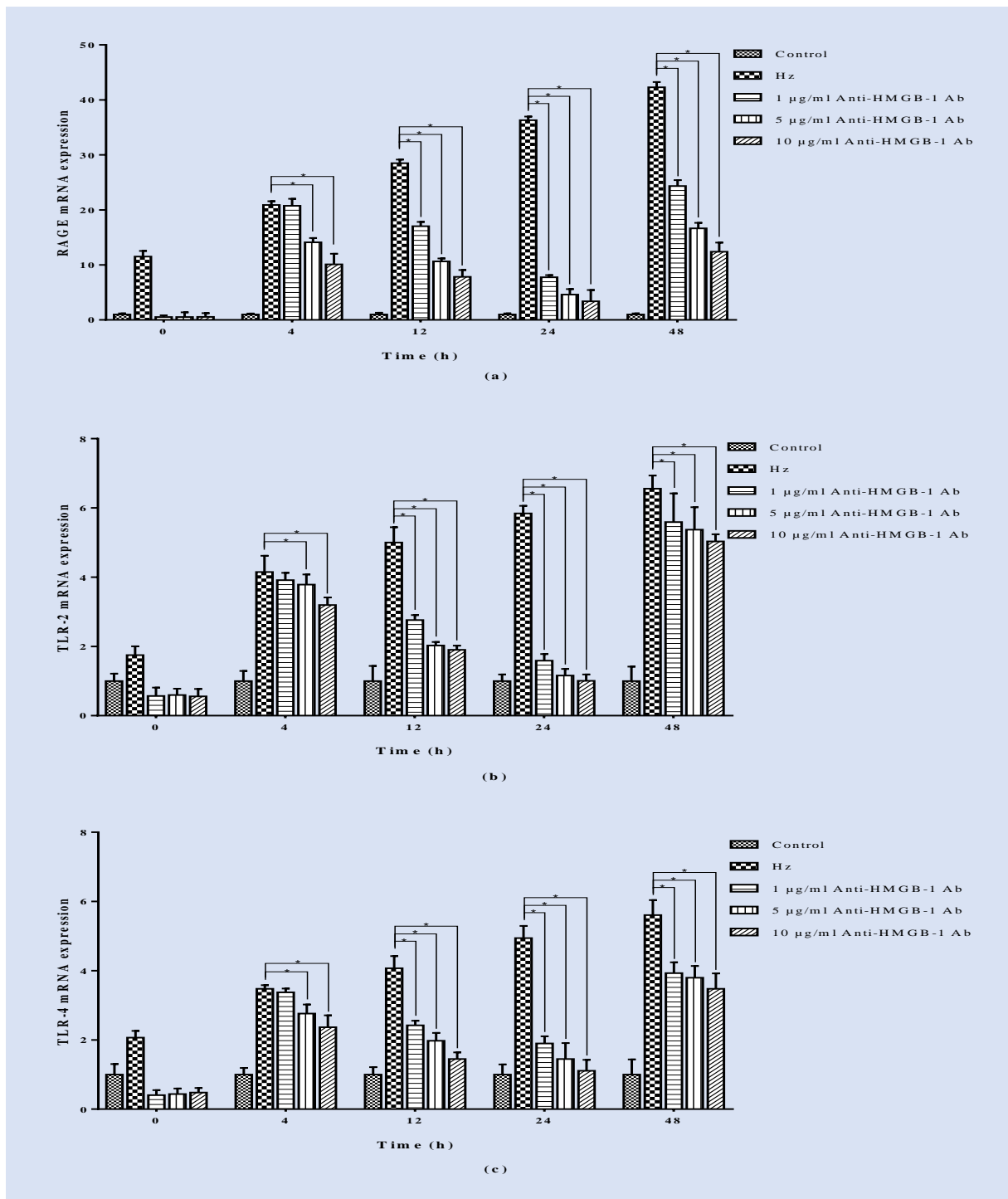
The levels of TNF- $\alpha$  and IFN- $\gamma$  were significantly decreased in the supernatant of HPAEpiCs treated with 1, 5, and 10  $\mu$ g/ml anti-HMGB-1 monoclonal antibody for 4, 12, 24, and 48 h compared with those in the supernatants of HPAEpiCs stimulated with synthetic Hz alone ( $P < 0.05$ ). The levels of TNF- $\alpha$  and IFN- $\gamma$  were lower in the supernatant of HPAEpiCs treated with 10  $\mu$ g/ml anti-HMGB-1 monoclonal antibody at all-time points than in the culture supernatants of HPAEpiCs stimulated with synthetic Hz alone. After treatment with 10  $\mu$ g/ml HMGB-1 monoclonal antibody, the lowest levels of TNF- $\alpha$  occurred at 4 h, but the levels of IFN- $\gamma$  began to decrease gradually after 4 h, reaching a minimum at 12 h, which increased slightly at 48 h. These results indicate that the anti-HMGB-1 antibody could be an effective agent to inhibit the release of TNF- $\alpha$  and IFN- $\gamma$ .

#### ***Effect of the anti-HMGB-1 neutralizing antibody on the expression of receptors for HMGB-1***

To investigate the mRNA expression of HMGB-1 receptors (RAGE, TLR-2 and TLR-4) in HPAEpiCs after exposure to synthetic Hz and treatment with the anti-HMGB-1 monoclonal antibody, mRNA expression was analyzed and quantified by using real-time PCR. Our results demonstrated that the mRNA expression of RAGE, TLR-2 and TLR-4 was significantly increased in HPAEpiCs stimulated with 20  $\mu$ M synthetic Hz in a time-dependent manner (Fig. 3). After exposure to synthetic Hz, RAGE showed the highest expression compared to that of TLR-2 and TLR-4. Treatment with 1, 5, and 10  $\mu$ g/ml anti-HMGB-1 antibody significantly decreased the mRNA expression levels of RAGE, TLR-2, and TLR-4 at 24 h compared with their expression at 12 h. However, at 48 h of anti-HMGB-1 antibody treatment with all concentrations, the expression levels of RAGE, TLR-2, and TLR-4 were significantly increased compared with those at 24 h (Fig. 3).



**Fig. 2:** The levels of TNF- $\alpha$  and IFN- $\gamma$  in the culture supernatant of human pulmonary alveolar epithelial cells (HPAEpiCs) after stimulation with 20  $\mu$ M synthetic Hz and treatment with different concentrations (1, 5, and 10  $\mu$ g/ml) of anti-HMGB-1 monoclonal antibody for different times (0, 4, 12, 24, and 48 h). (a) The level of TNF- $\alpha$  in the culture supernatant. (b) The level of IFN- $\gamma$  in the culture supernatant. The data are expressed as the mean  $\pm$  SEM of 3 independent experiments



**Fig. 3:** The mRNA expression of RAGE, TLR-2 and TLR-4 in human pulmonary alveolar epithelial cells (HPAEPiCs) after stimulation with 20 µM synthetic Hz and treatment with different concentrations (1, 5, and 10 µg/ml) of anti-HMGB-1 monoclonal antibody for different times (0, 4, 12, 24, and 48 h). (a) The mRNA expression of RAGE. (b) The mRNA expression of TLR-2. (c) The mRNA expression of TLR-4. The data are expressed as the mean ± SEM of 3 independent experiments. \* $P < 0.05$



## Discussion

The present study demonstrated that synthetic Hz induced HPAEpiC injury and cytokine release. The levels of HMGB-1, TNF- $\alpha$  and IFN- $\gamma$  in the supernatant of HPAEpiCs stimulated with synthetic Hz were significantly increased compared with those of the control group. These results were similar to those of previous reports on the effect of Hz (4, 5, 21-23). Hz directly induced cell apoptosis in both in vitro (4, 5) and in vivo studies (23), resulting in the release of chemokines and proinflammatory cytokines (IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$ , and IFN- $\gamma$ ) (2, 4, 5, 21-23). Consistently, our study suggested that Hz induced alveolar epithelial cell injury and proinflammatory cytokine release into the supernatant. HMGB-1 is a protein that is predominantly found in the nuclei of all cell types (11, 12). However, the protein HMGB-1 is also released into the extracellular space in response to cell injury or cell death conditions (12, 24), resulting in high levels of extracellular HMGB-1. In early apoptosis, the levels of extracellular HMGB-1 are not significantly different, while in late apoptosis, high levels of extracellular HMGB-1 and proinflammatory cytokines such as IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and IFN- $\gamma$  are observed (6, 12, 25). This study suggested that HMGB-1 can also be released by apoptotic cells at a late stage, which might be stimulated by proinflammatory cytokines.

After administration of different concentrations of the anti-HMGB-1 neutralizing antibody, the levels of HMGB-1 and proinflammatory cytokines were significantly decreased in the supernatant of HPAEpiCs, indicating that a high concentration of anti-HMGB-1 neutralizing antibody has potent effects in preventing the release of HMGB-1 and proinflammatory cytokines. Anti-HMGB-1 treatment showed beneficial effects in several experimental models, such as sepsis and ALI/ARDS (25-28). An anti-HMGB-1 neu-

tralizing antibody downregulated the expression of HMGB-1, chemokines and proinflammatory cytokines (25-27). This study suggested that the reduction in HMGB-1 and proinflammatory cytokines in the supernatant may be due to anti-HMGB-1 neutralizing antibody-mediated blocking of HMGB-1, leading to inhibition of HMGB-1 binding to its receptors and activation of the transcription factor NF- $\kappa$ B (29).

Our results showed that the mRNA levels of RAGE, TLR-2 and TLR-4 were significantly increased in HPAEpiCs exposed to synthetic Hz compared with the control and anti-HMGB-1 neutralizing antibody treatment groups. In this study, the RAGE exhibited the highest mRNA expression in HPAEpiCs after stimulation with synthetic Hz. After anti-HMGB-1 neutralizing antibody treatment at all concentrations, the mRNA levels of RAGE, TLR-2, and TLR-4 were significantly downregulated compared with those of Hz treatment alone. These findings were consistent with previous studies demonstrating the reduction in RAGE, TLR-2 and TLR-4 mRNA expression after anti-HMGB-1 neutralizing antibody treatment (30-32). The interaction between HMGB-1 and its receptors (RAGE, TLR-2 and TLR-4) has been reported in earlier studies (15, 33, 34). It has been reported that the interaction between HMGB-1 and RAGE is involved in cell migration, pyroptosis, and the inflammatory response (12, 33). A previous study demonstrated that decreased mRNA expression of RAGE, TLR-2 and TLR-4 was observed after administration of anti-HMGB-1 antibody (30), leading to reduced secretion of proinflammatory cytokines. Taken together, these results suggest that the anti-HMGB-1 neutralizing antibody might exert its effects through HMGB-1 and its receptor signaling pathway.

Further studies are required to determine the basic mechanism underlying the prophylactic effects of the anti-HMGB-1 antibody. Addi-

tionally, the effect of an anti-HMGB1 neutralizing antibody on disease progression and outcome should be assessed in a model of malaria-associated ALI/ARDS.

## Conclusion

HPAEpiCs treated with 10 µg/ml anti-HMGB-1 monoclonal antibody showed a significant reduction in HMGB-1 release compared with those treated with lower antibody concentrations. The levels of TNF-α and IFN-γ were significantly decreased in the supernatant of HPAEpiCs treated with all concentrations of anti-HMGB-1 monoclonal antibody. The mRNA expression levels of RAGE, TLR-2, and TLR-4 were significantly decreased after 24 h of anti-HMGB-1 antibody treatment at all concentrations compared with those after 12 h of treatment. Therefore, this study indicates that the neutralization of HMGB-1 using an anti-HMGB-1 monoclonal antibody reduces the release of HMGB-1 from HPAEpiCs after stimulation with synthetic Hz and could be applicable for the treatment of malaria-associated ALI/ARDS.

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## Conflict of interest

The authors declared that there is no conflict of interest.

## References

1. Mohan A, Sharma S, Bollineni S. Acute lung injury and acute respiratory distress syndrome in malaria. *J Vector Borne Dis.* 2008; 45(3):179-93.
2. Deroost K, Tyberghein A, Lays N, et al. Hemozoin induces lung inflammation and correlates with malaria-associated acute respiratory distress syndrome. *Am J Respir Cell Mol Biol.* 2013; 48(5):589-600.
3. Milner D, Factor R, Whitten R, et al. Pulmonary pathology in pediatric cerebral malaria. *Hum Pathol.* 2013;44(12):2719-26.
4. Maknitikul S, Luplertlop N, Grau GER, et al. Dysregulation of pulmonary endothelial protein C receptor and thrombomodulin in severe falciparum malaria-associated ARDS relevant to hemozoin. *PloS One.* 2017; 12(7):e0181674.
5. Maknitikul S, Luplertlop N, Chaisri U, et al. Featured article: Immunomodulatory effect of hemozoin on pneumocyte apoptosis via CARD9 pathway, a possibly retarding pulmonary resolution. *Exp Biol Med (Maywood).* 2018;243(5):395-407.
6. Ding J, Cui X, Liu Q. Emerging role of HMGB1 in lung diseases: Friend or foe. *J Cell Mol Med.* 2017;21(6):1046-1057.
7. Ying S, Jiang Z, He X, et al. Serum HMGB1 as a potential biomarker for patients with asbestos-related diseases. *Dis Markers.* 2017;2017:5756102.
8. Angeletti D, Kiwuwa MS, Byarugaba J, et al. Elevated levels of high-mobility group box-1 (HMGB1) in patients with severe or uncomplicated *Plasmodium falciparum* malaria. *Am J Trop Med Hyg.* 2013;88(4):733-5.
9. Alleva LM, Yang H, Tracey KJ, et al. High mobility group box 1 (HMGB1) protein: Possible amplification signal in the pathogenesis of falciparum malaria. *Trans R Soc Trop Med Hyg.* 2005;99(3):171-4.
10. Higgins SJ, Xing K, Kim H, et al. Systemic release of high mobility group box 1 (HMGB1) protein is associated with severe and fatal *Plasmodium falciparum* malaria. *Malar J.* 2013;12:105.
11. Lee S, Kwak M, Kim S, et al. The role of high mobility group box 1 in innate immunity. *Yonsei Med J.* 2014;55(5):1165-76.

12. Yang H, Wang H, Chavan SS, et al. High mobility group box protein 1 (HMGB1): The prototypical endogenous danger molecule. *Mol Med.* 2015;21 Suppl 1:S6-S12.
13. Mantell LL, Parrish WR, Ulloa L. HMGB-1 as a therapeutic target for infectious and inflammatory disorders. *Shock.* 2006; 25(1):4-11.
14. Sohun M, Shen H. The implication and potential applications of high-mobility group box 1 protein in breast cancer. *Ann Transl Med.* 2016; 4(11):217.
15. Yang H, Wang H, Ju Z, et al. MD-2 is required for disulfide HMGB1-dependent TLR4 signaling. *J Exp Med.* 2015;212(1):5-14.
16. Magna M, Pisetsky DS. The role of HMGB1 in the pathogenesis of inflammatory and autoimmune diseases. *Mol Med.* 2014; 20(1): 138-146.
17. Takeda K, Akira S. Toll-like receptors in innate immunity. *Int Immunol.* 2005;17(1):1-14.
18. Ueno H, Matsuda T, Hashimoto S, et al. Contributions of high mobility group box protein in experimental and clinical acute lung injury. *Am J Respir Crit Care Med.* 2004; 170(12):1310-6.
19. Zaher TE, Miller EJ, Morrow DMP, et al. Hyperoxia-induced signal transduction pathways in pulmonary epithelial cells. *Free Radic Biol Med.* 2007;42(7):897-908.
20. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods.* 2001; 25(4):402-8.
21. Griffith JW, Sun T, McIntosh MT, et al. Pure hemozoin is inflammatory in vivo and activates the NALP3 inflammasome via release of uric acid. *J Immunol.* 2009;183(8):5208-5220.
22. Bobade D, Khandare AV, Deval M, et al. Hemozoin-induced activation of human monocytes toward M2-like phenotype is partially reversed by antimalarial drugs-chloroquine and artemisinin. *Microbiolopen.* 2019; 8(3):e00651-e00651.
23. Jaramillo M, Plante I, Ouellet N, et al. Hemozoin-inducible proinflammatory events in vivo: Potential role in malaria infection. *J Immunol.* 2004;172(5):3101-10.
24. Lu B, Wang H, Andersson U, et al. Regulation of HMGB1 release by inflammasomes. *Protein Cell.* 2013;4(3):163-167.
25. Yoshihara-Hirata C, Yamashiro K, Yamamoto T, et al. Anti-HMGB1 neutralizing antibody attenuates periodontal inflammation and bone resorption in a murine periodontitis model. *Infect Immun.* 2018;86(5):e00111-18.
26. Yang H, Ochani M, Li J, Qiang X, et al. Reversing established sepsis with antagonists of endogenous high-mobility group box 1. *Proc Natl Acad Sci U S A.* 2004; 101(1):296-301.
27. Gong Q, Xu JF, Yin H, et al. Protective effect of antagonist of high-mobility group box 1 on lipopolysaccharide-induced acute lung injury in mice. *Scand J Immunol.* 2009;69(1):29-35.
28. Tsung A, Sahai R, Tanaka H, et al. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. *J Exp Med.* 2005;201(7):1135-1143.
29. Andersson U, Tracey KJ. HMGB1 is a therapeutic target for sterile inflammation and infection. *Annu Rev Immunol.* 2011;29:139-62.
30. Wang D, Liu K, Wake H, et al. Anti-high mobility group box-1 (HMGB1) antibody inhibits hemorrhage-induced brain injury and improved neurological deficits in rats. *Sci Rep.* 2017;7:46243.
31. Kokkola R, Andersson A, Mullins G, et al. RAGE is the major receptor for the proinflammatory activity of HMGB1 in rodent macrophages. *Scand J Immunol.* 2005;61(1):1-9.
32. Yang H, Liu H, Zeng Q, et al. Inhibition of HMGB1/RAGE-mediated endocytosis by HMGB1 antagonist box A, anti-HMGB1 antibodies, and cholinergic agonists suppresses inflammation. *Mol Med.* 2019; 25(1):13.
33. van Zoelen MAD, Yang H, Florquin S, et al. Role of toll-like receptors 2 and 4, and the receptor for advanced glycation end products in high-mobility group box 1-induced inflammation in vivo. *Shock.* 2009;31(3):280-4.
34. van Beijnum JR, Buurman WA, Griffioen AW. Convergence and amplification of toll-like receptor (TLR) and receptor for advanced glycation end products (RAGE) signaling pathways via high mobility group B1 (HMGB1). *Angiogenesis.* 2008;11(1):91-9.