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Research article

High mobility group box-1 (HMGB-1) and its receptors in the pathogenesis of malaria-associated acute lung injury/acute respiratory distress syndrome in a mouse model

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ABSTRACT

The DNA-binding protein high mobility group box-1 (HMGB-1) mediates proinflammatory cytokines that contribute to acute lung injury (ALI). Although ALI is a frequent complication of malaria infection, the contribution of HMGB-1 and its receptors to the pathogenesis of malaria-associated ALI/acute respiratory distress syndrome (MA-ALI/ARDS) has not been investigated in a mouse model. Here, the malaria-infected mice were divided into two groups according to lung injury score: the ALI/ARDS and non-ALI/ARDS groups. The expression of HMGB-1 and its receptors (RAGE, TLR-2 and TLR-4) in lung tissues was investigated by using immunohistochemical staining and real-time polymerase chain reaction (PCR). Additionally, HMGB-1 and proinflammatory cytokine (TNF-α, IFN-γ, IL-1 and IL-6) levels in plasma and lung tissues were quantified by using enzyme-linked immunosorbent assays. Cellular expression of both HMGB-1 and its receptors (RAGE, TLR-2 and TLR-4) was significantly increased in the lung tissues of the ALI/ARDS group compared with those in the non-ALI/ARDS and control groups. The levels of HMGB-1, TNF-α, IFN-γ, IL-1 and IL-6 were significantly increased in both plasma and lung tissues of the ALI/ARDS group compared with those in the non-ALI/ARDS and control groups, which were similar to the results obtained by real-time PCR. Increased mRNA expression of RAGE, TLR-2 and TLR-4 was found in the lung tissues of the ALI/ARDS group. Furthermore, the plasma HMGB-1 level was positively correlated with TLR-4 mRNA expression in the ALI/ARDS group. HMGB-1 levels were significantly increased in plasma and lung tissues of MA-ALI/ARDS mice and were related to the upregulated expression of HMGB-1 and proinflammatory cytokines. In conclusion, this study demonstrates that HMGB-1 is an important mediator of MA-ALI/ARDS pathogenesis and may represent a target for therapeutic malaria interventions with ALI/ARDS.

1. Introduction

High mobility group box-1 (HMGB-1) is a nuclear DNA-binding protein that plays a role in several cellular processes, including inflammation, cell differentiation and tumour cell migration [1]. HMGB-1 can be released from the cell into extracellular space by active and passive pathways [2] after infection or stimulation of endogenous danger signals, such as bacteria, viruses, lipopolysaccharide (LPS), and extracellular adenosine triphosphate (ATP) [3, 4]. Subsequently, HMGB-1 proteins can interact with multiple cell surface receptors, including receptor for advanced glycation end products (RAGE), which is the most common binding site of the HMGB-1 protein, toll-like receptor-2 (TLR-2) and TLR-4 [5, 6, 7]. Previous studies implicated HMGB-1 in several conditions,

including infectious diseases, vascular diseases, neurodegenerative diseases, and cancer [2, 8, 9]. In the context of infectious diseases, a high level of HMGB-1 was found in the blood circulation of patients with pneumonia [10], leading to the upregulation of proinflammatory cytokines [11], lung tissue injury and pulmonary oedema [9]. In tuberculosis, the level of HMGB-1 was significantly increased in the bronchoalveolar lavage fluid in a mouse model [12], and the levels of HMGB-1 and interleukin-6 (IL-6) were significantly elevated in both plasma and sputum from tuberculosis patients [13]. Regarding malaria infection, elevated plasma levels of HMGB-1 were reported in malaria patients with *Plasmodium falciparum* infection and was associated with disease severity and a subsequent fatal outcome [5, 14]. In addition, an *in vitro* study demonstrated that activation of blood mononuclear cells by *Plasmodium*-infected red blood cells

Table 1. Histopathological changes and grading criteria used to evaluate lung tissues.

Histopathological changes	Histopathological grading			
	0	1	2	3
Alveolar fibrin	Absent	Present		
Alveolar oedema	Absent	Present		
Septal congestion	Absent	Present		
Leukocyte infiltration	Absent	1–5 cells/HPF	≥5 cells/HPF	
Malaria pigment in macrophages	Absent	≤10%/HPF	>10–40%/HPF	>40%/HPF
%Parasitized red blood cells	Absent	≤5%/HPF	>5–10%/HPF	>10%/HPF
Alveolar haemorrhage	Absent	≤25%/HPF	>25–50%/HPF	>50%/HPF
Septal thickness	Absent	≤25%/LPF	>25–50%/LPF	>50%/LPF

HPF = high power field (magnification 400×); LPF = low power field (magnification 100×).

Table 2. List of primers used in this study.

Gene	Primer sequence
HMGB-1	(F) 5′-GCTTATCCATTGGTGATGTT-3′ (R) 5′-CTCTGTAGGCAGCAATATCC-3′
RAGE	(F) 5′-ACAGCCAGTGTCCCTAATAA-3′ (R) 5′-TCCTTCACGAGTGTTTCTTT-3′
TLR-2	(F) 5′-GATGCCTTTGTTTCCTACAG-3′ (R) 5′-GTGGAGACACAGCTTAAAGG-3′
TLR-4	(F) 5′-ACCTCTGCCTTCACTACAGA-3′ (R) 5′-AGGGACTTCTCAACCTTCTC-3′
TNF-α	(F) 5′-GCCGATTTGCTATCTCATAC-3′ (R) 5′-TGGGTAGAGAATGGATGAAC-3′
IFN-γ	(F) 5′-GAGGTCAACAACCCACAGGT-3′ (R) 5′- GGGACAATCTCTTCCCCACC -3′
IL-1β	(F) 5′-CTAAAGTATGGGCTGGACTG-3′ (R) 5′-GGCTCTCTTTGAACAGAATG-3′
IL-6	(F) 5′-AATGATGGATGCTACCAAAC-3′ (R) 5′-TAGCCACTCCTTCTGTGACT-3′
β-actin	(F) 5′-GGACCTGACAGACTACCTCA-3′ (R) 5′-GTTGCCAATAGTGATGACCT-3′

could stimulate the release of HMGB-1 into the extracellular space [5, 14]. Based on published studies, several other authors have also agreed that extracellular HMGB-1 is an important mediator of the proinflammatory cytokine response and contributes to the development of acute lung injury/acute respiratory distress syndrome (ALI/ARDS) during malaria infection. Therefore, to understand the role of HMGB-1 and its receptors in the pathogenesis of malaria-associated ALI/ARDS (MA-ALI/ARDS), this study aimed to investigate HMGB-1 and its receptors in mice with MA-ALI/ARDS and demonstrate the relationship between HMGB-1, its receptors and proinflammatory cytokines.

2. Materials and methods

2.1. Experimental animal and sample collection

Male C57BL/6 mice weighing between 20-25 g were obtained from Nomura Siam International Co. Ltd. (Pathumwan, Bangkok, Thailand). The mice were maintained under specific pathogen-free conditions with a 12-hour light/12-hour dark cycle and received diet and water *ad libitum* for one week before the experiment began. Twenty-eight mice were intraperitoneally injected with 1×10^6 *Plasmodium berghei* (*P. berghei*) ANKA-infected red blood cells as previously described [15], and the

control mice (n = 10) were intraperitoneally injected with phosphate-buffered saline (PBS). After induction, parasitaemia was monitored every day by thin-blood smear staining with Wright's Giemsa. At day 14 post-infection, the mice were anaesthetized by intraperitoneal injection with pentobarbital sodium (60 mg/kg body weight) (Ceva Sante Animale, Netherlands), and blood samples were collected by cardiac puncture. Heparinized blood was centrifuged at 3,000 g for 10 min. Plasma samples were collected and stored at -80 °C until analysed for HMGB-1 and proinflammatory cytokine levels. In addition, lung tissues were harvested immediately for histopathological, immunohistochemical, and real-time polymerase chain reaction (PCR) examinations. Ethical clearance was reviewed and approved by the Animal Ethics Committee, Walialak University, Thailand (Protocol number 008/2018).

2.2. Histopathological examination

The formalin-fixed lung specimens were dehydrated in a graded series of ethanol, cleared in xylene, infiltrated and embedded in paraffin. Next, the embedded specimens were serially sectioned at a 5 μm thickness and stained with haematoxylin and eosin (H&E). The histopathological scoring method was used to quantify pathological alterations in lung tissue under a light microscope by two observers who were blinded to the experimental group allocations. The degree of the histopathological alteration was scored on the following 8 variables: alveolar oedema, alveolar haemorrhage, alveolar fibrin, leukocyte infiltration, malaria pigment, septal congestion, septal thickening and percentage of parasitized red blood cells (%PRBC) in blood vessels [16]. The details of the grading criteria of each variable that was used to evaluate the lung tissues are described in Table 1. Finally, to quantify the overall histopathological changes, the lung injury score (LIS) was calculated by adding all scores, for a total of 0–17. A score of 0 meant no injury, while a score of 17 meant maximum severity. According to the data analysis, a score of 11 points with 90.9% sensitivity and 88.2% specificity was determined as the optimum cut-off for group classification. A LIS greater than 11 was classified as the ALI/ARDS group, whereas a LIS less than 11 was considered the non-ALI/ARDS group. In this study, eleven (39.28%) malaria-infected mice were classified as the ALI/ARDS group, whereas seventeen mice (60.71%) were classified as mice without ALI/ARDS or the non-ALI/ARDS group, according to the LIS. The mean percentage of parasitaemia in the ALI/ARDS and non-ALI/ARDS groups was 29.49 ± 2.12% and 27.92 ± 1.56%, respectively.

2.3. Immunohistochemical staining

The lung paraffin sections were deparaffinized in xylene and rehydrated in a series of ethanol dilutions. To quench endogenous peroxidase, the sections were incubated in 3% hydrogen peroxide for 10 min (Sigma Aldrich Co. Ltd., USA). For antigen retrieval, the sections were incubated in a citrate-based solution, pH 6.0 (Vector Laboratories Inc., USA), and heated with a microwave. The sections were washed in PBS and blocked in normal goat serum for 30 min to inhibit non-specific proteins. Next, the sections were separately incubated overnight with rabbit anti-mouse HMGB-1 (dilution: 1:400; Cat. # ab18256, Abcam, UK), RAGE (dilution: 1:1000; Cat. # ab361, Abcam, UK), TLR-2 (dilution: 1:200; Cat. # ab213676, Abcam, UK) or TLR-4 (dilution: 1:200; Cat. # ab13556, Abcam, UK) primary antibodies. The sections were incubated with biotinylated secondary antibodies for 30 min, followed by incubation with VECTAS-TAIN ABC reagent (Vector Laboratories Inc., USA) for 30 min at room temperature. Diaminobenzidine/H_2O_2 was used as a substrate for the immune-peroxidase reaction. Then, the sections were counterstained with Harris haematoxylin (Merck, Germany) for 5 min, dehydrated with a series of ethanol dilutions and mounted for analysis under a light microscope. The negative control was processed in the same manner but without primary antibodies. To evaluate the expression of HMGB-1, RAGE, TLR-2 and TLR-4, each immunostained section was randomly counted in 10 microscopic fields at high magnification (400×) under a light microscope by two observers who were blinded to the experimental group allocations.
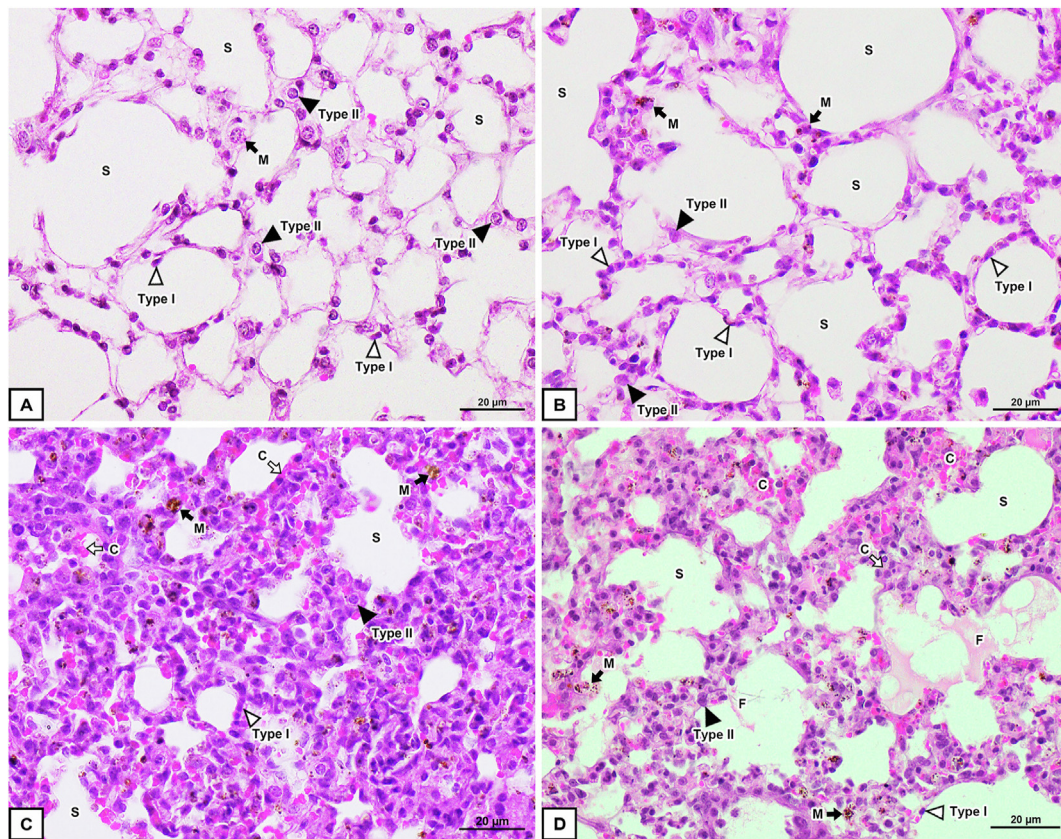
Figure 1. Histopathological changes in H&E-stained lung tissues in the control (A), malaria-infected without ALI/ARDS (B) and malaria-infected with ALI/ARDS (C and D) groups. Malaria-infected mice with ALI/ARDS showing alveolar oedema, thickening of alveolar septa and infiltration of leukocytes. Type I alveolar epithelial cell (Type I), Type II alveolar epithelial cell (Type II), alveolar macrophage (M), alveolar sac (S), capillary (C) and alveolar fibrin (F). All images were taken at 400× magnification. Bar = 20 μm.

Table 3. Histopathological assessment of lung injury in control mice (n = 10), malaria-infected mice without ALI/ARDS (n = 17) and malaria-infected mice with ALI/ARDS (n = 11).

Histopathological changes	Score (mean ± SEM)		
	Control (n = 10)	Non-ALI/ARDS (n = 17)	ALI/ARDS (n = 11)
Alveolar fibrin	0.02 ± 0.04	0.52 ± 0.09[a,c]	0.67 ± 0.09[b,c]
Alveolar oedema	0	0.03 ± 0.05[a,c]	1.60 ± 0.45[b,c]
Septal congestion	0.04 ± 0.07	0.58 ± 0.24[a,c]	0.87 ± 0.05[b,c]
Leukocyte infiltration	0.42 ± 0.08	1.39 ± 0.44[a,c]	1.60 ± 0.27[b]
Malaria pigment in macrophages	0	2.72 ± 0.25[a,c]	2.75 ± 0.33[b]
% Parasitized red blood cells	0	1.82 ± 0.64[a,c]	2.36 ± 0.50[b,c]
Alveolar haemorrhage	0	0.44 ± 0.10[a,c]	1.30 ± 0.87[b,c]
Septal thickening	0.21 ± 0.35	2.38 ± 0.36[a,c]	2.80 ± 0.32[b,c]
Lung injury score (LIS)	**0.69 ± 0.11**	**9.88 ± 0.24[a, c]**	**13.95 ± 0.22[b, c]**

Significant differences were identified at $p < 0.05$.
[a] control vs non-ALI/ARDS.
[b] control vs ALI/ARDS.
[c] non-ALI/ARDS vs ALI/ARDS.

The immunostaining of each target protein in endothelial cells, alveolar epithelial cells, alveolar macrophages and leukocytes was individually investigated to calculate the percentage of positively stained cells according to a previous study [17]. In addition, the intensity of staining was subjectively graded as follows: 0 = no staining, 1 = weakly positive, 2 = moderately positive, and 3 = strongly positive. Finally, the total score was calculated by multiplying the percentage of positive cells (%) and staining intensity (I), according to a previous study [17].

2.4. Protein extraction from lung tissue

The lung tissues were homogenized in cell lysis buffer (Cat. # 9803, Cell Signaling Technology, USA) containing phenylmethylsulfonyl fluoride (Calbiochem, CA) and incubated on ice for 5 min, followed by tissue scraping. Next, each sample was centrifuged at 14,000 g for 10 min at 4 °C. Then, the supernatant was collected, and the protein concentration was measured using a Bradford protein assay kit (Bio-Rad Laboratories, USA). The proteins were aliquoted and stored at -80 °C until use.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The levels of HMGB-1 and proinflammatory cytokines (Tumor necrosis factor (TNF)-α, Interferon (IFN)-γ, IL-1 and IL-6) were measured in lung tissues and plasma by using commercial ELISA kits (Cat. #E-EL-M0676, Elabscience, USA; Cat. #900-T54, PeproTech Asia, Israel; Cat. #900-T98, PeproTech Asia, Israel; Cat. # 88-5019, Thermo Fisher Scientific, USA; and Cat. #EK0411, Boster Biological Technology, USA) according to the manufacturers' protocols. Briefly, protein extracts and plasma (10:90) samples were added to a 96-well plate and incubated for 90 min at 37 °C. Next, the contents in the well plate were discarded without washing, and biotinylated antibody was added to each well and incubated for 60 min at 37 °C. After the plate was washed with PBS buffer three times, avidin biotin peroxidase was added to each well and incubated for 30 min at 37 °C. The plate was washed with PBS buffer five times. Tetramethylbenzidine solution was added and incubated for 15–20 min at 37 °C in the dark. Next, hydrochloric acid was added to each well to stop the reactions. Finally, optical density and absorbance were measured at 450 nm in a microplate reader within 30 min of adding the stop solution. Each sample was analysed in duplicate. The results are presented as the mean ± SEM.
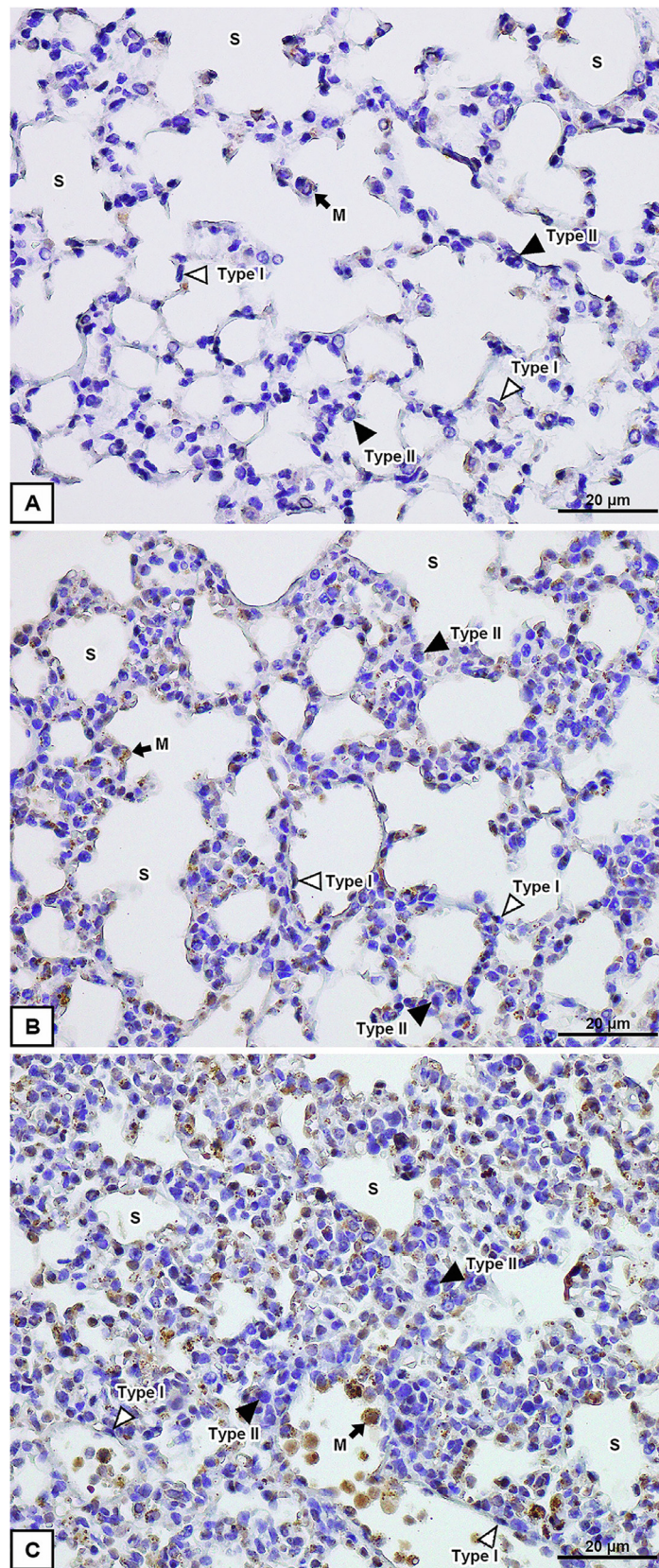
Figure 2. Immunoperoxidase staining for HMGB-1 in lung tissues of the control (A), malaria-infected without ALI/ARDS (B) and malaria-infected with ALI/ARDS (C) groups. Type I alveolar epithelial cells (Type I), Type II alveolar epithelial cells (Type II), alveolar macrophages (M), and alveolar sacs (S). All images are 400× magnification. Bar = 20 μm.
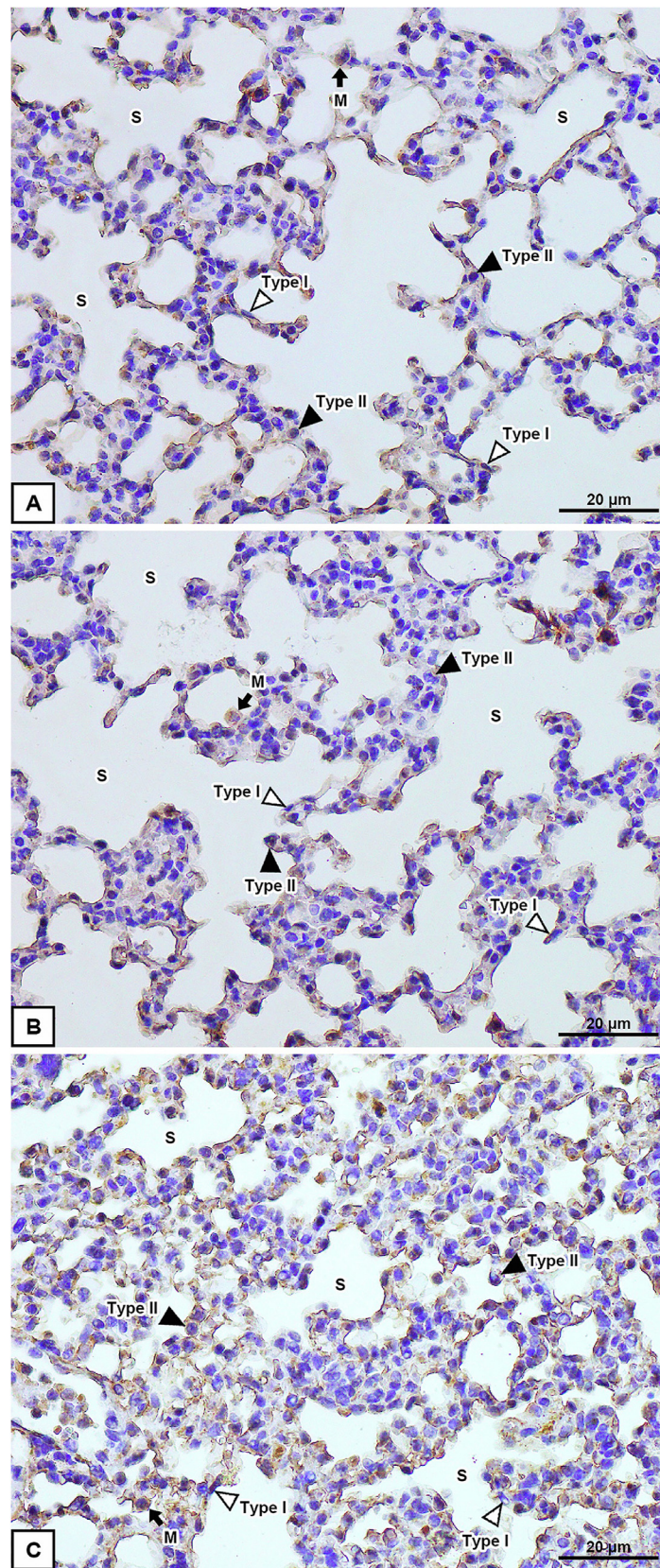
Figure 3. Immunoperoxidase staining for RAGE in lung tissues of the control (A), malaria-infected without ALI/ARDS (B) and malaria-infected with ALI/ARDS (C) groups. Type I alveolar epithelial cells (Type I), Type II alveolar epithelial cells (Type II), alveolar macrophages (M), and alveolar sacs (S). All images are 400× magnification. Bar = 20 μm.
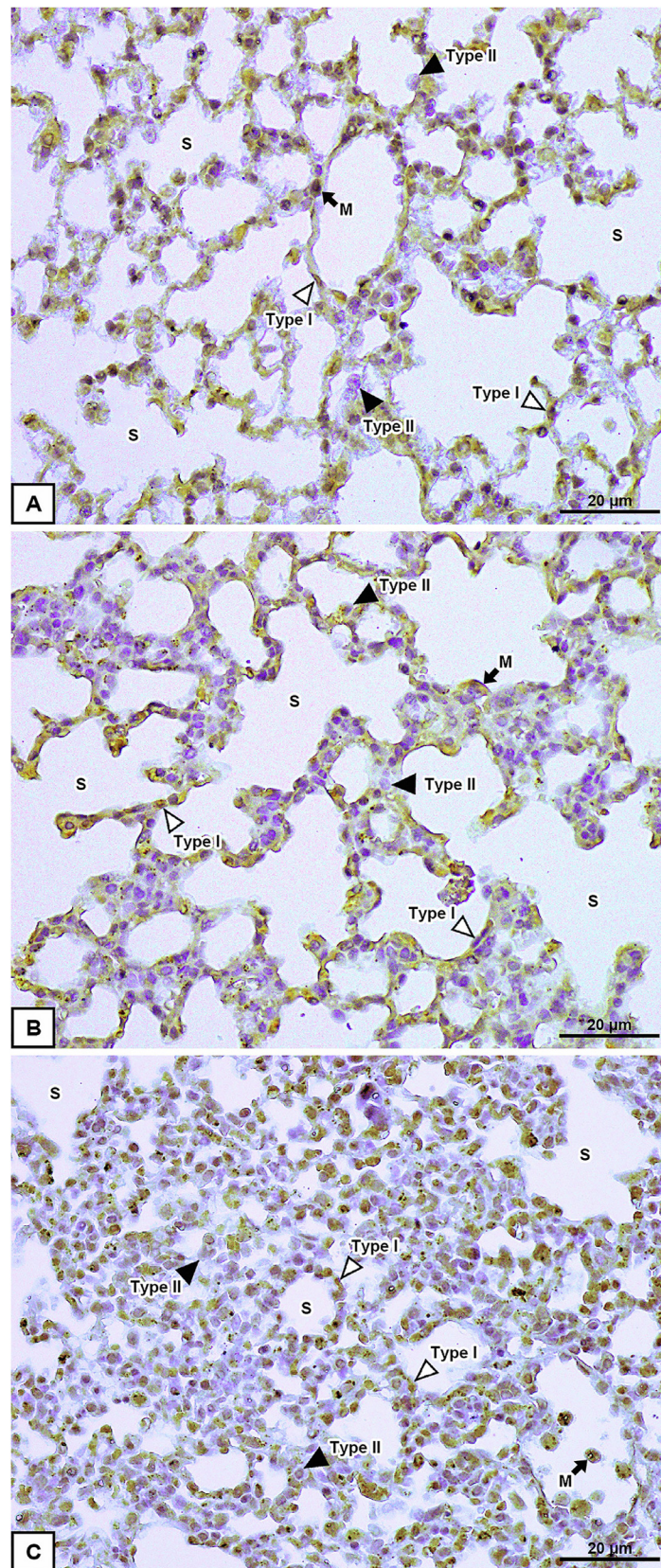
Figure 4. Immunoperoxidase staining for TLR-2 in lung tissues of the control (A), malaria-infected without ALI/ARDS (B) and malaria-infected with ALI/ARDS (C) groups. Type I alveolar epithelial cells (Type I), Type II alveolar epithelial cells (Type II), alveolar macrophages (M), and alveolar sacs (S). All images are 400× magnification. Bar = 20 μm.

Figure 5. Immunoperoxidase staining for TLR-4 in lung tissues of the control (A), malaria-infected without ALI/ARDS (B) and malaria-infected with ALI/ARDS (C) groups. Type I alveolar epithelial cells (Type I), Type II alveolar epithelial cells (Type II), alveolar macrophages (M), and alveolar sacs (S). All images are 400× magnification. Bar = 20 μm.
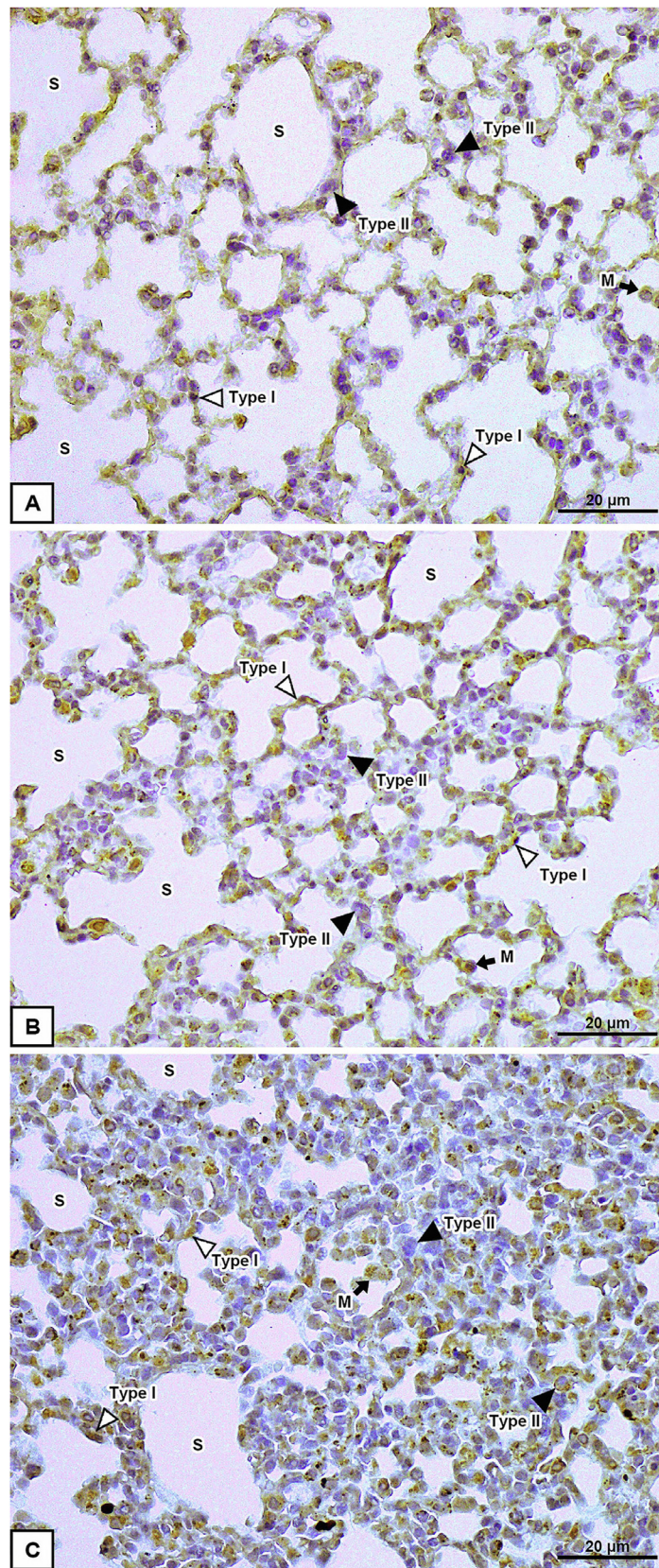
Table 4. The mean percentages of cells that were positively stained for HMGB-1 and its receptors in the lung tissues of control mice (n = 10), malaria-infected mice without ALI/ARDS (n = 17) and malaria-infected mice with ALI/ARDS (n = 11).

Marker	Group	Endothelium	Alveolar epithelium	Leukocytes	Macrophages
HMGB-1	Control	20.2 ± 2.89	24.0 ± 4.28	22.6 ± 2.69	21.8 ± 3.62
	Non-ALI/ARDS	25.5 ± 3.72[c]	31.4 ± 8.20[a,c]	27.8 ± 6.09[c]	28.0 ± 6.92[c]
	ALI/ARDS	31.2 ± 5.07[b,c]	48.8 ± 5.94[b,c]	40.2 ± 7.32[b,c]	45.6 ± 9.37[b,c]
RAGE	Control	ND	20.4 ± 1.74	ND	22.0 ± 3.34
	Non-ALI/ARDS	ND	26.0 ± 4.19[a,c]	ND	27.0 ± 3.37[c]
	ALI/ARDS	ND	43.2 ± 7.70[b,c]	ND	41.1 ± 8.13[b,c]
TLR-2	Control	ND	22.0 ± 2.96	ND	23.4 ± 3.35
	Non-ALI/ARDS	ND	29.8 ± 8.02[c]	ND	32.4 ± 8.56[c]
	ALI/ARDS	ND	51.2 ± 8.15[b,c]	ND	51.6 ± 7.57[b,c]
TLR-4	Control	ND	27.2 ± 2.40	ND	26.8 ± 3.37
	Non-ALI/ARDS	ND	40.8 ± 9.60[a,c]	ND	39.0 ± 10.74[c]
	ALI/ARDS	ND	51.4 ± 6.45[b,c]	ND	50.6 ± 9.84[b,c]

ND: not determined. Significant differences were identified at $p < 0.05$.
[a] control vs non-ALI/ARDS.
[b] control vs ALI/ARDS.
[c] non-ALI/ARDS vs ALI/ARDS.

2.6. Real-time PCR

HMGB-1, receptors for HMGB-1 (RAGE, TLR-2 and TLR-4) and proinflammatory cytokine genes (TNF-α, IFN-γ, IL-1 and IL-6) were amplified by specific primers (Table 2). The total cDNA was determined by real-time PCR using 5× HOT FIREPol® EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne, Estonia) with ABI Prism 7000 SDS software (Applied Biosystems, USA). The PCR amplification protocol comprised preheating at 95 °C for 15 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55–60 °C for 30 s, and extension at 72 °C for 30 s. Finally, the final extension was performed at 72 °C for 5 min. The relative expression of each gene was normalized using the beta-actin (β-actin) gene.

2.7. Statistical analysis

The results are presented as the mean ± SEM. Statistical analysis was performed using IBM SPSS Statistics version 23.0 software (SPSS, IL, USA). The distribution of all variables was tested by the Kolmogorov-Smirnov goodness of fit test. The Mann-Whitney U test was used to test for differences in HMGB-1, TNF-α, IFN-γ, IL-1, IL-6, RAGE, TLR-2, and TLR-4 expression between groups. Spearman's rank correlation test was used to analyse the correlations in the expression of HMGB-1, receptors for HMGB-1 and proinflammatory cytokines. Significant differences were determined at a p-value less than 0.05 ($p < 0.05$).

3. Results

3.1. Histopathological changes in lung tissues

The lung tissue of the control group showed normal lung histology composed of a thin alveolar septum, some leukocytes in the alveolar space and no evidence of congestion in the blood capillaries or alveolar fibrin (Figure 1A). Histopathological changes in the lung tissue from the non-ALI/ARDS group showed alveolar thickening with congested blood

capillaries and infiltration of alveolar fibrin and leukocytes into the alveolar sac (Figure 1B). In addition, a small number of alveolar macrophages containing malaria pigments and parasite-infected red blood cells were observed in the non-ALI/ARDS group (Figure 1B). In the lung tissue of the ALI/ARDS group, evidence of alveolar oedema, represented by the pink area, a large amount of leukocyte infiltration and alveolar fibrin, was detected in the alveolar sac (Figure 1C and D). The malaria pigment, which presented as black or brown dots, and parasite-infected red blood cells were also observed in whole lung tissue. In addition, the alveolar septa showed thickening with blood capillary congestion (Figure 1C and D). Semiquantitative analysis of lung injury showed that the mean LIS was significantly increased in the ALI/ARDS group (13.95 ± 0.22) compared with that in the non-ALI/ARDS (9.88 ± 0.24) and control groups (0.69 ± 0.11) (Table 3).

3.2. Localization of HMGB-1 and its receptors in lung tissues of malaria-infected mice

Using immunohistochemical staining, HMGB-1 was detected in both the nucleus and cytoplasm of cells (Figure 2). Immunopositive staining of HMGB-1 was also observed in the alveolar endothelium, the alveolar epithelium, leukocytes and alveolar macrophages (Figure 2). After malaria infection, the expression of HMGB-1 was strongly upregulated in the lung tissues of the ALI/ARDS group (Figure 2C) compared to that of the non-ALI/ARDS (Figure 2B) and control (Figure 2A) groups. HMGB-1 was predominantly located in the nuclei of lung cells. Positive staining for all HMGB-1 receptors, including RAGE (Figure 3), TLR-2 (Figure 4) and TRL-4 (Figure 5), was detected in the cell membrane and cytoplasm. Immunopositive staining for RAGE, TLR-2 and TLR-4 was detected in the alveolar epithelium and alveolar macrophages in all groups, and RAGE (Figure 3C) and TLR-4 (Figure 5C) were mostly located in the alveolar epithelium, whereas TLR-2 was frequently localized in alveolar macrophages (Figure 4C).

Semiquantitative analysis showed that the mean percentage of HMGB-1-positive cells was significantly increased in the alveolar endothelium (31.2 ± 5.07 vs 22.2 ± 0.86), the alveolar epithelium (48.8 ± 5.94 vs 36.0 ± 2.14), leukocytes (40.2 ± 7.32 vs 29.3 ± 1.23) and alveolar macrophages (45.6 ± 9.37 vs 26.1 ± 1.45) in the ALI/ARDS group compared with that in the control group.

In the lung tissues of the ALI/ARDS group, the highest percentage of HMGB-1-positive cells was found in the alveolar epithelium (Table 4). The mean percentage of RAGE-positive cells was significantly increased in the ALI/ARDS group compared with that in the control group (43.2 ± 7.70 vs 20.4 ± 0.00, 43.0 ± 6.14 vs 23.5 ± 0.66 and 41.1 ± 8.13 vs 28.6 ± 1.53). The mean percentage of TLR-2-positive cells was significantly increased in the ALI/ARDS group compared with that in the control group (51.2 ± 8.15 vs 22.0 ± 2.96, 49.4 ± 6.87 vs 21.8 ± 2.44 and 51.6 ± 7.57 vs 23.4 ± 3.35). The mean percentage of TLR-4-positive cells was significantly different in the ALI/ARDS group compared with that in the control group (51.4 ± 6.45 vs 27.2 ± 2.40, 47.2 ± 7.95 vs 24.0 ± 3.22 and 50.6 ± 9.84 vs 26.8 ± 3.37) (all $p < 0.05$). Among these HMGB-1 receptors, both TLR-2 and TLR-4 were mainly localized on the cell membrane of alveolar epithelial cells in the lung tissues of the ALI/ARDS group.

3.3. Levels of HMGB-1 and proinflammatory cytokines in plasma and lung tissue

The levels of HMGB-1 and proinflammatory cytokines, including TNF-α, IFN-γ, IL-1 and IL-6, was measured in both plasma and lung tissues by using ELISA (Figure 6). During malaria infection, the mean levels of plasma HMGB-1 were significantly increased in the ALI/ARDS group (3447.13 ± 161.55) compared with those in the non-ALI/ARDS (2878.03 ± 154.06) and control (2347.52 ± 175.63) groups ($p < 0.001$). Similarly, the mean levels of HMGB-1 were significantly increased in homogenized lung tissues of the ALI/ARDS group (12.78 ± 2.33) compared with those in the non-ALI/ARDS (8.21 ± 1.91) and control (7.22 ± 5.12) groups ($p <$
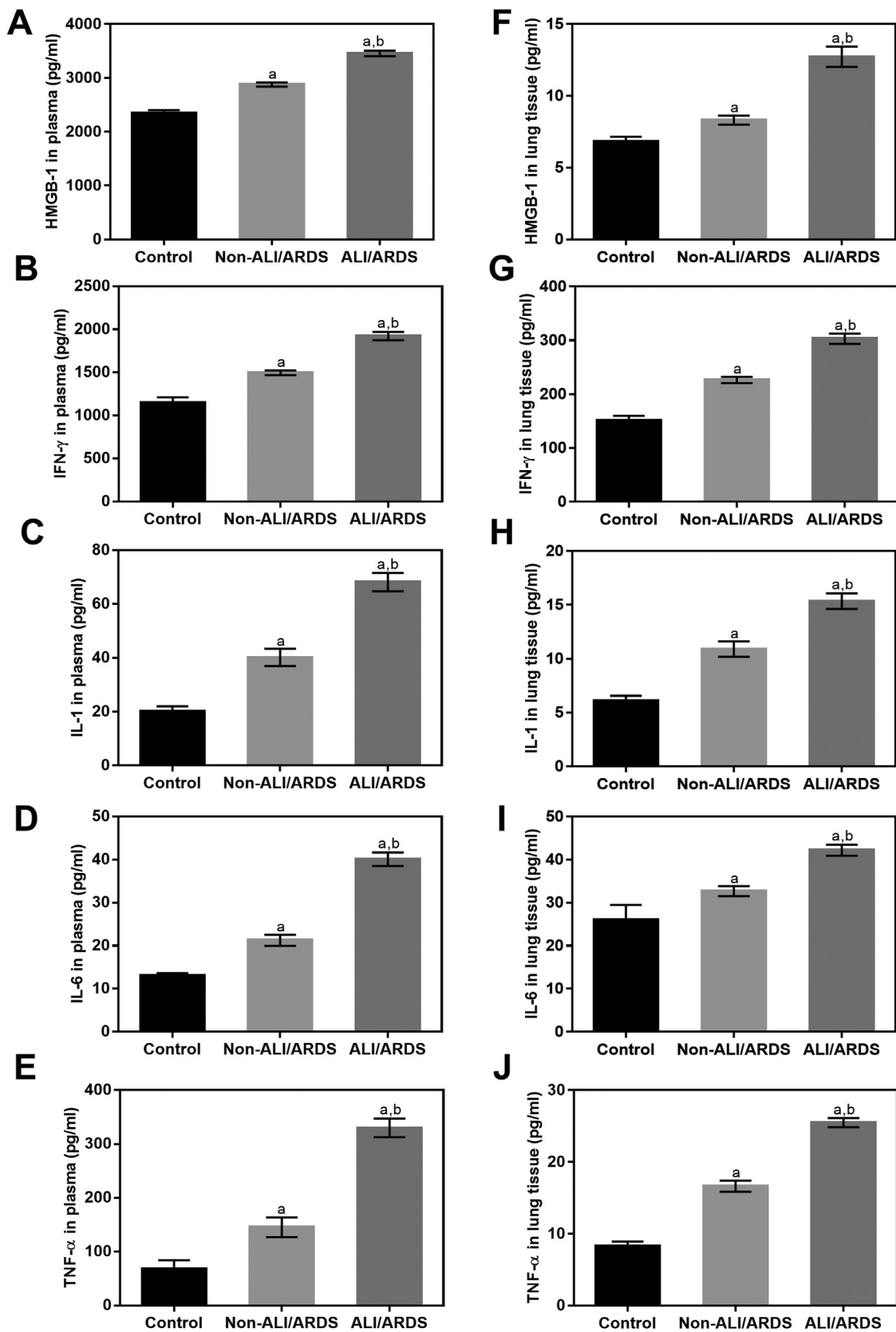
Figure 6. Levels of HMGB-1 and proinflammatory cytokines in the plasma and lung tissue of the control mice (n = 10), malaria-infected mice without ALI/ARDS (n = 17) and malaria-infected mice with ALI/ARDS (n = 11). The levels of HMGB-1 (A), IFN-γ (B), IL-1 (C), IL-6 (D), and TNF-α (E) were measured in plasma by ELISA. The levels of HMGB-1 (F), IFN-γ (G), IL-1 (H), IL-6 (I), and TNF-α (J) were measured in lung tissue by ELISA. [a]$p < 0.001$ compared with the control group. [b]$p < 0.001$ compared with the non-ALI/ARDS group. The data are presented as the mean ± SEM.

Table 5. The average mRNA expression and qPCR expression fold changes of differentially expressed genes in the control, malaria-infected without ALI/ARDS and malaria-infected with ALI/ARDS groups.

Gene	mRNA expression value[a]			*p*-value[b]
	Control	Non-ALI/ARDS	ALI/ARDS	
HMGB-1	1 ± 0.51	1.07 ± 0.41	4.26 ± 0.04	0.039
RAGE	1 ± 0.21	1.66 ± 0.02	3.97 ± 0.06	0.002
TLR-2	1 ± 0.29	1.12 ± 0.35	7.17 ± 0.66	0.034
TLR-4	1 ± 0.43	1.33 ± 0.21	3.18 ± 0.94	0.009
TNF-α	1 ± 0.18	2.04 ± 0.18	4.08 ± 0.51	0.002
IFN-γ	1 ± 0.19	1.40 ± 0.03	3.33 ± 0.67	0.009
IL-1	1 ± 0.06	11.26 ± 0.77	18.77 ± 1.10	0.002
IL-6	1 ± 0.30	3.95 ± 1.78	4.92 ± 1.00	0.044

Significant differences were identified at $p < 0.05$.

[a] The mRNA expression value was normalized to the β-actin gene (mean ± SEM).

[b] Statistical analysis of individual relative mRNA expression among malaria-infected mice and control mice.

Table 6. Correlations between plasma HMGB-1 levels, HMGB-1 receptors and proinflammatory cytokines in the ALI/ARDS group (n = 11).

	Correlation with plasma HMGB-1	
	Correlation coefficient (r_s)	*p*-value
Plasma HMGB-1	–	–
Lung injury score	0.862	0.001*
Plasma TNF-α	0.639	0.003*
Plasma IFN-γ	0.638	0.020*
Plasma IL-1	0.804	0.010*
Plasma IL-6	0.893	0.010*
Tissue HMGB-1	0.379	0.044*
Tissue TNF-α	0.857	0.010*
Tissue IFN-γ	0.835	0.010*
Tissue IL-1	0.710	0.010*
Tissue IL-6	0.727	0.010*
HMGB-1 mRNA	0.607	0.050
RAGE mRNA	0.335	0.581
TLR-2 mRNA	0.505	0.078
TLR-4 mRNA	0.621	0.024*
TNF-α mRNA	0.571	0.041*
IFN-γ mRNA	0.491	0.150
IL-1 mRNA	0.599	0.031*
IL-6 mRNA	0.949	0.051

* Significant correlations at $p < 0.05$ by using Spearman's rank correlation test.

0.001). In addition, plasma levels of proinflammatory cytokines were increased significantly in the ALI/ARDS group (TNF-α: 334.83 ± 62.28; IFN-γ: 1910.02 ± 173.76; IL-1: 66.82 ± 11.59; IL-6: 40.03 ± 5.00) compared with those in the non-ALI/ARDS (TNF-α: 139.57 ± 76.40; IFN-γ: 1495.60 ± 112.66; IL-1: 44.52 ± 14.34; IL-6: 21.26 ± 5.07) and control groups (TNF-α: 74.40 ± 63.20; IFN-γ: 1120.87 ± 186.49; IL-1: 20.47 ± 5.79; IL-6: 13.25 ± 1.79) ($p < 0.001$), which were similar to the results of the homogenized lung tissue, indicating significantly increased levels of TNF-α, IFN-γ, IL-1 and IL-6 in the ALI/ARDS group compared with those in the non-ALI/ARDS and control groups (Figure 6).

3.4. Gene expression of HMGB-1, its receptors and proinflammatory cytokines by real-time PCR

The relative mRNA expression of HMGB-1, its receptors and proinflammatory cytokines was determined by real-time PCR, and the relative

expression of each gene was normalized to the β-actin gene. Our results showed that the gene expression of HMGB-1 was significantly upregulated in malaria-infected mice in the ALI/ARDS group (4.26 ± 0.04) compared with that in mice in the non-ALI/ARDS (1.07 ± 0.41) and control groups (1.00 ± 0.51). The gene expression levels of RAGE, TLR-2 and TLR-4 were significantly increased in malaria-infected mice in the ALI/ARDS group compared with those of mice in the non-ALI/ARDS and control groups. In the ALI/ARDS group, TLR-2 gene expression (7.17 ± 0.66) was higher than RAGE (3.97 ± 0.06) and TLR-4 (3.18 ± 0.94) gene expression (Table 5). In addition, the gene expression of all proinflammatory cytokines (TNF-α, IFN-γ, IL-1 and IL-6) was higher in malaria-infected mice in the ALI/ARDS group than in mice in the non-ALI/ARDS and control groups. Among the proinflammatory cytokines, the highest gene expression of IL-1 was observed in malaria-infected mice in the ALI/ARDS group, which was up to 1.6-fold higher than that in mice in the non-ALI/ARDS group (Table 5).

3.5. Correlation between plasma levels of HMGB-1, its receptors and proinflammatory cytokine in malaria-infected mice with ALI/ARDS

As shown in Table 6, Spearman's rank correlation coefficients were used to demonstrate the correlations between plasma HMGB-1, its receptors and proinflammatory cytokines in the ALI/ARDS group. The results revealed that there were significant positive correlations between plasma HMGB-1 levels, the LIS and levels of proinflammatory cytokines (TNF-α, IFN-γ, IL-1 and IL-6 levels) in both plasma and lung tissues. In addition, there were also significant positive correlations between plasma HMGB-1 levels and the gene expression of TLR-4, TNF-α and IL-1. However, there was no correlation between plasma HMGB-1 levels and the gene expression of HMGB-1, RAGE, TLR-2, IFN-γ or IL-6 (Table 6).

4. Discussion

HMGB-1 is a DNA-binding protein that is expressed in almost all cells and functions as a proinflammatory cytokine. There are two major pathways for HMGB-1 release into the extracellular space, active and passive mechanisms [1, 2, 6, 18]. HMGB-1 appears to stimulate several receptors, including RAGE, TLR-4 and TLR-2, leading to upregulation of the production of proinflammatory cytokines [1, 2, 6, 18]. Many studies have reported that circulating HMGB-1 can trigger a devastating inflammatory response that promotes the progression of sepsis and ALI [19, 20, 21, 22].

In this study, the histopathological findings in the lung tissues of malaria-infected mice in the ALI/ARDS group showed alveolar thickening with capillary congestion, alveolar fibrin, alveolar oedema, alveolar haemorrhage and leukocyte accumulation in the alveolar sac, similar to the results of previous studies [23, 24, 25]. The key pathogenesis of MA-ALI/ARDS is associated with sequestration of parasitized red blood cells on the endothelium and exacerbation of the haemozoin-mediated inflammatory response, leading to apoptosis of endothelial and epithelial cells and resulting in impaired gas exchange and severe hypoxemia [26, 27]. As expected, the findings of the present study demonstrated that plasma concentrations of TNF-α, IFN-γ, IL-1 and IL-6 were significantly increased in malaria-infected mice in the ALI/ARDS group compared with those in the non-ALI/ARDS and control groups, which is consistent with previous studies showing elevated levels of cytokines in patients with malaria infection [28, 29] and in a mouse model of MA-ALI/ARDS [30, 31]. In addition, this study found that high levels of plasma HMGB-1 were positively correlated with levels of proinflammatory cytokines (TNF-α, IFN-γ, IL-1 and IL-6) in both plasma and lung tissues, indicating that activation of HMGB-1 is involved in the immune response to malaria infection.

To investigate the localization of HMGB-1 in the lung tissue, immunohistochemical studies were performed and revealed a significant increase in the number of HMGB-1-positive cells in the lung tissues of the malaria-infected ALI/ARDS group compared with that in the non-ALI/
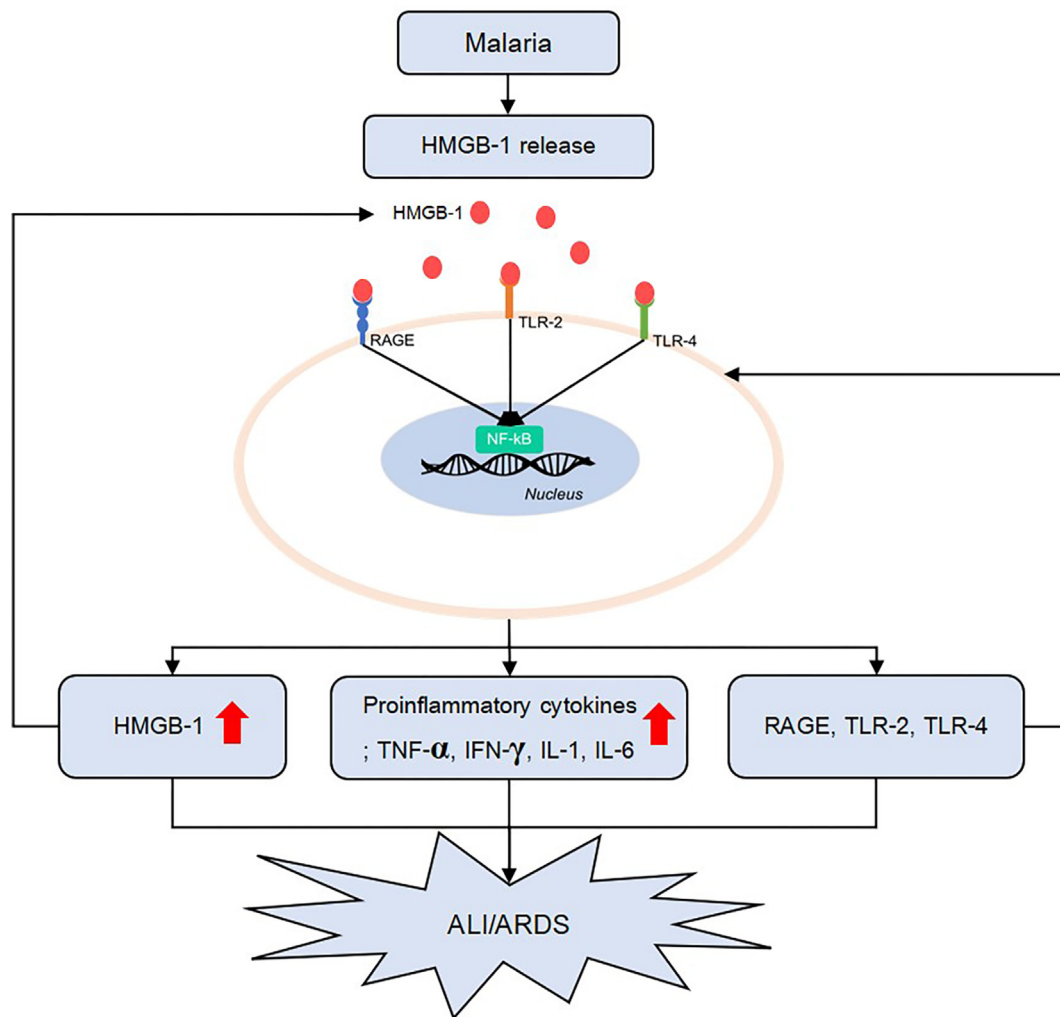
Figure 7. Schematic representation of HMGB-1 and its receptors in the pathogenesis of MA/ALIARDS.

ARDS and control groups. In the lung tissues from malaria-infected mice in the ALI/ARDS group, the increased expression of HMGB-1 was localized in the alveolar epithelium, endothelial cells, leukocytes and alveolar macrophages. This result was consistent with the findings from previous studies demonstrating that the distribution of HMGB-1 in the alveolar epithelium, endothelial cells and alveolar macrophages by using immunohistochemical analysis [9, 32, 33], suggesting that extracellular HMGB-1 can be either actively secreted or passively released from the alveolar epithelium, inflammatory cells and alveolar macrophages in lung tissue during malaria infection. In the present study, extracellular HMGB-1 was quantified by ELISA. Our study revealed that the plasma level of HMGB-1 was significantly increased in the malaria-infected ALI/ARDS group compared with that in the non-ALI/ARDS and control groups. This finding is similar to previous studies showing elevated levels of HMGB-1 in children with severe or uncomplicated *Plasmodium falciparum* malaria [5, 14] and in the *P. berghei* ANKA infection model of experimental severe/cerebral malaria [5]. These studies suggested that the elevated plasma levels of HMGB-1 observed in malaria-infected mice with ALI/ARDS may be due to activation of immune cells that can induce epithelial cell apoptosis, leading to HMGB-1 release into the blood circulation [34]. On the other hand, necrosis of both endothelial and epithelial cells also leads to HMGB-1 release [35]. Furthermore, this may be caused by the number of dead, dying or injured cells during malaria infection, which activates immune cells to release chemokines and cytokines into the blood circulation [36], leading to an increased level of HMGB-1 in the blood.

HMGB-1 is known to signal through RAGE and TLRs. Activation of these receptors results in activation of Nuclear factor κB (NF-κB), which regulates the expression of several genes, including cytokines, chemotactic proteins, adhesion molecules and matrix proteins involved in cell differentiation, growth, and death [37]. The real-time PCR results of this study demonstrated that the mRNA expression of RAGE, TLR-2 and TLR-4 was significantly increased in the MA-ALI/ARDS group compared with that in the non-ALI/ARDS and control groups. In addition, the high level of circulating HMGB-1 was positively associated with the gene expression of TLR-4 in the lung tissues of the ALI/ARDS group, suggesting that the release of HMGB-1 might be preferentially activated via TLR-4. However, further studies are required to elucidate the cell signalling pathways of HMGB-1 binding to its receptors in MA-ALI/ARDS.

RAGE was the first receptor that was demonstrated to bind to HMGB-1 [38], and this binding induces cell proliferation, growth, migration, pyroptosis, and cytokine secretion [39, 40] by activating the NF-kB and mitogen-activated protein kinase (MAPK) pathways [41]. HMGB-1 binds to TLRs to induce NF-kB and MAPK activation to regulate the gene expression of various immune and inflammatory mediators [42, 43, 44]. It has been reported that the interaction between HMGB-1 and its receptors can release various proinflammatory cytokines, such as TNF-α, IFN-γ, IL-1, and IL-6 [42, 45, 46]. Previous studies have shown that the interaction of HMGB-1 and RAGE on the cell surface induces direct intracellular signalling by Akt activation and nuclear NF-kB translocation, leading to the release of TNF [47]. In addition, it has been reported that the interaction of HMGB-1 and TLR-4 via MD-2, which is a

mandatory HMGB-1 receptor complex for cytokine production, can mediate NF-kB translocation [7, 42]. Therefore, this study provides evidence that HMGB-1 is an important mediator involved in the pathogenesis of MA-ALI/ARDS as summarized in Figure 7.

Some limitations of this study should be noted. First, all mice were sacrificed on the last day of the experiment (Day 14). Therefore, our study lacks kinetic information on circulating HMGB-1. Second, apart from the investigation of HMGB-1 and its receptors, we did not further investigate cell signalling in this study. For a better understanding of the contribution of HMGB-1 to development of ALI/ARDS in malaria infection, further works should focus on cell signalling and the protective effects of anti-HMGB-1 neutralizing antibodies and antagonists of HMGB-1 receptors.

5. Conclusion

This study shows that HMGB-1 is increased in plasma and lung tissues in the context of MA-ALI/ARDS and is associated with elevated levels of proinflammatory cytokines. This study supports that HMGB-1 is an important mediator involved in the pathogenesis of MA-ALI/ARDS via activation of the receptor TLR-4. Further *in vitro* and *in vivo* studies are required to better understand a new treatment strategy to inhibit the HMGB-1 or the TLR-4 signalling pathway to prevent the inflammatory response and development of ALI/ARDS during malaria infection.

Declarations

Author contribution statement

Tachpon Techarang: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Pitchanee Jariyapong, Parnpen Viriyavejakul, Chuchard Punsawad: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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