M1 macrophage activation in severe *Plasmodium falciparum* malaria patients with pulmonary oedema

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Abstract

Background

Pulmonary oedema (PE) is a serious complication of severe P. falciparum malaria which can lead to acute lung injury in severe cases. Lung macrophages are activated during malaria infection due to a complex host-immune response. The molecular basis for macrophage polarisation is still unclear but understanding the predominant subtypes could lead to new therapeutic strategies where the diseases present with lung involvement. The present study was designed to study the polarisation of lung macrophages, as M1 or M2 macrophages, in the lungs of severe P. falciparum malaria patients with and without evidence of PE.

Methods

Lung tissue samples, taken from patients who died from severe P. falciparum malaria, were categorised into severe malaria with PE and without PE (non-PE). Expression of surface markers (CD68- all macrophages, CD40- M1 macrophage and CD163- M2 macrophage) on activated lung macrophages was used to quantify M1/M2 macrophage subtypes.

Results

Lung injury was demonstrated in malaria patients with PE. The expression of CD40 (M1 macrophage) was prominent in the group of severe P. falciparum malaria patients with PE (p < 0.05), whereas there was no difference observed for CD163 (M2 macrophage) between PE and non-PE groups.

Conclusions

The study demonstrates M1 polarisation in lung tissues from severe P. falciparum malaria infections with PE. Understanding the nature of macrophage
characterisation in malaria infection may provide new insights into therapeutic approaches that could be deployed to reduce lung damage in severe P. falciparum malaria.

Background

Pulmonary oedema (PE) is one of the major complications and therapeutic challenges in severe P. falciparum malaria. This condition is associated with acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) [1]. PE occurs in approximately 21% of cerebral malaria patients and 40% of these develop into ARDS [2]. Furthermore, substantial fatality (70%) is observed in malaria patients with PE who subsequently progress to develop ARDS [2, 3]. In P. falciparum malaria, PE is associated with inflammatory infiltrates consisting of mainly mononuclear cells, hemozoin deposit, the accumulation of macrophages, as well as parasite sequestration [4]. Recruitment of macrophages to the lung signifies an important immune response in the pathogenesis of ALI/ARDS [5]. Activated macrophages have been described as two functional subsets, namely classically activated macrophages (M1) and alternatively activated macrophages (M2) [6, 7]. M1 macrophages are considered as pro-inflammatory macrophages that produce pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-a, interleukin-6 (IL-6) and other mediators. M2 macrophages are typically anti-inflammatory macrophages and produce anti-inflammatory cytokines, such as IL-10 and secrete growth factor such as transforming growth factor beta 1 (TGF-β1) for tissue repair [7].

The polarisation of M1 and M2 macrophages are important for disease regulation. Previous studies have documented that activated macrophages are prominent in lung infection with bacteria [8] and viruses [9-11] as well as in lungs from smokers
and chronic obstructive pulmonary disease (COPD) [12]. The main purpose of this study was to investigate the status of lung macrophages in severe *P. falciparum* malaria patients with and without PE. The outcome of the work highlighted the role of macrophages on PE/ALI and serve as a basis for drug adjuvant therapy for lung complications in severe *P. falciparum* malaria.

**Materials and methods**

**Tissue specimens**

Embedded human lung tissues from severe *P. falciparum* malaria infected patients and non-infected controls were retrieved from the Department of Tropical Pathology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Lung tissues were embedded and prepared for histopathological evaluation. Based on histopathological findings of oedematous fluid in the lung, lung tissues were divided into severe *P. falciparum* malaria with PE, non-PE and control lung tissues. Normal control lung tissues were obtained from patients who died from accidents, and showed no pathological changes in the lungs. The study protocol was reviewed and ethical clearance was obtained from the Ethics Committee of Faculty of Tropical Medicine, Mahidol University (MUTM 2017-054-01).

**Histopathology and evaluation**

Lung tissues were re-embedded with new paraffin medium, sectioned at 4 μm in thickness and routinely stained with haematoxylin and eosin (H&E). The pathological changes of lung tissues were interpreted based on eight histological criteria in twenty low power fields (LPF) (200X) per slide, namely septal congestion, alveolar haemorrhage, alveolar oedema, hyaline membrane formation, parasitised red blood cell (PRBC) sequestration, malarial pigment, lung macrophages and
infiltration of inflammatory cells [13]. Each variable was graded on a scale based on percentage of severity based on a previous study with modifications, as follows: no injury = 0, injury £ 25% /LPF = 1, injury > 25% and £ 50%/ LPF = 2, injury > 50% and £ 75%/ LPF = 3, and injury > 75% LPF = 4 [14]. Lung macrophages and white blood cells (WBC) were determined using a standard Z-score from the mean of cell count/high power field (HPF): -1.5 SD = 0, -0.5 SD = 1, mean = 2, +0.5 SD = 3 and +1.5 SD = 4. Subsequently, a lung injury score ranging from 0 to 32 points was calculated by adding the sum of each variable to determine the overall histopathological changes in lung tissues from malaria patients with *P. falciparum*. A score of 0 means the absence of histopathological changes while a score of 32 signified the most severe histopathological changes. All histopathological parameters were examined in a blinded manner, without prior knowledge of the patients’ clinical status.

**Immunohistochemical evaluation of lung macrophages**

The expressions of macrophage surface markers (CD68, CD40, and CD163) were detected by immunohistochemical staining [15, 16]. Lung sections of 4 μm thickness were placed on adhesive slides coated with poly-L-lysine and de-paraffinised through a series of xylene and re-hydrated through graded alcohol solutions. Antigen retrieval from lung tissues was performed by a microwave technique with 0.01 mol/L citrate buffer at pH 6.0 for 20 min. To reduce endogenous peroxidase activity, tissue sections were incubated with 1% H₂O₂ in PBS for 30 min at room temperature (RT) and rinsed in running tap water for 10 min. Lung sections were treated with normal goat serum for 30 min at RT to reduced non-specific background. To detect the surface makers on macrophages, lung sections were incubated with primary antibody: monoclonal mouse anti-human CD68 (1:100
optimal dilution, DakoCytomation®; Denmark), mouse monoclonal antibody CD163 (1:200 optimal dilution, Novocastra™; Leica Biosystems Newcastle Ltd UK.), and rabbit polyclonal antibody CD40 (1:200 optimal dilution, Santa Cruz Biotechnology, Inc., CA., USA.) in a moisture chamber overnight at 4 °C. Next day, tissue sections were extensively washed with PBS, and incubated with secondary antibody for 30 min at RT, then incubated with avidin-biotin complex (ABC) conjugated with horseradish peroxidase (HRP) (VECTASTAIN® ABC Kits; Vector Laboratories, CA., USA) for 30 min at RT. After the removal of non-reacted secondary antibodies, all sections were incubated with DAB (3,3′-diaminobenzidine) Peroxidase Substrate Kit (Vector Laboratories, CA, USA) for 3 min. Finally, sections were counterstained with Mayer’s haematoxylin, mounted with a coverslip and evaluated under a light microscope. Spleen was used as a positive control for CD68, CD40 and CD163 [17]. The anti-CD68 within the tissue sections were used as positive internal controls.

Positive cells stained brown pattern with different degrees of immunoreactive intensity.

**Evaluation of immunohistochemistry staining**

Quantitative assessments of macrophage subtypes were based on the number of positive stained cells for CD68, CD40 and CD163. Macrophages in lung tissues were evaluated randomly in twenty fields per slide, in both the septal area and within the alveoli under high magnification (400X). The numbers of surface markers specific for each macrophage subtype were counted and expressed as percentage of positive cells. The intensity of positive stained cells was graded as follows: 0 = negative; 1 = weak staining; 2 = moderate staining; 3 = strong staining. The total score (TS) for this immunohistochemical study was determined by obtaining the
product of the percentage of positive cells and the intensity of the staining [18, 19].
The sections were examined in a blinded manner, without prior knowledge of the patients, clinical status.

**Statistical analysis**

All quantitative data were expressed as means ± standard error of the mean (SEM). Statistical analysis was performed using SPSS version 18.0 software (SPSS, IL, USA). Data was tested with Kolmogorov-Smirnov test for normality of distribution. Difference of M1/M2 expression between groups was analysed by the Mann-Whitney U test. The correlations between M1/M2 expression and histological/clinical data were analysed by Spearman’s correlation. Statistical differences at \( p < 0.05 \) were considered to be statistically significant.

**Results**

**Summary of clinical and laboratory data from malaria patients**

Malarial lung tissues were obtained from 17 patients, 9 cases with PE and 8 cases with non-PE. Six normal lung tissues samples were used as control. No difference in clinical complications of severe malaria (ie. cerebral malaria, acidosis, hypoglycaemia, shock, disseminated intravascular coagulopathy) was observed between the two groups, except for the presence of acute kidney injury (\( p = 0.03 \)). The demographic data from the severe *P. falciparum* malaria patients is documented in Table 1. There was a significant difference in the patient haematocrit between non-PE and PE (\( p = 0.04 \)). No significant difference in age, blood urea nitrogen (BUN), creatinine, alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin, globulin, total bilirubin, direct bilirubin, WBC counts, and parasitaemia (all \( p > 0.05 \)) was observed.
Histopathological changes in the lungs of *P. falciparum* malaria patients

Common histopathological changes in the lungs of severe *P. falciparum* malaria included the presence of septal congestion and alveolar haemorrhage (Fig. 1A), alveolar oedema (Fig. 1B), hyaline membrane formation (Fig. 1C), PRBC sequestration in pulmonary capillaries (Fig. 1D), malarial pigment and an increase in the number of macrophages in alveolar spaces and septal area (interstitial area) (Fig. 1E) (all $p < 0.05$, compared to control lung, Fig. 1F). Mixed inflammatory cells are occasionally seen within the alveoli. Comparing non-PE and PE groups, significant difference in histological findings were noted in the presence of alveolar haemorrhage, accumulation of malarial pigment and the number of lung macrophages (all $p < 0.05$). The calculated lung injury score (based on histopathological criteria) was significantly higher in the PE group (19.22±0.90), compared to non-PE group (12.13±0.09, $p = 0.001$) (Fig. 2).

Immunohistochemistry study of M1/M2

Expression of CD68

CD68 expressing cells were detected as fine brown color in the cytoplasm of lung macrophages in both the septal area and within the alveoli (Fig 3, Panel A). The number of macrophage positive cells was significantly increased in PE group (49.23±5.16/HPF) compared to the non-PE group (31.22±3.81/HPF, $p = 0.016$) and the normal control group (6.40±1.39/HPF, $p = 0.001$). In septal areas, the number of CD68 positive cells was significantly higher in both the PE group (36.64±4.50/HPF), and the non-PE group (24.41±3.40/HPF), compared to the control group (3.58±0.99/HPF, $p < 0.05$). In terms of the number of macrophages per alveoli, the number of CD68 positive cells was significantly increased in the PE group (2.09±0.27) when compared to the non-PE group (1.20±0.21, $p = 0.025$) and the
control group (0.89±0.22, p = 0.007).

Expression of CD40

CD40 was used to detect activated M1 subtype lung macrophages. Positive cells expressed a fine brown color on the cell membrane of macrophages. The expression of CD40 was prominent in the group of severe *P. falciparum* malaria with PE (97.88±1.71%) compared to non-PE (72.81±7.17%, p = 0.001) and the control group (19.83±11.81%, p = 0.003) (Fig. 3, Panel B). Macrophages expressing CD40 in the alveolar septal area and within the alveoli show similar trends between PE and non-PE groups, as depicted in Fig. 4A.

Expression CD163

CD163 was used as a marker for the M2 subtype. Positive cells express a fine brown color in the cytoplasm of macrophages. Lung macrophages in severe *P. falciparum* malaria patients with PE and non-PE show an increase in CD163 expression, compared to the control group (p < 0.01). No significant difference in the mean percentage of CD163 positive cells was observed between PE and non-PE groups (Fig. 3, Panel C, Fig. 4B).

Correlations between M1/M2 surface markers and histopathological changes

There was a significant positive correlation between the ratio of M1/M2 and alveolar haemorrhage ($r_s = 0.775, p < 0.001$), alveolar oedema ($r_s = 0.672, p = 0.003$), presence of malarial pigments ($r_s = 0.605, p = 0.010$), lung macrophages ($r_s = 0.630, p = 0.007$), WBC infiltration ($r_s = 0.611, p = 0.009$) and acute lung injury score ($r_s = 0.713, p = 0.001$) (Fig. 5A-F).

Discussion
Acute lung injury (ALI) was demonstrated in the group of severe \textit{P. falciparum} malaria patients with pulmonary oedema (PE). The pathological findings of septal congestion, alveolar haemorrhage, alveolar oedema, hyaline membrane formation, malarial pigment, increase number of lung macrophages (both alveolar and interstitial), and white blood cells are commonly seen in lung tissues of fatal \textit{P. falciparum} malaria, in addition to the presence of PRBCs.

PE is a common finding in ALI and can cause the lungs to develop into acute respiratory distress syndrome (ARDS) [20]. The activation of lung macrophages and release of cytokines are important mechanisms contributing to lung damage in patients with ARDS [15, 21]. Under various triggers, lung macrophages can be polarised into either classically activated (or M1) or alternatively activated (or M2) subtypes [22]. M1 macrophages can be stimulated by interferon (IFN)-g, lipopolysaccharide (LPS) and granulocyte-macrophage colony stimulating factor, causing upregulation of genes involved in pathogen clearance and an inflammatory response. M2 macrophages, on the other hand, are induced by interleukin (IL)-4 and IL 13 and cause upregulation of genes involved in wound healing, phagocytic activity and an anti-inflammatory responses [22]. The main secreting cytokines from M1 macrophages which contribute to ALI include tumor necrosis factor (TNF)-a, IL-1\beta and reactive oxygen species [23]. In malaria, an essential factor that can contribute to the pathogenesis of lung injury is hemozoin pigment present in the lung tissue. A previous study in an animal model reported that hemozoin pigment can activate pro-inflammatory mediators and is related to the occurrence of ARDS in mice [24].

The subsequent influx of inflammatory cells into the lung tissue damages endothelial cells and results in increased vascular permeability [25]. It is possible that hemozoin activated pro-inflammatory chemokines (ie. IFN-g, CXCL 10), and
cytokines (ie. TNF-α, IL-6) [26] can contribute to M1 polarisation in malaria, resulting in ALI/ARDS. Consequently, pathological changes of PE, pulmonary haemorrhage, further recruitment of inflammatory cells and hyaline membrane formation may occur. In addition, macrophages can adhere and interact with the epithelium of alveoli resulting in an increase in cytosolic calcium levels which can lead to apoptosis of epithelial cells and the release of TNF-α. This mechanism can further damage the pulmonary barrier resulting in PE [21].

Malaria infection triggers recruitment of resident macrophages to the lungs as evident by an increase in CD68 expressing cells. Results demonstrated the accumulation of M1 macrophages in the lung of severe P. falciparum malaria patients, especially in the PE group as demonstrated by an increase in CD40 expressing cells. Similar findings were documented in mice lung infected with E. coli [27], in lung-induced chronic obstructive pulmonary disease (COPD) [12], and in viral infection in mice [9]. In addition to M1 being associated with accumulation of hemozoin and the occurrence of acute lung injury, polarisation of macrophages to the M1 subtype is responsible for controlling infectious diseases during the acute phase. In influenza virus infection, lung macrophages can polarize to M1 in the early stage and later present the M2b phenotype. Functionally, M1 macrophages are characterised by enhanced endocytic functions and enhanced ability to kill intracellular pathogens [28]. In Listeria monocytogenes infection, M1 macrophages were reported to be activated. These macrophages help prevent bacterial phagosome escape and stimulate intracellular killing of bacteria [27].

From this study, M1 macrophages predominate in the PE group and correlate directly with lung pathology, providing evidence for M1 associated damaged lung tissue in severe P. falciparum malaria. M2 polarisation (CD163) however, was similar
in both the PE and non-PE group. Previous reports documented an increase CD163 levels in uncomplicated malaria patients as compared to severe malarial anemia and cerebral malaria patients. This phenomenon is suggestive of a higher-level anti-inflammatory response in uncomplicated malaria in order to avoid disease complications [29]. Since lung samples from this study was available only once, at autopsy, no data on sequential M1/M2 shifts between early and late infection in human malaria is possible. Theoretically, at a later stage of malaria infection, after pathogenic factors are eliminated through therapeutic anti-malarial treatment and immune clearance of malaria parasites, lung macrophages may shift from a M1 phenotype to the anti-inflammatory M2 phenotype. The switching of macrophage polarity has been associated with transcriptional control [3]. M2 macrophages have an important role in lung tissue repair by limiting the levels of proinflammatory cytokines in the cellular space and producing anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonist [5]. M2 macrophages also remove necrotic cells and debris through phagocytic activity, which can be demonstrated in the malaria lung tissues as lung macrophages engulf hemozoin and fragmented PRBCs. Pulmonary macrophages are important factor in causing lung pathology in malaria. The study showed M1 macrophage activation in ALI in severe P. falciparum malaria, which indicates disease severity. As M1 polarisation prevails, more lung damage can occur. Further investigation on the pathways of the malaria-induced M1 phenotype is necessary to identify specific activators for M1 polarisation that could be attenuated and factors that promote the anti-inflammatory M2 phenotype, in order to suppress the inflammation and improve clinical ALI in severe malaria patients.

Abbreviations
**ABC:** avidin-biotin complex

**ALI:** acute lung injury

**ALP:** alkaline phosphatase

**ALT:** alanine transaminase

**ARDS:** adult respiratory distress syndrome

**AST:** aspartate aminotransferase

**BUN:** blood urea nitrogen

**CD:** cluster differentiation

**COPD:** chronic obstructive pulmonary disease

**CXCL:** C-X-C motif ligand

**DAB:** 3,3’-diaminobenzidine

**H&E:** haematoxylin and eosin

**HPF:** high power field

**HRP:** horseradish peroxidase

**IFN:** interferon

**IL:** interleukin

**LPF:** low power field

**LPS:** lipopolysaccharide

**M1:** classically activated macrophages

**M2:** alternatively activated macrophages

**p:** probability value

**PBS:** phosphate-buffered saline

**PE:** pulmonary oedema

**PRBC:** parasitised red blood cells

**r_s:** Spearman correlation coefficient
Availability of data and materials

All data generated or analysed during this study are included in this published article.

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**Contributions**

AK, SG and CS retrieved lung samples, prepared specimen for histopathological evaluation, performed immunohistochemical work, analysed the data, and drafted the manuscript. SA and SK supervised laboratory work, analysed the clinical data and revised the manuscript. SAW and PV formulated the research idea, designed the experiments, analysed the immunohistochemistry data and revised the final manuscript. All authors have approved the final version of this manuscript.

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**Ethics declarations**

**Ethics approval and consent to participate**

The study protocol was reviewed and ethical clearance was obtained from the Ethics Committee of Faculty of Tropical Medicine, Mahidol University (MUTM 2017-054-01).

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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**Table**

| Parameters                              | Severe malaria patients |
|-----------------------------------------|-------------------------|
|                                         | non-PE                  | PE                        |
| Age (years)                             | 24.00 ± 5.09            | 27.00 ± 2.53              |
| Haematocrit (%)                         | 32.20 ± 2.91            | 24.00 ± 1.53              |
| Blood urea nitrogen (BUN) (mg/dl)       | 21.30 ± 3.16            | 24.00 ± 1.53              |
| Creatinine (mg/dl)                      | 1.45 ± 0.29             | 2.61 ± 0.57               |
| Alanine transaminase (ALT) (U/l)        | 179.33 ± 150.42         | 42.75 ± 12.42             |
| Aspartate aminotransferase (AST) (U/l)  | 269.75 ± 193.05         | 58.25 ± 18.53             |
| Alkaline phosphatase (ALP) (U/l)        | 75.33 ± 36.75           | 17.65 ± 12.99             |
| Albumin (g/l)                           | 2.93 ± 0.36             | 2.50 ± 0.26               |
| Globulin (g/dl)                         | 2.75 ± 0.19             | 3.15 ± 0.38               |
| Total bilirubin (mg/dl)                 | 3.33 ± 1.75             | 6.87 ± 3.82               |
| Direct bilirubin (mg/dl)                | 8.09 ± 4.13             | 12.08 ± 6.21              |
| White blood cell (WBC) (/µl)            | 25,291 ± 15,400         | 25,200 ± 12,947           |
| Parasitaemia/µl on admission            | 401.00 ± 369.95         | 93.50 ± 56.28             |

*Significant difference of $p < 0.05$

**Figures**
Figure 1

Histopathological changes of lungs in P. falciparum malaria: A- alveolar congestion
Lung injury score in malaria patients. A significant difference was observed between PE and non-PE patients (p < 0.05). Data presented as mean ± SEM.
The immunohistochemical staining of lung macrophages in *P. falciparum* malaria.
Figure 4

Comparative mean percentages of lung macrophage positive cells for CD 40 (A) and CD 163 (B) between PE groups and non-PE groups.
Figure 5

Positive correlations between M1/M2 surface markers and histopathological changes.