CELLULAR-MEDIATED IMMUNE RESPONSES IN THE LIVER TISSUE OF PATIENTS WITH SEVERE *PLASMODIUM FALCIPARUM* MALARIA

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Abstract. The immune responses against *Plasmodium falciparum* malaria infections are complex and poorly understood. No published studies have yet reported the lymphocyte subsets involved in the human liver tissue of *P. falciparum* malaria patients. To understand the cellular-mediated immune responses in the liver during malaria infection, we determined the numbers of the various lymphocyte subsets in tissue samples obtained at autopsy from patients who died with *P. falciparum* malaria infection. All the liver tissue specimens had been stored at the Department of Tropical Pathology, Faculty of Tropical Medicine, Mahidol University, Thailand. On the basis of total bilirubin (TB) levels prior to death, patients were divided into 2 groups: those with hyperbilirubinemia [total bilirubin (TB) ≥51.3 µmol/l) ($n = 9$)] and those without hyperbilirubinemia (TB <51.3 µmol/l) ($n = 12$). Normal liver specimens ($n = 10$) were used as controls. An immunohistochemistry method was used to analyze the types and numbers of lymphocytes (T and B lymphocytes), and Kupffer cells, using specific antibodies against CD3+, CD4+, CD8+, CD20+, and CD68+. Our findings reveal the numbers of T lymphocytes (CD3+ T-cells) and their subsets (CD4+ and CD8+ T-cells) were significantly greater in the portal tracts and sinusoids of liver tissue obtained from *P. falciparum* malaria cases with hyperbilirubinemia than those without hyperbilirubinemia or controls. CD8+ T-cells were the major lymphocyte subset in the liver tissue of patients with severe falciparum malaria. A significant positive correlation was seen between the numbers of CD4+ and CD8+ T-cells and the liver enzyme levels among *P. falciparum* malaria patients. The number of CD68+ cells (Kupffer cells) was significantly greater in the liver sinusoids of *P. falciparum* malaria cases with hyperbilirubinemia than those without hyperbilirubinemia. These findings suggest T-cells, especially CD8+ T-cells and Kupffer cells are an important part of the cellular immune response in the liver tissue of *P. falciparum* infected patients.

Keywords: *Plasmodium falciparum*, malaria, liver, lymphocyte subsets, T-cells, B-cells, Kupffer cells, immunohistochemistry

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INTRODUCTION

The liver is the first organ targeted by *Plasmodium* sporozoites following an infective mosquito bite (Frevert, 2004). Hepatocytes are an initial obligatory site for schizogony, a process of plasmodial reproduction (Frevert, 2004; Frevert *et al*, 2005). Evidence suggests the host's immune response can contribute to the pathophysiology of malaria (Spence and Langhorne, 2012). This evidence includes the finding of proinflammatory cytokines released by T-cells and macrophages in response to malaria parasites and their products. These include: glycosylphosphatidylinositol (GPI), malaria pigment and plasmodium-derived nitric oxide synthase (NOS)-inducing factor (Malaguarnera and Musumeci, 2002). The liver is not only pivotal for the survival of the parasite, but crucial for inducing the anti-plasmodium protective immune response (Berenzon *et al*, 2003). Macrophages, other important cellular effectors, antigens and cytokines, are involved in activating the immune response against the pre-erythrocyte and blood-stages of malaria (Malaguarnera and Musumeci, 2002). The parasite can induce a specific immune response, stimulating the release of cytokines from human peripheral blood mononuclear cells (PBMCs), which may play an important role in activating the host's cellular immune response (Hensmann and Kwiatkowski, 2001; Malaguarnera and Musumeci, 2002). T-cells play a major role in protective immunity against pre-erythrocytic *Plasmodium* infections (Malaguarnera and Musumeci, 2002). CD4+ T-cells are crucial for the development of the CD8+ T-cell response to malaria-infected hepatocytes (Carvalho *et al*, 2002). Recent studies have shown that the interaction and balance between CD4+ and CD8+ T-cells dictates immunity and the genesis of malaria cytokines (Spence and Langhorne, 2012). Histopathological findings have shown that lymphocytes are the predominant cell type in inflammatory infiltrate in the portal tracts and sinusoidal spaces of the liver in patients infected with *P. falciparum* (Bhalla *et al*, 2006; Kochar *et al*, 2003; Viriyavejakul *et al*, 2014). Livers infected with *P. falciparum* have pronounced macrophage accumulations (Kochar *et al*, 2003; Prommano *et al*, 2005; Viriyavejakul *et al*, 2014), with hyperplastic characteristics (Prommano *et al*, 2005) and apoptotic changes (Viriyavejakul *et al*, 2014).

Since immunity in malaria appears to be T-cell dependent, it is important to determine the cellular elements associated with the immune mediated tissue response to *P. falciparum* infection in the liver. In this study we investigated the numbers of the various lymphocyte populations and Kupffer cells in the liver tissue of patients with *P. falciparum* infection. This knowledge could help us better understand the immune response to *P. falciparum* malaria in the human liver.

MATERIALS AND METHODS

Patients

The liver tissue samples from patients with malaria were obtained from autopsy specimens from patients who died from *P. falciparum* infection. The tissue was paraffin-embedded and had been stored at the Department of Tropical Pathology, Faculty of Tropical Medicine, Mahidol University, Thailand. The liver tissue was obtained from two types of patients: those who had a total bilirubin (TB) level of ≥51.3 µmol/l and those who had a TB level of <51.3 µmol/l prior to death. The control liver tissue samples were acquired from

autopsy specimens from patients who died from accidents and had no history of liver pathology. Specimens obtained from patients with a history of liver cirrhosis, alcoholic hepatitis, drug-induced hepatitis, autoimmune hepatitis, liver cancer, viral hepatitis or human immunodeficiency virus (HIV) infection were excluded from the study. The use of specimens and the research protocol were approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (MUTM 2013-013-01) and the Ethics Committee on Human Rights Related to Research Involving Human Subjects, Walailak University, Thailand (062/2012).

Immunohistochemical detection of lymphocyte subsets and Kupffer cells

Five micrometer thick histopathological sections were obtained from each paraffin-embedded specimen and placed on glass slides coated with 3-aminopropyltrietoxysline (Sigma, St Louis, MO) for immunohistochemistry staining for specific antibodies. Immunostaining was conducted using a Vecterstain ABC kit (Vector Laboratories, Burlingame, CA), according to the manufacturer's instructions. Briefly, after deparaffinization in xylene and rehydration in a series of ethanol dilutions, the sections were exposed to microwave antigen retrieval using a sodium citrate buffer (pH 6.0) at 800 watts for 15 minutes, then cooled for 20 minutes to room temperature. The sections were placed in 3% hydrogen peroxide for 30 minutes to inhibit endogenous peroxidation. After washing with phosphate-buffered saline (PBS, pH 7.4), the nonspecific binding site was blocked with normal goat serum for 30 minutes at room temperature. Sections were incubated overnight at 4°C with specific primary antibodies: CD3+ (all T-cells; polyclonal 1:200), CD4+ (mono-

clonal OPD4 1:200), CD8+ (monoclonal 1:100), CD20+ (mature B-cells; monoclonal 1:200) and CD68+ (macrophage; monoclonal 1:200). All antibodies were purchased from Dako Company, Glodtrup, Denmark. Next, the sections were washed three times with PBS and incubated with a secondary antibody for 30 minutes at room temperature and reacted with avidin-biotin complex (ABC) conjugated with horseradish peroxidase (HRP) (Vecterstain ABC kit, Vector Laboratories, Burlingame, CA). After washing with PBS, enzymatic activity was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB) for peroxidase (brown color). Finally, the sections were counterstained with hematoxylin, dehydrated and then mounted with a coverslip.

To confirm the specificity of the primary antibodies and validate the immunohistochemistry staining techniques, lymph node and thymus tissue samples were used as positive controls. A negative control was obtained by omission of the primary antibody and then stained. Liver sections were incubated with the immunohistochemistry substrate (DAB) to confirm the presence of endogenous peroxidase activity.

Evaluation of immunohistochemistry staining

Evaluations were conducted by two independent observers who were blinded to the patient disease category. Cell counts were performed manually at 400x magnification for immunoreactive cells (CD3+, CD4+, CD8+ and CD20+) and the total number of lymphocytes (separately in portal tracts and sinusoids) for 10 microscopic fields at 400x magnification. For each field, the percent immunoreactivity for the antigen was obtained by dividing the number of positive

T lymphocytes (CD3+, CD4+, and CD8+) or B lymphocytes (CD20+) by the total number of lymphocytes counted. The average scores were then calculated. The number of CD68+ cells was counted for each sinusoidal space field for 10 fields at 400x magnification. The mean number of CD68+ cells was calculated and expressed as the mean of number of positive cells per high-power field (HPF).

Statistical analysis

The mean ± standard error of the mean (SEM) were calculated for all data. The normality of distribution was tested using the Kolmogorov-Smirnov test. Differences between groups were analyzed with the Mann-Whitney U test. The Spearman's rank correlation coefficient was calculated and used to estimate the direction and strength of correlations between the mean percentage of positive cells and clinical data. Statistical analysis was performed using SPSS version 17.0 software (IBM, Armonk, NY). A p-value <0.05 was considered significant.

RESULTS

Lymphocyte subsets in liver tissue infected with *P. falciparum*

The liver tissue samples from malaria patients had significantly ($p <0.001$) more lymphocytes than the samples from the non-malaria cases. On immunohistochemistry staining of CD3+ T-cells and its subsets, CD4+ and CD8+ T-cells (Fig 1) and CD20+ B-cells (Fig 2), revealed these cells were mainly seen in the portal tracts of the liver in the falciparum malaria cases.

The portal tracts of the liver tissue samples from the malaria cases with hyperbilirubinemia had significantly higher ($p <0.001$) numbers of CD3+ (66.91 ± 0.87%), CD4+ (20.23 ± 0.24%), and CD8+ (45.73 ± 0.29%) T-cells and CD20+ (23.52 ± 0.61%) B-cells than those without hyperbilirubinemia (CD3+: 38.49 ± 1.05%; CD4+: 13.87 ± 0.34%; and CD8+: 24.1 ± 0.24%; CD20+: 19.97 ± 0.50%) or the control groups (CD20+: 13.90 ± 0.32%) ($p <0.001$) (Fig 3). Similarly, in the sinusoidal areas in the malaria patients with hyperbilirubinemia, there were significantly greater numbers of CD3+ (63.95 ± 0.21%), CD4+ (20.27 ± 0.63%) and CD8+ (45.43 ± 0.43%) T-cells, and CD20+ (45.43 ± 0.43%) B-cells compared to those without hyperbilirubinemia (CD3+: 44.34 ± 0.61%); CD4+: 12.14 ± 0.38%; CD8+: 33.92 ± 0.51% and CD20+: 28.67 ± 0.4%) ($p <0.001$) or the control group (14.87 ± 0.23%) ($p <0.001$). The CD4+/CD8+ ratio in the portal tracts of the malaria patients with hyperbilirubinemia (0.44 ± 0.00) was significantly lower than in the malaria patients without hyperbilirubinemia (0.57 ± 0.01) and the control group (2.35 ± 0.13) ($p <0.05$). In the sinusoidal areas, the CD4+/CD8+ ratio of the malaria patients with hyperbilirubinemia (0.45 ± 0.02) was significantly higher than in the malaria patients without hyperbilirubinemia (0.36 ± 0.01) and was significantly lower than the control group (2.08 ± 0.24) ($p <0.05$).

Quantification of Kupffer cells in *P. falciparum*-infected liver tissue

In the control livers, a few CD68+ Kupffer cells were elongated, thin and spindle-shaped and distributed in the sinusoidal areas, and throughout the lobules (Fig 4A). Prominently enlarged aggregations of Kupffer cells were seen in the sinusoids in the *P. falciparum* cases with hyperbilirubinemia. Accumulations of CD68+ Kupffer cells were seen in the sinusoids of *P. falciparum* patients with and without hyperbilirubinemia (Fig 4B and 4C). The mean number of CD68+
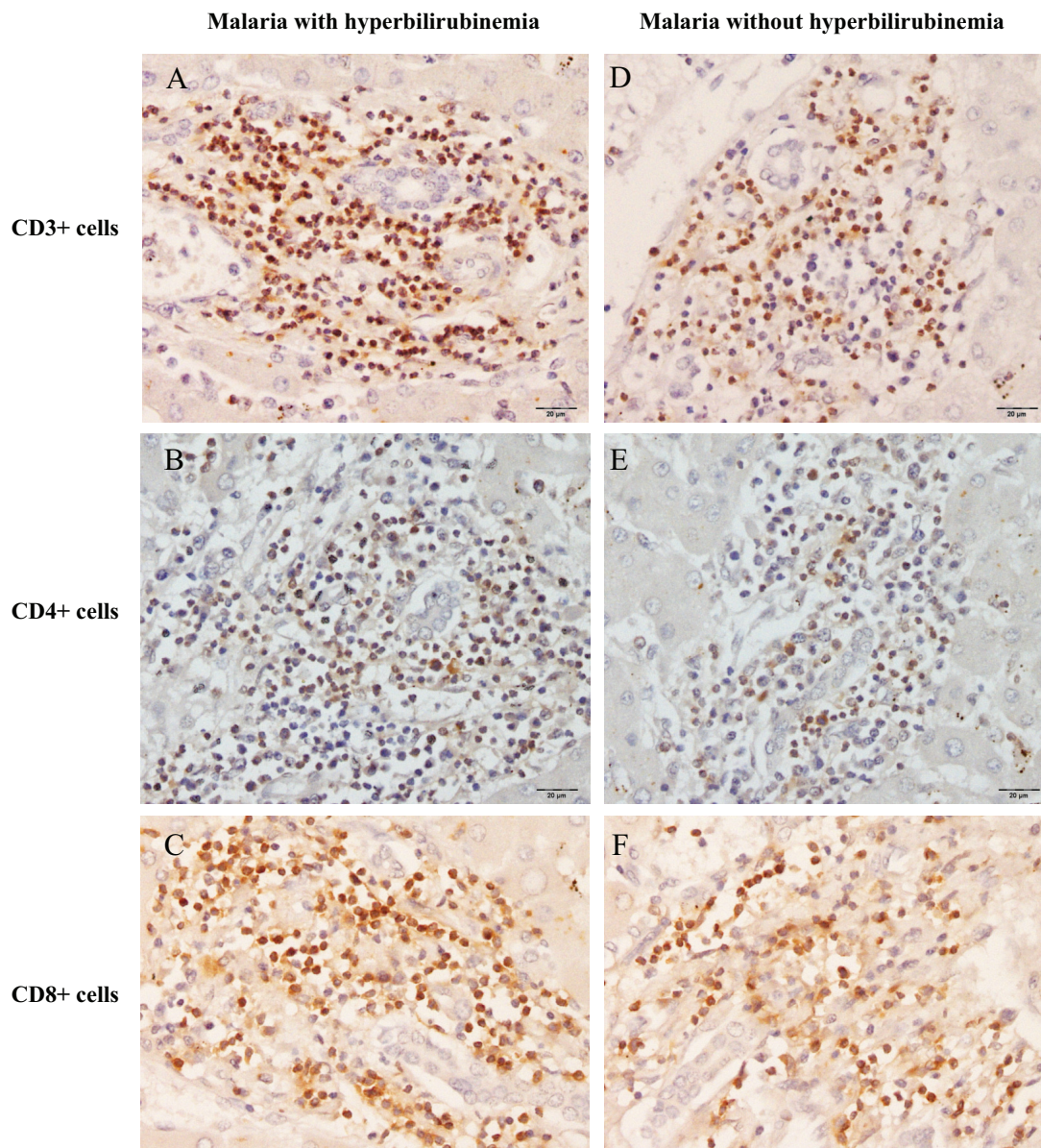
Malaria with hyperbilirubinemia   **Malaria without hyperbilirubinemia**

Fig 1–Immunohistochemical staining of T lymphocytes (CD3+ T-cells) and their subsets (CD4+ and CD8+ T-cells) in the liver tissue of falciparum malaria cases with hyperbilirubinemia (A, B, C) and without hyperbilirubinemia (D, E, F) (x400).

Kupffer cells was significantly higher in the liver tissue of *P. falciparum* cases with hyperbilirubinemia (51.57 ± 0.23 cells/HPF) than without hyperbilirubinemia (38.65 ± 0.52 cells/HPF; $p <0.005$) or in the controls (14.64 ± 0.30 cells/HPF; $p <0.005$).

Association between T lymphocytes, B lymphocytes and Kupffer cells and clinical data

A significant positive association was found between the mean number of CD3+ T-cells in the portal tract and the total bilirubin (TB) ($r_s = 0.591$, $p = 0.010$) and direct
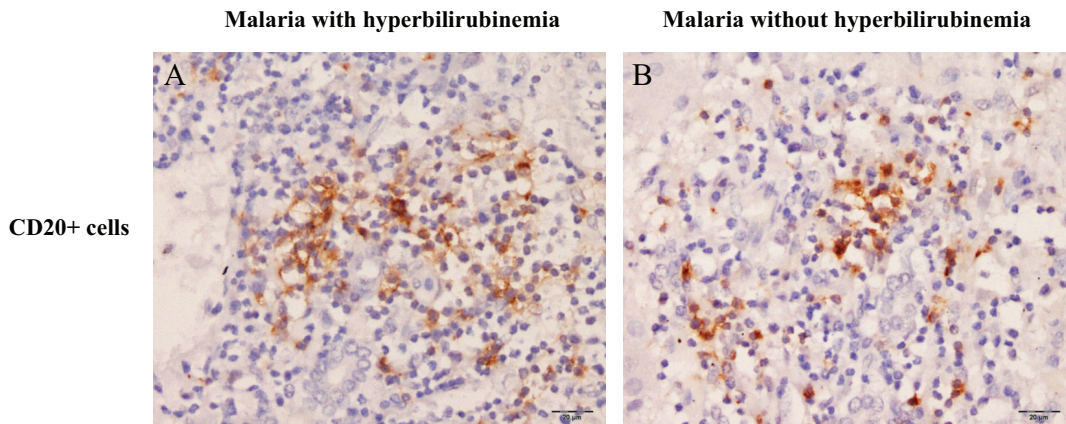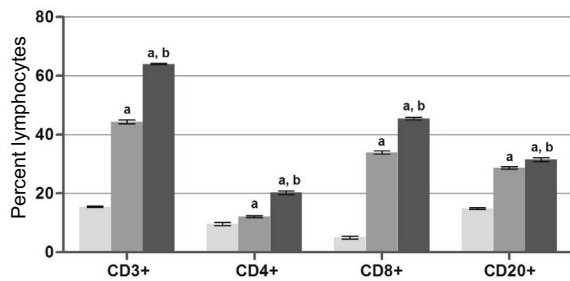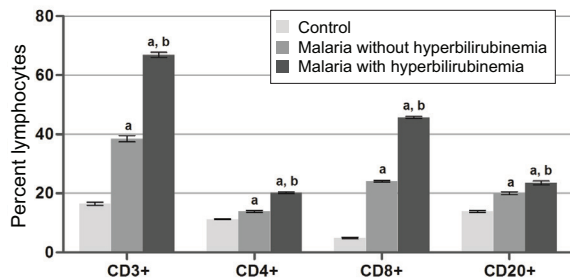
Malaria with hyperbilirubinemia **Malaria without hyperbilirubinemia**

CD20+ cells

Fig 2–Immunohistochemical staining of B lymphocytes (CD20+ B-cells) in the liver tissue of a severe falciparum malaria case with hyperbilirubinemia (A), and without hyperbilirubinemia (B) (x400).

A Portal tracts

B Sinusoids

aSignificant difference compared to normal.
bSignificant difference by malaria group.

Fig 3–Mean percentages and types of lymphocytes in the portal tracts (A) and sinusoids (B) of liver tissue samples from severe falciparum malaria cases with hyperbilirubinemia ($n = 12$), without hyperbilirubinemia ($n = 9$) and in normal controls ($n = 10$).

bilirubin (DB) ($r_s = 0.564$, $p = 0.015$). The number of CD4+ T-cells was significantly positively associated with aspartate aminotransferase (AST) ($r_s = 0.764$, $p < 0.001$), alanine aminotransferase (ALT) ($r_s = 0.569$ $p = 0.014$), TB ($r_s = 0.542$, $p = 0.020$) and DB ($r_s = 0.547$, $p = 0.019$) levels. In the sinusoidal spaces, a significant positive association was seen between the mean numbers of CD4+ and CD8+ T-cells and AST, TB and DB levels. The mean number of CD68+ Kupffer cells was positively associated with TB ($r_s = 0.618$, $p = 0.006$) and DB ($r_s = 0.640$, $p = 0.004$) levels. No significant association was found between the mean numbers of T lymphocytes (and their subsets), B lymphocytes and Kupffer cells and the clinical data (age, sex and parasitemia).

Association between lymphocyte subsets in the portal area and sinusoidal spaces

Significant associations were seen between the number of portal and sinusoidal CD4+ T-cells, ($r_s = 0.767$, $p < 0.001$) and the number of portal and sinusoidal CD8+T- cells ($r_s = 0.707$, $p < 0.001$) (Fig 5).
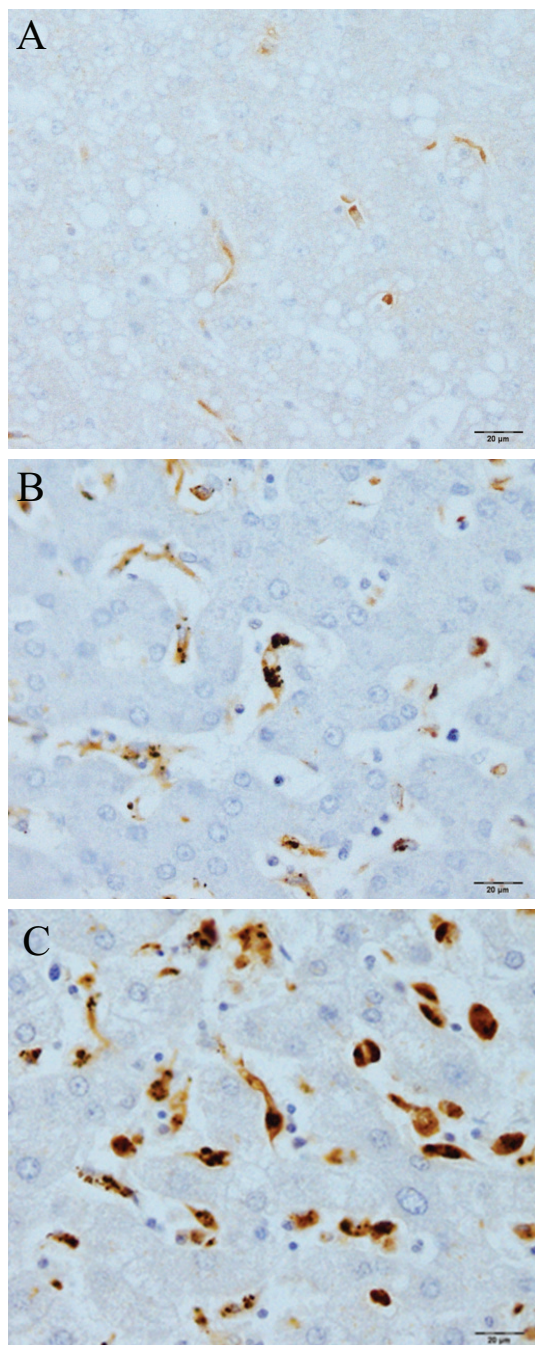
Fig 4–Immunohistochemistry staining of CD68+ in the liver tissue samples from: (A) control patient, (B) patient with *P. falciparum* malaria without hyperbilirubinemia and (C) patient with *P. falciparum* malaria with hyperbilirubinemia (x400).

DISCUSSION

The liver contains large resident and migratory populations of lymphocytes and macrophages, which provide immune surveillance against foreign antigens (Lalor *et al*, 2002). Lymphocytes are mobile immune cells that circulate within blood vessels and lymphoid organs, and migrate through tissues. Immunologically, there are two major types of lymphocytes: T-cells and B-cells. In malaria, T-cells are involved in eliminating the growth and development of both pre-erythrocytic and erythrocytic stages of Plasmodia, while B-cells provide protective immunity. In the present study, we found T lymphocytes (CD3+ T-cells) and their subsets (CD4+ and CD8+ T-cells) in significantly greater numbers in the portal tracts and sinusoids of the liver tissue of *P. falciparum* malaria patients with hyperbilirubinemia than those without hyperbilirubinemia. These greater numbers may be due to chemical mediators or antigen stimulation expressed during the pre-erythrocytic stage of plasmodium infection, such as glycosylphosphatidylinositol (GPI) (Nebl *et al*, 2005) or malaria antigens. The liver has the capacity to sequester activated CD8+ T-cells by removing them from the circulation (Mehal *et al*, 1999). This effect depends on the intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expressed in the hepatic vasculature (John and Crispe, 2004). Circulating lymphocytes can enter the liver at three different sites: 1) the vascular endothelium of the portal tract, 2) the sinusoids and 3) the central hepatic veins (Lalor and Adams, 2002; Lalor *et al*, 2002). Kupffer cells, as antigen-presenting cells (APC), can induce liver-specific CD8+ T-cell retention and tolerization (Knolle and Gerken, 2000; Limmer *et al*,
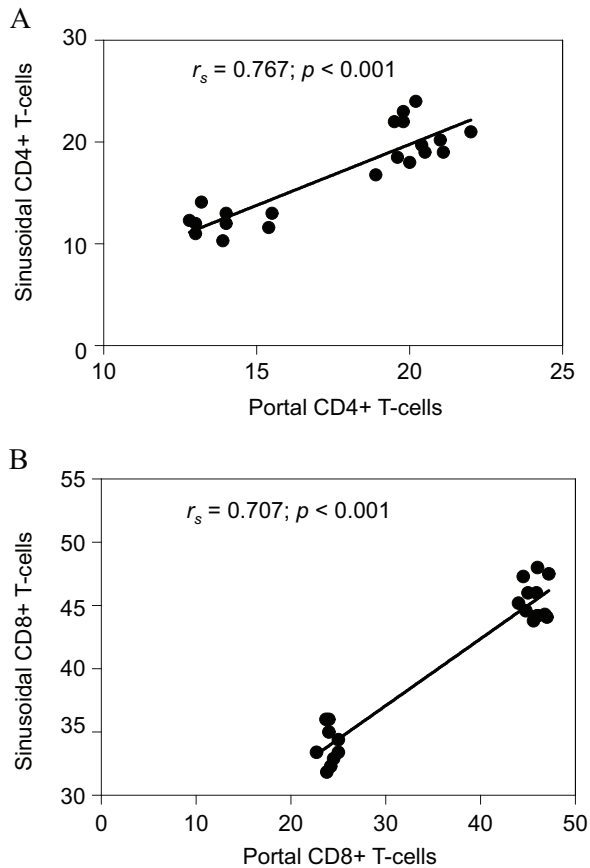
A

$r_s = 0.767; p < 0.001$

B

$r_s = 0.707; p < 0.001$

Fig 5–Association between CD4+ cells in portal and sinusoid areas of the liver (A) and between CD8+ cells in the portal and sinusoid areas of the liver (B).

2000; von Oppen *et al*, 2009). In an experimental animal model, the cytokines, interferon gamma (IFNγ) and tumor necrosis factor (TNF), induced by *P. berghei*, caused liver injury and activated lymphocytes in the liver (Adachi *et al*, 2001).

Greater production of T-cell activation markers has been seen among human with acute malaria than among non-infected controls (Riley *et al*, 1993; Elhassan *et al*, 1994). In contrast, significantly lower levels of circulating CD8+ cytotoxic lymphocytes have been seen among malaria patients living in malaria endemic areas than among non-infected controls (Srisurapanon *et al*, 2003). These lower numbers of circulating CD8+ T-cells may result from recruitment of circulating lymphocytes to the liver, which are associated with greater numbers of intrahepatic infiltrating CD8+ T-cells in patients with severe *P. falciparum* malaria (Srisurapanon *et al*, 2003). The greater number of T lymphocytes may compensate for the dysfunction of intrahepatic T-cells. This is consistent with a recent report demonstrating lymphocyte apoptosis in the liver during severe *P. falciparum* malaria with hyperbilirubinemia (Viriyavejakul *et al*, 2014). However, further study is needed to address the function of intrahepatic T-cells. The density of infecting parasites, duration of infection, and duration of the cytokine response may affect the numbers of intrahepatic T-cells in severe *P. falciparum* malaria. In this study, a significant association was seen between portal and sinusoidal CD8+ and CD4+ T lymphocytes. This association in our study may support that the theory of CD4+/CD8+ cross-talk is involved in the development of immune responses against malaria infection. A previous study proposed that interleukin-4 (IL-4) secreted by CD4+ T-cells is essential for the development of CD8+ T-cell responses to hepatocytes infected with malaria parasites (Carvalho *et al*, 2002).

An altered balance in T-cell subsets (increased proportion of CD8+ T-cells and decreased proportion of CD4+ T-cells) was found in the liver of *P. falciparum* malaria patients, which could be caused by recruitment of T-cells in response to protective immunity against *Plasmodium* infection in the liver (Tsuji and Zavala, 2003). Cytokine can mediate recruitment of T-cells into the liver in response to the process of elimination and clearance of

parasites (Spence and Langhorne, 2012). A significant positive correlation was seen between the number of CD4+ and CD8+ T lymphocytes and liver enzyme levels in *P. falciparum* malaria patients in our study. Elevated liver enzymes, along with hepatocellular necrosis is found in the liver of *P. falciparum* malaria patients (Kochar *et al*, 2003). Inflammatory cell infiltration of the liver is associated with elevated aminotransferase levels during dengue infection (de Macedo *et al*, 2006). The positive association between the number of CD4+ and CD8+ T lymphocytes in our study suggests possible intrahepatic infiltration involved in liver pathology during malaria infection.

In our study, greater numbers of Kupffer cells were found in the liver sinusoidal spaces of *P. falciparum* malaria patients with hyperbilirubinemia than without hyperbilirubinemia or controls. The greater number of Kupffer cells may be due to recruitment and activation of macrophages to eliminate parasitized infected red blood cells accumulated in the liver sinusoids. Macrophage can control malaria parasites through both antibody-dependent and independent phagocytosis (Chua *et al*, 2013; Malaguarnera and Musumeci, 2002) and secretion of soluble cytokines, such as interleukin 1 (IL-1) and TNF (Pichyangkul *et al*, 1994). A large number of resident macrophages containing hemozoin pigment has been found in the liver tissue of *P. falciparum* infected patients (Kochar *et al*, 2003; Prommano *et al*, 2005; Viriyavejakul *et al*, 2014). Accumulation of malaria pigment is associated with impairment of macrophage activation and function (Schwarzer *et al*, 1998). The recruitment and activation of macrophages for phagocytosis is necessary for clearance of malaria parasites (Chua *et al*, 2013). Several studies have proposed that the immunopathological role of macrophages may be implicated in the development of severe complications in malaria, such as severe malarial anemia, cerebral malaria and acute lung injury (Baratin *et al*, 2005; Chua *et al*, 2013) and in experimental models of malaria (Pais and Chatterjee, 2005). Our study confirmed hepatic Kupffer cells constitute the major immune cells in the liver during severe malaria infection. They are responsible for the phagocytosis of parasites, and are important cells contributing to parasite clearance.

In conclusion, the T-cell-dependent immune response in the liver during severe falciparum malaria is critical for parasitic clearance. CD8+ T-cells are important for the cellular immunity response in liver tissue during *P. falciparum* infection. Further studies are needed to determine the local immune mechanisms seen in the liver of a severe falciparum malaria patient, and to specify the functional role of CD8+ T-cells in malaria infection.

ACKNOWLEDGEMENTS

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