LOW MONOCYTE TO NEUTROPHIL RATIO IN PERIPHERAL BLOOD ASSOCIATED WITH DISEASE COMPLICATION IN PRIMARY *PLASMODIUM FALCIPARUM* INFECTION

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Abstract. Immunity to malaria can be acquired but only after repeat exposures to polymorphic *Plasmodium*. However, the development of clinical outcomes during *P. falciparum* infection is not clearly understood. This study elucidated whether monocytes, neutrophils and pro/anti-inflammatory cytokines were associated with clinical outcomes in single infection and prior repeated malaria infections. Two hundred and seventy-nine patients with complicated and uncomplicated malaria were investigated. Peripheral blood IFN-γ, TNF-α and IL-10 levels were measured by ELISA, and monocytes and neutrophils by an automated cell counter. On admission, in patients with uncomplicated malaria prior repeated infections, absolute neutrophil counts were positively and monocyte to neutrophil ratio negatively correlated significantly with parasitemia ($r = 0.358$, $p = 0.000$; $r = -0.356$, $p = 0.000$, respectively), while those with single infection absolute monocyte counts and monocyte to neutrophil ratio were significantly correlated negatively with IFN-γ ($r = -0.381$, $p = 0.001$; $r = -0.393$, $p = 0.000$, respectively), and positively with TNF-α levels ($r = 0.310$, $p = 0 .007$; $r = 0.227$, $p = 0.017$, respectively). In sharp contrast, in complicated malaria with single infection extremely high IFN-γ and IL-10 levels but significantly low percent monocyte counts and monocyte to neutrophil ratio were seen. After 7 days of treatment, absolute monocyte counts and monocyte to neutrophil ratio were significantly increased, while absolute neutrophil counts significantly decreased ($p = 0.000$, 0.000, and 0.001, respectively), similarly after 28 days of treatment ($p = 0.008$, 0.000 and 0.000, respectively). These results suggest different functions of monocytes, neutrophils and pro/anti-inflammatory cytokines in complicated and uncomplicated malaria with single *P. falciparum* infection or

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prior repeated infections in the context of disease severity. Low monocyte to neutrophil ratio may be regarded as a risk factor in developing complication in primary malaria infection.

Keywords: *P. falciparum*, cytokines, monocytes, neutrophils, malaria experiences, severity

INTRODUCTION

Severe malaria is responsible for approximately 0.7 million deaths in 2010, predominantly children under five years of age in endemic areas of *Plasmodium falciparum* infection (Murray *et al*, 2012). Multi-factors including impaired innate and limited malaria-specific immunity probably are causes for the progression to severe clinical malaria (Urban *et al*, 2001; Rovira-Vallbona *et al*, 2012). In areas with intense and stable malaria transmission, naturally acquired immunity is short lived, slow to develop, host and age dependent, incomplete and lost upon cessation of exposure (Kinyanjui *et al*, 2007). In low and seasonal malaria transmission, little or no acquisition of clinical immunity exists and subsequence of severe malaria can be fatal in all age groups (Baird, 1995).

When malaria re-emerged in Madagascar after a long period of eradication, people living 30 years earlier during malaria exposure were much more resistant to clinical disease than were younger individuals (Deloron and Chougnet, 1992). Specific memory-B cells have been detected in blood as long as 8 years after *P. falciparum* infection (Migot *et al*, 1995). Both cellular and humoral immunity to *P. falciparum* can persist in West African migrants after having spent up to 13 years outside malaria endemic areas (Chougnet *et al*, 1991), suggesting the existence of long-lived immunological memory.

In innate immunity, monocytes/macrophages and neutrophils are the main immune effectors for controlling malaria blood-stage infection via phagocytic activity (Serghides *et al*, 2003), particularly when cytokines, such as TNF-α and opsonizing antibodies, are present, which release reactive oxygen species (ROS), such as superoxide (Joos *et al*, 2010), highly toxic to intra-erythrocytic malaria parasites (Becker *et al*, 2004). Activation through pattern recognition receptors (PRRs) present on monocytes, dendritic cells and neutrophils induce release pro-inflammatory cytokines and chemokines, amplifying innate immune responses and thereby shape the development of acquired immunity (Kolli *et al*, 2013).

In falciparum malaria, analyses of cytokine levels alone can yield paradoxical results regarding protection and pathology of the underlying highly integrated immune responses (Baker *et al*, 2008). During acute infection, monocytes produce high levels of IL-1β, IL-12 and TNF-α (Fell and Smith, 1998). In a mouse model, depletion of neutrophils reduces expression of TNF-α, IFN-γ and IL-12 in the brain of mice with cerebral malaria (Chen *et al*, 2000), whereas malaria pigment, hemozoin (HZ), a rest product of hemoglobin induce high cytokine production by monocytes contributing to dendritic cell (DC) maturation (Jaramillo *et al*, 2004). In humans, an impaired function on the maturation of monocytes and DCs due to malaria has been indicated by the reduced numbers of blood DCs in adults (Pichyangkul *et al*, 2004) including pregnant women (Diallo *et al*, 2008). Low levels of *P. falciparum*

blood-stage infection cause DC apoptosis and dysfunction in healthy volunteers (Woodberry *et al*, 2012), possibly by HZ-induced inactivation of T cells, and secretion of immunosuppressive IL-10 instead of IL-12 (Urban *et al*, 2001). Although IFN-γ is a key inducer of immune effector mechanisms, essential for initial control of pre-erythrocytic and blood-stage infections (Good and Doolan, 1999) with higher concentrations in symptomatic than asymptomatic individuals (Mshana *et al*, 1991), their levels need to be carefully balanced to avoid immune pathology (Artavanis-Tsakonas *et al*, 2003). TNF-α has a role in clearance of malaria parasites and resolution of fever (Kremsner *et al*, 1995), but excessive TNF production may damage host tissues associated with severe malaria (Kwiatkowski, 1990). Regulatory cytokines including transforming growth factor beta (TGF-β) and IL-10 are important in dampening down T helper 1 (Th1) inflammatory responses (Day *et al*, 1999), and higher IL-10 to TNF-α ratios were reported in children with mild and high parasitemia than malaria anemia (Othoro *et al*, 1999). Thus, proper regulation of pro- and anti-inflammatory cytokines is of the utmost importance in malaria treatment.

Functional evaluation of immune cells in association with pro- and anti-inflammatory cytokines in single or repeated infections is essential in order to understand the development of immunocompetence and progression of malaria infection to the severe forms. In this study we analyzed levels of monocytes, neutrophils, IL-10, IFN-γ and TNF-α with clinical outcomes of the disease in patients with single *P. falciparum* infection and prior repeated infections. The numbers of monocytes and neutrophils in relation to parasitemia were also evaluated.

MATERIALS AND METHODS

Subjects

Description of the subjects and sample collections were reported previously (Tangteerawatana *et al*, 2009). In brief, 110 patients with complicated and 169 with uncomplicated *P. falciparum* malaria who had been living in malaria endemic areas along the Thai-Myanmar border in the west and Thai-Kampuchea border in the east of Thailand were recruited. These areas are considered as having low and seasonal malaria transmission. The two groups of patients were matched for age, gender, nationality and ethnic groups, *ie*, Thai, Burmese, Mon and Karen. The median age was 25 years (range of 14-67) in complicated and 24 years (range 13-65) in uncomplicated malaria. Based on clinical records and interview, 27% and 55% of patients with complicated and uncomplicated malaria, respectively, have had 1-2 malaria episodes (84/125), 3-8 episodes (27/125) and > 8 (12/125). Intervals from current and previous malaria episodes vary from 1 month to 2 years. However, both patient groups have acute infection upon admission.

In this study, patients without previous malaria experience are defined as those with single infection, and with previous malaria experience as with repeated infections. When complicated and uncomplicated malaria were separated according to single or repeated infections, all groups were matched for age, gender, nationality and ethnic groups. Clinical manifestations of complicated and uncomplicated malaria are defined according to World Health Organization criteria (WHO, 2000). This study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

Blood samples

Blood samples were collected in EDTA sterile tubes before and after treatment at admission (Day 0) and on Days 7 and 28 after treatment. Plasma and packed cells were separated and frozen at -20ºC.

Absolute and differential white blood cell (WBC) counts

Absolute and differential WBC counts were determined by an automated cell counter (Advia 120 Hematology System, Siemens Medical Solutions Diagnostics, Tarrytown, NY) at Days 0, 7 and 28. Absolute numbers of monocyte and neutrophil subsets were obtained by multiplication of the absolute WBC with their respective differential WBC counts.

Parasite density

Peripheral blood *P. falciparum* density was determined by thick and thin blood films stained with Giemsa. Parasite concentration was estimated by the number of asexual forms per 200 WBC on thick smear multiplied by WBC counts, or by the number of asexual forms per 1,000 erythrocytes on thin smear multiplied by RBC count. Parasite density is expressed per microliter (µl) of blood.

Confirmation of *Plasmodium* species

To confirm that the patients were infected with *P. falciparum*, the malaria species were determined by nested PCR. DNA was extracted using High Pure PCR Template Preparation Kit according to the manufacture's protocol (Roche, Mannheim, Germany) and subjected to nested PCR for *Plasmodium* spp as previously described (Snounou *et al,* 1993).

Determination of circulating pro- and anti-inflammatory cytokines

Mouse antibodies against human cytokines used as capture antibodies were anti- IL-10 (9D7), -IFN-γ (ID-1K) and -TNF-α (3/4) antibodies, and biotinylated antibodies used for detection were anti-IL-10 (12G8), -IFN-γ (7-B6-1) and -TNF-α (5) antibodies (Mabtech, Nacka, Sweden).

Plasma IL-10, IFN-γ and TNF levels were determined using double sandwich ELISA (Tangteerawatana *et al*, 2007) with some modifications. In brief, 96-well plates (Costar, Bloomington, MN) were coated with capture antibodies (1 µg/ml) by overnight incubation at 4ºC in carbonate buffer pH 9.6. Plates then were incubated with 0.1% milk powder in PBS for 1 hour at room temperature. Fifty microliters of test plasma was added and incubated overnight at 4ºC. Plates were washed with 0.1% tween in PBS and incubated for 1 hour at room temperature with 50 µl of 1:1,000 of appropriate biotinylated antibodies, washed and incubated with streptavidin-conjugated alkaline phosphatase for 1 hour at room temperature. The p-nitrophenyl phosphate (Sigma, St Louis, MO) was used as substrate. Optical density values were measured at 405 nm in a Biotek ELX 808™ plate reader (Bio-Tek Instuments, Winooski, VT) for IFN-γ and TNF-α, and in a Vmax Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA) for IL-10. Cytokine concentrations were calculated using standard curves generated from serial dilutions of recombinant IL-10, IFN-γ and TNF-α (R&D System, Minneapolis, MN). Cut-off value is 6.4, 25.0 and 14.4 pg/ml for IL-10, IFN-γ and TNF-α, respectively.

Statistical analysis

Data were analyzed using SPSS computer software. Mann-Whitney *U* test was applied for comparison between two patients' groups. Correlations between separate variables were examined by Spearman's rank correlation test. For

comparison of absolute and differential WBC counts and cytokine levels before and after treatment, Wilcoxon's matched pairs test was used. A $p < 0.05$ is considered significant.

RESULTS

WBC and monocyte to neutrophil ratio (MNR)

On admission before treatment (Day 0), the absolute WBC counts were significantly higher in patients with complicated malaria with single infection (SCM) than uncomplicated malaria with single infection (SUCM), or complicated malaria with prior repeated infection (RCM) or uncomplicated malaria with prior repeated infection (RUCM). Absolute monocyte counts (AMC) were significantly lower, whereas absolute neutrophil counts (ANC) were significantly higher in SCM than those with SUCM or RUCM, but AMC and ANC in SCM and RCM were not significantly different (Fig 1). On the other hand, percent monocyte in SCM was significantly lower than that of SUCM, RCM or RUCM, whereas percent neutrophil was significantly higher in SCM than SUCM, but not in RCM or RUCM. MNR in SCM was significantly lower than in SUCM, RCM or RUCM.

After treatment, on Day 7 and/or 28 significant increases in absolute WBC, AMC and MNR were observed in SCM, RCM, SUCM and RUCM (Fig 2). However, a significant decrease in ANC was seen only in SCM and RCM, but not in SUCM and RUCM.

Circulating IL-10, IFN-γ and TNF-α levels

The median plasma cytokine levels were analyzed separately based on disease outcomes and history of previous malaria experiences. On Day 0, IL-10 levels were significantly higher in SCM than in SUCM or RUCM, but not in RCM (Fig 3). IFN-γ levels were significantly higher in SCM than in SUCM, RCM or RUCM. However, TNF-α levels among SCM, SUCM, RCM or RUCM were not significantly different.

On Day 7 and/or 28 following treatment, significant decreases in IL-10 levels were observed only in SCM and RCM, but not in SUCM and RUCM (Fig 4). In contrast, a significant decrease in TNF-α was seen only in RCM and RUCM, but not in SCM and SUCM. Conversely, significant decrease in IFN-γ levels were noted in SCM, SUCM and RUCM, while there was no significant difference between Day 0 and Day 7 or Day 28 in RCM.

Relationship of monocytes/neutrophils and cytokine levels

Polymorphonuclear leukocytes (PMNs) and monocytes were involved in IL-10, IFN-γ and TNF-α production (Boeuf *et al,* 2012). In SCM, a significant inverse correlation was observed between IFN-γ levels and ANC, but not in RCM, whereas a significant inverse correlation exits between IFN-γ levels and AMC in RCM but not in SCM (Fig 5). However, no significant correlations were observed between AMC or ANC with IL-10 or with TNF-α levels either SCM or RCM. Conversely, no significant correlations between MNR and IL-10, IFN-γ or TNF-α were observed in SCM or RCM.

In SUCM, there was a significant inverse correlation between AMC or MNR with IFN-γ, whereas significant positive correlation was observed for AMC or MNR with TNF-α (Fig 5). However, in RUCM no significant correlation was noted for AMC or MNR with IL-10, IFN-γ and TNF-α.

Relationship of parasitemia and monocytes/neutrophils

Monocytes and neutrophils play im-

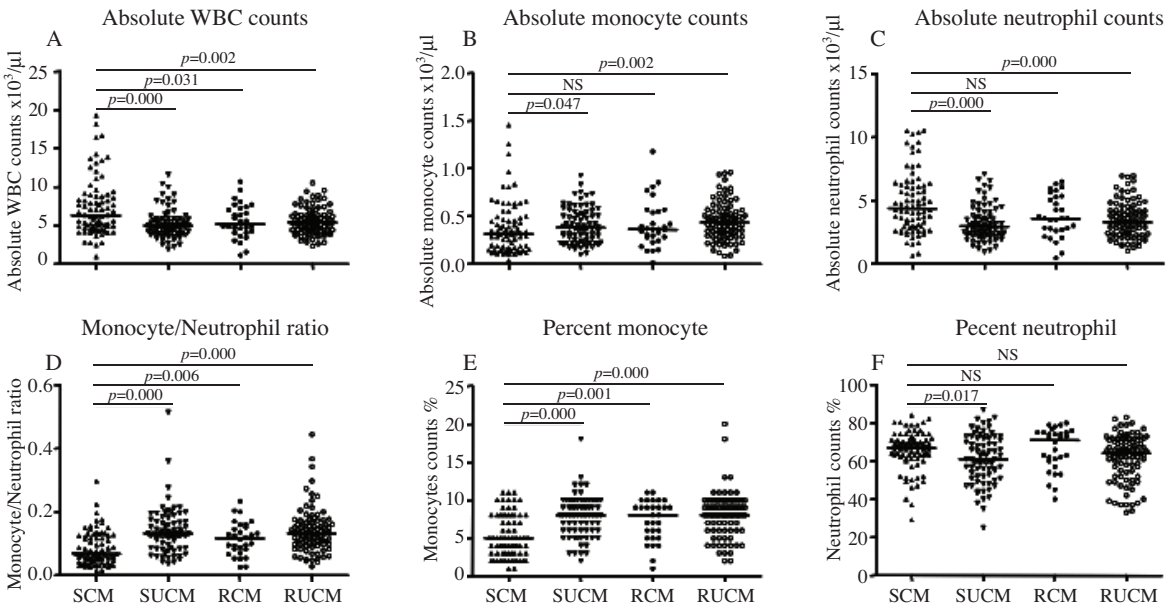

Fig 1–White blood cell (WBC) (A), monocyte (B) and neutrophil (C) counts, monocyte to neutrophil ratio (D) and percent monocyte (E) and neutrophil (F) in patients with complicated and uncomplicated *P. falciparum* malaria on the day of admission. (▲) Complicated malaria with single infection (SCM), (●) uncomplicated malaria with single infection (SUCM), (▽) complicated malaria with prior repeated infections (RCM), (○), uncomplicated malaria with prior repeated infections (RUCM). Each triangle or circle represents a single patient, with a horizontal line through each data set indicating median concentration. Statistical analysis was performed using Mann-Whitney *U* test. NS, not significant.

portant roles in controlling blood stage malaria parasites (Bouharoun-Tayoun *et al*, 1990; Fell and Smith, 1998). Parasitemia was significantly correlated with ANC and MNR only in RUCM but not in SUCM, whereas no significant correlation in these parameters in SCM or RCM was observed (Fig 6).

DISCUSSION

The relationships of circulating monocytes and neutrophils with their corresponding pro-/anti-inflammatory cytokines, IFN-γ, TNF-α, and IL-10 with clinical outcomes in naturally single or repeated complicated and uncomplicated falciparum infections were explored. The results indicated that very low monocyte to neutrophil ratio and extremely high circulating IL-10 and IFN-γ levels in primary complicated malaria were associated with pathogenesis, while the correlation of monocyte to neutrophil ratio and IFN-γ or TNF-α levels in primary, and of monocyte to neutrophil ratio and parasitemia in secondary uncomplicated malaria associated with reduced severe form of the disease.

In humans, macrophages mediate the control of blood-stage malaria parasites (Couper *et al*, 2007). However, their roles in naturally single or repeated falciparum infections have not been analyzed

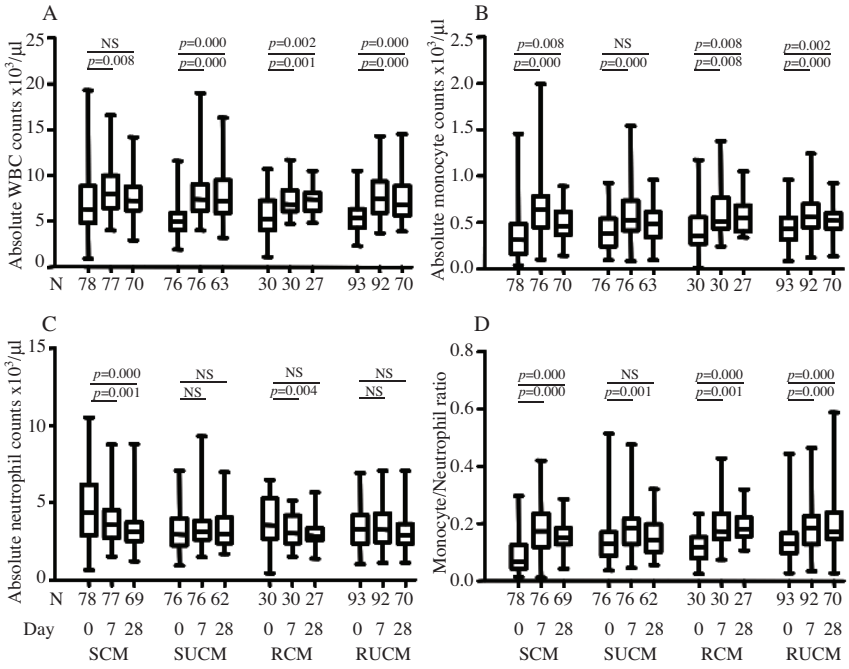

Fig 2–Time course of white blood (WBC) (A), monocyte (B) and neutrophil (C) counts, and monocyte to neutrophil ratio (D) in patients with *P. falciparum* infection. SCM, complicated malaria with single infection; SUCM, uncomplicated malaria with single infection; RCM, complicated malaria with prior repeated infections; RUCM, uncomplicated malaria with prior repeated infections; N, number of patients. The box represents values between 25% and 75% quartile, horizontal line median and vertical line 10% and 90% percentiles. Wilcoxon's matched pairs test was used for the analysis. NS, not significant.

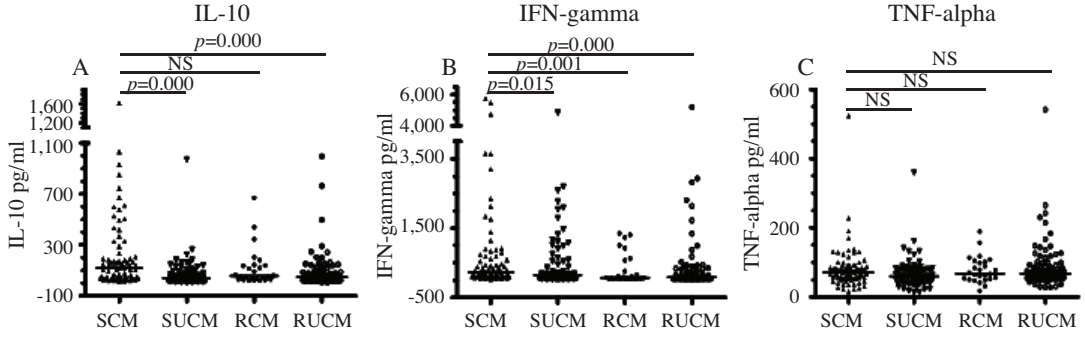

Fig 3–Plasma levels of IL-10 (A), IFN-gamma (B), and TNF-alpha (C) levels in patients with complicated and uncomplicated *P. falciparum* malaria on day of admittance. (▲) Complicated malaria with single infection (SCM), (▽) uncomplicated malaria with single infection (SUCM), (●), complicated malaria with prior repeated infection (RCM), (○) uncomplicated malaria with prior repeated infection (RUCM). Each triangle or circle represents a single patient, with a horizontal line through each data set indicating the median concentration. Statistical analysis was performed using Mann-Whitney *U* test. NS, not significant.

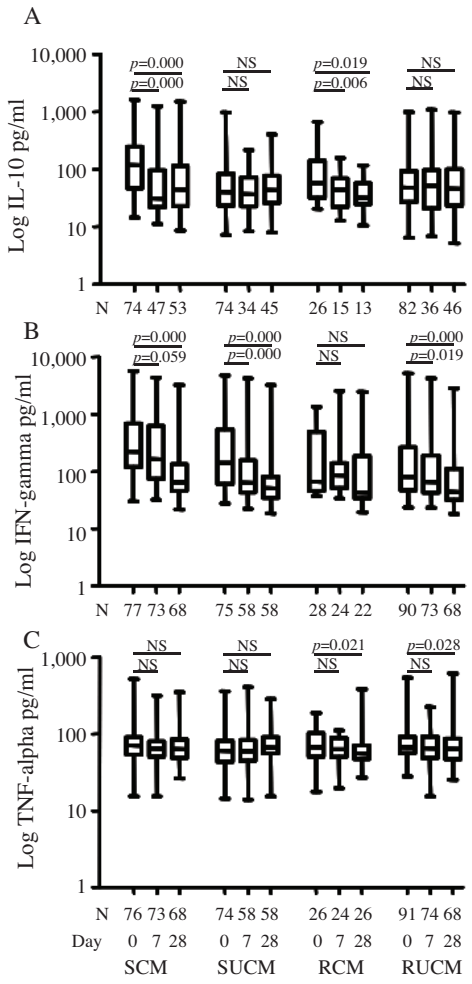

Fig 4–Time course of plasma IL-10 (A), IFN-gamma (B) and TNF-alpha (C) levels in patients with *P. falciparum* infection. SCM, complicated malaria with single infection; SUCM, uncomplicated malaria with single infection; RCM, complicated malaria with prior repeated infections; RUCM, uncomplicated malaria with prior repeated infections; N, number of patients. The box represents values between 25% and 75% quartile, horizontal line median and vertical line 10% and 90% percentiles. Wilcoxon's matched pairs test was used for the analysis. NS, not significant.

thoroughly. The crucial role of monocytes and neutrophils in controlling parasite multiplication in early phase of uncomplicated malaria infection of patients with previous repeated infections were noted in this study, as evidenced by the positive correlation of parasitemia and absolute neutrophil counts as well as the inverse correlation of parasitemia with monocyte to neutrophil ratio. It is possible that Th1 type memory cells stimulated in early infection secrete cytokines such as IFN-γ and TNF-α involved in macrophage and neutrophil activation, and may cooperate with specific opsonizing antibodies to produce nitric oxide leading to parasite killing (Groux and Gysin, 1990).

In another context, Th1 memory cells in uncomplicated malaria with prior repeated infection may produce pro-inflammatory cytokines, such as IFN-γ, to induce antibody production and activate phagocytes expressing Fc receptors for IgG (Kawano *et al*, 1994). Antigen uptake via these receptors may influence the expression of TNF-α, suggesting the role of memory $CD4^+$ T cells in regulating innate immune cells during the early phase of the disease (Strutt *et al*, 2011). On the other hand, the lack of relationship of parasitemia with monocyte and/or neutrophil counts in complicated malaria with prior repeated infections may be in part due to different engagement of IgG subclasses and $Fc_\gamma R$ on monocytes/neutrophils (van der Pol and van de Winkel, 1998). These results suggest different balances of immune responses in complicated and uncomplicated malaria with repeated infections, implying that complications of the disease are not due to the lack of prior exposure to malaria parasites (Erunkulu *et al*, 1992).

In fact, activated macrophages can also function as antigen-presenting cells

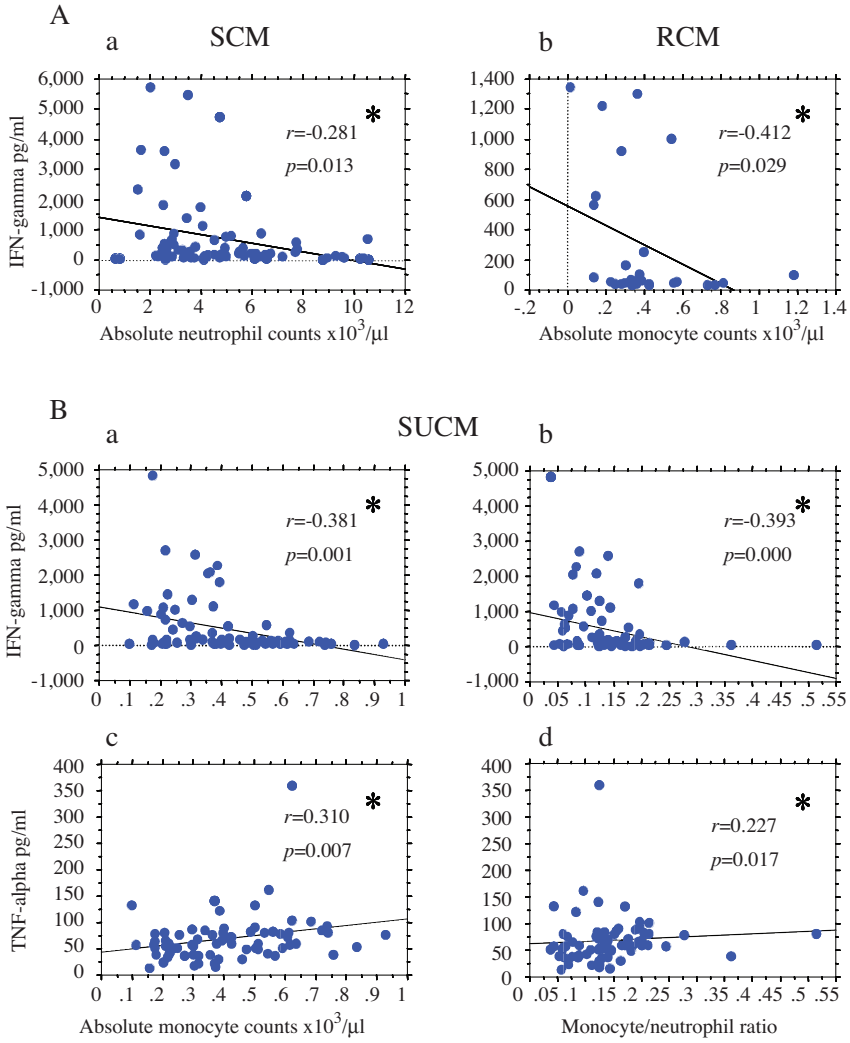

Fig 5–Correlations of cytokine levels and innate immune cell counts in patients with complicated and uncomplicated *P. falciparum* malaria. A, correlation of IFN-gamma with absolute neutrophil counts (ANC) (a) or absolute monocyte count (AMC) (b) in single (SCM) and prior repeated (RCM) complicated malaria, B, correlation of IFN-gamma, TNF-alpha with absolute monocyte counts (AMC) (a, c) or with monocyte to neutrophil ratio (MN ratio) (b, d) in single uncomplicated malaria (SUCM). Statistical analysis was performed using Spearman's rank correlation test. *Statistically significant

and contribute significantly to immunity by modulating both cellular and humoral immunity (Bouharoun-Tayoun *et al*, 1990; Lee *et al*, 2007). Among individuals with malaria, the numbers of blood dendritic cells are reduced in adults (Pichyangkul *et al*, 2004), but remain unchanged in children (Urban *et al*, 2006). *In vitro*, human monocyte-derived dendritic cells undergo apoptosis after exposure to high levels of malaria parasite-infected red blood cells (Elliott *et al*, 2007). Loss of dendritic cell numbers and function during onset of clinical symptoms in volunteers

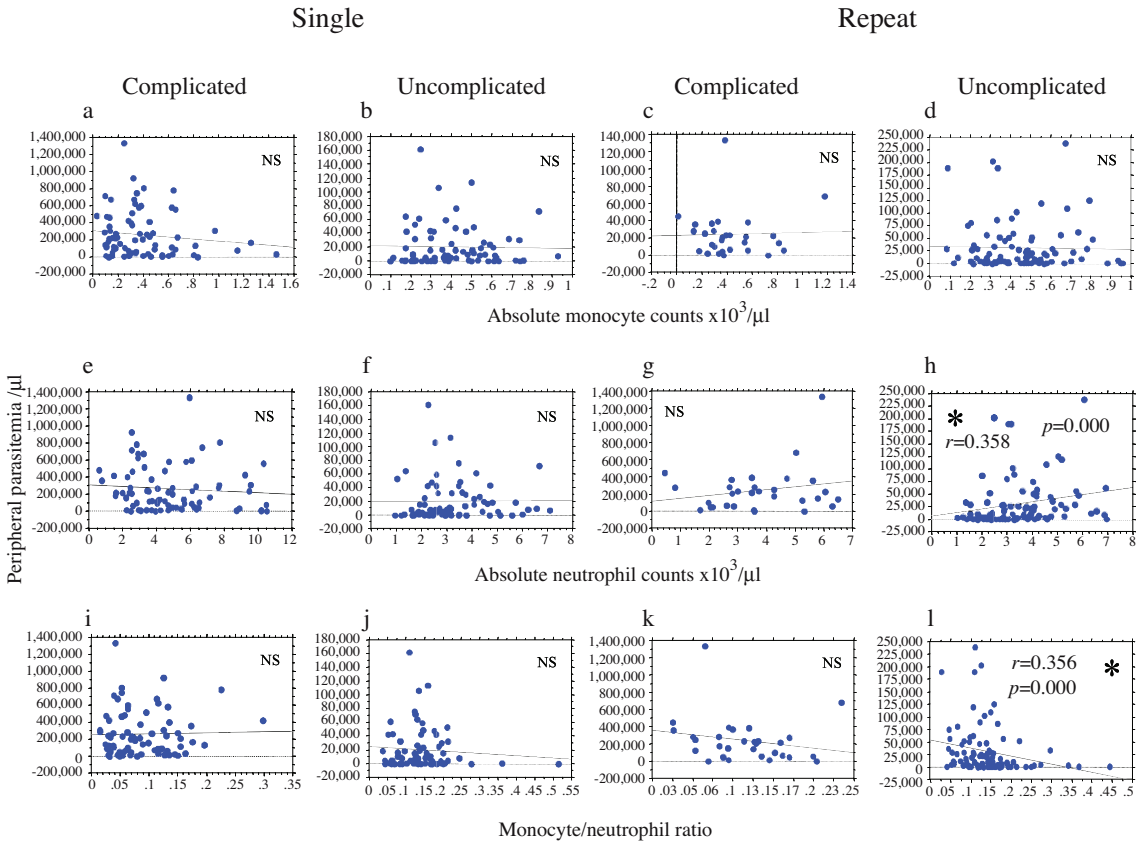

Fig 6–Correlations of parasitemia with monocyte counts, neutrophil counts and monocyte to neutrophil ratio in patients with complicated and uncomplicated *P. falciparum* malaria. Each circle represents a single patient. a, b, c, d indicate the correlations between circulating parasitemia and absolute monocyte counts; a = SCM, b = SUCM, c = RCM, d = RUCM. e, f, g, h indicate correlations between circulating parasitemia and absolute neutrophil counts; e =SCM, f = SUCM, g = RCM, h = RUCM. i, j, k, l indicate correlations between circulating parasitemia and monocyte to neutrophil ratio; i = SCM, j = SUCM, k = RCM, l = RUCM. Spearman's rank correlation test was used for the analysis; NS, not significant; *statistically significant.

with primary *P. falciparum* infection has been reported (Woodberry *et al*, 2012). Nevertheless, uptake of hemozoin triggers production of IL-10 in monocytes (Keller *et al*, 2006) and induces a state of monocyte "anergy/reprogramming", associated with"the deregulation of pro-inflammatory cytokines production, such as TNF-α (Schwarzer *et al*, 2008). Thus, the significantly lower monocyte counts and extremely high IL-10 levels of patients with single infection in the early stage of complicated malaria in this study might be associated with less effective clearance of parasites and pathogenesis, which may limit the development of specific immunity. However, the lack of relationship between monocyte counts and plasma IL-10 levels in early stage of disease in these individuals is in agreement with the recent findings of the lack of association between IL-10 levels and numbers of cir-

culating hemozoin-containing monocytes (Boeuf *et al*, 2012). Furthermore, the high IFN-γ levels in early stage of complicated malaria in patients with single infection may increase expression of adhesion receptors such as I-CAM-1, and induce high cytoadherence, which can lead to pathogenesis as well (Mazier *et al*, 2000). In contrast, in uncomplicated malaria with single infection, the results of neither low monocyte counts nor high IL-10 levels, or the association of TNF-α and IFN-γ with absolute monocyte counts and with monocyte to neutrophil ratio in early stage of the disease, possibly indicate that in these primary infections, macrophages and polymorphonuclear leukocytes are activated directly and release cytokines, such as IFN-γ and TNF-α, which contribute to fever and/or in mediating protective effects through increased phagocytosis and production of ROS and NO (Fell and Smith, 1998). IFN-γ could also enhance neutrophils to inhibit parasite growth and destroy parasites via phagocytosis (Kumaratilake *et al*, 1991). This is probably a protective host response designed to keep parasite levels under control until an effective and specific immunity develops.

The coordinated action of professional phagocytes including neutrophils, monocytes and macrophages are crucial for effective elimination of malaria parasites. On admission before treatment, patients with complicated malaria from single infection have low monocyte and high neutrophil counts, thus, the monocyte to neutrophil ratio should be a more important indicator than the absolute levels of specific cell types. The present study demonstrated that the monocyte to neutrophil ratios in complicated malaria are significantly lower than in uncomplicated malaria, with the lowest ratio in those with single infection. The increase in monocyte to neutrophil ratios after 7 and 28 days following treatment indicated the existence of the low ratios during the onset of clinical symptoms rather than the actual levels. Indeed, neutrophils are mobilized in high numbers and are extraordinarily rich in inflammatory mediators and in proteases and oxidants, which if released in excess, can damage many cell types (Weiss, 1989). Moreover, macrophages also contain tissue-damaging components, although to a lesser amount compared with neutrophils (Owen and Campbell, 1999). Thus, a balance between neutrophils and mononuclear phagocytes must be tightly regulated in order to avoid considerable collateral damage and pathogenesis. As monocyte or neutrophil counts are derived from full differential blood counts, determination of monocyte to neutrophil ratio may better reflect the capacity to mount an effective immune response and/or changes in disease progression.

In summary, complicated and uncomplicated malaria with single or prior repeated infections exhibited different levels of pro/anti-inflammatory cytokines, monocytes and neutrophils, suggesting the crucial role of primary and secondary immune responses in malaria pathogenesis. During the early stage of uncomplicated malaria resulting from single infection, interactions of IFN-γ and TNF-α with innate immune cells may result in the reduced disease severity, whereas the relationship of parasitemia with monocyte to neutrophil ratio in those with prior repeated infections might be associated with the reduced severe forms of the disease. In sharp contrast, the extremely high IFN-γ and IL-10 levels, but very low monocyte to neutrophil ratio in complicated malaria resulting from single infection may

indicate an association with pathogenesis. Notably, these results may facilitate a rationale design of vaccines, diagnostic and novel therapeutics. However, the observations in this study need to be confirmed in a larger study cohort in order to take into account such confounding factors as age, previous malaria episodes, parasite virulence and host genetics.

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