LATE-STAGES *P. FALCIPARUM* ANTIGEN LYSATE-INDUCED PHENOTYPIC CHANGES ASSOCIATED WITH MYELOID DENDRITIC CELL MATURATION VIA TOLL-LIKE RECEPTOR 2

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Abstract. *Plasmodium falciparum* infection is capable of altering immune competence of host possibly through effecting changes in dendritic cell (DC) functionality. Toll-like receptors (TLRs) on DCs recognize *P. falciparum* antigen to activate signaling of their maturation. This study aimed to elucidate *in vitro* whether late stages *P. falciparum* antigen lysates derived from cultured parasite isolates obtained from patients with severe (SM) or uncomplicated malaria (UCM) induce phenotypic changes associated with DC maturation, and whether this process was dependent on TLR2. SM and UCM antigen lysates were able to activate myeloid DC (MDC) maturation via TLR2 demonstrated by increase in percent MDCs expressing CD83 in a dose dependent manner, and by elevation in mean fluorescent intensity (MFI) of immune-labeled cell surface CD83 with significantly higher levels in samples stimulated by SM antigen lysates. Percent MDCs expressing HLA-DR and CD80 did not increase upon antigen lysates stimulation, but raised MFI levels were observed. High MFI levels of CD83 and HLA-DR but low CD80 were obtained in both types of antigen lysates stimulation indicating partial maturation of MDCs. SM antigen lysates stimulation demonstrated a negative correlation of percent CD83-expessing MDCs with corresponding patients' plasma IFN-γ and TNF-α levels, while those expressing CD80 a negative correlation with IFN-γ; however, there was a positive correlation of CD80 MFI with plasma IL-12, TNF-α and IFN-γ levels. For UCM antigen lysates stimulation, a positive correlation of CD80 MFI with plasma IL-6 level was observed. These results suggest that late stages *P. falciparum* antigen lysates derived from parasites isolates from severe or uncomplicated malaria patients could *in vitro* alter blood dendritic cell function via TLR2, which may impact on host immunity to malaria.

Keywords: *Plasmodium falciparum*, blood stage malaria antigen, myeloid dendritic cell maturation, pro-inflammatory cytokine, toll-like receptor 2

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INTRODUCTION

Malaria remains the major cause of morbidity and mortality in human, with an estimated 214 million new cases and approximately 600,000 deaths annually, the latter mostly in children younger than five years of age (WHO, 2016). Clinical manifestations of malaria are highly varied ranging from mild to severe forms.

The interaction of *Plasmodium* parasites and host immune system are complex as regards activation and regulation of different types of immune cells (Reily *et al*, 2006). In endemic areas, certain characteristics of immune response may contribute to successful persistence of parasites and slow generation of immunity against the disease (Reily *et al*, 2006). Inhibition of specific T cells response to malaria antigens and T cell depletion were identified in malaria-infected individuals (Goonewardene *et al*, 1990; Hviid *et al*, 1991). *In vitro* models using *P. falciparum* cultures and *in vivo* murine models of infection with different *Plasmodium* strains demonstrated that infection is associated with altered responses of macrophages (Martiney *et al*, 2000) and dendritic cells (DCs) (Millington *et al*, 2006, 2007; Wykes *et al*, 2007); however, normal DC response to *P. falciparum* has also been reported (Luyendyk *et al*, 2002; Ing *et al*, 2006; Sponaas *et al*, 2006).

DCs are key players involved in regulating both innate and adaptive immune responses, as evidenced by their unique capacity to activate naïve T cells and polarize $CD4^+$ T cell response (Banchereau *et al*, 2000). In malaria, parasites can influence and regulate DC function to promote a more permissive environment for their survival (Urban *et al*, 1999). Maturation and function of human monocyte-derived (M) DCs can be modulated by *P. falciparum*-infected erythrocytes *in vitro* (Urban *et al*, 1999; Elliott *et al*, 2007). This phenomenon is further supported by field evidence that peripheral DCs from children with acute *P. falciparum* infection demonstrate low expression of human leukocyte antigen (HLA)-DR on DC surface (Urban *et al*, 2001, 2006), suggesting a functional impairment of these DCs.

Certain immunologically active components of malaria parasite recognized by toll-like receptors (TLRs), resulting in up-regulation of pro-inflammatory cytokines, have been proposed to be involved in malaria pathogenesis (Adachi *et al*, 2001). In humans, *P. falciparum* could regulate TLRs expression on antigen-presenting cells (APCs). The glycosylphosphatidylinositol (GPI) moiety present in merozoite surface protein 1 (MSP-1) and MSP-2 is recognized mainly by TLR2 and to lesser extent by TLR4 (Krishnegowda *et al*, 2005), leading to expression of effector genes involved in inflammatory cytokines, antimicrobial peptides, costimulatory molecules and major histocompatibility complex (MHC) molecules (Akira and Takeda, 2004; Gazzinelli *et al*, 2004). Being a receptor for parasite-derived GPI, TLR2 is likely to be involved in the disease pathogenesis (Krishnegowda *et al*, 2005). Modulation of DC functions by *P. falciparum* contributes to both delayed acquisition of antimalarial immunity and immunosuppression associated with acute malaria infection.

Studies of malaria in mice have produced conflicting results of both activation (Leisewitz *et al*, 2004; Ing *et al*, 2006) and inhibition (Carapau *et al*, 2007; Millington *et al*, 2007) of DC function. Intact *P. yoelii*-infected erythrocytes induce a broad inhibitory effect, rendering DC non-responsive to ligands for TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9, while lysates of infected erythrocytes induce a MyD88-independent activity response in DCs (Bettiol *et al*, 2010).

In naïve volunteers, during acute falciparum malaria, TLR2/TLR1 response results in an increase in both pro- and anti-inflammatory cytokine production compared to baseline levels, while significant up-regulation of pro-inflammatory cytokines through TLR4 response is noted (McCall *et al*, 2007). There is evidence that peripheral blood DCs from patients with falciparum infection show impairment in their ability to up-regulate co-stimulatory and MHC class II molecules, antigen uptake and presentation to $CD4^+$ T cells, in association with significant levels of spontaneous DC apoptosis (Pinzon *et al*, 2013). In African populations, the collective appearance in genetic variations of TLR2, TLR4 and TLR9 and their signaling pathways can change their expression and potentially affect the inflammatory response and thus the disease outcome (Leoratti *et al*, 2008; Esposito *et al*, 2012; Greene *et al*, 2012; Omar *et al*, 2012). Although TLRs signaling is undoubtedly a significant aspect of pro-inflammatory responses, it also is important in disease susceptibility as documented above. However, the exact role of TLRs during severe malaria is poorly understood.

Due to the complex life cycle, malaria parasite antigens and/or metabolites released from ruptured parasite-infected red blood cells (pRBCs) activate host cellular components of the innate immunity system to produce high levels of circulating pro-inflammatory cytokines, which probably cause the progression to severe clinical malaria and fatal outcome (Schofield and Grau, 2005). *In vitro* binding of intact pRBCs to DCs inhibit their maturation leading to a reduction in their capacity to stimulate T cells (Urban *et al*, 1999; Clark *et al*, 2006; Eriksson *et al*, 2013). This notion is supported by observations of *Plasmodium* parasite surface molecules, such as *P. falciparum* erythrocyte membrane protein 1(PfEMP1) (Bull and Marsh, 2002), mediating adherence to DCs *via* scavenger receptor CD36 (Baruch *et al*, 1996) and chondroitin sulfate A (CSA), a proteoglycan (Fried and Duffy, 1996; Elliott *et al*, 2007). However, modulation of DC responses *in vitro* does not always require contact between DC and PfEMP1, and high dose of pRBCs can induce DC apoptosis (Elliott *et al*, 2007).

In mouse malaria models, recognition of parasite-derived factors from pRBCs by TLR2, TLR4 and TLR9 opens the possible role of innate immune response for parasite-soluble factors (Pichyangkul *et al*, 2004; Coban *et al*, 2005; Zhu *et al*, 2005). Although assessment of TLRs expression in relation to disease outcome is relatively limited, however, it was shown there is a significant increase in TLR2 and TLR4 expression on $CD14^+$ monocytes and MDCs in Thai patients with severe (SM) and uncomplicated (UCM) malaria, and conversely, a decrease in TLR9 expression on plasmacytoid DCs, suggesting their role in innate immune recognition in which the differential expression of TLRs on APCs could be regulated by *P. falciparum* (Loharungsikul *et al*, 2008). However, TLR2-induced response to malaria in association with malaria severity is not well understood.

Hence, this study aimed to elucidate whether lysate of *P. falciparum* late stages obtained from SM or UCM malaria patients induced the phenotypic changes associated with DC maturation *in vitro*, and whether this process was dependent upon MDC TLR2 signaling.

MATERIALS AND METHODS

Blood samples

Four ml of heparinized blood from

6 healthy individuals living in Bangkok where malaria is not endemic were collected for *in vitro* TLR2 activation assay. The subjects had no history of malaria exposure and had not traveled to any malaria endemic area in the past 2 years, and so they would be most unlikely to have been exposed to malaria. Stored anonymous plasma from 6 severe and 10 uncomplicated malaria cases from a previous study (Loharungsikul *et al*, 2008), from whom cultured *P. falciparum* isolates were used to prepare late stages parasite-infected erythrocyte lysates for cytokine determination.

The study was approved by the Ethics Committee of Faculty of Tropical Medicine, Mahidol University (approval no. MUTM 2012-007-02).

Preparation of late stages *P. falciparum*-infected erythrocyte lysates

Stored *P. falciparum* isolates from the previous study (Loharungsikul *et al*, 2008) were cultured in RPMI 1640 medium (Gibco Life Technologies, New York, NY) containing HEPES buffer, 10% human serum, 2 mM L-glutamine, 2.5 μg/ml gentamicin and 25 mM sodium bicarbonate, and incubated at 37°C in a gas mixture of 5% CO_2, 1% O_2 and 94% N_2 (Trager and Jensen, 1976). Parasite growth was monitored daily by Giemsa-stained thin blood smears until the culture reached high parasitemia (70%) of late stages-infected red cells. pRBCs were enriched by 60% Percoll® gradient centrifugation (Sigma-Aldrich, St Louis, MO), yielding > 90% pRBCs of trophozoite and schizont stages. This was followed by washing twice with RPMI 1640 medium and centrifugation at 1,500*g* for 5 minutes. PRBCs obtained from SM and UCM isolates were separately pooled, counted and lysed by sonication (pulse on 9.9 and pulse off 0.1 s) for 5 minutes (Sonics and Materials, Newtown, CT). Protein concentration was determined using a Nanodrop® spectrophotometer (NanoDrop Technologies, Wilmington, DE). Supernatants from SM and UCM parasite isolates, designated as SM and UCM antigen lysates, were used for whole blood stimulation assay.

Optimization for whole blood stimulation assay

Duration of *in vitro* peripheral blood MDC TLR2 activation was optimized using a TLR2 specific ligand, the peptidoglycan from *Staphylococcus aureus* (PGN) (Sigma-Aldrich) (Ida *et al*, 2006). In brief, 300 μl aliquot of blood was incubated with PGN (5, 10 and 20 μg/ml) at 37°C in 5% CO_2 humidified atmosphere at a 5° slant for 1 hour, and 3, 5, 10, 16 and 24 hours. The cell suspensions were subjected to flow cytometry analysis for the surface maturation marker (CD83) on MDCs. The stimulation time for 5 hours was the most appropriate for surface CD83 expression.

The optimal concentration of late stages *P. falciparum*-infected RBC antigen lysate for use in the *in vitro* whole blood stimulation assay was determined by incubating 300 μl of blood from healthy individuals with 1, 5, 15 and 150 μg/ml of SM or UCM antigen lysate for the optimal time at 37°C in a 5% CO_2 humidified atmosphere at a 5° slant. The surface maturation marker (CD83) on MDCs was analyzed by flow cytometry.

Determination of TLR2 activation markers on MDCs

Activation of TLR2 signaling pathway by *P. falciparum* antigen lysate was investigated by an *in vitro* whole blood stimulation assay (Ida *et al*, 2006). In short, 300 μl aliquot of blood from 6 healthy subjects was incubated with optimal concentration of SM or UCM antigen lysate for the optimal time period. Normal

blood incubated with 10 μg/ml of PGN and unstimulated blood was used as positive and negative control, respectively. Cell suspensions were subjected to flow cytometry analysis for the expression of cell surface molecules on MDCs.

TLR2 blocking assay

The expression of surface molecules induce through MDC TLR2 receptor was confirmed by a TLR2 blocking assay (Hertz *et al*, 2001). In brief, 300 μl aliquot of blood was incubated with 10 μg/ml of purified anti-human TLR2 mAb (clone TLR2.1; Biolegend) for 30 minutes before being treated with the optimal concentration of SM or UCM antigen lysate for the optimal time period at 37°C in 5% CO_2 humidified atmosphere at a 5° slant. Expression of surface molecules was analyzed by flow cytometry. The TLR2 blocking assay using 10 μg/ml of PGN served as positive control.

Flow cytometry analysis

The maturation and co-stimulatory markers, CD83, CD80, and HLA-DR on myeloid dendritic cells (MDCs) were assessed by standard flow cytometry using a five-color immunofluorescence technique. Direct immunofluorescence staining of MDCs was performed according to the manufacturer protocol (Becton Dickinson, Mountain View, CA). The following combination of fluorochrome-conjugated monoclonal antibodies (mAbs) were used: PE-labeled anti-BDCA1 (clone AD5-8E7; Miltenyi Biotec, Bergisch Gladbach, Germany), PE-labeled anti-BDCA3 clone (AD5-14H12; Miltenyi Biotec) and PerCP-labeled anti-CD14 (clone CD14 MøP9; Becton Dickinson) to select MDCs that are dual positive; and FITC-labeled anti-CD83 (clone HB15e; Biolegend, San Diego, CA), PE-Cy7-labeled anti-CD80 (clone 2D10; Biolegend) and APC-labeled anti-HLA-DR (clone L243; BD Biosciences, San Jose, CA) to select MDCs that are triple positive. Isotype controls were mouse isotype IgG1-FITC (eBioscience), mouse isotype IgG1-PE-Cy7 (Biolegend) and mouse isotype IgG2a-APC (BD Biosciences). After stimulation with whole blood, cell suspensions were stained with the mAb combinations and incubated in the dark at 4°C for 30 minutes. Red blood cells were lysed with 2 ml of FACS lysing solution (BD Biosciences) for 10 minutes, then centrifuged at 300*g* for 5 minutes. Cell pellets were washed with 2 ml of phosphate-buffered saline pH 7.4 (PBS) containing 2% fetal bovine serum, followed by centrifugation as described above and suspended in 300 μl of freshly prepared 1% paraformaldehyde in PBS.

Data acquisition and analysis were performed using a five-color immunofluorescence technique on an LSRII flow cytometer (BD Bioscience) equipped with FACSDiva software. Gating strategy for peripheral blood MDCs was identified using a two-dimensional dot plot of CD14 versus BDCA1 and BDCA3. Only $CD14^-$ $BDCA1^+$ $BDCA3^+$ cells were used for further analysis of the expression of CD83, CD80 and HLA-DR molecules. Induction of maturation marker (CD83), co-stimulatory molecule (CD80) and MHC class II molecule (HLA-DR) on MDCs through activation of TLR2 is shown in Fig 1. Forward- and side-scatter were used to gate mononuclear cells (both monocytes and lymphocytes) and to exclude the majority of granulocytes and cell debris. At least 30,000 events of MDCs were analyzed per individual blood sample. Isotype controls were used to define positive and negative populations.

Measurement of plasma cytokines

Levels of pro-inflammatory cyto-

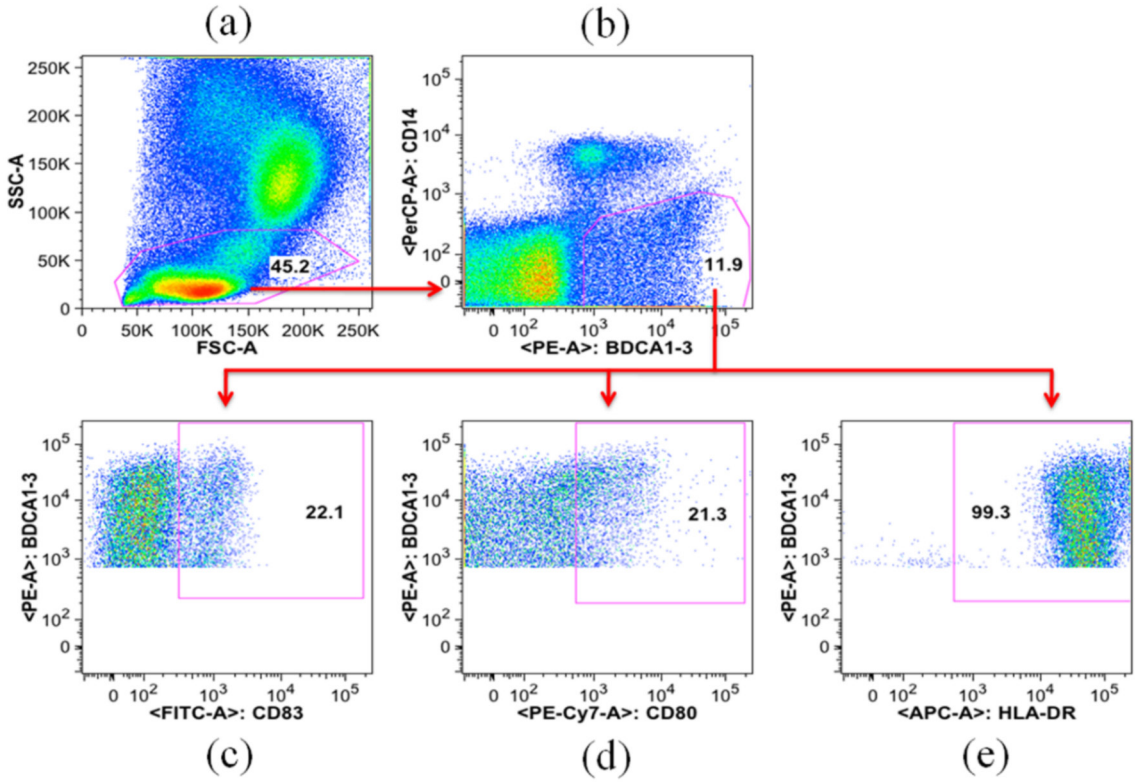

Fig 1–Gating strategy for quantifying blood myeloid dendritic cells (MDCs) expressing maturation marker (CD83), co-stimulatory molecule (CD80) and MHC class II molecule (HLA-DR). (a) Forward- and side-scatter was used to gate mononuclear cells and exclude the majority of granulocytes and cell debris. (b) Two-dimensional dot plots of CD14 versus BDCA1 and BDCA3 were used to identify MDCs. Only $CD14^{-}$ $BDCA1^{+}$ $BDCA3^{+}$ cells were used for further analysis of the expression of (c) CD83, (d) CD80 and (e) HLA-DR molecules.

kines, IFN-γ, TNF-a, IL-12p70 and IL-6 in stored plasma were measured using a sandwich ELISA (MABTECH, Stockholm, Sweden). In short, 96-microwell plates (Corning, New York, NY) were coated overnight at 4ºC with capture antibodies (diluted in 0.1 M carbonate-bicarbonate buffer pH 9.6) and, after washing with PBS containing 0.05% Tween 20, the plates were incubated with PBS containing 0.5% bovine serum albumin (BSA) for 90 minutes at room temperature. Test plasma (25 µl; 1:2 dilution) were added and incubated overnight at 4ºC. After washing, the plates were incubated at room temperature for 90 minutes with 25 µl of biotinylated anti-human cytokines monoclonal antibodies followed by streptavidin-conjugated alkaline phosphatase and p-nitrophenyl phosphate (Sigma Diagnostics, St Louis, MO). After 20 minutes, $A405_{nm}$ was measured by an ELISA reader (V-max, Molecular Devices, San Diego, CA). Experiments were performed in duplicate. Cytokine concentrations were calculated from standard curves obtained by incubating serial dilution of recombinant cytokines (MABTECH, Stockholm, Sweden). Detection limits of cytokines were based on the linear standard curves and the

background values of buffer controls. The lower detection limit was 1 U/ml IFNγ and 100 pg/ml TNF, IL-12p70 and IL-6.

Statistical analysis

Data were analyzed using SPSS software version 17 (SPSS, Chicago, IL). Differential expressions of CD83, CD80 and HLA-DR of MDCs stimulated by SM and UCM *P. falciparum* antigen lysates were compared by Wilcoxon signed-rank test. Median cytokine levels in SM and UCM were compared by Mann–Whitney *U* test. Correlations of differential expressions of MDC CD83, CD80 and HLA-DR and cytokine levels were determined by Spearman's rank test. A p-value < 0.05 is considered as statistically significant.

RESULTS

Optimal conditions for TLR2 activation

By stimulation whole normal blood with PGN, a specific ligand for TLR2 activation, MDCs expression at 5 hours (optimal time period) of CD83 increased to 21.6%, 28.2% and 28.1% after stimulation with 5, 10, and 20 μg/ml PGN, respectively, compared to unstimulated blood sample (7.8%). Using the optimal concentration of 10 μg/ml PGN, MDC expressing CD83 at 1 hour, and 3, 5, 10, 16, and 24 hours was 7.14%, 21.7%, 24.8%, 23.6%, 13.0%, and 17.8%, respectively, compared to unstimulated blood sample (0.6 %). The stimulation time of 5 hours was chosen in further studies.

When blood samples were stimulated for 5 hours with 1.5-150 μg/ml SM or UCM antigen lysates, mean percent MDCs expressing CD83 increased in a dose-dependent manner for both SM and UCM antigen lysates (Fig 2). Mean percent MDCs expressing CD83 in unstimulated blood samples and PGN positive control was 4 ± 1 and 25 ± 1, respectively. Thus, 150 μg/ml SM and UCM antigen lysates was chosen for stimulation experiments in subsequent studies.

Phenotypic changes associated with MDC maturation after *in vitro* stimulation with antigen lysates

Percent MDCs expressing of maturation marker CD83, co-stimulatory molecule CD80 and MHC class II molecule HLA-DR as well as their cell surface mean fluorescence intensity (MFI) following whole blood stimulation were analyzed by flow cytometry. Median percent MDCs expressing CD83 increases significantly by stimulation with SM (34; range 19.6 - 50.1) and UCM (28; range 24.7- 34.9) antigen lysates compared to non-stimulation controls (6; range 3.8 - 8.1) (p = 0.028 and 0.028, respectively), but not between SM and UCM antigen lysates stimulation groups (Fig 3a). Median mean fluorescent intensity (MFI) of CD83 expression by SM (1,307; range 1,140 - 1,520) but not UCM (1,073; range 729 - 1,311) antigen lysate stimulation is higher than non-stimulation controls (976; range 777 - 1,397) (p = 0.028) (Fig 3b). On the other hand, MDCs expressing CD80 by both SM and UCM antigen lysates stimulation are not significantly different from non-stimulation controls (Fig 3c), but MFI levels are decreased significantly (SM = 2,588; range 1,231 - 5,187, UCM = 1,861; range 1,451 - 2,647, control = 2,891; range 1,445 - 4,172, p = 0.6 and 0.075, respectively) (Fig 3d). However, although the numbers of MDCs expressing HLA-DR by both antigen lysates stimulation are not significantly different from non-stimulation controls (Fig 3e), MFI levels are elevated significantly (SM = 72,738; range 41,647 - 92,032, UCM = 67,834; range 43,135 - 101,000) over controls (47,468; range 39,743 - 88,431) (p = 0.028 and 0.046, respectively), but not between the two stimulation groups (Fig 3f).

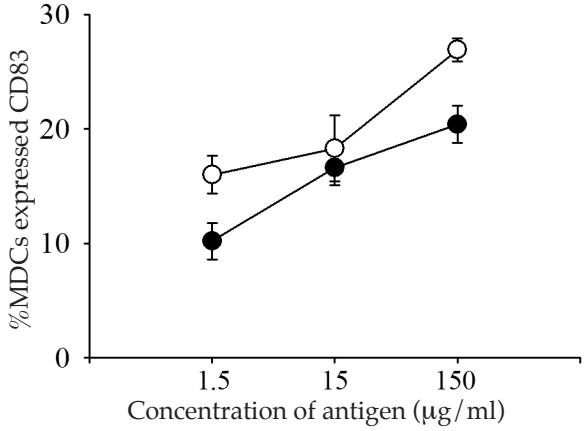

Fig 2–Concentration optimization of *Plasmodium falciparum* antigen lysates for *in vitro* induction of maturation marker CD83 on human blood myeloid dendritic cells (MDCs) by flow cytometry. Severe malaria (SM) and uncomplicated malaria (UCM) antigen lysate was prepared from late stages *P. falciparum* antigen lysate derived cultured parasites isolated from SM and UCM patient, respectively. (–●–) SM antigen lysate. (–o–) UCM antigen lysate.

Effect of TLR2 blocking on percent MDCs expressing CD83

In order to confirm that SM and UCM antigen lysates induced MDCs maturation and their co-stimulatory molecules via TLR2, whole blood samples were pre-treated with anti-TLR2 antibodies. This resulted in a significant reduction in median percent MDCs expressing CD83 compared with Ab-untreated controls in SM (pre-treated/untreated = 26; range 19 - 37) and UCM (pre-treated/untreated = 20; range 15 - 30.5) antigen lysates stimulation (Fig 4a), but no significant difference in median MFI levels between pre-treated and untreated samples in SM (pre-treated/untreated = 1,109; range 560 - 1,301) and UCM (pre-treated/untreated = 1,052; range 618 - 1,565) antigen lysates stimulation (Fig 4b). There are no significant differences in median percent MDCs expressing CD80 (Fig 4c) and median MFI levels (Fig 4d), and in median percent MDCs expressing HLA-DR (Fig 4e) and median MFI levels (Fig 4f) between TLR2 blocking and non-blocking samples stimulated by SM or UCM antigen lysates.

Relationship between malaria parasite antigen-stimulated MDCs and patients' plasma pro-inflammatory cytokines

Percent MDCs expressing CD83 are significantly inversely correlated only with corresponding patients' plasma IFN-γ (Fig 5a) and TNF-α levels (Fig 5b), while those expressing CD80 are significantly inversely correlated with plasma IFN-γ levels (Fig 5c); however, CD80 MFI levels correlated significantly with plasma IL-12 (Fig 5d), TNF-α (Fig 5e) and IFN-γ levels (Fig 5f). On the other hand, for blood samples stimulated with UCM antigen lysates, only CD80 MFI levels are significantly correlated with patients' plasma IL-6 levels (Fig 5g). Interestingly, for samples stimulated by SM and UCM antigen lysates there are no significant correlations between percent MDCs expressing HLA-DR or their MFI levels with all four plasma cytokines measured (data not shown).

DISCUSSION

Innate immune recognition in falciparum malaria requires (in part) differential expression of TLRs on APCs that can be induced by *P. falciparum* antigens. It has been demonstrated that there is a significant increase in TLR2 and TLR4 expression on $CD14^+$ monocytes and MDCs among SM and UCM patients, and conversely, a decrease in TLR9 expression on plasmacytoid dendritic cells (Loharungsikul *et al*, 2008). In the present

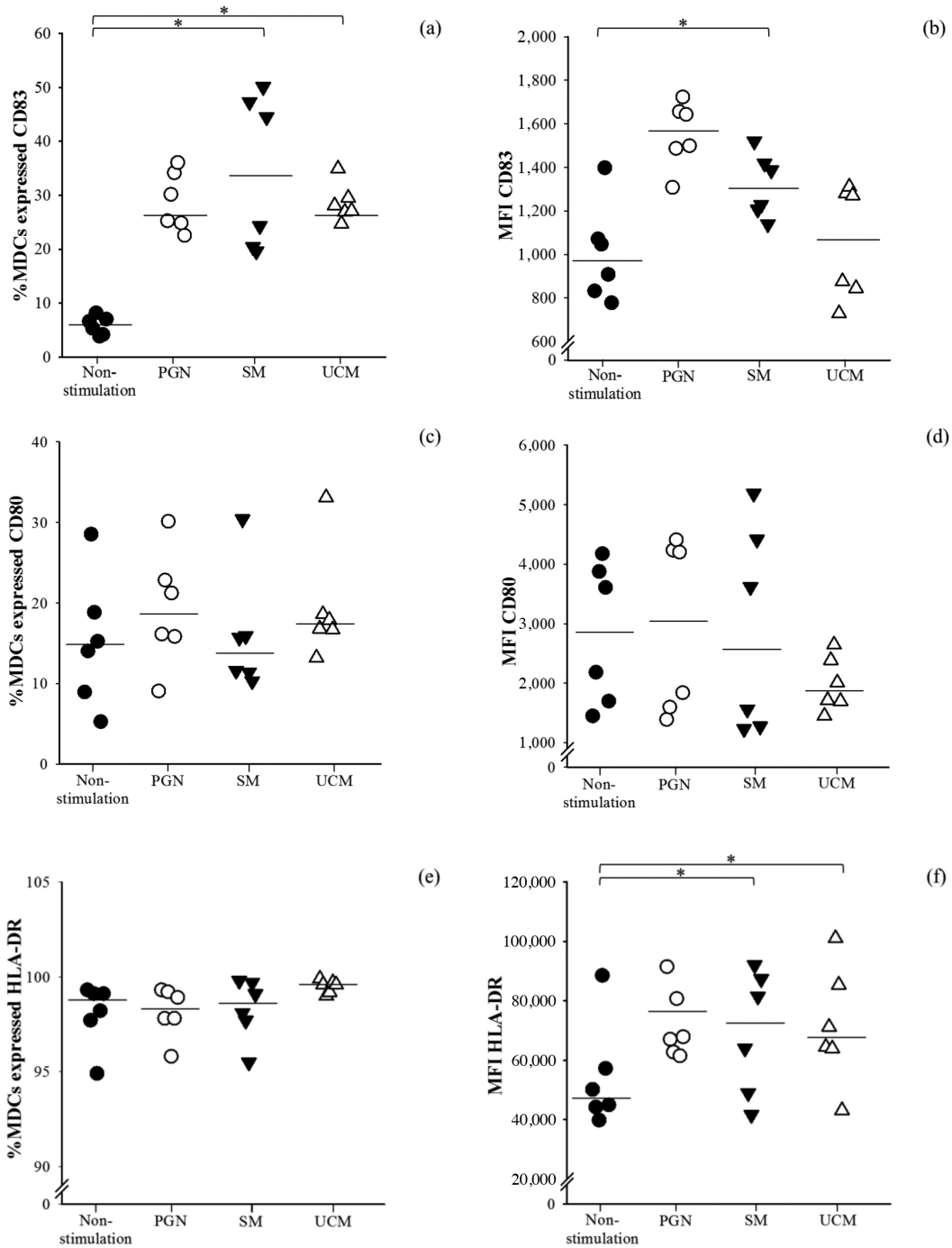

Fig 3–Percent myeloid dendritic cells (MDCs) expressing CD83, CD80 and HLA-DR and their mean fluorescent intensity (MFI) after stimulation with severe (SM) and uncomplicated malaria (UCM) antigen lysates. Antigen lysates were prepared as described in legend to Fig 2, and MDCs were identified and quantified by flow cytometry. (a), (c) and (e): percent MDCs. (b), (d) and (f): MFI. Each dot represents one individual sample. A horizontal bar indicates median of each group. PGN, peptidoglycan from *Staphylococcus aureus*. *$p < 0.05$.

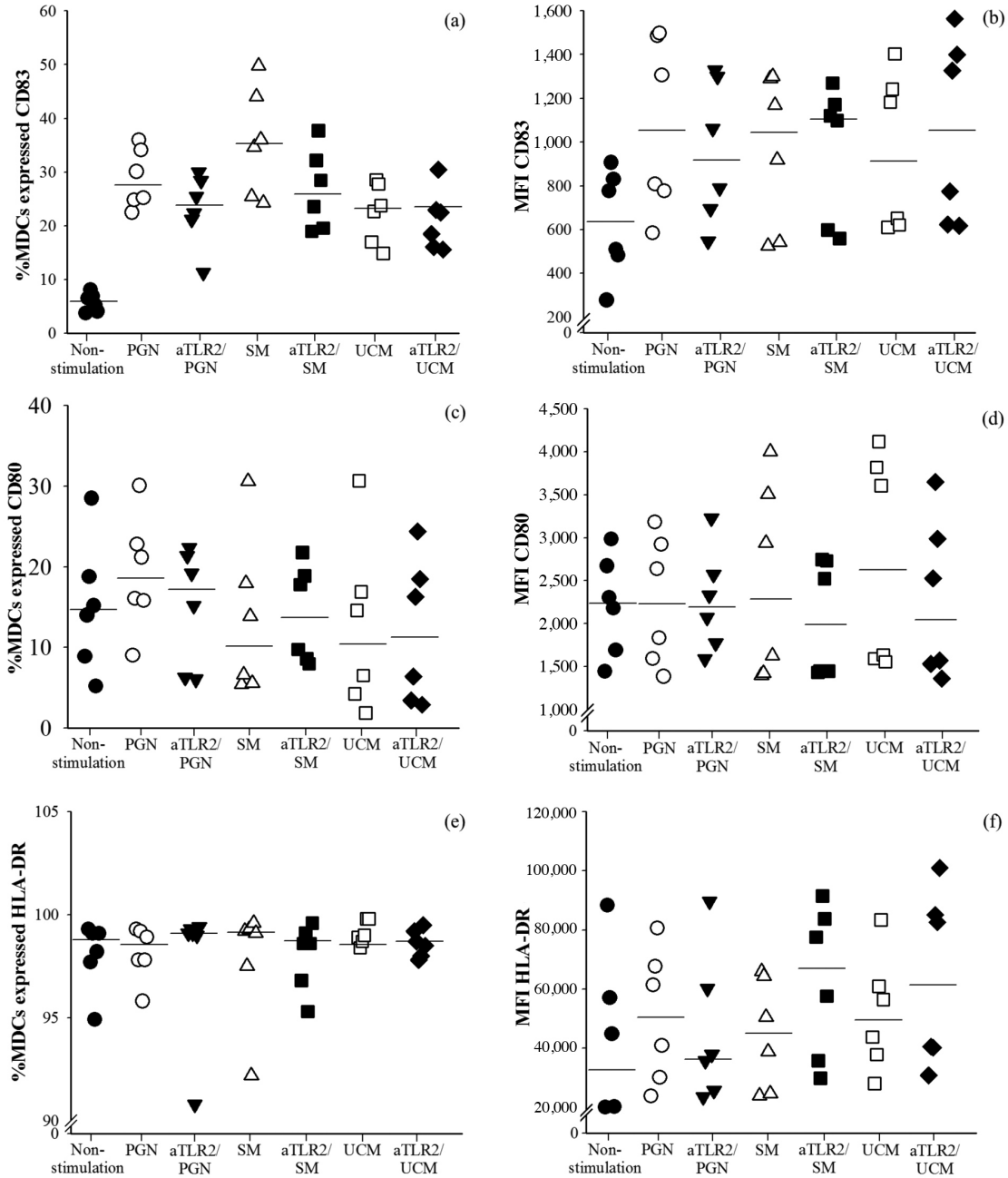

Fig 4–Percent myeloid dendritic cells (MDCs) expressing CD83, CD80 and HLA-DR and their mean fluorescent intensity (MFI) after blocking of TLR2 prior stimulation with severe malaria (SM) and uncomplicated malaria (UCM) antigen lysates. Antigen lysates were prepared as described in legend to Fig 2, and MDCs were identified and quantified by flow cytometry. TLR2 blocking was performed using anti-human TLR2 monoclonal antibody for 30 minutes before stimulation with SM or UCM antigen lysates. (a), (c) and (e): percent MDCs. (b), (d) and (f): MFI. Each dot represents one individual sample. A horizontal bar indicates median of each group. PGN, peptidoglycan from *Staphylococcus aureus*; aTLR2, TLR2 blocked.

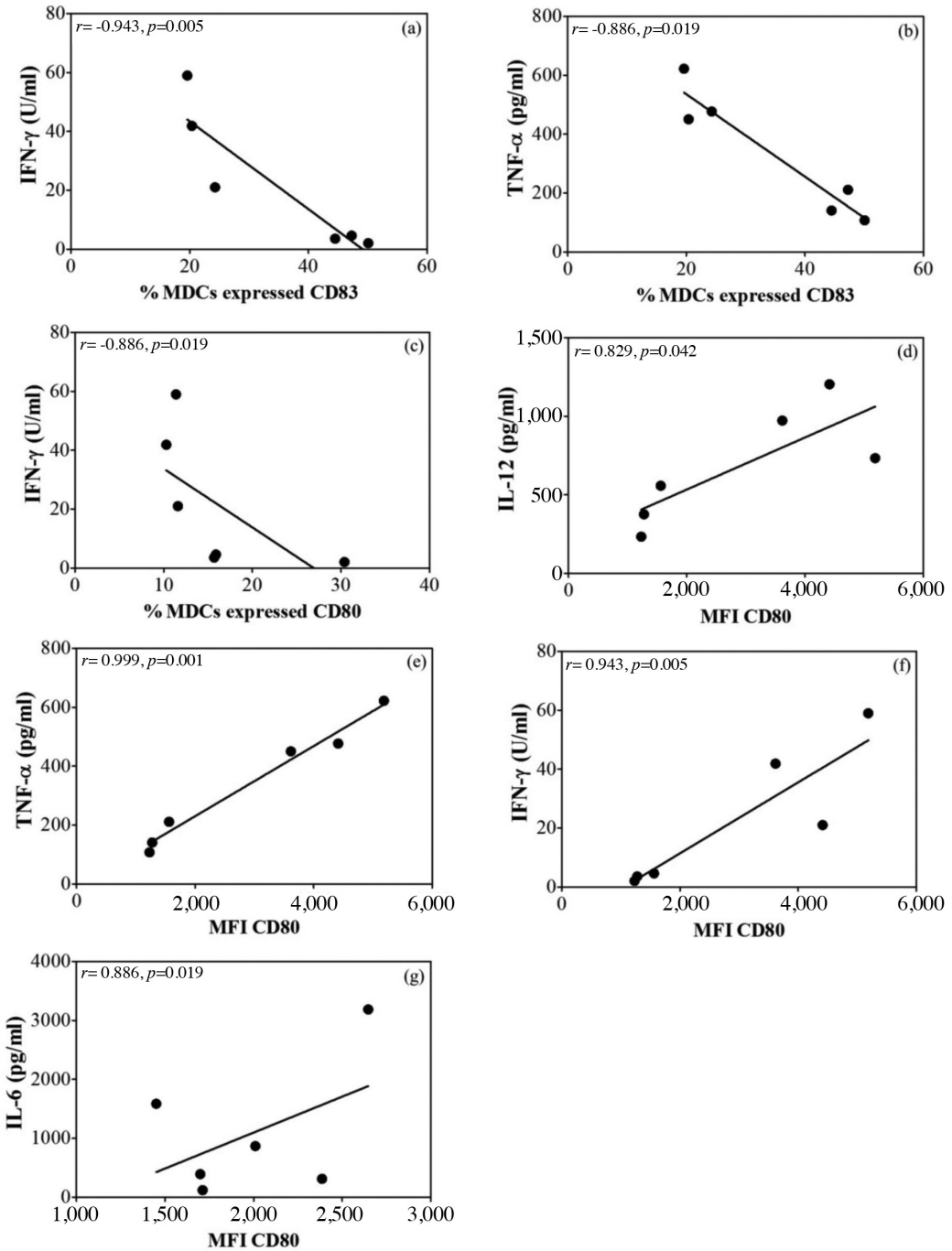

Fig 5–Correlation of *P. falciparum* antigen-stimulated myeloid dendritic cells (MDCs) and plasma pro-inflammatory cytokine levels. Antigen lysates were prepared as described in legend to Fig 2, and MDCs were identified and quantified by flow cytometry. Levels of pro-inflammatory in stored plasma samples were measured using a sandwich ELISA. (a), (b), (c): percent MDCs and plasma cytokine level. (d), (e), (f), (g): mean fluorescent intensity (MFI) and plasma cytokine level. Each dot represents one individual sample.

study, intra-erythrocytic late stages *P. falciparum* lysates derived from cultured isolates obtained from SM and UCM patients modulated MDC maturation in part through TLR2 by *in vitro* stimulation, demonstrated by significant increases in percent MDCS expressing CD83, CD80 and HLA-DR. Percent CD83-expressing MDCs are correlated significantly only with corresponding patients' plasma IFN-γ and TNF-α levels for samples treated with SM antigen lysates, and no correlations were observed with cytokines IL-12, IFN-γ, TNF-α and IL-6 for UCM antigen lysates stimulation.

The *in vitro* whole blood stimulation assay used to elucidate the activation of MDCs is considered a close approximation of the *in vivo* situation. Previously, analysis on dendritic cell function relied on cumbersome purification techniques, which require much *ex vivo* manipulation and the opportunity for artefactual results (Liu, 2005). The *in vitro* whole blood assay employing flow cytometry analysis is faster, cheaper and requires smaller blood volumes than use of peripheral blood mononuclear cells for functional study of peripheral blood MDCs in response to TLR activation (Ida *et al*, 2006).

The present results support the notion that malaria parasite components may affect host immune response during malaria infection through modulation of MDCs properties and function (Coban *et al*, 2002). Late stages *P. falciparum* antigen lysates derived from parasite cultures obtained from either SM or UCM patients were able to activate MDCs as demonstrated in the increased median percent MDCs expressing surface maturation markers in a dose dependent manner. This might be due to that late stages *P. falciparum* antigen lysates contain parasite antigens (proteins, membrane GPI and malarial hemozoin pigment) with varying immunostimulatory effects on DC responses (Ramachandra *et al*, 2000; Struik *et al*, 2004). Schizont lysates have the same ability as intact schizonts in stimulating IFN-α production in human plasmacytoid dendritic cell cultures (Pichyangkul *et al*, 2004). However, intact *P. yoelii*-infected red blood cells (iRBC) do not induce DC maturation *in vitro*, conversely, lysates of infected erythrocytes induced a MyD88-independent activating response in DCs, suggesting that accessibility of parasite inflammatory molecules to their receptors is a key issue in the activation of DCs by *P. yoelii* (Bettiol *et al*, 2010).

SM and UCM antigen lysates caused higher expression of MDC CD83 and HLA-DR than CD80. A possible explanation is that *P. falciparum* lysates might not be able to suppress the function in antigen presentation, but rather affect the co-stimulatory signal for T cell activation, consistent with a previous demonstration that infected-red blood cell lysates of parasite lines induce partial phenotypic maturation of monocyte-derived DCs as shown by the upregulation of CD86, but not CD83 or HLA-DR (Elliott *et al*, 2007); whereas schizont lysates can stimulate human plasmacytoid DCs to upregulate CD86 expression and activate γδ-T cells instead of $CD4^+$ T cells response (Pichyangkul *et al*, 2004). Such impairment in the natural ability of DCs is in line with the association of partial maturation of cord blood MDCs and plasmacytoid dendritic cells with hemozoin accumulation in placenta of pregnant women infected with *P. falciparum* (Fievet *et al*, 2009) and uric acid precipitates accumulated in *P. falciparum*-infected red blood cells (Hoef *et al*, 2013).

Due to the fact that the number of MDCs expressing CD83, CD80 and HLA-DR are overlapping populations, so that a

high number of MDCs expressing CD83, but not CD80 and HLA-DR, could be observed in both antigen lysates stimulation experiments, while a differential fluorescent intensity (MFI) of CD83, CD80 and HLA-DR expression were seen. It may be speculated that *P. falciparum* antigens could be able to trigger the production of a proportion of blood $CD83^+$ MDCs *in vitro* and induce their responsiveness by increasing or decreasing the expression of essential cell surface molecules for T cell activation. The present observation of the lack of correlation between percent MDCs expressing CD80 and HLA-DR and their MFI levels might be due to differences in the rates of expression of the two markers on the MDC surface (Ida *et al*, 2006). This notion subsequently was supported by measurements of the kinetics of CD83, CD80 and HLA-DR expression on human monocytes-derived DCs upon *in vitro* stimulation with β-hematin (hemozoin) and TNF-α/PGE_2 (Giusti *et al*, 2011). This phenomenon might be due to the compensation between percent blood DCs expressing maturation markers and level of surface markers as shown in circulating DCs of malaria-infected African children, (Urban *et al*, 2001; Arama *et al*, 2011).

Although DC TLR2, TLR4, TLR7 and TLR9 are activated by *Plasmodium* antigens and induce anti-malarial immune responses (Eriksson *et al*, 2013), a previous study demonstrated that there is a significant difference in the numbers of MDCs expressing TLR2 and its expression levels on MDCs and monocytes in malaria-infected patients in Thailand (Loharungsikul *et al*, 2008). In this study, TLR2 blockage reduced percent MDCs expressing CD83, but not CD80 or HLA-DR, in both SM and UCM antigen lysates stimulation. Blocking TLR2 association with malaria antigen might impair MDCs maturation. This supposition is consistent with the finding that blocking of TLR suppresses MDCs activation induced by *Mycobacterium bovis* BCG peptidoglycan (Uehori *et al*, 2003) and that *in vitro* blocking TLR reduces the ability of DCs to prime T cell response against *Encephalitozoon cuniculi* (Lawlor *et al*, 2010). However, the relationship in humans between DC activation and extent of TLR participation needs to be explored.

Surface phenotype of DCs and cytokine profile may have a modulatory influence on host immune response (Thomas and Lipsky, 1994). Our finding that when stimulated with SM antigen lysate, there was a strong correlation only between percent CD83-expressing MDCs and corresponding patients' plasma IFN-γ and TNF-α levels might reflect clinical manifestation or immune response during severe malaria phase. These results confirmed the following previous findings: (i) a strong correlation between high plasma IL-12p70 and IFN-γ levels with TLR2 expression on circulating blood MDCs from patients with SM and UCM (unpublished data presented at the Keystone Symposia Conference: Malaria: Immunology, Pathogenesis and Vaccine Perspectives, 2008), (ii) different phenotypes and function of DCs in lethal and non-lethal mouse malaria (Wykes *et al*, 2007, 2008), and (iii) correlation of blood DCs phenotypes and their cytokine profiles in humans (Pashenkov *et al*, 2000). The results on the stimulation of whole blood with UCM antigen lysates, in which only CD80 MFI levels are significantly correlated with patients' plasma IL-6 levels support the previous finding that purified hemozoin from *P. falciparum* induces maturation of DCs through MyD88-dependent pathway and secretion of TNF, IL-12 and IL-6 (Coban *et al*, 2005).

It was expected that late stage *P. falciparum* antigen lysates derived from severe or uncomplicated malaria isolates might induce differently the alterations of DCs function and subsequently control the outcome of immunoregulation or immunopathology during infection. Surprisingly no significant differences in phenotypic changes of human blood MDCs after *in vitro* stimulation with both types of antigen lysates. This might be due to the limited sample size of test *P. falciparum* antigen lysates, and a larger sample size is needed to test this supposition.

In conclusion, these preliminary results suggest that late stage *P. falciparum* antigen lysates derived from cultured parasites obtained from severe and uncomplicated malaria patients could *in vitro* alter myeloid dendritic cell function via (in part) TLR2, thereby modulating the phenotypic maturation of these blood dendritic cells and (*in vivo*) pro-inflammatory cytokine production, which may influence host anti-*Plasmodium* immunity.

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