Cytokine profiles in Malawian children presenting with uncomplicated malaria, severe malarial anemia and cerebral malaria

Wilson L. Mandala, PhD[1,2,3]

Chisomo L. Msefula, PhD[1,3,4]

Esther N. Gondwe, PhD[1,3]

Mark T. Drayson, FRCPath[5]

Malcolm E. Molyneux, FMedSci[1,3,6]

Calman A. MacLennan, FRCP[1,5,7]

[1]Malawi-Liverpool-Wellcome Trust Clinical Research Programme, College of Medicine, University of Malawi, Malawi

[2]Department of Basic Medical Sciences, College of Medicine, University of Malawi, Malawi

[3]Liverpool School of Tropical Medicine, Pembroke Place, University of Liverpool, UK

[4]Department of Microbiology, College of Medicine, University of Malawi, Malawi

[5]Institute of Immunology and Immunotherapy, College of Medicine and Dental Sciences, University of Birmingham, Birmingham, UK

[6]Department of Medicine, College of Medicine, University of Malawi, Malawi

[7]The Jenner Institute, Nuffield Department of Medicine, University of Oxford, UK

Corresponding Author:

Wilson Mandala, PhD

College of Medicine, P/Bag 360, Blantyre, Malawi

Tel: +265 995 450 785. Fax No.: +265 995 450 785

Email: wmandala2002@gmail.com

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Abstract

Pro-inflammatory cytokines are involved in clearance of *Plasmodium falciparum*, and very high levels of these cytokines have been implicated in the pathogenesis of severe malaria. In order to determine how cytokines vary with disease severity and syndrome, we enrolled Malawian children presenting with cerebral malaria (CM), severe malarial anaemia (SMA) and uncomplicated malaria (UCM), and healthy controls. We analysed serum cytokine concentrations in acute infection, and in convalescence. With the exception of IL-5, cytokine concentrations were highest in acute CM, followed by SMA, and were only mildly elevated in UCM. Cytokine concentrations had fallen to control levels when re-measured at one month of convalescence in all three clinical malaria groups. IL-10-to-TNF-α and IL-10-to-IL-6 ratios followed a similar pattern. Compared to concentrations in sera from healthy controls, children presenting with acute CM had significantly higher concentrations of TNF-α ($p<0.0001$), IFN-γ ($p=0.0019$), IL-2 ($p=0.0004$), IL-6 ($p<0.0001$), IL-8 ($p<0.0001$) and IL-10 ($p<0.0001$). Compared to those presenting with acute SMA, acute CM patients had significantly higher concentrations of IL-6 ($p<0.0001$) and IL-10 ($p=0.0003$). Our findings are consistent with the concept that high levels of pro-inflammatory cytokines, despite high levels of the anti-inflammatory cytokine IL-10, could contribute to the pathogenesis of CM.

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Introduction

Nearly 214 million clinical episodes of malaria were reported in 2015 leading to 438,000 deaths, the majority of which were among African children and attributable to *P. falciparum* malaria (1). Clinical *P. falciparum* malaria presents either as uncomplicated malaria (UCM) or as one of the following severe forms of the disease: cerebral malaria (CM), severe malarial anaemia (SMA), metabolic acidosis (MA) or respiratory distress (RD) and other complications including some overlap syndromes (1-2). Immunity to malaria is both humoral and cell-mediated and involves various mechanisms (3). Antibodies that develop through exposure to *P. falciparum* play a role (3), and the involvement of different lymphocyte subsets has been implicated in both protection against, and pathogenesis of malaria (4 - 6).

Cytokines are regulatory proteins or glycoproteins secreted by white blood cells and various other cells in response to a number of stimuli (7). 'Cytokine' is a general term, but cytokines have more specific names depending on the type of cells that produce them and on the functions they perform, such that lymphokines are produced by lymphocytes, and monokines by monocytes and macrophages (8). Lymphokines, such as interferon-gamma (IFN-γ), and interleukin 4 (IL-4) stimulate B cells to produce antibodies, attract and activate immune cells such as macrophages and other lymphocytes at sites of infection. (8 - 11). In contrast, monokines such as Tumor Necrosis factor alpha (TNF-α), IL-1, IL-6 and IL-8, play roles that are inflammatory in nature and also attract neutrophils by chemotaxis (9 - 10). However, it is clear now that the majority of cytokines can be produced by a range of different cell types, questioning the apparent specificity of 'lymphokine' and 'monokine'.

77

78 Cytokines can also be grouped based on the T cells that produce them when the T cells are stimulated
79 to differentiate. T helper 1 (Th1) cells are known to produce large quantities of IFN-γ, induce delayed
80 hypersensitivity reactions, activate macrophages and are crucial for the defence against intracellular
81 pathogens (11 – 12) whereas Th17 cells produce IL-17, IL-21 and IL-22 (11). Th2 cells produce IL-4
82 and are important in inducing IgE production, recruiting eosinophils to sites of inlammation and
83 helping clear parasitic infections (8, 11).

84

85 When categorised based on their effect on inflammation, cytokines can either be termed pro-
86 inflammatory, with the cytokines IL-1, TNF-α, IFN-γ, IL-12 and IL-18 included in this group, while
87 cytokines such as IL-4, IL-10, IL-13 and transforming growth factor beta (TGF-β) are reffered to as
88 anti-inflammatory cytokines (12 - 13). Pro-inflammatory cytokines are produced by a multiplicity of
89 cells including lymphocytes, monocytes, macrophages, fibroblasts, neutrophils, endothelial cells and
90 mast cells and are known to be involved in clearing the initial parasitaemia in the early stages of *P.*
91 *falciparum* infection (7, 14 - 15). Pro-inflammatory cytokines such as TNF-α (16), IFN-γ, IL-6 and IL-
92 1 (17 - 18), when produced in an unregulated manner, have been implicated in the pathogenesis of
93 cerebral malaria (19), and correlate with disease severity and death (20).

94

95 In contrast, anti-inflammatory cytokines such as IL-10 have been shown to down-regulate the pro-
96 inflammatory cytokines (15, 21). Experiments in which IL-10 was administered in mice models of
97 malaria resulted in production of lower TNF-α and lower incidence of experimental cerebral malaria
98 (ECM) (22 - 23), leading some to hypothesise that IL-10 counteracts the potentially pathological host
99 pro-inflammatory response to malaria (14).

100

101 Inflammatory cytokines also play an important role in the pathogenesis of SMA, with high levels of
102 TNF-α, but low levels of IL-10 (24), being associated with SMA in areas of high malaria endemicity
103 (24 -25). IL-12 has been shown to be involved in protective immunity against malaria by regulating
104 IFN-γ, TNF-α and nitric oxide responses in experimental studies (26) and enhancing erythropoiesis in
105 *P. chabaudi*-infected susceptible mice (27).

106

107 Although cytokines may act on the same cells that secrete them (autocrine action), or on cells within
108 close proximity (paracrine action) or in some cases at distant cells (endocrine action) (8), *in vitro*
109 assays can only either measure proportions of cytokine-producing cells by intracellular cytokine
110 staining or quantify cytokine concentration in serum or plasma samples extracted from stimulated or
111 unstimulated venous blood samples using commercially-available enzyme immunoassays (28). We
112 analysed serum samples of children presenting with different clinical presentations of malaria during
113 acute infection and in convalescence, together with samples from healthy children, in order determine
114 concentrations of different cytokines.

115

Materials and Methods

Study Area and Study Population

The study was conducted within the Malawi-Liverpool-Wellcome Trust Clinical Research Programme and Department of Paediatrics, College of Medicine, University of Malawi, and Blantyre Malaria Project. Participants were children admitted with acute malaria to Queen Elizabeth Central Hospital (QECH), and medically-well children attending surgical outpatient clinics at QECH and Beit Cure International Hospital, both in Blantyre, Malawi. Demographic and clinical features of the participants have been reported previously (6). In brief, children were enrolled during the rainy season (November 2005 to April 2006) after obtaining informed consent from the parent or guardian. Each child was examined by a research nurse and clinical officer, baseline demographic data were recorded and a venous blood sample was collected. Criteria defining clinical malaria were: fever, a clinical syndrome compatible with malaria without any apparent alternative cause, and a thick blood film positive for *Plasmodium falciparum* asexual parasites on microscopy. Children were assessed for level of consciousness using the Blantyre Coma Score (BCS) on admission and at two- to-four hourly intervals during intensive clinical care. Over forty children were prospectively enrolled into each of the four clinical groups defined by diagnoses of cerebral malaria (CM), severe malarial anemia (SMA) or uncomplicated malaria (UCM), or healthy controls.

Children with CM had a BCS of two or less at admission and four hours later, while children in all other groups had a score of five at both times (Table 1). Children with SMA had a blood haemoglobin concentration of 5 g/dl or less, and all other children had a haemoglobin concentration above 5 g/dl . Children who tested positive for HIV infection were excluded from the study and referred to the antiretroviral therapy clinic. Children who presented with UCM or SMA were treated with a standard

regimen of sulfadoxine-pyrimethamine (SP), which was the first line treatment for malaria in Malawi at the time the study was conducted. In contrast, children presenting with CM were treated with intramuscular (IM) quinine as recommended for CM patients at that time. Study participants in the UCM, SMA and CM groups were seen again approximately 30 days after treatment (convalescence or follow-up (F) visit), at which time a second blood sample was collected.

Malaria Microscopy

Thick and thin films were prepared for determining the density of malaria parasitemia. Preparation and reading of malaria slides were performed in accordance with standard WHO procedures (29). Briefly, two blood slides were prepared from each participant's blood sample. Each slide had a measured volume of 6 µl of blood for thick film and 2 µl for the thin film. A 3% working stock of Giemsa stain was prepared using a principal Giemsa-staining stock solution and Giemsa buffer prepared from buffer tablets. Thin and thick blood smears were stained with Giemsa after fixing the thin smear with absolute methanol. The stained slides were read by two competent, independent malaria microscopists. The entire smear was first screened at a low magnification (10X and 40X objective lens) to detect suitable fields with even distribution of white blood cells (WBC) (10–20 WBC/field). Smears were then examined using 100X oil immersion lens. At least 100 low power fields were examined before a thick smear was declared negative. A blood slide was declared positive when a concordant result was produced by the microscopists. *P. falciparum* parasites were counted per 200 or 500 leukocytes, in order to estimate the parasite density per microliter of blood. Discordant results were resolved by a third reading of the films. Thin films were examined to confirm the species of the infecting *Plasmodium*.

HIV and malaria tests

HIV testing was performed using two rapid tests, Determine (Abbott Laboratories, Tokyo) and UniGold (Trinity Biotech, Dublin). Discordant results and positive results in children under 18 months were confirmed by PCR as previously described (30).

Serum Collection and preservation

Whole blood samples from study participants were collected on admission and prior to administering antimalarial therapy and one month after treatment as previously described (6). An aliquot of the blood sample was collected in a plain tube and allowed to coagulate with serum separation by centrifugation. Serum was divided into aliquots and preserved at -80^{o}C until required for cytokine analysis.

Cytokine analysis

Concentrations of various cytokines were determined using Becton Dickson (BD) CBA (Cytokine Bead Array) kits. Sera were thawed and centrifuged at maximum speed for 10 minutes to remove fibrin deposits. 25µl of each sample was mixed with 25µl of the capture bead mixture and then with 25µl of detection reagent. Subsequent steps were performed according to manufacturer's instructions (BD CBA Instruction Manuals, 2006). The kit sensitivity (minimum detectable concentration) limits for the various cytokines are provided in Table S1.

182 **Statistical Analysis**

183 Statistical tests were performed using GraphPad Prism version 6.01 for Windows (GraphPad Software,
184 San Diego California USA). Kruskall Wallis Test was used to compare the medians of the different
185 cytokine concentrations (pg/ml) and ratios in different clinical groups. Between-group comparisons of
186 cytokine concentrations for the four groups (Controls, UCM, SMA and CM) were assessed with
187 Bonferroni's multiple comparison test and *p* value of <0.0125 was considered statistically significant.
188 The Wilcoxon matched pairs test was used to determine the statistical significance of the differences
189 concentrations and ratios observed during acute infection and in convalescence for each clinical
190 syndrome of malaria and a *p* value of <0.05 was considered statistically significant.

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192 **Ethical Approval**

193 The study was approved by the College of Medicine Research and Ethics Committee, University of
194 Malawi, and Ethics Committee of the Liverpool School of Tropical Medicine, UK.

195

196

Results

Characteristics of study children

The number of participants recruited in each group and their demographic and haematological characteristics have been published previously (6) and are presented in Table 1. Briefly, consent was obtained for 188 children aged 5 to 84 months to participate in the study. Blood samples from 33 children were excluded for the following reasons: HIV infection (n=14), malaria parasites in the blood of control subjects (n=14), BCS greater than 2 at 4 hours post-admission in children with suspected CM (n=4), and hemoglobin below 5 g/dL in one child with CM. Five children (four with CM and one with SMA) died days after therapy had been administered.

Cytokine concentrations during acute infection and in convalescence.

The median concentration of IFN-γ (Fig. 1A, Table S1) was significantly ($p=0.0019$) higher in acute CM cases (17.3 pg/ml) than in controls (2.32pg/ml) and in acute SMA ($p=0.025$) and acute UCM ($p=0.029$) which then decreased significantly ($p<0.001$) in convalescence (Fig. 2A). TNF-α levels during acute disease were higher in all types of clinical malaria compared to controls (Fig 1B) with significant ($p<0.001$) differences observed between CM patients (median 3.76 pg/ml) and controls (median 1.41 pg/ml), and between SMA patients (median 2.95 pg/ml) and controls. UCM patients also had significantly ($p=0.0012$) higher TNF-α levels (2.12 pg/ml) than controls (1.41 pg/ml), but significantly ($p=0.0006$) lower levels than CM cases (median 3.76 pg/ml). TNF-α levels decreased

significantly in convalescence (Fig. 2B) for both CM (median 3.76 pg/ml falling to 1.69 pg/ml; p=0.002) and in SMA (median 2.95 pg/ml falling to 1.80 pg/ml; p=0.0002).

Acute CM (2.48 pg/ml) patients had significantly (p=0.0009) higher median concentrations of IL-1β than controls (median 1.89 pg/ml) during acute infection (Fig. 1C), and surprisingly the level in the CM group remained elevated median (3.09 pg/ml) in convalescence (Fig 2C). SMA patients had significantly (p=0.0358) higher median concentrations of IL-1β during convalescence (median 3.34 pg/ml) than during acute disease (median 2.21 pg/ml) (Fig. 2C).

Both CM patients (median 3.34 pg/ml) and SMA patients (median 2.55 pg/ml) had significantly higher ($p<0.001$) levels of IL-2 in acute disease than in controls (2.12 pg/ml) (Fig.1D). Levels in both CM and SMA were significantly higher ($p<0.05$) than levels in acute UCM (2.02 pg/ml). IL-2 levels in convalescence in all malaria types were similar to controls (medians: 1.60 pg/ml for UCM, 1.30 pg/ml for SMA, 1.65 pg/ml for CM) (Fig. 2D).

On admission CM (median 3.62 pg/ml) and SMA (median 2.03 pg/ml) groups had significantly (p=0.0006 for CM and p=0.0037 for SMA) higher concentrations of IL-4 than controls (median 1.41 pg/ml) (Fig. 1E), levels in the disease groups decreasing in convalescence (Fig. 2E). IL-4 levels in acute CM were significantly ($p<0.001$) higher than those in acute SMA and UCM (median 1.49 pg/ml). Concentrations of IL-5 in acute infection of all three types of malaria were similar to those of healthy controls (Fig. 1F) although the levels of IL-5 in acute CM (median 1.74 pg.ml) were significantly higher ($p<0.001$) compared to the levels in UCM (median 1.54 pg.ml). IL-5 levels in all three malaria types were similar in acute infection and in convalescence (Fig. 2F).

IL-6 levels in all three malaria types (medians: 17.31 pg/ml for UCM, 12.20 pg/ml for SMA and 156.3 pg/ml for CM) were significantly ($p<0.0001$) higher during acute disease than in controls (2.37 pg/ml) (Fig G). Among the three malaria types, CM patients had the highest IL-6 levels and the difference between the levels in acute SMA and CM and between acute UCM and CM were significant ($p<0.0001$). All three malaria types had significantly ($p<0.0001$) lower IL-6 levels (medians: 1.90 pg/ml for UCM, 2.09 pg/ml for SMA and 1.87 pg/ml for CM) in convalescence (Fig. 2G) than in acute infection.

During acute illness (Fig. 1H) IL-8 levels were higher in all malaria types (medians: 8.47 pg/ml UCM, $p=0.031$; 13.03 pg/ml for SMA, $p=0.0002$; and 29.71 pg/ml for CM, $p<0.0001$) than in controls (median 6.55 pg/ml). IL-8 concentrations in CM patients were significantly ($p<0.0001$) higher than in both SMA and UCM patients. In convalescence IL-8 levels had significantly decreased in all three malaria types ($p=0.0059$ for UCM and $p<0.0001$ for SMA and CM) (Fig. 2H).

During acute illness median IL-10 levels were significantly ($p<0.0001$ for UCM, SMA and CM) higher in children presenting with each of the malaria syndromes than in controls (Fig. 1I). Similar to the trend for IL-8, levels of IL-10 in acute SMA and UCM patients were significantly ($p<0.0001$) lower than in acute CM. IL-10 levels in all malaria groups were significantly ($p<0.0001$) lower in convalescence (medians: 4.13 pg/ml UCM, 4.50 pg/ml for SMA and 4.64 pg/ml for CM) than in acute disease (Fig. 2I). Patients presenting with acute CM had significantly ($p=0168$) higher levels of IL-12p70 (median 2.09 pg/ml) than controls (median 1.52 pg/ml) (Fig. 1J). Levels of IL-12p70 were similar in acute infection and convalescence (Fig. 2J).

IL-12 is the main driver of the IFN-γ response in the T helper 1 pathway, and so, perhaps surprisingly, IL-12p70 was only significantly elevated in CM compared with controls, but only to a modest degree (medians: 2.13 pg/ml and 1.52 pg/ml, p=0.0168).

CM patients (n=4) and one SMA patient who had died had significantly ($p<0.05$) higher levels of all cytokines (Fig 3, Table 2) during acute illness than those who survived (n=25).

Comparison of IL-10/TNF-α and IL-10/IL-6 ratios between different groups

The IL-10-to-TNF-α ratio was significantly ($p<0.0001$) higher in acute UCM, SMA and CM (medians: 43.25, 25.16 and 140.2 respectively) patients than in controls (median 3.47), while the ratio in acute SMA was significantly ($p<0.0001$) lower than the ratio in acute CM (Fig. 1K). The IL-10-to-TNF-α ratios for all three malaria types (medians: 2.10 for UCM, 2.60 for SMA and 3.65 for CM) in convalescence were similar to ratios in controls (median 1.63) (Fig. 2K).

The IL-10-to-IL-6 ratios in acute UCM, SMA and CM (medians: 4.58, 3.92 and 2.93 respectively) were significantly ($p<0.0001$ for UCM, $p=0.0003$ for SMA and $p=0.0090$ for CM) higher than the ratio in controls (median 1.63) (Fig. 1L and Table 2). During convalescence, UCM (median 2.20) still had significantly ($p=0.0149$) higher IL-10-to-IL-6 ratio than controls (median 1.63) but SMA (median 2.20) and CM (median 2.20) ratios were just as low as those of controls. (Fig. 2L and Table S1).

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Discussion

Cytokine production by different cell types in response to foreign antigen is one of the defence mechanisms that characterise cellular immunity and can drive both normal and pathological immune responses (7). Previous studies have shown that when pro-inflammatory cytokines (produced by a variety of cells, including Th1 cells and macrophages) such as TNF-α (16), IFN-γ, IL-6 and IL-1 (17 - 18), are produced in an unregulated manner, they contribute towards pathogenesis of cerebral malaria (19), and to disease severity and death (20). In contrast, anti-inflammatory cytokines (produced by cells that include monocytes and Th2 cells) such as IL-10 and IL-13 have been shown to down-regulate production of pro-inflammatory cytokines (15, 21) and to reduce the incidence of experimental cerebral malaria (ECM) in mice models (22).

We analysed concentrations of serum cytokines in Malawian children presenting with CM, SMA and UCM in acute illness and in convalescence, and compared these levels with those in healthy controls (Table S1 and Table 2). We found that both pro-inflammatory (TNF-α, IFN-γ, IL-1, IL-6) and anti-inflammatory cytokine (mainly IL-10) concentrations were markedly elevated over control levels in Malawian children presenting with CM, moderately raised in SMA patients and minimally, but significantly increased in those children presenting with UCM. In all patient groups, cytokine concentrations decreased to control levels in convalescence. A similar trend was observed for IL-10-to-TNF-α and IL-10-to-IL-6 ratios. These results indicate that acute malaria, regardless of severity, is characterised by higher than normal levels of a broad range, but not all, of cytokines, whether in the 'Th1 group' (IFN-γ, TNF-α and IL-1) or 'Th2 group' (IL-4, IL-6 and IL-10). These high levels decrease significantly in convalescence.

In line with our findings, most studies that have determined cytokine concentrations in *Plasmodium* malaria, both in mouse models (22, 26-27) and in humans (14, 17, 21, 23, 12, 31-43), have reported highly elevated cytokine concentrations in symptomatic malaria of all clinical varieties. Although murine studies provide some insight into malaria-related cytokine perturbations, data from humans with various malaria syndromes are essential for understanding the pathogenesis of human disease.

Among the various studies that have investigated cytokine perturbation in *P. falciparum* malaria in other countries (12, 31-43), few have compared the levels in the different clinical types of malaria, namely UCM, SMA and CM (12, 14). TNF-α, IFN-γ, IL-1, IL-6, IL-8 and IL-10 have been found in increased levels in patients with severe malaria compared to healthy controls (14, 25) decreasing in convalescence to control levels, but in these studies the clinical syndromes of severe malaria were not fully described. Cytokine data from children presenting with strictly-defined UCM, SMA and CM and a month in convalescence in this study provide additional valuable information.

Interestingly, there are some apparent paradoxes between the cytokine concentrations reported in the current manuscript and monocyte intracellular cytokine staining (44) and immunophenotyping findings (6) that we have reported previously for the same study participants. We observed decreased IL-6 and TNFα production by monocytes in children with difference forms of malaria (44), This indicates that the elevated serum levels of these cytokines in the current report are produced by cells other than monocytes (or macrophages), most likely T cells and NK cells. Moreover, we reported panlymphopenia among children with cerebral malaria and uncomplicated malaria. Therefore, elevated cytokine production by lymphocytes in these groups would either have to come counterintuitively from

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a reduced number of peripheral blood lymphocytes, or from lymphocytes retained in secondary lymphoid tissues or sequestered in other vascular structures.

Not all malaria-infected children with high levels of Th1 pro-inflammatory cytokines, such as TNF-α, develop severe malaria (14), suggesting that the cytokine network as a whole, rather than a single cytokine, may contribute in different ways to severe disease (12, 25). Thus, severe *P. falciparum* malaria could be associated with an inadequate negative feedback response by Th2 anti-inflammatory cytokines such as IL-10. The timing of IL-10 production is likely to be important in determining its effectiveness as an anti-inflammatory cytokine with *in vitro* studies showing that TNF-α, IL-6 and IL-1β are produced within 2 to 4 hours of stimulation, while IL-10 is first detected after 8 hours supporting the concept that IL-10 counter-regulates the pro-inflammatory response to *P. falciparum* (12). Our *in vitro* observations that IL-10 was detected 7 hours after activation of monocytes with LPS and maximal IL-10 levels were only observed after 24 to 48 hours of stimulation with LPS (45) are consistent with this concept.

Since IL-10 serves to regulate both the production and functions of TNF-α and IL-6 (19, 45), it has been suggested that children with a low IL-10-to-TNF-α ratio may be more likely to develop severe malaria compared to children with a higher ratio (14). In a study from Kenya (25), children with severe malaria (type of severe malaria was not specified) had higher IL-10-to-TNF-α ratios compared to children presenting with mild disease. Here we found that Malawian children presenting with all forms of malaria had high IL-10-to-TNF-α and IL-10-to-IL-6 ratios, so high levels of TNF-α and IL-6 in CM could not be attributed to a lack of IL-10 response. Nevertheless, it is apparent that the IL-10 response observed in CM was unable to prevent these high pro-inflammatory cytokine levels since higher levels

of IL-6 and TNF-α in malaria patients who died than in those who survived, as found previously in adults in Vietnam (19), suggest that uncontrolled levels of these cytokines may have contributed to the demise of these children. In a separate study in Malawian children, those presenting with severe malaria had higher levels of IL-6 and TNF-α compared to those presenting with UCM although severe malaria was not further sub-categorised (31).

Overall, the observation that higher levels of the pro-inflammatory/Th1 cytokines were found in CM compared with SMA is consistent with the concept that CM results from an immunopathological response in which the production of pro-inflammatory cytokines are poorly regulated (19, 45). Other investigators have argued that early, as opposed to late, production of IFN-γ and TNF-α correlates well with protection since when produced early, over-production can more easily be kept in check by the presence of anti-inflammatory cytokines such as IL-10 (32).

The present study was limited in that the analysed blood samples were collected only at two time points, acutely and once in convalescence at time points which were roughly 30 days apart. It would be informative to conduct a longitudinal study recruiting children that present with different forms of malaria who are then followed closely to provide a time course curve for these cytokines as has been done before with blood samples from South African adults (39), although these were followed for only five days.

An unavoidable limitation of clinical studies of natural infection is that we do not know the point in time at which Plasmodial sporozoites are first inoculated by the mosquito, nor the time when

365 merozoites first emerge from the liver to invade erythrocytes. Although we have reported on the
366 proportion of cytokine-producing monocytes from each of these three malaria groups (44), inclusion of
367 an intracellular cytokine analysis for other cytokine-producing cells, could enable identification of the
368 main producers of the cytokines present in corresponding serum/plasma. Lastly, although this study
369 analysed serum samples for concentrations of some cytokines, analysis of additional cytokines that are
370 suspected to play some roles in malaria immunity (42-43) as well as concentrations of chemokines
371 such as RANTES and IP-10, which have been shown to vary with malaria severity (42), would provide
372 additional insight into their separate and/or synergistic roles in malaria. Subsequent studies should
373 combine analysis of serum or plasma samples for cytokines with intracellular cytokine staining, in
374 samples collected from children presenting with different forms of clinical malaria.

375 We have shown that, just as different clinical malaria syndromes are characterised by diverse
376 perturbations of leukocyte and lymphocyte subsets (6), they are also characterised by altered cytokine
377 patterns. While in acute CM there is a transient pan-lymphopenia (6), and lowest proportion of IL-6
378 and TNF-α producing monocytes (44), there is a paradoxical concomitant elevation of circulating
379 cytokine levels, with all perturbations normalising in convalescence (6, 44). Many studies of cytokine
380 levels in malaria have been published, yielding generally similar findings. In this paper, however, we
381 bring together various malarial syndromes, different classes of cytokines and admission and
382 convalescent time-points in the same group of children, in whom circulating leucocyte counts and
383 lymphocyte subsets have already been quantitated. The question as to why the cells that might have
384 been expected to increase in association with their secretory product actually decrease during acute
385 illness (6) could be addressed by the hypothesis that the secretory cells get sequestered in secondary
386 lymphoid tissue during acute disease. Whereas the observation of high cytokine levels in acute disease
387 but low proportion of cytokine producing monocytes (44) could be explained by the hypothesis that the

388 monocytes responsible for producing the observed high cytokine levels are anergic to further
389 stimulation during the phase of acute disease when a venous sample is collected.
390
391 Our findings support the suggestion that cytokines, particularly in CM, may promote the transient
392 sequestration of lymphocytes in secondary lymphoid tissue, potentially causing the observed
393 paradoxical lymphopenia (6) by contributing to the upregulation of CD69 that is recognized in CM
394 (46). The differences in cytokine levels between CM and other malarial syndromes may reflect the
395 severity of the disease (CM has the highest case fatality rate among these syndromes) and/or the
396 parasite burden, which in most studies that include these three syndromes, is greatest in CM (2).
397
398 With developing technologies for bedside diagnosis, patterns of circulating cytokine concentrations
399 may in due time contribute to the rapid differentiation between malaria and other causes of fever. For
400 this possibility to be realized, increased amounts of data on immunological and biochemical
401 parameters, including cytokine levels, will need to be gathered from clinically well-characterised
402 patients, so that new tests can be evaluated for their practical usefulness.
403

404 **Figure Legends**

405 **Figures 1A to 1J)**: Plots of log transformed concentrations (pg/ml) of different cytokines (IFN-γ, TNF-
406 α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and IL-12p70) in sera samples collected from healthy
407 controls (Control), acute uncomplicated malaria (UCM), acute severe malarial anaemia (SMA) and
408 acute cerebral malaria (CM). **Figures 1K and 1L** are plots of the ratio of log transformed IL10-to-
409 TNF-α and IL-10-to-IL-6 respectively during acute infection. (Medians and 10^{th} and 90^{th} percentiles)

410

411 **Figures 2A to 2J**: Plots of log transformed concentrations (pg/ml) of different cytokines (IFN-γ, TNF-
412 α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and IL-12p70) in sera samples collected from healthy
413 controls (Control) and from convalescent uncomplicated malaria patients (UCM-F), severe malarial
414 anaemia (SMA-F) patients and cerebral malaria (CM-F) patients. **Figures 2K and 2L** are plots of the
415 ratio of log transformed IL10-to-TNF-α and IL-10-to-IL-6 respectively during convalescence (Medians
416 and 10^{th} and 90^{th} percentiles).

417

418 **Figure 3**: **Cytokine levels in children with CM and SMA who died and survived**: Plot of log
419 transformed concentrations (pg/ml) of different cytokines (IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6,
420 IL-8, IL-10 and IL-12p70) in sera samples collected from five children who died (red dots) and those
421 children who survived (black dots) after presenting with acute CM and SMA (Medians and 10^{th} and
422 90^{th} percentiles)

423

424

425 **Contributors**

426 WLM, MEM and CAM conceived the study. CAM and MEM oversaw clinical aspects of the study.
427 WLM, CLM and ENG performed the investigations. WLM, and CAM analysed the data. WLM, CAM,
428 MEM and MTD wrote the report. CAM oversaw the research. All authors contributed to the study
429 design and reviewed the report.

430

431 **Conflict of Interest Declaration**

432 CAM was previously an employee of the Novartis Vaccines Institute for Global Health and recipient of
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439

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441 The funding sources played no role in the study design, data analysis or writing of the report. The
442 corresponding and senior author had full access to all data in the study and had final responsibility for
443 the decision to submit for publication.

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Clinical Group	Controls	Cerebral Malaria	Severe Malarial Anemia	Uncomplicated Malaria
Number	42	29	30	54
Died after Recruitment	-	4	1	0
Reviewed in Convalescence	-	18	21	34
Sex (M:F)	29:13	10:19	19:11	38:16
Age (months) (range)	20 (5 - 76)	30 (5 - 84)	23 (5 - 38)	27 (6 - 58)
Parasites/µl blood) (range)	0	41,800 (900 - 517,000)	3,500 (20 - 296,000)	52,300 (460 - 768,000)
Blantyre Coma Score (range)	5 -	1 (0-2)	5 -	5 -
Hemoglobin (g/dL) (range)	11.2 (7.0 - 14.1)	7.7 (5.3 - 12.5)	3.9 (2.4 - 4.9)	9.3 (5.0 - 13.0)

Table 1: Demographic and clinical details of study participants: Subjects were children with cerebral malaria, severe malarial anemia and uncomplicated malaria presenting to the Pediatric Accident and Emergency Clinic at Queen Elizabeth Central Hospital in Blantyre, Malawi. Control subjects were children admitted for elective surgical procedures who were medically well. Values are medians and range. These participants' details have been published previously (Mandala et al, CVI 2015 (6))

CVI

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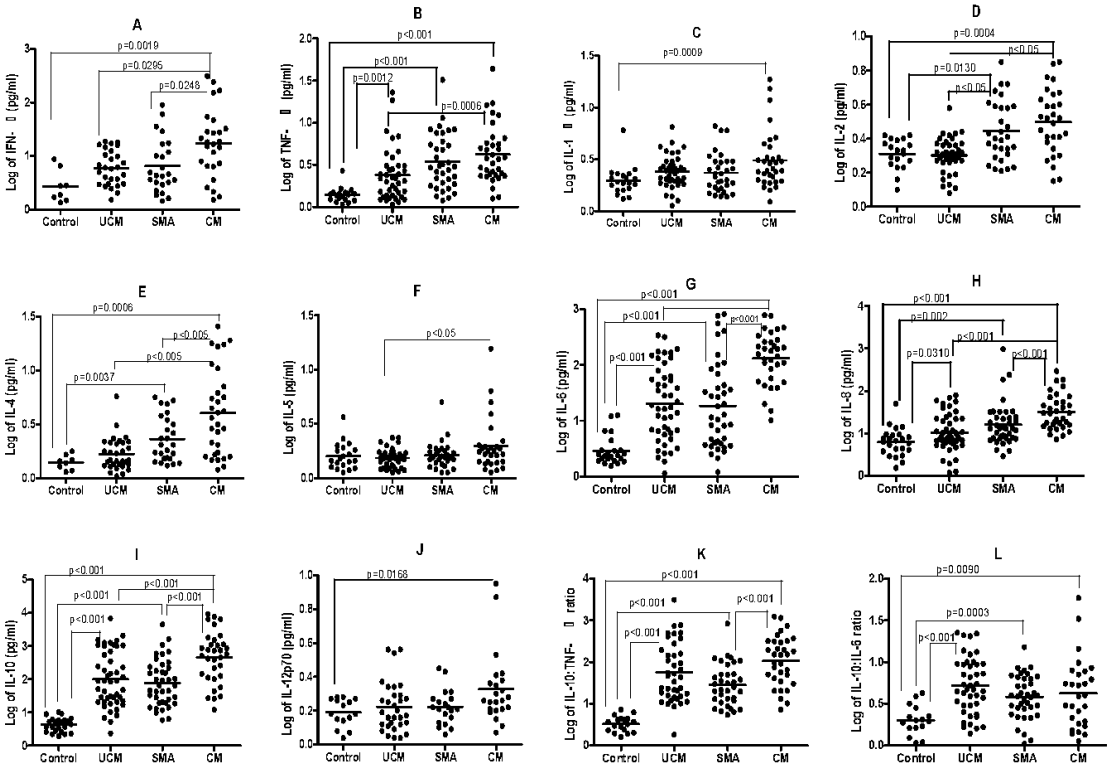
Table 2: Median concentrations (pg/ml) of different cytokines (IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and IL-12p70) in sera samples collected from five children who died (n=4 for CM and n=1 for SMA) and those children who survived after presenting with acute CM (n=11) and SMA (n=14). The p values for the differences in cytokine concentrations between those died and those who survived.

	Median Concentration (pg/ml)		***P* value**
Cytokine	**Dead (n=5)**	**Survivors (n=25)**	
IFN-γ	2.22	0.94	0.0030
TNF-α	1.23	0.76	0.0543
IL-1β	1.125	0.385	0.0010
IL-2	0.805	0.45	0.0012
IL-4	0.75	0.425	0.0065
IL-5	0.59	0.22	0.0009
IL-6	2.33	1.84	0.0061
IL-8	2.24	1.24	0.0007
IL-10	3.65	2.34	0.0039
IL-12p70	0.38	0.22	0.0035

Figure 1:



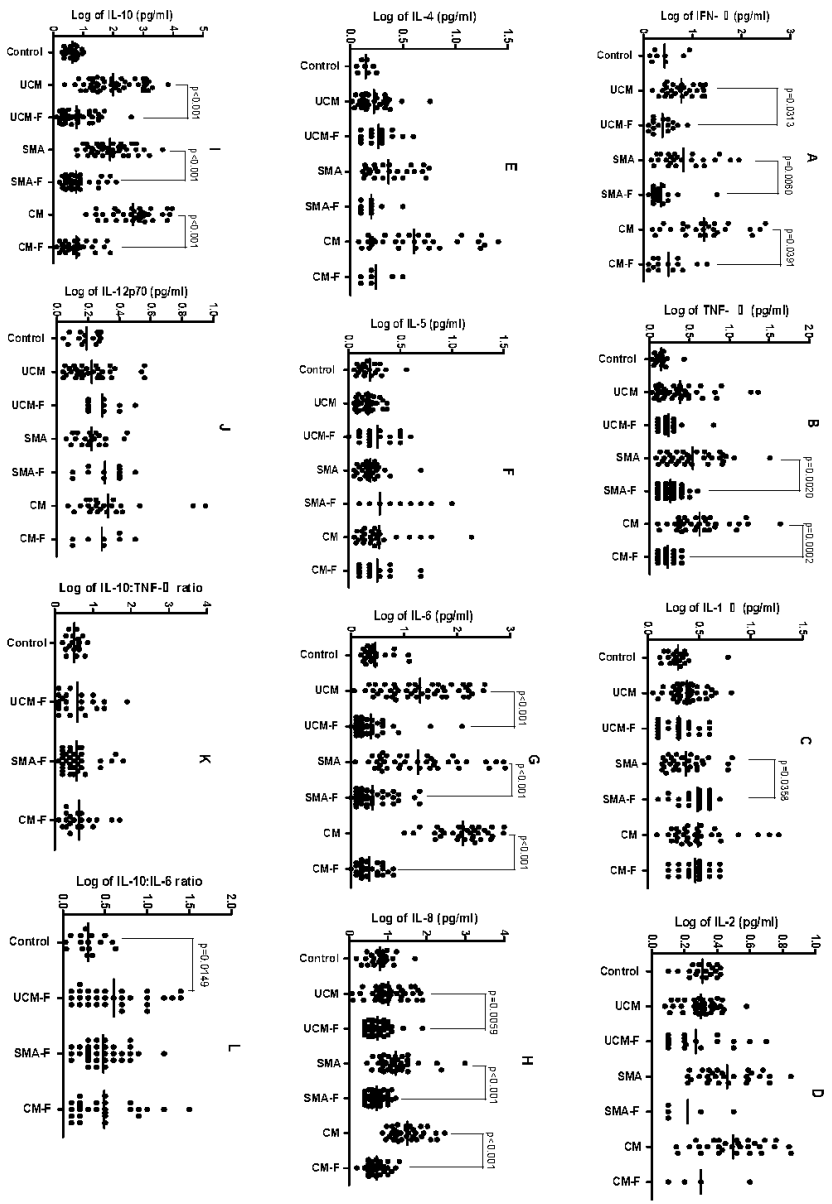


Figure 2:

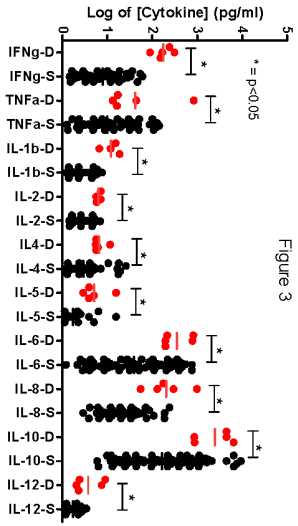


Figure 3