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Low antibodies against *Plasmodium falciparum* and imbalanced pro-inflammatory cytokines are associated with severe malaria in Mozambican children: a case–control study

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Abstract

Background: The factors involved in the progression from *Plasmodium falciparum* infection to severe malaria (SM) are still incompletely understood. Altered antibody and cellular immunity against *P. falciparum* might contribute to increase the risk of developing SM.

Methods: To identify immune responses associated with SM, a sex- and age-matched case–control study was carried out in 134 Mozambican children with SM (cerebral malaria, severe anaemia, acidosis and/or respiratory distress, prostration, hypoglycaemia, multiple seizures) or uncomplicated malaria (UM). IgG and IgM against *P. falciparum* lysate, merozoite antigens (MSP-1$_{19}$, AMA-1 and EBA-175), a Duffy binding like (DBL)-α rosetting domain and antigens on the surface of infected erythrocytes were measured by ELISA or flow cytometry. Plasma concentrations of IL-12p70, IL-2, IFN-γ, IL-4, IL-5, IL-10, IL-8, IL-6, IL-1β, TNF, TNF-β and TGF-β1 were measured using fluorescent bead immunoassays. Data was analysed using McNemar's and Signtest.

Results: Compared to UM, matched children with SM had reduced levels of IgG against DBLα (*P* < 0.001), IgM against MSP-1$_{19}$ (*P* = 0.050) and AMA-1 (*P* = 0.047), TGF-β1 (*P* < 0.001) and IL-12 (*P* = 0.039). In addition, levels of IgG against *P. falciparum* lysate and IL-6 concentrations were increased (*P* = 0.004 and *P* = 0.047, respectively). Anti-DBLα IgG was the only antibody response associated to reduced parasite densities in a multivariate regression model (*P* = 0.026).

Conclusions: The lower levels of antibodies found in children with SM compared to children with UM were not attributable to lower exposure to *P. falciparum* in the SM group. IgM against *P. falciparum* and specific IgG against a rosetting *Pf*EMP1 domain may play a role in the control of SM, whereas an imbalanced pro-inflammatory cytokine response may exacerbate the severity of infection. A high overlap in symptoms together with a limited sample size of different SM clinical groups reduced the power to identify immunological correlates for particular forms of SM.

Keywords: *Plasmodium falciparum*, Children, Severe malaria, Antibodies, Cytokines

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Background

In countries endemic for *Plasmodium falciparum*, severe malaria (SM) was responsible for approximately 0.7 million deaths in 2010, predominantly children under five years of age [1]. Reduction of case fatality rates and morbidity is hampered by an incomplete understanding of the parasite and host factors involved in the pathophysiology of life-threatening disease. Progression from infection to severe clinical malaria is probably multi-factorial, including sequestration of infected erythrocytes (IEs) in vital organs and mechanical obstruction of blood flow, limited malaria-specific antibody immunity and deregulated inflammatory responses to *P. falciparum* [2].

Antibodies targeting blood stage parasite antigens are acquired after exposure to *P. falciparum* [3] and may contribute to protection from SM by reducing parasite density [4]. Specifically, antibodies against *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) have been suggested to block formation of rosettes [5] and cytoadhesion of IEs to specific host receptors [6]. Several studies have reported associations between antibody responses and risk of SM in young children [7-12]. However, conclusions are contradictory, some showing low immunoglobulin G (IgG) levels in children with severe anaemia (SA) [7,10] compared to uncomplicated malaria (UM), others reporting no associations [9] or even opposite trends for cerebral malaria (CM) [7]. In addition, the role of immunoglobulin M (IgM) antibodies in children with SM has been much less explored [9,12], although reduced IgM responses have been observed in adults with CM [13,14].

In addition to antibodies, cytokines such as interleukin (IL)-12, interferon (IFN)-γ and tumor necrosis factor (TNF) have been shown to be involved in the control of *P. falciparum* infection [15-17]. However, the excessive production of pro-inflammatory cytokines, such as TNF and IL-6, may damage host tissues, increase the expression of adhesion molecules on endothelial cells and enhance parasite cytoadhesion [18]. The regulation of pro-inflammatory cytokines production by IL-10 or transforming growth factor (TGF)-β1 seems to be a key factor in preventing acute pathology [19-21] and, overall, the fine balance between pro-inflammatory versus immuno-regulatory cytokines is suggested to determine the outcome of *P. falciparum* infection [22].

Combined information on both antibody and cytokine responses is needed to understand the role of immunity in the progression of malaria infection to SM, and to develop rational strategies that reduce mortality and morbidity associated to SM. Remarkably, there is scarce data on antibody responses in children with signs of severity other than SA and CM such as prostration, acidosis and/or respiratory distress (ARD) or multiple seizures (MS), which are among the most prevalent forms of SM in some endemic areas [23]. In the present study, the association of SM with low antibody responses and an exacerbated pro-inflammatory cytokine response was tested in Mozambican children. IgG and IgM against parasite lysate, merozoite antigens and *P. falciparum* antigens on the surface of IEs, as well as plasma cytokines and chemokines, were compared between children with different clinical presentations of SM and children with UM, matched by sex and age.

Methods

Study area

The area of study was located in the Manhiça District, southern Mozambique. Detailed descriptions of the area have been reported elsewhere [24]. Briefly, Manhiça is characterized by a perennial malaria transmission with some seasonality and of moderate intensity, mostly attributable to *P. falciparum*. The majority of SM cases occur in children under five years of age. Prostration, ARD and SA are the most common clinical presentations, with coma being infrequent and slightly shifted to older children [23].

Study design and patients

Between April and November 2006, a sex and age (+/−3 months) matched case–control study was conducted, with a primary aim of characterizing the cytoadhesion phenotypes of *P. falciparum* isolates [25]. Children under five years of age attending the Manhiça District Hospital with a clinical diagnosis of *P. falciparum* malaria were recruited after written informed consent was given by their parents or guardians. Clinical malaria was defined as the presence of fever (axillary temperature ≥37.5 °C) with an asexual *P. falciparum* parasitaemia of ≥500/μL by microscopic examination of Giemsa-stained blood smears; this definition of malaria has a sensitivity and specificity of >90% in children from Manhiça [26]. Cases were children presenting with clinical malaria and at least one of the following definitions of SM [27]: CM (Blantyre Coma Score ≤2), SA (packed cell volume <15% or hemoglobin <5 g/dL), ARD (lactate >5 mM and/or chest indrawing or deep breathing), prostration (inability to sit or breastfeed in children old enough to do so), hypoglycaemia (blood glucose <2.2 mM) and MS (≥2 convulsions in the preceding 24 h) . Controls were outpatient children with malaria not showing any of the mentioned signs of severity and able to take oral medication (UM group). All patients were reviewed by the study pediatrician to confirm that malaria was the sole or principal cause of the disease. Children with positive bacteraemia were excluded from the study. Malnutrition was defined as the presence of marasmus or kwashiorkor by clinical examination or as a mid-upper arm circumference <12.5 cm in children

>12 months of age. SM patients were admitted and treated with intravenous quinine until able to switch to oral therapy, while UM controls were treated following Mozambican national guidelines at that time (artesunate plus sulphadoxine-pyrimethamine). The study was approved by the National Mozambican and the Hospital Clinic of Barcelona Ethics Review Committees.

Sample collection

Before treatment, peripheral blood was taken by venipuncture into a tube containing lithium heparin, and 2 drops of blood were spotted onto filter paper (Schleicher & Schuell; n° 903^{TM}). Biochemical determinations (alanine aminotransferase, bilirubin and creatinine) and full blood counts were performed using Vitros DT60 and Sysmex Kx21 analyzers, respectively. Lactate was determined using Lactate Pro$^®$ (Fact Canada) at the bed side. After centrifugation, plasma was stored at −80 °C, erythrocyte pellets were Buffy-coat depleted, washed 3 times in phosphate-buffered saline (PBS) and cryopreserved in glycerolyte solution.

Parasite density and multiplicity of infection

DNA was extracted from a 50 ml blood drop onto filter paper with QIAamp DNA Mini Kit (Qiagen), and resuspended in 150 ml of water. Five ml of DNA samples, tested in triplicate, were used to measure parasite density by a real-time quantitative PCR (qPCR) targeting the *P. falciparum* 18 S ribosomal RNA gene, as described elsewhere [28]. Multiplicity of infection (MOI) was determined by nested PCR-typing of the polymorphic regions of *msp-1* and *msp-2* genes [29], and estimated as the highest number of *msp-1* or *msp-2* alleles in the sample.

Enzyme-linked immunosorbent assays (ELISA)

IgG and IgM antibodies were measured against the recombinant proteins merozoite surface protein 1 (MSP-1$_{19}$, 19 kD fragment, 3D7 [30]), erythrocyte binding antigen 175 (EBA-175, F2 region, CAMP [31]), apical membrane antigen 1 (AMA-1, full ectodomain, 3D7 [32]) and a Duffy binding like alpha (DBLα) domain from a *Pf*EMP1 involved in rosetting through adhesion to complement receptor 1 (*R29var1* minimal domain, [33]), all produced at the International Centre for Genetic Engineering and Biotechnology (New Delhi, India). High-binding 96-well microplates (Nunc Maxisorp) were coated with 200 ng/well of recombinant protein in 0.05 M carbonate-bicarbonate buffer. Plates were washed with 0.05% Tween-20 in PBS (PBS-Tween), blocked with 2% bovine serum albumin (BSA) in PBS-Tween for 8 h at 4 °C and washed again. One hundred μl of plasma at 1/200 dilution along with positive (pool from 8 hyper-immune Mozambican adults) and negative

controls (9 unexposed Europeans) were added in duplicate to wells. After incubation overnight at 4 °C, peroxidase-conjugated goat anti-human IgG or IgM secondary antibodies (Sigma) were added at 1/30000 and 1/2000, respectively. After 1 h of incubation and washing, 100 μl/well of a phosphate solution with 0.012% of H_2O_2 substrate and *o*-phenylendiamine chromagen were added for 5 min and the colorimetric reaction was stopped with 25 μl/well of H_2SO_4. Specific reactivity of plasmas was obtained as optical density (OD) values measured at 492 nm (Multiskan EX, Labsystems) and normalized by dividing OD of each sample by the OD of the positive control run in each plate.

To measure antibodies to *P. falciparum* lysate, the 3D7 and HB3 clones (provided by the Malaria Research and Reference Reagent Resource Center, MR4) from asynchronous cultures at 5% parasitaemia were homogenized in PBS at 1% haematocrit by a minimum of three freeze-thawing steps and mixed (1:1). A lysate of uninfected erythrocytes was used to determine unspecific recognition. Coating was done with 50 μl of erythrocyte extract and plasma was added at 1/1600 dilution. Reaction was performed as described above. OD of uninfected lysate wells was subtracted from OD of infected lysate wells, and values were normalized against the positive control run in each plate.

Antibodies to IEs surface antigens

Five paediatric isolates (three from SM patients [MOZ1, MOZ2 and MOZ4] and two from UM patients [MOZ3 and MOZ5]) and two placental isolates from O blood group individuals [25,34], were tested for antibody recognition. MOZ2 was adapted to *in vitro* culture, whereas the remaining isolates were used in antibody measurements without *in vitro* expansion. IgG were also measured against four laboratory clones with different receptor binding phenotypes: R29 (rosetting, [35]), ITG$_{ICAM}$ (ICAM-1, [36]), FCR3$_{CSA}$ (CSA, [37]) and E8B$_{CD36}$ (CD36, [38]). The study samples, plus negative and positive controls, were tested blindly in a single assay against each parasite. Briefly, cryopreserved ring-stage parasites were thawed in a sorbitol gradient and cultured to late trophozoites following standard methods. Cells were washed three times in PBS and resuspended at 1% of haematocrit and 1-5% parasitaemia in 1% BSA-PBS solution. Five μl of plasma were added to 95 μl/well of erythrocyte suspension, incubated for 30 min at room temperature and stained for 30 min with 100 μl of polyclonal rabbit anti-human IgG (DakoCytomation) at 1/200. Subsequently, cells were incubated with 100 μl of AlexaFluor$^®$-conjugated donkey anti-rabbit IgG (Invitrogen) diluted at 1/1000 and 10 mg/mL ethidium bromide for 30 min in darkness. Samples were washed three times with PBS-BSA between incubations.

Data from 1000 positive events was acquired with a Becton-Dickinson FACSCalibur flow cytometer. Reactivity against IEs surface antigens was expressed as the difference between the mean fluorescence intensity (MFI) of IEs and the MFI of uninfected red blood cells.

Cytokines and chemokines

Concentrations (pg/mL) of IL-12p70, IL-2, IFN-γ, IL-4, IL-5, IL-10, IL-8, IL-6, IL-1β, TNF, TNF-β and TGF-β1 in plasma were measured using fluorescent bead immunoassays (Human Th1/Th2 11plex and Human TGF-β1 FlowCytomix Simplex kits, Bender MedSystems, Austria) following manufacturer's instructions. Beads fluorescence was acquired with a Becton-Dickinson FACSCanto II and analysed in FlowCytomix Pro2.2.1 software (Bender MedSystems). Concentration of each analyte was obtained by extrapolating fluorescence intensity to a 7-point dilution standard curve supplied by the manufacturer. Any value below the limits of detection was given a value of half the detection limit for that cytokine.

Statistical methods

Prevalence of recognition of parasites and recombinant proteins by antibodies in children was considered positive if MFI or normalized OD values were above the mean of the negative controls plus three standard deviations for each antigen. Spearman's rank correlations were performed to evaluate correlations between immunological parameters. Comparisons between matched case–control pairs for categorical variables were done using McNemar´s chi-squared, and reported as the number of pairs with a divergent result between SM and UM. Continuous variables were analysed using Sign test, and reported as the median difference between SM and UM values. Children were stratified by SM clinical presentations, and compared to their matched controls. Adjustment for multiple comparisons was done by Monte Carlo permutation tests with 1000 random permutations [39]. Associations between immune responses and parasite qPCR densities were assessed by multivariate linear regression with log-transformed parasite density as the outcome and log-transformed antibodies or cytokines as independent variables. All data collected were analysed using Stata version 10.0 (Stata Corporation). *P*-values <0.05 were considered statistically significant.

Results

Characteristics of the study participants

Among 142 children recruited, four tested negative for *P. falciparum* by qPCR and were excluded together with their matched pairs. Therefore, 134 children (67 case–control pairs) were finally analysed. The clinical characteristics of the patients are summarized in Table 1. Prostration, ARD, SA and MS were the most prevalent

symptoms of severity, whereas hypoglycaemia and CM were only described in five and three children, respectively. Forty-four (65%) children presented with two or more SM criteria. The 23 cases with a single criterion of severity distributed as follows: 13 prostration, 6 MS, 3 SA and 1 ARD. Three SM patients died yielding a case-fatality rate of 2%, and two were transferred to Maputo Central Hospital. Parasite densities by qPCR were higher in SM cases than in paired controls, but this difference was not statistically significant ($P = 0.087$). Children with SM or UM did not differ in malnutrition, indirect clinical indicators of AIDS (oral candidiasis) or in the number of days with clinical symptoms prior to recruitment. Pre-treatment with an anti-malarial was only reported for one patient with SM. Previous history of SM was more common among children with SM (11 [16%]) than in children with UM (3 [4%]; divergent pairs = 13 [18%], $P = 0.037$).

Antibodies and malaria severity

Prevalence and levels of IgG against DBLα were significantly lower in children with SM compared to their matched controls ($P = 0.032$ and $P < 0.001$, respectively), whereas no differences were found for IgG against merozoite antigens (Figure 1A). In contrast, IgG levels against *P. falciparum* lysate were significantly higher in children with SM than in matched controls ($P = 0.004$; Figure 1B). Recognition of antigens on the surface of IEs did not differ between SM and UM children for any of the parasites tested (Table 2). Overall, IgG prevalence was highest for R29 and E8B$_{CD36}$ IEs, and lowest for CSA binding IEs (FCR3$_{CSA}$) and placental isolates. A similar seroprevalence was found for IEs isolated from SM patients (MOZ1, MOZ2, MOZ4) or UM patients (MOZ3, MOZ5; Table 2). The number of IgM responders against MSP-1$_{19}$ and AMA-1 was lower in SM ($P = 0.038$ and $P = 0.024$, respectively), and a similar but not significant trend was found for DBLα (Figure 2A). Likewise, anti-MSP-1$_{19}$ and AMA-1 IgM levels were lower in SM compared to their UM pairs ($P = 0.050$ and $P = 0.047$, respectively). In general, different antibody responses correlated weakly (Additional file1), with the exception of IgG against EBA-175 and MSP-1$_{19}$ (Spearman's $rho = 0.8635$, $P < 0.001$) and IgM against EBA-175 and AMA-1 (Spearman's $rho = 0.8595$, $P < 0.001$).

A stratified analysis by SM clinical presentations showed that levels of IgG against DBLα were lower both for SA ($P = 0.031$), ARD ($P = 0.015$) and prostration ($P = 0.002$) compared to their respective matched controls (see Additional file2). Similar to the main analysis, no differences were found for other recombinant antigens or for IgG against IEs surface (data not shown). Levels of IgM were significantly lower in children with SA, ARD and prostration for at least one of the antigens

Table 1 Characteristics of the study population

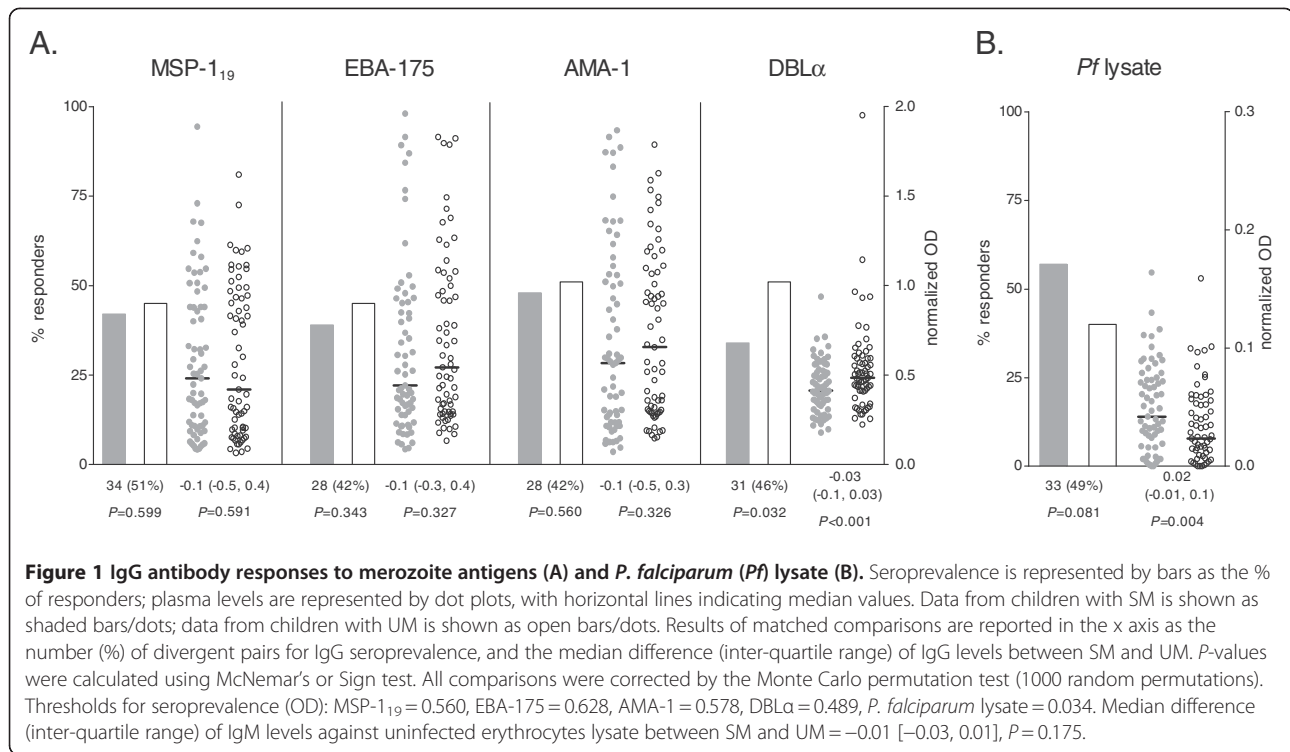| Parameter[a]	Clinical groups			
	SM (N = 67)	UM (N = 67)	Matched comparison[c]	P
Demographic data				
Age (months)	28 (16, 39)	29 (15, 38)	-	-
Males, n (%)	42 (63)	42 (63)	-	-
Physical findings				
Temperature (°C)	38.6 (37.9, 39.2)	38 (36.4, 39.5)	0.3 (−0.7, 2.2)	0.227
Weight (kg)	11.1 (8.9, 13)	10.9 (9.6, 12.8)	0 (−1.6, 1.2)	1.000
Malnutrition, n (%)	5 (8)	2 (3)	4 (6)	0.453
Oral candidiasis, n (%)	1 (1)	1 (1)	2 (3)	1.000
Hepatomegaly, n (%)	13 (19)	2 (3)	13 (19)	0.005
Splenomegaly, n (%)	35 (52)	12 (18)	35 (52)	<0.001
N° of days with symptoms[b]	1 (1, 3)	1 (1, 2)	0 (0, 1)	0.192
Laboratory parameters				
Parasitaemia by qPCR, (×10³/µL)	34.2 (7.5, 151.3)	13.3 (1.7, 51.6)	2.1 (−1.0, 21.8)	0.087
Parasitaemia by microscopy, (×10³/µL)	40.4 (17.3, 99.3)	34.7 (11.5, 71.3)	12.9 (−10.3, 58.3)	0.036
Multiplicity of infection	3 (2, 4)	3 (3, 4)	0 (−1, 2)	0.070
Packed cell volume (%)	26 (16, 31)	31 (28, 34)	−6 (−15, 0)	<0.001
Platelets (×10⁹/L)	117 (70, 185)	145 (92, 192)	−38 (−110, 43)	0.043
Glucose (mM)	5.8 (4.6, 7)	5.8 (5.3, 6.9)	−0.1 (−1.6, 1.2)	0.702
White blood cells (×10⁹/L)	10 (6.8, 12.7)	9.1 (7.2, 11.5)	0.7 (−2.8, 3.5)	0.110
Lymphocytes (%)	35.5 (24.7, 46)	40.6 (30.2, 50.8)	−7.6 (−19.0, 9.1)	0.008
Neutrophils (%)	56.9 (42.9, 67.1)	53.7 (36.9, 65.3)	5.8 (−5.1, 10.2)	0.043
Creatinin (U/L)	34 (30, 38)	34 (30, 39)	−1 (−6, 6)	0.470
Bilirubin (µM)	24 (14, 37)	13 (10, 21)	10 (−1, 23)	<0.001
ALT[c] (U/L)	31.5 (19, 39)	28 (1, 36)	4 (−11, 15)	0.376
Lactate (mM)	3.8 (2.3, 5.1)	2.3 (1.9, 3.2)	1.0 (−0.3, 2.4)	0.001
SM symptoms				
Prostration, n (%)	50 (75)			
ARD [c], n (%)	34 (51)			
Severe anaemia, n (%)	22 (33)			
Multiple seizures, n (%)	19 (28)			
Hypoglycaemia, n (%)	5 (8)			
Cerebral malaria, n (%)	3 (5)			
N° of SM symptoms, n (%):				
One	23 (34)			
Two	27 (40)			
≥ three	17 (25)			

[a] Presented as medians (inter-quartile range), except when indicated. qPCR, quantitative real-time polymerase chain reaction; ALT, alanine aminotransferase; ARD, acidosis and/or respiratory distress.
[b] Fever, cough or vomits.
[c] Matched comparison reports the median difference (inter-quartile range) between SM and UM values for continuous variables or the number (%) of divergent pairs for categorical variables. *P*-values calculated using McNemar´s or Sign test for matched pairs. All comparisons were corrected by the Monte Carlo permutation test (1000 random permutations).

tested. Immune responses in children with CM and hypoglycaemia could not be assessed separately due to the low prevalence of these groups (Table 1).

A linear regression model including all antibody responses showed that IgG against DBLα was the only antibody independently associated to lower *P.*

Figure 1 IgG antibody responses to merozoite antigens (A) and *P. falciparum* (*Pf*) lysate (B). Seroprevalence is represented by bars as the % of responders; plasma levels are represented by dot plots, with horizontal lines indicating median values. Data from children with SM is shown as shaded bars/dots; data from children with UM is shown as open bars/dots. Results of matched comparisons are reported in the x axis as the number (%) of divergent pairs for IgG seroprevalence, and the median difference (inter-quartile range) of IgG levels between SM and UM. *P*-values were calculated using McNemar's or Sign test. All comparisons were corrected by the Monte Carlo permutation test (1000 random permutations). Thresholds for seroprevalence (OD): MSP-1$_{19}$ = 0.560, EBA-175 = 0.628, AMA-1 = 0.578, DBLα = 0.489, *P. falciparum* lysate = 0.034. Median difference (inter-quartile range) of IgM levels against uninfected erythrocytes lysate between SM and UM = −0.01 [−0.03, 0.01], *P* = 0.175.

falciparum densities. The proportional reduction of parasite densities per a two-fold increment in IgG levels was 0.27 when both SM and UM children were included in the analysis ([95% confidence interval [CI] 0.08, 0.85], *P* = 0.026) and 0.11 including only SM cases ([95% confidence interval [CI] 0.02, 0.72], *P* = 0.022), but the effect was not observed among UM controls (1.34 [95% CI 0.28, 6.50], *P* = 0.712).

Cytokines and chemokines and malaria severity
Children with SM had significantly higher IL-6 concentrations in plasma than children with UM (*P* = 0.047,

Table 2 IgG antibody responses to infected erythrocytes surface antigens

Parasites[a]	Seroprevalence[b] (N = 67 pairs)				IgG levels[c] (N = 67 pairs)			
	SM	UM	Matched comparison	P	SM	UM	Matched comparison	P
R29	64 (96)	65 (97)	5 (7)	1.000	11.5 (8.7, 18.2)	9.9 (6.6, 20.2)	2.0 (−8.6, 9.1)	0.088
FCR3$_{CSA}$	8 (12)	11 (16)	15 (22)	0.608	7.6 (6.6, 9.3)	7.7 (6.7, 9.2)	−0.3 (−2.3, 1.6)	0.609
E8B$_{CD36}$	58 (87)	62 (93)	14 (21)	0.414	6.7 (4.2, 11.3)	6.9 (4.5, 10.7)	−0.8 (−4.3, 4.5)	0.636
ITG$_{ICAM1}$	11 (16)	15 (22)	24 (36)	0.300	1.1 (0.1, 2.1)	1.3 (0.2, 2.5)	−0.4 (−2.5, 1.6)	0.345
MOZ1	17 (25)	18 (27)	25 (37)	0.666	28.0 (23.6, 31.8)	26.9 (24.5, 33.9)	0.01 (−5.5, 4.9)	1.000
MOZ2	40 (60)	36 (54)	34 (51)	0.367	1.8 (0.8, 5.9)	1.6 (0.8, 4.8)	0.7 (−2.3, 3.8)	0.334
MOZ3	32 (48)	30 (45)	34 (51)	0.611	2.6 (1.6, 4.1)	2.4 (1.3, 4.9)	0.2 (−2.3, 2.3)	0.650
MOZ4	47 (70)	44 (66)	27 (40)	0.704	5.3 (−1.4, 24.9)	3.3 (−1.4, 13.1)	2.8 (−11.9, 19.1)	0.329
MOZ5	51 (76)	56 (83)	24 (36)	0.293	88.0 (53.1, 115.4)	83.4 (62.3, 129.9)	−7.4 (−45.7, 35.6)	0.727
PLC1	0 (0)	3 (4)	3 (4)	0.255	58.3 (53.2, 64.8)	60.7 (51.9, 67.5)	−1.1 (−8.7, 7.3)	0.547
PLC2	0 (0)	3 (4)	3 (4)	0.255	19.8 (12.3, 24.2)	18.6 (10.1, 24.5)	−0.7 (−8.2, 8.9)	0.907

[a] MOZ, isolates from Mozambican children; PLC, placental isolates from Mozambican women.
[b] Presented as the number (%) of responders. Matched comparison reports the number (%) of divergent pairs. *P*-values calculated using McNemar's test.
Thresholds for seroprevalence (MFI): R29 = 5.1, FCR3$_{CSA}$ = 10.1, E8B$_{CD36}$ = 2.6, ITG$_{ICAM}$ = 2.7, MOZ1 = 31.7, MOZ2 = 1.4, MOZ3 = 2.9, MOZ4 = 0.4, MOZ5 = 52.6, PLC1 = 107.6, PLC2 = 42.9.
[c] Presented as medians (inter-quartile range) of MFI values. Matched comparison reports the median difference (inter-quartile range) of IgG levels between SM and UM. *P*-values calculated using Sign test.
All comparisons were corrected by the Monte Carlo permutation test (1000 random permutations).
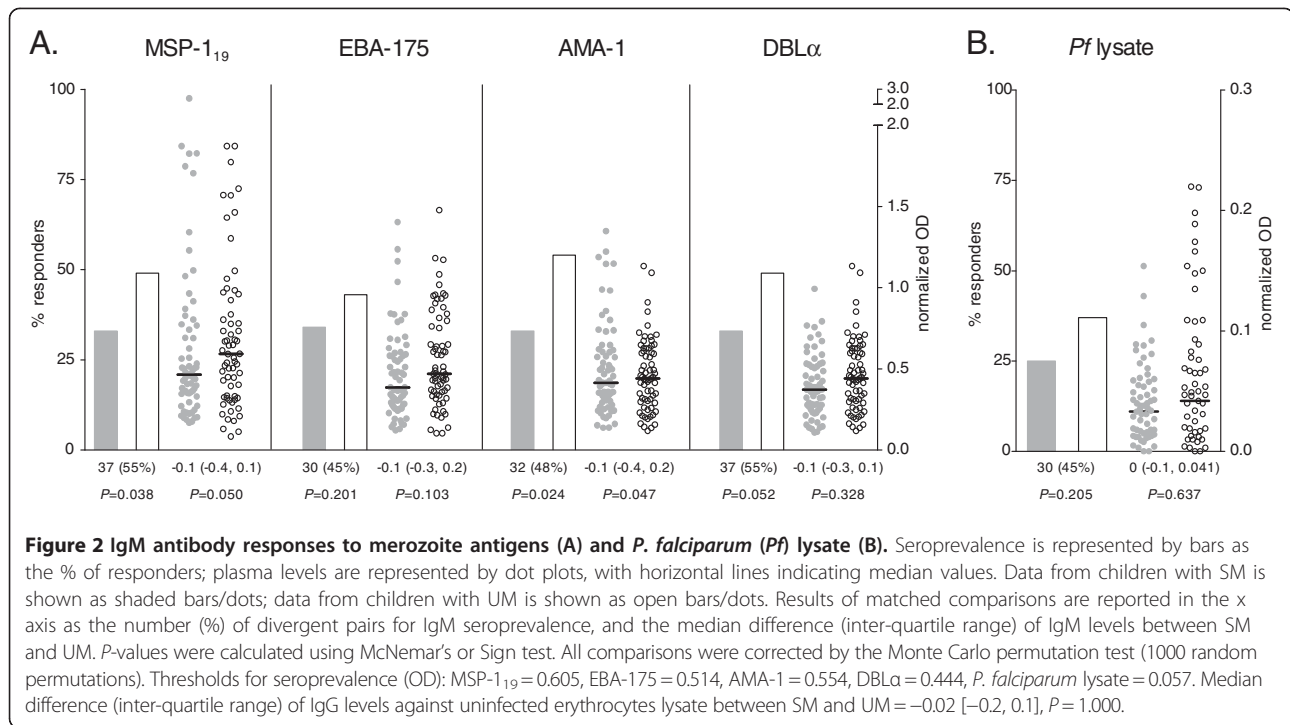
Figure 2 IgM antibody responses to merozoite antigens (A) and *P. falciparum* (*Pf*) lysate (B). Seroprevalence is represented by bars as the % of responders; plasma levels are represented by dot plots, with horizontal lines indicating median values. Data from children with SM is shown as shaded bars/dots; data from children with UM is shown as open bars/dots. Results of matched comparisons are reported in the x axis as the number (%) of divergent pairs for IgM seroprevalence, and the median difference (inter-quartile range) of IgM levels between SM and UM. *P*-values were calculated using McNemar's or Sign test. All comparisons were corrected by the Monte Carlo permutation test (1000 random permutations). Thresholds for seroprevalence (OD): MSP-1_{19} = 0.605, EBA-175 = 0.514, AMA-1 = 0.554, DBLα = 0.444, *P. falciparum* lysate = 0.057. Median difference (inter-quartile range) of IgG levels against uninfected erythrocytes lysate between SM and UM = −0.02 [−0.2, 0.1], *P* = 1.000.

Figure 3A). IL-1β was more frequently detected in children with SM ($P = 0.013$), although no significant differences were found for IL-1β concentrations. Conversely, children with SM had lower levels of TGF-β1 and IL-12 ($P < 0.001$ and $P = 0.039$, respectively; Figure 3B and 3C), and lower prevalence of detectable IL-12 concentrations than matched UM controls ($P = 0.011$; Figure 3C). IL-5 and TNF-β were excluded from the statistical analysis since levels in most samples were below the limit of detection (109/134 [81%] for IL-5 and 132/134 [99%] for TNF-β). Overall, there were weak or moderate correlations within cytokine responses (Additional file3), with the exception of IL-6 and IL-10 (Spearman's *rho* = 0.8682, $P < 0.001$). No significant correlations were found between antibodies and cytokines.

Stratification by severe clinical presentations showed that children with ARD had higher levels of IL-10 ($P = 0.032$) and IL-6 ($P = 0.002$) and lower levels of TGF-β1 ($P = 0.002$) compared to their controls (Additional file2). TGF-β1 concentrations were also reduced in the prostration group ($P = 0.007$).
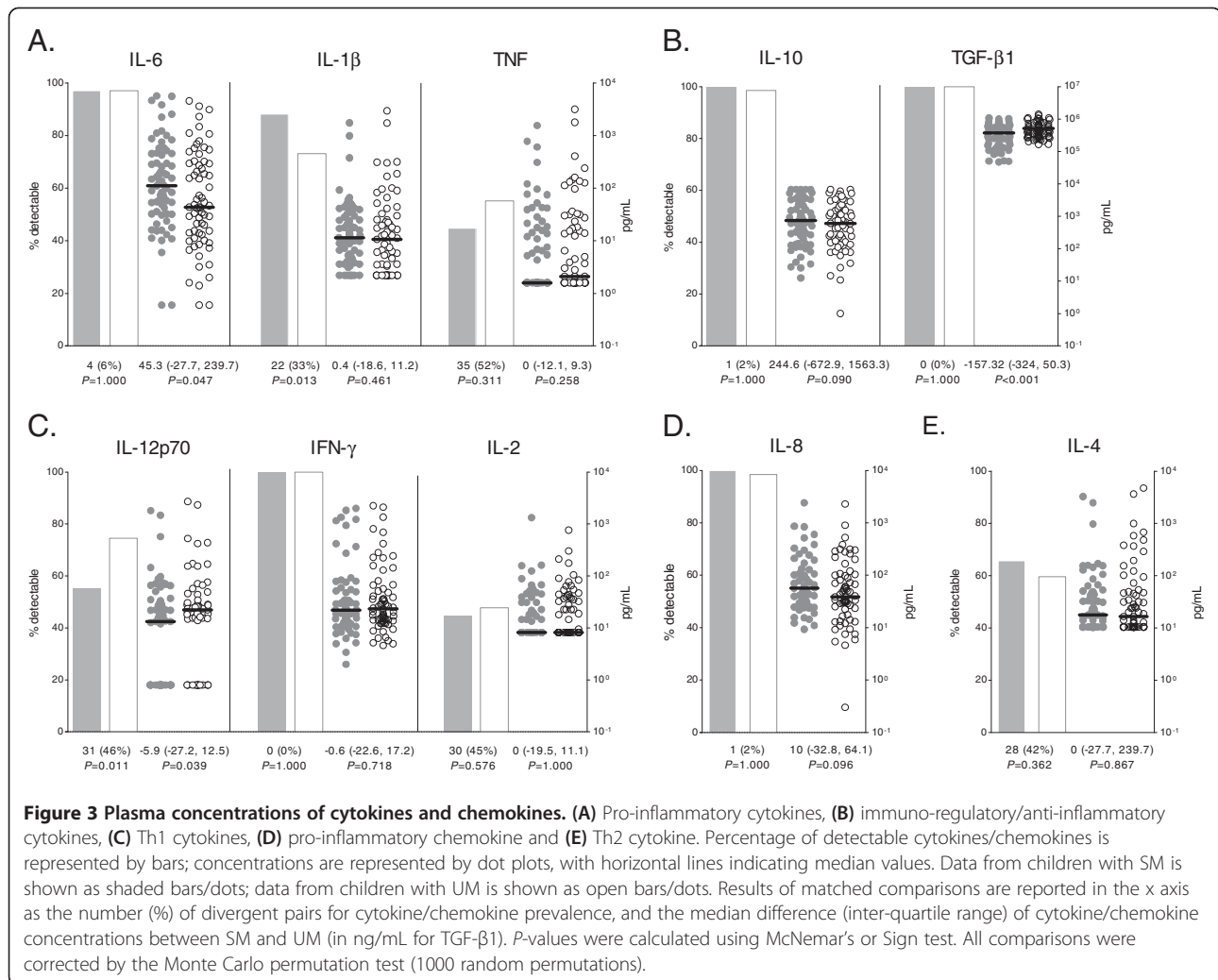
IL-12 concentrations were associated with a reduction of parasite densities when all children were included in the analysis (0.69 [95% CI 0.54, 0.90], $P = 0.006$) and including only children with SM (0.62 [95% CI 0.44, 0.89], $P = 0.010$), but the association was not found in children with UM (0.88 [95% CI 0.58, 1.33], $P = 0.528$). On the other hand, higher concentrations of IL-10 were associated with an increase in parasite densities in all groups (all children: 2.44 [95% CI 1.58, 3.77], $P < 0.001$; SM

cases: 2.59 [95% CI 1.37, 4.92], $P = 0.004$; UM controls: 2.62 [95% CI 1.38, 5.01], $P = 0.004$).

Discussion

Results of this case–control study show that SM in Mozambican children under five years of age is associated with low IgM responses to blood stage antigens and low IgG responses to a rosetting DBLα domain [33], as well as with high levels of pro-inflammatory IL-6 and low levels of TGF-β1 and IL-12. In contrast to previous reports, this study did not find differences in IgG responses against MSP-1_{19} [7], AMA-1 [40] or IEs surface antigens [10], possibly due to different definitions of SM clinical presentations and levels of endemicity in the study settings.

Although the observational approach of the study does not allow determining if lower levels of antibodies are the cause or the consequence of a severe infection, the fact that immunoglobulin reduction was not uniform strongly argues against a general impairment of immune response as a consequence of SM. The reduction of anti-DBLα IgG suggests that DBLα domains involved in rosetting are an important target of immunity against SM, both for presentations such as ARD, prostration and SA (and being the lack of association with MS probably attributable to low sample size). IgG antibodies to several DBLα domains have already been linked to natural acquisition of immunity [33,41] and to reduced risk of clinical malaria [42]. The finding is further supported by a negative association between IgG to DBLα and parasite

Figure 3 Plasma concentrations of cytokines and chemokines. (A) Pro-inflammatory cytokines, **(B)** immuno-regulatory/anti-inflammatory cytokines, **(C)** Th1 cytokines, **(D)** pro-inflammatory chemokine and **(E)** Th2 cytokine. Percentage of detectable cytokines/chemokines is represented by bars; concentrations are represented by dot plots, with horizontal lines indicating median values. Data from children with SM is shown as shaded bars/dots; data from children with UM is shown as open bars/dots. Results of matched comparisons are reported in the x axis as the number (%) of divergent pairs for cytokine/chemokine prevalence, and the median difference (inter-quartile range) of cytokine/chemokine concentrations between SM and UM (in ng/mL for TGF-β1). *P*-values were calculated using McNemar's or Sign test. All comparisons were corrected by the Monte Carlo permutation test (1000 random permutations).

densities. Antibodies against this *Pf*EMP1 domain may be able to reduce parasite densities through disruption of rosettes, a mechanism that has been associated with protection from SM [43]. Conversely, no difference was found for IgG against surface antigens of R29 IEs, the parasite from which DBLα was cloned [33], suggesting that other R29 *var1-Pf*EMP1 domains and other antigens on the surface of IEs may be masking epitope-specific DBLα responses. Despite the absence of differences between clinical groups, prevalence of recognition of R29 surface antigens was high. R29 predominantly expresses a group A *Pf*EMP1 [44], to which antibodies are rapidly acquired during early childhood [41,45]. On the other hand, no significant recognition of the pregnancy-specific CSA-binding parasites was detected, in contrast with a recent study that suggests recognition of VAR2CSA DBL5ε domain in young children [45].

This study also reports a reduction of IgM responses to blood stage antigens in children with SM compared to UM, in agreement with findings from previous studies

[12-14]. IgM levels have been associated with reduced risk of malaria in children and adolescents [46,47] and negatively correlated with parasite densities [46], suggesting that IgM are capable of controlling *Plasmodium* infection through early limitation of parasite growth [48] or through blocking of pro-inflammatory TNF production [49].

The differences in antibody levels between children with SM and UM observed in this study were not attributable to a lower exposure to *P. falciparum* in SM cases, since children with SM had similar levels of IgM and even higher IgG against total *P. falciparum* antigens compared to children with UM. Moreover, differences could not be explained by a longer progression of the disease in SM cases because the number of days with symptoms of malaria prior to enrolment was similar to that in the UM group. Importantly, a higher number of previous SM episodes were reported among cases. This observation may indicate that host genetic factors can predispose children to develop SM [50] or that children

in the SM group were subject to a higher *in utero* exposure to *P. falciparum* antigens, producing immunological tolerance and increasing susceptibility to subsequent malaria infections [51]. Also, an episode of SM may render children more susceptible to future severe episodes, maybe through an expansion of exhausted T cells or atypical B cells [52,53] which may impair the development of an adequate protective immune response.

SM was also found to be associated with alterations of the fine balance between pro-inflammatory and anti-inflammatory cytokines [22]. Children with SM showed higher levels of pro-inflammatory IL-6 [16,54], had more detectable levels of IL-1β and insufficient immuno-regulatory TGF-β1, which has been shown to counterbalance the pro-inflammatory response [22]. Although levels of immuno-regulatory IL-10 were significantly increased in children with ARD [55] this response did not seem to be sufficient to control the IL-6 imbalance. As previously reported by Lyke *et al.* in Mali [54], TNF did not differ between SM and UM in Mozambican children, an observation that is in contrast to other studies suggesting that TNF is the major cytokine involved in the pro-inflammatory response in SM [56,57]. It is possible that IL-6 and IL-10 levels reached by SM children at admission had already down regulated initial TNF pro-inflammatory response [19,20]. Children with SM were also characterized by lower levels of IL-12 [22,57], a cytokine previously associated with anti-malarial immunity [15,17,22]. Consistently, a negative association between IL-12 concentrations and parasite densities was found. Haemozoin phagocytosis by monocytes but also IL-10 may be acting as IL-12 inhibitors in SM [57,58]. Overall, this study suggests that children with SM may have an immature immune system typical of early childhood, consisting in a greater dependence on innate immune responses for protection against infections [59], lower Th1-polarizing cytokine responses (i.e. IL-12) [60,61] and a bias of Toll-Like receptor-mediated responses towards acute phase (i.e. IL-1β and IL-6) and IL-10 responses [60]. Therefore, an immune system still under development, together with an insufficient or impaired antibody response at this age, may limit the ability to contain infection, lead to an exacerbated inflammatory response and increase the risk of developing SM.

The present study is limited by a number of factors. First, antibodies and cytokines measured in peripheral blood at a single time point may not reflect the course of infection or the response in specific organs where parasite sequestration is occurring. Second, it was not possible to control for the effects that co-infection with HIV or helminthes may have on anti-malarial immunity [62,63]. Nevertheless, clinical data suggests that AIDS was infrequent in patients from the present study. Finally, the high degree of overlap in severe symptoms, which is otherwise common in endemic areas [64], together with the limited sample size of clinical groups, may have hampered the identification of immunological correlates that are particular to a clinical form of SM.

Conclusions

These data supports a role for specific IgM to *P. falciparum* and IgG to rosetting *Pf*EMP1 domains in controlling SM. Also, imbalanced pro-inflammatory cytokine responses may exacerbate the severity of infection in children. This information is of relevance to understand how immunity to SM is acquired in early childhood and also for the design of strategies to control life-threatening malaria. In particular, subunit vaccines containing DBLα epitopes from rosetting *Pf*EMP1 might potentially elicit functional antibodies that reduce the risk of SM in children. Prospective cohort studies assessing IgG isotypes and functionality of antibodies, together with detailed phenotype analysis of cytokine-producing cells will help to understand the mechanisms underlying natural acquisition of immunity to malaria severe disease.

Additional files

Additional file 1: Spearman's rank correlation coefficients between IgG and IgM levels in the study population. * $P < 0.05$.

Additional file 2: Matched comparisons of antibody levels and cytokine and chemokine concentrations in children with different severe malaria clinical presentations.

Additional file 3: Spearman's rank correlation coefficients between cytokine and chemokine concentrations in the study population. * $P < 0.05$.

Abbreviations

AMA: apical membrane antigen; ARD: acidosis and/or respiratory distress; CM: cerebral malaria; DBL: Duffy binding like; EBA: erythrocyte binding antigen; IEs: infected erythrocytes; IFN: interferon; IgG: immunoglobulin G; IgM: immunoglobulin M; IL: interleukin; MFI: mean fluorescence intensity; MOI: multiplicity of infection; MS: multiple seizures; MSP: merozoite surface protein; OD: optical density; *Pf*EMP: *Plasmodium falciparum* erythrocyte membrane protein; SA: severe anaemia; SM: severe malaria; TNF: tumor necrosis factor; TGF: transforming growth factor; UM: uncomplicated malaria.

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