

Cytokine and Antibody Responses to *Plasmodium falciparum* in Naïve Individuals during a First Malaria Episode: Effect of Age and Malaria Exposure

Gemma Moncunill^{1*}, Alfredo Mayor^{1,2}, Alfons Jiménez¹, Augusto Nhabomba², Laura Puyol¹, Maria N. Manaca², Diana Barrios¹, Pau Cisteró¹, Caterina Guinovart^{1,2}, Ruth Aguilar^{1,2}, Azucena Bardají¹, María-Jesús Pinazo¹, Evelina Angov³, Sheetij Dutta³, Chetan E. Chitnis⁴, José Muñoz¹, Joaquim Gascón¹, Carlota Dobaño^{1,2}

1 Barcelona Centre for International Health Research (CRESIB, Hospital Clínic-Universitat de Barcelona), Barcelona, Spain, **2** Centro de Investigação em Saúde de Manhiça, Maputo, Mozambique, **3** Walter Reed Army Institute of Research (WRAIR), Silver Spring, Maryland, United States of America, **4** International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India

Abstract

Age- and exposure-dependent immune responses during a malaria episode may be key to understanding the role of these factors in the acquisition of immunity to malaria. Plasma/serum samples collected from naïve Mozambican children (n = 48), European adults (naïve travelers, n = 22; expatriates with few prior malaria exposures, n = 15) and Mozambican adults with long-life malaria exposure (n = 99) during and after a malaria episode were analyzed for IgG against merozoite proteins by Luminex and against infected erythrocytes by flow cytometry. Cytokines and chemokines were analyzed in plasmas/sera by suspension array technology. No differences were detected between children and adults with a primary infection, with the exception of higher IgG levels against 3D7 MSP-1₄₂ (P = 0.030) and a *P. falciparum* isolate (P = 0.002), as well as higher IL-12 (P = 0.020) in children compared to other groups. Compared to malaria-exposed adults, children, travelers and expatriates had higher concentrations of IFN- γ (P \leq 0.0090), IL-2 (P \leq 0.0379) and IL-8 (P \leq 0.0233). Children also had higher IL-12 (P = 0.0001), IL-4 (P = 0.003), IL-1 β (P = 0.024) and TNF (P = 0.006) levels compared to malaria-exposed adults. Although IL-12 was elevated in children, overall the data do not support a role of age in immune responses to a first malaria episode. A T_H1/pro-inflammatory response was the hallmark of non-immune subjects.

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* E-mail: gemma.moncunill@cresib.cat

¶ These authors contributed equally to this work.

Introduction

The worldwide burden of disease from malaria remains a major public health problem, with approximately 216 million cases and 655,000 deaths in 2010, with the majority of deaths occurring in sub-Saharan Africa [1]. In areas where malaria is endemic, children together with pregnant women are the most susceptible populations. Children suffer more malaria episodes and are more prone to severe malaria compared to adults [2]. With increasing age, there is a shift from severe malaria to clinical malaria and eventually to asymptomatic parasitemia, but immunity is never sterilizing. Malaria exposure but also age may have an effect on the natural acquisition of immunity as the immune system of children is not totally mature [3,4]. After several decades of investigation in animal models and humans, the mechanisms involved in naturally acquired immunity and immunopathology

are still poorly defined, partly because it is difficult to separate the effects of age from those of repetitive exposure. There is no clear evidence for an effect of age at first exposure to *Plasmodium falciparum* in the development of immunity in African children [5,6], although previous studies in malaria-naïve migrants in Indonesia suggested that the more mature immune system from an adult may allow acquisition of immunity more rapidly than the less mature immune system from a child under the same conditions of exposure [7,8]. Totally naïve adults such as travelers or migrants never exposed to *P. falciparum* going to malaria-endemic areas are also more susceptible to clinical and severe malaria than adults from endemic areas [2,9,10]. Furthermore, it has been suggested that naïve adults are initially more susceptible to severe disease than children [2,11] and show different clinical presentations [12].

In this study, we aimed to determine (i) the effect of age on cytokine, chemokine and Immunoglobulin G (IgG) responses in

children and adults during the acute and convalescent phases of a first *P. falciparum* malaria episode and (ii) the effect of exposure on immune responses in adults during the acute and convalescent phases of a malaria episode. We hypothesized that the immune response of children during a first malaria episode would be quantitatively and qualitatively different from the immune response of naïve adults. Further, we hypothesized that during a malaria episode the immune response in naïve adults would be quantitatively and qualitatively different from immune responses in adults with different levels of prior exposure to *P. falciparum* infection.

Materials and Methods

Ethics Statement

Written informed consent was obtained from participants or their respective parents or guardians before sample collection. Parasitemic individuals were treated according to standard national guidelines at the time of the studies. Approval for the protocols was obtained from the National Mozambican Ethics Review Committee and the Hospital Clinic of Barcelona Ethics Review Committee.

Study design, patients and sample collection

All volunteers included in this study had an acute clinical malaria episode at the moment of recruitment and/or sample collection. There were four groups of patients: (i) naïve children from a malaria endemic area with a first episode (ii) naïve adults from a non-endemic area with a first episode (travelers) (iii) other non-immune adults temporarily resident in an endemic area (expatriates) and (iv) adults from an endemic area (malaria-exposed).

Children were recruited in the context of a study conducted at the Centro de Investigação em Saúde de Manhiça, Manhiça District, southern Mozambique, where transmission of *P. falciparum* is perennial, with some seasonality of moderate intensity. Forty eight children presenting a first malaria episode were chosen from a total of 287 followed up from birth to age 24 months in a three-arm randomized, double-blind, placebo-controlled trial with monthly chemoprophylaxis with sulfadoxine-pyrimethamine and artesunate between 2005 and 2009 [5]. Children had been followed up by a combination of active and passive case detection. Blood samples were collected into EDTA microtainers by finger-prick at acute episode (day 0) and at convalescence after treatment (day 28). Two blood smears and blood spots onto filter paper (Schleicher and Schuell; no. 903TM) were used to determine parasitemia by microscopy [5] and PCR, respectively.

Travelers and expatriates were European adults without previous malaria exposure (22 travelers) or who have lived in a malaria endemic area for a minimum of one year (15 expatriates). They were recruited at the Tropical Medicine Unit in the Hospital Clínic de Barcelona, Spain, between 2005 and 2009, and diagnosed with *P. falciparum* malaria by microscopy after traveling to an African country. Blood samples from acute episodes (day 0) and convalescence after malaria treatment (days 7 and 28) were collected by venipuncture into one heparin vacutainer for infected erythrocyte (IE) pellet cryopreservation in glycerolyte solution, and one vacutainer without anticoagulant for serum cryopreservation at -80°C . Two blood drops from each sample were spotted onto filter paper for parasite PCR analysis.

Malaria-exposed adults with life-long exposure to *P. falciparum* were non-pregnant women and men recruited from patients attending the Manhiça District Hospital, Mozambique, with clinical malaria between 2004 and 2005 [13]. Blood samples were collected by venipuncture into heparin tubes and plasma samples

were cryopreserved at -80°C . Two blood drops from each sample were spotted onto filter paper for parasite PCR detection.

Recombinant proteins

Apical membrane antigen 1 (AMA-1) from the 3D7 strain [14], the receptor-binding region F2 of the 175 kDa erythrocyte binding antigen from the CAMP strain (EBA-175; F2 region) [15], the Duffy binding-like alpha (DBL- α) domain of *PfEMP-1* [16] and the DBL3X domain of VAR2CSA *PfEMP-1* [17] were produced as recombinant proteins at ICGEB. AMA-1 from FVO strain and MSP-1₄₂ from 3D7 and FVO strains [18,19] were produced as recombinant proteins at WRAIR.

Antibody levels to recombinant proteins

A multiplex suspension array technology panel was established to quantify IgG responses to *P. falciparum* antigens using Luminex xMAP™ (Luminex Corp., Austin, Texas) and the Bio-Plex 100 platform (Bio-Rad, Hercules, California) [20]. Briefly, 3,000 microspheres per analyte were used per sample test. A pool from hyper-immune Mozambican adult volunteers at dilution 1/30,000 and samples from non-exposed European adults at dilution 1/1,000 were added in duplicates to each plate as positive and negative controls, respectively. Study samples were tested in duplicates at dilutions 1/1,000 and 1/30,000, but only 1/1,000 was chosen for the statistical analysis because of a wider quantitative dynamic range. In some wells no serum/plasma was added as a control of background. Beads were incubated with serum/plasma samples for 1 h. The plate was washed by pelleting microspheres (centrifuge at 800 *g* for 5 min) and resuspended with 0.05% Tween 20-PBS. One hundred μL of biotinylated anti-human IgG (Sigma, Tres Cantos, Spain) diluted 1/2,500 in assay buffer were applied and incubated for 45 min. The plate was washed as before, and 100 μL of streptavidin-conjugated R-phycoerythrin (Invitrogen, Carlsbad, California) diluted 1/1,000 in assay buffer were added and incubated for 25 min. All incubations were done at room temperature (RT) with plate agitation and protection from light. The plate was read using Bio-Plex Manager version 4.0, and at least 100 microspheres per analyte were acquired per sample. Mean fluorescent intensity (MFI) with background fluorescence subtracted was exported and arbitrary units (AU) concentration for each antigen was calculated by dividing MFI of each sample by the MFI of the positive control run in each plate.

Antibodies to IEs surface antigens

Two laboratory clones (CS2, R29), 3 isolates collected from travelers to endemic regions (IE_{Trav1} , IE_{Trav2} , IE_{Trav3}), 2 pediatric isolates from Mozambican children, one with uncomplicated malaria (IE_{Ch1}) and another with severe malaria (IE_{Ch2}) and 1 isolate from a pregnant Mozambican woman (IE_{Woman}), each of whom had the O blood group, were tested for recognition by IgG using flow cytometry. Study samples were tested blindly in a single assay against each parasite. A pool of plasma samples from immune Mozambican adults and a pool of plasma samples from non-exposed European adults were included as positive and negative controls, respectively. Cryopreserved ring-stage parasites were thawed in a sorbitol gradient and grown to late trophozoites. Cells were washed 3 times in PBS and resuspended at 1% hematocrit and 1–5% parasitemia in 1% BSA-PBS solution. Five μL of plasma were added to 95 μL /well of erythrocyte suspension, incubated for 30 min at RT 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 1/1,000

and 10 µg/mL of ethidium bromide (Ecogen) for 30 min in darkness at RT. Samples were washed 3 times with PBS-BSA between incubations. Data from 1,000 positive events was acquired with a Becton-Dickinson (BD) FACSCalibur flow cytometer. Reactivity against IE surface antigens was expressed as the difference between the MFI of IEs and the MFI of uninfected erythrocytes.

Cytokine/chemokine concentrations in plasma/serum

Concentrations (pg/mL) of interleukin (IL)-12p70, IL-2, IFN- γ , IL-4, IL-5, IL-10, IL-8, IL-6, IL-1 β , TNF and TNF- β in plasma and serum were measured using a commercial multiplex suspension array technology kit (Human Th1/Th2 11plex FlowCytomix kit, Bender MedSystems) and flow cytometry. Twenty-five µL of plasma or serum were tested following manufacturer's instructions. MFI from microspheres was acquired with a BD FACSCanto II and analyzed in FlowCytomix Pro2.2.1 software (Bender MedSystems). Concentration of each analyte was obtained by interpolating 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. TNF- β was excluded from the statistical analysis since levels in most samples (226/233, 97%) were below the limit of detection.

Statistical methods

Recognition of recombinant proteins and parasites by antibodies was considered positive if AU or MFI values were above the mean of the negative controls plus 2 standard deviations for each antigen. Threshold values for seroprevalence were: 238.02 AU for AMA-1 3D7; 1134.73 AU for AMA-1 FVO; 921.18 AU for MSP-1₄₂ 3D7; 638.33 AU for MSP-1₄₂ FVO; 3110.36 AU for EBA-175; 1572.55 AU for DBL- α ; and 217.44 AU for DBL3X. Comparisons between groups for categorical variables were done using χ^2 test or Fisher's exact test. Continuous variables were analyzed using the non-parametric Kruskal Wallis test or the Mann-Whitney U test. Correlations within groups were assessed by Spearman's rank coefficient. All *p*-values were two-sided and considered statistically significant when <0.05 . All data collected were analyzed using Stata version 11.0 (Stata Corporation, College Station, Texas, USA).

Results

Description of participants

Table 1 details the characteristics of the study subjects. There were significant differences in the median age between children, travelers, expatriates and malaria-exposed individuals and no significant differences in the gender distribution. Children with no documented prior malaria episodes and malaria-exposed adults were from malaria endemic areas of Mozambique, whereas travelers and expatriates were from non-malaria endemic countries. Travelers had never experienced malaria before, whereas expatriates had lived in malaria endemic areas and 12 out of 15 reported at least one previous malaria episode. Malaria-exposed adults were individuals living in *P. falciparum* malaria endemic regions presenting with an episode of clinical malaria. There were no differences in parasite densities between travelers and expatriates and between children and malaria-exposed adults. Some children were born to mothers with placental malaria infection defined as having malaria pigment or parasites in the placenta (10 out of 38 mothers, 10 unknown) and 3 children had cord blood that was PCR positive for *P. falciparum*.

IgG antibody responses during a first malaria episode

IgG antibody levels against recombinant proteins and *P. falciparum* IEs were measured in children and travelers presenting with a first malaria episode on the day of malaria diagnosis and at convalescence. Merozoite proteins AMA-1, MSP-1₄₂, EBA-175 were chosen because they are involved in essential parasite functions (erythrocyte invasion) and are targets of naturally acquired antibodies associated with malaria immunity and thus are leading vaccine candidates [21–25]. The R29 isolate was used because it forms rosettes, a mechanism involved in severe malaria, and that predominantly expresses a group A PfEMP-1 [13], to which antibodies are rapidly acquired in children [26]. DBL- α is a PfEMP-1 domain cloned from the R29 lab isolate that is involved in rosetting through adhesion to complement receptor 1 (R29var1 [27]). In addition, prior pilot studies with several laboratory parasite isolates in the Manhica area showed that R29 is the most immunogenic when measuring IgGs to variant surface antigens (VSA) (data not shown). The CS2 strain was included because it is a parasite isolate from pregnant women that binds to CSA, and an extensive and persistent recognition of VAR2CSA by plasma from children has been reported [28]. Finally, DBL3X was included because it is a VAR2CSA domain involved in CSA binding.

IgG antibody levels in plasma against recombinant proteins and IEs were not significantly different in children and travelers during a first malaria episode with the exception of MSP-1₄₂ 3D7 and IE_{Woman} isolate (Table 2). Children had higher levels of IgG against MSP-1₄₂ 3D7 and IE_{Woman} than travelers ($P=0.030$ and $P=0.002$, respectively). Similarly, there were no differences in antibody prevalences or in breadth of response (data not shown) with the exception of IgG prevalence against MSP-1₄₂ 3D7 that was higher in children than travelers ($P=0.027$) (Table 2).

At convalescence, there were no significant differences in IgG levels or prevalences to recombinant proteins (Table 3). Children born from mothers with placental malaria infection did not show any significant differences in antibody responses compared to those born from mothers without placental malaria infection (data not shown).

Cytokine and chemokine responses during a first malaria episode

Cytokine and chemokine concentrations in plasma or serum were measured in children and travelers with a first malaria acute episode and during convalescence. No differences were detected in any of the cytokines/chemokines tested with the exception of IL-12 (Figure 1A). In the acute phase, children had significantly higher levels of IL-12 than travelers (median {IQR} of 13.33 {0.75; 191.81} pg/mL and 0.75 {0.75; 17.98} pg/mL, respectively; $P=0.020$). During convalescence children had significantly higher levels of IL-10 (14.66 {6.09; 70.81} pg/mL) compared to travelers (0.95 {0.95; 0.95} pg/mL, $P=0.003$). The day 0/day 28 ratio was significantly higher for IL-12 in children compared to travelers (2.66 {0.89; 72.89} and 0.52 {0.03; 1.00}, respectively; $P=0.026$) and significantly lower for IL-10 (16.41 {4.6; 110.16} and 277.33 {100.77; 1406.74}, respectively; $P=0.015$) (Figure 1B).

IL-10 levels correlated moderately with parasite density in both groups whereas IL-8, IL-6 and IL-1 β concentrations correlated only in travelers (Table S2). There were no correlations between cytokine and antibody levels (data not shown). In children, no association was found between cytokine levels and being born from a mother with placental malaria (data not shown).

Table 1. Characteristics of the study participants.

Characteristics	Children	Travelers	Expatriates	Malaria-exposed	P-value
Day 0, n	48	22	15	99	
Day 7, n	0	12	10	0	
Day 28, n	28	6	4	0	
Age, median years (IQR)	1.1 (0.9; 1.4)	31 (28; 38)	53 (45; 60)	26 (19; 36)	<0.001 ^a
Male, n (%)	26 (54)	15 (75)	7 (58)	52 (53)	0.320 ^b
Origin, n (%)					
African	48 (100)	0 (0)	0 (0)	99 (100)	
European	0 (0)	17 (85)	15 (100)	0 (0)	
Others	0 (0)	3 (15)	0 (0)	0 (0)	
Previous malaria episodes, n (%)	0 (0)	0 (0)	12 (80)	99 (100) ^c	<0.001 ^b
Parasitemia by thin smear microscopy, %	ND	0.08 (0.01; 0.80)	0.06 (0.01; 0.19)	ND	0.888 ^d
Parasitemia by thick smear microscopy, parasites/ μ l ^e	26032 (9608; 45926)	ND	ND	35379 (14338; 61176)	0.091 ^d

Abbreviations: IQR, interquartile range; ND, not determined.

^aKruskal Wallis test.

^b χ^2 .

^cData presumed due to the long-lived exposure to malaria of malaria-exposed adults in the living area.

^dMann-Whitney test.

^eParasitemia data was available in 45 children, 20 travelers, 14 expatriates, 90 malaria-exposed adults.

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Table 2. Plasma IgG antibody levels and seroprevalences (number and % of responders) against recombinant proteins and IEs surface antigens in children and naïve adults in the acute phase of a first *P. falciparum* malaria episode.

Antigen	IgG levels (AU)				P-value ^a	Prevalences				P-value ^b
	Children n = 30		Travelers n = 20			Children n = 28		Travelers n = 20		
	Median	(IQR)	Median	(IQR)		n	%	n	%	
Proteins ^c										
AMA-1 3D7	758.74	(202.22; 8402.47)	287.84	(77.22; 1471.94)	0.075	19	68	9	45	0.144
AMA-1 FVO	1036.82	(477.63; 7260.11)	823.74	(293.71; 4171.51)	0.403	11	39	6	30	0.555
MSP-1 3D7	3369.76	(1578.10; 14002.25)	1172.45	(389.85; 8370.79)	0.030	23	82	10	50	0.027
MSP-1 FVO	1094.26	(301.89; 13641.75)	745.68	(147.21; 6985.65)	0.380	15	54	8	40	0.394
EBA-175	358.73	(21.30; 802.77)	344.32	(8.54; 663.42)	0.900	0	0	0	0	ND
DBL α	493.55	(220.83; 901.79)	849.98	(220.35; 1091.30)	0.452	4	14	3	15	1.000
DBL3X	114.06	(76.11; 309.35)	133.02	(50.62; 329.63)	0.786	8	29	6	30	1.000
IEs										
IE _{Trav1}	26.43	(22.87; 32.30)	25.3	(20.31; 33.22)	0.458	7	23	4	20	1.000
IE _{Trav2}	30.75	(26.69; 39.98)	32.56	(26.28; 43.48)	0.678	8	27	7	35	0.547
IE _{Trav3}	10.23	(8.00; 13.77)	9.23	(7.74; 11.70)	0.294	2	7	1	5	1.000
CS2	8.91	(8.09; 9.22)	9.62	(8.84; 10.46)	0.057	3	10	0	0	0.265
R29	13.65	(11.27; 15.93)	16.93	(12.81; 22.07)	0.083	6	20	7	35	0.327
IE _{Ch1}	3.21	(2.06; 5.12)	2.7	(1.89; 3.13)	0.148	3	10	1	5	0.641
IE _{Woman}	3.76	(3.32; 4.45)	3.16	(2.68; 3.44)	0.002	3	10	1	5	0.641
IE _{Ch2}	0.73	(0.47; 1.83)	0.62	(0.13; 1.18)	0.201	6	20	2	10	0.450

Abbreviations: AU, Arbitrary units; IQR, interquartile range; ND, not determined; IEs, infected erythrocytes.

^aMann-Whitney test.

^bFisher's exact test.

^cDeterminations were done in children n = 29.

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Table 3. Plasma IgG levels and seroprevalences in children and travelers in convalescence (day 28) after a first episode of *P. falciparum* malaria.

Antigens	IgG levels (AU)				<i>P</i> -value ^a	Prevalences				
	Children n = 28		Travelers n = 6			Children n = 28		Travelers n = 6		<i>P</i> -value ^b
	Median	(IQR)	Median	(IQR)		n	%	n	%	
Proteins										
AMA-1 3D7	12459.59	(2442.78; 25005.45)	7211.44	(516.28; 11636.50)	0.5571	25	89	6	100	1.000
AMA-1 FVO	13248.19	(1622.11; 23681.75)	12544.35	(7112.02; 21767.80)	0.7861	21	75	5	83	1.000
MSP-1 3D7	21629.75	(17970.65; 30033.10)	21027.6	(16816.20; 22336.90)	0.4981	27	96	6	100	1.000
MSP-1 FVO	24619.3	(17512.80; 33060.50)	26869.15	(20308.50; 30278.10)	0.6191	25	89	6	100	1.000
EBA-175	595.58	(38.64; 1251.93)	753.07	(290.80; 1070.08)	0.7181	3	11	6	100	1.000
DBL α	410.65	(313.82; 993.56)	821.74	(271.98; 1430.16)	0.4421	3	11	5	83	1.000
DBL3X	137.11	(82.10; 656.53)	94.51	(74.86; 131.44)	0.3201	9	32	0	0	0.559

Abbreviations: AU, Arbitrary units; IQR, interquartile range.

^aMann-Whitney test.

^bFisher's exact test.

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IgG antibody responses in differently malaria-exposed individuals

To study how exposure to malaria is affecting the IgG antibody responses to different antigens, we compared IgG levels (Table 4) and prevalences (Table S1) in children, travelers and expatriates versus life-long malaria-exposed adults. Individuals with a primo-infection (children and travelers) had significantly lower levels of IgG than malaria-exposed adults ($P < 0.001$) regardless of the antigen. Expatriates with previous few exposures to malaria still had significantly lower levels of antibodies compared to life-long malaria-exposed individuals although the levels were higher than naïve subjects with the exceptions of IgG to MSP-1₄₂ FVO, EBA-175 and DBL- α . Breadth of response was also significantly lower in primo-infected individuals or expatriates compared to malaria-exposed adults (data not shown).

Cytokine and chemokine responses in differently malaria-exposed individuals

Cytokine measurements in individuals with an acute episode showed that children, travelers and expatriates had significantly higher levels of IFN- γ than malaria-exposed individuals (median {IQR} of 163.85 {51.33; 564.16}, 584.54 {77.17; 1446.56}, 44.03 {13.7; 199.72}, and 10.65 {0.8; 29} pg/mL, respectively; $P < 0.0001$, $P < 0.0001$, $P = 0.006$, respectively) (Figure 2). In addition, significantly higher levels of IL-2 were detected in children (8.2 {8.2; 54.93} pg/mL), travelers (17.03 {8.2; 27.42} pg/mL) and expatriates (15.77 {8.2; 19.7} pg/mL) compared to malaria-exposed adults (8.2 {8.2; 8.2} pg/mL; $P = 0.0379$, $P = 0.0003$, $P = 0.0144$, respectively). Children, travelers and expatriates also had significantly higher concentrations of IL-8 (41.97 {30.02; 55.7}, 52.2 {32.72; 114.69} and 95.31 {31.86; 126.26} pg/mL, respectively) in comparison with malaria-exposed adults (32.2 {14.69; 51.61}; $P = 0.0233$, $P = 0.0029$, $P = 0.0035$, respectively). Compared to malaria-exposed adults, children also had higher levels of IL-12 (13.33 {0.75; 191.81} vs. 0.75 {0.75; 17.98} pg/mL; $P = 0.0001$), IL-4 (10.94 {10.4; 333.6} vs. 10.4 {10.4; 14.99} pg/mL; $P = 0.003$), IL-1 β (2.1 {2.1; 132.18} vs. 2.1 {2.1; 7.3} pg/mL; $P = 0.024$) and TNF (1.6 {1.6; 56.97} vs. 1.6 {1.6; 1.6} pg/mL; $P = 0.006$).

In addition to the correlations between cytokines and parasitemia in children and travelers described above, IFN- γ , IL-4 and IL-1 β correlated significantly with parasitemia only in the expatriates group (Table S2). None of the cytokines/chemokines tested correlated significantly with parasitemia in malaria-exposed adults.

No correlations between cytokines and antibodies were observed in any study group (data not shown).

Discussion

Results comparing naïve adults and children indicate that age has no prominent role in the immune responses induced during a first malaria episode. Differences were found only in IgG responses to MSP-1₄₂ 3D7 and to a parasite from a pregnant woman, and in IL-12 concentrations in serum/plasma. All three responses were higher in children. In addition, at convalescence children had higher levels of IL-10 and there were no differences in antibody levels or seroprevalences, although for travelers only 6 samples out of 22 were available at day 28 of convalescence.

IL-12 has been associated with protection against malaria [29,30] and severe disease [31,32]. IL-12 is a T_H1 cytokine produced mainly by dendritic cells (DC) in response to Toll-Like-Receptor (TLR) stimulation. It induces and regulates DC maturation and function and promotes IFN- γ production by T cells and NK cells as well as their activation [33,34]. Here increased levels of IL-12 indicate a higher innate immune response in younger children, probably due to an age-differential response to TLR ligands [4]. This could contribute to the lower risk of severe complications and different clinical presentations described in naïve adults compared to children [2,12].

At convalescence, children had higher levels of the anti-inflammatory cytokine IL-10 than naïve adults. Therefore, the total change in magnitude of this cytokine in acute vs. convalescence was significantly lower in children compared to adults, suggesting different capacity of regulating the pro-inflammatory response. The observation that children had higher levels of TNF and IL-1 β in acute phase compared to life-long malaria-exposed adults also suggests a different ability to regulate pro-inflammatory and anti-inflammatory responses according to age and/or exposure. Nevertheless, most children were *P.*

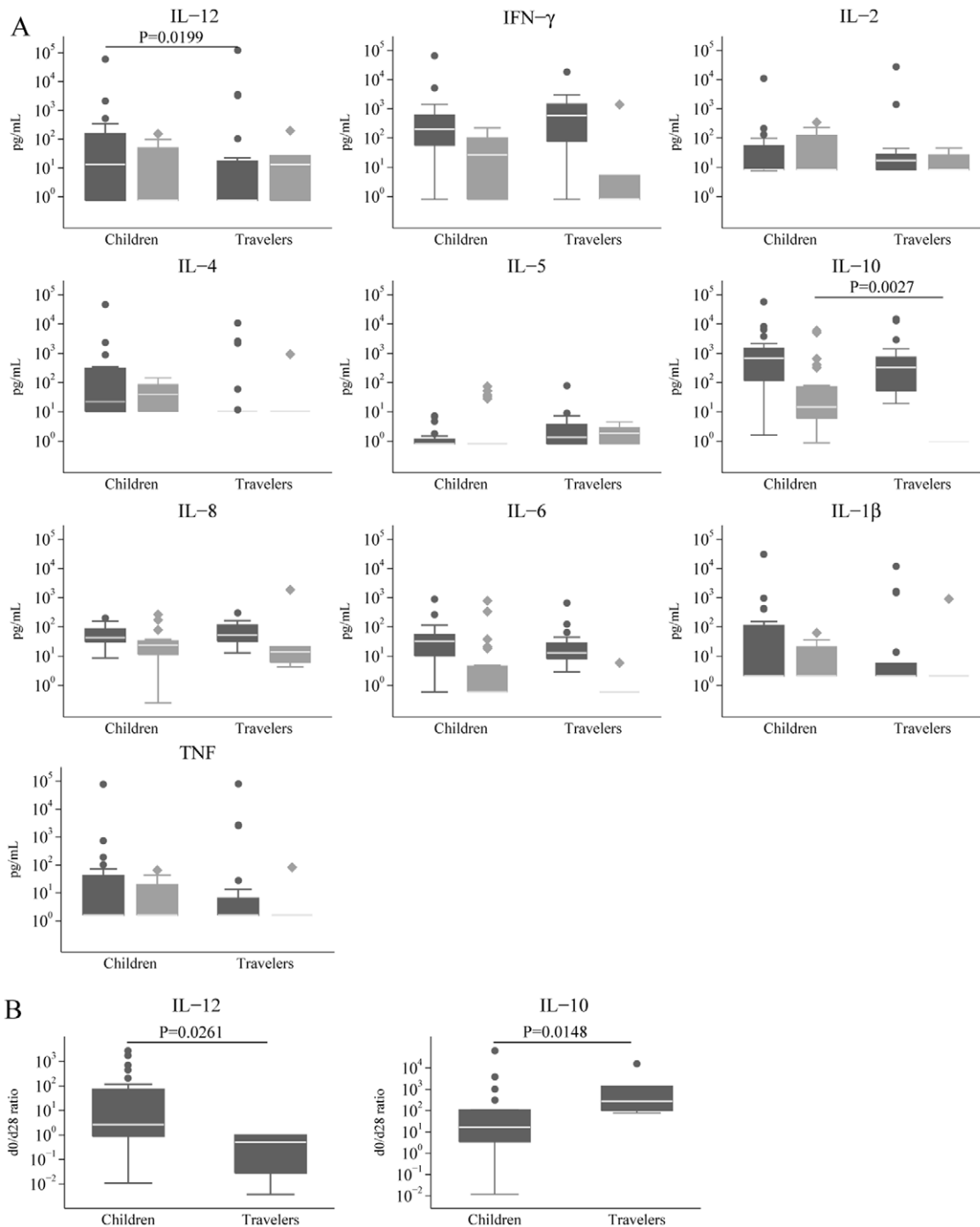


Figure 1. Plasma/serum cytokine levels in children and adults during a first malaria episode. A) Plasma/serum cytokine/chemokine concentrations in the acute phase (dark grey) and convalescence (light grey) were compared between children and adults using the Mann-Whitney test. B) Significantly different day 0/day 28 ratio cytokine levels in children and adults. doi:10.1371/journal.pone.0055756.g001

falciparum PCR positive at day 28 (data not shown), although they were treated with anti-malarial drugs and they did not have fever or microscopic parasitemia. Conversely, none of the adults were PCR positive at convalescence. It is possible that the higher IL-10 levels at convalescence were reflecting a response to submicroscopic parasitemia from uncleared/recrudescing or new infections. However, the most plausible explanation is that PCR positives

were due to residual parasite DNA in dead IEs remaining in circulation [35].

Comparisons between individuals with different levels of exposure to *P. falciparum* allowed us to identify IFN- γ , IL-2 and IL-8 as cytokines/chemokines involved in cellular immune responses to *P. falciparum*. As expected, increasing exposure showed to be associated to an increase of antibody levels against any antigen, but in this study we were unable to evaluate its role in

Table 4. Plasma IgG levels in an acute episode of differently exposed individuals.

Antigens	IgG levels, median AU (IQR)				P-value ^a		
	Children n = 30	Travelers n = 20	Expatriates n = 14	Malaria-exposed n = 50	Child-MalExp	Trav-MalExp	Expat-MalExp
Proteins ^b							
AMA-1 3D7	758.74 (202.22; 8402.47)	287.84 (77.22; 1471.94)	12128 (565.27; 22844.40)	24455.2 (19811.30; 27728.20)	<0.001	<0.001	0.001
AMA-1 FVO	1036.82 (477.63; 7260.11)	823.74 (293.71; 4171.51)	16090 (154.64; 25124.90)	23957.35 (19613.80; 26059.20)	<0.001	<0.001	0.020
MSP-1 3D7	3369.76 (1578.10; 14002.25)	1172.45 (389.85; 8370.79)	24565.2 (12456.30; 29953.00)	21651.4 (15681.70; 23614.60)	<0.001	<0.001	0.057
MSP-1 FVO	1094.26 (301.89; 13641.75)	745.68 (147.21; 6985.65)	25396.85 (10079.10; 28122.50)	22546.95(9320.43; 24863.10)	<0.001	<0.001	0.242
EBA-175	358.73 (21.30; 802.77)	344.32 (8.54; 663.42)	1220.22 (776.30; 6980.53)	4214.73 (837.81; 22594.80)	<0.001	<0.001	0.284
DBL α	493.55 (220.83; 901.79)	849.98 (220.35; 1091.30)	665.61 (409.81; 1980.22)	1353.96 (789.37; 2606.57)	<0.001	0.019	0.163
DBL3X	114.06 (76.11; 309.35)	133.02 (50.62; 329.63)	113.99 (52.13; 281.91)	306.06 (210.63; 1042.82)	<0.001	0.002	0.004
IEs ^c							
IE _{Trav1}	26.43 (22.87; 32.30)	25.3 (20.31; 33.22)	33.62 (21.40; 40.94)	207.26 (121.95; 396.35)	<0.001	<0.001	<0.001
IE _{Trav2}	30.75 (26.69; 39.98)	32.56 (26.28; 43.48)	36.86 (26.47; 44.68)	251.63 (135.58; 437.20)	<0.001	<0.001	<0.001
IE _{Trav3}	10.23 (8.00; 13.77)	9.23 (7.74; 11.70)	19.74 (7.04; 21.27)	169.19 (71.57; 286.32)	<0.001	<0.001	<0.001
CS2	8.91 (8.09; 9.22)	9.62 (8.84; 10.46)	9.63 (8.65; 10.16)	14.03 (11.63; 27.38)	<0.001	<0.001	0.006
R29	13.65 (11.27; 15.93)	16.93 (12.81; 22.07)	15.36 (15.08; 35.63)	214.29 (87.23; 393.92)	<0.001	<0.001	0.001
IE _{Ch1}	3.21 (2.06; 5.12)	2.7 (1.89; 3.13)	3.85 (3.39; 7.44)	63.28 (30.99; 123.09)	<0.001	<0.001	<0.001
IE _{Woman}	3.76 (3.32; 4.45)	3.16 (2.68; 3.44)	3.73 (2.82; 5.15)	21.43 (11.92; 46.10)	<0.001	<0.001	0.001
IE _{Ch2}	0.73 (0.47; 1.83)	0.62 (0.13; 1.18)	1.34 (-0.14; 1.49)	41.69 (22.40; 81.08)	<0.001	<0.001	<0.001

Abbreviations: AU, Arbitrary units; IQR, Interquartile range; MalExp, Malaria-Exposed; Trav, Travelers; Expat, Expatriates; IEs, Infected erythrocytes.

^aThe Mann-Whitney test was used.

^bDeterminations were done in children n = 28.

^cDeterminations were done in expatriates n = 5.

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immunity. Compared to antibodies, the more moderate cytokine responses may reflect naturally acquired immunity that allows a better control of pro-inflammatory responses rather than mere exposure. Although IFN- γ has been associated with malaria protection [29,36] and IL-2 may be key for the generation of effector responses to malaria [36,37], the strong T_H1 and inflammatory responses detected reflect innate rather than protective adaptive immune responses. Moreover, levels of IL-1 β and TNF were higher in children compared to malaria-exposed adults during acute infection despite having a trend to lower parasite density than malaria-exposed adults mounting a higher antibody response to infection. This reinforces the idea that naïve individuals have stronger pro-inflammatory responses that would be independent from the parasite biomass and may be key factors to the prognosis of clinical episodes. Children also presented significantly higher IL-4 levels compared to malaria-exposed adults. It has been described that IL-4 promotes IL-12 DC secretion [38], which is in line with the higher IL-12 levels found in children compared to adults. The higher T_H1/pro-inflamma-

tory responses observed in naïve individuals were not caused by higher parasite densities. Although we could not compare parasitemias between all groups because of different methods used to assess parasite density, there were no significant differences between children and malaria-exposed adults or between travelers and expatriates. Additionally, correlations between cytokines and parasitemia showed that cytokines differently expressed in naïve versus malaria-exposed individuals (IL-12, IFN- γ , IL-2, IL-1 β and TNF) did not show to be associated with parasitemia with the exception of IL-8 in naïve adults. These results suggest that these cytokines are not directly affected by parasitemia and the differences observed in cytokine concentrations may not be a direct effect of differences in parasite densities among the groups. Moreover, the fact that the correlations between cytokines and parasite densities were different among groups might be a reflection of different mechanisms of cytokine regulation depending on age and immunity.

Hence, our data may be reflecting development of tolerance, a recognized mechanism of acquisition of immunity to *P. falciparum*

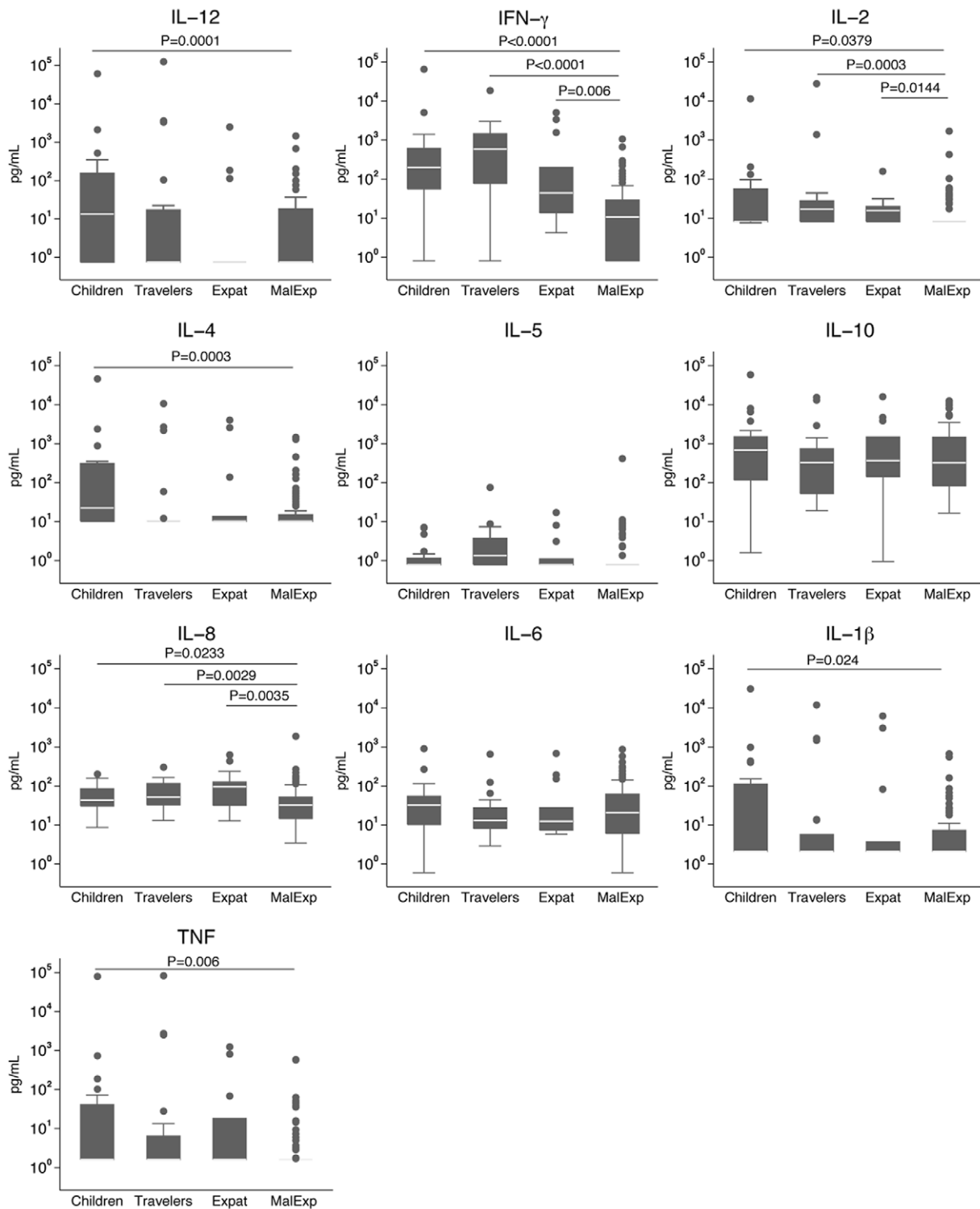


Figure 2. Plasma/serum cytokine/chemokine concentrations (pg/mL) in the acute phase of differently exposed subjects. Mann-Whitney test was used to compare children, travelers and expatriates (Expat) to malaria-exposed adults (MalExp). doi:10.1371/journal.pone.0055756.g002

[39]. Tolerance has been described to be associated with less expression of co-stimulatory molecules, less IL-6, IL-12, TNF and IFN- γ (pro-inflammatory cytokines) and an attenuation of cell signaling. Mechanisms may include TLR tolerance due to

repeated antigen stimulation [40,41], tolerogenic DC [42,43], regulatory T cells [44,45] and exhausted T cells [46].

Antibody seroprevalences in children and naïve adults were quite elevated with the exception of EBA-175, considering that IgG levels were determined at the acute phase of a first malaria

episode. Study children were from a malaria endemic area and it is plausible that they might have been previously exposed to *P. falciparum* infection (i.e. after birth or in utero [47]). However, they were followed up closely by active and passive morbidity surveillance and we are quite confident that we captured the first malaria episode. In addition, cytokine and antibody responses did not show any significant association with in utero exposure through maternal malaria infection (placental infection or cord infection). In fact, IgG responses to most antigens, except those described above, were not higher in children than adults. Regarding IgG responses to the pregnant woman parasite isolate, it could be due to passively transferred maternal antibodies against CSA-binding parasites, however it is unlikely that these children still had antibodies from the mother because they had a median age of 1.1 years with the youngest one being 10.8 months old. IgG detected in seropositive children and travelers are probably reflecting the beginning of the antibody response to blood-stage antigens even if few days had passed since the release of merozoites from the liver [48]. Antibody responses were quite low and both IgG levels and seroprevalences increased considerably at convalescence, and were consistently lower than those of expatriates or life-long malaria-exposed adults. In addition, several days may have passed before traveler patients attended the Tropical Medicine Unit.

Overall, antibody results are consistent with previous data of acquired immunity in which there are two levels of immunity, one strain-specific that is slowly acquired by consecutive exposures to polymorphic antigens and another transcending antigenic diversity [8]. In travelers, a single *P. falciparum* infection can be sufficient to induce antibodies reactive with several PfEMP-1 variants that are already detected at initial presentation to the hospital [48]. Also a quite extensive breadth of agglutination specificities was reported in migrants from a non-endemic to an endemic zone considering a relatively short time of exposure [49]. However, the degree of cross-reactivity among strains seems to correlate with age [8]. In our study, no differences in median breadth of response were detected between children and naïve adults against recombinant proteins or VSAs, which had a very low breadth response. In contrast, expatriates were responding to more antigens and malaria-exposed adults showed a significantly higher breadth of response compared to less exposed groups. The fact that travelers were exposed to geographically distinct strains of *P. falciparum* seems not to affect the antibody response to the tested antigens and VSAs from Mozambican strains as they responded similarly to children from Mozambique. Similar observations were reported in people with limited histories of exposure who showed broad agglutination activity against geographically diverse isolates [50] or in a study where IgG VSA recognition was independent of the geographical origin of parasites [51].

IgG seroprevalence to DBL- α in children was about 15%, lower than what has been described in Manhiça children [32], although in that study children were older and some have had previous malaria episodes. Antibodies against DBL- α have been linked to natural acquisition of immunity [26], to reduced risk of clinical malaria [52], and have been associated with protection from severe malaria [32]. The prevalence of antibodies against DBL3X was about 30% in children, which is similar to a recent study that

suggested recognition of the VAR2CSA DBL5 ϵ domain in young children [28]. Therefore our results contribute to the hypothesis that exposure to VAR2CSA-expressing parasites is probably common throughout early childhood [53]. Nevertheless, the recognition of pregnancy-specific CSA-binding parasites was about 10% in children and less in travelers.

To our knowledge only antibodies against MSP-2 3D7, RESA and GPI have been correlated with age independently of exposure [49,54]. Our study is limited by a number of factors that may mask the detection of other age-dependent differences. For instance, differences due to the African vs. European origin of the comparison groups may confound the results. IgM, IgG isotype levels and antibody affinity need to be determined in follow up studies. IgM responses are relevant in a first malaria episode and the protective effect of IgG isotypes has been attributed to the cytophilic subclasses IgG1 and IgG3 [22,55,56]. In addition, affinity seems to increase with age and is associated with protection [57].

In conclusion, age appeared not to be a significant factor in the immune response to a first malaria episode, particularly for the antibody responses examined. However, some observations such as higher levels of IL-12 or higher pro-inflammatory responses in children may indicate that some cellular age-dependent responses have remained undetected in this study. Exposure to *P. falciparum* was clearly associated with higher levels of IgG antibodies against merozoite antigens and IEs, although we could not dissect their role in immunity. Exposure to malaria and consequently, naturally acquired immunity, was associated with lower levels of T_H1 and pro-inflammatory cytokines, indicating a more regulated and balanced innate response related to mechanisms of tolerance.

Supporting Information

Table S1 Seroprevalence (number and % of responders) of IgG antibody responses in a malaria acute episode against recombinant proteins and IEs surface antigens in differently exposed individuals.

(PDF)

Table S2 Spearman correlations between cytokines/chemokines and parasitemia.

(PDF)

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Author Contributions

Produced the recombinant proteins: EA SD CEC. Designed and conducted the field trial: CG. Performed clinical diagnosis, management of malaria patients and collected clinical data: CG MJP JM JG AB. Coordinated data compilation and performed blood sample processing: RA LP DB PC MNM AN. Conceived and designed the experiments: CD JG AM GM. Performed the experiments: GM DB AJ. Analyzed the data: GM AM CD. Wrote the paper: GM AM CD.

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