CHEMOKINE LEVELS AND PARASITE- AND ALLERGEN-SPECIFIC ANTIBODY RESPONSES IN CHILDREN AND ADULTS WITH SEVERE OR UNCOMPLICATED *PLASMODIUM FALCIPARUM* MALARIA

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Chemokine and antibody response profiles were investigated in children and adults with severe or uncomplicated *Plasmodium falciparum* malaria; the aim was to reveal which profiles are associated with severe disease, as often seen in nonimmune children, or with mild and uncomplicated disease, as seen in semi-immune adults. Blood samples were obtained from children under 5 years of age as well as adults with falciparum malaria. Classification of malaria was performed according to parasite densities and hemoglobin concentrations. Plasma levels of chemokines (IL-8, IP-10, MCP-4, TARC, PARC, MIP-1δ, eotaxins) were quantified, and antibody responses (IgE, IgG1, and IgG4) to *P. falciparum*, *Entamoeba histolytica*-specific antigen, and mite allergen extracts were determined. In children with severe malaria proinflammatory, IL-8, IP10, MIP-1δ, and LARC were at highly elevated levels, suggesting an association with severe disease. In contrast, the Th2-type chemokines TARC, PARC, and eotaxin-2 attained in children the same levels as in adults suggesting the evolution of immune regulatory components. In children with severe malaria, an elevated IgG1 and IgE reactivity to mite allergens and intestinal protozoan parasites was observed. In conclusion, exacerbated proinflammatory chemokines together with IgE responses to mite allergens or *E. histolytica*-specific antigen extract were observed in children with severe falciparum malaria.

Keywords: *Plasmodium falciparum,* malaria, children, chemokine, antibody

Introduction

Induction, development, and maintenance of protective immunity against *Plasmodium falciparum* infection will start with the first parasite encounter; immune sensitization to *Plasmodium*-derived antigens may already occur prenatally and will, in any event, continue in children shortly after birth [1–3]. During the first months of life, maternal antibodies may mediate partial protection against *P. falciparum* infection [4, 5], while naturally acquired protective immunity against malaria is slow to develop, remains incomplete, and requires repeated exposure to infection which will generate memory B cells, which produce specific IgG subclasses able to neutralize the blood developmental stages of the parasite [6–10]. The secretion of cytokines and chemokines by T cells, monocytes, and NK cells is another essential prerequisite for the regulation of cellular effector mechanisms against *P. falciparum*

blood-stage parasites [11], but inflammatory cytokines and chemokines may also exacerbate disease manifestation and organ-specific pathogenesis [12].

Activation of Th1 cytokines, including IL-12 and the early production of gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) by T lymphocytes and natural killer (NK) are known to support resistance to infection and host survival [13]. Prominent Th2 response profiles have been reported in patients suffering from severe malaria, with an early IL-10 response being associated with higher susceptibility to infection; however, IL-10 will also exert anti-inflammatory effects, thus, limiting pathogenic and excessive Th1 cytokine responses [14, 15]. With repeated exposure to *Plasmodium* spp. and immune maturation, adequate and regulatory cytokine and chemokine responses may set off cellular effector mechanisms which will not induce severe inflammation and which will avoid excessive host tissue and organ damage [16]. Recent stud-

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ies have shown that, in children with *P. falciparum* malaria, the profile of cytokine and chemokine levels varied with disease severity; moreover, the proinflammatory chemokines MIP-1α/CCL3, MIP-1β/CCL4, and MIG/CXCL9 and cytokines IL-31 and IL-33 were elevated, while RANTES/CCL5 and IL-27 appeared suppressed in children with severe falciparum malaria [17, 18]. Heightened chemokine levels may contribute to neuropathology and, potentially, predict mortality in children with cerebral malaria [19]; however, with repeated exposure to *P. falciparum*, an immune response repertoire should evolve enabling better parasite control [11, 12]. Investigation and comparison of the chemokine responses in children and adults with severe or uncomplicated falciparum malaria have the potential to elucidate which response profiles are associated with progression to severe disease (as seen in nonimmune children) or with the capability to control infection (as seen in semi-immune adults).

In addition, with repeated exposure to *Plasmodium* spp., an immune response repertoire, chiefly consisting of cytophilic IgG1 and IgG3 subclasses, but also IgE, will progressively evolve in children, having the capacity to control and reduce parasite multiplication [9, 20–22]. Such responses may gradually approach, and come to resemble, the immune reactivity profiles observed in adults, where antibodies specific against multiple *P. falciparum* antigens mediate partial parasite control [23].

In human *P. falciparum* malaria, elevated IgE may be instrumental in the pathogenesis of this disease. The initial finding indicated that IgE elevation was most pronounced in sera from patients with cerebral malaria [22]. Later observations suggested that IgE may be considered a pathogenic factor in severe disease without cerebral involvement and, probably, in *P. falciparum* malaria in general [24]. In addition, allergic-type inflammatory mechanisms were suggested as enhancing the pathogenesis of severe forms of malaria disease [25]. Inflammatory responses may arise with the production of IgE against environmental allergens and parasitic worms; effector cells such as mast cells and granulocytes which express Fcε receptors may mediate this hyper-reactivity, but parasite-specific IgE antibodies, by cooperating with Fcε-receptor-bearing cells, may also protect against malaria infection [26]. Furthermore, malaria infection is a strong driver of IgE production irrespective of helminth coinfection, parasite density, and helminth egg output [27].

The present work revealed that, in children with severe falciparum malaria, proinflammatory chemokines, which activate monocytes and neutrophil granulocytes, were at highly elevated levels and such responses were not observed in children or adults with uncomplicated falciparum malaria, while Th2-type chemokines in children attained the same levels as in adults. Furthermore, in children with severe malaria, an elevated IgG1 and IgE reactivity to mite allergens and *Entamoeba histolytica*-specific antigen was observed, which was most likely induced following immune sensitization and infection during early postnatal life.

Materials and methods

Study population

This work was conducted at the Centre Hospitalier Regional (CHR) in Sokodé in the Central Region of Togo. The study was approved by the National Committee de Bioethique pour la Recherche en Santé in Togo and by the Ethics Committee of the University Clinics of Tübingen, Germany. Informed written consent was obtained from all adults and from all parents for the participation of their children in this study; to ensure complete understanding of the study's aims, investigations, risks, and procedures, explanations were given in the local languages by the medical personnel of the CHR. Children under 5 years of age as well as adults with falciparum malaria were recruited. Classification of malaria type was performed according to previously published criteria for severe malaria characterized by parasitemias of higher than 250,000 parasites/μl blood and/or the presence of severe anemia with hemoglobin concentrations of less than 5 g/dl. Matched uncomplicated malaria patients were defined by parasite counts of less than 250,000 parasites/μl blood and hemoglobin concentrations of higher than 5 g/dl plus the absence of any signs or symptoms of severe malaria. *P. falciparum*-exposed children testing negative for parasites in thick blood film and negative in rapid detection test kits for *P. falciparum* (Malaria *P.f* HRP-II Antigen Rapid Test, Standard Diagnostics Inc., Korea) were defined as those participants with previous malaria episode(s) and an absence of illness due to malaria within the last 2 weeks. Blood samples were obtained prior to treatment with antimalarials and/or antipyretics, and all children with malaria were given antimalaria and the appropriate supportive therapy, such as is required and recommended by the guidelines for malaria treatment issued by the Ministry of Health in Togo.

Synthetic P. falciparum *peptides*

Five synthetic peptides corresponding to highly conserved B-cell epitopes were used. These were the following: 1) the epitope (EENV)4 of the C-terminal part of the *P. falciparum* ring-infected erythrocyte surface antigen (RESA), which is immune-dominant, and antibodies against which are associated with resistance to clinical malaria [28]; 2) the epitope KLYQAQYDLSF representing amino acids 277 to 287 of the N-terminal conserved part of the *P. falciparum* merozoit surface protein 1 (MSP-1-Nt) [29], antibodies associated with protection against malaria disease [30]; 3) the epitope KAASNTFINNA representing amino acids 27 to 34 of the N-terminal conserved region of the *P. falciparum* merozoit surface protein 2 (MSP-2-Nt); 4) the epitope AAAQHGHMHGS representing amino acids 199 to 206 of MSP-2 (MSP-2-Ct1); and 5) the epitope AAANTSDSQKE representing amino acids 213 to 220 of MSP-2 (MSP-2-Ct2) [31].

Preparation of E. histolytica-*specific antigen, and* Dermatophagoides farinae *allergen extracts*

E. histolytica trophozoites (strain HM-1 axenic culture) were a gift from Dr. Brigitte Walderich (Institute for Tropical Medicine, University Clinics of Tübingen, Germany). *E. histolytica* trophozoites were transferred into a Ten-Broek tissue grinder and homogenized extensively on ice. The homogenate was then sonicated twice (30% intensity) for 3 min on ice and centrifuged at 16,000*g* for 30 min at 4 °C. The supernatants were collected and then sterile-filtered (0.22 mm), and the protein concentration was determined with a BCA protein assay (Pierce, Rockford, USA). *D. farinae* extracts were purchased from Allergopharma (Rheinbeck, Germany).

P. falciparum-, E. histolytica-, *and* D. farinae-*specific antibody ELISA*

Microtitre 96 well EIA/RIA plates (Corning Costar, NY, USA) were coated with *P. falciparum* peptides (final concentration 5 μg/ml), *E. histolytica* antigen (final concentration 1 μg/ml), or *D. farinae* antigen (final concentration 1 μg/ml) in PBS (Sigma Aldrich, St. Louis, USA) overnight at room temperature (RT). Plates were then blocked with 5% PBS-BSA (Sigma Aldrich, St. Louis, USA) for 1.5 h. Patients' sera were diluted to 1:20 in PBS, and 50 μl was added to each well. Patients' samples were applied in duplicates. After 1 h of incubation at RT, plates were washed three times with PBS. For subclass-specific antibody detection, HRP-conjugated antibodies against human IgG1, IgG4 (Invitrogen, CA, USA), and IgE (AbDSeroTec, Oxford, UK) (1:500) were applied and incubated for 1 h at RT. Following washing of the plates as described above, 50 μl of tetramethylbenzidine (TMB) substrate solution (Thermo Fisher Scientific, Waltham, USA) was added to each well. Plates were read at 405 nm in a BioTek (Winooski, VT, USA) microplate autoreader.

Chemokine and cytokine ELISA

Quantitative enzyme-linked immunosorbent assay (ELISA) was performed using commercially available assays (Duo-Set: R&D Minneapolis, MN, USA) to determine plasma levels of the chemokines NAP-1/CXCL8/IL-8 (assay detection limit [DL] as indicated by the manufacturer: 30 pg/ml), IP-10/CXCL10 (DL: 30 pg/ml), eotaxin-1/CCL11 (DL: 15 pg/ml), MCP-4/CCL13 (DL: 8 pg/ml), MIP-1δ/CCL15 (DL: 15 pg/ml), TARC/CCL17 (DL: 8 pg/ml), PARC/CCL18 (DL: 8 pg/ml), LARC/CCL20 (DL: 15 pg/ml), eotaxin-2/CCL24 (DL: 20 pg/ml), and eotaxin-3/CCL26 (DL: 60 pg/ml). Sample concentrations of each cytokine and chemokine were quantified from standard curves generated with recombinant chemokines/cytokines included in the DuoSet ELISA kit.

Statistical data analysis

The statistical package JMP version 5.0.1.2 was used for data analysis. For cytokine and for chemokine analysis, differences between groups were determined after logarithmic transformation to stabilize the variance of data [log (pg/ml + 1)]. The level of significance was adjusted according to Bonferroni–Holm ($\alpha = 0.0025$).

Results

Study subject characteristics

A total of 234 children ranging from 0 to 5 years of age were included in this investigation. *Table 1* shows the characteristics of the children patient groups; the endemic control group (NEG) was children in whom *P. falciparum* was not detectable by thick blood smear and rapid antigen detection kits. The uncomplicated malaria cases (uM) were children positive for *P. falciparum* infection, with parasite

Table 1. Age, hemoglobin levels, leukocyte count, and *Plasmodium falciparum* parasite density in children with uncomplicated (uM) or severe (sM) malaria, in children negative (NEG) for *P. falciparum* and in adults with uncomplicated falciparum malaria (uM). Differences between study groups are indicated as [**]$p < 0.0001$ when compared to all other groups; [*]$p < 0.05$ when compared to Child-uM, and [&]$p < 0.0001$ when compared to Child-uM. The level of significance was adjusted according to Bonferroni–Holm ($\alpha = 0.0025$)

Patient groups	*N*	Age (years) (mean; range)	Leucocytes/μl (mean; range)	Pf/μl (mean; range)	Hb (mean; range)
Child-NEG	79	2.6 (0.1–5)	11,175 (2800–4700)	0	10.1 (3–17.7)
Child-uM	78	2.3 (0.02–5)	11,361 (2900–6400)	23,887 (77–223,500)	8.8 (5.3–16.7)
Child-sM	77	2.1 (0–5)	13,930 (4100–6900)	44,536[*] (130–453,333)	4.1[**] (1.7–11.4)
Adult-uM	74	32.9 (18–65)	7608[**] (1800–35,400)	9255[**] (122–87,000)	11.7[&] (6.5–18.2)

densities not exceeding 250.000 parasites/µl blood and with hemoglobin levels equal and superior to 5 g/dl. The children group with severe malaria (sM: >250.000 parasite/µl; <5 g/dl hemoglobin) presented with significant lower age and leucocytes counts than did NEGs and sM children *(Table 1)*, and in both malaria groups, hemoglobin levels were significantly lower as compared to the NEG children ($p < 0.0001$).

Adult patients were $n = 74$, between 18 and 65 years of age and with uncomplicated falciparum malaria; i.e., they tested positive for *P. falciparum* infection with parasite densities not exceeding 250.000 parasites/µl blood and with hemoglobin levels equal or superior to 5 g/dl. Immediately following primary diagnosis, all *P. falciparum*-positive children and adult patients received treatment according to the recommended guidelines of the Togolese malaria control program. All hospitalized malaria cases,

both uncomplicated and severe, were followed until release from the hospital's paediatric ward.

Chemokine plasma levels in uncomplicated or severe malaria

Plasma levels of the neutrophil granulocyte-activating protein NAP-1/CXCL8/IL-8, interferon-induced protein IP-10/CXCL10, MIP-1δ/CCL15, and the liver and activation-regulated chemokine LARC/CCL20 were quantified by specific ELISA in NEG, uM, and sM children, but also in adults with uncomplicated malaria (Adult-uM) *(Fig. 1)*. IL-8, IP-10, and LARC were highly elevated in children with uncomplicated and severe malaria, and IP-10 and LARC progressively heightened in cases of enhanced malaria disease severity. In adults with uncomplicated ma-
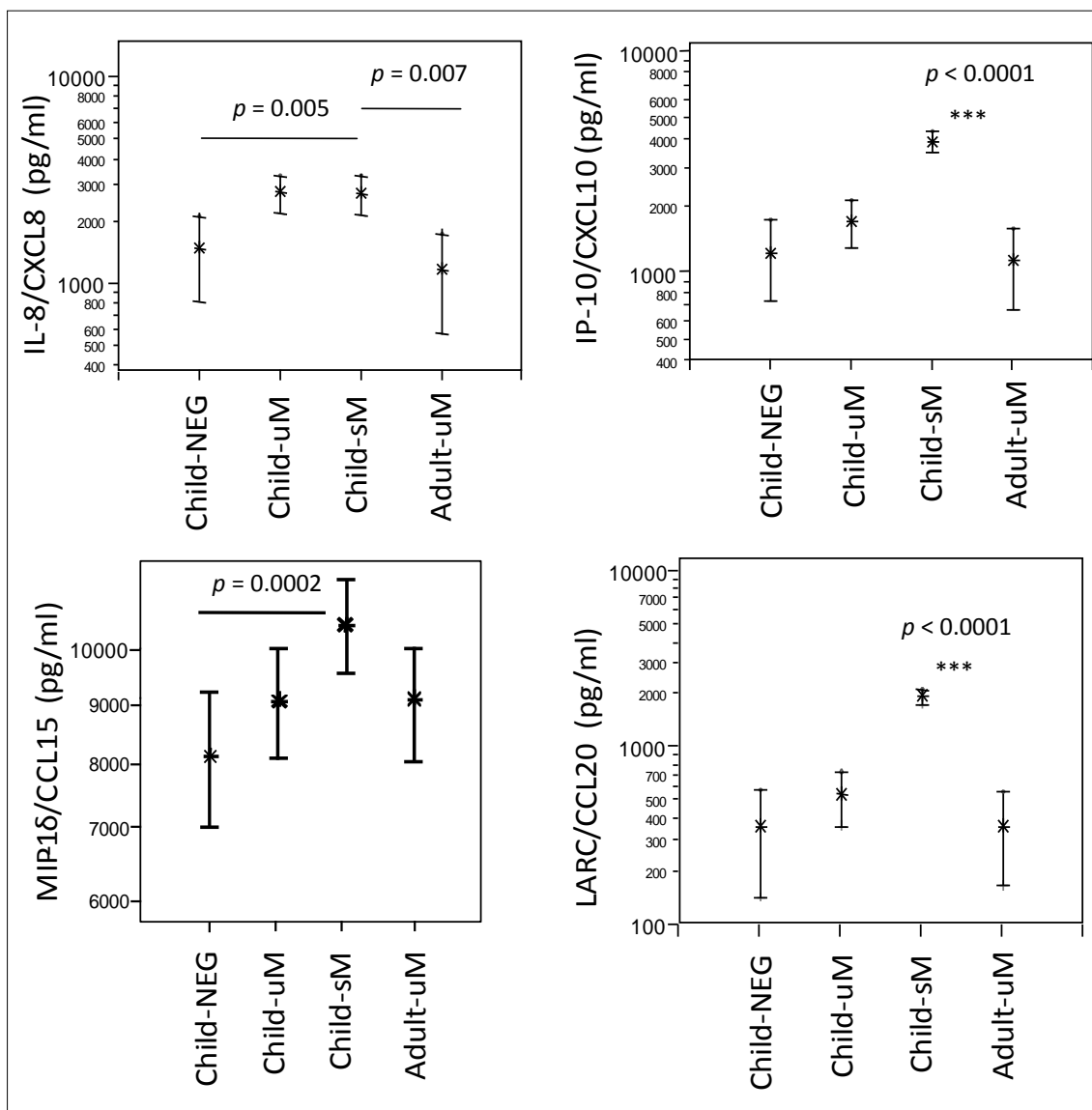

Fig. 1. CXCL and CC chemokines in children with uncomplicated (uM) or severe (sM) malaria in children negative (NEG) for *Plasmodium falciparum* and in adults with uncomplicated falciparum malaria (uM). Differences between study groups are indicated as [**]$p < 0.0001$ when compared to all other groups; otherwise, differences between groups are given as p values. The level of significance was adjusted according to Bonferroni–Holm ($\alpha = 0.0025$)
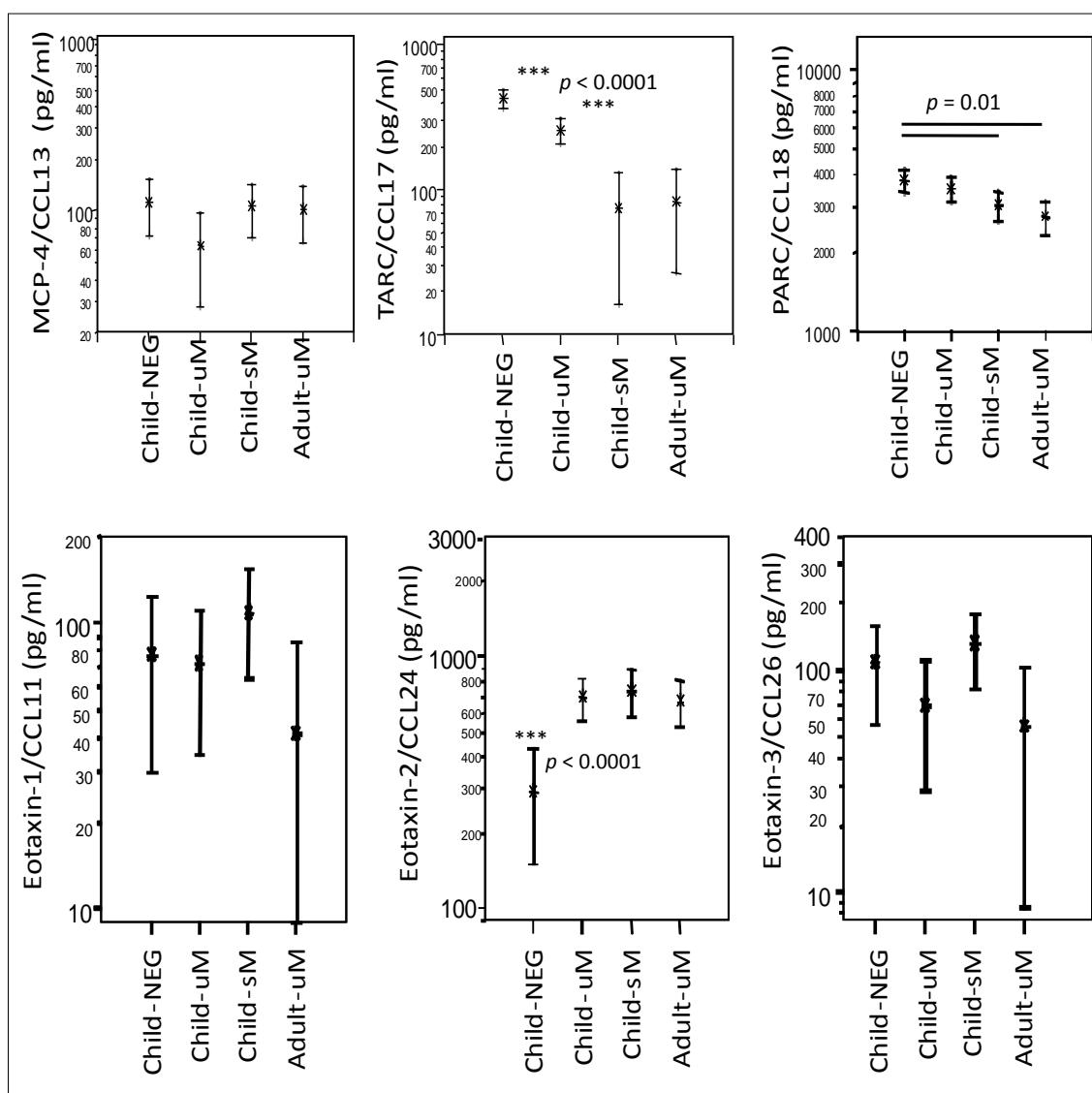
Fig. 2. CC chemokines in children with uncomplicated (uM) or severe (sM) malaria, in children negative (NEG) for *Plasmodium falciparum*, and in adults with uncomplicated falciparum malaria (uM). Differences between study groups are indicated as **$p < 0.0001$ when compared to all other groups; otherwise, differences between groups are given as *p* values. The level of significance was adjusted according to Bonferroni–Holm ($\alpha = 0.0025$)

laria, levels of IL-8, IP10, and LARC were as low as in NEG children. LARC levels were similar in the children and adults with uncomplicated malaria, but LARC/CCL20 presented highest in children with severe malaria. MIP-1δ/CCL15 plasma levels were highest in children with severe falciparum malaria ($p = 0.0002$ compared to NEG children) and slightly above those measured in children and adults with uncomplicated malaria.

In contrast, the plasma concentrations of the tissue and pulmonary chemokines TARC/CCL17 and PARC/CCL18, both regulated by activation, were highest in NEG children and progressively decreased with enhanced malaria severity, being similarly low in children with severe and in adults with uncomplicated disease *(Fig. 2)*. The levels of the monocyte inflammatory chemokine MCP-4/ CCL13 were similar in all study groups. The eosinophil granulocyte-attracting chemokines eotaxin-1/CCL11 and eotaxin-3/CCL26 exhibited similar plasma concentrations in the children and adult study groups. The eotaxin-2/ CCL24 was enhanced in all *P. falciparum*-infected groups to similar levels but was lowest in children negative for *P. falciparum* infection.

Chemokine level analysis

Chemokine–chemokine correlations and their plasma levels were analyzed for their associations with age, hemoglobin concentration, and parasite densities *(Table 2)*. With increasing age, the chemokine plasma concentrations of IL-8/CXCL8, IP10/CXCL9, LARC/CCL20, and TARC/CCL17 decreased (for all $p < 0.001$). Levels of the interferon gamma-induced chemokine IP10/CXCL9 and the liver- and activation-regulated chemokine LARC/

Table 2. Chemokine–chemokine correlations and their association with age, parasite densities, and hemoglobin concentrations in children with uncomplicated (uM) or severe (sM) falciparum malaria, in children negative (NEG) for *P. falciparum,* and in adults with uncomplicated falciparum malaria (uM)

Variable	Variable	n	R^2	p value
Hb (g/dl)	Age (months)	291	0.4610	<0.0001
Hb (g/dl)	Leucocytes (n/μl)	283	−0.3523	<0.0001
Leucocytes (n/μl)	Age (months)	282	−0.2205	0.0002
Parasite density (n/μl)	Hb (g/dl)	292	−0.2150	0.0002
Parasite density (n/μl)	IP10 (pg/ml)	292	0.3033	<0.0001
Parasite density (n/μl)	LARC (pg/ml)	290	0.2802	<0.0001
Parasite density (n/μl)	TARC (pg/ml)	288	−0.3787	<0.0001
IL8 (pg/ml)	Age (months)	291	−0.1931	0.0009
IL8 (pg/ml)	Hb (g/dl)	292	−0.2377	<0.0001
IL8 (pg/ml)	IP10 (pg/ml)	292	0.2536	<0.0001
IL8 (pg/ml)	LARC (pg/ml)	290	0.3143	<0.0001
IP10 (pg/ml)	Age (months)	291	−0.2628	<0.0001
IP10 (pg/ml)	Hb (g/dl)	292	−0.3374	<0.0001
IP10 (pg/ml)	MCP4 (pg/ml)	292	0.2778	<0.0001
IP10 (pg/ml)	LARC (pg/ml)	290	0.4869	<0.0001
LARC (pg/ml)	Age (months)	289	−0.2696	<0.0001
LARC (pg/ml)	Leucocytes (n/μl)	281	0.2499	<0.0001
LARC (pg/ml)	Hb (g/dl)	290	−0.4883	<0.0001
LARC (pg/ml)	MCP4 (pg/ml)	290	0.2321	<0.0001
TARC (pg/ml)	Age (months)	287	−0.2217	0.0002
TARC (pg/ml)	Hb (g/dl)	288	0.2184	0.0002
TARC (pg/ml)	MCP4 (pg/ml)	288	0.2014	0.0006

CCL20 correlated positively with parasite densities, while TARC/CCL17 (tissue- and activation-regulated chemokine) diminished with heightening parasite loads (for all p<0.0001). With lowering hemoglobin concentration the levels of IL-8, IP10, and LARC enhanced (for all $p < 0.0001$), and in opposite, TARC levels heightened with increasing hemoglobin values ($p = 0.0002$). IL-8/CXCL8 associated positively with IP10 and LARC ($p < 0.0001$), CXCL10/IP10 levels enhanced together with LARC/CCL20, and the monocyte chemoattractant chemokine MCP4 correlated positively with IP10 ($p < 0.0001$), LARC ($p < 0.0001$), and TARC ($p = 0.0006$).

Antibody responses to mite allergen and parasitic antigens in children and adults

P. falciparum peptide-specific antibody responses in children and adults

Figure 3A shows the subclass-specific antibody reactivity to *P. falciparum* peptides in children and adults with falciparum malaria as well as in children without *P. falciparum* infection. Highest IgE antibody responses against *P. falci-*

parum peptides were detected in infection-free controls. Antibody reactivity decreased in children with progression of disease severity. Adults with mild malaria presented with similar antibody responses as those in children with mild malaria. IgG1 specific for *P. falciparum* peptides was lowest in *P. falciparum*-negative children and slightly more elevated in children with mild malaria, while IgG1 responses were highest in children with severe malaria. In adults with mild malaria, *P. falciparum* peptide-specific IgG1 attained similar levels as observed in infection-free children. IgG4 reactivity to *P. falciparum* peptides was highest in adults and children with mild malaria, without any difference between the two groups, while both children with severe malaria and children without *P. falciparum* infection presented with reduced *P. falciparum* specific IgG4 responses.

E. histolytica antigen-specific antibody responses in children and adults

In *Fig. 3B*, the subclass-specific antibody reactivity to an antigen extract of the intestinal protozoan parasite *E. histolytica* antigen is shown for children and adults with falciparum malaria and for children without *P. falciparum*
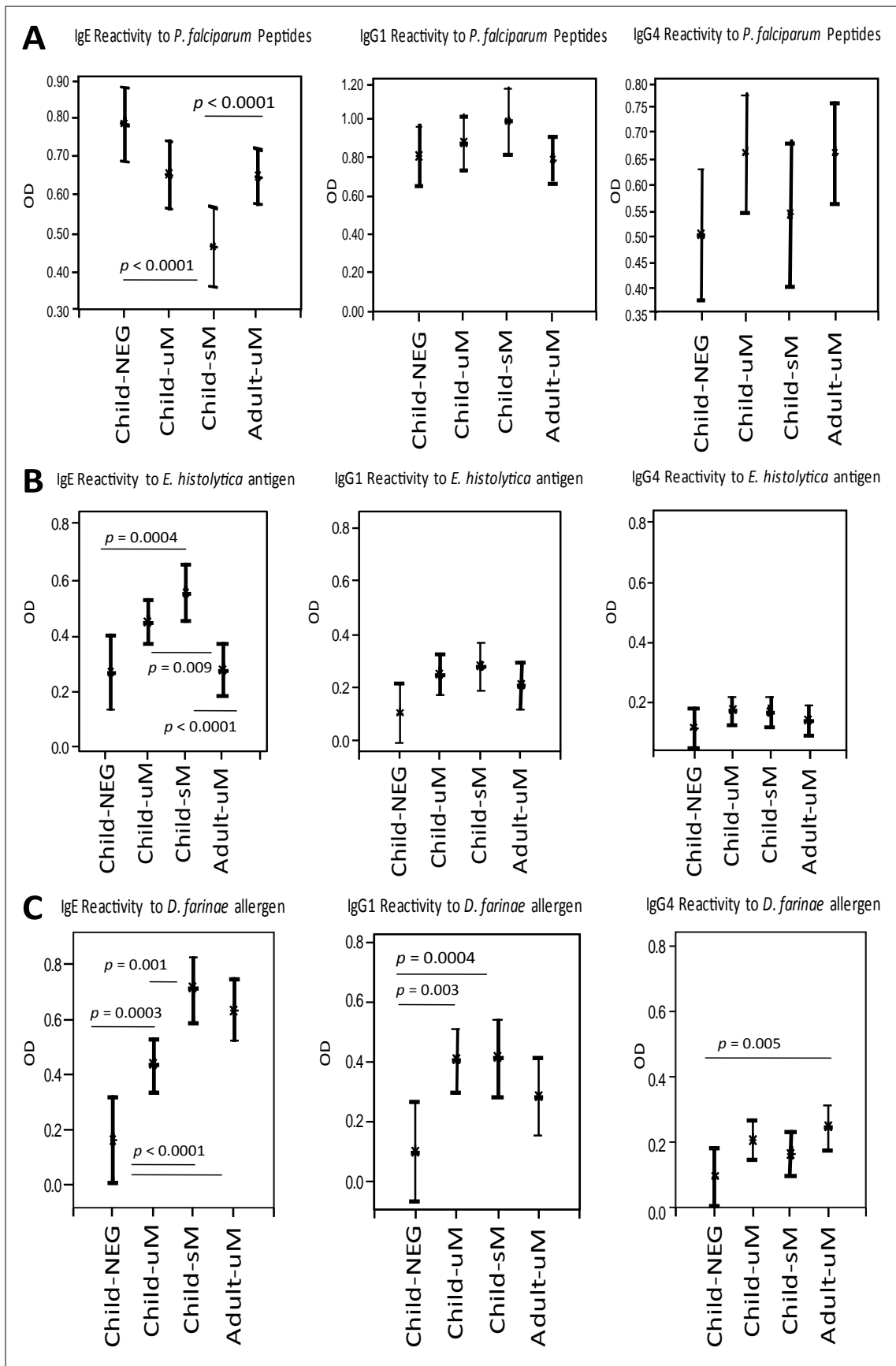
Fig. 3. Antibody subclass reactivity to *Plasmodium falciparum* peptides (Part A), *Entamoeba histolytica* antigen (HM1 axenic strain) (Part B), and *D. farinae* allergen extract (Part C) in children with uncomplicated (uM) or severe (sM) malaria, in children negative (NEG) for *P. falciparum*, and in adults with uncomplicated falciparum malaria (uM). Differences between study groups are indicated as *p* values. The level of significance was adjusted according to Bonferroni–Holm ($\alpha = 0.0025$)

infection. IgE reactivity to *E. histolytica* antigen was lowest in *P. falciparum* negative children and enhanced in uncomplicated cases of children, with the highest responses observed in children with severe malaria. IgE levels in adults with mild malaria did not differ from the *P. falciparum* negative children group. IgG1-mediated recognition of *E. histolytica* antigens was lowest in children without *P. falciparum* infection, and enhanced in children with mild and with severe malaria. Adults and children with mild malaria presented with similar *E. histolytica*-specific IgG1 responses. No major differences in *E. histolytica* specific IgG4 levels were observed between the malaria and *P. falciparum* negative groups.

D. farinae allergen-specific antibody responses

Figure 3C shows the subclass specific antibody reactivity to mite allergens *(D. farinae)* in children and adults with falciparum malaria as well as in children without *P. falciparum* infection. Lowest *D. farinae* specific IgE responses were observed in children without *P. falciparum* infection. In children, antibody levels heightened with increasing malaria severity, being highest in children with severe malaria. Adults with mild malaria presented with a slightly reduced IgE reactivity to mite allergens as compared to children with severe malaria. *D. farinae*-specific IgG1 responses were lowest in infection-free controls. Both in children with mild and with severe malaria, these *D. farinae*-specific IgG1 levels were clearly enhanced, without any difference between the groups. *D. farinae*-specific IgG1 levels were reduced in adults, as compared to both mild and severe children malaria groups, but adult responses were higher than those in infection-free controls. Both adults and children with *P. falciparum* infection presented with higher IgG4 responses to *D. farinae* than did children without infection.

Discussion

Cytokines and chemokines can act as central contributors to severe and life-threatening illness, and parallels exist between falciparum malaria and other illnesses such as sepsis, influenza [9, 32, 33] and allergic inflammatory responses [25, 34, 35]. Our present work showed that in children with severe malaria, chemokines activating granulocytes and monocytes (IL-8, IP10, MIP-1δ/CCL15, and LARC/CCL20) were at high levels disclosing pronounced innate inflammatory responses, which may predispose to malaria aggravation. In parallel, these children had chemokine plasma levels at magnitudes observed to an equal extent in adults with uncomplicated malaria, notably TARC/CCL17, PARC/CCL18, and eotaxin-2/CCL24, which may signify a gradual evolution of regulated immune responses during early postnatal life.

Resolution of *P. falciparum* infection will require immune responses which are of both proinflammatory and regulatory type; an early Th1-type gamma interferon (IFN-γ) cytokine response may support resistance [13]; and while high regulatory IL-10 levels were reported in children with severe malaria [18, 36, 37], IL-10 also exerts anti-inflammatory effects, thus, limiting pathogenic and excessive Th1 cytokine responses. In the present work, the neutrophil granulocyte-activating and proinflammatory chemokine NAP-1/CXCL8/IL-8 was at highest levels in children with severe malaria, while CXCL8/IL-8 was low in adults with uncomplicated disease. Neutrophil granulocytes inducing allergic-type reactions were shown to play a central role in the development of experimental cerebral malaria [25]. Similarly, the chemokines IP-10/CXCL10, MIP-1δ/CCL15, and LARC/CCL20, which attract and activate granulocytes and monocytes, were enhanced in children but not in adults. Notably high IP-10 levels were considered as biomarker and risk indicators for cerebral malaria and progression to lethal disease [19]. The neutrophil-activating CXCL8/IL-8 was previously found to be weakly elevated in children with severe malaria [38, 39] but present at higher concentrations in adults [40], and elevated IL-8 and also IP-10 in cerebral spinal fluid were predictive markers for cerebral malaria and lethality [19]. Expression of IL-8 [41] and also IP-10 [42] can be triggered following exposure to hemin or hemozoin. Released during rupture of *P. falciparum*-infected erythrocytes, hemozoin is phagocytized and will activate several proinflammatory chemokines; intravenous hemozoin injection correlates with malaria-associated acute respiratory distress syndrome and pulmonary inflammation in mice [42].

In the present work, plasma levels of IL-8 were elevated in children with uncomplicated or severe malaria, while interferon-induced protein IP-10 and liver- and activation-regulated chemokine LARC/CCL20 were highest in children with severe malaria, with no such amounts being measured in adults. This divergence may be due to unbalanced inflammatory innate responses in children, which only may become adequately regulated after repeated *P. falciparum* infections. This is supported by the negative correlation of IL-8/CXCL8 and IP10/CXCL10 with age, i.e., both will decrease with higher age *(Table 2)*. In children, several chemokines approached the magnitudes observed in infected adults, with TARC/CCL17 and PARC/CCL18 lessening and eotaxin-2/CCL24 enhancing, and both CC-chemokines being involved in effector cell activation and immune regulation. PARC and LARC were negatively correlated with age, disclosing that, during aging and with repeated exposure to *P. falciparum* infections, pulmonary (PARC) and tissue (TARC) activation regulated chemokines will diminish. The alternative macrophage activation-associated chemokine PARC/CCL18 is mainly produced by antigen-presenting cells induced by Th2 type cytokines and may exhibit dual functions, with pro- and anti-inflammatory properties [43]. PARC/CCL18 was found to enhance protective cell-mediated immunity in murine malaria suggesting this chemokine as a potential adjuvant to enhance adaptive immune responses against *Plasmodium yoelii* infection [44]. In our study, plasma levels of TARC/CCL17 and PARC/CCL18 were

similarly high in children with severe malaria and in adults with uncomplicated malaria, suggesting that even though being diminished in children to adult levels, PARC and TARC may not be the critical components which mediate an immune response preventive against severe malaria. In regard to the molecules able to generate such a disease-preventive immune responsiveness and to balance the pro-inflammatory IP-10, IL-8, and LARC chemokines, these regulators remain to be identified.

The eosinophil granulocyte-activating eotaxin-2/CCL24 was markedly elevated in all *P. falciparum*-infected cases and was low in negative children. Eotaxin-1/CCL11 and eotaxin-3/CCL26 were below 100 pg/ml in all study groups. This finding suggested a *P. falciparum*-induced eotaxin-2/CCL24 production, which may attract granulocytes and mast cells along CCL24 gradients into parasite-infested sites; after cross-linking of Fcε receptors with IgE, the subsequent degranulation of these cells may cause tissue damage, e.g., endothelial apoptosis. Such allergic-type inflammation was suggested as interacting with the outcome of malaria disease; moreover, the activation of effector mechanisms involving IgE and basophil [39] and neutrophil granulocytes with unconventional Fcε receptor expression [25, 45] was identified as components in the experimental induction of cerebral malaria in mice. Furthermore, the eotaxins CCL24 and CCL26 are mediators of persistent allergen-induced bronchial eosinophilic inflammation [46, 47]. We further observed in malaria children an IgE and IgG1 reactivity progressively enhancing with disease severity to mite allergens and *E. histolytica*-specific antigen; such antibody production was probably generated following allergen sensitization and *E. histolytica* infection during early postnatal life. The evolution of immunity to malaria results from broad and prominent antibodies responses to multiple plasmodium merozoite antigens [48], but parasite coinfections, e.g., helminthes or intestinal protozoan infections, may influence the antibody repertoire and its magnitude [49–51]. In consequence, parasite coinfections may hamper or bias the immune regulatory capacity, which may, in turn, serve to protect against disease or make it worse. In children concomitantly infected with *Schistosoma mansoni*, an increased number of malaria attacks were observed [52]; moreover, infection modulated *P. falciparum*-specific immune responses, potentially increasing the risk of plasmodial infection or disease [53]. Strong inflammatory chemokine responses to *P. falciparum* and *E. histolytica* antigens in newborns and 10-year-old children suggested that adequately balanced immune regulatory mechanisms may not yet have developed [3].

Conclusion

In children with falciparum malaria, prominent proinflammatory chemokines together with IgE responses to allergens or *E. histolytica*-specific antigens were observed; moreover, children generated immune regulatory-type chemokines to similar high levels as in adults, which suggested the evolution of regulatory immune responses; however, those may not yet suffice to mediate an immune response preventive against severe malaria. Repeated exposure to *P. falciparum* antigens during infancy and childhood is required to generate cytokine and chemokine responses which balance, prevent, or stop the progress of malaria pathogenesis, and these immune regulators need to be identified.

Conflicts of interest

The authors declare that they have no conflict of interest.

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