

Interleukin-21 is associated with IgG1 and IgG3 antibodies to erythrocyte-binding antigen-175 peptide 4 of *Plasmodium falciparum* in Gabonese children with acute *falciparum* malaria

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ABSTRACT. Interleukin-21 (IL-21) is a newly described, typical, four-helix cytokine showing significant homology with IL-2, IL-4 and IL-15. It regulates IgG1 production and co-operates with IL-4 in the production of multiple antibody classes *in vivo*. IgG1 and IgG3 are critically involved in the development of clinical immunity to *Plasmodium falciparum* malaria. However, the mechanisms driving class-switch recombination towards these specific isotypes remain to be elucidated. Seventy-three children with *P. falciparum*-positive, thick blood smears were recruited from the pediatric wards of the Albert Schweitzer Hospital and the General Hospital in Lambaréné. Children were grouped into two categories according to age: group A (1 to 5 years old) and group B (6 to 16 years old). Patients with severe (severe anemia and/or hyperparasitemia) and mild malaria were enrolled. Prevalence and level of IL-21, total IgG and subclass (IgG1, IgG2, IgG3 and IgG4) titers were determined in plasma by enzyme-linked immunosorbent assay (ELISA). Plasma IL-21 levels correlated with IgG1 and IgG3 levels. Additionally, plasma IL-21 levels correlated with hemoglobin levels in younger children and with parasite density. Here we describe the relationship between IL-21 and antibodies for erythrocyte-binding antigen-175 (EBA-175) peptide 4, a malaria vaccine candidate in Gabonese children with acute *falciparum* malaria. This study provides new insights into the field of malaria.

Keywords: interleukin-21 (IL-21), antibodies, *Plasmodium falciparum*, EBA-175, Gabon

Humoral immune responses are important in the control of malaria [1, 2]. Gamma globulins (IgG) play a critical role in these responses, as passive transfer of hyperimmune IgG from semi-immune individuals to malaria patients leads to the control of both clinical symptoms and parasitemia [3]. Characterization of IgG subclasses in semi-protected subjects has shown that cytophilic IgG1 and IgG3 isotypes predominate [4, 5], however, various studies have also associated non-cytophilic IgG2 and IgG4 with protection [6, 7]. We need to better understand anti-*Plasmodium falciparum* IgG subclass switching in order to develop efficient, blood-stage malaria vaccines. The IgG1/IgG3 pattern has been associated recently with target antigen, host genetic background, transmission intensity, cumulative exposure and host age [8-11]. However, factors that drive switching of IgG1 and IgG3 antibodies during

malaria infection are poorly understood. Interleukin-21 (IL-21) has been reported to drive the class switch recombination towards IgG1 and IgG3 *in vitro* and in an animal model [12, 13].

IL-21 is a pleiotropic type I cytokine produced by activated CD4⁺ T cells. It is involved in leukocyte activation, signaling through a receptor composed of IL-21R and the common cytokine receptor γ -chain complex [14].

Although the role of IL-21 in cancer and atopic diseases has been well studied, there are few data about its role in infectious diseases, and no data concerning IL-21 in malaria.

We investigated the relationship between IL-21 and humoral immune responses to malaria by measuring IL-21 and anti-erythrocyte binding antigen-175 (EBA-15) antibodies in plasma from children living in a malaria-endemic area. EBA-175 peptide 4 (1062-1103aa) is a vaccine candidate. It has a conserved region of 42 amino acids (aa)

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within regions III to V of the EBA-175 protein. This region is antigenic, eliciting antibodies that inhibit merozoite invasion of erythrocytes [15-17].

Here we report that plasma IL-21 level correlated with age, levels of anti-EBA-175 peptide 4 IgG and IgG subclasses, parasite load and hemoglobin level.

PATIENTS AND METHODS

Study site and participants

Between May 2005 and April 2006 we conducted a cross-sectional study, in the Medical Research Unit of Albert Schweitzer Hospital (MRU) in Lambaréné, Gabon, where *P. falciparum* is hyperendemic with a perennial mode of transmission and little seasonal fluctuation. The entomological inoculation rate is about 50 infective bites per person per year and the main vectors are *Anopheles gambiae* and *Anopheles moucheti* [18].

Seventy-three children with *P. falciparum*-positive, thick blood smears were recruited at the pediatric wards of the Albert Schweitzer Hospital and the General Hospital in Lambaréné. Children were grouped into two categories according to age: group A (1 to 5 years old) and group B (6 to 16 years old), in the knowledge that children under 5 are particularly vulnerable compared to older children. Patients with severe (severe anemia and/or hyperparasitemia) and mild malaria were enrolled. Severe malaria was defined as a hemoglobin level below 5g/dL, a hematocrit level less than 15% and/or a parasite load greater than 250 000 parasite/ μ L.

Children that had taken antimalarial drugs within the four weeks prior to presentation and those with mixed infections were excluded. Children were treated according to the national treatment guidelines for uncomplicated malaria or severe malaria.

Informed consent was obtained from parents or legal guardians of the children before inclusion in the study. The study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital in Lambaréné and conducted in accordance with the guidelines for human experimentation and clinical research of the Ministry of Public Health and Population, Libreville, Gabon.

Blood collection

Venous blood (2 mL) samples were collected in sterile and heparinized tubes, and immediately centrifuged for 10 min at 400 g with a Biofuge Pico centrifuge (Heraeus Instruments) to separate the pellets containing the packed erythrocytes from the plasma. Plasma samples were then frozen and stored at - 80°C until analysis.

Malaria diagnosis

P. falciparum asexual blood stage was assessed on Giemsa-stained, thick blood smears and examined by two microscopists following standard quality-controlled procedures. Parasite loads were quantified and expressed as the number of asexual forms of *P. falciparum*/ μ L blood [19].

Haemoglobin measurement

Hemoglobin levels were determined with the HORIBA ABX Diagnostics Pentra 60 automated analyzer (Axon lab GmbH).

Antibody enzyme-linked immunosorbent assay (ELISA)

Prevalence and level of total IgG and subclass (IgG1, IgG2, IgG3 and IgG4) titers were determined in plasma by enzyme-linked immunosorbent assay (ELISA) as described elsewhere [20] with few modifications. Briefly, 96-well plates (Nunc Maxisorp, Roskilde, Denmark) were coated with 42-mers of EBA-175 peptide 4 (1 μ g/mL) (Interactiva Biotechnology, Ulm Germany), diluted in carbonate-bicarbonate buffer (pH 9.6, Sigma-Aldrich, Germany) and incubated for 16 h at 4°C. Plates were washed with 0.05% Tween-20 in phosphate-buffered saline (PBST, 7.4) and blocked for 2 h with 5% skimmed milk/PBST. One-hundred-microliter aliquots of plasma (diluted [1/100] in 2.5% skimmed milk/PBST) were added to antigen-coated wells and the plates were incubated for 2 h. Horse-radish-peroxidase (HRPO)-conjugated, goat anti-human IgG was added to the wells and the plates were incubated for 1 h at room temperature. Bound antibodies were detected by the addition of ready-to-use TMB One (3, 3', 5, 5'-tetramethylbenzidine) (KEM-EN-TEC Diagnostic, Tastrup Denmark). The absorbance was read at 450 nm with an ASYS Hitech ELISA plate reader.

The following antibodies were used to detect IgG subclasses: mouse anti-human IgG1_clone HP6069 (1 μ g/mL), IgG2_clone HP6002 (0.25 μ g/mL), IgG3_clone HP6047 (0.5 μ g/mL) and human IgG4_clone HP6023 (0.25 μ g/mL). Antibodies were purchased from Invitrogen and diluted in 2.5% skimmed milk/PBST. HRPO-conjugated goat anti-mouse IgG (1/3 000) was used to detect bound antibodies. The detection threshold was assessed by adding positive and negative controls to each plate. A sample with an OD value which was at least two standard deviations (SD) away from that of the negative controls was considered positive.

IL-21 ELISA

One-hundred-microliter aliquots of polyclonal, goat anti-human IL-21 antibody (ABNOVA Corporation, Taiwan) (5 μ g/mL) diluted in 0.1 M sodium phosphate-buffered were loaded onto 96-well plates. The plates were incubated for 16 h at 4°C, washed with PBST and blocked with 1% BSA/PBS for 1 h at room temperature. Plates were then washed, seeded with 100 μ L diluted plasma (1/100) in 1% BSA/PBST and incubated for 2 h at room temperature. Chicken anti-human IL-21 (100 μ L) (catalog number I8442-03, Biomol GmbH, Hamburg Germany) was added for 1 h at room temperature followed by the addition of peroxidase-conjugated, goat anti-chicken IgG (H&L) (catalog number 603-103-002 Immunochemicals for Research, Rockland USA). Ready-to-use TMB One (100 μ L) was added to each well and the reaction stopped with 0.2 N H₂SO₄. Absorbance at 450 nm was measured with an ELISA plate reader controlled by MikroWin software.

Statistical analysis

StataCorp 2001 (Stata Statistical Software, Release 9.2; Stata Corporation, College Station, TX, USA) was used for data analysis. Demographic, clinical and laboratory data of patients were recorded on a medical form, entered into JMP Version 5. Data were cleaned before analysis. Chi-square or Fisher's exact tests for proportions and Student's t-test were used to determine differences between groups. Analysis of variance (ANOVA) was used for continuous, normally distributed variables. The nonparametric Kruskal-Wallis test was used to compare continuous, not normally distributed variables.

Spearman's test was used to determine correlations between continuous variables. The Bonferroni correction was used for multiple comparisons.

Two-tailed p values less than 0.05 were considered to be significant.

RESULTS

Characteristics of study patients

Mean age was 6.6 years, median hemoglobin concentration was 9.4 g/dL, median temperature was 37.4°C and geometric mean of parasite load was 15, 327.17 parasites/ μ L. Of the 73 recruited children, 33 belonged to group A and 40 to group B. Ten children had severe malaria (severe anemia and/or hyperparasitemia). Table 1 lists the characteristics of the two groups.

Plasma IL-21 level

We compared the plasma levels of IL-21 between the two groups of children according to age (figure 1). Children from group A had higher levels of IL-21 plasma levels than their group B counterpart. However, the difference was not significant.

Anti-EBA-175 peptide 4 antibody responses

The prevalence of total IgG, IgG1, IgG2, IgG3 and IgG4 were 60%, 51.38%, 1.38%, 9.58% and 8.82%, respectively (figure 2A).

Overall, the levels of total IgG and IgG subclasses antibodies against EBA-175 peptide 4 appeared to increase with age. With the exception of IgG2, and IgG4, total IgG

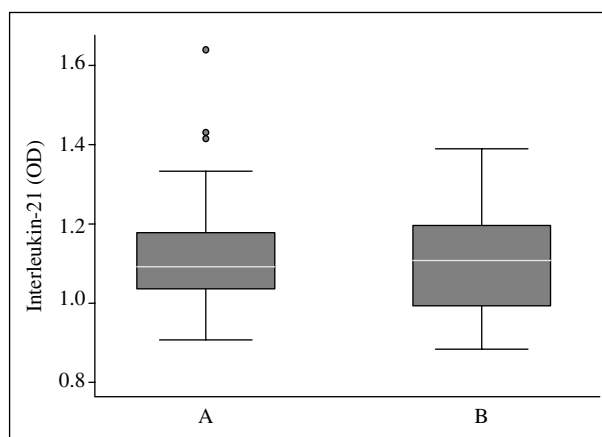


Figure 1

Box plots of plasma IL-21 concentrations. Comparison of plasma IL-21 concentrations between children aged from 1 to 5 years old (A) and children aged from 6 to 16 years old (B), $p = 0.9$. Box plots illustrate medians with 25th to 75th percentiles, with lines extending to 10th and 90th percentiles.

(Spearman $\rho = 0.51$, $p = 0.0001$), IgG1 (Spearman $\rho = 0.27$, $p = 0.32$) correlated significantly with age. Surprisingly, IgG3 (Spearman $\rho = 0.3$, $p = 0.15$) did not correlated with age after correction.

We looked at the prevalence of antibodies to EBA-175 peptide 4 in each group and further compared the prevalence between the two groups. The analysis showed that the prevalence and the levels of total IgG ($p = 0.0002$), IgG1 ($p = 0.0002$) and IgG3 ($p = 0.04$) were significantly higher in group B than in group A (figure 2B, C and E). There was no significant difference between groups for levels of IgG2 and IgG4 (figure 2D and F).

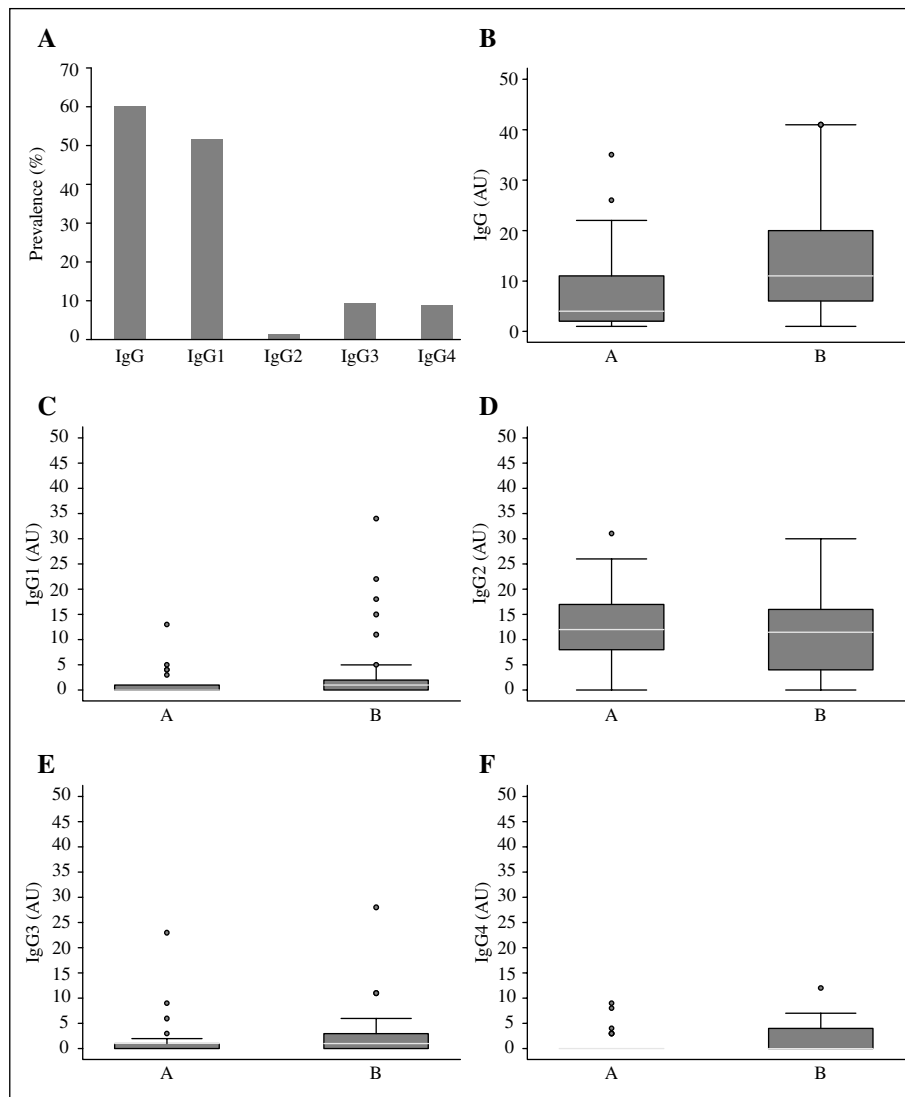
Correlation between IL-21 and specific antibodies to EBA-175 peptide 4

When investigating the relationships between the plasma level of IL-21 antibodies raised against EBA-175 peptide 4, we found that the IgG1 level (Spearman $\rho = 0.29$, $p = 0.02$) significantly correlated to the plasma IL-21 level (figure 3A); we found a trend of positive correlation between the plasma IL-21 level and IgG3 (Spearman $\rho = 0.22$, $p = 0.07$) (figure 3B). There was no significant correlation between the plasma IL-21 level and levels of total IgG, IgG2 and IgG4.

Table 1
Patient characteristics

Characteristics	Groups		p
	A	B	
Number	33	40	
Age* (SD)	2.9 (1.5)	9.7 (3.2)	0.0001
Sex ratio F/M	16/17	20/20	
Parasite load**			
Geometric mean	14 839.4	15 739.35	NS
[95% CI]	[7 213.9-30 525.2]	[9 742.5-25 427.4]	
Hemoglobin level g/dL& (SD)	8.4 (2.0)	10.1 (1.7)	0.0004
Axillary temperature °C& (SD)	37.5 (1.1)	37.8 (1.2)	NS
Severe cases (%)	8 (25.0)	2 (5.4)	-

* Mean; ** parasites/ μ L; & median; NS: not significant; SD: standard deviation.

**Figure 2**

Prevalence of IgG antibodies and IgG subclasses with specificity for EBA-175 peptide 4 in a cross-section of the Gabonese child population (A). Box plots of plasma levels of IgG and IgG subclasses. Comparison of concentrations of plasma IgG ($p = 0.0002$) (B), IgG1 ($p = 0.0002$) (C), IgG2 ($p = 0.9$) (D), IgG3 ($p = 0.04$) (E) and IgG4 ($p = 0.09$) (F) between children aged from 1 to 5 years old (A) and children aged from 6 to 16 years old (B). Box plots illustrate medians with 25th to 75th percentiles, with lines extending to 10th and 90th percentiles.

Further, we performed a within-group analysis to assess the relationship between the plasma level of IL-21 and antibodies to EBA-175 peptide 4 in each group. In the group A, with the exception of IgG4, total plasma IgG, IgG1, IgG2 and IgG3 levels appeared to increase with increasing IL-21 levels in each group. We found a significant correlation (Spearman $\rho = 0.39$, $p = 0.03$) between IgG1 and IL-21 levels in group A (figure 3C). The IL-21 plasma level and levels of total IgG and IgG subclasses were not significantly correlated in group B.

Interleukin-21 and parasite load

Hyperparasitemia is one of the factors contributing to severe malaria. Thus, we investigated the relationship between parasite load and the IL-21 plasma level. Our results show that the IL-21 plasma level was not significantly increased with parasite load.

We formed two subgroups according to parasite density (high parasite density: parasite density $\geq 10\,000$ parasites/ μL ; low parasite density: parasite density

$< 10\,000$ parasites/ μL) to see whether or not the difference in parasite load influences the production of IL-21. Based on the fact that cytophilic IgG subtypes are associated with the reduction of parasitemia, we first characterized the distribution of cytophilic IgG1 and IgG3 in each group, and secondly assessed the correlations between these antibody levels and IL-21 levels.

The plasma IgG1 level was higher in the low parasite density subgroup ($p = 0.04$). Plasma IL-21 and IgG3 levels were not significantly different between these subgroups. There was a slightly significant, positive correlation between plasma IgG1 and IL-21 levels in children with high parasite densities (Spearman $\rho = 0.29$, $p = 0.04$), but not in the low parasite density subgroup. There was no correlation between IL-21 and IgG3 levels in either of these subgroups.

Interleukin-21 and hemoglobin level

As the hemoglobin level differed significantly between groups A and B ($p = 0.004$), we examined the correlation

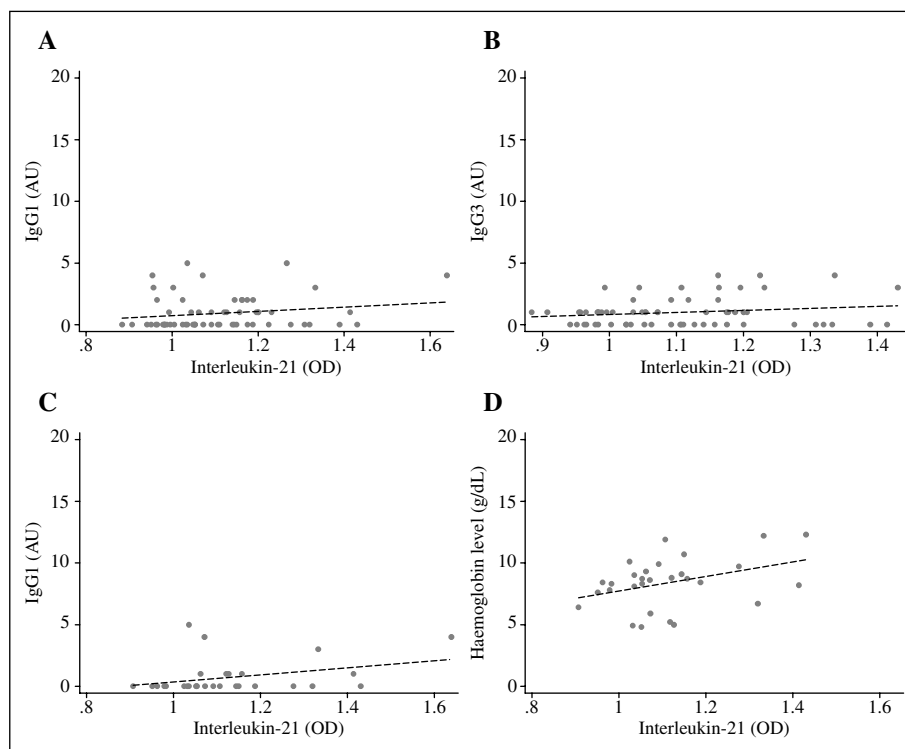


Figure 3

Correlation between IL-21 and IgG1 in all children (A), Spearman's $\rho = 0.28$ with $p = 0.02$. Correlation between IL-21 and IgG3 in all children (B) Spearman's $\rho = 0.21$ with $p = 0.07$. Correlation between IL-21 and IgG1 in children from the group A (C) Spearman's $\rho = 0.39$ with $p = 0.03$. Correlations between IL-21 and hemoglobin level in children from the group A (D), Spearman's $\rho = 0.36$ with $p = 0.04$.

between IL-21 and hemoglobin levels within groups. As shown in *figure 3D*, plasma IL-21 levels positively correlated with hemoglobin level in group A (Spearman $\rho = 0.35$, $p = 0.04$), but not in group B (Spearman $\rho = -0.06$, $p = 0.69$).

Severe anemia contributes greatly to malaria mortality and morbidity. We further characterized the relationships between plasma IL-21 levels and hemoglobin levels in study participants grouped into severely anemic (hemoglobin level ≤ 5 g/dL), moderately anemic ($5 < \text{hemoglobin level} < 10$ g/dL) and non-anemic (≥ 10 g/dL). We found that hemoglobin levels correlated positively with IL-21 levels in group A (Spearman $\rho = 0.9$, $p = 0.00001$) and in non-anemic children, and correlated only slightly in moderately anemic children.

In group B, we observed no difference in IL-21 levels between anemic, non-anemic and moderately anemic children.

DISCUSSION

Antibodies recognizing monomorphic epitopes [21] and polymorphic epitopes with clonal antigenic variation [22, 23] have been suggested to contribute to protection against malaria in areas of stable malaria transmission. The pattern of IgG isotypes in malaria patients showed that cytophilic IgG1 and IgG3 antibodies play a critical role in the protection against *falciparum* malaria [4, 5]. Nevertheless, the mechanisms that control class-switch recombination towards these specific antimalarial isotypes are unclear. Human B cells switch isotype from immunoglobulin M (IgM)-IgD to IgG1-4, IgA1-2, or IgE during antigen-

induced immune responses as a result of Ig isotype class-switch recombination [24]. This is a highly regulated process controlled by cytokines and cellular interactions, involving B cell-produced CD40 and its ligand, CD154 [25].

IL-21 is a γ -common chain cytokine produced by activated T helper lymphocytes [26] which regulates leukocyte functions [25, 26]. Furthermore, naturally acquired IgG1 and IgG3 specific for EBA-175 peptide 4 have been reported to contribute to the development of clinical protective immunity [20]. The major aim of the present study was to determine the relationship between IL-21 and plasma levels of IgG1 and IgG3. In particular, we investigated whether IL-21 in plasma from malaria-infected patients is involved in class-switching recombination of IgG1 and IgG3 isotypes. We targeted anti-EBA-175 peptide 4 total IgG and IgG isotypes for the quantification of the antibody response. We demonstrated that IL-21 levels correlated strongly with anti-EBA-175 peptide 4 IgG1 levels and, to a lesser extent, with IgG3 levels if the entire study population was considered. In contrast to our findings for IgG3, the correlation between IL-21 and plasma IgG1 levels persisted in younger children longer than in older children with acute *falciparum* malaria. IL-21 may not account for all of the switch-inducing activity of IgG3 and, particularly, IgG1. The prevalence of IgG1 is possibly due to another factor inducing or enhancing switching to IgG1. Indeed, switching of various Ig isotypes is controlled by redundant molecules, *e.g.* switching to human IgG4 and IgE in response to IL-4 or IL-13. However, this study raises the possibility that patients suffering from IgG3 and IgG1 deficiencies may secrete lower than normal levels of IL-21,

produce IL-21 antagonists or have an intrinsic B-cell impairment resulting in a defective IL-21 response.

Our study population had humoral immune responses to EBA-175 peptide 4 that were consistent with results reported previously. We found that 60% of the plasma samples tested contained antibodies directed to EBA-175 peptide 4. The predominant isotype was IgG1, found in 51.38% of these samples. The prevalence of IgG3 (9.58%) and IgG4 (8.82%) were similar and IgG2 had a very low prevalence. We found that levels of cytophilic IgG1 and IgG3 against EBA-175 peptide 4 correlated positively with age, as observed by others [4, 20]. However, our findings were different from those of Touré *et al.* who reported the predominance of IgG1 and IgG3 against EBA-175 peptide 4 in children living in malaria-endemic areas [20]. The difference between the two studies may be explained by the study design (cross sectional *versus* longitudinal study), the transmission intensity and the targeted age range.

Analysis of the entire study population and within-group analysis showed that IL-21 levels and parasite load were not significantly correlated. However, analysis of subgroups formed according to parasite load showed that plasma IgG1 level correlated positively with IL-21 levels in the subgroup with low parasite density. This finding suggests that IL-21 is indirectly involved in lowering parasite density.

We also assessed the relationship between plasma IL-21 levels and hemoglobin levels. We found that IL-21 correlated positively with hemoglobin levels in younger children and in non-anemic and moderately anemic participants, regardless of the age group. Our study suggests that IL-21 is beneficial for hemoglobin production. Only one study has investigated the role of IL-21 outside the immune system; the authors reported that overexpression of IL-21 induces the expansion of hematopoietic progenitor cells [27]. Additional studies are therefore needed to confirm the putative role of IL-21 in erythropoiesis in the context of malaria.

Both IL-21 and anti-CD40 are required for the secretion of IgM, IgG1 and IgG3 [13]; thus, our results do not discriminate between the roles of IL-21 and anti-CD40 in inducing isotype switch. Basic research efforts to distinguish between these two potential switch signals should include elucidation of the structural requirements for interaction between CD19⁺ splenic B cells from *P. falciparum*-infected individuals and anti-CD40 monoclonal antibody in the presence of various concentrations of recombinant IL-21. Studies at the molecular level are required to determine the respective contributions of IL-21 and CD40 triggering.

Induction of IgG1 production by naive B cells in humans is also under the control of IL-10, a cytokine that can induce switching by activation of $\gamma 1$ and $\gamma 3$ germline promoters [13]. Whereas IL-10 is anti-inflammatory and plays an important role in inflammatory disorders, including malaria [28, 29]. IL-21 is included in the growing list of CD132-dependent cytokines with inflammatory properties *in vivo* and *in vitro* [30]. Molecular studies should facilitate determination of the contribution of IL-21 to malaria-induced inflammation.

Our findings support the notion that IL-21 induces naive sIgD⁺ B cells to switch towards IgG1 and IgG3, as: (i) naive B cells exclusively synthesize IgG1 and IgG3 in

response to IL-21, and do not produce IgG2, and (ii) various sources of naive B cells may yield the same isotypes.

Our findings are consistent with previous studies showing that IL-21 regulates the production of IgG1 and IgG3 isotypes [14] and support the hypothesis that other mechanisms are involved in isotype switching towards IgG1 and IgG3 during malaria infection, as reported elsewhere [31]. Moreover, we found that IL-21 production was associated with low parasite density and normal hemoglobin levels. Attempts to design efficient malaria vaccines have emphasized the critical role of the IgG1/IgG3 pattern [32], and IL-21 has been shown to bridge innate and adaptive immunity [33]. On the basis of our findings, we believe that additional studies are needed to clarify the role of IL-21 as an immunological factor in the protection against malaria.

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