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Research Article

Profiles of Immunoglobulin G Antibody Subclass Responses Specific to MSP3 and UB05 in Plasma of Malaria Negative Children Living in Two Different Agro-ecological Settings of Cameroon

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Abstract

Introduction: In malaria endemic areas, antibody specific to promising asexual blood stage malaria vaccine candidates have been demonstrated to play a critical role in protection during sub-clinical malaria. In this context naturally acquired protective immunity is usually driven by blood stage antigen specific IgG antibody subclass responses among which the cytophilic antibody subclasses IgG1 and IgG3 remain the most relevant. Thus, we have assessed IgG antibody subclass responses specific to Plasmodium spp. derived MSP3 and UB05 malaria vaccine candidates, in plasma of children living in areas differing in malaria transmission intensity within Cameroon.

Methods: Using MSP3 and UB05 displayed upon the surface of recombinant RNA coliphage Qβ as previously described in our group, IgG antibody subclass responses specific to both immunogens were profiled in plasma from both *P. falciparum* (Pf) infected and uninfected malaria asymptomatic children.

Results: In malaria negative children living in low transmission areas the cytophilic antibody subclasses IgG1 and IgG3 specific to UB05 were significantly higher (P<0.0001) than those specific to MSP3. In contrast IgG1 and IgG3 antibody subclass responses specific to MSP3 were instead significantly higher (P<0.0001 for IgG1; P=0.0007 for IgG3) in their counterparts living in high malaria transmission settings. In asymptomatic Pf infected children living in both areas, whereas IgG1 antibody subclass responses specific to MSP3 was significantly higher (P<0.0001) than the responses specific to UB05, IgG3 antibody subclass responses specific to UB05 was significantly higher (P<0.0001).

Conclusion: Thus, there is a differential generation of cytophilic antibody subclasses specific (IgG1 and IgG3) to two classical asexual blood stage antigens in children living within these areas in a malaria endemic region. Whereas for Pf negative children living in low malaria transmission areas UBO5 specific IgG1 and IgG3 correlated best with naturally acquired immunity against malaria; elevated MSP3 targeted cytophilic antibodies were instead prominent in high malaria transmission areas. Thus, repeated exposure to malaria as it is the case with bimodal as against monomodal rainfall areas might be necessary for sustaining high levels of MSP3 specific cytophilic antibodies. This probably tags MSP3 as an unsuitable candidate to measure correlates of protective immunity against malaria.

Keywords: IgG Subclasses; Asymptomatic *Plasmodium Infected* Children; Bimodal; Monomodal; Rainfall; QβUB05, QβMSP3

Introduction

Cameroon is like most Sub Saharan African (SSA) countries continues to bear the brunt of malaria with SSA alone accounting for 94 % of all global malaria cases and 95 % of related deaths in 2022 alone. Children under 5 years' old and pregnant women remain highly vulnerable bearing the lion shar of the disease. In 2022, 249 million people contracted malaria in 85 countries, with approximately 608,000 deaths [1]. A staggering 77% of these deaths occurred in children under the age of 5 years with most of the reported casualties being from SSA. *Plasmodium falciparum* remains both the primary cause of malaria and also the predominant cause of malaria mediated mortality in SSA in Sub-Saharan Africa [2]. Within malaria endemic regions repeated exposure to infections builds up naturally acquired immunity over time, but hardly leads to total protective immunity from clinical episodes of malaria. People living within these regions would rather develop just partial immunity to clinical malaria which makes them continuously vulnerable to the disease [3]. Immunity to malaria could be clinical during which individuals are protected from clinical episodes of the disease, anti-parasite immunity and sterilizing immunity in which low-grade, asymptomatic parasitaemia is maintained especially in people living in disease endemic areas a have been described [4,5]. People living in such areas develop a partial Naturally Acquired Immunity (NAI) very early in life as a result exposure to the causative agent [5]. A critical component of such immunity is Plasmodium species induced IgG antibody responses targeting a number of parasite derived antigens. IgG and IgG antibody subclasses specific to several asexual blood stage antigens including Apical Membrane Antigen 1 (AMA-1), Erythrocyte Binding Antigen (EBA-175), Reticulocyte-Binding Protein Homologue (Rh5), Glutamate-Rich Protein (GLURP), Merozoite Surface Proteins (MSPs)and UB05 have been demonstrated to be essential components of naturally acquired malaria specific immunity [6-8]. NAI develops gradually starting from protection from clinical symptoms till complete immunity that results to parasite control after repetitive infections over long periods in long term inhabitants of endemic regions [9]. Target antigens diversity and consequent elevated levels of parasite specific humoral immunity have been associated with such NAI. However, due to their inherent polymorphism inducing effective immunity targeting the asexual blood stage antigens remains a daunting challenge. Never the less NAI to asexual stage antigens are an essential component of future malaria vaccine candidates. Some elements of *P. falciparum* antigen specific antibody-mediated immunity have been suggested to cause auto-immunity through targeting host brain cells. A universal malaria vaccine candidate incorporating asexual blood stage must take into consideration these challenges.

Malaria transmission in some endemic countries like Cameroon varies in intensity across geographical regions within the country as a result uneven distribution of rainfall and tropical forest. The bimodal rainfall areas in which two distinct wet seasons are separated by a dry season there is higher malaria transmission intensity in contrast to monomodal areas with just one wet season. As a result, children less than five years within bimodal rainfall areas are continuously exposed to a higher intensity of malaria transmission. Since repeated exposure to malaria parasite drives NAI development within both regions; it is not known whether children living in monomodal rainfall areas could build up similar levels of IgG antibody subclass responses to currently malaria vaccine candidates under optimization. MSP-119 specific IgG and IgG antibody subclass responses have recently been demonstrated by Kwenti, et al., to vary considerably between Cameroonian children based on different bio-ecological strata [6]. To optimize universally applicable malaria vaccine candidates, it is necessary to select candidates which should provoke the same outcome irrespective of transmission intensity and geographical areas.

As previously demonstrated in our group and others; IgG antibodies specific to *Plasmodium falciparum* derived UB05 is surrogate marker for protective immunity to malaria especially in semi-immuned adults living within the Cameroonian rainforest [7]. In animal studies using laboratory-bred albino BALB/c mice strains it has also been shown that antibodies induced after immunization with recombinant fusion proteins consisting of UB05 and UB09 (UB05-09), could block not only parasitemia but equally protected from a lethal challenge with Plasmodium yoelii 17XL [10]. On the other hand, cytolyphilic antibody subclasses IgG1 and IgG3 have been demonstrated as a protective biomarker of Plasmodium spp. infection [11,12]. Cytophilic antibody subclasses possessed a high affinity for most Fc receptors on diverse immune cells and therefore play vital roles during complement fixation and opsonization [13]. This gives them the ability to mediate protection against malaria through complement-mediated lysis and cell-mediated mechanisms, such as opsonic phagocytosis and antibody-dependent cellular inhibition (ADCI) [14,15]. IgG2 and IgG4 have been classically considered as non-protective antibodies against malaria [16,17].
We previously demonstrated a differential expression of UBO5 and MSP3 specific IgG antibodies in these children (manuscript

in press). However, to understand the relevance of each IgG antibody subclass as a biomarker of NAI in the same population we have compared the IgG antibody subclasses targeting the same vaccine candidates. Using the same plasma and the antigens previously described in our group; their antigenicity relative to IgG antibody subclasses was determined for all study participants [18]. As previously described the recombinant QβMSP3phage displays the conserved C-terminal 88 AA of the Merozoite Surface Protein 3 (MSP3) whilst QβUB05 bears the previously described malaria antigen UB05 [7,19].

Cameroon has a wide variety of soils and climates that permit the division of the country into 5 main agro-ecological zones consisting of a forest zone with monomodal rainfall and a rainforest zone with bimodal rainfall [20]. Whilst previous studies in Cameroon have shown that malaria transmission is perennial in the forested regions little is known about immune response to malaria vaccine candidates in plasma from children from different ecological settings of the country [21,22]. We compared the IgG antibody subclass responses specific to MSP3 and UB05, in plasma from children living in two different agro ecological regions with perennial malaria transmission in Cameroon. This consisted of Buea situated in a forest zone with monomodal rainfall and Bikop deep in a rainforest with bimodal rainfall. Our study is important for selecting effective malaria vaccine candidates which can give similar immune outcomes across varying malaria transmission intensities thereby providing model immunogens for measuring correlates of protective immunity. This should enable informed design of potentially more effective malaria vaccines applicable in all regions irrespective of climatic conditions or transmission intensities.

Material and Methods

Description of the Study Area

This study was carried out in a dense rainforest area with bimodal rainfall (Bikop) in comparison with second dense rainforest region but instead with a monomodal rainfall (Buea) characteristics.

Bikop (3°31'00.0"N 11°25'00.0"E), is a rural health district located in the Center Region of Cameroon (Fig. 1), 48 Km away from Yaounde, the City Capital of Cameroon. The Bikop health district covers 28 rural communities with an estimated population of 30,000 inhabitants. This area is moderately accessible in all weathers. The climate is typically equatorial with an average annual temperature of 23.5°C and mean annual rainfall of 831.7 mm. There are two seasons; the dry season from November to February and June to August; the wet season from March to May and September to November. Agriculture and fishing are the main sources of livelihood [23,24].

Buea (4°09' 34''N 9° 14' 12"E) is presently the head quarter of the South West Region of Cameroon (Fig. 1). Buea health district covers 67 communities with an estimated population of 200,000 inhabitants, on a surface area of 870 km^2. Buea has an equatorial climate with two major seasons. Rainy season which runs from February to October and Dry season, from November to May. Temperature ranges between 20°C to 28°C while, annual rainfall ranges between 3000 mm to 5000 mm. Buea Municipality is bounded to the North by a tropical forest on the slope of mount Cameroon (4100 m above sea level), to the South-West by the city of Limbe, to the South-East by the city of Tiko, to the East by the town of Muyuka and Idenau district to the West. Agricultural, administrative, business, tourism and the financial sector are the main sources of livelihood in Buea. Whereas 60% of the population have financial difficulties in accessing healthcare services, about 40% of the population do not have access to quality health care [24,25].

Study Design and Ethical Considerations

The study was carried out during the months of November 2017, February and November 2018. We obtained ethical clearance from the Cameroon national Ethics Committee for research of Human health (CNERSH) and an authorization from the Ministry of Public health of Cameroon to collect samples from the Bikop Catholic health facility and Buea health district. Meetings were organized with the parents, schools and health facilities staff, during which the project objectives, methods and possible benefits/risks was clearly explained. Thereafter, children were invited to participate in the survey. Children with parental assent were interviewed in the presence of parent and using a pretested questionnaire, socio-demographic information, history of previous malaria episodes, together with treatment history was collected. A general clinical evaluation was carried out for each child by a competent clinician. Children with acute malaria symptoms such, fever (>37.5°C) or chills or those recently sick (three months prior to data collection) or under antimalarial treatment were excluded from the study. All diagnosed cases of malaria were treated for free, using Artesunate-lumefantrine combination therapy according to recommended national treatment

strategies for malaria.

Data And Samples Collection and Processing

A total of 123 children in Bikop and 99 children in Buea was enrolled in the study. Based on the selection criteria, only 68 children in Bikop and 66 others in Buea respectively, were finally enrolled in the study. Temperature was recorded using an infrared body thermometer. Blood sample was collected using sterile disposable syringes into a 2.5 mL labelled Ethylene Diamine Tetra Acetate (EDTA) tubes, at the Bikop Catholic health facility and Buea Hospital. While the Rapid Diagnostic Test for malaria was immediately done on the site, the rest of the blood for each study participant were transported to CIRCB on ice in a cool box with temperature between +2 and +8°C for serological analyses.

Laboratory Investigations

Malaria Diagnosis

The SD Bioline Malaria antigen uses Histidine-Rich Protein 2 (HRP 2) to detect *P. falciparum* and Lactose Dehydrogenase (LDH) for *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* [26,27]. This test is a WHO prequalified test useful for the region where all malaria species are circulating, with a reported sensitivity of 99.7% for *P. falciparum* and 95.5% for *P. vivax* and a 99.3%specificity [28]. The test was used according to manufacturer's instructions. To ensure the validity of the results, RDTs were read within a 15 minutes timeframe by two independent laboratory technicians. We also used optical microscopy for the diagnostic of malaria during this study. Optical microscopy of thick and thin stained blood smears remains the standard method for diagnosing malaria. It involves Giemsa staining and examination of malarial parasites.

- Estimation of Parasite Density

We counted all parasites and white blood cells in the final field and recorded the numbers on an appropriate worksheet. When counting was completed, parasite densities for all participants were calculated using an estimated average white cell count of 8000/μL for children aged more than five years. In addition, we used the WBCs reference values established for children less than five years (9200/μL).

The following formula was used for the calculation [29]:

$$\text{Parasites / μL blood} = \frac{\text{Number of parasites counted} \times \text{Number of white blood cells/μL}}{\text{Number of white blood cells counted}}$$

Study Antigens

The antigens consisted of recombinant Qβ displaying *Plasmodium falciparum* 3D7 strains derived N-terminal part of MSP3 (QβMSP3) and UB05 (QβUB05) generated as previously described in our group [30].

Determination of IgG Antibody Subclass Responses Specific to QβUB05 and QβMSP3

The plasma levels of IgG1, IgG2, IgG3 and IgG4 antibodies specific to the malaria antigens QβUB05 and QβMSP3 were determined using ELISA assays as previously described in our group [30]. Briefly high binding ELISA plates were coated with 107 particles/well of each recombinant phage and incubated overnight at 4°C. The following day, Plates were washed 3x with PBST (PBS containing 0.05% Tween 20) and blocked with 3% BSA in PBS for one hour at 37°C. Heat inactivated plasma samples were diluted in PBS at 1:500 then 100 μl/well added in triplicates and incubated for two hours at 37°C. The plates were washed four times with PBST after which the bound antibodies were probed with the peroxidase-conjugated mouse anti-human IgG1 or IgG2 or IgG3 or IgG4 diluted 1:4000 in 1X PBS. Bound conjugate was detected using ABTS substrate and stop solution according to the manufacturer's protocol (Southern Biotech, Birmingham USA). The colorimetric signal was measured at 405 nm using a multiscan FC microplate reader (Thermo Fisher Scientific, USA).

Data Analysis

Data were entered using excel 2013 and analyzed with the prism graph pad 6.0 statistical software. Group means were compared using Analysis of Variance (ANOVA) and Student's -test. Statistical significance was confirmed when $P < 0.05$.

Results

IgG1 and IgG3 Antibody Responses Specific to UB05 and MSP3 in Malaria Negative Children Living in Monomodal and Bimodal Rainfall Areas

In Fig. 1 data is shown for IgG1 and IgG3 antibody responses specific to either recombinant QβUB05 or QβMSP3 in plasma of malaria negative children from both monomodal and bimodal rainfall areas. A comparison is made between UB05 and MSP3 IgG1 and IgG3 specific antibody responses. In Fig. 1 data is shown for IgG1 antibody subclass responses specific to recombinant UB05 in comparison to responses specific to MSP3. In Fig. 1 data is shown for IgG3 antibody responses specific to recombinant UB05 compared to response specific to MSP3.

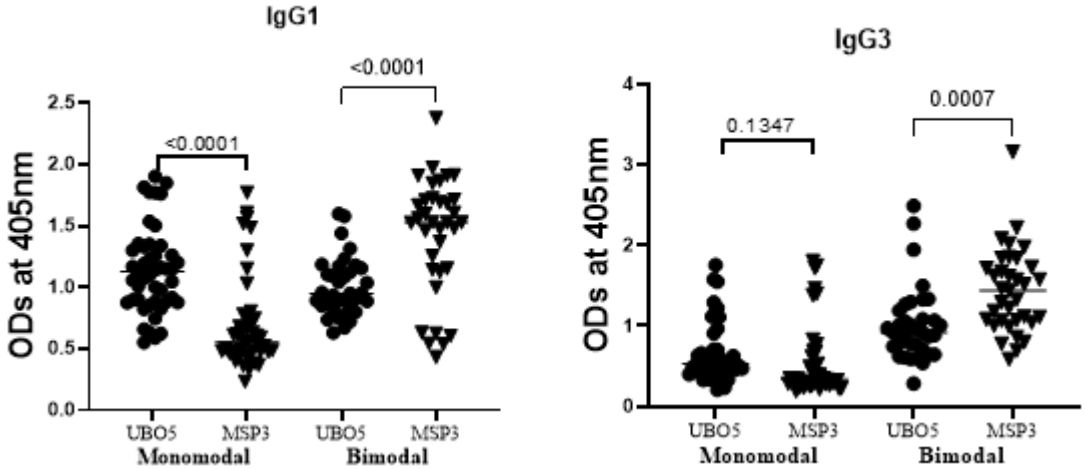

Figure 1: Comparison between IgG1 and IgG3 antibody subclass responses specific to UB05 and MSP3 in malaria negative children.

In Pf negative children living in monomodal rainfall areas (low transmission regions), IgG1 antibody subclass responses specific to UB05 were significantly higher than the responses specific to MSP3 ($p<0.0001$). The converse was true with their counterparts living in bimodal rainfall area (high transmission settings), as IgG1 antibody subclass responses specific to MSP3 was significantly higher ($p<0.0001$). With respect to IgG3, whereas the subclass antibody responses specific to MSP3 were higher than those specific to UB05 ($p=0.0007$) in Pf negative children living in bimodal rainfall area, no significant difference was observed in Pf negative children living in monomodal rainfall area ($p=0.1347$) (Fig. 1).

IgG1 and IgG3 Antibody Responses Specific to UB05 and MSP3 in Asymptomatic Pf Infected Children

In Fig. 2 data is shown for IgG1 and IgG3 antibody subclass responses specific to either recombinant UB05 or MSP3 in plasma of asymptomatic Pf infected children living in both monomodal and bimodal rainfall areas. A comparison is made between UB05 and MSP3 specific IgG1 and IgG3 antibody responses. In Fig. 2 data is shown for IgG1 antibody responses specific to recombinant UB05 in comparison with the response specific to MSP3. In Fig. 2 data is shown for IgG3 antibody responses specific to recombinant UB05 in comparison with response specific to MSP3.

In asymptomatic Pf infected children living in both monomodal and bimodal rainfall areas, IgG1 antibody response specific to MSP3 was significantly higher than the response specific to UB05 ($p<0.0001$ for both areas). The converse was true for the IgG3 antibody specific response, as the UB05 specific response was higher in both monomodal ($p<0.0001$) and bimodal ($p=0.0626$) rainfall areas (Fig. 2).

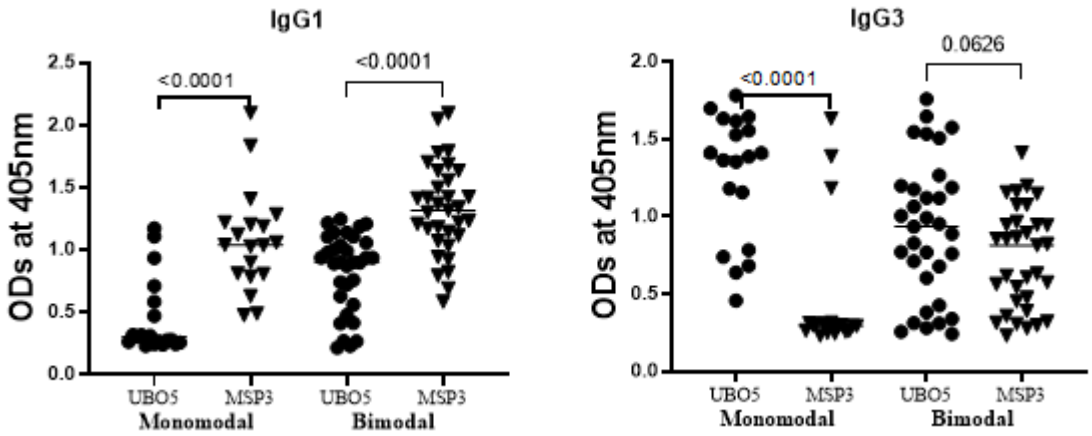

Figure 2: Comparison between IgG1 and IgG3 antibody subclass responses specific to UB05 and MSP3 in asymptomatic Pf infected children.

IgG2 and IgG4 Antibody Responses Specific to Both UB05 and MSP3 in Malaria Negative Children

In Fig. 3 data is shown for IgG2 and Ig4 antibodysubclass responses specific to either recombinant UB05 or MSP3 in plasma of malaria negative children from both monomodal and bimodal rainfall areas. A comparison is made between UB05 and MSP3 specific IgG2 and IgG4 antibody responses. In Fig. 3 data is shown for IgG2 antibody responses specific to recombinant UB05 in comparison with that specific to MSP3. In Fig. 3 data is shown for IgG4 antibody responses specific to recombinantUB05 compared to the response specific to MSP3.

In Pf negative children, IgG2 and IgG4 antibody subclass responses specific to UB05 was higher than the response specific to MSP3 ($p<0.0001$) in low transmission area. However, no significant differences were observed in the IgG2 and IgG4 subclass antibody responses specific to UB05 and MSP3 in Pf negative children living in bimodal rainfall areas ($p=0.3467$ for IgG2 and $p=0.6987$ for IgG4) (Fig. 3).

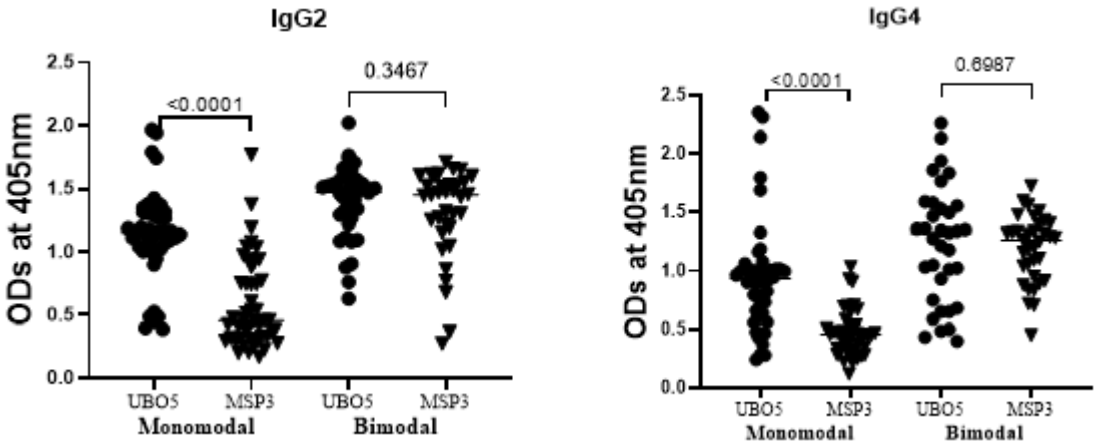

Figure 3: Comparison between IgG2 and IgG4 antibody responses specific to UB05 andMSP3 in malaria negative children.

IgG2 and IgG4 Antibody Subclass Responses Specific to UB05 and MSP3 in Asymptomatic Pf Infected Children

In Fig. 2 data is shown for IgG1 and IgG3 antibody subclass responses specific to either recombinant UB05 or MSP3 in plasma of asymptomatic Pf infected children living in both monomodal and bimodal rainfall areas. A comparison is made between UB05 and MSP3 specific IgG1 and IgG3 antibody subclass responses. In Fig. 4 data is shown for IgG1 antibody responses specific to recombinant UB05 in comparison with the response specific to MSP3. In Fig. 4 data is shown for IgG3 antibody subclass responses specific to UB05 compared to that specific to MSP3.

In asymptomatic Pf infected children, whereas IgG2 antibody response specific to UB05 was higher than that specific to MSP3 ($p<0.0001$) in bimodal rainfall area, no significant difference was observed in monomodal rainfall area ($p=0.6953$). On the other hand, IgG4 antibody subclass responses specific to MSP3 was significantly higher when compared to thatspecific to UB05 in both monomodal ($p<0.0001$) and bimodal ($p=0.0002$) rainfall areas (Fig. 4).

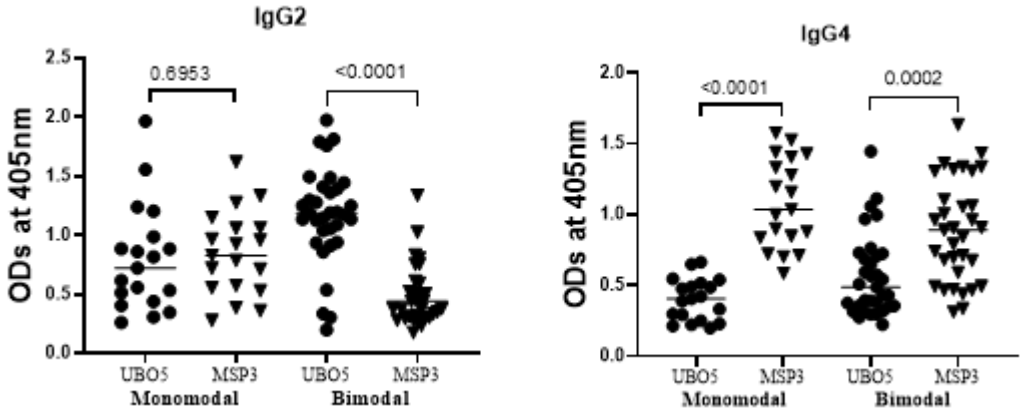

Figure 4: Comparison between IgG3 antibody subclass responses specific to UB05 compared to that specific to MSP3.

Discussion

A number of factors including changes in temperature, rainfall, humidity as well as the level of immunity in humans are associated with the transmission of the malaria parasites. The malaria parasite specific IgG antibody subclass mediating cytophilic IgG responses to *Plasmodium falciparum* merozoite antigens remain unclear. However, this response has been shown to play a role in protection against symptomatic and asymptomatic malaria. In fact, cytophilic antibodies (IgG1 and IgG3) are known to play vital roles in combating infection through reducing parasitaemia and clinical symptoms [31]. During this process malaria specific IgG antibody subclasses, are considered the most potent asternal as a result of their efficiency in activating complement and the also leukocytes through binding to FcγRI and FcγRIII [31,32]. For this reason, profiling naturally acquired immunity to malaria in infants living in endemic regions could enable the detection of desirable merozoite derived immunogens for potential vaccine candidate optimization. In this population based cross sectional study we profiled IgG antibody subclasses responses specific to MSP3 and UB05 in plasma from asymptomatic *Plasmodium falciparum* infected and negative children living in two regions of Cameroon differing in rainfall characteristics. In Pf negative children, whereas in children living in low transmission area IgG1 and IgG3 antibodies responses specific to UB05 was higher than the response specific to MSP3, the converse was true with their counterparts living in high transmission settings, as IgG1 and IgG3 antibodies responses specific to MSP3 were higher. This probably suggest that cytophyllic antibody responses specific to UBO5 could be a better correlate of naturally acquired immunity against malaria in low transmission settings, while cytophyllic antibody responses specific to MSP3 are a better correlate of naturally acquired immunity against malaria in high transmission settings. Nevertheless, such high levels of circulating pathogen specific antibody subclasses in the presence of high levels of infections could simply be an indication of an immune system exposure to uncontainable amounts of irrelevant antigens useless for infection eradication. Although immunity to malaria is usually seen to be species- and/or strain-specific, it has previously been demonstrated that differences in MSP3 and UB05 specific IgG and IgG antibody subclass responses are dependent upon the antigen type, malaria transmission intensity and malaria infection [7,33].

On the other hand, asymptomatic Pf infected children living in both low and high transmission settings, showed significantly higher IgG1 antibody response specific to MSP3 than the response specific to UB05 ($p<0.0001$) [34]. In contrast, IgG3 antibody specific responses to UB05 was higher in both areas. The rate of acquisition of antibodies to different merozoite antigens is known to vary and this may impact on the acquisition of complement fixing antibodies and also on the role they play in protective immunity to malaria [35]. Cytophilic antibody subclasses including IgG1 and IgG3, can potently activate complement withIgG3 antibody subclass know to have a greater activity than IgG1. In fact, IgG3 is known to have the longest hinge region which provides a flexible spacer between the Fc region and Antigen Binding fragment (Fab) facilitating its ability to activate complement as compared to the IgG1 antibody subclass [32-34]. The fact that IgG3 antibody subclass responses specific to UB05

in asymptomatic *Plasmodium falciparum* infected children was higher in both areas, might suggest probably a high sensitivity to complement pathway activation by cytophilic antibody subclasses specific to UB05. Thus, exposure to UB05 enable not only IgG1 response, but also strong IgG3 response which compensate for the shorter half-life of IgG3.

Our results indicate that the IgG subclass antibody responses specific to recombinant malaria vaccine candidates UB05 and MSP3 are induced early in children and could vary dependent upon the climatic conditions driving parasite transmision intensity [35]. Therefore, in Bikop areas with bimodal rainfall and two annual peaks of exposure to *Plasmodium falciparum* infection, children are more exposed and thereby more likely to develop a higher immune response to MSP3 and UB05 compared to children from Buea with monomodal rainfall and one annual peak of exposure to *Plasmodium falciparum* infection. Our data demonstrates that antibody subclass responses specific to UB05 could be more reliable index protective immunity to malaria irrespective of the climatic conditions and malaria transmission intensity. In addition, UB05 is also a *Plasmodium falciparum* immunodominant antigen, which had previously been reliably used to predict semi-immunity to malaria in adults living in a monomodal area of Cameroon [8,10]. Our work extends these findings not only to children but equally highlight its importance as a component for future malaria vaccine candidates applicable also in bimodal rainfall areas. The recombinant QβUB05 phage display platform hereby represent a promising reliable tool for monitoring protective immunity in children living in malaria endemic areas especially for sub-Saharan Africa.

Conclusion

Overall, the IgG antibody subclass responses specific to both antigens are relatively heterogeneous being certainly influenced by a number of factors including the nature of the antigen, rainfall conditions of the area and malaria parasite load. Nevertheless, there was a clear indication that IgG1 and IgG3 antibody subclass responses specific to UBO5 correlated better with naturally acquired protective immunity to Pf infection irrespective of the rainfall conditions. Thus, cytophilic IgG1 and IgG3 antibody subclass antibody responses specific to UBO5 should be further characterized as a probable correlate of naturally acquired immunity to malaria in Pf negative Cameroonian children. Nevertheless, continuous or repeated exposure to malaria as it is the case in bimodal rainfall area compared to monomodal rainfall area probably contributes to the shorter half-life of IgG3.

Conflict of Interests

The authors have no conflict of interest to declare.

Ethics Approval and Consent to Participate

This study received ethical approval from the Cameroon National Ethics Committee for Human Health Research (Reference numbers 2015/03/561/CE/CNERSH/SP and 2018/01969/CE/CNERSH/SP) and the CIRCB institutional review board (protocol number 14-11). All participants provided written informed consent. Data were processed using specific identifiers for privacy and confidentiality purposes. Clinical data generated during the course of this study was provided free of charge to all participants.

Availability of Data and Materials

All data are fully available without restriction. Data are available from the CIRCB Institutional Data Access/Ethics Committee for researchers who meet the criteria for access to confidential data." All request for Data should be addressed to the General Manager of CIRCB reachable by the following address:

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E-mail: andjolo@yahoo.com or andjolo@circb-cm

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Competing Interests
The authors declare that they have no competing interests.

Author Contributions
Conceived and designed the experiments: GWN, ABW, CE, EA, MO and HFO
Performed the experiments: HFO, LNN, AL, VE, GC and PEA
Technical assistance: JCT, GA, CGP, ABW and JNCA
Analyzed the data: GWN and HFO
Wrote the paper: HFO and GWN

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