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ORIGINAL RESEARCH

Expression of CD11a, CD11b, CD11c, and CD18 on Neutrophils from Different Clinical Types of Malaria in Malawian Children

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Background: Malaria in individuals who have never had an infection before is usually characterized by an inflammatory response that is linked to the expression of specific activation markers on cells of the innate immune system.

Methods: This study investigated absolute white blood cell (WBC) and neutrophil counts and expression of several adhesion markers on neutrophils from HIV-uninfected children who were suffering from cerebral malaria (n=35), severe malarial anemia (SMA, n=39), and uncomplicated malaria (n=49) and healthy aparasitemic children (n=33) in Blantyre, Malawi.

Results: All clinical malaria groups had higher WBC and neutrophil counts compared to healthy controls, with the acute SMA group having significantly ($p<0.0001$) higher WBC counts than the controls. These elevated counts normalized during recovery. Surprisingly, in all clinical malaria groups, the surface expression of CD11b, CD11c, and CD18 on neutrophils was lower than in healthy controls, again normalizing during convalescence.

Conclusion: In areas where *Plasmodium falciparum* malaria is hyperendemic, such as where this study was conducted, neutrophils have reduced expression of adhesion molecules and activation markers during acute stages of the infection, regardless of the clinical type of malaria. This reduced expression could be due to an individual's past exposure to *P. falciparum* or other parasite-related factors that manifest during active malaria that still need to be investigated.

Keywords: white blood cells, neutrophils, malaria, integrins, receptors

Introduction

Despite significant gains achieved in mitigating the detrimental effect of malaria globally,[1,2] malaria caused by the *Plasmodium falciparum* parasite still causes death in thousands of children annually, with a majority of these occurring in sub-Saharan Africa.[3] Although the actual mechanistic cause of death is yet to be fully elucidated, it is now known that clinical *P. falciparum* malaria can manifest either as uncomplicated malaria (UM) or one of the severe forms of the disease — cerebral malaria (CM), severe malarial anemia (SMA), or respiratory distress — and other complications, with a number of possible overlaps among these syndromes.[4,5] Of these syndromes, CM is associated with the worst outcomes, contributing to the highest number of deaths,[4] severe neurological sequelae, and debilitating neurological impairments in survivors.[6]

Neutrophils make up the highest proportion of peripheral leukocytes — 40%–60%.[7] These cells are known to play a crucial role in the innate immunoresponse during the early stages of infectious diseases, mainly through clearance of pathogens by

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phagocytosis.[8,9] Studies conducted in Africa have shown that peripheral neutrophil counts increase by as much as 40% during acute UM, with the degree of increase being associated with the level of parasitemia.[10] Furthermore, it is now known that neutrophils are not only the primary circulating cells to respond to an invading pathogen[9] but that once they are activated, these cells are capable of phagocytosing infected red blood cells (iRBCs).[11] Like other circulating immune-system cells, neutrophils too need to express molecules involved in migration (chemotaxis) and adhesion.[9,12]

During an inflammatory response, as is the case during acute malaria, adhesion molecules work to facilitate pairing between various receptors and their respective ligands and also transmit intrinsic signals, which in turn confer unique effector functions.[13] It is estimated that four superfamilies of adhesion molecules are involved in these events, some of which include the selectins (L-selectin, E-selectin, and P-selectin), the integrins, some members of the immunoglobulin superfamily, and cadherins.[14] A large family of heterodimeric glycoproteins, integrins are grouped according to the particular β subunit they possess. Based on this, β_2 integrins are expressed exclusively on leukocytes and participate in leukocyte adhesion to the endothelium. They are also involved in subsequent transmigration through interactions with molecules expressed on the surface of endothelial cells, including ICAM1. These heterodimeric adhesion molecules consist of a common βsubunit (CD18) that associates with one of four αsubunits: CD11a (LFA1), CD11b (MAC1), CD11c (p150), or CD11d.[15] Based on their amino-acid homology, CD11b and CD11c are quite similar proteins, with close to 50% amino-acid similarity. Several ligands have been described for CD11b/CD18, including iC3b, fibrinogen, heparin, ICAM1 (CD54), ICAM2 (CD102), and ICAM4.[16] However, less is known about CD11c/CD18, which is expressed mainly on monocytes, granulocytes, and activated T and B cells and has been identified as the ligand for ICAM1.[15]

During the life-cycle stages of *P. falciparum* that occur inside RBCs, hemoglobin is degraded, but the parasite is unable to catabolize heme. The unprocessed heme ends up aggregating, forming an insoluble polymer called malarial pigment or hemozoin.[17] Various studies have demonstrated that monocytes and macrophages ingest hemozoin or hemozoin-containing parasitized RBCs, which results in a large proportion of these cells being loaded with hemozoin during malaria infection and sometimes for months postinfection.[18] Other studies have further shown that phagocytosis of hemozoin can actually interfere with the expression of CD11c and CD11b, a receptor for complement components C3b and C4b.[19] In addition, hemozoin-fed monocytes have been observed to be viable but functionally impaired, with hemozoin promoting suspected of contributing to cell dysfunction, downregulation of phagocytosis, and reduced expression of MHC2 and CD54.[19]

Although neutrophil numbers are known to increase during acute malaria, little is known about the expression of various migration and adhesion receptors by neutrophils in peripheral. More specifically, not much is known on how neutrophil-adhesion molecules, such as CD11a, CD11b, and CD18, are affected during malaria infection. The goal of this study was thus to determine the absolute neutrophil numbers and characterize the surface expression of leukocyte-adhesion molecules CD11a, CD11b, CD11c, and CD18 on neutrophils in peripheral blood of children acutely suffering from different clinical types of *P. falciparum* malaria in comparison with blood samples from healthy controls.

Methods

Study Participants

Details of the study design and study participants have been reported previously.[20] In summary, the study was conducted at the Malawi Liverpool Wellcome Trust Clinical Research Programme and Department of Paediatrics, College of Medicine, University of Malawi, and Blantyre Malaria Project. Study participants were children who were either acutely suffering from malaria at the time of recruitment or had been admitted to Queen Elizabeth Central Hospital with malaria, and children who were medically well at the time of recruitment and were attending surgical outpatient clinics at Queen Elizabeth Central Hospital and Beit Cure International Hospital, both located in Blantyre, Malawi. Study participants were recruited mainly during the malaria season, which usually coincides with the rainy season (November 2005 to April 2006).

Firstly, informed written consent was obtained from the parent or guardian of each participant, then each potential participant was examined by a research nurse and/or a clinical officer. Once the participant had fulfilled the recruitment criteria, baseline demographic data were recorded and a 5 mL peripheral blood sample collected. All participants were then assessed for level of consciousness using the Blantyre Coma Score. This was done

several times, starting from admission and then at 2- to 4-hourly intervals during intensive clinical care. In summary, well over 40 children were prospectively enrolled into each of the three clinical malaria groups, defined by diagnoses of CM, SMA, and UM, and matched the criteria for the healthy control group.

For this study and a previous one on the same participants,[20] malaria was defined as a clinical syndrome without any other apparent possible cause, and was confirmed by a thick blood film positive for *P. falciparum* asexual parasites on microscopy. Study participants who presented with CM had a Blantyre Coma Score of two or less at admission and 4 hours later, while participants in the other malaria groups (SMA and UCM) and healthy controls had a score of five at both times.[20] Study participants with SMA had a blood-hemoglobin concentration of 5 g/dL or less, whereas those in the other groups had a hemoglobin concentration above this level. All participants who tested positive for HIV infection during the screening stage were excluded from the study and immediately referred to the antiretroviral therapy clinic. Following exclusions, 113 children with malaria (54 with UM, 30 with SMA, and 29 with CM) and 42 healthy controls were recruited. Of the children with malaria, 73 (34 UM, 21 SMA, and 18 CM) were successfully followed up a month after treatment, baseline demographic data recorded, and a 5 mL peripheral blood sample collected for laboratory investigations. Ages and sex ratios of the study participants for each group are provided in Supplementary Table 1.

Ethics Review and Approval

Ethics review and approval for the study was obtained from College of Medicine Research and Ethics Committee. Written informed consent was obtained from the parent or guardian of every child before the child was recruited into the study. A 5 mL venous blood sample was taken at the time of recruitment and during the recovery period. Blood was collected in tubes that already had EDTA anticoagulant in them, and these samples was used for immunophenotyping. This study was conducted in accordance with the Declaration of Helsinki.

Blood-Sample Analyses

Samples were analyzed as previously reported.[20] For each sample, 25 μL EDTA blood was mixed with 1 μL of three directly conjugated antibodies, as listed in Table 1. In one tube, 1 μL of each of the antibodies anti-CD11a-FITC, anti-CD11b-PE, and anti-CD14-PerCP was mixed. In another tube, 1 mL of anti-CD18-FITC, anti-CD11c-PE, and anti-CD14-PerCP was mixed. A third tube was included that was labeled with isotype controls. Samples were incubated for 15 minutes in the dark at room temperature. FACS lysing solution (500 μL; Becton Dickinson) was added to each tube and incubated in the dark for 10 minutes at room temperature. Cells were washed twice with 2 mL PBS and fixed with 100 μL PBS–1% formaldehyde. Data were acquired on a BD FACSCalibur flow cytometer and analyzed using CellQuest Pro software. CD14-PerCP was used to set a gate for monocytes, and this simplified the gate setting for neutrophils, which were distinguished by light- and forward-scatter characteristics (Figure 1).

Data Analysis

Results from the four groups (healthy controls, UCM, SMA, and CM) were analyzed using GraphPad Prism 6.01 (GraphPad Software, San Diego, CA, USA). $p<0.05$ was considered statistically significant. The Kruskal–Wallis test was employed in the comparison of geometric mean fluorescent intensity (GMFI) values for the markers CD11a, CD11b, CD11c, and CD18 on neutrophils.

Table 1 Monoclonal antibodies and their corresponding cell population. In each tube, 25 μL EDTA blood was mixed with 1 μL of the indicated antibodies. In tube 1, representing the isotype controls, the blood sample was mixed with 1 μL of each of anti-moGI-FITC, anti-G2a-PE, and anti-CD14-PerCP. In tube 2, the blood sample was mixed with 1 μL of each of anti-CD11a-FITC, anti-CD11b-PE, and anti-CD14-PerCP. In tube 3, the blood sample was mixed with 1 μL of each of anti-CD18-FITC, anti-CD11c-PE, and anti-CD14-PerCP. In tube 4, the blood sample was mixed only with anti-CD14-PerCP, since this was the negative control

	FITC	PE	PerCP	
Tube 1	moGI	moG2a	CD14	Isotype controls
Tube 2	CD11a	CD11b	CD14	CD11a- and CD11b-expressing neutrophils
Tube 3	CD18	CD11c	CD14	CD18- and CD11c-expressing neutrophils
Tube 4	—	—	CD14	Negative control

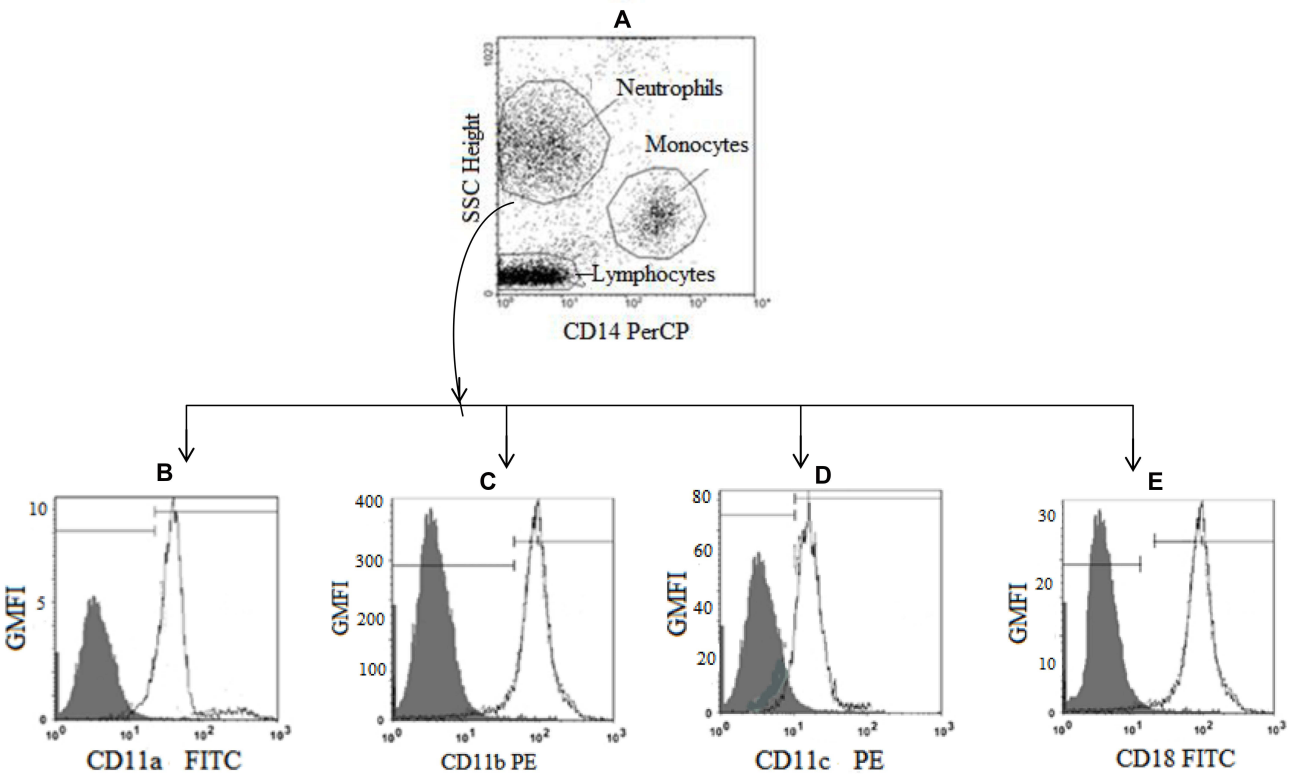

Figure 1 Gating strategy for determining the neutrophil population in a side-scatter/CD14 plot (**A**), with the neutrophil gate set to exclude monocytes and lymphocytes (**A**), geometric mean fluorescent intensity (GMFI) values for neutrophil surface markers CD11a (**B**), CD11b (**C**), CD11c, (**D**) and CD18 (**E**). The gray histograms represent the GMFI of the isotype controls, and the white histogram represents the GMFI of the different surface markers.

Wilcoxon matched-pair tests was used to determine the statistical significance of differences in GMFI values observed during acute infection and the recovery stage for UCM, SMA, and CM. The results in Figures 2–4 are presented as medians and 10th and 90th percentiles of absolute cells counts for white BCs (WBCs) and neutrophils and GMFI values for CD11a, CD11b, CD11c, and CD18 on neutrophils during the acute and convalescent stages of UCM, SMA, and CM. For these scatterplots, the bottom and lines through the middle of the dots correspond to the median value (10th percentile at the bottom to 90th percentile at the top).

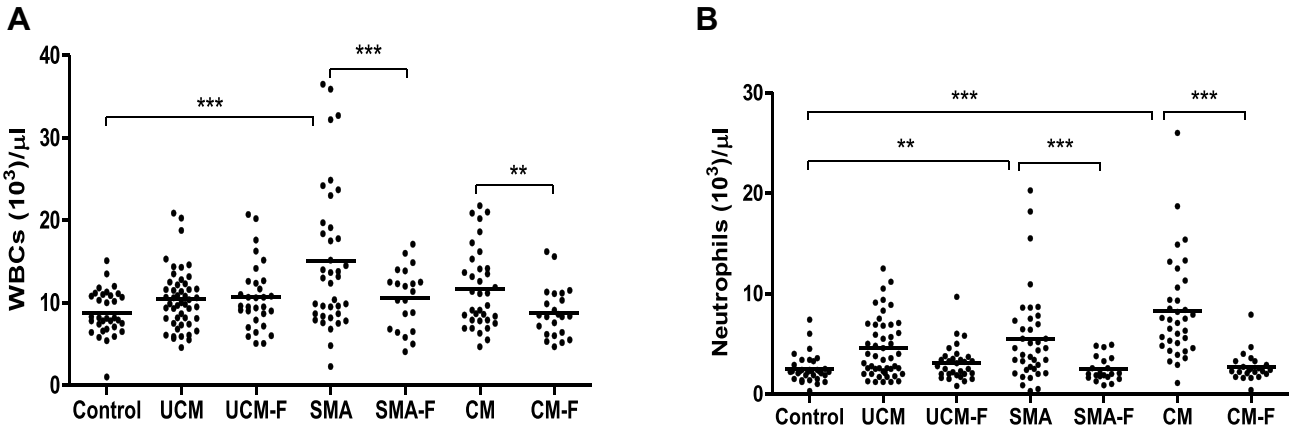

Figure 2 Medians (10th and 90th percentiles) of absolute white blood–cell counts (**A**) and neutrophil (**B**) counts in healthy controls and different types of malaria during acute stages of infection (UCM, SMA, CM)* and during convalescence (UCM-F, SMA-F,CM-F)# at 30 days after treatment. $p<0.05$ considered statistically significant. **$p<0.01$; ***$p<0.0001$.

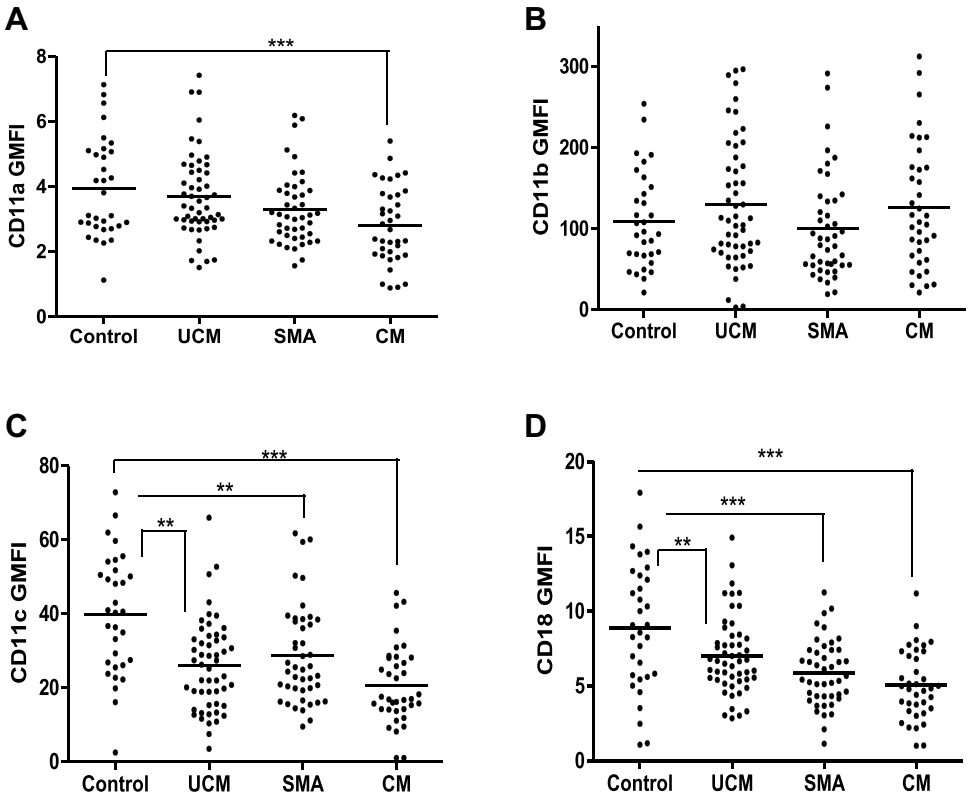

Figure 3 Medians (10th and 90th percentiles) of GMFI of CD11a (**A**), CD11b (**B**), CD11c (**C**), and CD18 (**D**) on neutrophils during acute stages of different clinical types of malaria (UCM, SMA, and CM)* and in healthy controls. $p<0.05$ considered statistically significant. **$p<0.01$; ***$p<0.0001$.

Results

Absolute Cell Counts

WBCs (Figure 2A) were higher in all three malaria types when compared to counts in the healthy controls, but the difference was statistically significant ($p=0.0001$) only between the median counts for SMA (13.1×10^3/μL) and for healthy controls (8.2×10^3/μL). Comparing cells counts during acute and convalescent stages, WBC counts were significantly ($p=0.027$) lower (11.4×10^3 cells/μL) in the convalescent stage of SMA than in acute infection (13.1×10^3 cells/μL), but this count was still higher than WBC counts in controls, although the difference between the medians was not statistically significant ($p=0.053$). Neutrophils (Figure 2B) were significantly ($p<0.0001$) higher (7.4×10^3 cells/μL) in CM patients than controls (2.30×10^3 cells/μL), UCM patients (3.7×10^3 cells/μL), and in SMA patients (4.48×10^3 cells/μL). The median of neutrophil counts in SMA participants was also significantly ($p=0.016$) higher than controls. The high neutrophil counts (7.4×10^3 cells/μL) observed in the acute-CM patients normalized in convalescence (during follow-up, 2.74×10^3 cells/μL), and the difference between counts in acute disease and convalescence was significant ($p<0.0001$, Figure 2B). The high neutrophil counts (4.48×10^3 cells/μL) observed in acute SMA normalized in convalescence (2×10^3 cells/μL), and this difference was significant ($p=0.0036$).

CD11a, CD11b, CD11c, and CD18 Expression on Neutrophils During Acute Malaria

In acute infection, the expression of CD11a (Figure 3A) — but not CD11b (Figure 3B) — CD11c (Figure 3C), and CD18 (Figure 3D) decreased in all malaria types. The decrease in the expression of CD11a was significant ($p=0.0023$) in acute CM, and the expression of CD18 was also significantly lower for acute UCM ($p=0.0319$), SMA ($p=0.0006$) and CM ($p<0.0001$) than healthy controls. The expression of CD11c (Figure 3C) was significantly lower for UCM and CM (both $p<0.0001$) and for SMA ($p=0.0015$) than healthy controls. There were no significant differences in expression of CD11b among any of the three malaria types (Figure 3B) or in comparison with healthy controls.

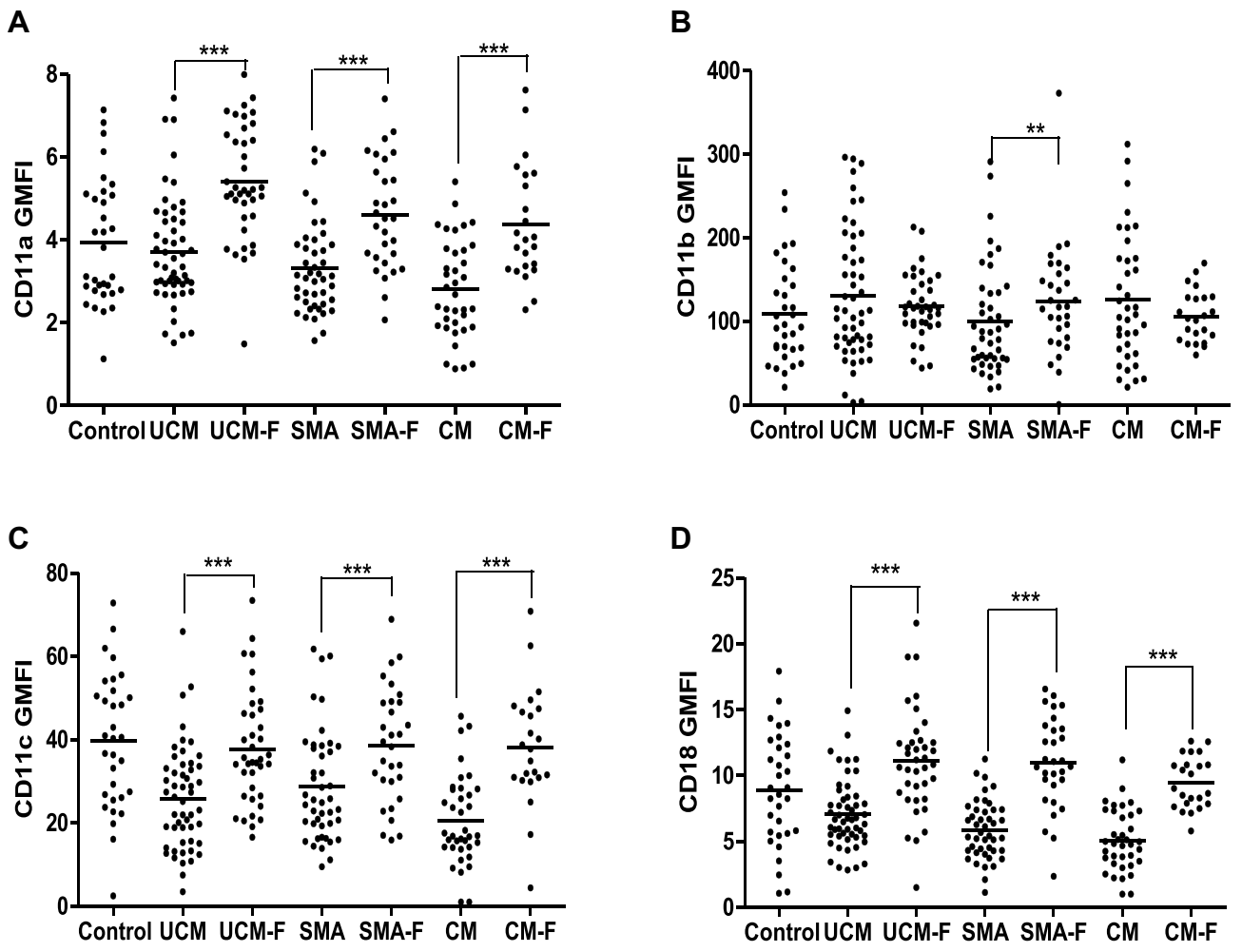

Figure 4 Medians (10th and 90th percentiles) of GMFI of CD11a (**A**), CD11b (**B**), CD11c (**C**), and CD18 (**D**) on neutrophils in healthy controls and in acute stages of infection (UCM, SMA, and CM)* and during convalescence (UCM-F, SMA-F and CM-F)# when at 30 days after treatment. $p<0.05$ considered statistically significant. **$p<0.01$; ***$p<0.0001$.

Abbreviations: UCM, uncomplicated malaria; CM, cerebral malaria; SMA, severe malarial anemia; UCM-F, convalescent stage of uncomplicated malaria, CM-F, convalescent stage of cerebral malaria; SMA-F, convalescent stage of severe malarial anemia.

CD11a, CD11b, CD11c, and CD18 Expression on Neutrophils During Recovery Stage

The expression of CD11a (Figure 4A) — but not CD11b (Figure 4B) — CD11c (Figure 4C), and CD18 (Figure 4D) was significantly ($p<0.0001$) higher during recovery for all malaria types than in acute malaria. Although increases were observed in the expression of CD11b in convalescence (Figure 4B), the difference between acute and convalescent stages was significant ($p=0.0380$) only in SMA patients. Overall, the expression of all these markers in convalescence increased to almost identical levels to those observed in healthy controls (Figure 4A–D).

Discussion

In a previous study on a Malawian cohort,[21] WBC counts were observed to decrease with age from 18 months to adulthood. The observation in the current study of higher WBC counts in all malaria groups than controls suggests that the increase in WBC counts was in response to acute malaria infection. The high WBC counts observed in severe malaria (CM and SMA) were not consistent with studies conducted elsewhere,[22,23] which reported lower-than-normal total WBC counts during acute severe *P. falciparum* malaria. However, this study's results are consistent with other studies[24–26] that also reported higher-than-normal WBC counts in *P. falciparum* patients.

One of those studies[24] found higher leukocyte counts in Kenyan children admitted with malaria than aged-matched healthy controls. It also found higher-than-normal leukocyte counts were linked to severe anemia and death and that children who died as a result of malaria had significantly higher numbers of leukocytes than those who survived. Studies finding decreased leukocytes in acute *P. falciparum* malaria patients[22,23,27] had recruited patients aged >15 years who presented with UCM, while studies that found similar results to those of this report[24–26] had investigated *P. falciparum* malaria in children aged ≤5 years who presented with severe malaria, similar to this report. Although other factors, such as geographical location, malaria endemicity,[28] and different malaria strains,[29] may play a crucial role, our results suggest that the age of the malaria patient is a crucial factor that determines how leukocytes are affected by malaria infection. Similarly, although neutrophil counts increase with age in healthy individuals,[30,31] the neutrophil counts observed in acute CM were much higher than the highest count (2,800 cells/μL) observed in healthy children aged 3–5 years. This observation also suggests that the neutrophilia in acute SMA and CM could be as a result of the malaria infection itself. The neutrophilia observed in children with acute CM in this study was similar to what had been observed in children who presented with severe malaria in the Gambia,[32] although the clinical malaria type was not clearly stated.

Results of work in mice depleted of peripheral neutrophils and infected with *P. berghei* ANKA showed that the depletion of neutrophils much earlier in the infection ended up preventing the development of experimental CM (ECM) and greatly reduced the level of microhemorrhage in the brain,[33] suggesting that neutrophils are somehow involved in the development of ECM although this possible link has not been proven in human CM as yet. However, results of another study have shown that neutrophils, or possibly a subset of neutrophils, interact with *P. falciparum* iRBCs through the PfEMP1 and ICAM1 receptors.[34] Considering the role these two markers play in the interaction between iRBCs and the endothelium of the brain microvasculature,[35,36] it is possible that activated neutrophils are involved directly or indirectly in the cytoadherence of iRBCs in brain blood vessels, which is one of the signature characteristics of human CM,[37] or in some way contribute to malaria-associated pathology.[38,39]

One study reported that the neutrophil-specific activation marker lipocalin was elevated in plasma collected from patients presenting with severe malaria.[40] Others observed that lipocalin was upregulated in the brains of mice just before the onset of ECM syndrome.[41] This has led some to the hypothesis that neutrophils recruited to the inflammation site are a likely source of such cytokines as IL12, IL18, IFNγ, and TNFα, which contribute to malaria pathogenesis.[41] Various studies,[27,42] including one of ours,[20] have shown that T cells and other immune-system cells are highly activated during acute malaria. These results are in support of the hypothesis that malaria infection stimulates cells of the innate and acquired immune systems to secrete proinflammatory cytokines with the aim of clearing the malaria parasites from the circulation.[43] It is thuse surprising to observe a general downregulation of the expression of integrins by neutrophils in acute stages of all clinical malaria types.

However, the significantly lower expression of CD11c in acute malaria we observed in this study has been observed by other investigators[19] who investigated how membrane antigens directly involved in the immunoresponse and phagocytic process are expressed. That investigation involved mixing PBMCs prepared from healthy unexposed adults with *P. falciparum* hemozoin extracts. The investigators found that expression of CD54 (ICAM1) and CD11c was decreased in hemozoin-loaded monocytes, although expression of CD11b was not affected in any way.[19] This observation led them to suggest that hemozoin loading might contribute to impairment of the immunoresponse, especially antigen presentation.[19] A previous study by our group[44] showed lower expression of CD11a, Cd11b, and CD18 on monocytes from blood samples of Malawian children suffering from acute CM, SMA, and UCM, which then normalized in convalescence.

Contrary to the results of this study, in vitro exposure of leukocytes from naïve unexposed adults to isolated malaria pigment derived from ruptured schizonts has resulted in a significant increase in the expression of CD11b/CD18.[18] The earliest response of CD11b/CD18 expression was detected 10 minutes following exposure to infected malaria pigment, a plateau reached after 60 minutes, and a decline starting after 90 minutes.[18] The low expression of CD11b and CD18 observed in this study may be due to the fact that we were studying a population that had already been exposed to *P. falciparum* antigens on a number of occasions prior to the infection, which resulted in the children being recruited. In addition, an in vitro study[18] assessed the expression of these markers over 90 minutes, whereas the children in this study presented most likely after they

had been infected with blood-stage malaria for hours or days. Therefore, the period of exposure may explain the marked differences between the results of this study and those of the in vitro study. Surprisingly, there seems to be no studies that have assessed the expression of these markers in vivo in different clinical types of malaria, as this study has done. Other studies have found that neutrophils isolated from malaria-infected children are capable of phagocytosing schizonts in vitro,[45] whereas neutrophils from US servicemen infected with *P. falciparum* but no documented previous exposure to *P. falciparum* malaria were not.[46,47] One study measured the expression of CD11a/CD18 on lymphocytes in peripheral blood of Sudanese malaria patients, finding that expression of these markers was significantly higher on lymphocytes of malaria patients than lymphocytes from healthy controls. However, the study did not determine the expression of these markers on neutrophils.

This study has several limitations, one of which is the failure to include a neutrophil-specific marker in the antibody cocktail used in labeling the blood samples, as others have done using such markers as CD66b.[48,49] However, other studies[9,50] have used a similar gating strategy to that used in this study for characterizing neutrophils in whole blood. Secondly, we were restricted to studying only the expression of a limited number of neutrophil receptors due to the type of monoclonal antibodies available at the time. Future studies could include more markers, such as FcγRI, FcγRII, and FcγRI, and complement receptors (CR1 and CR3). Lastly, if this study measured hemozoin loading in the neutrophils in the different groups, as we had previously done with monocytes,[44] we could have established a more convincing link between hemozoin load and the lower-than-normal expression of the molecules observed.

Conclusion

Expression of the integrins CD11a, CD11b, CD11c, and CD18 on neutrophils was markedly lower during the acute stages of all three malaria types than in blood samples from controls. The expression of all four integrins was higher in convalescence than during acute infection. One possible explanation for these observations is that the hemozoin loading that occurs during acute infection contributes to a reduction in expression of the surface integrins, since the reduction in molecule expression decreased with malaria severity.

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Disclosure

The author declares no conflicts of interest.

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