IAI Accepts, published online ahead of print on 8 October 2012
Infect. Immun. doi:10.1128/IAI.00945-12

Polymorphisms in Fc gamma receptor (FcγRIIIA-176 F/V) and Toll-like receptor (TLR9[-1237 T/C]) are associated with protection against severe malarial anemia and changes in circulating IFN-γ

Elly O. Munde[1], Winnie A. Okeyo[1], Samwel B. Anyona[2], Evans Raballah[3], Stephen Konah[4], Wilson Okumu[2], Lilian Ogonda[1], John Vulule[5] and Collins Ouma[1]

1; Department of Biomedical Sciences, School of Public Health and Community Development, Maseno University, Maseno, Kenya.

2; Department of Medical Biochemistry, School of Medicine, Maseno University, Maseno, Kenya.

3; Department of Medical Laboratory Sciences, School of Health Sciences, Masinde Muliro University of Science and Technology, Kakamega, Kenya.

4; Department of Mathematics and Applied Statistics, School of Mathematics and Actuarial Sciences, Maseno University, Maseno, Kenya.

5; Center for Global Health Research, Kenya Medical Research Institute, Kisumu, Kenya.

Running Title: FcγRIIIA, TLR9, SNPs combinations and SMA
Key Words: FcγRIIIA, TLR9, IFN-γ, SNPs combination, susceptibility
Abstract word count: 250
Text Word Count: 4,816

FOOTNOTE PAGE

The study was approved by the Ethics Review Committee of the Kenya Medical Research Institute. Informed written consent was obtained from the parent or legal guardian of all children participating in the study.

Financial support: This work was supported by grants from the National Institute of Health (7R01-TW008306-05).

Please address any correspondence to:

Collins Ouma, PhD

Director,
Research, Publication and Consultancies,
Maseno University,
Kisumu-Busia Road
Private Bag,
Maseno, Kenya
Phone: +254-57351622
Fax: +254-57351221
E-mail: collinouma@yahoo.com

ABSTRACT

Understanding the immunogenetic basis of naturally acquired immunity to *Plasmodium falciparum* infection would aid in designing a rationally-based malaria vaccine. Variants within the Fc gamma receptors (FcγRs) mediate immunity through engagement of immunoglobulin (Ig)G and other immune mediators such as interferon gamma (IFN-γ), resulting in erythro-phagocytosis and production of inflammatory cytokines in severe malarial anemia (SMA). The toll-like receptors (TLRs) trigger transcription of pro-inflammatory cytokines and induce adaptive immune responses. Therefore, these receptors may condition malaria disease pathogenesis through alteration in adaptive and innate immune responses. To further delineate the impacts of FcγRIIIA and TLR9 in SMA pathogenesis, the association between FcγRIIIA -176 F/V, TLR9 (-1237 T/C) variants, SMA (Hb<6.0g/dL) and circulating IFN-γ levels were investigated in children (n=301) with acute malaria from western Kenya. Multivariate logistic regression analysis (controlling for potential confounders) revealed that children with FcγRIIIA -176V/TLR9 -1237C (VC) variant combination had a 64% reduced odds of developing SMA (OR, 0.36, 95% CI 0.20-0.64, p=0.001) while carriers of FcγRIIIA -176V/TLR9 -1237T (VT) variant combination were twice more susceptible to SMA (OR, 2.04, 95% CI 1.19-3.50, p=0.009). Children with SMA had higher circulating IFN-γ levels compared to non-SMA (p=0.008). Hemoglobin levels were negatively correlated with IFN-γ levels (r=-0.207, p=0.022). Consistently, the FcγRIIIA -176V/TLR9 -1237T (VT) carriers had higher levels of circulating IFN-γ (p=0.011) relative to non-carriers supporting the observation that higher IFN-γ levels are associated with SMA. These results demonstrate that FcγRIIIA-176 F/V and TLR9 (-1237 T/C) variants condition susceptibility to SMA and functional changes in circulating IFN-γ levels.

INTRODUCTION

Plasmodium falciparum malaria is a complex clinical syndrome comprising a milieu of life-threatening conditions including severe malarial anaemia (SMA), cerebral malaria (CM), metabolic acidosis, high density parasitemia (≥10,000parasites/μL), respiratory distress, hypoglycaemia and other less frequent complications such as hypotension (32). Globally, falciparum malaria accounts for the greatest degree of malaria-related morbidity and mortality (63). The majority of this morbidity and mortality occurs in immune-naïve African children under five years of age (11). In western Kenya, SMA (Hb<6.0g/dL with any density parasitemia) is the most common clinical manifestations of severe falciparum malaria in pediatric populations resident in holoendemic transmission regions (9, 43).

Changes in human genome has been influenced by pressure due to malaria endemicity, for example, the observed increase in sickle cell allele (HbAS) in malaria-exposed populations despite its fatal consequences (58). Even though not completely understood, the pathological mechanisms that underlie SMA may include lysis of infected and uninfected erythrocytes (20, 51), erythrocyte sequestration in the spleen (12, 21), imbalanced cytokine production in bone marrow suppression (26) and consequently, dyserythropoiesis (1, 49).

Fc gamma receptors (FcγR) are a heterogeneous group of hematopoietic cell surface glyco-proteins that facilitate the efficiency of antibody-antigen interactions with effector cells of the immune system (18, 27, 52). FcγR genes are mapped to chromosome 1q on 1q21-q23 (18, 27, 52). These receptors regulate a variety of humoral and cellular immune responses including phagocytosis, degranulation, antibody-dependent cellular cytotoxicity (ADCC), regulation of cytokine expression,

activation of B cells and clearance of immune complexes (23). FcγR family consist of FcγRI, FcγRII and FcγRIII (61).

The FcγRs have functional allelic polymorphisms that influence their effector capabilities (61). The FcγRIIIA is expressed predominantly on macrophages monocytes, natural killer (NK) cells and gamma/delta (γ/δ) T cells where they function as phagocytic and cytotoxic trigger to antigens (15). It has two co-dominantly expressed alleles, the -176V and -176F that differ in amino acid at position -176 in the extracellular domain (valine or phenylalanine, respectively). The existence of dimorphism in the amino acid position -176(F/V) of the FcγRIIIA has been shown to influence the binding of IgG subtypes, with the -176V variant displaying a higher binding affinity for IgG_1 and IgG_3 compared to the -176F (29). In *P. falciparum* infections, IgG_1 and IgG_3 antibodies have been shown to be associated with low parasitemia and low risk of malaria infection (6). Despite these investigations, the functional role of FcγR variants in the regulating IFN-γ during malaria disease pathogenesis still remains elusive.

Toll-like receptors (TLRs) are type 1 trans-membrane proteins differentially expressed among immune cells (4, 28). TLRs recognize and bind to conserved pathogen-associated molecular patterns (PAMPs), triggering activation of signal transduction pathways that induce cytokine production (5). TLR9 occupies 5kb on chromosome 3p21.3 and consists of two exons and encodes 1,028 amino acids (22). The PAMPs for TLR9 are hemozoin and unmethylated CpG-DNA (8, 50). Hemozoin, a heme metabolite secreted during malaria infection, activates the innate immune system via a TLR9-mediated MyD88-dependent pathway, resulting into signals that up-regulate tumor necrosis factor-alpha (TNF-α), interleukin (IL)-12p40, monocyte chemo-attractant protein 1 (MCP-1), and IL-6 production by dendritic cells

(16). Hemozoin is also a carrier that facilitates entry of unmethylated CpG-DNA of plasmodial into the host cell, where the latter can bind to, and stimulate TLR9 (48).

Several single-nucleotide polymorphisms (SNPs) that alter susceptibility to infectious and inflammatory diseases have been identified in TLRs. For instance, a study carried out in Ugandan children (aged 3-12 years) showed that carriers of a C allele at TLR9 −1237CC or the G allele at TLR9 1174GG was associated with an increased risk to cerebral malaria (CM) since these alleles enhanced production of IFN-γ following severe *P. falciparum* infection (53). Studies in pregnant Ghanaian women with *P. falciparum* infection showed that variants in the TLR9 -1486CC increased the risk of maternal malaria (35). Other studies investigating the development of premalignant gastric changes induced by *Helicobacter pylori* have identified the TLR9 (-1237 T/C) polymorphism as a risk factor (37). Taken together, these studies demonstrate that TLRs have the capacity to mount acute inflammatory responses against invading pathogens through induction of inflammation.

The IFN-γ is a multi-functional cytokine produced by T lymphocytes, B cells and natural killer cells (NKs). It plays an important role in inflammatory responses and is often associated with the development of overt Th1-like cell-mediated immune responses (25), and hence forms an important part of the immune system. Previous studies in animal models indicated that early production of IFN-γ is necessary for parasitemia resolution and stimulation of phagocytic cells, leading to clearance of infected erythrocytes (54). Moreover, elevated levels of IFN-γ at the acute phase of uncomplicated *P. falciparum* malaria has been shown to limit progression to clinical malaria (59). Studies in Thai adults demonstrated significantly higher IFN-γ levels in uncomplicated malaria than in individuals presenting with complicated malaria (56). In addition, a previous longitudinal study in pediatric population in western Kenya

demonstrated that high levels of circulating IFN-γ were associated with enhanced SMA severity in pediatric population (46). Furthermore, studies in animal models have shown that long-term immunity to malaria infection may be affected by an IFN-γ-mediated depletion of parasite-specific CD4+ T cells during infection (64), further demonstrating the critical role of IFN-γ in malaria pathogenesis.

Although FcγRIIIA -176 F/V and TLR9 -1237 T/C polymorphisms have been implicated in inflammatory diseases (31, 35, 37), to date, no studies have examined the associations between these variants and malaria disease outcomes, specifically in pediatric population resident in Siaya District, a *P. falciparum* holoendemic transmission area of western Kenya. Since previous genetic-based studies have thoroughly investigated SNPs (41, 47) and haplotypes (40, 46), we investigated the effect of cross SNP combinations in conditioning SMA. We examined the associations between FcγRIIIA (-176 F/V) and TLR9(-1237 T/C) promoter variant combinations and susceptibility to SMA (Hb<6.0g/dL) in children (aged 3-36 months) residing in this holoendemic *P. falciparum* transmission area of western Kenya. In addition, we investigated the functional role of these variants in mediating circulating IFN-γ concentrations in children with malaria. The results presented here show that co-inheritance of FcγRIIIA -176 F/V and TLR9 -1237 T/C is associated with susceptibility to SMA and functional changes in circulating IFN-γ levels.

MATERIALS AND METHODS

Study site. The study was conducted in Siaya District Hospital, western Kenya, and the surrounding community, a *P. falciparum* holoendemic transmission region (43). The region is inhabited by the Luo ethnic tribe (>96%), hence a homogenous population for genetic-based studies. Falciparum malaria prevalence is ~83% in children aged <4 years, with severe disease manifesting as SMA and/or high density parasitemia (HDP) (39, 43).

Study participants. Children [n=301] of both sexes were recruited in Siaya District Hospital (SDH) in western Kenya during their initial hospitalization for treatment of malaria using questionnaires and existing medical records. Recruitment followed a two-phase tier of screening and enrolment. The parent/guardian of the child received detailed explanation of the study. Enrolment decision was made after initial HIV-1 screening of the child and obtaining informed consent. Questionnaires and written informed consent were administered in the language of choice (i.e. English, Kiswahili or Dholuo). The children with acute malaria were stratified into two categories: non-severe malarial anemia (non-SMA) group: Children with a positive smear for asexual *P. falciparum,* parasitemia (of any density) and Hb≥6.0 g/dL and SMA group: Children with a positive smear for asexual *P. falciparum,* parasitemia (of any density) and Hb<6.0 g/dL (33). Venous blood samples (<3.0 mL) were collected in EDTA-containing vacutainer tubes at the time of enrollment, prior to provision of treatment or any supportive care. Blood samples were used for malaria diagnosis, hematological measurements, HIV testing, bacterial culture and genetic analyses. Children were excluded from the study for any one of the following reasons; children

with CM (rare in this holoendemic area); history of any HIV-1 related symptoms such as oral thrush; clinical evidence of acute respiratory infection; prior hospitalization; intent to relocate during the study period; and unwillingness to enroll child in the study. Participants were treated according to the Ministry of Health (MOH)-Kenya guidelines, which included the use of oral artemether/lumefantrine (Coartem®) for uncomplicated malaria and intravenous quinine (and in rare occasion, blood transfusion) for severe malaria. The study approval was obtained from the Ethics Review Committee of the Kenya Medical Research Institute (KEMRI).

Laboratory procedures. Hemoglobin levels and complete blood counts were determined using the Beckman Coulter ACT diff2™ (Beckman-Counter Corporation, Miami, FL, USA). To determine parasitemia, 10% Giemsa-stained thick blood smears were prepared and examined under a microscope on high power magnification. *P. falciparum* parasites per 300 white blood cells (WBC) were determined and parasitemia (/μL) estimated using total WBC count. In order to delineate severe anemia caused by malaria versus other anemia-promoting conditions, human immunodeficiency virus (HIV)-1, bacteremia, sickle-cell trait (HbAS) status and glucose-6-phosphate dehydrogenase (G6PD) deficiency were determined. Pre- and post-test HIV counseling was provided for all participants. HIV-1 exposure and infection were determined serologically (i.e., Unigold™ and Determine™) and through HIV-1 proviral DNA PCR testing, respectively, according to previously published methods (45). Bacteremia was determined using the Wampole Isostat Pediatric 1.5 system (Wampole Laboratories), and blood was processed according to the manufacturer's instructions. API biochemical galleries (bioMerieux, Inc.) and/or serology were used for identification of blood-borne

bacterial isolates. The presence of the sickle cell trait (HbAS) was determined by cellulose acetate electrophoresis, while Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency as previously described (47).

Genotyping. Blood spots were made on FTA Classic® cards (Whatman Inc., Clifton, NJ, USA), air dried, and stored at room temperature until use. DNA was extracted using the Gentra System (Gentra System Inc., Minneapolis, MN, USA) according to the manufacturer's recommendations. The FcγRIIIA-176 F/V (rs396991, assay ID: C__25815666_10) and TLR9-1237C/T (rs5743836, assay ID: C__32645383_10) promoter polymorphism were genotyped using the high-throughput TaqMan® 5' allelic discrimination Assay-By-Design method based on the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA).

Quantification of IFN-γ levels. Plasma samples were obtained from venous blood and stored at -80°C. Batch analysis was performed to restrict experimental variability between assays. Circulating IFN-γ concentrations were determined using human cytokine 25-plex Ab Bead Kit, (BioSource™ International) according to the manufacturer's instructions. Plates were read on Luminex 100™ system (Luminex Corporation) and analyzed using the Bio-plex Manager Software (Biorad Laboratories). The detection limit for IFN-γ was 2.0pg/mL.

Data analyses. SPSS® statistical software package version 19.0 (IBM SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Chi-square analysis was used to examine differences between proportions. Across group comparisons was determined by Kruskal-Wallis test, while Mann-Whitney U test was used for comparisons of demographic, clinical characteristics and circulating IFN-γ levels

between the two clinical groups and SNP combinations. FcγRIIIA (-176 F/V) and TLR9 (-1237C/T) SNP combinations were constructed using HPlus software program (Version 2.5). The relationship between genotypes, SNP combinations and SMA was determined by multivariate logistic regression, controlling for the confounding effects of age, gender, HIV-1 status [including HIV-1 exposed and definitively HIV-1(+) results], Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency, sickle cell trait (HbAS) and bacteremia. Correlation between IFN-γ concentrations and Hb levels in parasitemic children was determined by Spearman's correlation coefficient. Statistical significance was set at $p \leq 0.05$.

RESULTS

Clinical, demographic and laboratory characteristics of the study participants: A cross-sectional analysis in children (n=301, aged 3-36 months) presenting with acute *P. falciparum* malaria (any density parasitemia) was performed. Clinical stratification of the study groups was done based on previous age- and geographically-defined reference population from western Kenya (33), i.e. non-severe malaria (non-SMA; Hb≥6.0g/dL; n=163) and severe malaria anemia (SMA; Hb<6.0g/dL; n=138). The distribution of gender, parasitemia (parasites/μL), proportions of those with high density parasitemia (HDP≥10,000 parasites/μL) and axillary temperature (ºC) were not significantly different between the groups (p=0.668, p=0.508, p=0.456 and p=0.109, respectively; Table 1). Children presenting at hospital with SMA were younger than those with non-SMA (p=0.010). With reference to previous grouping, Hb (g/dL) concentration and erythrocyte counts ($x10^{12}$/L) were lower in SMA group (p<0.001) for the two clinical parameters (Table 1).

Distribution of FcγRIIIA (-176 F/V) and TLR9 (-1237 T/C) genotypes and alleles in the clinical groups: To investigate the role played by the polymorphic variation in FcγRIIIA (-176 F/V) and TLR9 (-1237 T/C) promoters in conditioning susceptibility to SMA, their allelic distributions were compared between the clinical groups (Table 2). FcγRIIIA -176 F/V genotypes in the overall population were 54.5% FF, 36.5% FV and 9.0% VV, with overall allele frequency of 0.72 for F and 0.28 for V. However, the overall allele frequency for FcγRIIIA -176 F/V did not deviate from the Hardy-Weinberg Equilibrium (HWE) (χ^2=1.83, p=0.180). The prevalence of FcγRIIIA -176 F/V genotypes in non-SMA was 52.2% FF, 36.8% FV and 11.0% VV with allele

frequency of 0.71 for F and 0.29 for V while the genotypic distribution of the FcγRIIIA -176 F/V in SMA group was 57.3% FF, 36.2% FV and 6.5% VV with an allele frequency of 0.75 for F and 0.25 for V. The allele frequencies in non-SMA (χ^2=2.20, *p*=0.140) and SMA group (χ^2=0.07, *p*=0.800) for the FcγRIIIA -176 F/V did not significantly deviate from HWE.

The distribution of TLR9 (-1237 T/C) genotype in the overall study population was 40.9% TT, 49.8% TC and 9.3% CC (Table 2), with an overall allele frequency of 0.70 for T and 0.30 for C. There was no significant departure from the HWE in the overall study group (χ^2=0.60, *p*=0.350). The genotypic distribution in non-SMA group was 36.8% TT, 54.4% TC and 9.8% CC (Table 2) with allele frequency of 0.63 for T and 0.36 for C. The allele frequency in the non-SMA group demonstrated a significant departure from the HWE (χ^2=4.00, *p*=0.040). The genotypic prevalence in SMA group was 45.7% TT, 45.7% TC and 8.6% CC (Table 2) and allele frequency of 0.69 for T and 0.31 for C. However, there was no departure from HWE in the SMA group (χ^2=0.60, *p*=0.400).

Additional χ^2 analysis showed that the distribution of the individual FcγRIIIA -176 F/V and TLR9 (-1237 T/C) genotypes were comparable between the SMA and non-SMA groups (*p*=0.356 and *p*=0.297, respectively).

Influence of polymorphic variability in FcγRIIIA -176 F/V and TLR9 (-1237 T/C) on SMA. The investigation on the association between individual genotypes of FcγRIIIA -176 F/V and TLR9 (-1237 T/C) and susceptibility to SMA was determined using multivariate logistic regression analyses while controlling for the confounding

effects of age, gender, HIV-1 status, sickle cell trait (HbAS), bacteremia and G6PD deficiency (3, 45, 62). No significant associations were observed between the variations at individual loci of FcγRIIIA (-176 F/V), TLR9 (-1237 T/C) and susceptibility to SMA (Table 3).

Distribution of FcγRIIIA -176 F/V and TLR9 (-1237 T/C) SNPs combinations in the clinical groups. As shown in Table 4, cross-gene SNP combination for the receptor polymorphisms yielded the following overall prevalence in the non-SMA and SMA group (33); FcγRIIIA -176F/ TLR9 -1237T (FT), 80.1% (241/301); FcγRIIIA -176 F/ TLR9 -1237C (FC), 38.2% (115/301); FcγRIIIA -176V/ TLR9 -1237T (VT), 26.6% (80/301) and FcγRIIIA -176V/ TLR9 -1237C (VC), 24.0% (74/301). Additional comparison showed a higher proportion of carriers of the -176V/ -1237T (VT) SNP combination in SMA group (33.3%) compared to the non-SMA (20.9%; p=0.015). Consistent with this observation, the VT carriers presented with significantly lower Hb levels [median (IQR); 5.70g/dL (3)], relative to the non-VT carriers [median (IQR); 6.70g/dL (3)], (p=0.014). Further analysis revealed a significantly lower proportion of the -176V/ -1237C (VC) SNPs in SMA group (15.2%) compared to non-SMA (32.5%; p=0.001). In agreement with this finding, carriers of this SNP combination also had significantly higher Hb levels [median (IQR); 6.70g/dL (3)] compared to non-carriers [median (IQR); 5.60g/dL (3)], p=0.002). Further analysis revealed comparable proportions and Hb levels for the -176F/ -1237T (p=0.887 and p=0.588), -176F/ -1237C (p=0.209 and p=0.064) (Table 4). These results show that carriage of FcγRIIIA -176 F/V and TLR9 (-1237 T/C) SNP combinations may condition susceptibility to SMA in children with acute malaria.

Associations between FcγRIIIA -176 F/V and TLR9 -1237 T/C gene SNP combinations and SMA. Prior to determining the influence of the SNP combinations on circulating IFN-γ plasma levels, multivariate logistic regression analysis controlling for covariates (3, 45, 62) was performed. The analysis demonstrated that carriers of -176V/ -1237T (VT) SNPs combination were at an increased risk of developing SMA (OR, 2.04, 95%CI 1.19-3.50, *p*=0.009) relative to non-carriers while carriers of -176V/ -1237C (VC) were at reduced risk of SMA (OR, 0.36, 95%CI 0.20-0.64, *p*=0.001; Table 5). Further analysis did not reveal any association between the -176F/ -1237T (OR, 0.94, 95%CI 0.52-1.68, *p*=0.830) and the -176F/ -1237C (OR, 1.30, 95%CI, 0.80-2.10, *p*=0.288) SNP combinations and SMA (Table 5). Given a possible diluting effect of each SNP combination in heterozygous individuals, additional construction of SNPs was carried out based on the carriage of F/V (at -176 F/V) and T/C (at -1237 T/C) i.e. FV/TC and associations with SMA. Results revealed that heterozygous individuals were peripherally at an increased risk to the development of SMA (OR, 1.89, 95%CI, 0.99-3.64, *p*=0.055). However, low numbers could not allow determination of associations between the dominant (FF/TT) and recessive (VV/CC) SNP combination models and SMA.

Relationship between circulating IFN-γ and SMA. To determine whether the changes in the circulating levels of IFN-γ are associated with severity of acute malaria, the levels were compared between the SMA (n=69) and non-SMA (n=70) groups. It is critical to note that after the first screening (to determine Hb levels), we were unable to collect additional blood samples to carry out measurements of IFN-γ levels in some study participants due to the fact that either the children were too

anemic or were too sick to ethically allow collection of additional blood sample, hence the reduction in numbers in this analysis. As presented in Figure 1, the results demonstrates that children with SMA had significantly higher levels of circulating IFN-γ plasma concentrations [median (IQR); 21.5(34.9) pg/mL] compared to non-SMA [14.8(27.1) pg/mL] (*p*=0.008). Additional analyses demonstrated that IFN-γ levels was negatively correlated with Hb levels (r=-0.207, *P*=0.022).

Association between circulating IFN-γ and FcγRIIIA (-176 F/V) and TLR9 (-1237 T/C) promoter polymorphisms. To determine whether these genotypes were associated with functional changes in concentrations of IFN-γ levels, plasma levels of IFN-γ were compared across the genotypic groups. As presented in Figure 2 (A) and (B), there were no significant differences in the concentrations of plasma IFN-γ across genotypes for both FcγRIIIA (-176 F/V; FF=77, FV=53 and VV=9) (*p*=0.480) and TLR9 (-1237 T/C; TT=65, TC=65 and CC=9) (*p*=0.559). The distribution of IFN-γ in the FcγRIIIA -176 F/V genotype were; FF [median (IQR); 19.6 (30.6)], FV [median (IQR); 15.9 (28.1) and VV [median (IQR); 19.6 (27.3) while the distribution of IFN-γ in TLR9 (-1237 T/C) genotype were; TT [median (IQR); 16.2(34.6), TC [median (IQR); 19.6(33.6) and CC [median (IQR); 14.0(27.5)].

Functional associations between FcγRIIIA -176 F/V and TLR9 -1237 T/C SNPs combinations and circulating IFN-γ levels. To determine whether co-inheritance of these receptor polymorphisms were associated with changes in concentrations of IFN-γ levels, circulating concentrations of IFN-γ were compared within SNP combinations in the FcγRIIIA -176 F/V and TLR9 -1237 T/C. As shown in Figure 3,

the individuals with FcγRIIIA -176V/TLR9 -1237T SNPs combination had significantly higher levels of IFN-γ [median (IQR); 19.6pg/mL (32.1)] relative to those without this combination [median (IQR); 13.4 pg/mL (16.9)], (p=0.011). However, the concentration of IFN-γ was comparable between those with FcγRIIIA -176F/ TLR9 -1237T (p=0.450), FcγRIIIA -176F/ TLR9 -1237C (p=0.775) and FcγRIIIA -176V/ TLR9 -1237C (p=0.188) SNP combinations.

DISCUSSION

To describe the role of receptors in susceptibility to child severe malarial anemia, we performed a cross-sectional analysis of the impacts of FcγRIIIA -176 F/V and TLR9 (-1237 T/C) promoter variants in a phenotypically well-defined cohort of children aged 3-36 months resident in a *P. falciparum* holoendemic transmission region. The results presented here demonstrate that carriage of the FcγRIIIA -176V and TLR9 -1237C (VC) confers protection against SMA (Hb<6.0 g/dL) (33), and is associated with significantly higher Hb levels in this population whereas the carriage of FcγRIIIA -176V and TLR9 -1237T (VT) increases susceptibility to SMA and produces significantly higher levels of circulating IFN-γ. Consistent with previous observations, children with SMA had significantly high levels of circulating IFN-γ (46).

Since FcγRIIIA is mainly expressed on the macrophages and monocytes, they play a primary role in phagocytosis of unparasitized erythrocytes and induction of pro-inflammatory cytokines, which play a role in SMA pathogenesis (10). On macrophages, FcγRIIIA is also involved in the clearance of immune complex (15). Consistent with a previous study in Thai adults (41), individual FcγRIIIA -176 F/V polymorphism failed to show any association with SMA. However, it is important to note that this polymorphism influences preferential binding of immunoglobulins (Ig)G in which the FcγRIIIA (-176V/V) has higher binding affinity to IgG_1 and IgG_3 which are associated with low parasitemia and low risk of malaria (6, 57). We are currently exploring this model to test whether carriage of this variant is associated with higher IgG binding in children naturally exposed to *P. falciparum* malaria in holoendemic region of western Kenya, in which the primary clinical outcome of severe malaria is SMA.

There is accumulating evidence on the potential role of TLR9 polymorphisms in clinical malaria (13, 30, 65). Consistent with our study, two separate studies, carried out in Brazil and Iran, have recently revealed no impact of individual TLR9 (-1237 T/C) promoter polymorphism on susceptibility to mild malaria in their respective populations (30, 65). Moreover, the TLR9 (-1237TT) genotype has only been associated with low parasitemia but not increased susceptibility to clinical malaria in Ghanaian children aged 3-11 years (40). Investigations in the Gambian and Malawian children less than 5 years characterized by mixed clinical phenotypes (cerebral and/or severe malaria anemia) did not show any association between the TLR9 (-1237 T/C) polymorphisms and severe malaria (13). However, a study in Ugandan children (aged 4-12 years), showed that TLR9 (-1237CC) genotype was associated with elevated levels of plasma IFN-γ and enhanced cerebral malaria (53) emphasizing the fact that these variants may individually be associated with CM rather than SMA. Moreover, other studies have revealed that individuals infected by malaria have up-regulated TLR9 and elevated IFN-γ, and that mouse with TLR9 gene knockout produce low IFN-γ levels in response to *Plasmodium chabaudi* AS (24). The discrepancies observed between our study and others may in part be explained by the difference in clinical phenotypes since in our study population, the main clinical manifestation is SMA in pediatric populations while the earlier studies focused on heterogeneous populations in which the most severe clinical manifestation was CM. In addition, pathways of TLR9 signalling involve polymorphisms in the downstream molecules, for instance NF-*κ*B and MyD88 that were not investigated in the current study. Furthermore, due to high prevalence of malaria in our population and that TLRs only act in recognition and induction of immune response, we assume that polymorphisms in the TLRs are not the primary

443 determinants of clinical malaria. We are currently investigating additional genes that
444 may significantly alter TLR pathways and alter malaria disease susceptibility.

445 Since susceptibility to infectious disease occurs through multi-factorial,
446 complex and even contradictory selective pressures (7), our laboratory constructed
447 cross SNP combination between FcγRIIIA (-176 F/V) and TLR9 (-1237 T/C), in an
448 attempt to determine whether co-inheritance of these receptor SNPs combinations
449 could influence susceptibility to SMA. Based on results from this study, carriage of
450 the -176V/ -1237C (VC) SNP combination were associated with reduced
451 susceptibility in the development of SMA relative to non-carriers. Consistent with
452 this observation, carriers of VC had concomitant higher levels of haemoglobin,
453 suggesting a potential protective role against SMA pathogenesis through increased
454 erythropoietic responses. In addition, the carriers of the -176V/ -1237T (VT) SNPs
455 combination were almost twice at risk of developing SMA and had relatively lower
456 haemoglobin levels. In the current study, we demonstrated that SMA in this
457 population is characterised in part by elevated circulating IFN-γ levels as previously
458 shown (46). Furthermore, the -176V/-1237T SNP combination which was associated
459 with an increased risk to SMA was also associated with higher circulating IFN-γ
460 levels. This is not surprising given that elevated circulating IFN-γ levels is associated
461 with SMA in this population. As such, any gene combination that may be associated
462 with higher circulating IFN-γ levels may promote SMA. It would be plausible to
463 explore how different cytokine milieu in relationship to IFN-γ levels and IgG
464 production promote the development of SMA over time in this pediatric population
465 resident in western Kenya. This approach will address the inherent limitation in
466 examining cytokine production at a single time point (time of admission) and in
467 circulation rather than in the local microenvironments which in essence complicates

the clear understanding of the exact role of immune mediators such as IFN-γ in SMA (34). This study underscores the importance of the use of cross-SNP combinations in genetic association studies of infectious diseases such as malaria because it reveals associations that are not identifiable with just single gene polymorphisms since such disease outcome(s) are dictated by genes functioning in concert (2).

It is worth noting that substitution of a T to a C in the TLR9 -1237 in the SNP combination (VC *vs.* VT) significantly determined whether individuals were increasingly susceptible or had reduced risk to SMA in children who presented with acute malaria. Activation of gene transcription depends upon the binding of regulatory and transcription factors to specific recognition sequences in the promoter. As such, variation in the TLR9 -1237 T/C promoter sequences likely alters specific transcription factor recognition sites and consequently affects transcriptional activation of IFN-γ and other effector production during acute disease. For example, the presence of a T at the TLR9 -1237 locus (VT in the SNP combination) may favor enhanced binding of transcriptional factors (or cause disruption of repressor binding sites) that lead to higher IFN-γ production, whereas, the presence of the C in the promoter (VC in the SNP combination) may create sites for enhanced binding of repressors that favor reduced IFN-γ production. Although the impact of the surface receptor SNP combinations examined here on promoter binding elements is largely unknown, our laboratory is currently investigating the mechanism(s) by which these across SNP combinations may alter IFN-γ production.

Despite continued investigations, the exact role of IFN-γ in the pathogenesis of SMA continues to be baffling. For example, high early IFN-γ production has been shown to confer protection against symptomatic malaria episodes in children aged 5-14 years from malaria endemic region of Papua New Guinea (17). An additional study

in holoendemic perennial falciparum malaria transmission area in southern Ghana reported that malaria-specific production of IFN-γ was associated with reduced clinical malaria and fever (19). Collectively, these studies implicate increased IFN-γ production in clinical malaria. However, certain studies have reported association between higher levels of IFN-γ with severe malaria. For instance, a study in Uganda reported positive association between increased IFN-γ levels and CM (53). Furthermore, a previous report in children population resident in western Kenya demonstrated that IFN-γ was a positive predictor of SMA (42). Results presented in the current versus previous study (42) likely differ due to differences in the stratification of the cohort groups. In the current study, we stratified our study population into SMA (Hb<6.0 g/dL, and any density parasitemia) and non-SMA groups (Hb≥6.0g/dL, and any density parasitemia), while the previous study (42), further stratified the overall non-SMA (Hb>6.0 g/dL, and any density parasitemia) group into uncomplicated malaria (UM; Hb levels of >11.0 g/dL; n = 31) and Non-SMA (Hb levels of 6.0 to 10.9 g/dL; n = 37) for the Least-Angle Regression (LAR) analyses. In addition, potential underlying genetic variation that may potentially contribute to differences in functional changes (e.g. IFN-γ) during disease in the population were never controlled for as a variable in the LAR analyses. In the current study, we demonstrate that children with SMA had significantly higher IFN-γ concentrations, a finding consistent with a previous study in the same population (46). Even though not explicitly explored, the pathogenic mechanisms of elevated IFN-γ in SMA may in part be as a consequence of over-stimulation of monocytes by IFN-γ to secret TNF-α (44). This stimulation would lead to the formation of toxic oxides and free radicals, such as reactive oxygen species (H_2O_2 and iNOS) by liver cells against intra-hepatic parasites and erythrocytic-stage parasite (38, 55, 60), as

well as enhanced phagocytic activities of monocytes/macrophages against parasitized and non-parasitized erythrocytes (36). Moreover, overproduction of IFN-γ, also promote enhanced malarial anemia pathogenesis through bone marrow suppression, dyserythropoiesis, and erythro-phagocytosis (14). However, for enhanced immunity to be accomplished, milieus of both pro-inflammatory and anti-inflammatory cytokines balance are involved (19) and should be considered in future study designs.

In summary, our results demonstrate that SMA in this pediatric population is conditioned by functional variations in FcγRIIIA (-176 F/V) and TLR9 (-1237 T/C) promoter polymorphisms. To exhaustively describe the impacts of surface receptors in development of naturally acquired immunity against malaria, further longitudinal studies aimed at examination of an all inclusive panel of receptor polymorphisms that influence innate immune response and disease outcome are required as this may provide an immunogenetic basis for the development of vaccines that modulate receptor functions.

ACKNOWLEDGEMENTS

We are grateful to the Siaya District Hospital for clinical support. We are indebted to the parents/guardians of the study participants and children who took part in the study. This work is published with the approval of the Director, KEMRI.

References

1. **Abdalla, S., D. J. Weatherall, S. N. Wickramasinghe, and M. Hughes.** 1980. The anaemia of P. falciparum malaria. Br J Haematol **46:**171-183.
2. **Adler, A. J., A. Scheller, and D. M. Robins.** 1993. The stringency and magnitude of androgen-specific gene activation are combinatorial functions of receptor and nonreceptor binding site sequences. Mol Cell Biol **13:**6326-6335.
3. **Aidoo, M., D. J. Terlouw, M. S. Kolczak, P. D. McElroy, F. O. ter Kuile, S. Kariuki, B. L. Nahlen, A. A. Lal, and V. Udhayakumar.** 2002. Protective effects of the sickle cell gene against malaria morbidity and mortality. Lancet **359:**1311-1312.
4. **Akira, S.** 2003. Mammalian Toll-like receptors. Curr Opin Immunol **15:**5-11.
5. **Akira, S., S. Uematsu, and O. Takeuchi.** 2006. Pathogen recognition and innate immunity. Cell **124:**783-801.
6. **Aribot, G., C. Rogier, J. L. Sarthou, J. F. Trape, A. T. Balde, P. Druilhe, and C. Roussilhon.** 1996. Pattern of immunoglobulin isotype response to *Plasmodium falciparum* blood-stage antigens in individuals living in a holoendemic area of Senegal (Dielmo, west Africa). Am J Trop Med Hyg **54:**449-457.
7. **Balaresque, P. L., S. J. Ballereau, and M. A. Jobling.** 2007. Challenges in human genetic diversity: demographic history and adaptation. Hum Mol Genet **16 Spec No. 2:**R134-139.
8. **Bauer, S., C. J. Kirschning, H. Hacker, V. Redecke, S. Hausmann, S. Akira, H. Wagner, and G. B. Lipford.** 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc Natl Acad Sci U S A **98:**9237-9242.
9. **Bloland, P. B., D. A. Boriga, T. K. Ruebush, J. B. McCormick, J. M. Roberts, A. J. Oloo, W. Hawley, A. Lal, B. Nahlen, and C. C. Campbell.** 1999. Longitudinal cohort study of the epidemiology of malaria infections in an area of intense malaria transmission II. Descriptive epidemiology of malaria infection and disease among children. Am J Trop Med Hyg **60:**641-648.
10. **Brattig, N. W., K. Kowalsky, X. Liu, G. D. Burchard, F. Kamena, and P. H. Seeberger.** 2008. Plasmodium falciparum glycosylphosphatidylinositol toxin interacts with the membrane of non-parasitized red blood cells: a putative mechanism contributing to malaria anemia. Microbes Infect **10:**885-891.
11. **Breman, J. G., M. S. Alilio, and A. Mills.** 2004. Conquering the intolerable burden of malaria: what's new, what's needed: a summary. Am J Trop Med Hyg **71:**1-15.
12. **Buffet, P. A., I. Safeukui, G. Milon, O. Mercereau-Puijalon, and P. H. David.** 2009. Retention of erythrocytes in the spleen: a double-edged process in human malaria. Curr Opin Hematol **16:**157-164.
13. **Campino, S., J. Forton, S. Auburn, A. Fry, M. Diakite, A. Richardson, J. Hull, M. Jallow, F. Sisay-Joof, M. Pinder, M. E. Molyneux, T. E. Taylor, K. Rockett, T. G. Clark, and D. P. Kwiatkowski.** 2009. TLR9 polymorphisms in African populations: no association with severe malaria, but evidence of cis-variants acting on gene expression. Malar J **8:**44.
14. **Clark, I. A., and W. B. Cowden.** 2003. The pathophysiology of falciparum malaria. Pharmacol Ther **99:**221-260.
15. **Clarkson, S. B., R. P. Kimberly, J. E. Valinsky, M. D. Witmer, J. B. Bussel, R. L. Nachman, and J. C. Unkeless.** 1986. Blockade of clearance of immune

592 complexes by an anti-Fc gamma receptor monoclonal antibody. J Exp Med
593 **164:**474-489.
594 16. **Coban, C., K. J. Ishii, T. Kawai, H. Hemmi, S. Sato, S. Uematsu, M.**
595 **Yamamoto, O. Takeuchi, S. Itagaki, N. Kumar, T. Horii, and S. Akira.**
596 2005. Toll-like receptor 9 mediates innate immune activation by the malaria
597 pigment hemozoin. J Exp Med **201:**19-25.
598 17. **D'Ombrain, M. C., L. J. Robinson, D. I. Stanisic, J. Taraika, N. Bernard, P.**
599 **Michon, I. Mueller, and L. Schofield.** 2008. Association of early interferon-
600 gamma production with immunity to clinical malaria: a longitudinal study
601 among Papua New Guinean children. Clin Infect Dis **47:**1380-1387.
602 18. **Daeron, M.** 1997. Fc receptor biology. Annu Rev Immunol **15:**203-234.
603 19. **Dodoo, D., F. M. Omer, J. Todd, B. D. Akanmori, K. A. Koram, and E. M.**
604 **Riley.** 2002. Absolute levels and ratios of proinflammatory and anti-
605 inflammatory cytokine production in vitro predict clinical immunity to
606 *Plasmodium falciparum* malaria. J Infect Dis **185:**971-979.
607 20. **Dondorp, A. M., B. J. Angus, K. Chotivanich, K. Silamut, R.**
608 **Ruangveerayuth, M. R. Hardeman, P. A. Kager, J. Vreeken, and N. J.**
609 **White.** 1999. Red blood cell deformability as a predictor of anemia in severe
610 falciparum malaria. Am J Trop Med Hyg **60:**733-737.
611 21. **Dondorp, A. M., K. T. Chotivanich, S. Fucharoen, K. Silamut, J. Vreeken,**
612 **P. A. Kager, and N. J. White.** 1999. Red cell deformability, splenic function
613 and anaemia in thalassaemia. Br J Haematol **105:**505-508.
614 22. **Du, X., A. Poltorak, Y. Wei, and B. Beutler.** 2000. Three novel mammalian
615 toll-like receptors: gene structure, expression, and evolution. Eur Cytokine
616 Netw **11:**362-371.
617 23. **Fanger, M. W., L. Shen, R. F. Graziano, and P. M. Guyre.** 1989. Cytotoxicity
618 mediated by human Fc receptors for IgG. Immunol Today **10:**92-99.
619 24. **Franklin, B. S., P. Parroche, M. A. Ataide, F. Lauw, C. Ropert, R. B. de**
620 **Oliveira, D. Pereira, M. S. Tada, P. Nogueira, L. H. da Silva, H.**
621 **Bjorkbacka, D. T. Golenbock, and R. T. Gazzinelli.** 2009. Malaria primes
622 the innate immune response due to interferon-gamma induced enhancement
623 of toll-like receptor expression and function. Proc Natl Acad Sci U S A
624 **106:**5789-5794.
625 25. **Gajewski, T. F., J. Joyce, and F. W. Fitch.** 1989. Antiproliferative effect of
626 IFN-gamma in immune regulation. III. Differential selection of TH1 and TH2
627 murine helper T lymphocyte clones using recombinant IL-2 and recombinant
628 IFN-gamma. J Immunol **143:**15-22.
629 26. **Helleberg, M., B. Q. Goka, B. D. Akanmori, G. Obeng-Adjei, O. Rodriques,**
630 **and J. A. Kurtzhals.** 2005. Bone marrow suppression and severe anaemia
631 associated with persistent Plasmodium falciparum infection in African children
632 with microscopically undetectable parasitaemia. Malar J **4:**56.
633 27. **Indik, Z. K., J. G. Park, S. Hunter, and A. D. Schreiber.** 1995. The
634 molecular dissection of Fc gamma receptor mediated phagocytosis. Blood
635 **86:**4389-4399.
636 28. **Janeway, C. A., Jr., and R. Medzhitov.** 2002. Innate immune recognition.
637 Annu Rev Immunol **20:**197-216.
638 29. **Koene, H. R., M. Kleijer, J. Algra, D. Roos, A. E. von dem Borne, and M.**
639 **de Haas.** 1997. Fc gammaRIIIa-158V/F polymorphism influences the binding
640 of IgG by natural killer cell Fc gammaRIIIa, independently of the Fc
641 gammaRIIIa-48L/R/H phenotype. Blood **90:**1109-1114.

30. **Leoratti, F. M., L. Farias, F. P. Alves, M. C. Suarez-Mutis, J. R. Coura, J. Kalil, E. P. Camargo, S. L. Moraes, and R. Ramasawmy.** 2008. Variants in the toll-like receptor signaling pathway and clinical outcomes of malaria. J Infect Dis **198:**772-780.
31. **Li, X., T. S. Ptacek, E. E. Brown, and J. C. Edberg.** 2009. Fcgamma receptors: structure, function and role as genetic risk factors in SLE. Genes Immun **10:**380-389.
32. **Marsh, K., D. Forster, C. Waruiru, I. Mwangi, M. Winstanley, V. Marsh, C. Newton, P. Winstanley, P. Warn, N. Peshu, and et al.** 1995. Indicators of life-threatening malaria in African children. N Engl J Med **332:**1399-1404.
33. **McElroy, P. D., A. A. Lal, W. A. Hawley, P. B. Bloland, F. O. Kuile, A. J. Oloo, S. D. Harlow, X. Lin, and B. L. Nahlen.** 1999. Analysis of repeated hemoglobin measures in full-term, normal birth weight Kenyan children between birth and four years of age. III. The Asemobo Bay Cohort Project. Am J Trop Med Hyg **61:**932-940.
34. **Mirghani, H. A., H. G. Eltahir, A. E. TM, Y. A. Mirghani, M. I. Elbashir, and I. Adam.** 2011. Cytokine profiles in children with severe Plasmodium falciparum malaria in an area of unstable malaria transmission in central Sudan. J Trop Pediatr **57:**392-395.
35. **Mockenhaupt, F. P., L. Hamann, C. von Gaertner, G. Bedu-Addo, C. von Kleinsorgen, R. R. Schumann, and U. Bienzle.** 2006. Common polymorphisms of toll-like receptors 4 and 9 are associated with the clinical manifestation of malaria during pregnancy. J Infect Dis **194:**184-188.
36. **Naotunne, T. S., N. D. Karunaweera, G. Del Giudice, M. U. Kularatne, G. E. Grau, R. Carter, and K. N. Mendis.** 1991. Cytokines kill malaria parasites during infection crisis: extracellular complementary factors are essential. J Exp Med **173:**523-529.
37. **Ng, M. T., R. Van't Hof, J. C. Crockett, M. E. Hope, S. Berry, J. Thomson, M. H. McLean, K. E. McColl, E. M. El-Omar, and G. L. Hold.** 2010. Increase in NF-kappaB binding affinity of the variant C allele of the toll-like receptor 9 -1237T/C polymorphism is associated with Helicobacter pylori-induced gastric disease. Infect Immun **78:**1345-1352.
38. **Nussler, A. K., L. Renia, V. Pasquetto, F. Miltgen, H. Matile, and D. Mazier.** 1993. In vivo induction of the nitric oxide pathway in hepatocytes after injection with irradiated malaria sporozoites, malaria blood parasites or adjuvants. Eur J Immunol **23:**882-887.
39. **Obonyo, C. O., J. Vulule, W. S. Akhwale, and D. E. Grobbee.** 2007. In-hospital morbidity and mortality due to severe malarial anemia in western Kenya. Am J Trop Med Hyg **77:**23-28.
40. **Omar, A. H., M. Yasunami, A. Yamazaki, H. Shibata, M. F. Ofori, B. D. Akanmori, M. N. Shuaibu, M. Kikuchi, and K. Hirayama.** 2012. Toll-like receptor 9 (TLR9) polymorphism associated with symptomatic malaria: a cohort study. Malar J **11:**168.
41. **Omi, K., J. Ohashi, J. Patarapotikul, H. Hananantachai, I. Naka, S. Looareesuwan, and K. Tokunaga.** 2002. Absence of association between the Fc gamma receptor IIIA-176F/V polymorphism and the severity of malaria in Thai. Jpn J Infect Dis **55:**167-169.
42. **Ong'echa, J. M., G. C. Davenport, J. M. Vulule, J. B. Hittner, and D. J. Perkins.** 2011. Identification of inflammatory biomarkers for pediatric malarial anemia severity using novel statistical methods. Infect Immun **79:**4674-4680.

43. **Ong'echa, J. M., C. C. Keller, T. Were, C. Ouma, R. O. Otieno, Z. Landis-Lewis, D. Ochiel, J. L. Slingluff, S. Mogere, G. A. Ogonji, A. S. Orago, J. M. Vulule, S. S. Kaplan, R. D. Day, and D. J. Perkins.** 2006. Parasitemia, anemia, and malarial anemia in infants and young children in a rural holoendemic *Plasmodium falciparum* transmission area. Am J Trop Med Hyg **74:**376-385.

44. **Oswald, I. P., T. A. Wynn, A. Sher, and S. L. James.** 1992. Interleukin 10 inhibits macrophage microbicidal activity by blocking the endogenous production of tumor necrosis factor alpha required as a costimulatory factor for interferon gamma-induced activation. Proc Natl Acad Sci U S A **89:**8676-8680.

45. **Otieno, R. O., C. Ouma, J. M. Ong'echa, C. C. Keller, T. Were, E. N. Waindi, M. G. Michaels, R. D. Day, J. M. Vulule, and D. J. Perkins.** 2006. Increased severe anemia in HIV-1-exposed and HIV-1-positive infants and children during acute malaria. AIDS **20:**275-280.

46. **Ouma, C., G. C. Davenport, S. Garcia, P. Kempaiah, A. Chaudhary, T. Were, S. B. Anyona, E. Raballah, S. N. Konah, J. B. Hittner, J. M. Vulule, J. M. Ong'echa, and D. J. Perkins.** 2011. Functional haplotypes of Fc gamma (Fcgamma) receptor (FcgammaRIIA and FcgammaRIIIB) predict risk to repeated episodes of severe malarial anemia and mortality in Kenyan children. Hum Genet **131:**289-299.

47. **Ouma, C., C. C. Keller, D. A. Opondo, T. Were, R. O. Otieno, M. F. Otieno, A. S. Orago, J. M. Ong'Echa, J. M. Vulule, R. E. Ferrell, and D. J. Perkins.** 2006. Association of FCgamma receptor IIA (CD32) polymorphism with malarial anemia and high-density parasitemia in infants and young children. Am J Trop Med Hyg **74:**573-577.

48. **Parroche, P., F. N. Lauw, N. Goutagny, E. Latz, B. G. Monks, A. Visintin, K. A. Halmen, M. Lamphier, M. Olivier, D. C. Bartholomeu, R. T. Gazzinelli, and D. T. Golenbock.** 2007. Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9. Proc Natl Acad Sci U S A **104:**1919-1924.

49. **Phillips, R. E., S. Looareesuwan, D. A. Warrell, S. H. Lee, J. Karbwang, M. J. Warrell, N. J. White, C. Swasdichai, and D. J. Weatherall.** 1986. The importance of anaemia in cerebral and uncomplicated falciparum malaria: role of complications, dyserythropoiesis and iron sequestration. Q J Med **58:**305-323.

50. **Pichyangkul, S., K. Yongvanitchit, U. Kum-arb, H. Hemmi, S. Akira, A. M. Krieg, D. G. Heppner, V. A. Stewart, H. Hasegawa, S. Looareesuwan, G. D. Shanks, and R. S. Miller.** 2004. Malaria blood stage parasites activate human plasmacytoid dendritic cells and murine dendritic cells through a Toll-like receptor 9-dependent pathway. J Immunol **172:**4926-4933.

51. **Price, R. N., J. A. Simpson, F. Nosten, C. Luxemburger, L. Hkirjaroen, F. ter Kuile, T. Chongsuphajaisiddhi, and N. J. White.** 2001. Factors contributing to anemia after uncomplicated falciparum malaria. Am J Trop Med Hyg **65:**614-622.

52. **Ravetch, J. V., and J. P. Kinet.** 1991. Fc receptors. Annu Rev Immunol **9:**457-492.

53. **Sam-Agudu, N. A., J. A. Greene, R. O. Opoka, J. W. Kazura, M. J. Boivin, P. A. Zimmerman, M. A. Riedesel, T. L. Bergemann, L. A. Schimmenti, and C. C. John.** 2010. TLR9 polymorphisms are associated with altered IFN-

gamma levels in children with cerebral malaria. Am J Trop Med Hyg **82:**548-555.

54. **Stevenson, M. M., M. F. Tam, S. F. Wolf, and A. Sher.** 1995. IL-12-induced protection against blood-stage *Plasmodium chabaudi AS* requires IFN-gamma and TNF-alpha and occurs via a nitric oxide-dependent mechanism. J Immunol **155:**2545-2556.
55. **Su, Z., and M. M. Stevenson.** 2000. Central role of endogenous gamma interferon in protective immunity against blood-stage Plasmodium chabaudi AS infection. Infect Immun **68:**4399-4406.
56. **Tangteerawatana, P., S. Pichyangkul, M. Hayano, T. Kalambaheti, S. Looareesuwan, M. Troye-Blomberg, and S. Khusmith.** 2007. Relative levels of IL4 and IFN-gamma in complicated malaria: association with IL4 polymorphism and peripheral parasitemia. Acta Trop **101:**258-265.
57. **Taylor, R. R., S. J. Allen, B. M. Greenwood, and E. M. Riley.** 1998. IgG3 antibodies to Plasmodium falciparum merozoite surface protein 2 (MSP2): increasing prevalence with age and association with clinical immunity to malaria. Am J Trop Med Hyg **58:**406-413.
58. **Tishkoff, S. A., and S. M. Williams.** 2002. Genetic analysis of African populations: human evolution and complex disease. Nat Rev Genet **3:**611-621.
59. **Torre, D., F. Speranza, M. Giola, A. Matteelli, R. Tambini, and G. Biondi.** 2002. Role of Th1 and Th2 cytokines in immune response to uncomplicated *Plasmodium falciparum* malaria. Clin Diagn Lab Immunol **9:**348-351.
60. **Tsuji, M., Y. Miyahira, R. S. Nussenzweig, M. Aguet, M. Reichel, and F. Zavala.** 1995. Development of antimalaria immunity in mice lacking IFN-gamma receptor. J Immunol **154:**5338-5344.
61. **van Sorge, N. M., W. L. van der Pol, and J. G. van de Winkel.** 2003. FcgammaR polymorphisms: Implications for function, disease susceptibility and immunotherapy. Tissue Antigens **61:**189-202.
62. **Were, T., G. C. Davenport, J. B. Hittner, C. Ouma, J. M. Vulule, J. M. Ong'echa, and D. J. Perkins.** 2011. Bacteremia in Kenyan children presenting with malaria. J Clin Microbiol **49:**671-676.
63. **WHO.** 2010. WORLD MALARIA REPORT. WHO Press, Geneva Switzertland.
64. **Xu, H., J. Wipasa, H. Yan, M. Zeng, M. O. Makobongo, F. D. Finkelman, A. Kelso, and M. F. Good.** 2002. The mechanism and significance of deletion of parasite-specific CD4(+) T cells in malaria infection. J Exp Med **195:**881-892.
65. **Zakeri, S., S. Pirahmadi, A. A. Mehrizi, and N. D. Djadid.** 2011. Genetic variation of TLR-4, TLR-9 and TIRAP genes in Iranian malaria patients. Malar J **10:**77.

Tables

Table 1: Clinical, demographic and laboratory characteristics of the study participants

Characteristics	non-SMA (Hb≥6.0g/dL)	SMA (Hb<6.0g/dL)	*P* value
No. of participants (n=301)	163	138	
Gender, n (%)			
Male	82 (50.3)	66 (47.8)	0.668[a]
Female	81 (49.7)	72 (52.2)	
Age, months	11.0 (10.0)	8.0 (8.0)	**0.010[b]**
Hemoglobin level, g/dL	7.9 (3.0)	4.9 (1.0)	**<0.001[b]**
Parasite density, parasite/μL	18957.0 (43921.5)	17261.55 (36272.0)	0.508[b]
HDP (≥10,000 parasites/μL no. %)	106/163 (65.0)	84/138 (60.9)	0.456[a]
Red Blood Counts, $x10^{12}$/L	3.65 (1.2)	2.13 (0.8)	**<0.001[b]**
Axillary temperature, ºC	37.6 (2.0)	37.4 (2.0)	0.109[b]

Data are the median (interquartile range; IQR) unless otherwise noted. Children with parasitemia (n=301) were stratified according to a modified definition of SMA based on age- and geographically-matched Hb concentrations (i.e., Hb<6.0g/dL, with any density parasitemia) (33), into non-SMA (n=163) and SMA (n=138). HDP, (high density parasitemia).

[a] Statistical significance determined by the χ^2 analysis.

[b] Statistical significance determined by the Mann-Whitney U test.

800 **Table 2: Distribution of FcγRIIIA (-176 F/V) and TLR9 (-1237 T/C) genotypes in**
801 **the clinical groups**
802

	Genotype	Non-SMA (Hb≥6.0g/dL)	SMA (Hb<6.0g/dL)	*p-value*
No. of participants		163	138	N/A
FcγRIIIA-176 F/V	FF, n (%)	85 (52.2)	79 (57.3)	
	FV, n (%)	60 (36.8)	50 (36.2)	0.356[a]
	VV, n (%)	18 (11.0)	9 (6.5)	
TLR9 (-1237 T/C)				N/A
	TT, n (%)	60 (36.8)	63 (45.7)	
	TC, n (%)	87 (53.4)	63 (45.7)	0.297[a]
	CC, n (%)	16 (9.8)	12 (8.6)	

803
804 Data are presented as n (%) of children. Children with parasitemia were categorized
805 on the basis of presence or absence of severe malarial anemia SMA based (defined
806 as Hb<6.0g/dL, with any density parasitemia) (33).
807 [a] Statistical significance determined by the χ^2 analysis.
808

809 **Table 3: Association between individual FcγRIIIA -176 F/V and TLR9 (-1237 T/C)**
810 **genotypes and severe malarial anemia (SMA)**
811

		SMA (Hb level ≤6.0g/dL)	
Genotypes	OR	95%CI	*p-value*
FcγRIIIA -176 F/V			
FF	1.00 (reference)		
FV	0.88	0.53-1.45	0.163
VV	0.58	0.24-1.40	0.235
TLR9 -1237 T/C			
TT	1.00 (reference)		
TC	0.67	0.41-1.10	0.110
CC	0.72	0.31-1.68	0.450

812
813 Children with acute malaria (n=301) were stratified according to the modified
814 definition of SMA based on age- and geographically matched Hb concentration (i.e.,
815 Hb<6.0g/dL, with any density parasitemia) (33). Odds ratios(OR) and 95%
816 confidence intervals (CI) were determined using multivariate logistic regression
817 controlling for age, gender, HIV-1 infection, sickle cell trait (HbAS), bacteremia, and
818 G6PD deficiency. The reference groups in the multivariate logistic regression
819 analysis were the homozygous wild-type genotypes.

820
821
822
823
824
825

Table 4: Distribution of FcγRIIIA -176 F/V and TLR9 (-1237 T/C) SNPs combinations in the clinical groups

FcγRIIIA/TLR9 -1237 T/C	Hb (g/dL)			non-SMA	SMA	
		Median(IQR)	*p*			*p*
				163	138	
-176F/-1237T (n=241)	1	6.20(3.00)	0.588[b]	131(80.6)	110(79.7)	0.887[a]
	0	6.25(3.00)		32(19.6)	28(20.3)	
-176F/-1237C (n=115)	1	5.90(3.00)	0.064[b]	57(35.0)	58(42.0)	0.209[a]
	0	6.30(3.00)		106(65.0)	80(58.0)	
-176V/-1237T (n=80)	1	5.70(3.00)	**0.014[b]**	34(20.9)	46(33.3)	**0.015[a]**
	0	6.30(3.00)		129(79.1)	92(66.7)	
-176V/-1237C (n=74)	1	6.70(3.00)	**0.002[b]**	53(32.5)	21(15.2)	**0.001[a]**
	0	5.60(3.00)		110(67.5)	117(84.8)	

Data are presented as proportions (n, %), the Hb levels are medians (IQR) and the comparisons between carriers and non-carriers of the SNPs combinations were computed using Mann-Whitney U test. SMA (Hb<6.0g/dL with any density parasitemia), (33). Non-SMA, (Hb≥6.0g/dL with any density parasitemia). 1=carrier of SNPs combination, 0=non-carrier.

[a] Statistical significance determined by the χ^2 analysis.

[b] Statistical significance determined by the Mann-Whitney U test.

Values in bold are statistically significant at $p \leq 0.005$.

Table 5: Relationship between FcγRIIIA -176 F/V and TLR9 -1237 T/C SNP combinations and severe malarial anemia (SMA)

SNP Combination	SMA(Hb<6.0g/dL)		
	OR	95% CI	*p-value*
FcγRIIIA-176F/TLR9-1237T	0.94	0.52-1.68	0.830
FcγRIIIA-176F/TLR9-1237C	1.30	0.80-2.10	0.288
FcγRIIIA-176V/TLR9-1237T	2.04	1.19-3.50	**0.009**
FcγRIIIA-176V/TLR9-1237C	0.36	0.20-0.64	**0.001**

Children with acute malaria (n=301) were stratified according to the modified definition of SMA based on age- and geographically matched Hb concentration (i.e., Hb<6.0g/dL, with any density parasitemia) (33). Odds ratios (OR) and 95% confidence intervals (CI) were determined using multivariate logistic regression controlling for age, gender, sickle cell trait (HbAS), bacteremia, and G6PD deficiency. The reference groups in this multivariate logistic regression analysis were those without the respective SNPs combinations.

851 **FIGURE LEGENDS**

852

853 **Figure 1. Relationship between circulating IFN-γ levels and SMA (Hb<6.0g/dL).**
854 Data are represented in box-plots for non-SMA (n=70) and SMA (n=69) groups. The
855 boxes represent interquartile range; the line through boxes is the median while the
856 whiskers show the 10th and the 90th percentiles. Shaded boxes show children with
857 SMA while open boxes are non-SMA. Non-SMA children had significantly lower
858 circulating IFN-γ levels relative to SMA (*p*=0.008; Mann-Whitney U test).

859

860

861 **Figure 2 (A) and (B): Association between circulating IFN-γ and FcγRIIIA -176**
862 **F/V and TLR9 (-1237 T/C).** Data are represented in box-plots for FcγRIIIA -176 F/V
863 (FF=77, FV=53 and VV=9) and TLR9 -1237 T/C (TT=65, TC=65 and CC=9). The
864 boxes represent interquartile range; the line through boxes is the median while the
865 whiskers show the 10th and the 90th percentiles. Across group comparisons were
866 determined using Kruskal-Wallis test. The circulating IFN-γ levels were comparable
867 across the FcγRIIIA -176 F/V (*p*=0.480) and TLR9 -1237 T/C (*p*=0.559) genotypic
868 groups.

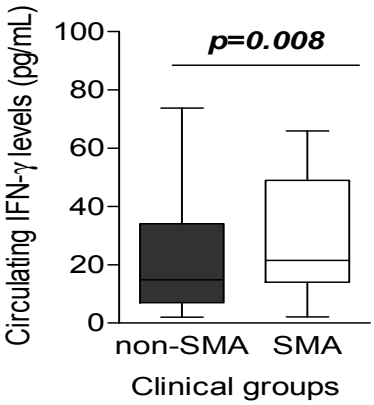
869

870 **Figure 3. Association between circulating IFN-γ levels and FcγRIIIA -176 F/V**
871 **and TLR9 (-1237 T/C) SNPs combinations.** Data are represented in box-plots for
872 FcγRIIIA -176F and TLR9 -1237T (FT=121), FcγRIIIA -176F and TLR9 -1237C
873 (FC=45), FcγRIIIA -176V and TLR9 -1237T (VT=104) and FcγRIIIA -176V and TLR9
874 -1237C (VC=106). The boxes represent interquartile range; the line through boxes is
875 the median while the whiskers show the 10th and the 90th percentiles. Pair-wise

876 comparisons in those with combination and those without the combination was done
877 using Mann-Whitney U test. Shaded boxes show children with the indicated SNPs
878 combination while open boxes show those without the combination. Carriage of
879 FcγRIIIA-176V/TLR9 -1237T (VT) SNP combination was associated with significantly
880 higher levels of circulating IFN-γ levels relative to non-carriers (p=0.011).
881
882

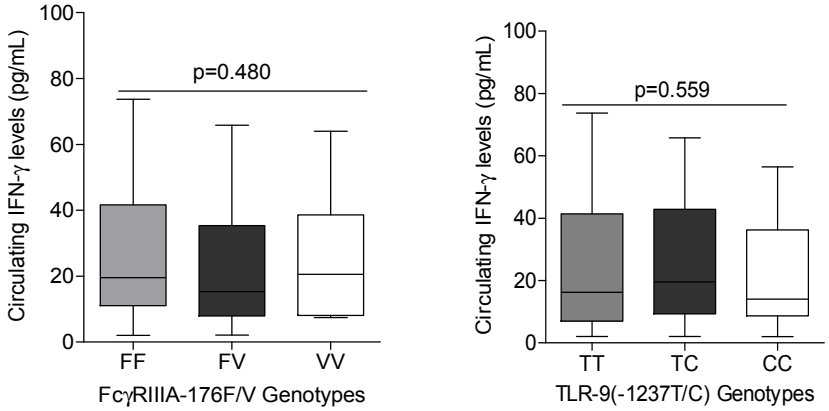
883

FIGURE 1



884
885

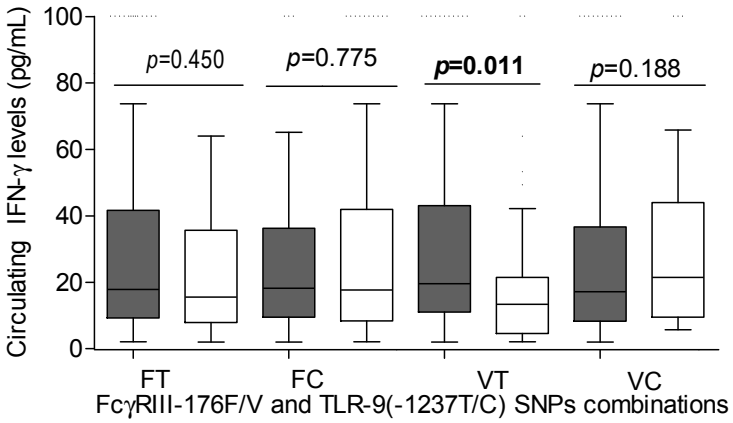
886 **FIGURE 2**
887



888
889

890
891

FIGURE 3



892