

Fc Gamma Receptor IIIB (Fc γ RIIIB) Polymorphisms Are Associated with Clinical Malaria in Ghanaian Children

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Abstract

Plasmodium falciparum malaria kills nearly a million people annually. Over 90% of these deaths occur in children under five years of age in sub-Saharan Africa. A neutrophil mediated mechanism, the antibody dependent respiratory burst (ADRB), was recently shown to correlate with protection from clinical malaria. Human neutrophils constitutively express Fc gamma receptor-Fc γ RIIA and Fc γ RIIIB by which they interact with immunoglobulin (Ig) G (IgG)-subclass antibodies. Polymorphisms in exon 4 of *FCGR2A* and exon 3 of *FCGR3B* genes encoding Fc γ RIIA and Fc γ RIIIB respectively have been described to alter the affinities of both receptors for IgG. Here, associations between specific polymorphisms, encoding Fc γ RIIA p.H166R and Fc γ RIIIB-NA1/NA2/SH variants with clinical malaria were investigated in a longitudinal malaria cohort study. Fc γ RIIA-p.166H/R was genotyped by gene specific polymerase chain reaction followed by allele specific restriction enzyme digestion. *FCGR3B*-exon 3 was sequenced in 585 children, aged 1 to 12 years living in a malaria endemic region of Ghana. Multivariate logistic regression analysis found no association between Fc γ RIIA-166H/R polymorphism and clinical malaria. The A-allele of *FCGR3B*-c.233C>A (*rs5030738*) was significantly associated with protection from clinical malaria under two out of three genetic models (additive: $p=0.0061$; recessive: $p=0.097$; dominant: $p=0.0076$) of inheritance. The Fc γ RIIIB-SH allotype (CTGAAA) containing the 233A-allele (in bold) was associated with protection from malaria ($p=0.049$). The Fc γ RIIIB-NA2*03 allotype (CTGCCGA), a variant of the classical Fc γ RIIIB-NA2 (CTGCAA) was associated with susceptibility to clinical malaria ($p=0.0092$). The present study is the first to report an association between a variant of Fc γ RIIIB-NA2 and susceptibility to clinical malaria and provides justification for further functional characterization of variants of the classical Fc γ RIIIB allotypes. This would be crucial to the improvement of neutrophil mediated functional assays such as the ADRB assay aimed at assessing the functionality of antibodies induced by candidate malaria vaccines.

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Introduction

Plasmodium falciparum malaria kills nearly a million people annually and over 90% of deaths occur in children under five years of age in sub-Saharan Africa [1]. Several sero-epidemiological studies have associated *P. falciparum* antigen-specific cytophilic immunoglobulin (Ig) G (particularly IgG1 and IgG3) with protection from clinical malaria [2–4] suggesting a critical role for immune effector cells in malaria immunity. Monocytes, upon activation by IgG opsonized infected erythrocytes are thought to release certain, as yet uncharacterized, factors that inhibit intra-erythrocytic parasite growth [5,6]. This mechanism, termed antibody dependent cellular inhibition (ADCI), has been widely studied in *in vitro* systems but has so far not shown any significant correlation with protection against clinical malaria. On the other hand a correlation has been reported between neutrophil

mediated antibody dependent respiratory burst (ADRB) and protection from clinical malaria in two Senegalese populations which differ in malaria transmission intensity [7]. Human neutrophils constitutively express two receptors, namely Fc gamma receptor (Fc γ R)- Fc γ RIIA and Fc γ RIIIB [8], which bind the Fc domain of IgG. These receptors complement each other functionally [9]. Fc γ RIIA is a transmembrane protein while Fc γ RIIIB has no transmembrane domain and is anchored in the plasma membrane through a C-terminus linked glycosylphosphatidylinositol moiety [10,11]. In addition to neutrophils, most cells of the immune system, including monocytes, macrophages, eosinophils, basophils, Langerhans cells, platelets, placental endothelial cells and some T cells subpopulations are known to express Fc γ RIIA while Fc γ RIIIB is expressed exclusively on neutrophils [12]. Crosslinking of Fc γ RIIA on neutrophils induces phagocytosis of IgG-opsonised particles [13] while Fc γ RIIIB

crosslinking leads to neutrophil degranulation and generation of reactive oxygen species (ROS), which have been shown to alter FcγRIIA avidity and efficiency in an allele-specific manner [14]. ROS are known to be highly toxic to intra-erythrocytic malaria parasites [5,15,16] and high ROS production by neutrophils has been correlated with fast *P. falciparum* clearance in Gabonese children [17] and protection from clinical malaria in two Senegalese populations [7].

Single nucleotide polymorphisms (SNPs) which alter the affinities of both FcγRIIA and FcγRIIIB in binding IgG subclasses have been described. In the present study, we hypothesised that the implications of these polymorphisms in the described neutrophil mediated immune correlate of clinical protection from malaria (the ADRB mechanism) by Joos and colleagues [7], could be critical to the outcome of *P. falciparum* infection. In the *FCGR2A* gene (NM_021642.3), a non-synonymous variant, c.497G>A (rs1801274) in exon 4, which specifies the ligand binding domain of the receptor, causes an arginine (R) substitution of an histidine (H) amino acid at position 166 of the polypeptide (p.H166R) [18]. The FcγRIIA-H166 allele has higher affinity for human IgG2 and IgG3, compared to FcγRIIA-R166 which binds weakly [19]. In a recent study, Schuldt and colleagues found FcγRIIA-R166/R166 homozygosity to be associated with severe malarial anaemia in Ghanaian children [20]. FcγRIIIB bears the neutrophil antigen (NA) polymorphism in its membrane-distal Ig-like domain and is found in three polymorphic forms, called human neutrophil antigen (HNA)-1a (or NA1), HNA-1b (or NA2) and HNA-1c (or SH), which are encoded by *FCGR3B*1*, *FCGR3B*2* and *FCGR3B*3* alleles, respectively [21]. The *FCGR3B*1* and *FCGR3B*2* alleles differ in five nucleotide positions; c.108C>G; c.114T>C; c.194A>G; c.244A>G and c.316A>G [22] in exon 3 of the *FCGR3B* gene (NM_00570.4) which results in four amino acid changes at positions; p.S36R; p.N65S; p.N82D and p.I106V of the peptide chain with the c.114T>C being a synonymous coding SNP [23]. FcγRIIIB-NA1 facilitates phagocytosis of IgG1- and IgG3-opsonized particles more efficiently than FcγRIIIB-NA2. This may be due to the presence of two additional N-linked glycosylation sites in FcγRIIIB-NA2 compared to FcγRIIIB-NA1 [24]. The nucleotide sequence of the FcγRIIIB coding region of *FCGR3B*3* is identical to the *FCGR3B*2* sequence except for a SNP c.233C>A (rs5030738) encoding p.A78D resulting in the expression of the FcγRIIIB-SH allo-antigen [25]. This allotype has been associated with about a third of FcγRIIIB alleles and higher expression levels of FcγRIIIB but its influence on receptor function is unclear [26]. Furthermore, in individuals whose neutrophils lack the FcγRIIIB molecule (NA_{null}) the corresponding gene deletion has been described [27]. Sequencing analysis has identified variants of the classical FcγRIIIB-NA1/NA2 allotypes but their functional significance have, as yet, not been characterised [28–30]. The FcγRIIIB-NA2 allotype in combination with the FcγRIIA-166H allele have been associated with cerebral malaria in Thai individuals [31] and severe malarial anaemia in Kenyan children [32]. In general, malaria immunogenetic studies have so far mainly focused on severe forms of malaria using either cross-sectional and/or case-control study data [20,32–34]. In the present study, we successfully elucidated associations between *FCGR3B* and *FCGR2A* polymorphisms and clinical malaria using data from a well characterised longitudinal cohort study.

Results

Demographic and clinical characteristics of study population

Of the 669 out of 798 children who successfully completed the 42 week longitudinal follow up, DNA was available for 585 (87.4%). These children were distributed across six villages as

follows: Asutsuare (ASU) (169), Kewum (KEW) (138), Avakpo (AVA) (33), Mafikorpe (MAF) (36), Osuwem (OSU) (71) and Volivo (VOL) (138) (Table 1). A total of 329 (56.2%) children were ≤5 years of age and 88 (15.0%) were sickle cell positive. The study population consisted predominantly of the Ga-Adangbe (n = 430, 73.5%) and Ewe (n = 76, 13.0%) ethnic groups. The remaining (n = 79, 13.5%), children belonged to the Akan, Hausa or Fulani ethnic groups. Children who used bed net constituted 42.1% of the study population. The gender distribution was not significantly different between the villages ($p=0.75$, χ^2 analysis) while the distribution of age, blood group, ethnicity and sickle cell status were significantly different between the villages ($p\leq 0.0007$, χ^2 analysis) (Table 1).

P. falciparum infections in the study cohort

The incidence of clinical malaria during the follow up period was low (52 cases, 8.9%). These individuals were considered susceptible to clinical malaria while those who never had clinical malaria were considered protected. The protected group was sub-categorized into two: (1) any individual with no malaria episode and (2) only individuals with no malaria episodes but with parasites detectable by microscopy at any time point during the follow up period. The overall number of susceptible and protected

Table 1. Demographics and clinical characteristics of study participants.

	ASU	KEW	AVA	MAF	OSU	VOL	p-value [†]
N	169	138	33	36	71	138	
Age group (years)							
1–5	122	66	17	15	50	59	
6–12	47	72	16	21	21	79	<0.0001
Sex							
Male	83	71	19	17	32	62	
Female	86	67	14	19	39	76	0.75
Sickle cell							
Negative	152	129	12	23	68	113	
Positive	17	9	21	13	3	25	<0.0001
Blood group							
O	96	64	10	11	37	62	
A	33	31	14	7	8	18	
B	32	36	9	13	19	46	
AB	8	7	0	5	7	12	0.0007
Bed net use							
Yes	63	65	21	26	26	45	
No	106	73	12	10	45	93	<0.0001
Ethnic group							
Ga-Adangbe	124	103	15	14	67	107	
Ewe	15	7	14	18	1	21	
Other	30	28	4	4	3	10	<0.0001
Clinical malaria status							
Susceptible	12	15	2	0	9	14	
Protected	157	123	31	36	62	124	0.24

[†]p-values refer to chi-square tests. ASU: Asutsuare; KEW: Kewum-Atrobinyina; AVA: Avakpo; MAF: Mafikorpe; OSU: Osuwem; VOL: Volivo. doi:10.1371/journal.pone.0046197.t001

individuals did not differ significantly ($p = 0.24$, χ^2 analysis) among the six villages (Table 1). Logistic regression analyses investigating the association of the covariates, age groups, sex, blood group, ethnicity and sickle cell status with clinical malaria, found an association with ethnicity. Individuals of the Ewe ethnic group had a significantly reduced risk of clinical malaria compared to other ethnic groups (likelihood ratio test: $p_{(LR \text{ test})} = 0.013$) (Table 2).

Distribution of FCGR2A and FCGR 3B genotype in Ghanaian children

The Fc γ RIIA-p.H166R (c.497A>G) genotypes were determined by gene specific polymerase chain reaction amplification followed by allele specific restriction enzyme digestion (PCR-ASRED). The minor allele frequency (which is this population was the A-allele) was 41.0% and there was no deviation from HWE ($p = 0.690$, χ^2 analysis). Nucleotide sequencing of exon 3 of *FCGR3B* identified the six polymorphisms (c.108C>G, c.114T>C, c.194A>G, c.233C>A, c.244A>G and c.316A>G), defining the Fc γ RIIB-NA1/NA2/SH allotypes. The previously described c.197T>G, and c.297G>T polymorphisms were also found in 87 (14.9%) and 46 (7.9%) individuals respectively. A new SNP, c.232G>A was identified in five (0.85%) of the study participants. All the SNPs were in HWE, $p > 0.05$ (Table 3). The distribution of the genotypes was not significantly different between the ethnic groups except for the *FCGR2A*-c.497A>G ($p = 0.038$, χ^2 test) and *FCGR3B*-c.194A>G ($p = 0.017$, χ^2 test) SNPs (Table S1).

FCGR2A and FCGR 3B genotypes and clinical malaria

The distribution of the *FCGR2A*-c.497A>G (Fc γ RIIA-p.H166R) genotype frequencies among the susceptible and protected groups did not differ significantly in a multivariate analysis and had no influence on the outcome of *P. falciparum* infection ($p_{(LR \text{ test})} = 0.80$) (Table S2). The *FCGR3B*-c.233C>A (Fc γ RIIB-p.A78D) polymorphism showed a statistically significant association with the outcome of *P. falciparum* infection ($p_{(LR \text{ test})} = 0.009$) (Table S1). The A-allele was significantly associated with protection from clinical malaria under two out of the three genetic models of inheritance (additive: $p = 0.0061$, recessive: $p = 0.097$ and dominant: $p = 0.0076$) analysed using a control group that included all individuals with no clinical malaria during follow up (Table 4). The confounding effect of possible heterogeneity in exposure was investigated by repeating the analysis with a redefined control group comprising only individuals with no malaria episodes but with a definitive evidence of exposure i.e. with parasites detected by microscopy during follow up. The same marker (c.233A-allele) was significantly associated with protection from malaria (Table S3) under the same genetic models of inheritance previously observed, confirming the initial observation. Disease association analyses for *FCGR3B* were restricted to the six SNPs encoding the Fc γ RIIB-NA1/NA2/SH allotypes. Of the three additional SNPs, only the T-allele of c.297G>T was significantly associated with protection from clinical malaria (OR = 0.307, 95%CI = 0.16–0.61, $p = 0.0016$, Fisher's exact test).

Table 2. Covariates association with clinical malaria.

Covariates	Susceptible (n = 52)	Protected (n = 533)	OR(95%CI) ^a	p-value ^a	LR test p-value
Age group (years)					
1–5	29	300	1		
6–12	23	233	1.01 (0.56–1.81)	0.97	0.97
Sex					
Male	25	259	1		
Female	27	274	0.96 (0.54–1.72)	0.89	0.89
Sickle cell					
Negative	45	452	1		
Positive	7	81	0.94 (0.37–2.07)	0.89	0.89
Blood group					
O	26	254	1		
A	6	105	0.59 (0.22–1.42)	0.27	
B	15	140	1.08 (0.54–2.11)	0.81	
AB	5	34	1.58 (0.50–4.16)	0.39	0.46
Bed net use					
Yes	20	226	1		
No	32	307	1.06 (0.58–1.96)	0.85	0.85
Ethnic group					
Other	8	71	1		
Ga-Adangbe	43	387	0.97 (0.46–2.33)	0.95	
Ewe	1	75	0.12 (0.01–0.67)	0.047	0.013

Odds Ratios (OR) and 95% confidence intervals (CI) were determined using multivariate logistic regression.

^aAnalysis for each covariate was adjusted by the other remaining covariates: age groups, sex, sickle cell status, blood group, bed net use and ethnic group. The likelihood ratio (LR) test result compares the adjusted model to a model which only includes the adjusting factors and thereby tests if the variable has an effect on susceptibility.

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Table 3. *FCGR3B* allele frequencies and Hardy-Weinberg (HW) estimations in protected individuals.

Variation ID	Alleles ^a	Amino acid change ^a	Minor allele (Frequency)	HW <i>p</i> -value ^a
rs403016	c.108C>G	p.S36R	G (0.491)	1.00
rs447536	c.114T>C	Synonymous coding	C (0.471)	0.62
rs448740	c.194A>G	p.N65S	A (0.390)	0.61
rs5030738	c.233C>A	p.A78D	A (0.214)	0.49
rs428888	c.244A>G	p.N82D	A (0.491)	0.066
rs2290834	c.316A>G	p.I106V	G (0.251)	0.29

^aAllele and amino acid numberings refer to positions in *FCGR3B* transcript ENST00000367964.

^bHW estimations based on children (*n* = 267) who had diploid copies of *FCGR3B*, were the first sibling in a family and were not susceptible to clinical malaria in the observation period.

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FCGR3B haplotype association with clinical malaria

The six SNPs defining the FcγRIIIB-NA1/NA2/SH were investigated for association with clinical malaria. Pairwise r^2 values between these SNPs ($0.08 \leq r^2 \leq 0.86$) from linkage analysis revealed a significant linkage between most of the SNPs (Figure 1A). A haplotype block was defined to include all SNPs in order to estimate the frequencies of FcγRIIIB allotypes in the entire study population (Figure 1A). The haplotype analysis identified the three major FcγRIIIB allotypes (FcγRIIIB-NA1/NA2/SH), two variants of FcγRIIIB-NA1 (FcγRIIIB-NA1*02 and FcγRIIIB-NA1*06) and two variants of FcγRIIIB-NA2 (FcγRIIIB-NA2*02 and FcγRIIIB-NA2*03) (Figure 1B). The FcγRIIIB-NA2*03 was significantly associated with susceptibility to clinical malaria (OR = 2.67, 95%CI = 1.27–5.59, $p = 0.0092$) while the FcγRIIIB-SH allotype showed a borderline significant association with protection (OR = 0.42, 95%CI = 0.18–1.00, $p = 0.049$) (Figure 1B). When only individuals who did not have malaria but with detectable parasites during the follow up period were considered as controls in the haplotype association analysis, logistic regression could not be performed because the risk haplotype FcγRIIIB-NA2*03 was completely absent in the control group (*n* = 57).

Population differences of FCGR3B-c.233C>A polymorphism

Exon 3 of *FCGR3B* was sequenced in 132 native Danish blood donors and genotype distributions compared to the Ghanaian

population to investigate differences and possible selection. Sequence analysis found all the six SNPs defining the FcγRIIIB-NA1/NA2/SH system. All SNPs were in HWE ($p > 0.05$). One person carried synonymous variant (c.201C>T). The c.233C>A genotype frequencies were significantly different ($p < 0.0001$, χ^2 analysis) between the Ghanaian and Danish populations (Figure 2). Although the A-allele remained the minor allele in both populations, none of the 132 Danes was homozygous compared to the 8.5% homozygosity in the Ghanaian population. Only 10 individuals were heterozygous in the Danish population. The Tajima's D statistic estimated for *FCGR3B* exon 3 showed a significant positive deviation from the values expected under neutrality in both the Ghanaian ($D = 2.9$, $p < 0.05$) and Danish ($D = 2.2$, $p < 0.05$) populations. Comparisons with data from the 1000 Genomes Project database showed that the A-allele frequency for the African (AFR) (17.9%) super population was similar to that in the Ghanaians (21.4%) in this study. Similarly, the A-allele frequency for the Ad Mixed American (AMR) (3.3%) and European (EUR) (0.8%) super populations were comparable to the Danes (3.8) (Table 5). Thus, the A-allele, which was associated with protection from *P. falciparum* malaria, was more frequent in malaria endemic populations than in non-endemic populations. To further evaluate the extent of divergence with respect to this polymorphism, pairwise F_{ST} indices were calculated for all five populations (Ghanaians, Danes, AFR, AMR and EUR). The AFR and the Ghanaian population shared a pairwise F_{ST} value of 0.004 while all comparisons between the malaria endemic (Ghanaians and AFR) and the malaria non-endemic (Danes, AMR

Table 4. Single marker association of *FCGR3B* alleles with clinical malaria.

SNP ID	Minor Allele	MAF		Additive model		Recessive model		Dominant model	
		Protected ^F	Susceptible	OR (95% CI)	<i>p</i> -value	OR (95% CI)	<i>p</i> -value	OR (95% CI)	<i>p</i> -value
rs403016	C	0.49	0.50	1.04 (0.71–1.54)	0.84	1.38 (0.74–2.53)	0.31	0.82 (0.44–1.53)	0.53
rs447536	C	0.47	0.46	0.98 (0.67–1.45)	0.94	1.11 (0.57–2.13)	0.77	0.88 (0.47–1.63)	0.68
rs448740	A	0.40	0.38	0.95 (0.64–1.42)	0.82	1.16 (0.56–2.40)	0.69	0.80 (0.45–1.44)	0.46
rs5030738	A	0.22	0.10	0.43 (0.23–0.78)	0.0061	0.18 (0.02–1.36)	0.097	0.37 (0.18–0.77)	0.0076
rs428888	A	0.50	0.40	0.74 (0.51–1.06)	0.10	0.52 (0.25–1.07)	0.075	0.74 (0.40–1.36)	0.34
rs2290834	G	0.27	0.22	0.81 (0.52–1.27)	0.36	0.87 (0.34–2.20)	0.77	0.69 (0.37–1.27)	0.23

Odds ratio (OR) and 95% confidence intervals (CI) were determined using multivariate logistic regression controlling for age, gender, ethnicity, sickle-cell status, *FCGR3B* copy number, blood group, family structure and use of bed net. MAF: minor allele frequency.

^FAll individuals who never had malaria despite parasitaemia at any time point (monthly blood slide) during the study, plus all individuals who never had malaria but without detectable parasitaemia by microscopy.

doi:10.1371/journal.pone.0046197.t004

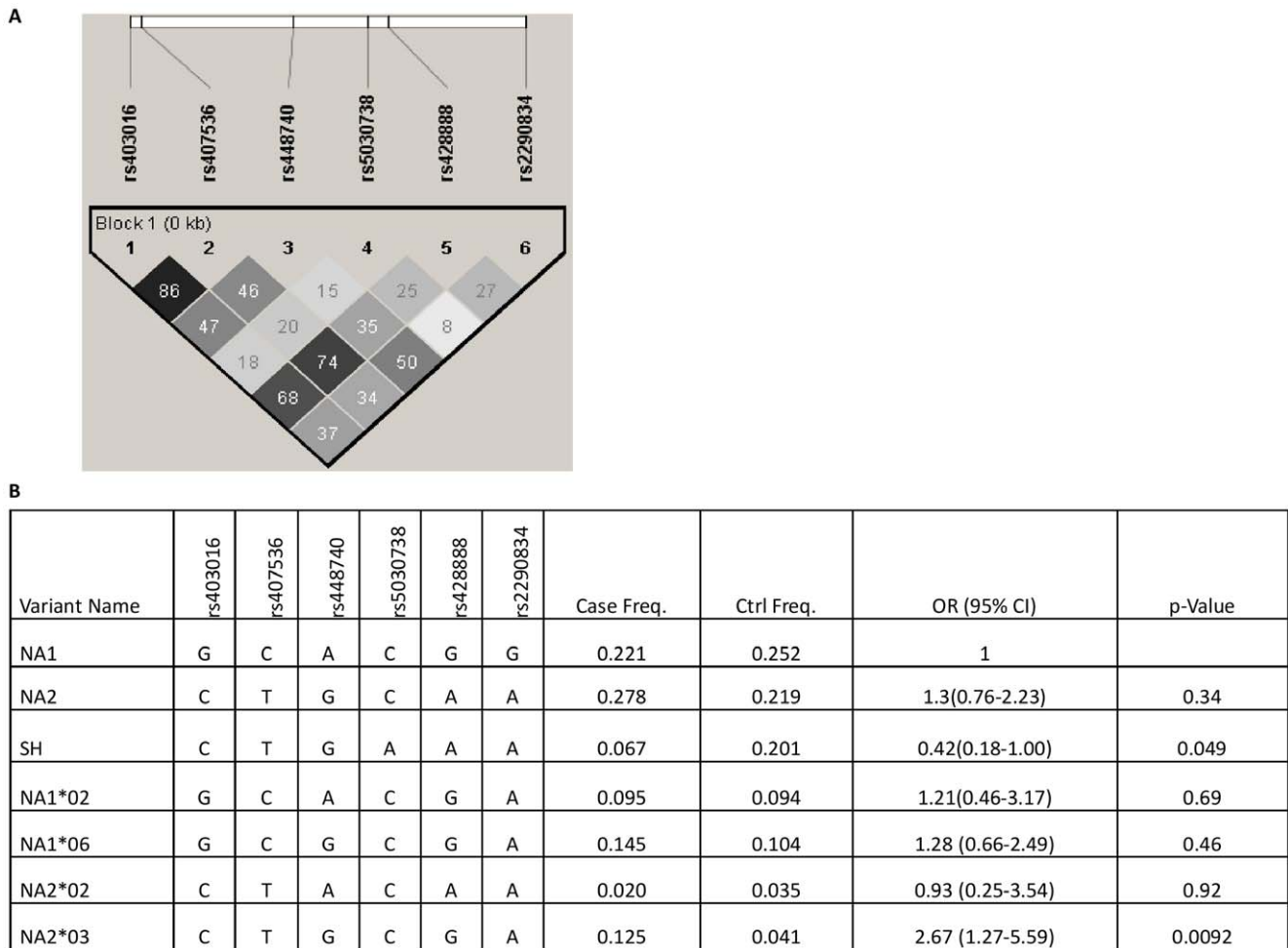


Figure 1. Studied SNPs in the *FCGR3B* gene, linkage disequilibrium (LD) patterns and haplotype association analysis. **A**) A schematic of exon 3 *FCGR3B* gene (NM_000570.4) and LD plot of the respective SNPs visualised using Haploview v4.2. The LD plot shows pairwise r^2 values ($\times 100$) given in the squares for each comparison between the SNPs. White squares represent r^2 values equal to 0. Different shades of grey represent r^2 values between 0 and 1. **B**) Haplotype associations with susceptibility to clinical malaria compared to clinically protected individuals. Odds ratio (OR) and 95% confidence intervals (CI) were determined using multivariate logistic regression controlling for age, gender, ethnicity, sickle-cell status, *FCGR3B* copy number, blood group and use of bed net. The haplotype with the highest frequency in the study population was considered the reference group in the multivariate logistic regression analyses. [‡]Variant first reported in this study, the associated gene for NA1*06 is *FCGR3B*01A194G, G316A*; [‡] Variants first reported by Matsuo et al, [28], the associated genes for NA1*02, NA2*02 and NA2*03 are *FCGR3B*01G316A, FCGR3B*02G194A* and *FCGR3B*02A244G* respectively. doi:10.1371/journal.pone.0046197.g001

and EUR) populations yielded consistently higher F_{ST} values ($0.098 \leq F_{ST} \leq 0.194$) (Table 6).

Discussion

Using PCR-ASRED and exon 3 nucleotide sequencing data for *FCGR2A* and *FCGR3B* respectively, the association between SNPs which alter the affinity of these receptors for IgG subclasses and clinical malaria were studied in a cohort of Ghanaian children. There was no association between the *FCGR2A*-c.497A>G (Fc γ RIIA-p.H166R) polymorphism and clinical malaria while the A-allele of *FCGR3B*-c.233C>A polymorphism (rs5030738) was strongly associated with protection from clinical malaria. Haplotype analysis identified the Fc γ RIIIB-SH allotype (CTG**AAA**) containing the c.233A-allele (in bold) to be associated with protection while a variant of Fc γ RIIIB-NA2, the Fc γ RIIIB-NA2*03 allotype (CTGCGA) was associated with susceptibility to clinical malaria. Individuals of the Ewe ethnic group showed a

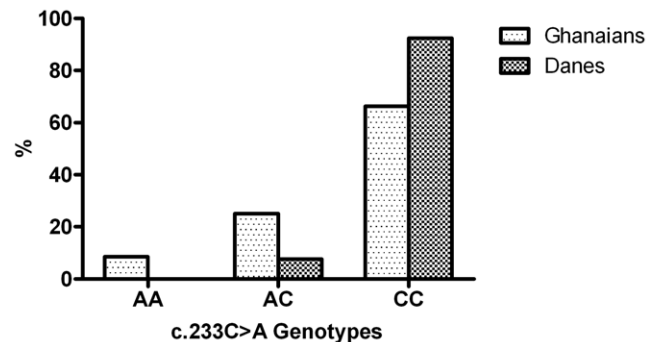


Figure 2. *FCGR3B*-c.233C>A genotypes. Genotype distribution compared between malaria endemic (Ghanaian) and malaria non-endemic (Danish) populations. doi:10.1371/journal.pone.0046197.g002

Table 5. rs5030738 (c.233C>A) allele distribution among malaria endemic and malaria non-endemic populations.

rs5030738 c.233C>A	Malaria endemic		Malaria non-endemic		
	Ghanaians (n = 585)	AFR* (n = 248)	Danes (n = 132)	EUR* (n = 381)	AMR* (n = 181)
A-allele %	21.4	17.9	3.8	0.8	3.3
C-allele %	78.6	82.1	96.2	99.2	96.7

*Allele frequency data from the 1000 Genomes project data base. AFR: African; AMR: Ad Mixed American; EUR: European.
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reduced risk to clinical malaria compared to other ethnic groups, however, the distribution of genotypes was not different between the ethnic groups except for *FCGR2A*-497A>G and *FCGR3B*-194A>G. These genotypes had no association with clinical malaria. Also, while the ethnic group distribution was different between the villages, the number of susceptible and protected individuals was not different between the villages. Thus, the genetic associations with protection and susceptibility observed here could not be due to a bias caused by ethnicity. Heterogeneity in malaria exposure is an important confounder in association studies [35]. In the current study, the outcome of the association analysis did not differ whether or not the control group included individuals with no malaria and no detectable asymptomatic parasitaemia by microscopy. This suggests exposure may have been homogeneous and that asymptomatic malaria infection in some of the individuals went undetected due to immunity resulting in sub-microscopic parasitaemia [35]. The protective allele (c.233A), though a minor allele in all populations in this study, still had a significantly higher frequency in malaria endemic populations compared to the non-endemic populations, possibly because of positive selection by *P. falciparum* malaria. The Tajima's D estimates show that *FCGR3B* exon 3 is under strong selection pressure in the Ghanaian populations and also to a lesser extent in the Danish population.

Both sero-epidemiological [2–4] and *in vitro* studies [5,36] have demonstrated that cytophilic IgG (1 and 3) subclasses are the most important in controlling parasites multiplication and/or disease, emphasizing the importance of effector cells such as monocytes and neutrophils in malaria immunity. Neutrophils are the most abundant leucocytes and known to have both high phagocytic and cytotoxic capabilities through the generation and release of potent cytotoxic mediators such as reactive oxygen species (ROS) and proteases [37]. They are the main effector cells in the ADRB

mechanism recently shown to correlate with protection from clinical malaria [7]. Neutrophils engage IgG1 and IgG3 immune complexes (ICs) through FcγRIIA and FcγRIIB constitutively expressed on the cell surface. Although these receptors function synergistically, it has been shown that phagocytosis by neutrophils is primarily dependent on FcγRIIA crosslinking [13] while ROS release is by FcγRIIB crosslinking with ICs [14]. The lack of association between clinical malaria and any of the genotypes of FcγRIIA-p.H166R in this study suggests that, neutrophil ROS activity may be paramount to phagocytic activity in protecting against clinical malaria since only FcγRIIB polymorphisms could explain the outcome of *P. falciparum* infection. This notion is supported by both *in vitro* studies of ROS toxicity on intra-erythrocytic malaria parasites [5,15,16] and field studies where high ROS production by neutrophils correlated with fast *P. falciparum* clearance [17] and protection from clinical malaria [7].

The c.233A-allele of *FCGR3B* which was associated with protection from clinical malaria in the present study causes a replacement of the hydrophobic amino acid alanine (A) with the negatively charged aspartic acid (D) at position 78 in the NA2 protein. This substitution results in the SH allotype which also showed association with protection. The functional implications of this p.A78D substitution in receptor-ligand (antibody) interactions have not been conclusively shown. Koene and colleagues [26] suggest it may influence a ligand epitope possibly located in the membrane distal Ig-like domain. In the same study, the SH allotype was associated with high expression levels of FcγRIIB. Thus, protection from clinical malaria seen in individuals with the A-allele and SH allotype may be due to increased ROS production as a result of both enhanced antibody-receptor interaction and increased FcγRIIB density on neutrophil cell surface. The maximum statistical power for A-allele association with protection was observed under the dominant model with homozygous individuals having over 60% reduced risk to acquiring clinical malaria compared to the other models tested. In genetic association studies, maximum power to detect significant association is reached when the 'true' mode of inheritance and the genetic model used in the analysis are concordant [38]. Thus, the dominant model best explains the mode of inheritance of the c.233C>A polymorphism in the Ghanaian cohort studied.

Association studies based on Polymerase Chain Reaction-Sequence Specific Primer (PCR-SSP) data of *FCGR2A* and *FCGR3B* have found individuals carrying the NA2 allotype in combination with FcγRIIA-166H have an increased risk of developing cerebral malaria [31] and severe malarial anaemia [32]. Here, using *FCGR3B*-exon 3 nucleotide sequencing data, we find a previously reported variant of FcγRIIB-NA2, FcγRIIB-NA2*03 (associated gene: *FCGR3B**02A244G, [28]) to be associated with susceptibility to clinical malaria. While the PCR-SSP technique [39] is an established method for genotyping *FCGR3B* and has been extensively used, sequencing data [28–30,40] have consistently shown that the *FCGR3B* gene is more polymorphic

Table 6. Pairwise genetic distances between malaria versus non-malaria population with respect to c.233C>A (rs5030738) polymorphism.

Population	AFR [†]	Danes	EUR [†]	AMR [†]
Ghanaian	0.004	0.131	0.194	0.141
AFR [†]		0.098	0.159	0.106
Danes			0.020	0.000
EUR [†]				0.015

F_{ST} distance (Latter et al., 1972).

[†]Allele frequency data from the 1000 Genomes Project database (http://browser.1000genomes.org/Homo_sapiens/Variation/Population?db=core;g=ENSG00000162747;r=1:161592986-161601753;t=ENST00000531221;v=rs5030738;vdb=variation;vf=8673417).
AFR: African; AMR: Ad Mixed American; EUR: European.
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than was previously thought. Variations at the allotype defining sites could result in aberrant PCR-SSP typing [30,40]. More importantly, the PCR-SSP method cannot differentiate between the classical NA2 and the variant NA2*03 and will simply type both as NA2. Thus, the PCR-SSP method alone may not be enough to sufficiently characterise Fc γ RIIIB allotypes in disease association studies. It is not clear how exon 3 sequencing data would have affected the conclusions from PCR-SSP typing association studies particularly where NA2 was associated with disease outcome. The risk associated allotype NA2*03 found in the Ghanaian population, differs from the classical NA2 by an asparagine (N) to aspartic acid (D) substitution at position 82 of the Fc γ RIIIB protein. This N82D substitution results in a loss of one potential N-glycosylation site of NA2. It is conceivable, that the NA2*03 variant would have one-less (ie. 5) potential N-glycosylation sites. NA2 shows a reduced capacity to facilitate phagocytosis, respiratory burst and degranulation responses compared to NA1, a property attributed to the extensive glycosylation in NA2 than in NA1 [9,41]. Thus, it would be expected that the NA2*03 variant possessing one-less glycosylation site compared to NA2 should have a higher affinity for IgG1 and IgG3 than NA2 and hence be the more efficient receptor. However, no study has as yet investigated the binding affinity of the NA2*03 variant for IgG1 and IgG3 or the possible conformational changes due to the replacement of a potential glycosylated group (p.82N) with an unglycosylated charged group (p.82D). N-glycosylation is a common feature of many membrane-bound and extracellular proteins in animals and the carbohydrate groups are considered crucial for biological functions such as proper protein folding [42], protein stability and solubility [43], ligand binding affinity [44], signal transduction [45], and immunogenicity [46]. Thus, it is plausible that the p.N82D substitution in the NA2*03 variant may alter an important biological mechanism critical for neutrophil respiratory burst and hence predispose to clinical malaria. Further studies are needed to delineate the possible functional consequences of the NA2*03 polymorphism in Fc γ RIIIB.

The Tajima's D analysis showed exon 3 of *FCGR3B* to be under selection pressure in both the Ghanaian and the Danish populations; however, it is not clear what may be driving the selection in these different populations. Malaria has been considered the strongest known selective pressure in the recent history of the human genome [47]. There was a higher frequency of the c.233A allele in malaria endemic populations and high pairwise F_{ST} indices ($0.098 \leq F_{ST} \leq 0.194$) between the malaria versus non-malaria endemic populations. These observations support the notion that malaria may be a significant contributor to the selection pressure, at least in the Ghanaian and African (AFR) populations. However, other possible factors that could explain the selection in both the malaria and non-malaria endemic populations may include inflammatory diseases such as rheumatoid arthritis [48] or periodontal diseases (PD). PD is a widespread condition and several studies have associated Fc γ RIIIB polymorphisms with PD in different populations [49]. In animal studies, PD has been shown to contribute to perinatal mortality [50]. A recent study in humans concluded that, in cases of extreme prematurity, maternal PD may be a significant contributor to perinatal mortality [51]. Given the contribution of PD in perinatal mortality and the association of Fc γ RIIIB polymorphisms in the pathogenesis of PD, we speculate that PD may contribute to the selection pressure acting on exon 3 of *FCGR3B*. It is however, worth noting that the present data does not clearly show which modes of natural selection may be at play in these two populations. Further studies are needed to clearly define the forces of selection on *FCGR3B*-exon 3 in these populations.

In conclusion, the present study has identified the c.233A allele of *FCGR3B*-c.233C>A (rs5030738) and the Fc γ RIIIB-SH (CTGAAA) haplotype to be associated with protection from clinical malaria. The Fc γ RIIIB-NA2*03 (CTGCGA) variant of Fc γ RIIIB-NA2 (CTGCAA) was associated with susceptibility to clinical malaria in the Ghanaian population. The study provides the justification for a more detailed functional characterisation of the Fc γ RIIIB-SH and Fc γ RIIIB-NA2*03 haplotypes in relation to neutrophil functionality especially in respiratory burst activity.

Materials and Methods

Ethics Statement

Ethical approval for the study was given by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research (NMIMR) of the University of Ghana, Accra, Ghana. Written informed consent was given by the parents and guardians of children before they were enrolled into the study. Ethical approval for Danish blood donor samples was given by the Scientific Ethics Committee of Copenhagen and Frederiksberg, Denmark. DNA samples from a total of 132 anonymous Danish blood, obtained for control purposes from Copenhagen University Hospital, were analysed in order to allow population comparisons of genotype distributions. These individuals are resident of central Copenhagen and provided written consent to have a small portion of their blood stored, anonymously, and used for research purposes. Blood donors in Denmark must be between the ages of 18 and 60. All data were analysed anonymously.

Study area, population and baseline sampling

The study was conducted in Asutsuare (ASU) (about 120 km north-east of Accra) and five neighbouring villages: Kewum-Atrobinya (KEW), Avakpo (AVA), Mafikorpe (MAF), Osuwem (OSU) and Volivo (VOL) of the Dangbe West District of the Greater-Accra Region of Ghana. The villages are only about 2-5 km apart. Like many other parts of Ghana, the climate of the area is characterised by two major seasons: a dry or the harmattan season (December to March) and a wet or the rainy season (June to August), however, there are also some few rains in November and early December, just before the onset of the harmattan season. Malaria transmission occurs throughout the year but peaks during and after the rains (September and January). *P. falciparum* constitutes 98% of all infection with the remaining 2% due to *P. malariae* and *P. ovale* [52]. The population is predominantly of the Ga-Adangbe ethnic origin but is interspersed with other ethnic groups such as the Ewes and the Akans. There are two health centres serving all these communities: Osudoku Community Health Centre at Asutsuare and the Osuwem Community Health Centre. In addition, the Akuse Hospital, about 10 km away serves as a referral hospital for cases beyond the capacity of the community health centres.

Altogether, 798 children (aged 1 to 12 years old) were enrolled and were followed up actively and passively for malaria case detection in a 42 week longitudinal cohort study. Genomic DNA for analysis was available for 585 of the 669 children who successfully completed the follow up without missing at most three successive weekly visits. Of these 585 children, there were 316 singletons, 91 families with two children enrolled, 17 families with 3 children enrolled and 9 families with 4 children enrolled. At baseline (enrolment), 5 ml EDTA-anticoagulated venous blood and thick and thin film blood slides were obtained from all individuals prior to the malaria transmission season (May 2008) for baseline immunological and parasitological determinations. Blood group and sickle cell status of each individual were determined by

a commercial blood grouping kit (Biotec Laboratories Limited, UK) and the sodium metabisulphite test respectively and haemoglobin (Hb) level was measured using the Hemocue-Hb 201 (Angelholm, Sweden). Blood was centrifuged to separate plasma and peripheral blood mononuclear cells (PBMCs) and stored at -80°C and in liquid nitrogen respectively. The thick and thin blood film slides were stained with Giemsa and examined for baseline asymptomatic parasitaemia. A slide was negative if no parasite was visualised in 200 oil fields of a thick film. For slides that were positive, parasites were counted per 200 white blood cells (WBCs) and parasite densities calculated by assuming 8,000 WBCs/ μl blood. A standardised questionnaire was used to obtain epidemiological, anthropometrical and clinical data of all study participants during the enrolment.

Danish Donors

DNA samples from a total of 132 anonymous Danish blood donors, obtained for control purposes, were analysed in order to allow population comparisons of genotype distributions. These individuals are resident of central Copenhagen and provided consent to have a small portion of their blood stored, anonymously, and used for research purposes. Blood donors in Denmark must be between the ages of 18 and 60 [33].

Parasitological and Clinical Surveillance

Parasitological surveillance for malaria infection was carried out monthly for each study participant during the follow-up period. This involved obtaining thick and thin blood film slides from finger pricks by trained medical personnel. In addition, about 500 μl of blood was collected during the monthly finger pricking to obtain plasma for immunological analyses. The remaining packed cells were stored at -80°C for DNA purification for genetic analyses. The active case detection surveillance comprised weekly visits to each participant's home, where a morbidity questionnaire (investigating symptoms occurring in the preceding week) was administered by trained Field Assistants. The presence or absence of fever (measured axillary temperature of $\geq 37.5^{\circ}\text{C}$, or reported) was ascertained. Study participants complaining of symptoms suggestive of malaria were referred for treatment at the respective health centres. In the passive case detection surveillance, visits by participants to the health centres without prior referral from a field assistant were documented. In both the active and passive case detection, Hb level was measured and thick and thin blood films were obtained from study participants with febrile temperature ($>37.5^{\circ}\text{C}$) or reported febrile temperature prior to treatment. Clinical malaria was defined as slide positive for any asexual *P. falciparum* parasitaemia with at least one other sign of malaria such as vomiting, diarrhoea, or malaise. Malaria was treated with artesunate-amodiaquine combined dose therapy which was the recommended standard treatment for malaria in Ghana. At the end of the study, on the basis of the clinical and parasitological data obtained, the study population was divided into three groups: (1) those susceptible, in which parasitaemia was associated with febrile disease, and (2) those apparently protected against clinical manifestation despite parasitaemia and (3) those apparently protected against clinical manifestation without detectable parasitaemia by microscopy.

FCGR2A genotyping

Genomic DNA was purified from packed cell samples using the Maxwell[®]16 system (Promega, Madison, USA) following manufacturer's guidelines. Genotyping of Fc γ RIIA-p.H166R was done by the gene specific polymerase chain reaction (PCR) amplification followed by allele specific restriction enzyme digestion

(ASRED) method [53]. The final BstUI restriction digestion products were visualized as 343 bp (H allele) and 322 bp (R allele) bands on 3% agarose (SeaKem[®]GTG[®] Agarose, ME) with ethidium bromide (AppliChem, Damstadt, Germany) staining. Both fragments were present for heterozygous individuals.

FCGR3B sequencing

We designed a protocol to specifically amplify and sequence exon 3 of the *FCGR3B* gene from genomic DNA. First, an approximately 4.3-kb fragment of the *FCGR3B* gene using the sense primer (5'-CTCCATTGCGAGACTTCAGAT-3') placed in exon 1 and the antisense primer (5'-CGTGGTTTCTAAGGTGTCACAGG-3'), positioned within intron 3. A 30-cycle amplification process consisting of denaturation at 95°C for 30 s, annealing at 63°C for 30 s and extension at 72°C for 5 mins was performed using *PfuUltra*[®] high-fidelity DNA polymerase (Stratagene, USA). The product was gel purified using E.Z.N.A[®] Gel Extraction Kit (Omega Bio-Tek, Inc., GA) and used as template in a nested PCR to amplify exon 3 of *FCGR3B* with the M13 tagged (in lower case) sense (5'-tgtaaacgacggccagtGTCAGCTTCATGGTCTTG-GATTG-3') and antisense (5'-caggaaacagctatgaccACACATTCA-CATTGTATGCACTCCA-3') primers. The 38 cycle amplification consisted of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 45 sec using TEMPase hotstart DNA polymerase (Biomol, Germany). The nested PCR product was then sequenced with M13 primers. The low affinity *FCGR* locus contains regions of copy number variation (CNV) which can alter receptor expression and leukocyte responses to IgG. However, previous *FCGR3B* CNV data determined by the SALSA[®] multiplex ligation probe amplification (MLPA[®]) kit P110-B1/P111-B1 FCGR (Lot 0210, 0409; v.08) (MRC Holland) on the same samples in the present study found no association with and clinical malaria (Adu *et al.*, unpublished).

Statistical analysis

Demographic and clinical characteristics of the study population were compared across village of residence (χ^2 -test). Since the risk of clinical malaria is known to be high in children aged 5 years and below, age was modelled as a categorical variable with two levels (1–5 years, and 6–12 years). Logistic regression analyses and likelihood ratio tests were carried out to evaluate the association of the variables, age group, sex, sickle cell status, blood group, use of bed net and ethnic group with clinical malaria. The genotype distribution of the SNPs was compared across ethnic groups (χ^2 -test). To test Hardy Weinberg equilibrium (HWE), we used the data of $n = 267$ children who had diploid copies of *FCGR3B*, were the first sibling in a family and were not susceptible to clinical malaria in the observation period. The exact test (HWE.exact) implemented in the R-package 'genetics v.1.3.6' (<http://CRAN.R-project.org/package=genetics>) was used. For each of the SNPs, the minor allele (single marker) association with clinical malaria was calculated under the three genetic models of inheritance (additive, recessive and dominant) using logistic regression adjusting for age group, sex, sickle cell status, blood group, use of bed net and ethnic group. Two different control groups were defined: (1) all individuals with no malaria episodes but with definitive exposure as indicated by parasites detectable by microscopy during the follow up, plus all individuals with no malaria episodes but without parasites detectable by microscopy and (2) only individuals with no malaria episodes but with definitive exposure as indicated by parasites detectable by microscopy during the follow up. The second analyses involving control group (2) was to account for any possible confounding due to heterogeneity in exposure. This was because, the group

comprising individuals apparently protected but without detectable parasitaemia by microscopy may also include individuals who did not have malaria due to lack of exposure to the parasite during the follow up period. Additional sensitivity SNP association analyses (logistic regression) were performed with a generalized estimating equation (GEE) approach to correct all confidence limits and *p*-values for the family structure in the data. Linkage disequilibrium (LD) in the *FCGR3B* SNPs and visualisation of pairwise r^2 LD values were evaluated using Haploview v. 4.2 [54]. Association of *FCGR3B* haplotypes with clinical malaria was analysed using the Hapassoc package v. 1.2–4 in R [55]. The pooling tolerance was set to 0.03 in order to restrict the association analysis to haplotypes whose frequency exceeded 3% in the population. The haplotype with the highest frequency was used as the reference. Comparison to the other haplotypes was performed using nested logistic regression adjusting for age group, gender, sickle cell status, blood group, use of bed net and ethnic group. Except for the LD and pairwise r^2 visualization, all statistical analyses were performed using R v. 2.13.2 (<http://www.R-project.org>). DnaSP v. 5.10 (<http://www.ub.edu/dnasp/>) was used to estimate Tajima's D for exon 3 of *FCGR3B* the Ghanaian and Danish populations. The window length for the Tajima's sliding window analysis was 50 with step size of 10 nucleotides. Genotyping data for rs5030738 was retrieved from the 1000 Genomes Project database (http://browser.1000genomes.org/Homo_sapiens/Variation/Population?db=core;g=ENSG00000162747;r=1:161592986-161601753;t=ENST00000531221;v=rs5030738;vdb=variation;vf=8673417); for the African (AFR); Ad Mixed American (AMR) and European (EUR) super populations and allele frequencies compared among all 5 populations. Pairwise F_{ST} distances were calculated for all 5 populations using the POPTREE2 software [56].

Supporting Information

Table S1 Distribution of *FCGR2A* and *FCGR3B* genotypes among the ethnic groups in the study population.

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* Comprises individuals from Akan, Hausa and Fulani ethnic groups (DOC)

Table S2 Univariate analyses of *FCGR2A* and *FCGR3B* genotypes association with clinical malaria. ^a encodes the FcγRIIA-166H/R polymorphism. ^b adjusted for age groups, sex, sickle cell status, blood group, bed net use and ethnic group (DOC)

Table S3 Single marker association of *FCGR3B* alleles with clinical malaria using sub-set of controls. Odds ratio (OR) and 95% confidence intervals (CI) were determined using multivariate logistic regression controlling for age, gender, ethnicity, sickle-cell status, *FCGR3B* copy number, blood group, family structure and use of bed net. MAF: minor allele frequency. [‡]All individuals who never had malaria despite parasitaemia at any time point during the study (DOC)

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Author Contributions

Conceived and designed the experiments: BA DD MT. Performed the experiments: BA SA. Analyzed the data: BA TAG SOL MT. Contributed reagents/materials/analysis tools: DD PLH FKNA TAG MC. Wrote the paper: BA MT.

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