ORIGINAL ARTICLE

Genome-wide association study of antibody responses to *Plasmodium falciparum* candidate vaccine antigensJ Milet^{1,2}, A Sabbagh^{1,2}, F Migot-Nabias^{1,2}, AJF Luty^{1,2}, O Gaye³, A Garcia^{1,2} and D Courtin^{1,2}

We conducted a genome-wide association study (GWAS) of antibody responses directed to three *Plasmodium falciparum* vaccine candidate antigens (MSP1, MSP2 and GLURP) previously associated with different patterns of protection against malaria infection in Senegalese children. A total of 174 950 single-nucleotide polymorphisms (SNPs) were tested for association with immunoglobulin G1 (IgG1) responses directed to MSP1 and to GLURP and with IgG3 responses to MSP2 FC27 and to MSP2 3D7. We first performed a single-trait analysis with each antibody response and then a multiple-trait analysis in which we analyzed simultaneously the three immune responses associated with the control of clinical malaria episodes. Suggestive associations ($P < 1 \times 10^{-4}$) were observed for 25 SNPs in MSP1 antibody response analysis or in multiple-trait analysis. According to the strength of their observed associations and their functional role, the following genes are of particular interest: *RASGRP3* (2p22.3, $P = 7.6 \times 10^{-6}$), *RIMS1* (6q13, $P = 2.0 \times 10^{-5}$), *MVB12B* (9q33.3, $P = 8.9 \times 10^{-5}$) and *GNPTAB* (12q23.2, $P = 7.4 \times 10^{-5}$). Future studies will be required to replicate these findings in other African populations. This work will contribute to the elucidation of the host genetic factors underlying variable immune responses to *P. falciparum*.

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INTRODUCTION

Despite a recent marked decline in deaths because of malaria, the latest available figures still show 584 000 individuals dying annually worldwide (range: 367 000–755 000), with at least 198 million clinical cases (range: 124–283 million).¹ Most of those dying are young African children below 5 years of age who lack adequate acquired protective immunity. Epidemiological and experimental evidence points to the essential role played by asexual blood-stage parasite antigen-specific antibodies in effective antimalarial immunity.^{2–4} However, no immunological markers have so far been identified as representing reliable, validated correlates of protective immunity. This situation largely reflects the dearth of relevant biological and/or mechanistic information concerning the nature or identity of the parasite antigens that may function as targets of naturally acquired immunity.

Generally, infectious diseases present with a wide spectrum of clinical syndromes, varying from entirely asymptomatic to severe acute illnesses, and this degree of variability also applies to malaria.⁵ Among the numerous factors that can separately explain parts of this variability, the host immune response is one of the most important.^{6–8} In this context, human genetic polymorphisms located in the genes involved in the control of immunological responses^{9–11} are of obvious interest.

Although numerous studies have explored the genetic associations between host polymorphisms and several different outcomes of malaria infections,^{12–16} the genetic control of antimalarial antibody (Ab) responses has been poorly studied. Stirnadel *et al.*¹⁷ documented familial aggregation of immunoglobulin G (IgG) responses directed against several antigens explained partially by both genetic and environmental factors. The same team showed that the variability in the IgG3 response to

the RESA (ring-infected erythrocyte surface antigen) and in the IgG1 and IgG2 responses directed to MSP2-FC27 was partly explained by human leukocyte antigen (HLA) class II genotypes under a complex genetic model.¹⁸ Other studies conducted in two different countries have shown that members of the Fulani ethnic group have higher levels of antimalarial antibodies than those of non-Fulani sympatric tribes¹⁹ and that the *IL4-524T* allele is present at a higher frequency and is associated with elevated Ab levels.²⁰ A family-based association study showed significant association between *IL1RN* alleles and total IgE, and a suggestive association between polymorphisms in the *IL10* promoter region and anti-*Plasmodium falciparum* IgE and IgG4.²¹ We recently reported two independent signals in the *HLA-G* 3' untranslated region that were associated with a modulation of, respectively, IgG1 and IgG3 responses directed to the *P. falciparum* vaccine candidate antigens GLURP and MSP2.²² More recently, a whole-genome linkage analysis using 400 microsatellites as markers revealed three chromosomal regions (8p22-p21, 9q34 and 20q30) as of potential interest in the control of IgG subclass responses directed to *P. falciparum* blood stage extracts.²³ At last, a candidate gene study was used to investigate single-nucleotide polymorphism (SNP) associations with Ab levels (AMA1, MSP1, MSP2, (NANP)₄ and total IgE) in a large-scale multicenter sample. On all the 202 SNPs described as playing a role in malaria and Ab production, most important effects were seen for hemoglobin S (HbS) and a SNP located in *CD36* but the study failed to detect a significant effect for any of them except HbS.

Genome-wide assessment of human genetic determinants of Ab responses to infectious agents is still scarce,^{24–26} and to our knowledge the present study is the first genome-wide association

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study (GWAS) focusing on Ab responses that have been previously associated with clinical or parasitological antimalarial protection. Indeed, we have shown in the population under study that elevated levels of defined IgG subclasses directed to specific *P. falciparum* antigens (MSP1, MSP2 and GLURP) were associated with a decreased risk of developing malaria or of high parasitemia.²⁷ Considering that an effective immune response is likely to be multifactorial and directed to multiple antigens, we performed in addition to single-trait analyses (that is, each individual Ab response considered separately), a multiple-trait analysis in which the three immune responses associated with the control of clinical malaria episodes (IgG1 against GLURP and IgG3 against both MSP2 3D7 and FC27 serogroups) were analyzed simultaneously.

RESULTS

The GWAS was conducted on 528 Sereer individuals including 290 children for whom IgG responses were measured, and their parents. The sample comprised 130 families including 1 to 5 children, with most having ≤ 3 children. Children were aged from 7 to 19 years with a mean age (s.d.) of 11.9 years (2.8). The study comprised more boys than girls (173 versus 117).

Before association testing, Ab measurements were adjusted on a set of covariates including age, gender, red blood cell polymorphisms (HbS and G6PD) and the delay between blood sampling and the last positive thick blood smear. The covariates and their effect on each immune response are presented in Table 1. The phenotypes further considered in the GWAS, still referred to as 'Ab responses' hereafter, were the residuals from multiple regression models.

Moderate linear correlations were found between the Ab responses (Table 2). As expected, the strongest correlation ($r=0.43$) was found between IgG3 directed to MSP2 FC27 and IgG3 directed to MSP2 3D7. Heritability values, estimated through a variance component linkage analysis, ranged between 28% and 49% for IgG3 directed to MSP2 3D7 and IgG1 directed to GLURP R0, respectively.

Genome-wide association of individual IgG subclass Abs

A genome-wide association analysis was performed for each of the four Ab responses. Each individual SNP was tested for association using additive, recessive and dominant models. No SNP achieved the statistical significance threshold of 2.47×10^{-7} required to control the genome-wide type I error rate. However, note that for low-density arrays whose design has been based on HapMap data, such as the Affymetrix 250K Nsp Array (Santa Clara, CA, USA), such a threshold is more difficult to achieve in African populations than in Europeans or Asians because of the generally lower levels of linkage-disequilibrium (LD) between causal variants and the SNPs that are genotyped. We therefore followed the example of previous GWASs performed in Africa^{12,16,28–31} and considered a cutoff value for identifying interesting signals. Given the low density of the array (250 K SNPs) and the low LD observed in African populations, a threshold of 10^{-4} was applied as in Jallow *et al.*¹²

Results of single-trait association analyses are presented for IgG1 directed to MSP1 associated with protection against high-density parasitemia in Table 3 and for the three Ab responses associated with protection against clinical malaria in Table 4. The top five SNPs with the lowest *P*-values are shown for each Ab response. *P*-values for the best SNP ranged from 9.26×10^{-6} for IgG1 directed to MSP1 to 2.4×10^{-5} for IgG1 directed to GLURP R0. It is noteworthy that among the regions containing the top five SNPs, the 1q41 region appears twice, for IgG3 directed to MSP2 3D7 and IgG3 directed to MSP2 FC27. Nevertheless, the two SNPs showing association with these two Ab responses (rs453079 and rs6675815, respectively) are located 1.1 Mb distant from each

Table 1. Effects of covariates on antibody levels

Covariates	Description ^a	IgG1 to MSP1 Coefficient (95% CI)	P	IgG3 to MSP2 3D7 Coefficient (95% CI)	P	IgG3 to MSP2 FC27 Coefficient (95% CI)	P	IgG1 to GLURP R0 Coefficient (95% CI)	P
Age (in years)	11.86 ± 2.79	0.88 (–0.16; 1.91)	0.096	–	–	0.77 (0.006; 1.54)	0.048	2.27 (0.71; 3.82)	0.0048
Gender (male/female)	182/122	–5.66 (–11.50; 0.19)	0.057	2.99 (0.62; 5.36)	0.013	–	–	7.89 (–0.97; 16.76)	0.081
Hemoglobin S (AS/AA)	32/272	–	–	–	–	–6.34 (–13.29; 0.62)	0.074	–9.3 (–23.21; 4.61)	0.19
Time since last infection (in months)	2 (0–15)	–	–	–0.46 (–0.69; –0.23)	< 0.0001	–0.57 (–0.99; –0.15)	0.007	–0.87 (–1.71; –0.02)	0.043

Abbreviation: CI, confidence interval. Effects of covariates were defined from the 304 children included in the seroepidemiological study. One multiple linear regression analysis was computed for each antibody measurement. This table shows the effect of covariates that were included in the final model of adjustment ($P < 0.20$). ^aValues are mean ± s.d., number of individuals per category or median (range).

other with no LD between them ($r^2 = 0.011$). Therefore, they most likely represent independent signals of association in the population under study. For each Ab response, SNPs that achieved

the predefined threshold of 10^{-4} are presented in Supplementary Tables S1–S3 (for IgG3 directed against MSP2 3D7 only the 5 presented SNPs reached the threshold of 10^{-4}).

Table 2. Correlation and heritability of antibody responses

	IgG3 to MSP2 3D7	IgG3 to MSP2 FC27	IgG1 to GLURP R0	h^{2a}
IgG1 to MSP1	0.331	0.318	0.307	33.45
IgG3 to MSP2 3D7	–	0.430	0.282	27.97
IgG3 to MSP2 FC27	–	–	0.367	31.09
IgG1 to GLURP R0	–	–	–	48.79

Pearson's correlation coefficients are given. All coefficients are highly significant, with P -values comprising between 7.1×10^{-7} and 7.1×10^{-15} . ^aHeritability estimated through a variance component linkage analysis.

Table 3. Top five associations ($P < 10^{-4}$) with the level of IgG1 to MSP1 (previously associated with parasite density)

Chr.	SNP	Minor allele	Minor allele frequency	Families ^a	Z ^b	P-value	Model	Nearby genes ^c
3q26.1	rs1157705	T	0.495	60	4.433	9.3×10^{-6}	Rec	<i>SPTSSB</i> (> 100 kb)
6q13	rs1204331	G	0.233	65	–4.264	2.0×10^{-5}	Add	<i>RIMS1</i>
2p24.3	rs4669869	T	0.437	77	–4.194	2.7×10^{-5}	Dom	<i>TRIB2</i>
7q22.2	rs9987005	T	0.196	67	–4.117	3.8×10^{-5}	Dom	<i>LHFPL3</i> ^d
4p14	rs6819520	T	0.388	65	–4.077	4.6×10^{-5}	Dom	<i>NWD2</i> (> 100 kb)

Abbreviations: Add, additive; Chr., chromosome; Dom, dominant; Rec, recessive; SNP, single-nucleotide polymorphism. Top five SNPs considering the lowest P -value among additive, dominant and recessive models for each SNP. The rs number in bold is a SNP found associated at $P < 0.05$ in the genome-wide association study performed previously using clinical and parasitological phenotypes.¹³ The rs4669869 was associated with the prevalence of *Plasmodium falciparum* infection ($P = 0.0097$). ^aNumber of informative families. An informative family is a family that has a non-zero contribution to the FBAT statistic. ^bZ-score. The sign of Z-score (+/–) indicates whether the allele is associated with a higher or a lower level of antibody responses. ^cRefSeq genes spanning or flanking the SNP (GRCh37/hg19 assembly). ^dIndicating that SNP is intronic; when there was no gene in the defined region, the closest gene (< 1 Mb away from) is specified (> 100 kb).

Table 4. Top five associations ($P < 10^{-4}$) with the level of IgG responses previously associated with clinical protection against malaria

Ab response, Chr.	SNP	Minor allele	Minor allele frequency	Families ^a	Z ^b	P-value	Model	Nearby genes ^c
<i>IgG to GLURP R0</i>								
9p21.2	rs10757705	G	0.324	72	–4.221	2.4×10^{-5}	Add	<i>LINGO2</i> ^d
15q22.2	rs16942900	T	0.251	61	–4.164	3.1×10^{-5}	Dom	<i>RORA</i> ^d
3p21.1	rs3821869	A	0.424	71	–4.057	5.0×10^{-5}	Dom	<i>IL17RB</i> ^d , <i>ACTR8</i> , <i>CHDH</i> , <i>CACNA1D</i> , <i>SELK</i>
7p15.3	rs971990	G	0.426	51	4.035	5.0×10^{-5}	Rec	<i>LINC01162</i>
4q26	rs7694946	C	0.492	55	3.972	7.1×10^{-5}	Rec	<i>NDST4</i> (> 100 kb)
<i>IgG3 to MSP2 FC27</i>								
16q23.1	rs17640520	C	0.111	42	–4.077	4.6×10^{-5}	Add	<i>WWOX</i> ^d
17q22	rs12946796	G	0.271	74	4.07	4.7×10^{-5}	Dom	<i>MSI2</i>
1q41	rs6675815	C	0.241	71	3.999	6.4×10^{-5}	Add	<i>LOC101929771</i>
11q13.4	rs7123288	G	0.434	62	–3.966	7.3×10^{-5}	Rec	<i>SHANK2</i> ^d
2q14.1	rs11679441	C	0.192	63	–3.959	7.5×10^{-5}	Add	<i>DDX18</i> (> 100 kb)
<i>IgG3 to MSP2 3D7</i>								
10p14	rs10905522	A	0.107	33	–4.028	5.6×10^{-5}	Dom	<i>LOC101928272</i> , <i>LINC00709</i>
	rs17145352	G	0.082	30	–3.997	6.4×10^{-5}	Dom	<i>LOC101928272</i> , <i>LINC00709</i>
1q41	rs453079	G	0.161	61	4	6.3×10^{-5}	Add	<i>MARC2</i> ^d
5q35.1	rs4868247	G	0.388	64	–3.984	6.8×10^{-5}	Dom	<i>NKX2-5</i> , <i>STC2</i> , <i>BNIP1</i>
	rs4868246	G	0.39	66	–3.962	7.4×10^{-5}	Dom	<i>NKX2-5</i> , <i>STC2</i> , <i>BNIP1</i>

Abbreviations: Ab, antibody; Add, additive; Chr., chromosome; Dom, dominant; Rec, recessive; SNP, single-nucleotide polymorphism. Top five SNPs considering for each SNP the lowest P -value among additive, dominant and recessive models. The rs number in bold is a SNP found associated at $P < 0.05$ in the genome-wide association study performed previously using clinical and parasitological phenotypes.¹³ The rs10757705 was associated with the level of parasitemia during asymptomatic infection ($P = 0.0083$); rs16942900 was associated with the number of mild malaria attacks ($P = 0.028$). In 10p14 region, SNPs rs10905522 and rs17145352 are distant from each other by 14 kb, and in 5q31.1 region SNPs rs4868247 and rs4868246 are distant from each other by 32 bp. ^aNumber of informative families. An informative family is a family that has a non-zero contribution to the FBAT statistic. ^bZ-score. The sign of Z-score (+/–) indicates whether the allele is associated with a higher or a lower level of antibody responses. ^cRefSeq genes spanning or flanking the SNP (GRCh37/hg19 assembly). ^dIndicating that SNP is intronic; when there was no gene in the defined region, the closest gene (< 1 Mb away from) is specified (> 100 kb).

Multiple-trait genome-wide association

Multiple-trait association analysis of the three Ab responses previously associated with protection against clinical malaria episodes revealed lower *P*-values than single-trait analyses. Figure 1 shows the plot of *P*-values for each SNP with the combined trait. Two SNPs showed a *P*-value of $<10^{-5}$, rs36093844 located in the 11q41.1 region ($P=6.2 \times 10^{-6}$) and rs4258776 located in the 2p23 region ($P=7.6 \times 10^{-6}$). Both SNPs are intronic and are located in the *CCDC83* and *RASGRP3* genes, respectively. A total of 17 SNPs were associated with the combined trait at a *P*-value of $<10^{-4}$ (Table 5). Among them, we noticed a SNP near the *RIMS1* gene (rs7341323) in the 6q13 region. This region appears of particular interest as another SNP (rs1204331) was also observed in the top five SNPs associated with

IgG1 directed to MSP1. These two SNPs are located on both sides of the *RIMS1* gene, at precisely 150 kb upstream (rs1204331) and 100 kb downstream (rs7341323) of the gene.

DISCUSSION

To our knowledge, until today there has been just one published genome-wide study that explored the genetic control of the immune response against *P. falciparum*, by using 400 micro-satellites as markers and exploring the Ab levels against *P. falciparum* blood-stage extracts.²³ Here, we present the first GWAS of Ab responses previously associated in the study population with different patterns of protection against either malarial disease or high parasitemia²⁷ using a multi-trait approach.

Figure 1. Manhattan plot of the multiple-trait genome-wide association. Genome-wide association signals for joint analysis of malaria IgG responses (IgG3 to MSP2 3D7, IgG3 to MSP2 FC27 and IgG1 to GLURP R0). Plot of the $-\log_{10} P$ -values for the 174 950 SNPs along the genome. Each point represents a SNP and the *P*-value corresponds to the most significant result among additive, dominant and recessive models. Dotted line indicates the predefined threshold of 10^{-4} .

Table 5. Results of the multiple-trait association analysis at $P < 10^{-4}$

Chr.	SNP	Minor allele	Minor allele frequency	Families ^a	FBAT-GEE ^b	P-value	Model	Nearby genes ^c
11q14.1	rs36093844	T	0.292	28	26.878	6.2×10^{-6}	Rec	<i>CCDC83</i> ^d (12b from exon2), <i>PICALM</i>
2p22.3	rs4258776	G	0.485	58	26.481	7.6×10^{-6}	Rec	<i>RASGRP3</i> ^d , <i>FAM98A</i>
2p25.5	rs17356557	C	0.098	39	24.225	2.2×10^{-5}	Dom	<i>SOX11</i> (> 100 kb)
11q12.1	rs7130252	A	0.49	89	24.123	2.4×10^{-5}	Add	<i>APLN</i> , <i>TNKS1BP1</i> , <i>SSRP1</i> , <i>LLRC55</i>
	rs1892964	C	0.48	88	23.579	3.1×10^{-5}	Add	
6q27	rs6605524	A	0.312	90	23.535	3.1×10^{-5}	Add	<i>THBS2</i> , <i>LOC101929504</i>
10p11.23	rs2488005	A	0.365	91	22.762	4.5×10^{-5}	Add	<i>LOC101929279</i>
1q42.2	rs853459	A	0.427	47	22.624	4.8×10^{-5}	Rec	<i>COG2</i>
15q21.3	rs538170	T	0.429	58	21.989	6.6×10^{-5}	Rec	<i>GCOM1</i> , <i>POLR2M</i>
6q13	rs7341323	G	0.299	72	21.845	7.1×10^{-5}	Dom	<i>RIMS1</i> , <i>KCNQ5</i>
4q32.1	rs12511271	G	0.263	28	21.816	7.1×10^{-5}	Rec	<i>CTSO</i> (> 100 kb)
8q12.1	rs4738494	G	0.317	32	21.772	7.3×10^{-5}	Rec	<i>PENK</i> , <i>SDR16C5</i> , <i>LOC101929415</i> , <i>LINC</i> ,
12q23.2	rs10860812	G	0.121	47	21.747	7.4×10^{-5}	Add	<i>DRAM1</i> ^d , <i>GNPTAB</i>
10p13	rs2026754	C	0.22	72	21.449	8.5×10^{-5}	Add	<i>FRMD4A</i> ^d
9q33.3	rs4837098	C	0.38	93	21.343	8.9×10^{-5}	Add	<i>MVB12B</i> , <i>LMX1B</i>
13q33.1	rs9518426	T	0.31	80	21.275	9.2×10^{-5}	Add	<i>ITGBL1</i> ^d , <i>NALCN</i>
8q24.23	rs2223027	G	0.242	79	21.189	9.6×10^{-5}	Add	<i>FAM135B</i> (> 100 kb)

Abbreviations: Add, additive; Chr., chromosome; Dom, dominant; Rec, recessive; SNP, single-nucleotide polymorphism. SNPs associated with the combined trait at $P < 10^{-4}$. In 11p12.1 region, SNPs rs7130252 and rs1892964 are distant from each other by 17.7 kb. The rs numbers in bold are SNPs found associated at $P < 0.05$ in the previous genome-wide association study on clinical and parasitological phenotype (rs4258776 associated with the prevalence of *Plasmodium falciparum* infection, $P=0.0077$; rs538170 and rs4738494 associated with the number of mild malaria attacks, $P=0.028$ and $P=0.029$ respectively; rs4837098 associated with the level of parasitemia during asymptomatic infection, $P=0.034$ respectively). ^aNumber of informative families. An informative family is a family that has a non-zero contribution to the FBAT statistic. ^bFBAT-GEE χ^2 , constructed using the multivariate score based on the generalized estimating equation approach. The test has 3 degree of freedom, the number of traits. ^cRefSeq genes spanning or flanking the SNP (GRCh37/hg19 assembly). ^dIndicating that SNP is intronic; when there was no gene in the defined region, the closest gene (< 1 Mb away from) is mentioned (> 100 kb).

Thus, in this cohort, the IgG1 response against GLURP and the IgG3 response against MSP2 (FC27 and 3D7) were associated with the control of clinical malaria episodes, whereas the IgG1 response against MSP1 was associated with protection against high-density parasitemia. The continuous and very close active surveillance of children during the follow-up allowed an accurate definition of the immunological phenotypes of interest in the present study.

In this GWAS of Ab responses against *P. falciparum* we identified 25 SNPs that showed suggestive association with MSP1 Ab responses or with the combination of responses linked to the control of clinical malaria episodes (MSP2 3D7/FC27 and GLURP R0). A test for association was also performed for these SNPs with the clinical or parasitological phenotypes described previously.¹³ Five of them were associated with these phenotypes: rs4258776 in *RASGRP3* (2p22.3), rs4669869 near *TRIB2* (2p24.3), rs4738494 near *PENK* (8q12.1), rs4837098 near *MVB12B* (9q33.3) and rs538170 near *GCOM1* (15q21.3). Then, a systematic review of links between nearby genes (in a region ± 100 kb around the SNP) and immune responses, and more particularly Ab responses, enabled us to identify the following genes as possibly involved in the genetic control of immune responses: *RASGRP3* (2p22), *DEK* (6p22.3), *RIMS1* (6q13), *MVB12B* (9q33.3), *KIRREL3* (11q24.2) and *DRAM1* and *GNPTAB* (12q23.2). Considering all our observations together, *RASGRP3* and *MVB12B* among the genes highlighted appeared to us as the most promising for further investigation. *RIMS1* and *GNPTAB* also deserve particular attention because the *RIMS1* locus was identified in both analyses (MSP1 response and multi-trait analyses) and *GNPTAB* plays a role in the control of B-cell functions.

The *RASGRP3* gene (*RAS guanyl releasing protein*) has been described as transmitting B-cell signals after B-cell receptor ligation, with a potential impact on immunoglobulin production and B-cell proliferation.³² It has also been recently described as being upregulated in natural killer cells in response to *P. falciparum*-infected red blood cells and associated with a proinflammatory response mainly mediated by type I interferon.³³ Another polymorphism (rs13385731) in this gene has been associated with a higher risk of developing systemic lupus erythematosus in Asian populations.³⁴ There are multiple hypotheses concerning the mechanisms underlying the complex relations between susceptibility/resistance to systemic lupus erythematosus and malaria.^{35–37}

The protein encoded by the *MVB12B* gene (*multivesicular body subunit 12B*) is a component of the ERCRT-I (endosomal sorting complex required for transport-I) complex that is implicated in the sorting of ubiquitinated cargo protein from the plasma membrane to the endosomal protein. Interestingly, the ubiquitin pathway and ESCRT machinery seem to play a key role for endocytic trafficking of an Fc receptor to the lysosomes where degradation of the complexes can take place. Very recently an association between FcγRIIA (CD32) gene polymorphism and malaria has been shown underlying the important role of Fc receptors in *P. falciparum* infection. As lysosomes may also contribute to FcγRIIA degradation,³⁸ our finding argues for investigations of the role of the ubiquitin pathway and ESCRT in FcγRIIA endocytic trafficking in the context of malaria.

The protein encoded by *RIMS1* (*Rab-interacting molecules*) is part of the Ras super family of small GTPases that have emerged as central regulators of distinct steps not only in membrane trafficking pathways³⁹ but also at the plasma membrane of endocytic structures.⁴⁰ In cells of the immune system, intracellular organelle function and transport are crucial and therefore highly regulated,⁴¹ and there is evidence that specific cytokines such as interferon- γ or tumor necrosis factor- α regulate Rab protein expression (for a review see Pei et al.⁴²). Parasites and viruses can also affect Rab expression levels after infection as has been previously shown for *Trypanosoma cruzi*⁴³ and for HIV-1. It was suggested that HIV-1 could utilize Rab expression in the infected

cell to downregulate CD4 expression and thus protect virus-infected cells against cytotoxic T cells.⁴⁴ Plausibly, then, the modulation of *RIMS1* expression may play a role in Ab responses to *P. falciparum* through a modulation of the organization of immune cells such as B cells. Another interest of this gene is that functional interactions between voltage-gated Ca²⁺ channels and Rab-interacting molecules have been shown recently.^{45,46} The involvement of calcium channels has been demonstrated in the mouse model of infection with *Plasmodium berghei*⁴⁷ and also during *P. falciparum* infection.⁴⁸ Moreover, a recent GWAS pointed out the putative role of a gene involved in Ca²⁺ metabolism during severe malaria infection.¹⁶ Taken together, all these lines of evidence support *RIMS1* as an interesting candidate to be further explored to investigate its role in *P. falciparum* infection and particularly in the genetic control of immune responses against this pathogen.

Another interesting signal was observed with rs10860812 located near the *GNPTAB* gene. *GNPTAB* encodes both the α - and β -subunits of GlcNAc-phosphotransferase that is involved in the synthesis of the mannose 6-phosphate determinant required for efficient intracellular targeting of newly synthesized hydrolases to the lysosome. The absence of phosphotransferase activity in *Gnptab* knock-in mice showed an impairment of B-cell proliferation, differentiation and antigen presentation, resulting in reduced immunoglobulin production.⁴⁹ In humans, the presence of mutations in *GNPTAB* responsible for aberrant splicing was associated with mucopolipidosis II.⁵⁰ Defective humoral immunity was also observed in mucopolipidosis II patients. A decreased percentage of B cells and lower concentrations of total IgG, IgA, and IgM were observed in mucopolipidosis II patients compared with matched controls.⁴⁹ Moreover, the specific Ab response to vaccination was poor or not detectable in these mucopolipidosis II patients.⁴⁹ The involvement of *GNPTAB* in IgG production and malarial disease merits further examination.

The threshold of 10^{-4} applied in this study corresponds to the threshold used by Jallow et al.¹² in the first GWAS of severe malaria. Two main reasons justify to consider a less stringent threshold in studies carried out in African populations.⁵¹ First, SNP arrays whose design has been based on HapMap genotyping data (500K array and lower), before 1000 Genomes projects sequencing data became available, provide considerably lower levels of genome coverage in Africans than in Europeans or Asians, and therefore a lower power to detect a genome-wide association signal. Moreover, recruiting large numbers of patients and ensuring accurate phenotypic data is difficult in a resource-poor setting and tend to limit until now the sample size of GWAS. Recent GWAS in Africa^{16,28–31} carried out with high-density arrays (≥ 600 K SNPs) considered a threshold of 10^{-5} or slightly higher (5.5×10^{-5}).

The multiple-trait approach used in this study has recently become popular in GWAS as simulations showed that joint analyses of several related phenotypes substantially outperformed approaches limited to single traits.^{52–55} This approach appears particularly adapted to the case of the study of the complex immune response against several *P. falciparum* antigens. Indeed, even if true correlates of immune protection have not been identified to date, studies on antibodies directed against blood-stage antigens showed that critical levels for different blood-stage antigens must be achieved to observe a degree of protection against malaria.⁵⁶ Thus, analyses of several Ab responses combined could help to identify genetic factors that globally enhanced the immune response to malaria and could be of particular interest in the perspective of vaccine development.

Several environmental and behavioral factors associated with different risks of infection may obviously be responsible for the variability in Ab levels. This is for example the case of exposure to infection.⁵⁷ Transmission rates in the Niakhar area have been shown to be homogeneous,⁵⁸ and mosquito catches performed

by our team during the follow-up confirmed this homogeneity (Senghor P, personal communication). Concerning the behavior of the population with respect to the risk of malaria, the use of bednets was estimated to be very low in the Niakhar area at the time of the study (just 5.7% of children reportedly slept under a bednet). To take into account the possible difference of exposure, before association analysis, Ab levels were adjusted for age, gender and the delay between blood sampling and the last positive Thick Blood Smear. The Ab levels were furthermore adjusted on HbS and G6PD A- polymorphisms.

There is no overlap between our results and those of Brisebarre *et al.*²³ who identified by means of a linkage analysis three chromosomal regions (8p22-p21, 9q34 and 20q13) potentially involved in the control of the levels of IgG and IgG subclasses directed against *P. falciparum* antigens. The SNP identified in the 9q33.3 region is located 6.8 Mb from the ABO gene (9q34 region) and it is unlikely that this SNP would be in LD with the region identified by linkage analysis. The absence of overlap between the two studies could be explained by methodological differences. IgG directed to total parasite extract (schizont stage) were quantified by Brisebarre *et al.*,²³ whereas IgG directed to several *P. falciparum* candidate vaccine antigens were quantified in the present study. Furthermore, Brisebarre *et al.*²³ used a microsatellite approach to perform a linkage analysis, whereas our study is based on a SNP array that may not be dense enough to contain the variants responsible for linkage signals. None of the SNPs that showed a suggestive association with Ab response to MSP2 and MSP1 (*CD36*, *LOC441108* and *RTN3*) in the study of Shelton *et al.*⁵⁹ were present in our array and therefore it was not possible to replicate their finding.

We did not find any association with a SNP located in the 5q31-q33 region that we have previously shown to be associated with the intensity of infection.^{13,60,61} This region contains a cluster of genes encoding interleukins (ILs; *IL3*, *IL4*, *IL5*, *IL9* and *IL12B*) and other genes involved in immunity. However, the phenotypes used in this and the former analyses are different and although the involvement of antibodies in the control of infection is undeniable, there are obviously other important effectors involved in the response to *P. falciparum* infection.

In conclusion, this study is based on a close follow-up of children and a precise definition of the phenotypes of interest. It is the first GWAS of Ab responses against *P. falciparum* and as such it highlighted some interesting candidate genes that obviously need to be replicated in another cohort and in other African populations. Further efforts are needed to enlarge the sample size of seroepidemiological studies or to group together studies in meta-analyses, but despite its potential limitations this study shows that GWAS could be a successful strategy to identify genes implicated in the control of antimalarial immune responses. A better understanding of the immune mechanisms involved in controlling *P. falciparum* infection will support the implementation of new tools to fight malaria.

MATERIALS AND METHODS

Study area and subjects

The Sereer families enrolled in this study lived in two Senegalese villages located in the Niakhar district located 135 km from the capital Dakar. The study area, study design and local epidemiology of malaria have been described in detail elsewhere¹³ and are briefly summarized below.

Malaria transmission in this area is seasonal from September to December, following the rainy season, with an estimate of between 9 and 12 infective bites per person per year. Transmission is due exclusively to the complex *Anopheles gambiae* s.l. of which 97% were represented by *Anopheles arabiensis*.⁵⁸ Mosquito catches performed in 2002 by our team confirmed a homogeneous distribution of malaria vectors among the study villages (unpublished data).

In a longitudinal malaria survey, the aims of which were to study the genetics of susceptibility to malaria, 504 children were followed from June

2001 to December 2003.¹³ Repeated cross-sectional surveys were conducted to identify subclinical parasitemia during both nontransmission (January, April, June) and transmission (September, October, December) seasons and malaria attacks were detected under an active surveillance.

Furthermore, during the last year of the follow-up in 2003, a subset of 305 children participated in a seroepidemiological study that aimed to assess the role of IgG responses directed against *P. falciparum* asexual blood stages.²⁷ For this purpose, IgG directed to AMA1, MSP1, MSP3, GLURP, MSP2 FC27 and MSP2 3D7 were measured from venous blood samples collected in December 2003, and the protective effect of antibodies was assessed through retrospective analyses, taking into account the parasitological and clinical data collected during the 2003 follow-up. The present study focused on the children ($n=290$) for whom both immunological and genetic data were available.

The two studies were explained in detail to all participants and their parents, and either they or their parents gave their signed informed consent. The ethics committee of the Health Ministry of Senegal approved studies protocols (No. 000526/MS/DERF/DER).

Phenotype definition

Phenotypes were restricted to the four IgG responses that were previously shown to be associated with antimalarial protection:²⁷ IgG3 directed to MSP2 3D7, IgG3 directed to MSP2 FC27, IgG1 directed to GLURP R0 and IgG1 directed to MSP1. The levels of IgG3 directed to one or the other FC27 or 3D7 allelic forms of the MSP2 antigen and the levels of IgG1 directed to GLURP were significantly higher in children harboring asymptomatic parasitemia (without fever) than in symptomatic children (identified by an axillary temperature $>37.5^{\circ}\text{C}$, a parasite density ≥ 2500 per μl and no other apparent cause of fever). The level of IgG1 to MSP1 displayed a significant association with protection against high-density parasitemia in asymptomatic children.

Antibody measurement

An enzyme-linked immunosorbent assay following a standardized methodology (Afro-immunoassay network standard operating procedures: procedure number AIA-001-02) was used to assess IgG responses to recombinant proteins of the *P. falciparum* asexual stage antigens (MSP1, MSP2 3D7, MSP2 FC27 and GLURP).²⁷ Positive-control plasma samples from Gabonese individuals and negative-control plasma samples from Dutch individuals were included in each plate and results were expressed in arbitrary units (AU) calculated from the formula: $100 \times [\ln(\text{OD test plasma}) - \ln(\text{OD negative control plasma})] / [\ln(\text{OD positive control plasma}) - \ln(\text{OD negative control plasma})]$. The positivity thresholds were determined from the mean reactivity ± 2 s.d. of 30 plasma samples from Dutch nonimmune volunteers.

Genotyping and quality control

A genome-wide scan was performed using the Affymetrix Gene Chip Human Mapping 250K Nsp that contains 262 264 SNPs spanning the entire genome. Genotyping was performed at the CNG (Centre National de Génomique, Evry, France) using automated high-throughput methods. Moreover, red blood cell polymorphisms responsible for sickle-cell trait and G6PD deficiency (*HbS* and *G6PD A* variants) were detected using procedures described elsewhere⁶² to take into account the effect of these potential confounders in the analysis.

Rigorous quality control was performed before statistical analyses to ensure reliable genotyping data, as described in Milet *et al.*¹³ Briefly, SNPs were filtered according to the following criteria: a sample call rate $< 95\%$, a minor allele frequency $< 5\%$, a deviation from Hardy-Weinberg equilibrium at P -value of $< 10^{-4}$ and more than eight Mendelian errors in the whole sample of children. For remaining Mendelian errors, genotypes were deleted in members of the respective families. Non-Mendelian errors (that is, unlikely genotypes) were also detected and automatically removed. Finally, a LD-based SNP pruning was performed to remove all SNPs in complete LD ($r^2 = 1.0$) with a nearby SNP (16 876 SNPs). In all, a total of 1 74 950 common SNPs from autosomal chromosomes and pseudo-autosomal regions of the X and Y chromosomes passed quality control criteria.

After removing samples with a genome-wide call rate $< 95\%$, 528 individuals were eligible for analyses, including 290 children with IgG responses measurement and their parents. For each IgG subclass, the Ab levels did not differ significantly between children included ($n=290$) and

those not included because of a low genome call rate ($n=15$) in further analyses (all $P>0.05$; data not shown).

Statistical analysis

Before performing association testing, IgG responses were adjusted on the covariates previously shown to influence acquisition of IgG responses against *P. falciparum*:²⁷ age (in years), sex, HbS and *G6PD* A polymorphisms,^{27,63,64} and the delay between blood sampling and the last positive thick blood smear (in months). The Ab responses were systematically adjusted for all covariates associated at $P<0.20$ using a multiple linear regression. Heritability of phenotypes was estimated using a variance components linkage analysis as implemented in the MERLIN program.^{65,66}

Association analyses were performed by means of the FBAT v2.0.2c software package⁶⁷ using the following strategy. First, a genome-wide scan aiming to test association of individual SNPs with each of the four immunological phenotypes was performed. This stage of analysis was referred to as single-trait analyses. The family-based association test statistic was computed using the empirical variance (option -e) and the mean of the trait as the offset value.

As we were particularly interested in detecting genetic variants that globally enhance the immune response to *P. falciparum* infection, a multiple-trait analysis was performed considering simultaneously the three Ab responses associated with the control of clinical malaria episodes (IgG1 directed to GLURP and IgG3 directed to both MSP2 responses). Indeed, simulation studies have shown that the joint analysis of phenotypes of the same etiology can substantially outperform approaches limited to single traits.⁶⁸ Multiple-trait analyses were performed using FBAT-GEE that is an extension of the original FBAT method to multiple phenotypes based on GEE (generalized estimating equations) scores.⁶⁸ The FBAT-GEE method can be influenced by the presence of outliers or by a highly skewed distribution of some traits. Therefore, the highly positively skewed distributions of IgG3 responses directed to MSP2 3D7 and IgG3 directed to MSP2 FC27 were transformed to normal scores before analysis. The three IgG responses were also centered as recommended because FBAT-GEE does not handle different offsets for the selected traits. Missing values were set to 0; thus individuals with missing values could be included in analysis without these values participating in the test statistics.

Both single-trait and multiple-trait analyses were performed under standard genetic models (additive, dominant and recessive). The minimum number of informative families necessary to perform a test was set to 10.

To reduce the number of false-positive associations arising from multiple testing, it is necessary to impose a rigorous threshold for statistical significance that takes into account the large number of SNPs tested for association with the four immunological phenotypes. The genome-wide significance threshold was set to 2.47×10^{-7} as advocated by Li et al.⁶⁹

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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