

Original article

Impact of the IL-4 -590 C/T transition on the levels of *Plasmodium falciparum* specific IgE, IgG, IgG subclasses and total IgE in two sympatric ethnic groups living in Mali

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Abstract

This study aimed to examine the effect of IL-4 -590 T/C polymorphism on the levels of malaria-specific IgE, IgG, IgG (1–4) subclasses as well as total IgE in the Fulani and their sympatric ethnic group, the Dogon, in Mali. Asymptomatic individuals, of the Fulani and the Dogon ethnic groups, were included in the study. IL-4 is involved in the regulation of IgE and IgG4 subclass. In line with this we found that within the Fulani, the T allele was associated with increased levels of total and anti-malarial IgE ($P = 0.02$ and $P = 0.04$, respectively). The Fulani T allele carriers had slightly higher levels of malarial specific IgG4 as compared to those with the CC genotype ($P = 0.08$). No such differences were observed amongst the Dogon individuals. Taken together, these data indicate that the impact of IL-4 -590 variants on antibody levels may vary in different ethnic populations, and that this might affect the Ig-class and subclass distributions.

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1. Introduction

Malaria remains one of the most deadly diseases worldwide. Individuals residing in malaria endemic areas naturally acquire immunity against this fatal disease. Naturally acquired immunity relies mainly on humoral response. Antibodies are now known to be important in the clearance of the blood stage parasite [1]. Protective anti-malarial antibodies are claimed to belong to certain antibody classes and subclasses, with IgG being the most important isotype in this respect. Cytophilic IgG1 and IgG3 are usually considered as protective subclasses, whereas IgG2 and IgG4 are known as non-protective ones [2].

Antibodies of the IgE isotype seem to be involved in both protection against- and pathogenesis of malaria disease [3,4].

During the last decade, it has been evident that susceptibility to malaria disease differs amongst individuals with different genetic backgrounds. Reports from Mali [5–7] and Burkina Faso [8–11] have shown that the Fulani ethnic group is more immunologically responsive to malaria as compared to the sympatric ethnic groups (the Mossi and Rimaibé in Burkina Faso and the Dogon in Mali). Lower frequencies of *Plasmodium falciparum* infection and fewer malaria attacks have been reported within the Fulani than the other sympatric ethnic groups, which has been suggested to be the result from a stronger malaria-specific humoral response seen in this group.

IL-4 plays a critical role in regulating the antibody response induced by *Plasmodium* parasite [12]. In vitro production of IL-4 by CD4⁺ T cell from malaria exposed donors has been

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associated with elevated levels of serum antibodies specific for the antigen used to stimulate T cells [13]. IL-4 induces antibody isotype switching from IgM and IgG to IgE [14].

A C to T exchange at -590 position in the promoter region of IL-4 gene has been shown to enhance IL-4 production [15]. A number of studies has correlated IL-4 -590 T/C single nucleotide polymorphism (SNP) to variations in the antibody levels in different diseases [15–18].

Studies in Burkina Faso [19] and Mali [20] have revealed a significant variation in IL-4 -590 T/C allele and genotype frequencies between the Fulani and non-Fulani ethnic groups. Similar frequencies of the IL-4 -590 T/C genotypes have been reported in the Fulani from Mali and Burkina Faso, whereas these frequencies were significantly different between those found in the non-Fulani ethnic groups from the two countries [19,20]. In Burkina Faso, the IL-4 -590 T allele has been correlated to elevated levels of anti-malarial IgG only within the Fulani but not non-Fulani groups, suggesting that the IL-4 -590 T/C on its own is not functional and may be linked to other genetic factor(s). The other factor(s) would then be actual element involved in susceptibility/resistance to malaria infection [19].

The aim of the present study was to investigate the influence of IL-4 -590 T/C polymorphism on anti-malarial IgG, IgG subclasses, IgE and total IgE levels in asymptomatic individuals of the Fulani and sympatric ethnic group, the Dogon, living in Mali.

2. Materials and methods

2.1. Study area

This study took place in four villages (Mantéourou, Naye, Binédama and Anakédié) in the Mopti area, 850 km northeast of Bamako, the capital of Mali. Malaria is mesoendemic in this area and transmission is seasonal from July to October. *P. falciparum* is the main cause of infection and entomological inoculation rates (EIR) of 0.38 infective bites per person per night has been estimated [5]. The Fulani and the Dogon ethnic groups live in sympatry in the study area [5,6,20].

2.2. Sample collection

Age matched asymptomatic subjects of the Dogon and the Fulani (165 and 160, respectively), participating in a cross-sectional malariometric survey in September 2005, were included in this study. Finger prick blood was collected on filter papers to extract human and parasite DNA. Plasma samples were separated from blood collected either in heparinized capillaries (finger prick blood) or Vacutainer tubes (venous blood). Parasite density was counted from Giemsa stained thick blood films and was defined as the number of parasite per microliter of blood. Spleen enlargement was determined by palpation and graded between 0 and 4 according to the Hackett score [21]. Informed consent was given by all participants or their parents, and the study was

approved by the Ethical Committee of Mali and National Ethics Committee of Sweden.

2.3. DNA extraction

Human and parasite DNA were extracted from blood on filter papers using Chelex-100, as previously described [20].

2.4. Human genotyping

The C/T single nucleotide polymorphisms (SNPs) at position -590 in the IL-4 gene promoter region was determined by polymerase chain reaction (PCR) followed by enzyme digestion and visualization on Ethidium Bromide (EtBr) stained 2% agarose gel. Amplification was done in 5 µl reaction mixture containing 1.5 µl genomic DNA, iProof™ High-Fidelity Master Mix (BIO-RAD Laboratories, Hercules, CA) and 500 nM of primer pairs. A fragment with the length of 328 bp was amplified using 5'-GAA ACC TCA GAA TAG ACC TAC-3' and 5'-TTG TTG TAA TGC AGT CTC C TG-3' primer pairs and following program; DNA was denatured at 98 °C for 1 min; then PCR was performed for 40 cycles of 98 °C/10 s, 63 °C/20 s, 72 °C/10 s, with a final 5 min extension. The PCR amplicons were incubated for 2 h at 37 °C with *Faq I* endonuclease (Fermentas GMBH, Germany) in buffer that was provided by the manufacturer. Enzyme treatment resulted in 80- and 210-bp bands for IL-4*C and 80- and 248-bp products for IL-4*T.

2.5. Parasite genotyping

Nested PCR was performed to determine the number of *msp2* (FC27 and 3D7) clones, as previously described [22]. Amplifications were done in 10 µl reaction mixture containing DNA template, iProof™ High-Fidelity Master Mix (BIO-RAD Laboratories, Hercules, CA) and 500 nM of primer pairs. The sequences of used primers have been presented elsewhere [23]. The number of products, corresponding to the number of infecting FC27 and IC/3D7 clones, was counted after visualization on Ethidium Bromide (EtBr) stained 2.5 and 1.5% agarose gels, respectively. Clone/strain numbers in the text refers to total numbers of *msp2* alleles detected in each individual.

2.6. Malaria antigen

P. falciparum strain F32 was continuously cultured as described before [24]. Late stage infected erythrocytes were enriched by 60% Percol, sonicated and used as antigen in ELISA [25].

2.7. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to examine the levels of *P. falciparum* specific IgG and IgE and total IgE antibodies as previously described [3]. Briefly, ELISA plates (Costar EIA/RIA, Corning, NY) were coated with 50 µl of crude *P. falciparum*

antigen (5 µg/ml) and incubated at 4 °C for overnight. For total IgE, plates were coated with affinity-purified goat anti-human IgE (Vector Laboratories, Burlingame, USA) (5 µg/ml). Diluted sera (1:100 for malaria-specific IgE and 1:1000 for anti-malarial IgG and total IgE) were added to the plates in duplicates and incubated overnight at room temperature for malaria-specific IgE, and 1 h at 37 °C for anti-malarial IgG and total IgE. To detect the bound antibodies, alkaline phosphatase–conjugated goat anti-human IgG (Mabtech, Stockholm, Sweden) (1:2000) or biotinylated goat anti-human IgE (Vector Laboratories, Burlingame, USA) (1:8000) were added and incubated at 37 °C for 1 h. ALP-conjugated Streptavidin (Mabtech, Stockholm, Sweden) in dilution of 1:1000 was added to the biotinylated antibodies and incubated for 1 h at 37 °C. The assay was developed using p-nitrophenyl phosphate disodium salt (Sigma, St. Louis, MO) as substrate. The optical densities were read at 405 nm in ELISA plate reader (Vmax™ Kinetic Microplate Reader, Menlo Park, CA). The antibody concentrations were calculated from standard curves using proteins with known concentrations and expressed as ELISA-units (equivalent to µg/ml).

To determine IgG subclasses' levels the test sera were diluted 1:500 for IgG1 and IgG3, and 1:50 for IgG2 and IgG4. Biotin-conjugated mouse anti-human monoclonal antibodies (IgG2: clone G18–21, 1:3000 dilution, PharMingen, San Diego, CA. IgG3: clone HP6047, 1:1000 dilution, Caltag Laboratories, Burlingame, CA. IgG4: clone HP6025, 1:2000 dilution, Sigma, St. Louis, MO) were used to detect IgG2, IgG3 and IgG4. ALP-conjugated Streptavidin (Mabtech, Stockholm, Sweden) in dilution of 1:1000 was then added and incubated 1 h at 37 °C. IgG1 was detected using mouse monoclonal anti-human IgG1 antibody (50 µl of 1:1000 dilution, clone NL16-purified, SkyBio Limited, Bedfordshire, U.K) followed by alkaline phosphatase-conjugated goat anti-mouse Ig (Dako Denmark A/S, Glostrup, Denmark).

Myeloma proteins of the IgG1–4 isotypes in known concentrations (Serotec, Oxford, UK), were used to draw standard curves. The antibodies concentrations were then calculated from standard curves. No cross reactivity between the different subclass reagents was detected in this system.

Sera from Swedish donors, non-exposed to malaria were used as negative controls.

2.8. Statistical analysis

Data were analysed using StatView 5.0.1 (SAS Institute Inc., Cary, NC) software. Spleen rate, sex ratio, *P. falciparum* prevalence, as well as genotype and allele frequencies between the two ethnic groups were compared using the χ^2 -test. Kruskal–Wallis and Mann–Whitney *U*-tests were used to compare parasite density and number of clones between ethnic groups and genotypes. Antibody levels were evaluated using ANOVA and unpaired *t*-test after log transformation. Logistic regression was applied to model the associations between antibody levels and the IL-4 -590 T allele. Subjects were categorized into six age groups (1–5, 6–10, 11–15, 16–25,

26–40 and 41–60 years). Adjustment was then done for sex and age. A *P* value less than 0.05 was considered as statistical significance. Correction for the number of tests has not been done, so the risk of overestimation of significances should be considered.

3. Results

3.1. Study population and malariometric characteristics

The study population consisted of 165 Dogon and 160 Fulani, all asymptomatic at the time of survey. Table 1 presents individual and malariometric characteristics of the subjects.

There was no difference in median age or between the ratio of males to females in the two study groups. Spleen enlargement was significantly more frequent amongst the Fulani individuals as compared to the Dogon (36% vs 11%, $P < 0.0001$). The Fulani was less frequently parasitized, as detected by PCR, than the Dogon (42% and 58%, respectively, $P = 0.003$). The mean number of concurrent clones was significantly lower within the Fulani (4.46) when compared to the Dogon (5.63, $P = 0.01$). The difference in median parasite density between the two communities did not reach statistical significance (Table 1).

3.2. Total IgE and *P. falciparum* specific IgE, IgG and IgG subclasses antibody levels

The antibody response was evaluated in 160 Dogon and 137 Fulani, from whom plasma samples were available. Significantly higher levels of malaria-specific IgG, IgG1–3, IgE and also total IgE (median 102 vs 34.1 for IgG, 135.1 vs 22.2 for IgG1, 8.6 vs 0.75 for IgG2, 11.4 vs 4.8 for IgG3, 1.27 vs 0.46 for *P. f* specific IgE and 207 vs 132 for total IgE, $P < 0.0001$ for all) were seen in the Fulani subjects as compared to individuals of the Dogon ethnic group. No significant difference in the level of IgG4 (median 0.4 in the Dogon and 0.5 in the Fulani, $P = 0.3$) was noted between the two groups (not shown).

Table 1
Malariometric indexes in the Dogon and Fulani ethnic groups participating in a cross-sectional survey.

	Dogon	Fulani	<i>P</i> value
Age-median (range)	16 (1–58)	15 (1–60)	NS
Sex (male/female)	85/80	80/80	NS
Spleen rate (%) ^a	11	36	<0.0001
Parasite prevalence (%) ^b	58	42	0.003
Parasite density (range) ^c	1225 (175–19875)	825 (175–24475)	NS
Number of clones ^d ± SD	5.63 ± 2.85	4.46 ± 2.71	0.01
Number of subjects	165	160	

^a Percentage of individuals with enlarged spleen.

^b Proportion of infected individuals, detected by PCR.

^c *P. falciparum* density (parasites/µl)-median.

^d Number of clones identified as *msp2* alleles, excluding PCR negatives.

Table 2
Allele and genotype frequencies of IL-4 polymorphism within the Fulani and the Dogon in Mali.

IL-4 -590 T/C	Genotype <i>n</i> (%)			Allele (%)	
	CC	CT	TT	C	T
Dogon (165)	11 (7)	52 (31)	102 (62)	22	78
Fulani (159)	49 (31)	84 (53)	26 (16)	57	43
<i>P</i> value	<0.0001			<0.0001	

Correlations between antibody response and malariometric indexes are reported elsewhere [26].

3.3. IL-4 -590 T/C allele and genotype frequency

The frequency of the IL-4 -590 T/C polymorphism was determined in 165 and 159 individuals of the Dogon and the Fulani, respectively. The allele and genotype frequency in both groups followed Hardy–Weinberg equilibrium (not listed). Allele and genotype frequencies differed significantly between the two ethnic groups ($P < 0.0001$ for both). A T/C distribution of 78/22 and 43/57 percent was observed in the Dogon and the Fulani, respectively. The frequency of CC/CT/TT genotypes was 7/31/62 percent in the Dogon and 31/53/16 percent in the Fulani (Table 2).

3.4. Antibody levels and malariometric indexes amongst IL-4 -590 T/C genotypes

Antibody levels in different genotypes of both groups are shown in Table 3. Within the Fulani, the difference in total IgE levels between IL-4 genotypes tended to be significant. No such tendency was seen amongst the Dogon. No significant differences in malaria-specific IgG, IgG subclasses and IgE between different genotypes were noted in any of the studied groups (Table 3).

To study the impact of the T allele on antibody levels, individuals carrying the CT and TT genotypes were considered as a single group and were compared to the CC genotype carriers. As can be seen in Table 4, within the Fulani, total IgE was significantly higher in individuals carrying the T allele than those with the CC genotype (median 244.5 vs 141.5,

$P = 0.02$). Malaria-specific IgG4 and IgE also tended to be higher in the Fulani T allele than the CC genotype carriers (Table 4). An age and sex adjusted model revealed a weak association between elevated levels of total and malaria-specific IgE and the T allele in the Fulani group, OR 4.14 (95% CI: 1.3–13.21; $P = 0.02$) and 2.6 (95% CI: 1.1–6.4; $P = 0.04$), respectively. A trend for relation between enhanced anti-malarial IgG4 levels and the T allele, was seen after adjustment, OR 2.51 (95% CI: 0.89–7.13; $P = 0.08$) (not listed).

No correlations between anti-malarial IgG or IgG1–3 and IL-4 -590 T allele, before and after adjustment, were noted.

Within the Dogon ethnic group, the T allele and the CC genotype carriers did not differ significantly in the level of the studied antibodies (Table 4).

Intra-ethnic comparisons of malariometric characteristics between different genotypes showed no significant differences in any of the studied indexes amongst any of the communities (not shown).

4. Discussion

Our results revealed a weak association between increased total and malaria-specific IgE levels and the IL-4 -590 T allele within the Fulani group. IgG4 levels were slightly higher in the Fulani T allele carriers. No significant differences in the levels of anti-malarial IgG and IgG1–3 subclasses were observed between the IL-4 -590 alleles or genotypes, in any of the studied ethnic groups.

We have earlier shown that the T allele was correlated to higher parasite prevalence in asymptomatic Fulani, suggesting that the T allele carriers are less successful in clearing the infection as compared to the CC genotype carriers [20], and that increased levels of total IgE, in this group, were associated to the presence of malaria parasites [26]. Thus, whether the increase in IgE levels, seen in the T allele carriers, is a result of genetic regulation, or if it is a consequence of the higher parasite prevalence observed in the T allele carriers, is not clear and remains to be clarified.

Earlier studies have indicated that IgE might be involved in both pathogenesis and protection against malaria disease [3,4]. The mechanisms whereby IgE exerts its action is believed to

Table 3
Antibody levels (median) among IL-4 -590 genotypes of the Fulani and the Dogon.

	Dogon					Fulani			
	CC <i>n</i> = 11	CT <i>n</i> = 51	TT <i>n</i> = 98	<i>P</i>		CC <i>n</i> = 40	CT <i>n</i> = 71	TT <i>n</i> = 25	<i>P</i>
IgG ^a	50.1	26.1	34.1	NS		104.5	105.5	91.4	NS
IgG1 ^a	12.4	17.3	29.6	NS		27.1	42.2	31.7	NS
IgG2 ^a	1.2	0.43	0.8	NS		8.9	7.4	6.2	NS
IgG3 ^a	4.4	5.4	4.8	NS		10.8	11.8	8.7	NS
IgG4 ^a	0.33	0.37	0.47	NS		0.44	0.51	0.53	NS
IgE ^b	0.35	0.47	0.49	NS		0.99	1.44	1.41	NS
Total IgE ^c	135.8	140.9	125.1	NS		141.5	236.4	265.1	0.07

^a Malaria specific, Unit (U)/ ml.

^b Malaria specific, U $\times 10^{-3}$ /ml.

^c U $\times 10^{-3}$ /ml.

Table 4
Antibody levels (median) among IL-4-590 CC genotype and T allele carriers.

	Dogon			Fulani		
	CC <i>n</i> = 11	T allele <i>n</i> = 148	<i>P</i>	CC <i>n</i> = 40	T allele <i>n</i> = 96	<i>P</i>
IgG ^a	50.1	33.6	NS	104.5	101.3	NS
IgG1 ^a	12.4	24.5	NS	27.1	37.8	NS
IgG2 ^a	1.2	0.7	NS	8.9	7.1	NS
IgG3 ^a	4.4	4.8	NS	10.8	11.4	NS
IgG4 ^a	0.33	0.4	NS	0.44	0.51	0.08
IgE ^b	0.35	0.48	NS	0.99	1.44	0.06
Total IgE ^c	135.8	131.5	NS	141.5	244.5	0.02

^a Malaria specific, Unit (U)/ ml.

^b Malaria specific, U × 10⁻³/ml.

^c U × 10⁻³/ml.

be that IgE, in the form of immune complexes, will induce nitric oxide (NO) and TNF production in monocytes through cross linking of the CD23 (the low affinity IgE receptors) on their surface, and this will lead to parasite killing. NO and TNF are two important inflammatory effector molecules in malaria immunity which could imply a role of IgE in immunity against the disease. However, overproduction of these molecules might lead to pathological reactions [27], suggesting a dual role of IgE in malaria.

Studies in Burkina Faso have shown that the T allele is correlated with higher total IgE levels in the non-Fulani (Mossi) children with severe malaria and not in those with uncomplicated malaria, while the T allele by itself was not associated with severe malaria [16]. These data suggest that other genetic/epigenetic/epistatic or environmental factors might act in concert with IL-4 and IgE and thereby affecting the outcome of the disease. One such factor could be the interferon regulatory factor (IRF)-1, which recently has been shown to be associated with the control of *P. falciparum* infections in the Fulani and the Mossi in Burkina Faso. Importantly, the two ethnic groups exhibited different patterns of polymorphisms, probably reflecting their different haplotype architecture [28]. IRF-1 triggers expression of IL-12p40 [29] and up-regulates the production of IL-12 [30]. IRF-1 deficiency has been shown to result in failure to mount a Th1 response [31]. IRF-1 is, therefore, critical for modulating the balance between Th1 and Th2 types of T-cell responses, known to be important for IgE production in malaria [6].

Studies on associations between IL-4 -590 T/C and IgG levels are limited and contradictory [17–19]. In this study, no significant intra-ethnic variation in IgG levels amongst different IL-4 -590 genotypes was observed. This contradicts studies in Burkina Faso, where the IL-4 -590 T allele was correlated with elevated levels of anti-malarial IgG in the Fulani ethnic group [19]. The reason for this discrepancy is not clear, but might be because the regulation of antibody production by IL-4 could be influenced by the time after the last malaria exposure, and/or by co-infections with other parasitic or bacterial pathogens, or by other regulatory factors.

The impact of IL-4 -590 T/C SNP on IgG subclasses has so far not been investigated. The production of IgG4 has been shown to be promoted by IL-4 and IL-13 [32]. In the present

study, anti-malarial IgG4 levels tended to be higher in individuals with the T allele than the CC genotype within the Fulani. This tendency was not seen in the Dogon. This might be due to general low levels of malaria-specific IgG4. Therefore, whether or not IL-4 -590 variants have any impacts on IgG4 production, and if this differs between the two groups, need to be addressed in larger study population.

No influence of IL-4 -590 T/C polymorphism on the production of malaria-specific IgG1, IgG2 and IgG3 subclasses was noted in this study.

The lack of correlations between IL-4 -590 variants and any of the studied antibody levels amongst the Dogon, may be reflected by the low frequency of the CC genotype in this group and should be confirmed including larger study groups.

All together these data suggest that the importance of IL-4 in response to malaria may not mainly depend on its role in regulation of IgG antibody responses. Rather, other implications of IL-4 in immunity, such as its impact on IgE levels and/or on inflammatory responses may be of more importance. These data emphasize that further studies are required to better understand the roles of IL-4 and IgE in malaria immunity.

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