A functional promoter variant in *IL12B* predisposes to cerebral malaria

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The role of the Th1 pathway in the pathogenesis of severe malaria is unclear. We recently reported that a polymorphism with increasing IFNG transcription is associated with protection against cerebral malaria (CM). Interleukin-12 is required for Th1 cell differentiation, which is characterized by the production of interferon-γ. We investigated 21 markers in *IL12*-related genes, including *IL12A* and *IL12B* encoding the two IL-12 (IL12p70) subunits, IL12p35 and IL12p40. We performed a family-based association study using a total sample set of 240 nuclear families. The IL12Bpro polymorphism was associated with susceptibility to CM. The CTCTAA allele and the GC/CTCTAA genotype are over-transmitted to children with CM (P < 0.0002 and 0.00002, respectively). We estimated the odds ratio to be 2.11 for risk of CM in heterozygous children ([95% confidence interval, 1.49–2.99]; P < 0.0001). Although the CTCTAA allele had a dominant effect on CM susceptibility, this effect is much stronger in heterozygous children, consistent with the functional effects of this allele in a heterozygous form. Heterozygosity for this polymorphism has been associated with reduced expression of the gene encoding IL12p40 and a low level of IL12p70 production. These results, together with the findings from immunological studies of low interferon-γ and IL-12 levels in CM, support a protective role for the Th1 pathway in CM.

**INTRODUCTION**

*Plasmodium falciparum* infection and its clinical manifestations are complex traits due to interactions between environmental and multiple host genetic susceptibility factors. Cerebral malaria (CM), the most frequent severe complication of *P. falciparum* infection, is a reversible encephalopathy characterized by seizures and loss of consciousness. It occurs mostly in young children. It is currently believed that the development of CM is associated with the sequestration of parasites in the small blood vessels of the brain (1,2) and local development of cytokine-mediated inflammation. Thus, severe clinical malaria can be determined by the balance between pro-inflammatory [IL-6, tumour necrosis factor (TNF) and IL-1β] (3–5) and anti-inflammatory (IL-10 and TGF-β) (6,7) cytokines.

Although a number of observations support a contribution of Th1 responses to brain vascular pathology in experimental and human CM, our previous findings suggest a protective role of interferon (IFN)-γ (8). Indeed, we have previously demonstrated that plasma IFN-γ concentrations are lower in Malian children with CM than in those with uncomplicated malaria.

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and that the IFN-183T allele resulting in increased gene transcription is associated with protection from CM (8,9). Hence, we investigated genes that are involved in the IFN-γ response to gain further understanding of the pathological mechanisms involved in CM susceptibility. As such, the cytokine IL-12 is essential in the induction of the Th1 response.

Interleukin-12 (IL-12) is a heterodimeric cytokine composed of p40 and p35 subunits to yield IL-12p70. It is produced primarily by antigen-presenting cells and exerts immunoregulatory effects on T and natural killer (NK) cells. A number of immunological functions are mediated by IL-12, in particular, the polarization of T cells towards the Th1 phenotype, which is characterized by the production of IFN-γ. IL-12 plays a major role in cell-mediated immunity against a variety of pathogens by rapid induction of IFN-γ production, not only from T cells, but also from NK cells. IL-12 acts as a growth factor for activated NK and T cells, enhances the cytotoxic activity of NK cells and promotes the generation of cytotoxic CD8+ T cells (10). Its biological activities are mediated through high-affinity binding to the IL-12 receptor (IL12R). IL12R is composed of two subunits: IL-12Rb1, encoded by \textit{IL12RB1} (MIM 601604), and IL-12Rb2, encoded by \textit{IL12RB2} (MIM 601642). It is primarily expressed on activated T and NK cells. IL-12 activity is dependent on the production of the transcription factor T-bet. T-bet upregulates IL-12Rb2, making cells responsive to IL-12, which can then promote IFN-γ release (11,12). Prior to the induction of IL-12-mediated activity, IL-27—a member of the IL-12 super-family of cytokines—stimulates naive T cells to become Th1 effectors, directly inducing T-bet expression and thereby rendering cells responsive to IL-12 (12,13). Furthermore, IL-27 suppresses GATA-3 transcription, thereby suppressing Th2 responses (14). The signal transducer and activator of transcription 4 (STAT4) is also known to be essential for IL-12 signal transduction in lymphocytes and for regulating the differentiation of T helper cells. STAT4 induces the transcription of the gene encoding IFN-γ, in response to the interaction between IL-12 and IL-12R (15,16). Homozygosity for the CTCTAA allele of the IL12B promoter variant (IL12Bpro) was previously associated with increased mortality in Tanzanian children with CM (17); this finding was not replicated for Kenyan subjects.

In this study, we examined whether polymorphisms located in genes encoding IL-12 [\textit{IL12B} (MIM 161561) and \textit{IL12A} (MIM 161560)], its receptors \{\textit{IL12RB1} (MIM 601604) and \textit{IL12RB2} (MIM 601642)\} and signal transducer molecule \{\textit{STAT4} (MIM 600558)\} affect the risk of CM in Malian children.

**RESULTS**

The univariate analysis of 21 polymorphic markers (Table 1) in the first cohort of 123 nuclear families \((n = 381)\) was performed using the family-based association test package (FBAT; version 1.7) (18,19). The analysis of individual markers revealed significant associations with disease for five polymorphisms: \textit{rs7709212}, \textit{rs17860508} (IL12Bpro), \textit{rs2546890}, \textit{rs568408} and \textit{rs404733} (Table 2). The CTCTAA allele of the IL12Bpro variant was over-transmitted to CM children \((P = 0.003)\) under the dominant model (Table 2). The GC/CTCTAA heterozygous genotype \((P = 0.0006)\) was more likely to be transmitted to children with CM than was expected under the null hypothesis using the FBAT analysis, whereas the GC/GC homozygote genotype is under-transmitted \((P = 0.003)\). The overall \(P\)-value obtained from the genotype model and multi-allelic test for the IL12Bpro polymorphism is less than 0.0001 \((df = 2, \chi^2 = 18.6)\). Thus, the CTCTAA allele and the GC/CTCTAA genotype are associated with an increased risk of CM. Moreover, the heterozygous genotypes of IL12B polymorphisms, \textit{rs2546890} \((P = 0.02)\) and \textit{rs7709212} \((P = 0.02)\), were more frequently transmitted to children with CM than expected under the null hypothesis in 83 and 73 informative families, respectively. An association test indicated that the rare allele T of the IL12B1 polymorphism, \textit{rs404733}, was over-transmitted from parents to affected offspring and associated with CM \((P = 0.04)\) in 50 informative families (Table 2). The G allele of \textit{rs568408} was less likely to be transmitted to children with CM than expected \((P = 0.03)\) (Table 2).

To follow-up these results, we studied 117 additional families, who were independent of the initial study sample, and were from the same population. Only one association between IL12Bpro polymorphism and CM was replicated in this second cohort. The CTCTAA allele and the GC/CTCTAA genotype were over-transmitted in CM subjects \((P = 0.03\) and 0.008, respectively) (Table 2). An association analysis of the total sample set of 240 nuclear families was performed. Among 134 CM children born to heterozygous parents (father and/or mother), 97 received the CTCTAA allele, whereas the expected number—in the absence of association between this allele and CM—was 76.5 \((P = 0.0002)\). Thus, the allelic analysis revealed an overtransmission of CTCTAA allele to CM children; however, the effect was even more significant using the genotypic analysis. The FBAT analysis revealed that the GC/CTCTAA genotype for IL12Bpro was more likely to be transmitted to CM children than expected under the null hypothesis (101 CM children with the GC/CTCTAA genotype versus an expected number of 74.8, \(P = 0.00002\)). Heterozygous GC/CTCTAA children have a higher risk of CM than children homozygous for either allele. This was further investigated using a case-pseudo-control data set and conditional logistic regression analysis, as described previously (20). The results confirmed \((P < 0.0001)\) that heterozygous children had a higher risk of CM [odds ratio \((OR)\), 2.11 (95% CI, 1.49–2.99)] than either GC/GC or CTCTAA/CTCTAA homozygous children.

To search for additional polymorphisms in linkage disequilibrium (LD) with IL12Bpro, further analysis of variant in the 100 kb genomic region of the IL12B gene was performed (chromosomal position 158632213–158732212). Owing to the absence of IL12Bpro genotype information in HapMap, single-nucleotide polymorphism (SNP) selection is based on LD (as measured by \(r^2\)) between \textit{rs7709212} (since this variant is in strongest LD with IL12Bpro in our Malian population, \(r^2 = 0.88\)) and markers from the HapMap data set. LD analysis has been performed both in YRI (Yoruba Sub-Saharan African) and CEU (CEPH European) populations. HapMap data show strong LD with \textit{rs1422876} \((r^2 = 0.91)\).
The position for each variant is given as the nucleotide number from the sequences NT_023133.12 (IL12B), NW_921807.1 (IL12A), NW_927195.1 (IL12RB1), NW_921351.1 (IL12RB2) and NW_921585.1 (STAT4), according to the National Center for Biotechnology Information public database (http://www.ncbi.nlm.nih.gov), genome build 128.

in YRI and with three polymorphisms rs11135059 ($r^2 = 0.76$), rs7253339 ($r^2 = 0.77$) and rs6556412 ($r^2 = 0.76$) in the CEU population. Therefore, to further explore the LD findings in our population, 30 Malian trios randomly selected from cohort 1 were genotyped for these four SNPs. The rs1422876 was the only polymorphism in LD with IL12Bpro. To investigate whether this is associated with CM, the whole sample set from the first cohort was analysed. The FBAT analysis indicated that C allele (under the dominant model) and C/T genotype are over-transmitted to CM children ($P = 0.02$ and 0.01, respectively). To follow-up this finding, rs1422876 was tested in the second cohort; however, no significant association has been observed. Hence, IL12Bpro polymorphism showed the most significant association with CM susceptibility.

**DISCUSSION**

The aim of this study was to evaluate whether polymorphisms in genes involved in the IL-12 signalling pathway were associated with susceptibility or protection against CM. Our initial findings revealed that IL12Bpro confers risk for CM. Children with the CTCTAA allele or the GC/CTCTAA genotype have an increased risk of CM; this remains significant after correction for multiple testing. The IL12Bpro polymorphism is an attractive candidate for CM susceptibility. It is located in the promoter region of the **IL12B**. Analyses of the effect of this polymorphism on gene expression and the cytokine production revealed that the heterozygous (GC/CTCTAA) state is associated with reduced gene transcription and/or decreased functional cytokine secretion *in vitro* (21–24). This finding is not replicated in response to whole-blood stimulation with LPS (25,26); however, cell types and methods of cell stimulation used were different in these studies. Although the CTCTAA allele had a dominant effect on CM susceptibility, this effect was strongest in heterozygous children, which is consistent with the functional effect of the heterozygous form of this variant. The result that heterozygous individuals for IL12Bpro polymorphism display a different phenotype is unusual, but there are previous examples (27–29). At a molecular level, it appears counterintuitive; however, there is growing evidence that molecular ‘heterosis’ is common in humans and may occur in up to 50% of all gene associations as reviewed by Comings and MacMurray (30). Although such observations remain obscure, three explanations are proposed: (i) an inverted U-shaped response curve; (ii) an independent third factor causing a hidden stratification of the sample; (iii) greater fitness in half heterozygous because they show a broader range of gene expressions (30).

Interestingly, IL12Bpro heterozygosity was associated with protection from tuberculosis in BCG-vaccinated individuals (26) and with asthma severity in a cohort of Australian children (22). In another study, the association between IL12B gene and asthma susceptibility was confirmed by Randolph et al. (31); however, they found no evidence to support the presence of IL12Bpro heterozygote effect on asthma severity. Moreover, association between IL12Bpro and increased mortality was observed in Tanzanian children with CM, but not in Kenyan subjects (17). Different LDs between markers and epistatic interactions may reflect the interethnic divergent results.

Thus, our findings demonstrate that low levels of IL12 production are associated with an increased risk of CM. Our findings are consistent with other studies, suggesting that the IL12B signalling pathway is involved in the pathogenesis of severe malaria. IFN-$\gamma$ and IL-12 levels are lower in children with severe malaria than in those with mild malaria (32). The levels of IL-12 are also inversely related to malaria

<table>
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<th>Nucleotide change or repeat</th>
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disease severity in Gabonese children (7). Moreover, IL-12 has a protective role in malarial infection (33), and human IFN-γ levels are correlated with protection against reinfection after treatment (34). We have previously shown that protection against CM is associated with the IFNG allele (8) and that expression of this allele results in the increased IFNG transcription (9). Hence, it is possible that the reduced production of plasma IFN-γ observed in CM Malian children could also result from IL-12 down-regulation. Overall, Th1 responses may reduce the parasite load, thereby decreasing the risk of severe malaria. Therefore, the deficiency of IL-12, which is an important regulator of TH-2 immune responses, may lead to the production of pathogenic TH-2 cytokine pattern and susceptibility to CM. In particular, IL-12 deficiency could markedly increase the IL-4-mediated IgE production by human peripheral blood monocytes. Indeed, children with severe malaria have elevated IgEs, and deposits of IgE have been observed in brain vessels where massive sequestration had occurred (35–37). Moreover, an exaggerated IL-4 cytokine response as observed in severe malarial children could contribute to CM by inhibiting the destruction of blood parasites (38). It also has specific effect on the vascular endothelium by acting synergistically with TNF to increase the VCAM-1 expression on endothelial cells (39,40), which could promote sequestration and CM.

Together with the previous findings, these data suggest that a decreased Th1 response during \textit{P. falciparum} infection may be harmful in CM.

### MATERIALS AND METHODS

#### Subjects

We investigated a first cohort of 123 nuclear families (\(n = 381\)), with one CM child hospitalized between 2000 and 2001 in the paediatric department of the Gabriel Toure Hospital in Bamako (Mali). The second cohort consisted of 117 nuclear families (\(n = 389\)) recruited at the Gabriel Toure Hospital between 2002 and 2003. These families were independent of the 123 described earlier. All these families were prospectively recruited; detailed descriptions of the subjects are provided elsewhere (8). The criteria used to define the CM phenotype were subjects in coma (Blantyre score \(\geq 3\)) and with a thick blood film positive for \textit{P. falciparum}. Meningitis was ruled out by lumbar puncture. Informed consent was obtained from the parents, and the study was approved by the Ethics Committee of the Faculty of Medicine, Pharmacy and Odontostomatolomy, University of Bamako, Bamako, Mali.

#### Polymorphic markers and genotyping

Twenty-one polymorphic markers were analysed (Table 1); the majority of which are associated with disease (17,22,41–43) or are functional variants (22,23,41,44,45). Among them, there are 19 SNPs, a short tandem repeat (ATT; \textit{rs10631390}) and a
complex insertion–deletion polymorphism (rs17860508, referred to as IL12Bpro). The IL12Bpro polymorphism, located 2.7 kb upstream of the transcription initiation site, results in a 4 bp difference between the two alleles. It appears to result from a micro-insertion of ‘CTCTAA’ combined with a ‘GC’ deletion in allele 1 and a ‘GC’ insertion/‘CTCTAA’ deletion in allele 2.

Genomic DNA was extracted, and parental inconsistencies were screened for, as described previously (8). A two-stage genotyping process was performed on extracted DNA using three different methods: PCR-restriction fragment length polymorphism assay, sequence length analysis and TaqMan SNP genotyping assays (Applied Biosystems). We analysed a second independent sample set from the same population to confirm the significant associations found in the initial analysis.

To search additional polymorphisms in LD with IL12Bpro, 30 Malian trios randomly selected from cohort 1 were genotyped for four markers (rs1422876 C→T, rs11135059 A→G, rs7725339 G→T and rs6556412 A→G) extracted from HapMap LD information. These markers are located in the promoter region at chromosomal positions 158693877 (rs1422876), 158703915 (rs11135059), 158709579 (rs7725339) and 158719963 (rs6556412).

Statistical analysis

Genotype distribution was tested for the Hardy–Weinberg equilibrium for each polymorphism using Genepop (web version 3.4) (46). None of the 21 polymorphisms in the parent group deviated from the Hardy–Weinberg equilibrium, with a significance level of .05.

The univariate analysis of 21 polymorphic markers (Table 1) in the first cohort of 123 nuclear families (n = 381) was performed using the family-based association test package (FBAT; version 1.7) (18,19). This programme compared with cerebral and uncomplicated malaria. Lancet, 351, 1768–1772.


