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A promoter polymorphism in the gene encoding interleukin-12 p40 (IL12B) is associated with mortality from cerebral malaria and with reduced nitric oxide production

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Interleukin-12 (IL-12) is an important regulatory cytokine in infection and immunity. Administration of IL-12 may reduce complications of severe malaria in rodents. Polymorphisms in IL12B, the gene encoding the IL-12 p40 subunit, influence the secretion of IL-12 and susceptibility to Type 1 diabetes. We therefore investigated whether IL12B polymorphisms may affect the outcome of severe malaria. Homozygosity for a polymorphism in the IL12B promoter was associated with increased mortality in Tanzanian children having cerebral malaria but not in Kenyan children with severe malaria. Furthermore, homozygotes for the IL12B promotor polymorphism had decreased production of nitric oxide, which is in part regulated by IL-12 activity. These studies suggest that IL12B polymorphisms, via regulation of IL-12 production, may influence the outcome of malaria infection in at least one African population.
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Introduction

Childhood malaria is a major cause of global morbidity and mortality.[1] Syndromes of severe and fatal malaria include cerebral malaria, metabolic acidosis, anemia and hypoglycemia.[1] One method of dissecting the complex pathophysiological processes involved in severe malaria is to identify genetic traits that confer protection from severe and fatal malaria and use these associations to investigate the role of particular mechanisms in acute infection. In this context, one particularly interesting gene to investigate is *IL12B*, which encodes the p40 subunit of interleukin-12 (IL-12), a heterodimer in which p35 is the second subunit (see ref. 2 for review). Studies have suggested that IL-12 has a protective role in malaria,[3,4] possibly through its ability to promote interferon-γ production and development of Th1 cells. Thus, administration of IL-12 may prevent malaria-induced anemia[3] and IL-12 levels are decreased in severe malaria in African children.[5] However, the role of functional genetic polymorphisms of the genes encoding IL-12 in determining the outcome of clinical infection has not been described.

We have defined polymorphisms in and around *IL12B*,[6] and have shown that a polymorphism in the *IL12B* 3′ untranslated region (3′UTR) is associated with susceptibility to development of the autoimmune disease, Type 1 diabetes (T1D) in British and Australian families.[7] The polymorphism associated with T1D susceptibility ('A' at position 16974 of the genomic sequence with Genbank code AY008847; referred to hereafter as the *IL12B* 3′UTR allele 1) was associated with higher levels of basal IL-12 expression *in vitro*.[7] Therefore, individuals with this genotype may have an increased likelihood of mounting immune responses with a bias toward the Th1 phenotype. In contrast, diseases characterized by lower IL-12 production may show an association with the alternative *IL12B* 3′UTR allele. That is, the allele that confers susceptibility to T1D may confer resistance to other diseases.[6] Recently, we have described another polymorphism, which resides upstream of the *IL12B* gene in a region which may have promoter activity. We therefore examined the influence of these two polymorphisms of the *IL12B* gene in the outcome of severe malaria in two independent case–control studies of severe malaria in children from East Africa.[8,9]

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Given our previous findings in T1D, we hypothesized that *IL12B* 3′UTR-2 homozygosity, associated with low levels of IL-12 secretion, would increase the risk of death from cerebral malaria. We were unable to measure IL-12 levels in these cohorts; so to investigate the functional significance of the *IL12B* polymorphisms, we examined whether different genotypes correlated with production of nitric oxide (NO), a downstream mediator of IL-12 activity.[10]

Results

Association between *IL12B* polymorphisms and outcome from cerebral malaria in Tanzanian subjects

Genotyping data were generated for the 178 Tanzanian subjects for whom DNA samples were available (Table 1). Each was typed for the *IL12B* 3′UTR polymorphism[6] and for a complex insertion–deletion polymorphism 3 kb upstream of the transcriptional start site (see the Methods section and Morahan *et al*, submitted for publication), referred to here as the *IL12B* promoter polymorphism, *IL12Bpro*. The longer allele *IL12Bpro* is designated as *IL12Bpro-1*; *IL12Bpro-2* is 4 bp shorter. There were no significant differences in *IL12B* genotypes between the two control groups (ie children exposed to malaria but not currently affected, and children who were experiencing a malaria attack without more severe complications; see the Methods section). However, the *IL12Bpro* polymorphism was associated with a significantly different likelihood of death from cerebral malaria (CM) in a 3×2 χ^2 analysis ($P = 0.024$). Individuals homozygous for *IL12Bpro-1* were over-represented amongst the children who died from CM. As there was no difference in the likelihood of death from CM between *IL12Bpro* heterozygotes and *IL12Bpro-2* homozygotes, these subjects were considered together as a single group. The unadjusted odds ratio (OR) for death from CM in *IL12Bpro-1* homozygotes was 5.04 ($P = 0.013$ (Table 1)). This finding remained significant after controlling for potentially confounding variables such as *IL12B* 3′UTR genotype, age and parasitemia (adjusted OR 5.09; 95% CI: 1.03–25.3; $P = 0.046$).

Considering the *IL12B* 3′UTR polymorphism, the OR for death from CM in 3′UTR-2 homozygotes was 3.2 (95% CI: 1.0–10.7; $P = 0.055$) after controlling for age and parasitemia. When the promoter genotype was added to the multivariate model, the OR for 3′UTR-2 homozygosity was reduced to 1.81 (95% CI: 0.45–7.21; $P = 0.40$), suggesting that the 3′UTR effect may be explained by linkage disequilibrium with *IL12Bpro-1*. If so, it would be important to consider haplotypes based on the two *IL12B* polymorphisms typed.

There are four potential haplotypes. Based on the above analyses, it was hypothesized that homozygosity for the *IL12Bpro*-1/3′UTR-2 haplotype would confer greater susceptibility to death from CM. Seven of the nine individuals (ie 88%) with CM having this genotype died, compared to 15 deaths from 72 (21%) CM cases having all other genotypes (Table 2). Controlling for age and parasitemia, the OR for death from CM in subjects homozygous for the *IL12Bpro-1*/3′UTR-2 haplotype was 18.1 (95% CI: 2.87–115; $P = 0.002$).

Association between *IL12B* polymorphisms and NO production

NO_x excretion, an indicator of NO production by NOS, has previously been shown to be significantly inversely associated with disease severity in these children, with levels lowest in children with CM (both fatal and non-fatal).[8] iNOS production is stimulated in part by the Th1 cytokine interferon-γ. *IL12B* genotype could have an effect on NO production via increased *IL12B* gene expression, Th1 cell development and interferon-γ production. Therefore, fasting urinary excretion of the

Table 1 Frequency of *IL12B* polymorphisms and genotypes within each disease category in Tanzanian children and odds ratio for death from CM

Subjects tested	*Total*	*IL12Bpro genotype*		*IL12B 3′UTR genotype*	
		1.1	1.2 or 2.2	1.1 or 1.2	2.2
Controls					
Healthy currently	43	6	36	30	9
Uncomplicated malaria	53	6	47	40	9
Total	96	12	83	70	18
Cerebral malaria					
Survival	59	5	54	47	12
Fatal outcome	23	7	15	13	9
Total	82	12	69	60	21
% CM survival	72	42	78	78	57
CM death odds ratio		5.04		2.71	
95% confidence interval		1.40–18.17		0.94–7.83	
P		0.013		0.065	

Genotypes were determined for *IL12B* promoter and 3′UTR polymorphism in Tanzanian children who were malaria-exposed but otherwise healthy at the time of study (HC); from children diagnosed with uncomplicated malaria (UM); and from children with cerebral malaria (CM). The latter were divided into subgroups based on survival of the CM episode. Unadjusted odds ratios and *P* values were determined with Intercooled Stata 6.0. Data were unobtainable for the promoter polymorphism in one HC subject, and for the *IL12B* 3′UTR in four subjects from each of the HC and UM groups. Disease outcome was not recovered for one CM subject who was thus excluded from survival analyses.

Table 2 Effect of *IL12B* haplotype on CM survival

Subjects tested	*IL12B haplotype*	
	Pro-1/3′UTR-2	*Others*
Controls		
Currently healthy	3	35
Uncomplicated malaria	2	46
Total	5	81
Cerebral malaria		
Total	9	72
Survival	2	57
Fatal outcome	7	15
% CM survival	22	79
CM death odds ratio	13.3	
95% confidence interval	2.5–70.7	
P	0.002	

Haplotypes formed by the *IL12B* promoter and 3′UTR polymorphisms were determined for each individual. Those homozygous for the haplotype formed by promoter allele 1 and 3′UTR allele 2 were compared with all other haplotype combinations. Odds ratio and *P* values were determined as in Table 1.

NO metabolites, nitrate+nitrite (NO_x), was measured in all Tanzanian subjects. In a univariate analysis, the geometric mean urinary NO_x/creatinine ratio was 0.11 (95% CI: 0.07–0.15) in *IL12pro-1* homozygotes compared to 0.16 (95% CI: 0.14–0.19) in all other subjects ($P = 0.027$). *IL12BPro-1* homozygosity was independently predictive of lower NO_x excretion after controlling for the previously described disease association[8] ($P = 0.012$; Figure 1A). The significant predictive effect of *IL12B* genotype was maintained after controlling for additional potentially confounding variables (age, parasitemia, plasma creatinine and *IL12B* 3′UTR genotype; $P = 0.042$). Mean urinary NO_x excretion was lowest in *IL12B* 3′UTR-2 homozygotes in each disease category (Figure 1B), but this was not statistically significant, either in a univariate model ($P = 0.066$) or after controlling for disease severity ($P = 0.077$).

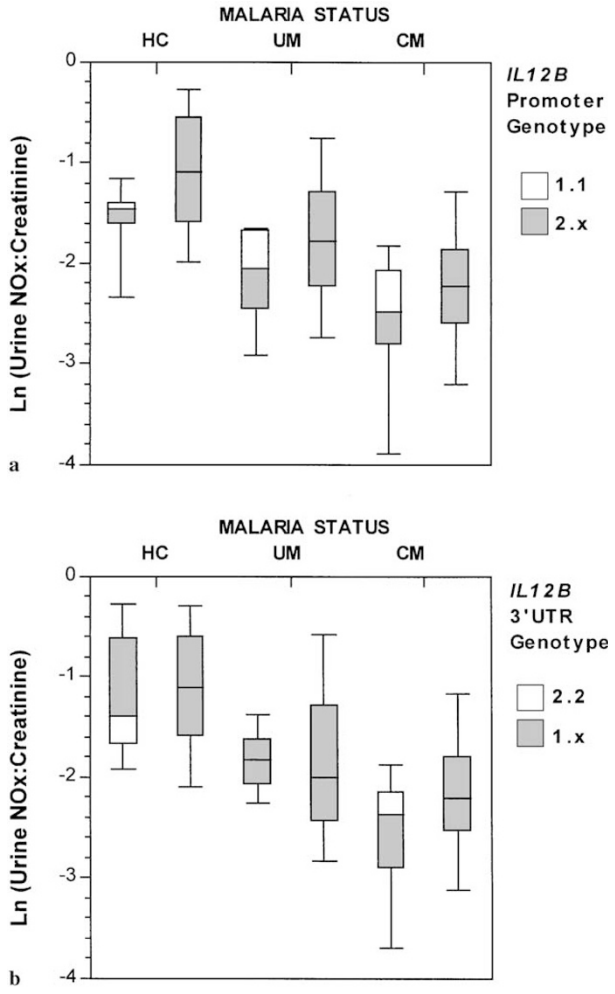

Figure 1 Association between *IL12B* polymorphisms and NO production (as reflected in urinary NO_x excretion, expressed as the natural logarithm of urinary NO_x:creatinine ratio), in Tanzanian children in each disease category:healthy malaria-exposed controls (HC), uncomplicated malaria (UM) and cerebral malaria (CM). The top, bottom, and middle lines of each box correspond to the 75th percentile, 25th percentile, and median, respectively; the mean for each group is also indicated (■). Whiskers extend to the top 90th percentile and to the 10th percentile. **(A)** Individuals homozygous for *IL12B* promoter allele 1 (*IL12Bpro*-1; unshaded bars) compared to all other genotypes (shaded bars). **(B)** Individuals homozygous for 3′UTR allele 2 (unshaded bars) *vs* all other genotypes (shaded bars).

Association of IL12B polymorphisms with outcome from severe malaria in Kenyan subjects

In the Kenyan children, no significant association was found between mortality from severe malaria and either the *IL12Bpro*-1 or *IL12B* 3′UTR-2 genotypes (data not shown). Similarly, there was no association between these *IL12B* genotypes and outcome in the subgroups with cerebral and non-cerebral severe malaria (data not shown). However, there was a weak association of the *IL12Bpro-1/3′*UTR-2 haplotype and another manifestation of severe disease, hypoglycemia: of 130 children who developed hypoglycemia, 16 (12%) were homozygous for this haplotype, while amongst those who did not become hypoglycemic, 31 of 477 (6%) had this genotype ($P = 0.03$).

Discussion

The association we have found between the *IL12Bpro* genotype and cerebral malaria mortality in Tanzanian children supports a role for differential expression of IL-12 affecting outcome in cerebral malaria. How may this effect be mediated? IL-12 production has been associated with protective immune responses to both exoerythrocytic and erythrocytic stages of *P. falciparum*.[4,11] Administration of IL-12 ameliorates disease in a number of rodent models.[3,4,12] Levels of IL-12 are also inversely related to malaria disease severity in Gabonese children.[5] IL-12 influences the development of interferon-γ secreting T cells, which are associated with a 'Th1'

cellular immune response and the recruitment and activation of mononuclear cells[2] including induction of inducible nitric oxide synthase (iNOS). Mononuclear cell iNOS expression and systemic NO production have previously been associated with protection from cerebral malaria in Tanzania[8] and with protection from severe malaria in other populations.[13,14]

We have previously demonstrated that children in the Tanzanian cohort with CM produced less NO than healthy control children or children with uncomplicated malaria.[8] In these same children, *IL12Bpro-1* homozygosity was functionally associated with significantly lower NO production. The magnitude of the effect of *IL12Bpro* genotype on NO production in children with uncomplicated or cerebral malaria may reflect the influence of parasite factors[15] and other cytokines such as IL-10 that negatively regulate NO production, as indices of NO production were markedly lower in these disease groups.[8]

Our results suggest that differential expression of genetically variable IL-12 production may be one of the mechanisms underlying the differential NO production and outcome seen in these Tanzanian children. Although *IL12B* genotypes did not affect increased risk of having CM, the findings are consistent with the hypothesis that once CM occurs, homozygosity of the *IL12Bpro-1* allele increases risk of fatal outcome through impaired production of mediators such as NO.

Although there was a strong association between *IL12B* genotype and fatal outcome in the Tanzanian children, no such association was found in the Kenyan cohort. We cannot exclude an effect due to chance but we think this unlikely because of the stronger association that was observed with a particular *IL12B* haplotype: if the original observation was due to chance there was no reason for *IL12Bpro-1* alleles that showed association with CM susceptibility to also show allelic association with *IL12B* 3′UTR allele 2. Furthermore, the functional correlation of protective genotype and NO production, which has been associated with disease protection,[8] supports a role for *IL12B* genotypes in CM outcome. Similar differences in genetic associations with severe malaria susceptibility/outcome have been shown previously among different African populations and polymorphisms for ICAM-1, TNF-α and haptoglobin 1-1, and with HLA types (reviewed in ref. 16). Although geographically close, the two populations studied here are ethnically distinct. It is possible that the *IL12Bpro-1* polymorphism is in linkage disequilibrium with one or more polymorphisms that also influence gene expression and downstream NO production. It was not possible to determine the functional relationships between *IL12B* promoter polymorphisms and NO production in the Kenyan case–control study. Other functional mutations in or around the *IL12B* gene may also exist in the Kenyan population, and further parallel functional and genetic studies are required in both populations. It is also possible that *IL12B* alleles interact differentially with other genetic elements, especially the genes encoding HLA. The effect of *IL12B* in T1D was observed in HLA identical but not mismatched sib pairs,[7] suggesting that a recessive HLA-linked gene might interact with *IL12B*. HLA types are known to differ between Kenya and Tanzania[17] so this could conceivably account for some of the difference.

Differences in malaria epidemiology have also been proposed to account for temporal and geographic variation in protective effect of host polymorphisms[16] yet the different observations of *IL12Bpro* polymorphism occurred here in coastal areas with similar malaria epidemiology. Both sites have rainfall-related seasonal peaks, and a relatively high proportion of CM, although with higher transmission in Kilifi than in urban Dar es Salaam. It is possible that unknown parasite-determined virulence factors may also differ between the two study areas.

The different explanations of the associations of the *IL12B* polymorphisms in the outcome of malaria in two distinct African populations are not mutually exclusive, and a number of effects may be operating. How should we proceed with the investigation of the role of *IL12B* genotypes in malaria? The strong association of the *IL12Bpro-1* polymorphisms in the outcome of malaria infection in Tanzania and the functional association of this polymorphism with urinary NO_x excretion and thus, by inference, IL-12 and NO production are consistent with the hypothesis that decreased IL-12 production may be harmful in severe malaria. Lack of an association of the same polymorphisms in Kenya can be explained in a variety of ways, as we have indicated. Perhaps the most plausible explanation in the light of all the data, including the observations of the role of IL-12 in animal malaria and other studies of acute malaria, is that the critical *IL12B* polymorphism(s) which regulate susceptibility to severe malaria are in linkage disequilibrium with different marker alleles in different populations. Such a crucial susceptibility allele should be found on the *IL12B* haplotype we have defined in the Tanzanian CM subjects. It will be important to determine if the alleles studied here, or other *IL12B* polymorphisms, influence the outcome of malaria infection in other populations, as well as the relationship of *IL12B* genotypes to IL-12 secretion and NO production.

Complex diseases with polygenic resistant factors will not easily be understood by single case–control studies. We therefore believe it is more helpful to present all the available data to allow the significance of the positive finding in a single study to be honestly evaluated and, crucially, to allow the planning of future studies in the light of as much relevant data as possible. In particular, our studies emphasize the importance of combining genetic and functional studies wherever possible and to examine the putative associations in multiple studies and/or distinct populations whenever and wherever possible. Only with such an integrated approach can we hope to progress from descriptive epidemiology to a reliable appreciation of pathophysiology.

Materials and methods

Subjects

DNA was genotyped from two previously described cohorts of subjects from Tanzania[8] and Kenya.[9] The Tanzanian cohort included 191 children admitted to Muhimbili Medical Centre, Dar es Salaam, with and without CM. Of these, 86 had cerebral malaria (CM) with unrousable coma, 55 had uncomplicated clinical malaria (UM) and 50 were asymptomatic malaria-exposed healthy control (HC) children (with or without subclinical

parasitemia at the time of sample collection).[8] The Kenyan case–control study[9] comprised 693 cases of severe malaria and 693 ethnically matched community controls from consecutive primagravidae attending the antenatal clinic in Kilifi. Of those with severe malaria, 413 had CM, and 280 had severe anemia, respiratory distress and/or hypoglycemia as previously defined.[9] Blood samples (and urine samples in Tanzania) were obtained with informed consent from parents of children and from adult controls. The Ethics Committee of each institution approved these studies.

Genotyping

DNA from Tanzanian children was amplified from blood filter spots using whole genome amplification (Hobbs *et al*., submitted for publication).[18] DNA samples were genotyped as described previously for the single nucleotide polymorphism at the *IL12B* 3′UTR.[6] The *IL12Bpro* polymorphism was found by extending our previous search for *IL12B* polymorphisms.[6] This variant is an insertion/deletion polymorphism (Morahan *et al*, submitted for publication). Alleles were genotyped as follows. DNA samples were amplified using the following primers (5′→3′: TCAGACACATTAACCTTGCA and TAATGTGGTCATTGGCAGGT, one of which was radiolabelled with ^{32}P-ATP) in PCR reactions with the following conditions: 50 ng DNA, 25 ng each primer, 200 nM dNTP, 2 mM $MgCl_2$, 0.3 U Taq polymerase (Gibco); DNA was denatured at 95°C for 3 min; then PCR was performed for 35 cycles of 95°C 20 s, 55°C 20 s, 72°C 30 s, with a final 2 min extension. Alleles were detected after separation on polyacrylamide gels. Allele 1 was designated as the 4 bp larger (ie insertion) allele; the smaller product was designated as allele 2.

No production

Levels of fasting urinary excretion of the NO metabolites, nitrate+nitrite (NO_x), had been previously measured on a low-nitrate diet in each Tanzanian disease group.[8] Because of variability in urine concentration among subjects, NO_x levels were expressed as a ratio of [NO_x] to [creatinine].

Statistical analyses

Predicted outcomes from CM were modelled using logistic regression, controlling for factors expected to influence outcome including age and level of parasitemia. Linear regression was used to model the relationship between *IL12B* genotypes and NO production (log-transformed urine NO_x/creatinine ratio), controlling for factors expected to influence urine NO_x excretion including age group (0–3.5, 3.5–7, >7 year), parasitemia and renal function (plasma creatinine as percentage of normal for age). P values of <0.05 were considered to indicate statistical significance. Analyses were performed using Intercooled Stata 6.0 (Stata Corporation, TX).

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