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ORIGINAL PAPER

Haplotypes of *IL12B* promoter polymorphisms condition susceptibility to severe malaria and functional changes in cytokine levels in Thai adults

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Abstract Polymorphic variability in immune response genes, such as *IL12B*, encoding the IL-12p40 subunit is associated with susceptibility to severe malaria in African populations. Since the role of genetic variation in conditioning severe malaria in Thai adults is largely unexplored, the functional association between *IL12B* polymorphisms [i.e. *IL12B*pro (rs17860508) and *IL12B* 3′ UTR T/G (rs3212227)], severe malaria and cytokine production was examined in patients with *Plasmodium falciparum* infections (n=355) recruited from malaria endemic areas along the Thai–Myanmar border in northwest Thailand. Circulating IL-12p40 (p=0.049) and IFN-γ (p=0.051) were elevated in patients with severe malaria, while only IL-12p40 was significantly higher in severe malaria patients with hyperparasitaemia (p=0.046). Carriage of the *IL12B*pro1.1 genotype was associated with enhanced severity of malaria (OR, 2.34; 95% CI, 0.94–5.81; p=0.066) and hyperparasitaemia (OR, 3.42; 95% CI, 1.17–9.87; p=0.025) relative to the *IL12B*pro2.2 genotype (wild type). Individuals with the *IL12B*pro1.1 genotype also had the lowest IL-12p40 (p=0.002) and the highest IFN-γ (p=0.004) levels. Construction of haplotypes revealed that carriage of the *IL12B*pro-2/3′ UTR-T haplotype was associated with protection against severe malaria (OR, 0.51; 95% CI, 0.29–0.90; p=0.020) and reduced circulating IFN-γ (p=0.06). Thus, genotypic and haplotypic variation at *IL12B*pro and *IL12B* 3′ UTR in this population influences susceptibility to severe malaria and functional changes in circulating IL-12p40 and IFN-γ levels. Results presented here suggest that protection against severe malaria in Thai adults is associated with genotypic variants that condition enhanced IL-12p40 and reduced IFN-γ levels.

Keywords *IL12B* · *P. falciparum* · Severe malaria · Single nucleotide polymorphism · Haplotype

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Abbreviations

SNP	Single nucleotide polymorphism
IL-12	Interleukin-12
IL-12p40	40-kDa subunit of IL-12
IFN-γ	Interferon gamma
IL12B	Gene encoding 40-kDa subunit of IL-12, *IL12B* 3′ UTR:A→C SNP located in the 3′ untranslated region (UTR) at position 1188 of the IL12B gene
*IL12B*pro	A compound polymorphism involving a GC/TT transition combined with an AGAG micro-insertion within the promoter region of the *IL12B*pro

Introduction

Infection with *Plasmodium falciparum* displays a wide spectrum of disease manifestations ranging from asymptomatic presentation to severe disease. The host immune response to malaria is coordinated by the release of pro- and anti-inflammatory cytokines (Kurtzhals et al. 1999; Luty et al. 2000; Othoro et al. 1999; Perkins et al. 2000; Prakash et al. 2006). Depending on the temporal expression and magnitude of production, pro-inflammatory (type-1) cytokines, such as interleukin (IL)-12 and interferon (IFN)-γ, promote both protective and pathogenic responses in malaria (McDevitt et al. 2004). IL-12 is produced primarily by antigen presenting cells (Ing et al. 2006) and promotes IFN-γ release from cells of the innate immune system, including natural killer (NK) cells (Artavanis-Tsakonas and Riley 2002; Hansen et al. 2007), NK-T cells (Schmieg et al. 2003) and γδ T cells (D'Ombrain et al. 2007). IL-12 is also important in adaptive immunity through its ability to augment IFN-γ production from $CD4^+$ T cells (Stephens and Langhorne 2006) and $CD8^+$ T cells (Schmidt et al. 2009). As such, IL-12-promoted release of IFN-γ is a primary mechanism for limiting intracellular pathogen growth (Chehimi and Trinchieri 1994).

IL-12 plays an important protective role against malaria in murine (Mohan and Stevenson 1998b; Sam and Stevenson 1999) and human systems (Boutlis et al. 2003; Keller et al. 2006; Perkins et al. 2000; Prakash et al. 2006; Wroczynska et al. 2005). Reduced circulating levels of IL-12 are associated with enhanced malaria pathogenesis in African children (Luty et al. 2000; Perkins et al. 2000) and non-immune adults (Wroczynska et al. 2005), while elevated plasma concentrations are associated with increased severity of malaria in Asian adults (Gosi et al. 1999; Prakash et al. 2006).

In addition, IFN-γ is an essential mediator of protective immunity against erythrocytic malaria (Favre et al. 1997; Stevenson et al. 1995). Early release of IL-12, IFN-γ and TNF-α in murine models of malaria promotes resistance to infection (De Souza et al. 1997; Favre et al. 1997; Mitchell et al. 2005; Shear et al. 1989; Stevenson et al. 1995). Results from studies in humans also show that enhanced cellular IFN-γ responses are associated with protective immunity against clinical malaria (Iriemenam et al. 2009; Luty et al. 1999; Migot-Nabias et al. 1999; Robinson et al. 2009). In contrast, other investigations have found that elevated circulating levels of IFN-γ are associated with increased pathophysiology of falciparum malaria (Day et al. 1999; Prakash et al. 2006).

Susceptibility to malaria infections and the clinical course of disease once an individual becomes infected are influenced by host genetic variation (Verra et al. 2008, 2009; Weatherall 2008). Association studies in malaria have shown that variation in both innate and adaptive immune pathways condition disease susceptibility and outcomes (Kwiatkowski 2005). Since IL-12 is important for mediating malaria disease outcomes, the current study investigated the role of genetic variation in *IL12B* in conditioning susceptibility to severe malaria in Thai adults infected with *P. falciparum*. IL-12 is a heterodimer composed of IL-12p35 and IL-12p40 subunits, encoded by *IL12A* and *IL12B* genes located on chromosomes 3p12-q13.2 and 5q31-33, respectively (Sieburth et al. 1992). A number of single nucleotide polymorphisms have been identified in the *IL12B* gene, including an *IL12B* promoter polymorphism (*IL12B*pro, a bi-allelic promoter polymorphism located at −2,703 bp from the transcription initiation site, rs17860508) and a TaqI polymorphism (a T to G transition at 1188) in the *IL12B* 3′ untranslated region (referred to as *IL12B* 3′ UTR from hence forth, rs321227) (Huang et al. 2000). These variants have been shown to be important for conditioning susceptibility to a number of infectious (Marquet et al. 2008; Morahan et al. 2002; Mueller et al. 2004; Tso et al. 2004) and inflammatory diseases (Cargill et al. 2007; Zwiers et al. 2004). Although the relationship between genetic variability in *IL12B* and susceptibility to severe malaria has shown mixed results (Barbier et al. 2008; Marquet et al. 2008; Morahan et al. 2002), homozygosity for the CTCTAA (*IL12B*pro1) allele of the *IL12B* promoter variant is associated with increased mortality in Tanzanian children with cerebral malaria, but not Kenyan children with severe malaria (Morahan et al. 2002). Variability in *IL12B* was further associated with *P. falciparum* parasitaemia in Burkina Faso (Flori et al. 2003; Rihet et al. 1999); however, subsequent familial-based studies failed to show a significant correlation between *IL12B*pro and a polymorphism in the 3′ untranslated region of *IL12B* (*IL12B* 3′ UTR) and hyperparasitaemia (Barbier et al. 2008).

The aim of the present study was to determine the role of *IL12B*pro and *IL12B* 3′ UTR variants in conditioning

susceptibility to severe malaria and functional changes in IL-12 and IFN-γ levels in Thai adults with falciparum malaria. The primary hypothesis of the study is that haplotypes of *IL12B* form functional blocks that mediate susceptibility to severe malaria by altering the levels of two critical cytokines that regulate innate and adaptive immune responses: IL-12 and IFN-γ. Determining the role of genetic variability in conditioning susceptibility to severe malaria in Thai adults, particularly in terms of haplotypic blocks, is significant, since the genes and gene pathways that mediate disease outcomes are largely unexplored in this population. Results presented here demonstrate that genotypes/haplotypes of *IL12B*pro and *IL12B* 3′ UTR are associated with differing susceptibilities to severe malaria and altered circulating levels of IL-12 and IFN-γ.

Methods and materials

Study subjects

Patients with falciparum malaria (n=355; age 18 to 67 years, mean ± SD=28.3±10.5) admitted to the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Bangkok were enrolled in an unmatched case–control study. The patients included those with severe malaria (n=103, cases) and those with uncomplicated malaria (n=252, controls) who had been living along the Thai–Myanmar border in the northwest of Thailand where malaria is endemic. These areas are considered to be of low malaria endemicity with two peak seasonal transmissions in May–July and November–January (Luxemburger et al. 1997; Nacher et al. 2001). Incidence rates of malaria were two to six cases per 1,000 population in 2001 (Socheat et al. 2003). In this region, adults are most at risk for the complications of severe malaria including hyperparasitaemia, jaundice, renal dysfunction and cerebral malaria, with severe malarial anaemia occurring only rarely. All patients with non-falciparum malaria infections, mixed *Plasmodium* infections, and the signs and symptoms of AIDS defining illness and/or meningitis were excluded from the study.

All patients were positive for *P. falciparum* infection by microscopic examination of thin and thick blood smears stained with Giemsa. Severe and uncomplicated malaria were defined according to World Health Organization criteria (WHO 2000). Cerebral malaria was defined as an unarousable coma with positive asexual forms of *P. falciparum* in peripheral blood. Severe malaria, in the absence of cerebral malaria, was defined as individuals with one or more of the following signs: hyperparasitaemia (>250,000 parasites/μL), hypoglycaemia (glucose <2.2 mmol/L), severe anaemia (haematocrit <20% or haemoglobin <7.0 g/dL) or increased serum levels of creatinine >3.0 mg/dL. Study participants with positive *P. falciparum* blood films who lacked these signs of severe malaria were categorised as uncomplicated malaria (controls). Patients with severe malaria were further stratified into hyperparasitaemia (≥250,000 parasites/μL) and non-hyperparasitaemia (<250,000 parasites/μL). The study was approved by the Ethical Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, with informed written consent obtained from all study participants.

Laboratory measures

Venipuncture blood (3–5 mL) was collected aseptically in EDTA-containing tubes prior to any treatment interventions. Plasma and packed cells were separated by centrifugation and stored at −20°C until use. Laboratory measures included parasite density determination, complete blood count and clinical biochemistry tests. Parasite densities were determined, and the number of parasites per 1,000 erythrocytes (in thin blood films) or parasites per 200 leukocytes (in thick films) were calculated and expressed as *P. falciparum* parasites per microlitre of blood.

DNA extraction and whole genome amplification

Genomic DNA was extracted using a FlexiGene DNA extraction kit (QIAGEN, Valencia, CA, USA). Prior to genotyping, whole genome amplification was performed by isothermal strand displacement using GenomiPhi V2 DNA Amplification kit (GE Healthcare, Piscataway, NJ, USA).

Genotyping

IL12B 3′ UTR (rs3212227) polymorphisms were genotyped using TaqMan® 5′ Allelic Discrimination Pre-Designed Assays (Applied Biosystems, USA). *IL12B*pro (rs17860508) genotyping was performed by polymerase chain reaction (PCR)–restriction fragment length polymorphism as described previously (Khoo et al. 2004) with some minor modifications. Briefly, the primers were 5′-TACAGCCTGTCTCCGAG AGAA-3′ and 5′-GAGGAAG TGGTTCTCGTACTTTAGC-3′. The PCR reaction was performed in a 25-μL reaction mixture containing 100 ng DNA, 2.5 mmol/L $MgCl_2$, 200 mmol/L dNTPs, 12.5 ng and 1 unit of Taq DNA polymerase (Promega, Medison, WI, USA) in buffer (10 mmol/L Tris–HCl, pH 9.0 and 50 mmol/L KCl). Underlined bases (indicted in the forward and reverse primers) were inserted to create an *Alu*I site in allele 1 (CTCTAA) or in allele 2 (GC). PCR conditions consisted of an initial 3-min denaturation at 95°C, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at

62°C for 30 s, and extension at 72°C for 45 s with a final extension at 72°C for 7 min. PCR products (15 μL) were further digested in a 20-μL reaction volume containing 1.5 units of *Alu*I (New England Biolabs, USA) at 37°C for 14 h. Digested products were separated on 4% high resolution agarose gel (Agarose SFR, Amresco, Solon, OH, USA) containing ethidium bromide and visualised under UV illumination.

Determination of circulating IL-12p40 and IFN-γ levels

Plasma IL-12p40 levels were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (BD Biosciences Pharmingen, San Diago, CA, USA). Circulating levels of IFN-γ were determined by ELISA (R&D systems, Minneapolis, MN, USA) as previously described (Tangteerawatana et al. 2007). Optical densities were measured at 405-nm wavelength, and cytokine concentrations were calculated from the standard curve using recombinant human IL-12 p40 and IFN-γ, respectively. Lower limits of detection for IL-12p40 and IFN-γ were 10 and 25 pg/mL, respectively.

Statistical analyses

Statistical analyses were performed using Minitab® 15 Statistical Software (Minitab Inc., State College, PA, USA) and SPSS (Version 15.0, SPSS Inc., Chicago, IL, USA). Deviation from Hardy–Weinberg equilibrium was determined using web-based calculations (http://www.kursus.kvl.dk/shares/vetgen_Popgen/genetic/appleys/kitest.htm). Chi-square (χ^2) analyses were used to compare proportions. Students's *t* test was used to compare demographic and clinical characteristics in uncomplicated vs. severe malaria. Across-group comparisons were determined by Kruskal–Wallis tests, and where significant, Mann–Whitney *U* tests were used for post hoc comparisons. *IL12B* haplotypes (*IL12B*pro and *IL12B* 3′ UTR T/G) were constructed using HPlus software (Version 2.5). The association between individual polymorphisms (and haplotypes) and malaria clinical outcomes were determined by multivariate logistic regression analysis, controlling for the confounding effects of age and gender. Statistical significance was defined as $p \leq 0.05$.

Results

Demographic clinical and laboratory characteristics of the study subjects The demographic, clinical and laboratory characteristics of the study subjects are shown in Table 1. No significant differences were observed in the distribution of age ($p=0.082$) between the severe (SM) and the uncomplicated malaria (UM) groups. Gender differed significantly between the two groups ($p=0.002$). Peripheral parasitaemia was also significantly different between the groups ($p<0.001$) with the highest parasitaemia present in the severe malaria group. Although haemoglobin (Hb) concentrations were not significantly lower in the SM group ($p=0.091$), the number of red blood cells was significantly reduced ($p=0.007$) in these patients. The white blood cell count was elevated in the SM group ($p<0.001$), while lymphocyte counts and platelets were significantly lower ($p<0.001$ and $p<0.001$, respectively). Total bilirubin and creatinine concentrations were significantly higher in patients with severe malaria ($p<0.001$ and $p=0.029$, respectively).

Circulating levels of IL-12p40 and IFN-γ in malaria-infected patients Prior to determining the influence of *IL12B* genotypes/haplotypes on susceptibility to severe malaria and functional changes in cytokine production, circulating levels of IL-12p40 and IFN-γ were determined in the UM and SM groups and in patients with severe malaria with and without hyperparasitaemia. As shown in Table 2, patients with severe malaria had significantly higher circulating IL-12p40 levels [median (IQR) 95.1 (27.6–133.1)] than those with uncomplicated malaria [median (IQR) 27.0 (10.0–98.8), $p=0.049$]. In addition, circulating IFN-γ levels were also higher in patients with severe malaria [median (IQR) 387.0 (144.2–632.0)] compared to the uncomplicated malaria group [median (IQR) 283.5 (118.5–473.5), $p=0.051$, Table 2].

Analysis of IL-12 and IFN-γ in patients with severe malaria, stratified according to hyperparasitaemia, demonstrated that individuals with hyperparasitaemia had significantly higher circulating IL-12p40 levels [median (IQR) 121.9 (43.3–163.3)] than those with non-hyperparasitaemia [median (IQR) 35.1 (12.5–103.9)], $p=0.046$ (Table 2). However, circulating IFN-γ levels were not significantly different in severe malaria patients with and without hyperparasitaemia (Table 2).

Distribution of IL12Bpro and IL12B 3′ UTR genotypic frequencies The distribution of genotypes for *IL12B*pro and *IL12B* 3′ UTR are presented in Table 3. The distribution of the *IL12B*pro1.1 and *IL12B* 3′ UTR-GG genotypes was significantly different between the UM and the SM groups ($p=0.017$ and $p=0.021$, respectively). None of the other genotypes showed any significant differences in frequency between the UM and SM groups.

Further investigation of the *IL12B*pro polymorphism revealed significant departure from Hardy–Weinberg equilibrium (HWE) in the UM ($\chi^2=63.28$, $p<0.001$) and SM ($\chi^2=16.41$, $p<0.001$) groups, respectively. However, the *IL12B* 3′ UTR polymorphism failed to display significant

Table 1 Demographic, parasitological and clinical characteristics of patients with *P. falciparum* malaria

Characteristic	Uncomplicated malaria	Severe malaria	*p*
Number, *n*	252	103	
Age, years	28.63 (0.63)	27.20 (1.13)	0.082[a]
Gender, %F:%M	85:15	71:29	0.002[b]
Parasitaemia, /μL	27,511 (2,586)	286,302 (26,485)	<0.001[a]
Haemoglobin, g/dL	12.12 (0.14)	11.56 (0.25)	0.091[a]
RBC, $\times 10^6$/μL	4.56(0.05)	4.28 (0.09)	0.007[a]
WBC, $\times 10^3$/μL	5.28 (0.17)	7.43 (0.51)	<0.001[a]
Lymphocyte, $\times 10^3$/μL	8.34 (0.43)	4.48 (0.48)	<0.001[a]
Platelet, $\times 10^3$/μL	98.12 (3.93)	50.56 (3.92)	<0.001[a]
Total bilirubin, mg/dL	1.33(0.06)	6.36 (0.83)	<0.001[a]
Creatinine, mg/dL	0.89 (0.02)	1.35 (0.12)	0.029[a]

Data are the means (SEM, standard error of mean) unless otherwise noted

RBC red blood cell, *WBC* white blood cell

[a] Statistical significance determined by pairwise comparisons with Student's *t* test

[b] Statistical significance determined by Chi-square analysis

departure from HWE in both the UM (χ^2=1.28; p>0.05) and SM (χ^2=2.68; p>0.05) groups, respectively.

Association between IL12B polymorphisms and susceptibility to severe malaria and hyperparasitaemia Multivariate logistic regression analyses were used to determine the association between variability in each of the two *IL12B* polymorphisms and severe malaria, controlling for the confounding effects of age and gender. In addition, since hyperparasitaemia is a prominent feature of severe malaria in Thailand, multivariate modelling (controlling for identical co-factors) was used to examine the relationship between variation in *IL12B* genes and hyperparasitaemia in patients with severe malaria. Presence of the *IL12B*pro1.1 genotype was associated with increased susceptibility to severe malaria (OR, 2.34; 95% CI, 0.94–5.81, p=0.066) and hyperparasitaemia (OR, 3.42; 95% CI, 1.17–9.97, p=0.025, Table 4).

In addition, heterozygous (TG) individuals at the *IL12B* 3′ UTR locus were 39% less likely to develop severe malaria (OR, 0.61; 95% CI, 0.33–1.00, p=0.096), while homozygous polymorphic individuals (GG) were 30% less likely to develop severe malaria (OR, 0.60; 95% CI, 0.36–1.39, p=0.311, Table 4). Variation at *IL12B* 3′ UTR did not show any prominent relationships with hyperparasitaemia.

Association between IL12B haplotypes and susceptibility to severe malaria and hyperparasitaemia Following analyses of the individual variants, haplotypes were constructed using HPlus software. The following haplotypic distributions were generated: 68.9% (184/267) *IL12B*pro-2/3′ UTR-T; 26.2% (70/267) *IL12B*pro-2/3′ UTR-G; 12.7% (34/267) *IL12B*pro-1/3′ UTR-T; and 68.9% (184/267) *IL12B*pro-1/3′ UTR-G. Multivariate logistic regression analyses, controlling for the confounding effects of age and gender, were used to determine the association of *IL12B* haplotypes with severe malaria and hyperparasitaemia. The model was constructed such that those *with* vs. those *without* presence of the haplotype were compared. The *IL12B*pro-*2*/3′ UTR-T haplotype was associated with significant protection against severe malaria (OR, 0.51; 95% CI, 0.29–0.90, p=0.020) and non-significant protection against hyperparasitaemia (OR, 0.57; 95% CI, 0.28–1.18, p=0.132, Table 5). Additional analyses failed to show any significant haplotypic associations with susceptibility to either severe malaria or hyperparasitaemia for

Table 2 Levels of IL-12p40 and IFN-γ in uncomplicated and severe malaria and non-hyperparasitaemia vs. hyperparasitaemia

	All study participants			Severe malaria patients		
	UM	SM	*p*	Non-hyperparasitaemia	Hyperparasitaemia	*p*
IL-12p40 (pg/mL), median (IQR) sample size	27.0 (10.0–98.8) 56	95.1 (27.6–133.1) 25	0.049	35.1 (12.5–103.9) 14	121.9 (43.3–163.3) 11	0.046
IFN-γ (pg/mL), median (IQR) sample size	283.5 (118.5–473.5) 106	387.0 (144.2–632.0) 75	0.051	333.0 (136.7–580.2) 42	486.0 (153.0–710.5) 33	0.228

Patients with *P. falciparum* infection were categorised as uncomplicated or severe malaria according to WHO criteria (WHO 2000). Patients with severe malaria were further stratified as non-hyperparasitaemia (<250,000 parasites/μL) and hyperparasitaemia (≥250,000 parasites/μL). IL-12p40 and IFN-γ were measured in those subjects with adequate volumes of available plasma samples. Results are presented as median and interquartile range (IQR), with $p \leq 0.05$ considered statistically significant. Mann–Whitney *U* test was used to compare cytokine levels between the disease conditions

Table 3 Distribution of *IL12B* genotypes in patients with uncomplicated and severe malaria

	Genotype frequency			P(X)
	n (%)	*n* (%)	*n* (%)	
*IL12B*pro (rs17860508)	1.1	1.2	2.2	
UM	17 (6.8)	185 (74.0)	48 (19.2)	P(2.2)=0.56
SM	14 (13.6)	72 (69.9)	17 (16.5)	P(2.2)=0.51
Total	31 (8.8)	257 (72.8)	65 (18.4)	P(2.2)=0.55
p (UM vs. SM)	*p*=0.017	*p*=0.288	*p*=0.224	
IL12B 3′ UTR (rs3212227)	TT	TG	GG	
UM	61 (27.1)	104 (46.2)	60 (26.7)	P(G)=0.50
SM	20 (23.8)	34 (40.5)	30 (35.7)	P(G)=0.56
Total	81 (26.2)	138 (44.7)	90 (29.1)	P(G)=0.51
p (UM vs. SM)	*p*=0.259	*p*=0.128	*p*=0.021	

Data are presented as proportions, *n* (%). *IL12B*pro did not amplify in one subject with UM and one subject with SM, while *IL12B* 3′ UTR did not amplify in 37 subjects with UM and nine subjects with SM. Statistical significance for differences in genotypic frequencies between the UM and SM groups was determined by Chi-square analysis

UM uncomplicated malaria (*n*=252), *SM* severe malaria (*n*=103), *P(X)* frequency of the wild allele in the population

*IL12B*pro-*1/3′* UTR-G, *IL12B*pro-*2/3′* UTR-G and *IL12B*-pro-*1/3′* UTR-T (Table 5).

Functional relationship between the IL12B polymorphisms and circulating levels of IL-12p40 and IFN-γ Since variation in *IL12B* may influence IL-12p40 and IFN-γ production, circulating levels of IL-12p40 and IFN-γ were compared across the UM and SM groups stratified according to genotype for their respective polymorphisms (Table 6). There was a significance difference in IL-12p40 levels across the UM group (*p*=0.002), but not in individuals with SM (*p*=0.919). Post hoc testing of the UM group revealed that carriage of the *IL12B*pro1.2 genotype was associated with significantly higher IL-12p40 levels [median (IQR) 75.4 (13.5–112.5)] compared to the *IL12B*pro1.1 genotype [median (IQR) 10.0 (10.0–10.0), *p*=0.002] and the *IL12B*pro2.2 genotype [median (IQR) 20.0 (10.0–69.3), *p*=0.040]. In addition, patients with the *IL12B*pro2.2 genotype also had higher IL-12p40 levels than those with *IL12Bpro*1.1 genotype (*p*=0.002).

Stratification according to *IL12B*pro genotypes showed that IFN-γ levels were also significantly different across the UM group (*p*=0.004), but not across the SM group (*p*=0.508). Post hoc analyses of the UM group revealed that carriage of the *IL12B*pro1.1 genotype [median (IQR) 438.5 (227.7–585.2)] was associated with significantly higher IFN-γ levels compared to the *IL12B*-pro*2.2* genotype [median (IQR) 140.0 (43.0–314.0), *p*= 0.002]. In addition, individuals with the *IL12B*pro1.2 genotype [median (IQR) 338.5 (125.5–522.2)] also had

Table 4 Association between *IL12B* polymorphisms and severe disease outcomes

Genotype	Severe malaria			Hyperparasitaemia (≥250,000/μL)		
	Odds ratio	95% CI	*p* value	Odds ratio	95% CI	*p* value
*IL12B*pro (rs17860508)						
2.2 (*n*=65)	Reference			Reference		
1.2 (*n*=257)	1.04	0.55–1.94	0.910	0.77	0.33–1.80	0.541
1.1 (*n*=31)	2.34	0.94–5.81	0.066	3.42	1.17–9.97	0.025
IL112B 3′ UTR (T/G) (rs3212227)						
TT (*n*=90)	Reference			Reference		
TG (*n*=138)	0.61	0.33–1.00	0.096	0.80	0.35–1.79	0.583
GG (*n*=81)	0.70	0.36–1.39	0.311	0.99	0.40–2.46	0.983

Patients with *P. falciparum* infection were categorised according to WHO criteria (WHO 2000) for severe malaria. Patients with severe malaria were also categorised as hyperparasitaemia (≥250,000 parasites/μL) vs. non-hyperparasitaemia (<250,000 parasites/μL). Multivariate logistic regression analyses were performed, controlling for the confounding effects of age and gender. Results are presented as odds ratio (OR) and 95% confidence interval (CI), with $p \leq 0.05$ considered statistically significant

Table 5 Haplotypic association between *IL12B* polymorphisms and severe disease outcomes

Haplotype	Severe malaria			Hyperparasitaemia (≥250,000/μL)		
	Odds ratio	95% CI	*p* value	Odds ratio	95% CI	*p* value
*IL12B*pro-2/3′ UTR-T	0.51	0.29–0.90	0.020	0.57	0.28–1.18	0.132
*IL12B*pro-2/3′ UTR-G	1.49	0.83–2.69	0.181	0.90	0.40–2.03	0.795
*IL12B*pro-1/3′ UTR-T	1.52	0.70–3.30	0.292	2.15	0.84–5.53	0.112
*IL12B*pro-1/3′ UTR-G	0.87	0.49–1.57	0.645	0.76	0.35–1.64	0.487

Patients with *P. falciparum* infection were categorised according to WHO criteria (WHO 2000) for severe malaria and stratified based on their *IL12B* haplotypes (*IL12B*pro/3′ UTR). Patients were also categorised as non-hyperparasitaemia (<250,000 parasites/μL) and hyperparasitaemia (≥250,000 parasites/μL). Multivariate logistic regression analyses were performed, controlling for the confounding effects of age and gender. Results are presented as odds ratio (OR) and 95% confidence interval (CI), with $p \leq 0.05$ considered statistically significant for those with vs. those without the haplotype

significantly higher IFN-γ levels than those with the *IL12B*pro*2*.2 genotype ($p=0.004$).

Comparison of IL-12p40 and IFN-γ levels across the UM and SM groups stratified according to the *IL12B* 3′ UTR polymorphisms did not reveal any significant differences.

Functional relationship between IL12B haplotypes and circulating IL-12 and IFN-γ levels To determine if haplotypes were associated with functional changes in IL-12 and IFN-γ production, circulating concentrations of these mediators were compared across the haplotypic groups. Although differences in IFN-γ levels approached significance between individuals with the *IL12B*pro-2/3′ UTR-T haplotype [median (IQR); 267.5 (117.5–527.0)] relative to those with the non-*IL12B*pro-2/3′ UTR-T haplotype [median (IQR); 387.0 (164.0–597.0), $p=0.06$], none of the other haplotypes showed any functional association with circulating IL-12 and IFN-γ levels (Table 6).

Discussion

Although the literature is replete with studies investigating genetic susceptibility to severe malaria in African populations, much less information is available about how variation in immune response genes condition severe disease outcomes in Southeast Asia. As such, we utilised a candidate gene approach to focus on the role of IL-12 in shaping severe malaria outcomes in Thai adults with falciparum malaria. IL-12 was selected for investigation, since this type I cytokine plays a critical role in protection against malaria in both animal models (Mohan and Stevenson 1998a; Sam and Stevenson 1999) and in humans (Boutlis et al. 2003; Gosi et al. 1999; Keller et al. 2006; Luty et al. 2000; Perkins et al. 2000; Wroczynska et al. 2005). We hypothesised that exploration of variation in two previously identified polymorphisms in *IL12B* (i.e. *IL12B*pro and *IL12B* 3′UTR) may provide insight into the role of IL-12 in mediating susceptibility to severe malaria and another primary feature of severe falciparum malaria in Thai adults: hyperparasitaemia. The cross-sectional study design utilised for the current investigation included only patients with *P. falciparum* parasitaemia, so that we could explore the role of IL-12 in mediating severe disease outcomes once an individual acquires malaria.

Prior to determining the effect of *IL12B* genotypes and haplotypes on conditioning disease outcomes and functional changes in IL-12p40 and IFN-γ production, we examined these cytokines in parasitized individuals stratified according to uncomplicated and severe malaria and in severe malaria patients with and without hyperparasitaemia. Circulating levels of IL-12p40 and IFN-γ were highest in patients with severe malaria and hyperparasitaemia, respectively. The patterns of cytokine expression observed here are both similar and different from a number of previous studies conducted in populations with differing ages and levels of malaria endemicity. For example, results presented here in the largely less immune adult population to malaria most closely resemble studies in non-immune adults undergoing experimental infection with *P. falciparum* in which there was an early release of plasma IL-12p40 and IFN-γ (Hermsen et al. 2003). Our results differ somewhat from previous studies in Cameroon in which plasma levels of IL-12p40 and IL-12p70 were not significantly elevated in children with severe malaria, while circulating concentrations of IFN-γ were significantly higher in this group (Hermsen et al. 2003). In addition, results presented here differ from investigations in Gabon and Kenya demonstrating that severe childhood malaria is characterised by suppression of circulating IL-12 p40/p70 levels (Keller et al. 2006; Luty et al. 2000; Perkins et al. 2000). An additional explanation for the heterogeneity in IL-12p40/p70 and IFN-γ responses in the differing populations may be, at least in part, due to host genetic factors (Artavanis-Tsakonas and Riley 2002; D'Ombrain et

Table 6 Functional relationship between *IL12B* genotypes/haplotypes and IL-12p40 and IFN-γ

	Uncomplicated malaria				Severe malaria			
*IL12B*pro (rs17860508)	1.1	1.2	2.2	*p*	1.1	1.2	2.2	*p*
IL-12p40 (pg/mL), median (IQR) sample size	10.0 (10.0–10.0) 7	75.4 (13.5–112.5) 30	20.0 (10.0–69.3) 19	0.002	37.6 (16.8–229.8) 4	99.2 (27.1–135.0) 16	95.1 (35.9–131.4) 5	0.919
IFN-γ (pg/mL), median (IQR) sample size	438.5[a] (227.7–585.2) 10	338.5 (125.5–522.2) 94	140.0 (43.0–314.0) 19	0.004	550.5 (204.5–692.7) 12	340.5 (164.7–629.7) 62	242.0 (105.0–677.0) 15	0.508
IL12B 3′ UTR (rs3212227)	TT	TG	GG		TT	TG	GG	
IL-12p40 (pg/mL), median (IQR) sample size	22.1 (10.0–87.8) 20	41.1 (10.0–104.8) 28	26.7 (10.0–108.3) 13	0.622	95.1 (26.6–131.4) 9	65.6 (21.0–143.5) 6	76.7 (27.0–148.0) 10	0.892
IFN-γ (pg/mL), median (IQR) sample size	160.0 (88.0–413.0) 23	329.0 (125.0–450.0) 55	374.5 (230.5–593.0) 26	0.083	382.5 (113.5–623.2) 16	265.0 (170.5–633.0) 29	432.5 (162.5–623.5) 28	0.921
Haplotypes	*IL12B*pro-2/3′ UTR-T		*IL12B*pro-2/3′ UTR-G		*IL12B*pro-1/3′ UTR-T		*IL12B*pro-1/3′ UTR-G	
	0	1	0	1	0	1	0	1
IL-12p40 (pg/mL), median (IQR) sample size	32.2 (10.0–112.9) 27	49.8 (10.2–103.5) 54	38.1 (10.0–102.5) 62	74.3 (15.6–118.6) 19	39.9 (12.8–108.0) 70	10.0 (10.0–92.4) 11	27.1 (10.0–95.1) 33	39.9 (11.8–116.9) 48
p value	0.596		0.354		0.263		0.219	
IFN-γ (pg/mL), median (IQR) sample size	387.0 (164.0–597.0) 65	267.5 (117.5–527.0) 116	321.5 (128.0–542.0) 128	338.0 (130.0–495.0) 53	314.0 (125.0–517.0) 157	406.5 (160.5–619.5) 24	199.0 (108.5–537.5) 51	343.5 (159.0–541.0) 130
p value	0.06		0.953		0.252		0.158	

Patients with *P. falciparum* infection were categorised according to severe malaria according to WHO criteria (WHO 2000). Patients were categorised as hyperparasitaemia (≥250,000 parasites/μL) vs. non-hyperparasitaemia (<250,000 parasites/μL). IL-12p40 and IFN-γ were measured in those subjects with adequate volumes of available plasma samples. Across-group comparisons were determined by Kruskal–Wallis test, while pairwise comparisons were determined by Mann–Whitney *U* test. Results are presented as median and interquartile range (IQR), with $p \leq 0.05$ considered statistically significant. For the haplotypic constructions, results are presented as those without (code=0) vs. those with (code=1) the particular haplotypes

al. 2007; D'Ombrain et al. 2008; Mueller et al. 2004; Peng et al. 2006; Shimokawa et al. 2009). Genotyping of the overall population in the study revealed frequencies for the *IL12B*pro polymorphism at 8.8% (*IL12B*pro1.1), 72.8% (*IL12B*pro1.2) and 18.4% (*IL12B*pro2.2), while frequencies for the *IL12B* 3′ UTR polymorphism were 26.2% (TT), 44.7% (TG) and 29.1% (GG). Among patients with severe malaria, there were significantly elevated frequencies of *IL12B*pro1.1 and *IL12B* 3′ UTR-GG compared to the uncomplicated malaria group, while none of the other distributions significantly differed. Frequencies of the *IL12B*pro polymorphism in the Thai population investigated here differ from those observed in Chinese (Tso et al. 2004) and Spanish (Orozco et al. 2005) populations. Distributions of the *IL12B* 3′ UTR polymorphism were comparable to those reported previously in Thai (Nair et al. 2000) and Japanese (Yang et al. 2006) populations, but different from observations in Caucasian Americans and African Americans (Ma et al. 2003).

Multivariate analyses, controlling for the confounding effects of age and gender, revealed that carriage of the *IL12B*pro1.1genotype was associated with a non-significant increase in the risk of severe malaria and a significant increase in susceptibility to hyperparasitaemia. However, variation at the *IL12B* 3′ UTR was not associated with susceptibility to either severe malaria or hyperparasitaemia. Since haplotypes can identify important associations with disease outcomes that may not be reflected by analysis of individual loci, we performed haplotypic analyses. Construction of haplotypes for the two *IL12B* polymorphisms revealed that carriage of the *IL12B*pro-2/3′ UTR-T haplotype was associated with a 49% reduction in severe malaria ($p=0.020$) and a 43% reduction in hyperparasitaemia ($p=0.132$). Additional haplotypic analyses did not reveal any significant associations with severe malaria or hyperparasitaemia. Although not statistically significant, it is important to note that individuals with the *IL12B*pro-1/3′ UTR-T haplotype had a 115% increase in susceptibility to hyperparasitaemia relative to those without the haplotype. The lack of statistical significance for this haplotype likely reflects reduced sample size issues in the current study that may deserve closer inspection in a larger cohort.

Recent reports illustrate that the *IL12B*pro and *IL12B* 3′ UTR polymorphisms affect IL-12 gene expression (Shimokawa et al. 2009) and IL-12 production in vitro (Muller-Berghaus et al. 2004; Peng et al. 2006; Seegers et al. 2002). Results presented here demonstrate that carriage of the heterozygous (i.e. *IL12B*pro1.2) genotype was associated with significantly higher IL-12p40 levels in patients with uncomplicated malaria. In individuals with severe malaria, carriage of the *IL12B*pro2 allele was associated with the highest levels of IL-12p40, suggesting that this allele may functionally influence increased IL-12 production. These results are consistent with the fact that the *IL12B*pro2 allele appears to influence IL-12 production by altering Sp1-mediated transcription activity (Shimokawa et al. 2009). These results also parallel the studies in Papua New Guineans and Africans showing that the *IL12B*pro1.1 genotype was associated with reduced circulating levels of IL-12p40 and elevated parasitaemia (Boutlis et al. 2003). In contrast to IL-12, IFN-γ levels were highest in individuals with the *IL12B*pro1.1 polymorphism in both uncomplicated and severe malaria. Thus, although enhanced IL-12 production is typically associated with elevated IFN-γ levels, the results presented here illustrate that this relationship is not present in Thai adults with malaria and that this relationship is conditioned by variation in the *IL12B* promoter. Analysis of cytokine production stratified according to haplotypes revealed that the *IL12B*pro-2/3′ UTR-T that conditioned decreased susceptibility to severe malaria was also associated with reduced IFN-γ levels. These results are consistent with our finding that individuals with severe malaria have the highest levels of circulating IFN-γ, suggesting that over-expression of IFN-γ in this population may be important for enhancing the development of severe malaria.

In conclusion, results presented here in Thai adults with falciparum malaria illustrate that elevated expression of IL-12 and IFN-γ are associated with severe disease and a primary clinical feature of severe disease (i.e. hyperparasitaemia) in this population. This study further illustrates that haplotypes of the *IL12B*pro and 3′ UTR polymorphisms, namely the *IL12B*pro-2/3′ UTR-T, is associated with decreased susceptibility to severe malaria and reduced circulating levels of IFN-γ. As such, it appears that one primary effect of variation in the *IL12B* promoter on conditioning disease severity in this population may be, at least in part, mediated through IFN-γ. Future studies aimed at examining additional variation in *IL12B*, as well as *IFN-γ* genes, may provide important insight into the complex genetic pathways that condition susceptibility to severe malaria in Thai adults.

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Authors' contributions SK, DJP and CP designed the study. CP collected blood samples, genotyped the *IL12B*pro and *IL12B* 3′ UTR polymorphisms, participated in data analysis and writing of the manuscript. CO assisted in genotyping of *IL12B*pro (rs17860508) polymorphisms, data analysis and writing of the manuscript. PT collected blood samples and determined the circulating IFN-γ levels. JT determined circulating IL-12p40 levels. TW assisted in genotyping of *IL12B* 3′ UTR polymorphisms and data analysis. DW and YM assisted in data analysis. SK and DJP financed the study, performed data analyses and co-wrote the manuscript. All authors have read and approved the final version of the manuscript.

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