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Haplotypes of IL-10 promoter variants are associated with susceptibility to severe malarial anemia and functional changes in IL-10 production

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Abstract

Plasmodium falciparum malaria is one of the leading global causes of morbidity and mortality with African children bearing the highest disease burden. Among the various severe disease sequelae common to falciparum malaria, severe malarial anemia (SMA) in pediatric populations accounts for the greatest degree of mortality. Although the patho-physiological basis of SMA remains unclear, dysregulation in inflammatory mediators, such as interleukin (IL)-10, appear to play an important role in determining disease outcomes. Since polymorphic variability in innate immune response genes conditions susceptibility to malaria, the relationship between common IL-10 promoter variants (-1082A/G, -819T/C, and -592A/C), SMA (Hb<6.0 g/dL), and circulating inflammatory mediator levels (i.e., IL-10, TNF-α, IL-6 and IL-12) were investigated in parasitemic Kenyan children (n=375) in a holoendemic *P. falciparum* transmission area. Multivariate logistic regression analyses demonstrated that the -1082G/-819C/-592C (GCC) haplotype was associated with protection against SMA (OR; 0.68, 95% CI, 0.43-1.05; *P*=0.044) and increased IL-10 production (*P*=0.029). Although none of the other haplotypes were significantly associated with susceptibility to SMA, individuals with the -1082A/-819T/-592A (ATA) had an increased risk of SMA and reduced circulating IL-10 levels (*P*=0.042). Additional results revealed that the IL-10:TNF-α ratio was higher in the GCC group

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(*P*=0.024) and lower in individuals with the ATA haplotype (*P*=0.034), while the IL-10:IL-12 ratio was higher in ATA haplotype (*P*=0.006). Results presented here demonstrate that common IL-10 promoter haplotypes condition susceptibility to SMA and functional changes in circulating IL-10, TNF-α, and IL-12 levels in children with falciparum malaria.

Keywords

IL-10; polymorphisms; falciparum; malarial anemia

INTRODUCTION

Malaria remains a leading cause of global morbidity and mortality resulting in ~350-500 million clinical cases and up to 2 million deaths annually (WHO 2005;Snow et al. 1999). Severe malarial anemia (SMA) in infants and young children accounts for the greatest proportion of malaria-associated morbidity and mortality worldwide (Breman et al. 2001), and is the most common clinical manifestation of severe malaria in *Plasmodium falciparum* holoendemic transmission areas, such as western Kenya. In such regions, cerebral malaria (CM), hyperparasitemia, hypoglycemia, and renal insufficiency occur only rarely in pediatric malaria cases (Bloland et al. 1999;Ong'echa et al. 2006).

Despite the fact that pediatric SMA results from both enhanced red blood cell (RBC) destruction and inefficient RBC production, the patho-physiological basis of disease remains largely undefined. Interleukin-10 (IL-10) is an anti-inflammatory (type 2) cytokine produced primarily by monocytes, and to a lesser extent by lymphocytes, that has pleiotropic effects on immunological regulation (Lalani et al. 1997). In addition, IL-10 is important for down-regulating expression of the pro-inflammatory (type 1) immune response (e.g., TNF-α, IL-1, IL-6, IL-8, IL-12, and IFN-γ) (Lalani et al. 1997). We and others have demonstrated that dysregulation in the balance of type 1 and type 2 cytokines is associated with the immuno-pathogenesis of SMA and CM (Kurtzhals et al. 1999; Luty et al. 2000; McDevitt et al. 2004; Othoro et al. 1999; Perkins et al. 2000; Prakash et al. 2006). These investigations suggest that appropriate regulation of IL-10 is essential for controlling *P. falciparum* infection and that dysregulation in IL-10 may cause enhanced pathogenesis.

Since pathogenesis in polygenic infectious diseases, such as malaria, is conditioned by variability in the promoter and/or coding region(s) of inflammatory genes (Burgner et al. 2003; Cramer et al. 2004; Hobbs et al. 2002; Ouma et al. 2008), a better understanding of disease processes can be achieved by identifying unique functional polymorphisms in those critical genes that mediate the development and clinical course of disease. The IL-10 gene is located on chromosome 1q31-32 with the promoter region spanning at least 5 kb with more than 27 polymorphic sites (Giordani et al. 2003). Variability in the IL-10 promoter includes single nucleotide polymorphisms (SNPs) at -1082A/G (rs1800870), -819T/C (rs1800871), and -592A/C (rs1800872), and two microsatellite repeats with several allelic forms (Eskdale and Gallagher 1995). Previous studies have linked IL-10 promoter polymorphisms with differential production and expression of IL-10 in a number of disease states (Crawley et al. 1999; Eskdale et al. 1998; Gibson et al. 2001; Keijsers et al. 1997; Lim et al. 1998; Turner et al. 1997; Upperman et al. 2005; Yao et al. 2008). Although our previous investigations focused largely on the role of individual SNPs in conditioning susceptibility to severe malaria (Awandare et al. 2006; Ouma et al. 2006), our recent strategy involves identification of haplotypes since: 1) multi-site haplotypes are highly informative allelic markers that can reveal associations with disease outcomes not identifiable with single polymorphisms; and 2) combinations of different functional polymorphic alleles in a haplotype indicate how these polymorphisms interact to amplify, or moderate, their individual effects (Ouma et al. 2008). Consistent with this notion,

previous studies demonstrated that the -1082A/-819T/-592A (ATA), ACC, and GCC haplotypes were associated with low, intermediate, and high IL-10 transcriptional activity, respectively (Wilson et al. 2005). A recent study in Tanzania established borderline associations between SNPs in the IL-10 promoter (-1082A/G and -592A/C) and both anti-*P. falciparum* IgG4 and IgE antibodies, respectively (Carpenter et al. 2007). Additional investigations in The Gambia showed an association between a haplotypic block in the IL-10 gene (comprised of five SNPs spanning region +5941 to +919) and susceptibility to CM and severe anemia (Wilson et al. 2005). Based on these previous studies, we hypothesized that haplotypic constructions of common functional IL-10 promoter variants (i.e., -1082A/G, -819T/C and -592A/C) may be important in mediating susceptibility to SMA in children residing in holoendemic *P. falciparum* transmission areas. Results presented here in a cohort (n=375) of phenotypically well-characterized Kenyan children (aged <3 yrs) exposed to holoendemic *P. falciparum* transmission demonstrates that polymorphic variability in the IL-10 promoter (-1082A/G, -819T/C and -592A/C) is associated with susceptibility to SMA and functional changes in circulating IL-10, TNF-α, and IL-12 concentrations.

METHODS

Study participants

In an un-matched case-control study, parasitemic children <3 years of age (n=375) presenting at hospital for their first documented visit for acute malaria were recruited at Siaya District Hospital, a rural setting in western Kenya with holoendemic *P. falciparum* transmission (McElroy et al. 1999). The study was carried out in a homogenous population from the Luo ethnic group. A full description of the study site and manifestations of pediatric malaria is presented in our recent publication (Ong'echa et al. 2006). In brief, a questionnaire was used to collect relevant demographic and clinical information. Since the goal of the study was to enroll children with minimal prior exposure to malaria, children visiting hospital for the first time were recruited. As such, the majority of children in the study were <1 year of age at the time of enrollment. If the child had previously been hospitalized or had reported antimalarial use within the previous 2 weeks, he/she was not eligible for participation in the study. Hence, the primary variables for recruitment into the study were Hb concentrations and peripheral parasitemia prior to antimalarial treatment. Based on a previous longitudinal study examining the distribution of >10,000 Hb measurements in an age- and geographically-matched reference population in western Kenya, SMA was defined as Hb<6.0 g/dL with any density parasitemia (McElroy et al. 1999). This definition of SMA is appropriately defined by Hb distributions according to age, gender, and geographic context. In addition, children were also classified according to WHO definition of SMA (Hb ≤ 5.0 g/dL with any density parasitemia) (CDC 2000) to place the current findings into a global context. None of the children included in the study had CM or malaria from non-*P. falciparum* species. Since our previous studies, and those of others, demonstrated that HIV-1 and bacterial co-infection impact on the development and severity of malarial anemia (Berkley et al. 1999; Otieno et al. 2006), all children were tested for these co-pathogens (see procedures listed below). Pre- and post-test HIV counseling was provided for the parents/guardians of all study participants. Written informed consent in the language of choice (i.e., English, Kiswahili, or Dholuo) was obtained from the parents/guardians of participating children. The study was approved by the ethical and scientific review committees at the Kenya Medical Research Institute and the Institutional Review Board at the University of Pittsburgh.

Laboratory procedures

Venous blood samples (<3.0 mL) were collected in EDTA-containing Vacutainer tubes, prior to administration of antimalarials and/or any antipyretics. Asexual malaria parasites (trophozoites) were counted against 300 leukocytes in peripheral blood smears stained with

3% Giemsa. Parasite density was estimated using the following formula: parasite density/μL = white blood cell (WBC) count/μL × trophozoites/300. Complete hematological parameters were determined with a Beckman Coulter® AcT diff2™ (Beckman-Coulter Corporation). Sickle-cell trait (HbAS) was determined by Hb electrophoresis according to manufacturer's instructions (Helena Laboratories). HIV-1 exposure and HIV-1 infection were determined by serological and PCR testing, respectively, according to our published methods (Otieno et al. 2006). Trimethoprim-sulfamethoxazole was administered to all children (~22%) that were positive for one or both serological HIV-1 tests. At the time of sample collection, none of the HIV-1(+) study participants had been initiated on antiretroviral treatment. For determination of bacteremia (with a prevalence of ~12% in this population), ~1.5 mL of blood was collected aseptically in sterile pediatric isolator microbial tubes (Wampole Laboratories). Blood samples were inoculated directly onto chocolate agar plates and incubated for 18 hours at 37 °C 5% CO_2; followed by subculture for 18-24 hours in an inverted position. If no growth were obtained the sub-cultures were continued for 4 days. The plates were inspected daily for signs of microbial growth and bacterial colonies growing on positive plates were identified by Gram stain, colonial characteristics, appearance, and biochemical tests. API biochemical galleries (Biomerieux, Louvres) and/or serology were used to confirm suspected blood-borne bacterial pathogens.

IL-10 genotyping

DNA was extracted from blood spotted on FTA® Classic cards (Whatman Inc.) using the Gentra System (Gentra System, Inc.). IL-10 -592A/C genotyping was carried out as previously described (Yao et al. 2008). IL-10 -819T/C and -1082A/G SNPs were genotyped using a Taqman 5' allelic discrimination Assay-By-Design method according to manufacturer's instructions (Assay IDs: C_1747362_10 and C_1747360_10, respectively; Applied Biosystems).

Determination of circulating IL-10, TNF-α, IL-6, and IL-12 levels

Plasma samples were obtained from venous blood and stored at -80°C until use. IL-10, TNF-α, IL-6, and IL-12 concentrations were determined using the Cytokine 25-plex Ab Bead Kit, Hu (BioSource™ International) according to the manufacturer's protocol. Plates were read on a Luminex 100™ system (Luminex Corporation) and analyzed using the Bio-plex Manager Software (Biorad Laboratories). The detection limit for IL-10, TNF-α, IL-6, and IL-12 was 5.0 pg/mL, 9.0 pg/mL, 2.0 pg/mL and 4.0 pg/mL, respectively.

Statistical analyses

Statistical analyses were performed using SPSS (Version 12.0). Chi-square analyses were used to examine differences between proportions. Across group comparisons were determined by Kruskal-Wallis tests, while Mann-Whitney U tests were used for comparisons of demographic, clinical characteristics and circulating immune mediator levels. IL-10 haplotypes (-1082A/G, -819T/C and -592A/C) were constructed using HPlus software (Version 2.5). The association between the IL-10 promoter genotypes/haplotypes and SMA were determined by multivariate logistic regression, controlling for the confounding effects of age, gender, HIV-1 status [including both HIV-1 exposed and definitively HIV-1(+) results], bacteremia, and sickle-cell trait (HbAS). Co-efficient (D') of pairwise linkage disequilibrium (LD) between the SNPs was calculated using the Multiallelic Interallelic Disequilibrium Analysis Software (MIDAS). Statistical significance was defined as $P \leq 0.05$.

RESULTS

Demographic, clinical, and laboratory characteristics of the study participants

To investigate the role of variability in the IL-10 promoter (i.e., -1082A/G, -819T/C and -592A/C) in conditioning susceptibility to SMA, children (n=375, aged <3 years) presenting at hospital with acute *P. falciparum* malaria were stratified into non-severe malarial anemia (non-SMA, Hb≥6.0 g/dL, any density parasitemia; n=238) and severe malarial anemia (SMA, Hb<6.0 g/dL, any density parasitemia; n=137). The demographic, clinical, and laboratory characteristics of the study participants are presented in Table 1. There were no significant differences in gender distribution between the groups (*P*=0.524). Age differed significantly between the groups with the non-SMA group being older than those in SMA group (*P*=0.009). Axillary temperature was comparable between the groups (*P*=0.989). Peripheral parasitemia (MPS/μL) and the prevalence of HDP (≥10,000 parasites/μL) were not significantly different between the non-SMA and SMA groups (*P*=0.852 and *P*=0.429, respectively), suggesting that significantly different levels of anemia in parasitemic children from this region is largely independent of concomitant parasite density.

Distribution of individual IL-10 promoter variants

Genotypic distributions of individual IL-10 promoter variants (-1082A/G, -819T/C, and -592A/C) in the non-SMA (n=238) and SMA (n=137) groups, and the combined cohort, are presented in Table 2. There was no departure from HWE for the -1082A/G variant in either the non-SMA (χ^2=0.827, *P*=0.950) or SMA (χ^2=2.320, *P*=0.100) group. In addition, no significant departures from HWE were observed for either the -819T/C and -592A/C variants in the non-SMA group (χ^2=0.746, *P*=0.970 and χ^2=3.288, *P*=0.100, respectively) or the -819T/C variant in the SMA group (χ^2=2.801, *P*=0.250). However, there was a significant departure from HWE for the -592A/C variant in the SMA group (χ^2=9.674, *P*=0.005). Additional analyses demonstrated that distribution of the -1082A/G, -819T/C, and -592A/C genotypes was not significantly different between the non-SMA and SMA groups (*P*=0.156, *P*=0.522 and *P*=0.300, respectively, Table 2).

Association between IL-10 promoter polymorphisms and SMA

To determine the role of individual IL-10 promoter loci in conditioning susceptibility to SMA, multivariate logistic regression analyses were conducted controlling for the confounding effects of age, gender, HIV-1 status, bacteremia, and sickle-cell trait (Aidoo et al. 2002; Berkley et al. 1999; Otieno et al. 2006). As shown in Table 3, these analyses demonstrated that variation at individual IL-10 promoter loci was not significantly associated with susceptibility to SMA (Hb <6.0 g/dL) at -1082A/G (AA vs AG, *P*=0.066, and AA vs GG, *P*=0.902), -819T/C (TT vs TC, *P*=0.399 and TT vs CC, *P*=0.484), and -592A/C (AA vs AC, *P*=0.205 and AA vs CC, *P*=0.417).

To place the current findings into the commonly used WHO definition of SMA (Hb<5.0 g/dL) (CDC 2000), additional multivariate logistic regression analyses were performed controlling for the appropriate co-factors (Aidoo et al. 2002;Berkley et al. 1999;Otieno et al. 2006). Consistent with the analyses in which SMA was defined by Hb <6.0 g/dL, analyses using Hb<5.0 g/dL as the cut off for SMA failed to show any significant associations between individual genotypes and SMA at -1082A/G (AA vs AG, *P*=0.296, and AA vs GG, *P*=0.136), -819T/C (TT vs TC, *P*=0.113 and TT vs CC, *P*=0.074), and -592A/C (AA vs AC, *P*=0.388 and AA vs CC, *P*=0.074). Taken together, the multivariate analyses demonstrated that individual IL-10 promoter loci were not significantly associated with susceptibility to SMA.

Distribution of IL-10 promoter haplotypes and linkage disequilibrium

Further stratification of children into haplotypic groups based on the three promoter polymorphisms yielded the following distribution: 64.3% (153/238) GCC (IL-10 -1082G/-819C/-592C); 35.3% (84/238) ACC; 4.2% (10/238) ATC; and 59.7% (142/238) ATA in the non-SMA group and 55.5% (76/137) GCC; 37.2% (51/137) ACC; 5.8% (8/137) ATC; and 59.1% (81/137) ATA in the SMA group (Table 4). Data were not presented for the other haplotypes since their overall frequencies were <0.05% in the population. As shown in Table 4, distribution of the GCC haplotype was significantly different between non-SMA and SMA groups (P=0.048), while the ACC, ATC and ATA haplotypes were not significantly different between the groups (P=0.395, P=0.316 and P=0.502, respectively).

Additional analyses demonstrated linkage disequilibrium between allele A at locus -1082 and both allele T at locus -819 (D'=0.950) and allele A at -592 (D'=0.950). Complete linkage disequilibrium was observed between locus -819 and locus -592 (D'=1.000).

Association between IL-10 promoter haploytpes and SMA

Prior to analyzing the associations between IL-10 promoter haplotypes and SMA, preliminary analyses examined the relationship between the different haplotypes and Hb levels. Presence of the GCC haplotype, median, IQR [8.00 (7.01-8.63)] was associated with significantly higher Hb levels relative to non-GCC group [6.55 (6.61-6.68), P=0.023]. However, Hb levels were comparable between the ACC vs. non-ACC (P=0.996), ATC vs. non-ATC (P=0.763), and ATA vs. non-ATA (P=0.831) groups.

Since multi-site haplotypes are highly informative allelic markers for identifying associations with disease outcomes, not identifiable with single polymorphisms, the influence of IL-10 promoter haplotypes on susceptibility to SMA (Hb<6.0 g/dL) was further assessed in a multivariate logistic regression model controlling for the confounding effects of age, gender, HIV-1 status, bacteremia and sickle-cell trait (Aidoo et al. 2002; Berkley et al. 1999; Otieno et al. 2006). These analyses revealed that individuals with the GCC haplotype had a 32% decreased susceptibility to SMA (OR; 0.68, 95% CI, 0.43-1.05; P=0.044) relative to the non-GCC group (Table 5). None of the other haplotypes showed a strong association with susceptibility to SMA using a cut-off of 6.0 g/dL (Table 5). In addition, no significant relationships were identified between any of the haplotypic structures and WHO definition of SMA (CDC 2000), (GCC, OR; 0.63, 95% CI, 0.47-1.10, P=0.274; ACC, OR; 1.07, 95% CI, 0.60-1.91, P=0.808; ATC, OR; 2.59, 95% CI, 0.91-7.34, P=0.073 and ATA, OR; 1.39, 95% CI, 0.79-2.42, P=0.090).

Given the high prevalence of the GCC and ATA haplotypes in the population and a possible diluting effect of each haplotype in heterozygous individuals (i.e. GCC/ATA), haplotypes were constructed based on the dominant model (i.e., presence of GCC/GCC and ATA/ATA) in association with SMA. The model demonstrated that individuals with the IL-10 GCC/GCC haplotype were significantly protected against SMA (Hb<6.0 g/dL) relative to the non-GCC/GCC haplotype (OR; 0.60, 95% CI, 0.57-0.97; P=0.039), further supporting our finding that the presence of GCC haplotype significantly reduces the risk of developing SMA (even though the OR included 1 above). The model further demonstrated that there was a tendency towards enhanced susceptibility to SMA in individuals with the ATA/ATA haplotype (OR; 1.36, 95% CI, 1.01-2.32; P=0.257) relative to those with non-ATA/ATA haplotypes.

Functional relationship between IL-10 promoter haplotypes and circulating IL-10, TFN-α, IL-6 and IL-12 levels

To determine if haplotypes were associated with functional changes in IL-10 production, circulating concentrations of IL-10 were compared across the haplotypic groups. There was a

significant difference in IL-10 levels across the groups (*P*=0.041, Figure I). Further analyses revealed that individuals with the -1082G/-819C/-592C (GCC) haplotype [median (IQR); 254.46 (103.03-726.16)] had significantly higher circulating IL-10 levels than those without this haplotype [223.95 (121.41-658.25), *P*=0.029, Figure I]. In addition, individuals with the ATA haplotype [median (IQR); 192.03 (88.90-884.32)] had significantly lower circulating IL-10 levels than those without this haplotype [280.32 (142.60-861.20), *P*=0.042, Figure I]. Even though carriage of ACC and ATC haplotypes are associated with intermediate IL-10 production (Wilson et al. 2005), no significant differences in IL-10 production were observed for individuals with and without either the ACC (*P*=0.104) or ATC (*P*=0.381) haplotypes. Results presented here illustrate that the GCC and ATA haplotypes are associated with significant differences in circulating IL-10 levels.

Furthermore, since IL-10 can exert its effects through down-regulation of TFN-α, IL-6 and IL-12 (Cai et al. 1999; Conti et al. 2003), cytokine ratios of IL-10:TFN-α, IL-10:IL-6, and IL-10:IL-12 were investigated in individuals with the GCC and ATA haplotypes. The IL-10:TFN-α ratio was significantly higher in the GCC group [median (IQR); 12.81 (4.73-32.22)] relative to the non-GCC group [9.06(3.73-17.05), *P*=0.024]. However, although the GCC group had a higher IL-10:IL-6 ratio [6.84(1.63-8.77)] than the non-GCC group [3.13 (1.82-8.54)], and a lower IL-10:IL-12 ratio [0.48(0.25-1.69)] than the non-GCC group [0.49 (0.18-1.40), these relationships were not statistically significant (*P*=0.567 and *P*=0.507, respectively). Further analysis revealed that the IL-10:TFN-α ratio in the ATA group [9.30 (3.80-17.24)] was significantly lower than the non-ATA group [12.87(4.99-39.29), *P*=0.034], while the IL-10:IL-12 ratio in individuals with the ATA haplotype [0.68 (0.31-2.16)] was significantly higher than the non-ATA group [0.44(0.19-1.19) *P*=0.006]. The IL-10:IL-6 ratio was non-significantly lower in ATA group [2.82 (1.40-5.36) relative to non-ATA individuals [7.01 (5.77-11.43), *P*=0.591]. Taken together, these results demonstrate that the GCC and ATA haplotypes are associated with differential levels of cytokines known to be regulated by IL-10, particularly TFN-α and IL-12.

DISCUSSION

Our hypothesis predicted that common IL-10 promoter variants (-1082A/G, -819T/C and -592A/C) would be associated with malaria disease outcomes and functional changes in circulating IL-10 levels. Cross-sectional results presented here in a phenotypically well-defined cohort of children less than 3 years of age demonstrate that presence of the IL-10 promoter haplotype -1082G/-819C/-592C (GCC) was associated with protection against SMA (Hb<6.0 g/dL) (McElroy et al. 1999). Children with GCC haplotype also had significantly higher circulating IL-10 levels relative to the non-GCC group. Additional results revealed that presence of haplotype -1082A/-819T/-592A (ATA) was non-significantly associated with increased susceptibility to SMA and significantly reduced IL-10 production. Consistent with these observations, the presence of the GCC haplotype was associated with significantly higher Hb levels relative to non-GCC haplotype, while presence of ATA haplotype was associated with non-significantly reduced Hb levels relative to non-ATA group. Further analyses revealed that the IL-10:TFN-α ratio was significantly higher in the GCC group and lower in the ATA haplotype. In addition, the IL-10:IL-12 ratio was significantly higher in the ATA group relative to the non-ATA group. Taken together, these results suggest that protection against SMA requires enhanced production of IL-10 with concomitant downregulation on TFN-α that is conditioned by haplotypic combinations of common promoter variants. To our knowledge, this is the first report demonstrating that the common IL-10 promoter variants (-1082A/G, -819T/C and -592A/C) condition susceptibility to SMA and functional changes in IL-10, TFN-α and IL-12 production in children with malaria.

Although none of the individual variants were significantly associated with susceptibility to SMA, a number of the genotypes approached significance, suggesting that a larger sample size may be required to identify the importance of individual polymorphisms using both 6.0 and 5.0 g/dL as the cut-off for SMA. For example, several of the IL-10 haplotypic combinations approached significance when using the WHO criteria of <5.0 g/dL to define SMA. These results suggest that there may be a difference in the association of the investigated haplotypic combinations with SMA when using lower Hb levels for the definition of SMA. We are currently collecting samples on 1,400 additional children with falciparum malaria that will provide the statistical power required to determine if there is a change in susceptibility to SMA when defined by progressively lower Hb levels.

In the current study, the frequencies of the -1082G, -819C and -592C alleles in the cohort were 38%, 58%, and 60%, respectively. These frequencies are significantly higher than those observed in Caucasians populations for the -1082G, -819C, and -592C alleles (i.e. 12%, 35%, and 35%, respectively) (Ide et al. 2002; Lee et al. 2005; Yao et al. 2008), but similar to those previously reported in African populations (Meenagh et al. 2002). The -1082A/G, -819T/C, and -592A/C loci were in strong linkage disequilibrium. The overall frequency of the major haplotype ATA in this cohort was 61%, which was significantly higher than those observed in Caucasian populations (>25%) (Mangia et al. 2004; Scassellati et al. 2004). Taken together, these investigations suggest that frequencies of IL-10 promoter alleles and haplotypes vary widely across different ethnic groups, possibly as a result of differential exertion of selective pressure on the human genome, particularly in host-immune response genes that mediate susceptibility and clinical outcomes of diseases such as malaria (Kwiatkowski 2005).

The IL-10 gene contains three common polymorphisms (i.e. -1082, -819 and -592) that have been reported to influence gene expression (Turner et al. 1997; Yao et al. 2008). Consistent with those studies, results presented here in children with falciparum malaria demonstrate that the -1082G/-819C/-592C (GCC) haplotype is associated with increased IL-10 production, while the -1082A/-819T/-592A (ATA) haplotype is associated with a reduced production of IL-10. In support of previous observations (Wilson et al. 2005), the GCC, ACC, and ATA individuals produced high, intermediate, and low circulating IL-10 levels, respectively. Activation of gene transcription depends upon the binding of regulatory factors to specific recognition sequences in the promoter. A number of putative recognition sites are present in the IL-10 promoter, including AP1, PEA1, and ETS-like element (Kube et al. 1995). Variation in cytokine promoter sequences, such as the IL-10 promoter, likely alter specific transcription factor recognition sites and consequently affect transcriptional activation and cytokine production. For example, presence of the GCC promoter haplotype may favor enhanced binding of transcriptional factors that lead to higher IL-10 production, while presence of the ATA haplotype may create sites for enhanced binding of repressors that favor reduced IL-10 production. Although the impact of the haplotypes examined here on promoter binding elements is currently unknown, our laboratory is currently investigating the mechanism(s) by which these haplotypes favor increased and decreased IL-10 production, respectively.

The importance of IL-10 variants has been documented in several disease processes by a number of independent studies (Gibson et al. 2001; Lim et al. 1998; Upperman et al. 2005; Wunderink and Waterer 2003; Yao et al. 2008). However, reports on the role of IL-10 in regulating the inflammatory response to malaria remain conflicting since several studies suggest that enhanced IL-10 is associated with increased pathogenesis, while others suggest that enhanced IL-10 is associated with protection, depending on the stage of disease (Jason et al. 2001; Keller et al. 2006; Kurtis et al. 1999; Kurtzhals et al. 1999; May et al. 2000; Ong'echa et al. 2008; Othoro et al. 1999; Winkler et al. 1998). A previous cross-sectional study carried out in a Gambian population in a combined case-control and intra-familial group demonstrated that the IL-10 haplotype +919C/-627G/-1117C/-3585T was weakly associated with protection

against a mixed clinical phenotype of severe malaria (i.e., CM and severe anemia) (Wilson et al. 2005). In addition, this observation lacked support from transmission disequilibrium analysis (Wilson et al. 2005). Consistent with findings presented here showing that the ATA haplotype was associated with a tendency towards increased susceptibility to SMA and significantly reduced IL-10 production, previous studies demonstrated that IL-10-deficient mice infected with *P. chabaudi chabaudi* succumb to severe disease and have higher mortality than either heterozygotes or normal mice, respectively (Linke et al. 1996). These results were attributed to persistence of a type 1 immune response in IL-10-deficient mice throughout the course of infection, while there was a predominant type 2 immune response in heterozygote and wild type mice with favorable outcomes.

Previous studies in children with malaria (Kurtzhals et al. 1999; Othoro et al. 1999; Perkins et al. 2000) support the notion that production of IL-10 is important for preventing over-expression of TNF-α that can promote enhanced malaria pathogenesis. In addition, the relative expression of IL-10 to TNF-α in children with malaria is conditioned by polymorphic variants in the TNF-α promoter (May et al. 2000). These studies are consistent with the fact that IL-10 inhibits the production of pro-inflammatory cytokines such as TNF-α in T-cells and monocytes (Fiorentino et al. 1991). Although induction of TNF-α induces fever and aids in suppressing parasitemia, sustained production of TNF-α appears to promote enhanced disease severity (Miller et al. 1994; Perkins et al. 2000). One important mechanism by which prolonged expression of TNF-α may cause enhanced malaria pathogenesis is through suppression of erythropoiesis (Clark and Chaudhri 1988). In the population investigated here, the IL-10:TNF-α ratio was significantly higher in GCC haplotype who had a reduced risk to SMA relative to non-GCC, while the IL-10:TNF-α ratio was significantly lower in ATA haplotype relative to non-ATA individuals. These results suggest that *P. falciparum*- infected children with the GCC haplotype produce higher amounts of IL-10 that can successfully regulate excessive TNF-α production and offer protection against the development of SMA. In contrast, the ATA haplotype associated with reduced IL-10 production may allow over-expression of TNF-α that could result in increased susceptibility to SMA.

Additional results revealed a significantly higher IL-10:IL-12 ratio in ATA group that had increased susceptibility to SMA relative to the non-ATA group. These results are consistent with our previous results in Gabonese children showing that severe malaria is associated with an increased IL-10:1L-12 ratio (Perkins et al. 2000). We are currently performing additional studies to elucidate the molecular basis through which dysregulation in the production of IL-10, TNF-α, IL-12 and IL-6 can alter erythropoietic maturation. In addition, examination of an inclusive panel of genetic variation in innate immune response genes may offer insight into the complex cytokine milieu that promotes development of SMA in children with falciparum malaria.

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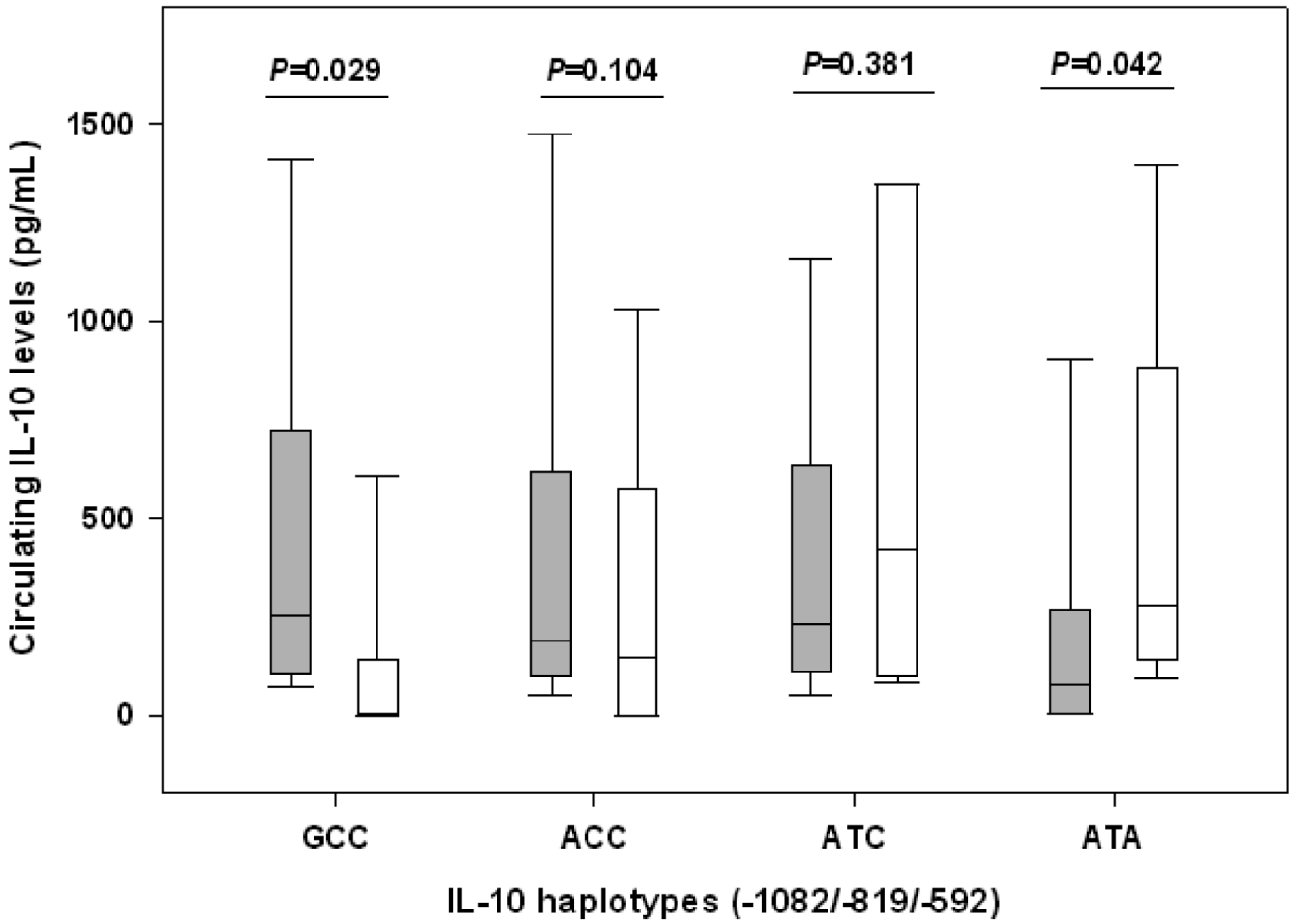

Figure I. Functional association between IL-10 promoter haplotypes and circulating IL-10 levels

Circulating IL-10 levels for children aged less than 3 years with malaria were measured using the Human Cytokine Twenty-Five-Plex Antibody Bead Kit and results are presented for haplotypic combinations of the IL-10 -1082, -819, and -592 promoter variants. Data are presented as box-plots where the box represents the interquartile range, the line through the box is the median, and whiskers show the 10^{th} and 90^{th} percentiles. Shaded boxes represent individuals with respective haplotypic combinations, while open boxes represent those without the haplotype. The nomenclature for each haplotypic combination is (for example) IL-10 -1082G, -819C and -592C is equivalent to GCC. Data are shown as presence or absence of GCC, ACC, ATC, and ATA haplotypes. Differences in circulating IL-10 levels were considered significant at $P<0.05$ (Mann-Whitney U tests).

Table 1

Demographic, clinical, and laboratory characteristics of the study participants

Characteristic	Non-SMA	SMA	*P*
No. of participants	238	137	N/A
Gender (n, %)			
Male	121 (50.8)	70 (51.1)	0.524[a]
Female	117 (49.2)	67 (48.9)	
Age (mos.)	11.00 (7.00-16.00)	8.00 (6.00-14.00)	**0.009**[b]
Axillary temperature (°C)	37.5 (36.8-38.7)	37.5 (36.8-38.5)	0.989[b]
Hemoglobin (g/dL)	8.30 (7.60-10.60)	5.00 (4.60-5.60)	
Parasite density (/μL)	22,628 (4,031-63,359)	25,738 (6, 848-56, 123)	0.852[b]
HDP (n, %)	165 (69.3)	137 (67.9)	0.429[a]

Data are median (interquartile range, IQR) unless otherwise stated.

Abbreviations: HDP, high-density parasitemia (≥10,000 parasites/μL); SMA, severe malarial anemia (Hb<6.0 g/dL, with any density parasitemia) (McElroy et al. 1999); Non-SMA, non-severe malarial anemia (Hb≥6.0 g/dL, with any density parasitemia.

[a] Statistical significance determined by Chi-square analysis.

[b] Statistical significance determined by Mann-Whitney U test. Statistical testing for differences in Hb levels between SMA and non-SMA was not provided because stratification of these groups was based on Hb levels and presence of parasitemia.

Table 2

Distribution of IL-10 promoter genotypes in children with malaria

	Genotype	Non-SMA	SMA	Total	*P*
No. of participants		238	137	375	
IL-10-1082A/G	AA, n (%)	85 (35.7)	60 (43.8)	145 (38.7)	
	AG, n (%)	120 (50.4)	55 (40.1)	175 (46.7)	0.156[a]
	GG, n (%)	33 (13.9)	22 (16.1)	55 (14.7)	
		P(A)=0.61	P(A)=0.64	P(A)=0.62	
IL-10-819T/C	TT, n (%)	44 (18.5)	32 (23.4)	76 (20.3)	
	TC, n (%)	109 (45.8)	58 (42.3)	167 (44.5)	0.522[a]
	CC, n (%)	85 (35.7)	47 (34.3)	132 (35.2)	
		P(T)=0.41	P(T)=0.44	P(T)=0.42	
IL-10-592A/C	AA, n (%)	43 (18.1)	33 (24.1)	76 (20.3)	
	AC, n (%)	100 (42.0)	49 (35.8)	149 (39.7)	0.300[a]
	CC, n (%)	95 (39.9)	55 (40.1)	150 (40.0)	
		P(A)=0.39	P(A)=0.42	P(A)=0.40	

Data are presented as proportions (n, %).

Abbreviations: SMA, severe malarial anemia (Hb<6.0 g/dL, with any density parasitemia) (McElroy et al. 1999); Non-SMA, non-severe malarial anemia (Hb≥6.0 g/dL, with any density parasitemia); IL-10, Interleukin-10; P(X), frequency of wild allele in the population.

[a]Statistical significance determined by Chi-square analysis.

Table 3

Relationship between individual IL-10 promoter loci and SMA

Genotype	SMA (Hb<6.0 g/dL)		
	OR	**95% CI**	***P***
IL-10-1082A/G			
AA	1.00		
AG	0.56	0.27-1.02	0.066
GG	0.94	0.54-1.78	0.902
IL-10-819T/C			
TT	1.00		
TC	0.88	0.72 - 1.28	0.399
CC	1.24	0.67 - 2.26	0.484
IL-10-592A/C			
AA	1.00		
AC	0.86	0.51 - 1.42	0.205
CC	1.27	0.71 - 2.28	0.417

Parasitemic children (n=375) were categorized according to a modified definition of SMA based on age- and geographically-matched Hb concentrations (i.e., Hb<6.0 g/dL, with any density parasitemia) (McElroy et al. 1999). Odd Ratios (OR) and 95% confidence interval (CI) were determined using multivariate logistic regression controlling for age, gender, HIV-1 status, bacteremia, and sickle-cell trait (HbAS).

Table 4

Distribution of IL-10 promoter haplotypes in children with malaria

Haplotype (-1082/-819/-592)		Non-SMA (n=238)	SMA (n=137)	*P*
GCC	1	153 (64.3)	76 (55.5)	**0.048**[a]
	0	85 (35.7)	61 (44.5)	
ACC	1	84 (35.3)	51 (37.2)	0.395[a]
	0	154 (64.7)	86 (62.8)	
ATC	1	10 (4.2)	8 (5.8)	0.316[a]
	0	228 (95.8)	129 (94.2)	
ATA	1	142 (59.7)	81 (59.1)	0.502[a]
	0	96 (40.3)	56 (40.9)	

Parasitemic children (n=375) were stratified based on their IL-10 promoter haplotypes (-1082/-819/-592). 1=Presence of haplotype, 0=Absence of haplotype. Data are presented as proportions (n, %).

Abbreviations: SMA, severe malarial anemia (Hb<6.0 g/dL, with any density parasitemia) (McElroy et al. 1999); Non-SMA, non-severe malarial anemia (Hb≥6.0 g/dL, with any density parasitemia).

[a]Chi-square analysis.

Table 5

Relationship between IL-10 promoter haplotypes and SMA

Haplotype (-1082/-819/-592)	SMA (Hb<6.0 g/dL)		
	OR	**95% CI**	***P***
GCC	0.68	0.43 - 1.05	**0.044**
ACC	1.15	0.72 - 1.82	0.544
ATC	1.40	0.52 - 3.78	0.498
ATA	1.92	0.62 - 2.52	0.921

Parasitemic children (n=375) were categorized according to a modified definition of SMA based on age- and geographically-matched Hb concentrations (i.e., Hb<6.0 g/dL, with any density parasitemia) (McElroy et al. 1999). Odd Ratios (OR) and 95% confidence interval (CI) were determined using multivariate logistic regression controlling for age, gender, HIV-1 status, bacteremia, and sickle-cell trait (HbAS).