Tumor Necrosis Factor and Lymphotoxin-α Polymorphisms and Severe Malaria in African Populations

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The tumor necrosis factor gene (TNF) and lymphotoxin-α gene (LTA) have long attracted attention as candidate genes for susceptibility traits for malaria, and several of their polymorphisms have been found to be associated with severe malaria (SM) phenotypes. In a large study involving >10,000 individuals and encompassing 3 African populations, we found evidence to support the reported associations between the TNF −238 polymorphism and SM in The Gambia. However, no TNF/LTA polymorphisms were found to be associated with SM in cohorts in Kenya and Malawi. It has been suggested that the causal polymorphisms regulating the TNF and LTA responses may be located some distance from the genes. Therefore, more-detailed mapping of variants across TNF/LTA genes and their flanking regions in the Gambian and allied populations may need to be undertaken to find any causal polymorphisms.

Malaria is a complex disease with many genetic and environmental determinants influencing the observed variation in the response to infection and the progression and severity of infection. To develop effective vaccines and antimalarial therapies, which are needed to control the serious toll of malaria-associated morbidity and mortality, it is crucial to understand the mechanisms of protective immunity against malaria, as well as the immunopathology of the disease. Cytokines are critical to the functioning of both innate and adaptive immune responses, and they are key regulators of the host response to infection. Identification of the polymorphisms regulating the cytokine response will assist in understanding the variation in response to infection.

Tumor necrosis factor (TNF) and lymphotoxin-α (LTA) have long attracted attention as candidate genes for susceptibility traits for malaria. TNF-α was the first cytokine shown to be present in elevated levels in humans with severe malaria (SM) [1, 2], and polymorphisms of the promoter region of the TNF gene have been extensively investigated [3, 4]. The TNF −376 polymorphism acts to modulate transcription factor Oct-1 in human monocytes in vitro, and it is associated with susceptibility to SM [4]. In particular, in a Gambian population, carriers of the TNF −376A allele were found to be at an increased risk for cerebral malaria (CM), and this observation was confirmed in a study performed in Kenya [4]. Additional variants TNF −308 and TNF −238 have been found to be associated with clinical sub-
phenotypes of malaria. Gambian children who are homozygous for the \( \text{TNF} - 308A \) allele also have an increased susceptibility to CM [3] but not to severe malarial anemia (SMA). Further research in the same population suggested that the \( \text{TNF} + 238A \) allele is associated with SMA, leading to speculation that SMA and CM are associated with different \( \text{TNF} \) promoter alleles (\( \text{TNF} + 238 \) and \( \text{TNF} + 308 \)) [5]. The results from additional studies (some of which were underpowered) in other populations have not been consistent (table 1). The functional significance of \( \text{TNF} + 238 \) and \( \text{TNF} + 308 \) is controversial, because some evidence is derived from underpowered studies and could be confounded by haplotypic heterogeneity (for a summary of the functional evidence, see [16, 17]).

\( \text{TNF} \) and \( \text{LTA} \) are neighboring genes that are in high linkage disequilibrium (LD) with each other [18]. In particular, \( \text{TNF} - 308 \) forms an extended haplotype with polymorphisms in \( \text{LTA} \). Genetic variation in the \( \text{LTA} \) locus in humans has been found to be associated with susceptibility to myocardial infarction [19], bacterial infection [20], asthma [21], leprosy [22], and other diseases [23]. It has also been shown that \( \text{LTA} \) is a principal mediator of murine CM [24]. The common \( \text{LTA} \) haplotype is defined uniquely by 2 markers, one of which, the \( \text{LTA} + 80 \) polymorphism, shows evidence of allele-specific binding by a transcriptional repressor, ABF-1 [17]. There is also some evidence that the other marker, the \( \text{LTA} + 252 \) polymorphism, modulates reporter gene expression and, possibly, protein-DNA binding [17].

In the present study, we investigated whether a number of \( \text{LTA} \) and \( \text{TNF} \) single-nucleotide polymorphisms (SNPs), including the functional \( \text{LTA} + 80 \) polymorphism and the potentially functional \( \text{TNF} - 376, -308 \), and \( -238 \) polymorphisms, are associated with SMA. This investigation considered multiple African populations (in The Gambia, Kenya and Malawi) by using both family- and population-based studies. We sought to clarify the previous association discrepancies in the \( \text{TNF}/\text{LTA} \) region by using the largest sample collections of SM available to date.

**SUBJECTS, MATERIALS, AND METHODS**

**Sample preparation and genotyping.** Genomic DNA samples underwent whole-genome amplification through either primer extension preamplification [25] or multiple displacement amplification [26], before genotyping was done on a Sequenom MassArray genotyping platform [27, 28]. The following SNPs were genotyped: (1) hemoglobin variant S (\( \text{HbS} \)) (rs334), (2) \( \text{LTA} + 252 \) (T252C; rs909253), (3) \( \text{LTA} + 80 \) (G80T; rs2239704), (4) \( \text{TNF} + 851 \) (A851G; rs3093662), (5) \( \text{TNF} - 238 \) (G238A; rs361525), (6) \( \text{TNF} - 308 \) (G308A; rs1800629), (7) \( \text{TNF} - 376 \) (G376A; rs1800750), and (8) \( \text{TNF} - 1031 \) (T1031C; rs1799964). \( \text{TNF} \) and \( \text{LTA} \) SNP selection was based on haplotype tags [29] and a previous association with malaria. Figure 1 shows a genetic map for the \( \sim 4\)-kb region in which the \( \text{LTA}/\text{TNF} \) polymorphisms that are being considered reside. All \( \text{TNF} \) polymorphisms are located within the promoter region, except \( \text{TNF} + 851 \), which is located in the exonic region of the gene. The \( \text{HbS} \) polymorphism is used to calibrate our results, because it is known to be under heterozygous advantage for SM.

**Study participants.** Patient samples were collected as part of ongoing epidemiologic studies of SM at the Royal Victoria Hospital in Banjul, The Gambia (2491 case patients with SM, 3881 cord blood controls, and 1055 family trios); Kilifi District Hospital in Kilifi, Kenya (927 case patients with SM and 953 cord blood controls); and the Queen Elizabeth Central Hospital in Blantyre, Malawi (1629 case patients with SM and 3578 cord

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**Table 1. Studies of malaria and lymphotoxin-α (LTA) and tumor necrosis factor (TNF) genes.**

The table is available in its entirety in the online edition of *The Journal of Infectious Diseases*.

![Figure 1](https://academic.oup.com/jid/article-abstract/199/4/569/2192188) The lymphotoxin-α gene (LTA) and tumor necrosis factor gene (TNF) region and the polymorphisms genotyped.
blood controls). The set of nuclear family trios comprised a child affected with SM and its 2 (biological) parents, and they were assessed as “true trios” by use of the NucStar software package (version 1.0) [30]. All DNA samples were collected and genotyped after approval was provided by the relevant research ethics committees and written informed consent was provided by the participants.

Phenotypic definition. All cases were children admitted to the hospital with evidence of Plasmodium falciparum on blood film and clinical characteristics of SM [31, 32]. Subjects were defined as having had CM if their Blantyre coma score was ≥2 at presentation or early during admission. A second phenotypic subset of individuals with SMA was defined as those subjects who had a hemoglobin concentration of <5 g/dL or a hematocrit of <15%. Participants with coexisting severe or chronic medical conditions (e.g., bacterial pneumonia or kwashiorkor) unrelated to a SM were excluded. The ratio of case patients with CM to case patients with SMA was ~2:30, 0.95, and 3.38 to 1 in the Gambian, Kenyan, and Malawian populations, respectively. Control samples were cord blood samples obtained from birth clinics in the same hospital as the case patient samples, and they were thus thought to approximate a random sample of the population, reflecting the true population allele frequency.

Statistical analysis. Genotypic deviations from Hardy-Weinberg equilibrium (HWE) were assessed using a χ² statistical test. Case-control association analysis using SNP alleles/genotypes and haplotypes was undertaken by logistic regression and included the following covariates: ethnic group and HbS polymorphism. Haplotypes were estimated using an expectation-maximization algorithm [33], and score tests [34] were applied to assess the level of evidence of both global and individual haplotype associations with malaria. Family-based association analysis was performed on SNPs and haplotypes by use of the transmission disequilibrium test (TDT) [35]. All analyses were performed using the R statistical package [36].

Table 2. Minor allele frequencies.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Allele</th>
<th>Gambia</th>
<th>Kenya</th>
<th>Malawi</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbS</td>
<td>S</td>
<td>0.075</td>
<td>0.064</td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td>LTA</td>
<td>0.373</td>
<td>0.500</td>
<td>0.506</td>
</tr>
<tr>
<td></td>
<td>TNF</td>
<td>0.064</td>
<td>0.058</td>
<td>0.069</td>
</tr>
</tbody>
</table>

Table 3. Allelic tests of association with severe malaria in case-control studies.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Alleles</th>
<th>The Gambia</th>
<th>Kenya</th>
<th>Malawi</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbS</td>
<td>S vs. A</td>
<td>0.16 (0.12–0.21)</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
</tr>
<tr>
<td></td>
<td>LTA +252</td>
<td>0.95 (0.88–1.02)</td>
<td>0.176</td>
<td>&lt;.001</td>
</tr>
<tr>
<td></td>
<td>LTA +80</td>
<td>0.97 (0.88–1.06)</td>
<td>0.015</td>
<td>&lt;.001</td>
</tr>
<tr>
<td></td>
<td>TNF +851</td>
<td>0.023</td>
<td>0.015</td>
<td>&lt;.001</td>
</tr>
<tr>
<td></td>
<td>TNF −238</td>
<td>0.160</td>
<td>0.128</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

NOTE. HbS, hemoglobin variant S; LTA, lymphotoxin-α gene; TNF, tumor necrosis factor gene.

* Polymorphisms with genotypes that deviate from Hardy-Weinberg equilibrium.
RESULTS

Figure 1 shows the LTA and TNF polymorphisms considered in the Gambian, Kenyan, and Malawian populations. The minor allele frequencies for the HbS, LTA, and TNF polymorphisms are presented in table 2. Only the genotypes for the TNF−1031 and TNF−376 polymorphisms in the Gambian and Malawian controls, respectively, show any evidence of distortion from Hardy-Weinberg equilibrium ($P < .001$). Most heterogeneity in allele frequencies appears in the LTA +80T, LTA +252C, and TNF−1031C alleles, where there are differences between the Gambian and non-Gambian populations. Table 3 shows the results of the tests of allelic association. The HbS S allele has a significant protective effect on SM across all studies ($P < 10^{-8}$). An analysis of HbS genotypes confirmed the protective effect of the heterozygous state with a $\sim 90\%$ reduced risk of SM across all populations. In particular, in The Gambia, Kenya, and Malawi, the odds ratios (ORs) for the AS vs. AA/SS genotypes are 0.09 (95% confidence interval [CI], 0.06–0.13; $P < .001$), 0.16 (95% CI, 0.10–
Table 4. Haplotype association analysis of tumor necrosis factor (TNF) polymorphisms and severe malaria in the case-control studies.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Frequency</th>
<th>The Gambiaa</th>
<th>Frequency</th>
<th>Kenyaa</th>
<th>Frequency</th>
<th>Malawi a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Case patients</td>
<td>OR (95% CI)</td>
<td>P</td>
<td>Controls</td>
<td>Case patients</td>
</tr>
<tr>
<td>AGGG</td>
<td>0.742</td>
<td>0.722</td>
<td>1.00</td>
<td>.019</td>
<td>0.796</td>
<td>0.819</td>
</tr>
<tr>
<td>GGGG</td>
<td>0.045</td>
<td>0.040</td>
<td>0.93 (0.78–1.11)</td>
<td>.232</td>
<td>0.045</td>
<td>0.034</td>
</tr>
<tr>
<td>GAGA</td>
<td>0.022</td>
<td>0.020</td>
<td>0.91 (0.71–1.18)</td>
<td>.413</td>
<td>0.047</td>
<td>0.048</td>
</tr>
<tr>
<td>AGAG</td>
<td>0.148</td>
<td>0.163</td>
<td>1.13 (1.02–1.26)</td>
<td>.025</td>
<td>0.094</td>
<td>0.085</td>
</tr>
<tr>
<td>GGGG</td>
<td>0.043</td>
<td>0.053</td>
<td>1.26 (1.06–1.49)</td>
<td>.013</td>
<td>0.017</td>
<td>0.009</td>
</tr>
</tbody>
</table>

NOTE. CI confidence interval; df, degrees of freedom; OR odds ratio.

- Based on TNF +851, TNF −238, TNF −308, and TNF −376. TNF −1031 was excluded because it deviated from Hardy-Weinberg equilibrium in The Gambia.
- Global statistic, 20.997; df, 6; P = .0018.
- Global statistic, 7.222; df, 6; P = .301.
- Global statistic, 6.117; df, 6; P = .410.

0.27; P < .001), and 0.11 (95% CI, 0.06–0.22; P < .001), respectively. These results were consistent when considering CM and SMA cases separately.

The allelic test for the LTA +252C polymorphism in the Malawian population suggested a marginal protective effect. A genotype analysis (for LTA +252, CC/CT vs. TT) in this population confirmed the marginal protective effect (OR, 0.86; 95% CI, 0.76–0.99; P = .038). There was a similar genotypic effect (for LTA +252, CC vs. TC/TT) in the Gambian population (OR, 0.87; 95% CI, 0.75–1.02), but it did not reach statistical significance (P = .080). There is some evidence to suggest that the TNF −238A allele increases the risk of SM in both the Gambian case-controls (P = .061) and the trios (P = .028) but not in other populations. Genotype analysis (AA/GA vs. GG) in the Gambian case-control study suggested an increase in the risk of SM (OR, 1.21; 95% CI, 1.04–1.42; P = .016). This result was consistent across populations with CM (OR, 1.21; 95% CI, 1.04–1.42; P = .016) and SMA (OR, 1.29; 95% CI, 1.01–1.65; P = .042). From the trio analysis, there is some evidence that the TNF −376A allele leads to disease susceptibility (OR, 1.85; 95% CI, 1.07–3.19; P = .027). However, these results were not replicated in the Gambian case-control study (P = .853), and, in the present study, there was no evidence of genotypic effects (AA/GA vs. GG) across all individuals with SM (OR, 1.05; 95% CI, 0.81–1.38; P = .704) and clinical subphenotypes (for CM, P = .626; for SMA, P = .997). In the Gambian case-control study, allelic-based tests of the TNF −308 polymorphism indicated possible disease susceptibility in association with the A allele (P = .032). This result was not confirmed in the genotype analysis (AA/GA vs. GG) of all individuals with severe disease (OR, 1.10; 95% CI, 0.98–1.23; P = .123) and clinical subphenotypes (for CM, P = .207; for SMA, P = .442).

The pairwise linkage disequilibrium (LD) patterns (using D’ and r² metrics) for the population controls are presented in figure 2. There is a high degree of LD within populations, and its patchy nature is consistent with previous reports [25]. There are differences in the patterns between populations—for example, the LD between LTA polymorphisms is higher in Malawian controls, but, between LTA and TNF polymorphisms, it is comparatively lower in the same population. On the basis of the Gambian results in table 4, we performed a haplotype analysis of the TNF region (TNF +851, TNF −238, TNF −308, and TNF −376) in this population, and we found that there was some evidence of global association (P < .002). In particular, compared with the common AGGG haplotype, both AGAG and GAGG are associated with a 10%–30% increased risk of SM (P < .03). Similar haplotype analyses failed to find strong associations in the Kenyan (P = .301) and Malawian (P = .410) populations (table 3). Analysis of the Gambian trios suggested that the common AGGG haplotype was associated with a reduction in the risk of SM (relative risk [RR], 0.88; 95% CI, 0.77–1.00), whereas the GAGG haplotype was associated with an increase in risk (RR, 1.32; 95% CI, 1.01–1.74) (results not shown). In addition, there was no strong evidence of global association in LTA (LTA +80 and LTA +252) haplotypes and SM (for The Gambia, P = .295; for Kenya, P = .207; and for Malawi, P = .134) (results not shown).

DISCUSSION

We conducted a genetic association study investigating the possible effect of candidate LTA and TNF polymorphisms and the HbS polymorphism on SM in 3 African populations. The HbS polymorphism is under the strongest selective genetic pressures known in the human genome [37], and, as expected, there was a strong heterozygous protective effect on SM across all 3 populations. We found no consistent association signals for the TNF and LTA polymorphisms across all 3 populations. This result coincides with the interstudy discrepancies found in previously reported association studies of the candidate TNF and LTA polymorphisms (table 1). These apparent discrepancies may be the result of a number of study characteristics, including: (1) the...
differences in sample size and the resulting statistical power, (2) phenotypic differences, (3) haplotypic diversity between populations and the effects of population structure, and (4) differences in study design (e.g., village surveys or hospital-based studies). In the present study, we considered some of the largest sample sets collected to date and a standardized protocol with a definition of SM. In addition, we used only cord blood controls and attempted to adjust for the potential effects of population structure by adjusting for ethnicity and location in case-control studies, as well as by using the robust study design including affected child–parent trios.

The TNF and LTA genes have been implicated in the host defense and pathogenesis of SM (reviewed in [38, 39]). Our analyses reveal that there is some evidence that TNF haplotypes (constructed from the SNPs considered) are associated with SM in the Gambian population but not in the closely related populations of Kenya and Malawi. This result may be explained by the occurrence of a selective event in the Gambian population that has not spread to or arisen in East Africa; the effect of such a variant is being detected because it is in LD with the TNF/LTA polymorphisms. Indeed, it has been suggested that the causal polymorphisms regulating the TNF and LTA response may be located some distance downstream of the genes [29, 40].

Furthermore, African populations have a high degree of diversity, and there may be allelic heterogeneity in the TNF/LTA region. Therefore, it is possible that the polymorphisms considered in the present study are not in sufficiently strong LD with any actual causal variants in Kenya and Malawi. Similar interpopulation haplotype heterogeneity has been demonstrated in the G6PD region [41], and there is strong evidence to suggest that the sickle trait arose on different haplotype backgrounds [37]. If the same effects are present for TNF/LTA, it would decrease the power of a study and could account for the inability to detect a clear signal of association in our study and others. This issue of malaria disease association mapping on the background of African genetic variation remains the main challenge facing this field of study. Further dense mapping in the major histocompatibility complex (MHC) class III region in the Gambian and allied populations may be necessary to identify any functional variants of TNF and LTA for SM.

Acknowledgments

We thank the patients from the Gambian, Kenyan, and Malawian malaria study populations, as well as the many investigators involved in the original studies in these populations, for their contributions.

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