TNF -308G>A Single Nucleotide Polymorphism Is Associated With Leprosy Among Brazilians: A Genetic Epidemiology Assessment, Meta-Analysis, and Functional Study

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Leprosy is an infectious disease caused by Mycobacterium leprae. Tumor necrosis factor (TNF) plays a key role in the host response. Some association studies have implicated the single nucleotide polymorphism TNF -308G>A in leprosy susceptibility, but these results are still controversial. We first conducted 4 association studies (2639 individuals) that showed a protective effect of the -308A allele (odds ratio [OR] = 0.77; P = .005). Next, results of a meta-analysis reinforced this association after inclusion of our new data (OR = 0.74; P = .04). Furthermore, a subgroup analysis including only Brazilian studies suggested that the association is specific to this population (OR = 0.63; P = .005). Finally, functional analyses using whole blood cultures showed that patients carrying the -308A allele produced higher TNF levels after lipopolysaccharide (LPS) (6 hours) and M. leprae (3 hours) stimulation. These results reinforce the association between TNF and leprosy and suggest the -308A allele as a marker of disease resistance, especially among Brazilians.

Leprosy is a human infectious disease that affects >200 000 individuals per year worldwide [1]. It is caused by Mycobacterium leprae, an obligate intracellular bacterium with a unique tropism for macrophages in skin and Schwann cells in peripheral nerves [2]. Although M. leprae is the primary cause of the disease, it is well known that leprosy outcome depends on additional risk factors related either to the host or to the environment, in a way that only about 1%-3% of the exposed individuals develop clinical disease [3]. Furthermore, the recently described low variability of M. leprae [4] contrasts with the wide range of susceptibilities from the successful blockage of M. leprae uptake to a clinical form exhibiting high bacillary loads, suggesting that disease variation could be mainly attributed to the genetic diversity of the host.

Leprosy has been extensively studied among twins and families, which have confirmed the genetic component in leprosy [5–7]. Subsequent association and linkage studies have implicated several genes such as HLADRBI, PARK2/PACRG, IL10 and LTA and chromosomal regions such as 10p13 in the susceptibility to leprosy per
Meanwhile, genes coding for ninjurin and toll-like receptors were implicated with disease phenotypes such as neuritis and leprosy reactions [11, 12]. Most of the genes consistently associated with leprosy per se until now are clustered in the 6p21 region [3, 13, 14]. The importance of the TNF locus in leprosy susceptibility has been demonstrated both by family or population-based studies [3] and afterwards, genomewide association (GWA) studies confirmed the previously reported associations of the human leukocyte antigen (HLA)/tumor necrosis factor (TNF) genes in the Chinese [15] and Indian populations [16].

The TNF/lymphotoxin-alpha (LTA) genes are juxtaposed within class III HLA and code for the cytokines TNF and LTA, which were clearly implicated in immunity against M. leprae. A recent study using knockout mice showed that these cytokines have complementary roles in the granuloma formation and maintenance. More specifically, LTA seems to regulate granuloma formation, whereas TNF is responsible for its integrity [17]. In a Mycobacterium marinum granuloma model, TNF abrogation caused increased mortality either when innate or adaptive immunity is at work [18].

The single nucleotide polymorphism (SNP) located at the -308 site of the TNF promoter region (TNF -308G>A), has been the most common target of the association studies. Nevertheless, these results are controversial, and the association between this SNP and leprosy still needs further investigation [3]. This study provides new evidence of an association between TNF -308A and protection against leprosy, especially among Brazilians. Functional studies also showed that the -308A carriers produced higher TNF levels in cultures stimulated with M. leprae or lipo-polysaccharide (LPS).

**MATERIALS AND METHODS**

**Participants and Study Design**

We conducted 4 association studies between the SNP TNF -308G>A (rs1800629) and leprosy susceptibility using both family-based and case-control study designs. The family-based studies included initially a total of 65 nuclear families (195 individuals) recruited from Duque de Caxias, a hyperendemic municipality of the Rio de Janeiro state (RIO), located in the southeastern region of Brazil. Then, an additional 42 pedigrees (comprising 54 nuclear families, 168 individuals) were recruited from the Prata Colony, located in the Pará state in the northern region of Brazil. For the case-control analyses, 2 independent populations from the southeastern region of Brazil were selected. The first study was an extension of the case-control study reported by Santos and colleagues (2002) and included a total of 778 leprosy cases diagnosed and treated at the Souza Araújo Clinic at Fiocruz, also located in RIO. The control group comprised 661 unrelated individuals selected among blood donors who lived in the same endemic area as the cases. The second case-control study included 368 cases selected from an outpatient unit at Lauro de Souza Lima Institute, Bauru, located in São Paulo state. The control population (375 individuals) was selected following the same criteria described for the RIO study. All studies were conducted concomitantly. Leprosy diagnosis was determined by experienced professionals according to Ridley and Jopling classification [19]. The patients were also classified as paucibacillary (PB) or multibacillary (MB) and treated according to the World Health Organization (WHO) specifications. All individuals were ethnically classified after careful inspection of facial morphological features, hair type, and skin color of the individual and his/her family. Written informed consent was obtained from all individuals included in this project, as approved by the local ethics committees. General characteristics of the populations are described in Table 1. All samples were genotyped for the SNP TNF -308G>A and the control populations were also genotyped for LTA +80C>A and LTA +252A>G to define the linkage disequilibrium patterns. All analyses were performed using leprosy per se as outcome regardless the clinical form. Results of the 4 studies were represented independently and combined in an overall analysis.

**SNP Genotyping**

DNA extraction was performed by the “salting-out” method. The TNF -308G>A SNP was genotyped by real-time PCR using a TaqMan “Assay by Design” (Applied Biosystems) according to the manufacturer’s instructions. Amplifications were performed in a final volume of 5 μL containing 2.5 μL of the TaqMan Universal Master Mix (Applied Biosystems), 0.125 μL of the TaqMan mix (primers and probes), and 10–50 ng of template. The forward (F) and reverse (R) primers used for amplification were 5’-CCA AAA GAA ATG GAG GCA ATA GGT T-3’ and 5’-GGA CCC TGG AGG CTG AAC-3’, respectively. Allelic discrimination was enabled by the use of the TaqMan probes 5’-CCC GTC CCC ATG GG-3’ (-308G, labeled with VIC dye), and 5’-CCC GTC CTC ATG CC-3’ (-308A, labeled with 6-carboxy-fluorescein [FAM dye]). Polymerase chain reactions (PCRs) were performed in the ABI Prism 7000 and 7500 Sequence Detection Systems using the standard cycling conditions (Applied Biosystems). The SNPs at LTA +80 and +252 sites were genotyped by PCR–restriction fragment length polymorphism (RFLP). PCR reactions were conducted in a final volume of 25 μL containing 1 U of Taq DNA polymerase (Invitrogen) in 1X of the supplied buffer, 0.3 μM of each primer, 0.2 mM of deoxyribonucleotide triphosphates (dNTPs), 1.5 mM of magnesium chloride, and 50–100 ng of genomic DNA. The primers used for the amplification were 5’-AAG GTG AGC AGA GGG AGA CA-3’ (LTA+80F), 5’-GCT TCG TGC TTT GGA CTA CC-3’ (LTA+80R), 5’-CCG TGC TTC GTG TTT GGA CTA CC-3’ (LTA+252F), and 5’-AGA GGG GTG GAT GCT TGG GGT C-3’ (LTA+252R). The cycling conditions were: 94°C (3 minutes) followed by 35 cycles of 94°C (45 seconds); 62°C for LTA +252...
or 53°C LTA +80 (45 seconds) and 72°C (1 minute) and 72°C (5 minutes). PCR products were digested overnight with 3 U of the restriction enzymes BseYI and NcoI (New England Biolabs), which cleaves the +80A and +252G alleles, respectively. SNP genotyping was performed by restriction fragment analysis in a 3.5% agarose gel.

**Literature Review and Identification of Eligible Studies for the Meta-Analysis**

We searched for studies that examined the association of the SNP TNF -308G>A with susceptibility to leprosy using both case–control and family-based association analyses. A literature search was made in Medline using PubMed citations to identify all available articles up to July 2010. Related articles to the selected papers (using PubMed search) and references in the studies were also reviewed to identify additional studies. The keywords “TNF,” “polymorphism,” “SNP,” and “leprosy” were entered as text words. No language restrictions were applied. Inclusion and exclusion criteria were applied as described by Pacheco et al [20], with some modifications to allow the inclusion of family-based studies. A study was included in the analysis if (1) it was published up to July 2010, (2) it was not related to previous publications, and (3) it provided enough data (total counts of each genotype in the case–control studies and transmissions of each allele in the family-based designs) to determine odds ratios (ORs). We excluded (1) studies that contained overlapping data, (2) studies in which the number of genotypes or transmissions could not be ascertained, and (3) case–control studies in which the control group deviated from the Hardy–Weinberg Equilibrium (HWE).

**Functional Analyses of TNF Secretion According to -308G>A Genotypes**

We included a total of 30 borderline tuberculoid (BT) patients in the functional analysis. Whole blood was diluted 1:4 and 1 mL was plated in 24-well plates in duplicates. We stimulated the cultures with LPS (1 ng/mL) and whole irradiated *M. leprae* (1 µg/mL) kindly provided by Dr Patrick Brennan (Colorado State University). We incubated cultures for 1 hour, 3 hours, 6 hours, 12 hours, and 20 hours. Then, we transferred cellular suspensions to eppendorf tubes and harvested them by centrifugation at 1500 rpm for 10 minutes at 4°C. We stored cell-free supernatants at −20°C until further use. We determined the TNF concentration in culture supernatants using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) according to manufacturer specifications.

**Statistical Analyses**

**Case–Control Study.** We tested deviations from HWE with chi-square tests. Frequencies of each genotype, allele, and carriers of the minor allele in cases and controls were compared using unconditional logistic regression models controlling for gender, ethnicity, and age. We measured linkage disequilibrium by the *r*² statistics [21]. We performed all analyses using the statistical software R for Windows version 2.9.2 [22] with the “genetics” and “haplo.stats” packages.

**Family-Based Studies.** Association with leprosy in the family-based design was assessed by the transmission-disequilibrium test (DTD), which considers the transmission of a marker allele from heterozygous parents to the affected offspring. We tested the transmission probability of each allele to determine the deviation from the expected frequency of 50%. We used sibling pairs to estimate parental genotypes when these...
RESULTS

Case–Control Studies

Results of the family-based analyses are shown in Table 2. No association was found when the frequencies of the alleles transmitted and nontransmitted to the affected individuals were compared by TDT in the RIO population (P = .55).

<table>
<thead>
<tr>
<th>Population</th>
<th>Allele</th>
<th>Frequency</th>
<th>Transmitted</th>
<th>Nontransmitted</th>
<th>Z (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIO</td>
<td>A</td>
<td>0.12</td>
<td>19</td>
<td>20</td>
<td>-0.6 (.55)</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>0.88</td>
<td>20</td>
<td>19</td>
<td>0.6 (.55)</td>
</tr>
<tr>
<td>Prata Colony</td>
<td>A</td>
<td>0.07</td>
<td>5</td>
<td>14</td>
<td>-2.065 (.04)</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>0.93</td>
<td>14</td>
<td>5</td>
<td>2.065 (.04)</td>
</tr>
</tbody>
</table>

Abbreviations: RIO, Rio de Janeiro, Brazil; SD, standard deviation; WHO, World Health Organization.

* Results of the transmission-dis-equilibrium tests (Z score and P value) are shown for the additive model.

Nevertheless, results from the Prata Colony demonstrated an association between -308A and protection against leprosy (5 transmitted vs 14 nontransmitted; P = .04). Similar results were observed in the case–control studies (Table 3). In the RIO population, the OR values suggested a protective effect for -308A in all comparisons performed, especially for the -308A carriers (OR = 0.75; P = .02). However, these results did not reach statistical significance after adjustment for nongenetic covariates. In the particular case of the Bauru case–control, despite the higher frequency of the -308A allele in the control population and the OR values suggesting protection, results were not statistically significant either before or after adjustment. On the other hand, when the data of the 4 studies were analyzed together using a meta-analysis model, the statistical power was increased and the association between the -308A allele and protection against leprosy was clearly observed, with a pooled OR of 0.77 (95% CI, .65–.92; P = .005).

Systematic Review and Meta-Analysis

We found a total of 9 studies in which the association between the TNF -308G>A SNP and leprosy susceptibility was analyzed. From these, 7 were population-based studies (case–control) conducted among Indians [25], Brazilians [26–28], Malawians [29, Thais [30], and Nepalese [31] and 2 studies used the family-based design [32, 33] to define the association between this SNP and leprosy in Brazilian families. Because the previous data of our group [26, 27] were partially overlapped, the study by Santos et al 2000 [26] was excluded. The Indian TDT study [33] was also excluded because the crude data were not available. All other studies were considered eligible for meta-analysis according to our established criteria, and results of the 7 selected studies are summarized in Supplementary Table 1. We detected no statistical evidence of publication bias when we applied the Egger test for funnel plot asymmetry (P = .28).

Results of the meta-analysis are summarized in Table 4 and Figure 1. Pooled risk estimates were defined using the random-effects model because of the statistically significant evidence of heterogeneity across studies. As observed, although the risk estimates suggested a protective effect for the -308A allele, the results were not statistically significant (pooled OR = 0.72; P = .16; Figure 1A). Next, the meta-analysis was repeated including data from the 4 additional studies reported here. Because this case–control study of the RIO population was designed as an extension of the 2002 study by Santos and colleagues [27], the latter study [27] had to be excluded to avoid the analysis of overlapping data. Results of this meta-analysis confirmed the protective effect of the -308A allele (Figure 1B), with statistically significant OR values showing an association of the -308A allele with protection against leprosy (OR = 0.55; P = .04). When a subgroup analysis was conducted using only studies of the Brazilian population (Table 4), a significant association between the -308A allele and protection against leprosy...
was observed either before (pooled OR = 0.44; P = .009; Figure 1C) or after inclusion of the present data (OR = 0.63; P = .005; Figure 1D).

Results of the pairwise linkage disequilibrium analysis conducted in the control groups of Bauru and RIO populations indicated that the SNPs TNF -308, LTA +80 and LTA +252 belong to different bins (r² < 0.5 in all pairwise comparisons). Similar results were found in the family-based designs. Furthermore, results of multivariate logistic regression models including LTA +80 and TNF -308 also indicated that these SNPs have independent effects (data not shown).

Finally, we tested the functionality of TNF -308G>A SNP to affect TNF secretion in vitro. We stimulated whole blood samples from BT patients and found that the -308A carriers produced higher TNF levels in cultures stimulated with either LPS at 6 hours (3982 ± 717 pg/mL vs 2053 ± 310 pg/mL; P = .02; Figure 2A) or M. leprae at 3 hours (544 ± 67 pg/mL vs 357 ± 53 pg/mL; P = .04; Figure 2B).

**DISCUSSION**

TNF exhibits a broad range of actions participating either in the resistance or in the immunopathology observed in leprosy. Whereas a high TNF:IL-10 ratio was shown to be higher among resistant household contacts, supporting a role for TNF in the resistance to *M. leprae* [34], exacerbated TNF levels were also correlated with inflammatory reactions and neuropathy along the natural course of the disease [35, 36].

Linkage studies have strongly suggested the genetic influence of the 6p21 region, especially the TNF/LTA locus in leprosy susceptibility [32, 37]. Despite the conflicting results obtained from candidate-gene approaches, results of the 2 GWA studies performed for leprosy [15, 16] showed that the HLA/TNF region provided the highest number of associated SNPs in both screenings. Nevertheless, no information about the TNF -308G>A SNP (rs1800629) was available in these studies.

The main objective of this paper was to clarify the ambiguity and describe a definitive role for the SNP TNF -308G>A in leprosy outcome. For this purpose, we have developed 4 independent association studies using not only different populations but also different study designs as a way to validate our results. Case–control studies frequently have higher statistical power compared with that of family-based studies simply because it is easier to collect samples from unrelated individuals and because a family is not informative for the TDT test unless the parents are heterozygotes. This latter issue is particularly challenging when the SNP under investigation has a low frequency, as does -308G>A. On the other hand, the results of

### Table 3. Case-Control Analyses of the Single Nucleotide Polymorphism (SNP) TNF -308G>A in Rio de Janeiro and Bauru Populations

<table>
<thead>
<tr>
<th>Allele</th>
<th>Cases, total counts (frequency)</th>
<th>Controls, total counts (frequency)</th>
<th>OR (P value)</th>
<th>OR (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>13 (0.02)</td>
<td>12 (0.02)</td>
<td>0.86 (.72)</td>
<td>0.53 (.22)</td>
</tr>
<tr>
<td>AG</td>
<td>137 (0.18)</td>
<td>148 (0.22)</td>
<td>0.74 (.02)</td>
<td>0.78 (.15)</td>
</tr>
<tr>
<td>GG</td>
<td>628 (0.81)</td>
<td>501 (0.76)</td>
<td>reference</td>
<td>reference</td>
</tr>
<tr>
<td>Allele A</td>
<td>778 (0.10)</td>
<td>661 (0.12)</td>
<td>(P = .07)b</td>
<td>(P = .20)b</td>
</tr>
<tr>
<td>Allele G</td>
<td>163 (0.10)</td>
<td>172 (0.13)</td>
<td>0.78 (.14)</td>
<td>0.76 (.19)</td>
</tr>
<tr>
<td>A carriers</td>
<td>150 (0.19)</td>
<td>160 (0.24)</td>
<td>0.75 (.02)</td>
<td>0.76 (.09)</td>
</tr>
</tbody>
</table>

**Abbreviations:** OR, odds ratio; RIO, Rio de Janeiro, Brazil.

a OR and P value calculated by logistic regression models and adjusted for the covariates gender, ethnicity and age.

b Overall P values for genotype comparisons (2 degrees of freedom).

### Table 4. Summary of the Results of the Meta-Analyses and Subgroup Analyses

<table>
<thead>
<tr>
<th>Studies included in the analysisa</th>
<th>Meta-analysis</th>
<th>Heterogeneity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pooled OR</td>
<td>95% Cl</td>
</tr>
<tr>
<td>Published studies</td>
<td>0.72</td>
<td>.46–1.14</td>
</tr>
<tr>
<td>Published studies and present data</td>
<td>0.74</td>
<td>.55–.99</td>
</tr>
<tr>
<td>Brazilian studies (only published)</td>
<td>0.44</td>
<td>.38–.71</td>
</tr>
<tr>
<td>Brazilian studies including present data</td>
<td>0.63</td>
<td>.45–.87</td>
</tr>
</tbody>
</table>

Results are shown for the random-effects model. n = 7, 10, 3, and 6 studies and ~3599, 5846, 1172, and 3419 individuals, respectively. Abbreviations: Cl, confidence interval; OR, odds ratio

a When data from this study were included, the study by Santos et al [27] was excluded to avoid the inclusion of overlapping data.
a case–control study are more prone to selection bias, especially because the definition of the control population is not always clear. Thus, as a rule, when the same effect is observed in both case–control and family-based designs, this concordance improves the reliability of the results.

Although the association of -308A allele with leprosy resistance was more prominent in the Prata Colony, the estimates showed a protective effect for the allele in almost all samples analyzed. The only exception was the TDT analysis from RIO, in which no association was observed. Despite this discrepant result, the risk estimates obtained from an overall analysis of the 4 studies indicated a clear protective effect for -308A. Then, a meta-analysis was conducted to combine all published data (n=3599). After the inclusion of the 2639 individuals from our case–control and family-based studies (n=5843 after exclusion of the data by Santos [27]), results of the meta-analysis

![Figure 1](image1)

**Figure 1.** Meta-analysis of the studies in which the association between the single nucleotide polymorphism TNF-308G>A and leprosy susceptibility was assessed. First, a meta-analysis was performed including all published data (A) and the present data (B). Then, a subgroup analysis was conducted including the Brazilian studies available in the literature either alone (C) or combined with the present data (D). Results are shown for the random-effects model. Bars represent 95% confidence interval and boxes represent the odds ratio values. The size of each box indicates the weight of the study in the pooled results. CI, confidence interval; Fb, family-based; OR, odds ratio; Pb, population-based.

![Figure 2](image2)

**Figure 2.** Tumor necrosis factor (TNF) production by whole blood cells (WB) in -308A carriers and noncarriers. WB was cultured for 3–20 hours with: lipopolysaccharide (LPS) from *Escherichia coli* (1 ng/mL) (A) or, sonicated Mycobacterium leprae antigen (1 µg/mL) (B). Results are represented as the TNF concentration in pg/mL. Bars represent the mean value plus standard error of each group. TNF-308A carriers (G/A + A/A, white bars, n=14) and noncarriers (G/G, black bars, n=16) were tested. Statistically significant differences were observed 6 hours after LPS stimulation and 3 hours after *M. leprae* stimulation. *P < .05 according to the Welch Two Sample t test.
confirmed the protective effect, with a P value of .04. Nevertheless, most Brazilian studies provided OR values pointing toward protection in the meta-analysis, suggesting a population-specific effect. Thus, we conducted a subgroup analysis including only Brazilian participants and found a clear protective effect of the -308A allele before and after inclusion of the present data. In our experience meta-analysis is a very important tool to decrease ambiguity of underpowered studies [20, 38].

We recently demonstrated that the IFNG +874T allele is important to leprosy resistance [39], and now we show that TNF-308A also has a protective effect. Although there are different genes associated with leprosy outcome, it seems that they are clustered in overlapping pathways (TNF/LTA or IFN-γ/IL-12 axis) indicating that protective responses to different infectious diseases share the same evolutionary strategies.

A possible explanation for the -308A association with leprosy could be the LD with another causative SNP. Indeed, the variation LTA +80C>A was associated with early-onset leprosy in different populations [40]. Nevertheless, our LD analyses showed that the TNF and LTA SNPs are not in the same bin, indicating that the -308G>A association with leprosy is independent.

In tuberculosis it is suggested that children and adults exhibit different diseases. In this scenario, a different set of SNPs in genes of the same pathways could be playing different roles in each disease outcome [41]. The age-specific effect of LTA +80A associated with leprosy was not detected among Brazilians [40] probably because most of case-control studies in Brazil recruit few young individuals. In fact, a review of the studies used in our meta-analysis showed that the mean age of participants among Brazilian studies was around 40 years old. Thus, assuming an age-dependent model similar to the observed in tuberculosis, if the age indeed influences the effect of the SNPs at TNF/LTA locus, the more prominent effect observed among Brazilians could be attributed to the mean age of the populations under analysis. In this case, the TNF-308A SNP might be a marker of leprosy resistance among adults whereas LTA +80A exerts its effect among children.

The functional analyses using whole blood cells from BT patients showed higher TNF levels among -308A carriers after M. leprae and LPS stimulus. Some reports have associated the -308A allele with higher TNF releases, whereas other studies indicated the -308A allele had no effect [3, 42–44]. Nevertheless, it has been suggested that TNF-308A may affect the binding of transcription factors [44–47]. Despite the clear limitations of the functional studies using single TNF promoter SNPs, especially considering the LD between TNF, LTA and HLA alleles, it is clear that the -308A allele is a marker for higher levels of TNF secretion in some in vitro assays such as ours. Moreover, we had previously demonstrated that TNF-308A carriers had a stronger inflammatory response to M. leprae antigens in the skin [48]. Thus, it is likely that a genetic variation slightly increasing TNF levels could be advantageous by maintaining granulomas and restricting M. leprae growth [49].

The genetic and epidemiological results presented here indicate consistent protective effect of the -308A allele in leprosy, denoted by an association with resistance and increased production among patients. Despite the clear evidence of a genetic heterogeneity component in leprosy susceptibility, our data showed an evident role of TNF variations in leprosy outcome.

### Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://www.oxfordjournals.org/our_journals/jid/).

Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

### Notes

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**Potential conflicts of interest.** All authors: No reported conflicts.

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