Genetic and Immunological Evidence Implicates Interleukin 6 as a Susceptibility Gene for Leprosy Type 2 Reaction

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In leprosy, type 1 reaction (T1R) and type 2 reaction (T2R) are major causes of nerve injury and permanent disabilities. A previous study on plasma levels of 27 cytokines in patients with T1R or T2R and controls with nonreactional leprosy identified the gene for interleukin 6 (IL-6) as a candidate for genetic analysis. Two nested case-control studies were built from a cohort of 409 patients with leprosy from central Brazil, monitored for T1R and T2R. There was evidence for association between T2R and IL-6 tag single-nucleotide polymorphisms rs2069832 ($P = 0.002$), rs2069840 ($P = 0.03$), and rs2069845 ($P = 0.04$), with information on the entire IL-6 locus, as well as functional IL-6 variant rs1800795 ($P = 0.005$). Moreover, IL-6 plasma levels in patients with T2R correlated with IL-6 genotypes ($P = 0.04$). No association was found between IL-6 variants and T1R. Identifying genetic predictive factors for leprosy reactions may have a major impact on preventive strategies.

Leprosy is a chronic, dermatoneurological, disabling disease caused by Mycobacterium leprae that, in 2009, affected approximately 245 000 individuals worldwide. Prevalence of the disease has remained stable since the mid-1990s despite global implementation of efficient multidrug therapy (MDT) [1]. Leprosy presents as a broad clinical, bacilloscopic, histopathological, and immunological spectrum, ranging from the localized tuberculoid (TT) form, associated with a granulomatous T-helper 1 (Th1)–type immune response, to systemic T-helper 2 (Th2)–type lepromatous (LL) disease. Immunologically unstable borderline forms (borderline tuberculoid [BT], borderline borderline [BB], and borderline lepromatous [BL]) lie between the poles [2].

Leprosy reactions are immune inflammatory–related phenotypes that may occur before diagnosis, during treatment, or after MDT. Leprosy reactions require immediate treatment to prevent permanent nerve impairment, motor disability, and deformity. Cohort studies estimate that disability in leprosy ranges from 16% to 56% [3], mainly owing to the occurrence of reactional episodes. Because leprosy reactions may occur months or even years after MDT completion, related disabilities are expected to continue to occur even under the unlikely scenario of leprosy eradication.

There are 2 major types of leprosy reactions. Type 1 reaction (T1R) affects 20%–30% of patients [4] and is characterized by acute inflammation of preexisting skin lesions or by the appearance of new lesions and/or neuritis. Systemic symptoms are rare. Type 1 reaction occurs predominantly among patients with leprosy presenting unstable immune response against the bacilli (BT, BB, BL forms) [5] or in patients with TT leprosy [6, 7]. Approximately 95% of T1R cases are diagnosed simultaneously with leprosy or during the first 2 years of MDT [8]. Even with adequate treatment, 40% of
patients with T1R may present permanent nerve damage [9]. Erythema nodosum leprosum is the main presentation of type 2 reaction (T2R), which predominantly affects patients with leprosy showing Th2 immune response and high bacterial load, which is therefore classified toward the lepromatous pole (BB, BL, and LL) of the disease. Most T2Rs occur during MDT [10], and the majority of patients usually experience several acute or chronic episodes over the years. Type 2 reaction presents as disseminated painful erythematous tender nodules with or without neuritis, usually followed by systemic symptoms such as fever and malaise. Type 2 reaction is characterized by increased levels of tumor necrosis factor α and immune complex–associated vasculitis, panniculitis, and uveitis. Treatment for T1Rs is based on corticosteroids, and T2R therapy includes corticosteroids and/or thalidomide [5].

Genomic sequence studies of M. leprae strains of worldwide distribution have shown very low genetic diversity, suggesting that differences in susceptibility to disease and to clinical manifestations, including reactional episodes, are influenced by the host defense genes [11]. Only recently genetic epidemiology studies have identified predictive susceptibility factors for leprosy reactions. Most of these studies focused on variants on genes for Toll-like receptors 1 and 2 (TLR1, TLR2), important mediators of mycobacterium recognition during innate immune response. Alleles of both the single-nucleotide polymorphism (SNP) rs3804099 and a microsatellite intragenic to TLR2 were associated with T1R in an Ethiopian population [12]. Nonsynonymous SNP rs5743618 (I602S) of TLR1 was found to be associated with protection against T1R in a Nepali population [13]. The same variation was subsequently associated with leprosy per se in Indian and Turkish populations [14]. Another nonsynonymous polymorphism of TLR1 (N248S) was associated with leprosy per se and T2R, with the S and the N alleles more frequent among all patients with leprosy and patients with T2R, respectively [15]. Finally, the first genome-wide association study in leprosy revealed 4 SNPs intragenic to the NOD2 gene associated with leprosy [16]. A subsequent study showed variants of the same gene associated with leprosy per se, T1R, and T2R [17]. However, associated variants were not the same in the 2 studies.

A recent analysis of 27 plasma factors, including cytokines, chemokines, and growth factors, by our group revealed interleukin 6 (IL-6) as the only biomarker of both T1R and T2R when compared with leprosy-affected individuals without reaction [18]. Previous studies have found higher IL-6 levels associated with LL leprosy, which is associated with occurrence of T2R [19–21]. A constitutive gene expression analysis using nonstimulated peripheral blood mononuclear cells from patients with reactional or nonreactional leprosy revealed that IL-6 was expressed in most of the patients with T1R or T2R. Among individuals with nonreactional leprosy, IL-6 messenger RNA was detected only in patients with TT or BT leprosy.

When skin biopsy specimens were analyzed, expression of IL-6 was detected in most lesions of both T2R and T1R patients [19]. Nevertheless, it is known that messenger RNA expression and the actual level of the secreted protein may be different in vivo. The production of IL-6 by peripheral blood mononuclear cells in response to anti-CD3 T-cell polyclonal stimulant was observed in a significantly higher proportion of patients with LL leprosy, compared with TT leprosy [20]. In a comparative study, lepromatous patients had the highest serum levels of IL-6, and patients with T2R had higher levels of IL-6 than those with nonreactional leprosy [21]. As a continuation of our previous study [18], here we present the results of a follow-up, prospective, nested, case-control study of association between leprosy reactions and tag polymorphisms of the IL-6 gene in an expanded population sample of affected individuals recruited at the same Brazilian reference center for leprosy diagnosis and treatment.

MATERIALS AND METHODS

Population Sample

A cohort of patients with leprosy was recruited at the time of leprosy diagnosis (newly detected cases) at a public health reference center (Centro de Referencia en Diagnostico e Terapeutica) located in Goiania city, central western Brazil, between February 2006 and March 2008. Leprosy diagnosis/classification was defined after detailed dermatoneurological examination by a clinician with expertise in leprosy diagnosis, bacilloscopy, and histopathology of skin lesion biopsy specimens, according to Ridley-Jopling criteria [2]. Case definitions of leprosy, T1R, and T2R have been described elsewhere [21]. Inclusion criteria were newly detected patients, without history of previous leprosy treatment, presenting or not presenting leprosy reactions (T1R or T2R). Patients with leprosy were closely followed up for reactional episodes during MDT (6 months for paucibacillary patients and 12 months for multibacillary patients). On completion of MDT, patients were strongly advised to return to the reference center if any sign or symptom of reaction occurred. Overall, patients have been followed up for ≥2 years since diagnosis; leprosy reactions were observed at diagnosis, during MDT, and after MDT. For patients who came for diagnosis during a reactional episode, blood was collected before the start of MDT and antileprosy reaction therapy. For patients with leprosy who developed reactions during MDT, blood was collected during MDT. For patients who had a reactional episode after completion of MDT, by the time blood was collected patients were taking neither MDT nor any other specific drug therapy for leprosy reaction.

Exclusion criteria for the genetic analysis were uncertain classification of leprosy or type of leprosy reaction, clinical classification inconsistent with the type of reaction, and clinical signs and symptoms compatible with pure neuritis. Two nested
case-control studies were built from this cohort: the first included patients with leprosy at their first episode of T1R, and the second included patients with leprosy at their first episode of T2R.

Controls were patients with newly detected leprosy who did not have reaction at the time of initial diagnosis or during follow-up. Patients with T1R were compared with controls presenting with polar TT or intermediary BT, BB, or BL leprosy. Patients with T2R were compared with controls with polar LL or BL and BB disease. All patients were treated for leprosy according to World Health Organization MDT guidelines and for leprosy reaction with the appropriate therapy. Demographic and clinical variables were compared using χ² and t tests; differences were considered significant at an α error of <.05 (P value). This study was approved by the Brazilian National Committee for Ethics in Research (CONEP) (protocol 650/2006/12638). All patients signed an informed consent; for patients <18 years old, the informed consent was signed by one of the parents or the legal guardian.

Marker Selection
Tag SNP markers capturing the entire information of the IL-6 locus were selected according to the information available at the International HapMap Project website (release 24/phase 2, Nov08). All selected markers presented a minor allele frequency of 0.2 or higher in the CEU HapMap population (northern or western European ancestry). Two markers were considered in linkage disequilibrium (LD) when presenting a pairwise r² > 0.8. Following these criteria, tag SNPs rs2069832, rs2069840, and rs2069845 were selected for genotyping.

DNA Extraction and Genotyping
Genomic DNA was obtained from peripheral blood by classic salting-out protocol [22] and was then purified and quantified. Tag SNPs rs2069832, rs2069840, and rs2069845 were genotyped with a polarized fluorescence, Taqman probe–based method [23], using the ABI 7500 platform. Genotypes were automatically assigned using the appropriate software with subsequent manual checking of accuracy. Functional SNP rs1800795 was genotyped by restriction fragment length polymorphism analysis, as described elsewhere [24].

Plasma IL-6 Levels
Plasma levels of IL-6 were measured for all patients with leprosy for whom a biological sample was available. The IL-6 levels were determined by enzyme-linked immunosorbent assay (eBioscience Human IL-6 kit) according to the manufacturer’s instructions, using undiluted samples. An exploratory stem-and-leaf test was carried out to identify outliers.

Statistical Analysis
Hardy-Weinberg equilibrium estimates were carried out using Haplview 4.2 software [25]. Genotyping frequencies for cases and controls were compared by univariate analysis under both dominant and additive model using χ² test or Student’s t tests when appropriate. Adjustment for sex, clinical form, and age was performed using stepwise multivariate logistic regression analysis. The P value threshold for rejection of the null hypothesis was fixed at .05; for comparisons that reached statistical significance, odds ratios were estimated with the appropriate test. The Mann–Whitney test was used to compare IL-6 plasma levels across controls and patients with leprosy reactions as well as across genotypes under a dominant model. Analyses were performed with the statistical software SPSS 13.0.

RESULTS
A total of 429 leprosy-affected individuals were recruited for the study. Based on exclusion criteria, 20 individuals were removed from the analysis: 10 with pure neuritis and 10 for inconsistencies or uncertain diagnosis of either leprosy and/or type of reaction. During the 2 years of follow-up since diagnosis, 37.7% of patients with leprosy (154 of 409) developed T1R, 9.5% (39 of 409) developed T2R, and the remaining 52.8% (216 of 409) did not present any sign of reaction. Regarding T1R, 8.5% of patients (13 of 154) came for diagnosis during the first T1R episode, 56.5% (87 of 154) presented the first TR episode during MDT, and the remaining 36% (54 of 154) developed T1R after completion of MDT, at a median of 3 months (range, 1–14) after MDT. Among patients with T2R, 10.2% of patients (4 of 39) came for diagnosis during the first reactional episode, 63.8% (25 of 39) presented T2R during MDT, and 26% (10 of 39) presented T2R after MDT, at a median of 4 months (range, 1–13) after MDT.

For the genetic analysis, the T1R subgroup was compared with 188 controls with nonreactional leprosy presenting with TT, BT, BB, or BL leprosy; the T2R subgroup was compared with 70 controls with nonreactional leprosy presenting BB, BL, or LL leprosy. Comparison between patients with T1R and controls revealed statistically significant differences in the distribution of the leprosy classification (P = 8.1 × 10⁻⁵), sex (P = .04) and age (P = 1.3 × 10⁻⁵). Type 1 reaction was more frequent among individuals with BT leprosy (51.3% of patients) and male patients (61.7%); the mean age for patients with T1R was higher (51.0 ± 15.2 years) than for controls (41.7 ± 16.6 years) (P = 1.3 × 10⁻⁵). For the T2R subgroup, the only difference observed between patients and controls was related to the leprosy classification (P = .005): the LL classification was more frequent among patients with T2R (71.8%) than among controls (40%). Description and analysis of the Ridley-Jopling classification and epidemiological data from T1R and T2R controls are summarized in Table 1.

To replicate the association between IL-6 and occurrence of leprosy reactions observed previously in a smaller subsample of the studied population [18], we compared plasma levels of IL-6 among 16 reaction-free, leprosy-affected control...
individuals, 33 patients with T2R, and 54 patients of T1R from
our expanded data set (Figure 1). Results confirmed increased
levels of IL-6 among patients with both T1R ($P = 5.009$) and
T2R ($P = 2.4 \times 10^{-8}$) when compared with reaction-free
leprosy controls. Interestingly, the magnitude of the effect was
much more pronounced for T2R than for T1R ($P = 1.0 \times 10^{-8}$).

All genetic markers tested were in Hardy-Weinberg equilib-
rium. Genetic analyses failed to detect association between
IL-6 variants and the occurrence of T1R. No association was
observed even after the removal of the 22 controls with TT
leprosy from this subpopulation (data not shown), because
these patients present a relatively stable immune response and
are less susceptible to developing T1R. Positive evidence for
association between all 3 tag SNPs and occurrence of T2R
(Table 2) was observed for both dominant and additive
models after correction for leprosy clinical form. Linkage
disequilibrium analysis confirmed independence of all 3 SNPs
(Figure 2).

A comprehensive search on the HapMap database (build
24/phase 2_Nov08) revealed that SNP rs2069832 was tagging
a bin that contained variant rs1800795, a known, functional
polymorphism initially localized at a negative regulatory domain
of the promoter region of IL-6 [26]. Therefore, SNP rs1800795
was genotyped exclusively in the T2R subpopulation. Analysis
confirmed strong LD between rs1800795 and rs2069832 in our
population sample ($r^2 = 0.69$) (Figure 1). Moreover, association
between rs1800795 functional variant of IL-6 and occurrence
of T2R was also observed. Odds ratios were estimated at 4.0
(95% confidence interval [CI], 1.64–9.76) and 3.7 (95% CI, 1.47–9.34)
for risk alleles A and C of markers rs2069832
and rs1800795, respectively. Allele G of marker rs2069840
showed very weak LD with both rs2069832 and rs1800795
SNPs and was associated with protection against T2R (odds
ratio, 0.27; 95% CI, .22–.91).

Multivariate analysis including all 4 SNPs confirmed in-
dependent association of 2 bins: the first captured by tag SNP
rs2069832 and including markers rs2069845 and rs1800795
($P = .01$) and the second an rs2069840 singleton, the latter
with borderline significance ($P = .06$). Haplotypic analysis
also revealed a statistically significant association between
T2R and haplotypes composed by the risk alleles of rs2069840
and both functional SNP rs1800795 and tag SNP rs2069832
($P = .02$ and .04, respectively) (Supplementary Table 1).

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<th>Ridley-Jopling classification</th>
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<th>Controls (n = 188)</th>
<th>$P^a$</th>
<th>T2R (n = 39)</th>
<th>Controls (n = 70)</th>
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Unless otherwise specified, data represent No. (%) of patients or controls.
Abbreviations: TT, tuberculoid leprosy; BT, borderline tuberculoid; BB, borderline borderline; BL, borderline leprosy; LL, lepromatous leprosy; SD, standard
deviation.

$^a$ $P$ values were determined with $\chi^2$ test for Ridley-Jopling classification and sex and with Student’s $t$ test for age.

Figure 1. Plasma levels (pg/mL) of interleukin 6 (IL-6) among 16
controls with reaction-free leprosy, 54 patients with type 1 reaction (T1R),
and 33 patients with type 2 reaction (T2R) from the genetic study data set.
$P$ represents nonparametric Mann–Whitney $P$ values.
Investigation of association between genotypes and plasma levels of IL-6 was performed using the entire subsample of 39 patients with T2R (Figure 3). Protective allele G of marker rs2069840 was associated with lower IL-6 plasma concentrations under a dominant model \( (P = 0.04) \). Higher plasma levels of IL-6 were observed among carriers of susceptibility alleles A and C of markers rs2069832 and rs1800795, although the differences did not reach statistical significance. Five individuals presented a plasma IL-6 concentration $\geq 56 \text{ pg/mL}$ (considered outliers) and were removed from the analysis after stem-and-leaf filtering.

**DISCUSSION**

This study presents combined genetic and serological evidence that implicates IL-6 gene variants as risk factors for susceptibility to the occurrence of leprosy T2R. The IL-6 gene was selected for genetic analysis based on results of a previous multiplex cytokine study from our group showing that, among 27 plasma factors tested, only IL-6 was identified as a potential biomarker of both T1R and T2R \[18\]. First, we replicated the original finding for both T1R and T2R in an expanded population sample of patients recruited from the same highly endemic region following a prospective design. However, the effect was less notable for the T1R group (Figure 1). Then, our strategy of comprehensive physical mapping of the IL-6 gene using tag SNPs followed by a genotype-phenotype correlation study led to an unbiased identification of a functional IL-6 variant associated with T2R in the expanded population sample. No association between IL-6 polymorphisms and leprosy T1R was observed. This is an intriguing finding that deserves further consideration. Occurrence of both types of leprosy reaction is clearly associated with increased production of pleiotropic inflammatory cytokine IL-6; however, in T1R, higher levels of IL-6 take place in a context of an already existing Th1 type of immune response. This increase of IL-6 in T1R could be due to nongenetic and/or genetic determinants located in different genes of the immune pathway. In contrast, a more exuberant increase of plasma IL-6 among individuals with T2R seems to be dependent on genetic determinants playing a central role.
because T2R occurs in a context of Th2 immune response background with only transient Th1 activation. This hypothesis is strongly reinforced by the results of both genetic association and genotype-phenotype correlation shown here. Altogether, our results argue in favor of different physiopathological mechanisms for T1R and T2R, in which genetic and non-genetic variants may have different effects on the phenotypes.

The small sample of patients with leprosy T2R is an acknowledged limitation of our study. Despite the large number of leprosy-affected individuals enrolled for follow-up, the number who developed T2R was small, as expected, reducing the power of the association analysis. Another limitation is the lack of replication of the genetic findings in an independent population sample. Nevertheless, these limitations are partially minimized by the fact that genetic association between at least 1 SNP bin, represented by tag SNP rs2069840, and occurrence of T2R was also observed in an independent experiment using plasma levels of IL-6 as a phenotype. The second associated bin, containing known functional SNP rs1800795, also correlated with different IL-6 plasma levels, but this association did not reach statistical significance, possibly because of the small sample size.

In humans, the IL-6 gene, located at 7p21 chromosome, produces a secreted 26-kDa glycoprotein arranged in 4 chains. There are several possible mechanisms by which higher IL-6 levels might affect the pathogenesis of T2R. Although IL-6 was first recognized by its property to induce antibody production, it is considered a key player in inflammation and in acute-phase reaction, which is one of the earliest responses to insults. Interleukin 6 can be produced by a broad spectrum of cell types, including T and B cells, macrophages, activated monocytes, mast cells, neutrophils, and eosinophils, and can exert its effects on virtually any cell. Interleukin 6 is known to be deregulated in many chronic autoimmune and inflammatory diseases, including rheumatoid arthritis, Crohn’s disease, juvenile arthritis, and multiple myeloma [26]. Interleukin 6 also participates in the adaptive immune response affecting T-cell differentiation into regulatory T cells or Th1 and Th2 and particularly T-helper 17 differentiation in humans. Although speculative, it seems reasonable to assume that higher levels of IL-6 could contribute to the development of leprosy T2R, mainly owing to the potent proinflammatory role of IL-6 and its capacity to stimulate antibody production. Together with extensive systemic inflammatory manifestations, immune complex–associated vasculitis is one of the hallmarks of leprosy T2R.

The results presented in this report are, to our knowledge, the first implicating IL-6 polymorphisms in leprosy T2R. Previous studies implicate IL-6 genetic variants with different diseases. In particular, SNP rs1800795 is a well-known functional IL-6 polymorphism located at position –174 of the promoter region of the gene, inside a negative regulatory domain. Alleles of rs1800795 have been described as risk factors for Grave’s disease [27], systemic-onset juvenile chronic arthritis [26], periodontitis [24, 28, 29], hepatitis C [30], and both types 1 and 2 diabetes mellitus [31, 32], with inconclusive results for type 2 diabetes mellitus [33]. As for tropical infectious diseases, the same allele C of rs1800795, identified here as a risk factor for T2R, has been associated with mucosal leishmaniasis in a Brazilian population [34]. Other studies failed to detect associations between IL-6 markers, including rs1800795, and Chagas disease and human immunodeficiency virus infection [35, 36].

Individuals homozygous for allele C of rs1800795 were shown to have a 6-fold higher risk of developing Crohn’s disease, a chronic inflammatory bowel disease that can also have erythema nodosum as a systemic complication [37]. Interestingly, this is not the first time that alleles of a gene have been associated with both Crohn’s disease and leprosy phenotypes. Several genetic epidemiology studies have independently detected variants of NOD2, TNFSF15, and IL12B as risk factors for both Crohn’s disease and leprosy [16, 38–40], leading authors to suggest an exciting hypothesis of a mycobacterial origin for Crohn’s disease [41]. In addition, NOD2 polymorphisms have been also associated with both types of leprosy reactions [17].

To date, no previous association of rs2069840 has been detected with any chronic inflammatory autoimmune or infectious disease. Because this SNP is not in LD with functional rs1800795 and seems to be independently associated with occurrence of T2R, these observations suggest the existence of additional IL-6 functional variants other than the rs1800795 LD bin that play a role in T2R susceptibility. In fact, marker rs2069840 seems to be of particular importance.
because it is associated with both T2R and plasma levels of IL-6. Interestingly, haplotypes composed of risk alleles of rs2069840 and both functional SNP rs1800795 and rs2069832 were also associated with T2R, even in low-power analyses due to low allele frequencies.

The essential role of IL-6 in leprosy T2R immunopathology needs to be explored further. The use of IL-6 as a biomarker in several diseases has been widely discussed, based on the multiple effects of this cytokine on the control of innate and adaptive immunity [42]. Here we propose genetic variants of IL-6 as possible biomarkers for T2R in leprosy. The results presented here add up to a solid body of evidence implicating IL-6 in the pathogenesis of leprosy T2R and point to this cytokine as a possible valuable predictive marker for this aggressive leprosy phenotype.

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 copyedited. 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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Figure 3. Plasma levels (pg/mL) of interleukin 6 (IL-6) according to IL-6 genotypes. Subsample was composed of 39 patients with type 2 reaction distributed into different genotype classes. P represents the Mann–Whitney P value for the IL-6 plasma levels across genotypes under a dominant model. Circles represent individuals with IL-6 plasma levels above the 95% confidence interval.