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The −308 bp *TNF* gene polymorphism influences tumor necrosis factor expression in leprosy patients in Bahia State, Brazil

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ABSTRACT

Leprosy or Hansen's disease is a debilitating chronic granulomatous disease caused by *Mycobacterium leprae*, with high incidence and prevalence in Brazil. The −308 bp G/A single nucleotide polymorphism (SNP rs1800629) in the tumor necrosis factor (*TNF*) gene promoter is a proposed risk factor for leprosy. In Brazil, Northern India, Egypt and Nepal, the common G allele was associated with leprosy. In Eastern India, Thailand and Malawi the minor A allele was the risk factor. Allele A was previously associated with high TNF. We genotyped rs1800629 in 326 leprosy cases from Bahia State, Brazil, including 72 paucibacillary (PB) and 47 multibacillary (MB) without reactions, and 69 reversal reaction (RR) and 78 erythema nodosum leprosum (ENL) with reactions. Logistic regression was used to compare patient groups with 331 healthy controls. Relative TNF mRNA was determined in peripheral blood leukocytes by QRTPCR, and serum TNF levels measured by ELISA. We found that TNF mRNA expression was higher ($P = 0.03$) in leprosy patients compared to endemic controls, but did not differ significantly between clinical subgroups. Carriage of the minor A allele was associated ($P = 0.003$) with low TNF mRNA across leprosy patients. Nevertheless, we found no evidence for either allele at this SNP as a risk factor for leprosy *per se* (OR = 1.12, 95% CI 0.79–1.60, $P = 0.52$), PB (OR = 0.99, 95% CI 0.54–1.81, $P = 0.97$), MB (OR = 0.86, 95% CI 0.40–1.83, $P = 0.70$), RR (OR = 1.37, 95% CI 0.79–2.38, $P = 0.27$) or ENL (OR = 0.76, 95% CI 0.40–1.45, $P = 0.42$) when compared to endemic controls. Further studies are required to determine whether the influence of the minor A allele on TNF mRNA levels determines response to treatment, particularly in the context of ENL reaction treatment with anti-TNF therapies and RR reactions where treatment with prednisolone is known to reduce TNF levels. Our findings contribute to understanding TNF as an important determinant of leprosy immunopathology in Brazil.

1. Introduction

Human leprosy (or Hansen's disease) is a chronic granulomatous infectious disease caused by the obligate intracellular organism *Mycobacterium leprae*. Although its prevalence was extensively reduced after the introduction of multidrug therapy (WHO, 2002) and BCG vaccination (Fine, 1996), leprosy is still considered a neglected disease and constitutes a major public health problem with 232,857 new cases reported globally in 2012 (WHO, 2013), 33,303 of which occurred in Brazil. Hence, leprosy remains a major concern in Brazil.

Our knowledge about the mechanisms underlying the variable clinical and immunological spectrum in leprosy remains limited, but genetic epidemiological studies have continued to contribute to our knowledge of immunopathology of disease. This genetic research is founded on the demonstration of high heritability for leprosy susceptibility and the conclusion that leprosy is largely a genetic disease (Alter et al., 2008, 2011). Indeed, genetic studies of leprosy have been particularly successful in identifying genetic risk factors in both genome-wide linkage (Mira et al., 2003, 2004) and association (Zhang et al., 2009, 2011) studies, with the human leukocyte antigen (HLA) region being the most significant association in the latter. This builds on a history of studies demonstrating associations at HLA for leprosy (reviewed (Blackwell et al., 2009)), a number of which have specifically focused on the role of polymorphisms at the HLA Class III region tumor necrosis

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factor (*TNF*) gene in Brazil (Cardoso et al., 2011; Franceschi et al., 2009; Santos et al., 2000, 2002; Shaw et al., 2001) and elsewhere (Fitness et al., 2004; Roy et al., 1997; Sapkota et al., 2010; Settin et al., 2007; Tarique et al., 2015), in particular on the role of a –308 bp G/A single nucleotide polymorphism (SNP rs1800629). In Brazil (Cardoso et al., 2011; Franceschi et al., 2009; Santos et al., 2000, 2002), Northern India (Tarique et al., 2015), Egypt (Settin et al., 2007) and Nepal (Sapkota et al., 2010), the common G allele at this −308 bp SNP has been associated with leprosy *per se*, and in stratified analysis of paucibacillary (PB) tuberculoid (Franceschi et al., 2009; Shaw et al., 2001) and multibacillary (MB) lepromatous leprosy (Franceschi et al., 2009; Santos et al., 2002; Settin et al., 2007; Shaw et al., 2001), while the minor A allele appears to be protective. Conversely, in Eastern India (Roy et al., 1997) and Thailand (Vejbaesya et al., 2007) the minor A allele was the risk factor for MB leprosy, while in Malawi (Cardoso et al., 2011; Fitness et al., 2004) the trend was also in this direction for leprosy *per se* and PB leprosy. The minor A allele has also been associated with a stronger delayed-type hypersensitivity skin-test response to *M. leprae* antigen in borderline tuberculoid leprosy patients (Moraes et al., 2001).

The *TNF* gene is located adjacent to *LTA* encoding lymphotoxin-alpha within the class III region of HLA. Both have essential but independent roles in the evolution of the granulomatous response in experimental leprosy (Hagge et al., 2009). In humans, circulating TNF is elevated in both PB and MB leprosy (Madan et al., 2011), and is found in granulomatous lesions (Lockwood et al., 2011). TNF also plays a part in acute inflammatory leprosy reactions, with higher circulating levels of TNF observed in erythema nodosum leprosum (ENL) type II reactions and in type I reversal reactions (RR) compared to cases not in reaction (Chaitanya et al., 2013; Madan et al., 2011). Down-regulation of TNF mRNA accompanies improved outcomes for type II reactional leprosy following administration of the TNF-specific drugs thalidomide and pentoxifylline (Moraes et al., 2000). Reduction in TNF mRNA (Moraes et al., 2000) and circulating TNF (Raju et al., 2014) is also observed following treatment of type I reversal reactions (RR) with the corticosteroid prednisolone.

Associations between infectious and autoimmune diseases and the −308 bp G/A (rs1800629) SNP in the *TNF* promoter (reviewed (Bayley et al., 2004; Qidwai and Khan, 2011)) has led to many investigations questioning whether the SNP is functional in determining TNF levels. A number of investigators have reported higher levels of induced (*e.g.* lipopolysaccharide or *M. leprae* antigen) levels of TNF in whole blood or peripheral leukocytes in individuals carrying the minor A allele compared to GG homozygotes (Cardoso et al., 2011; Louis et al., 1998). The resulting rationale is that high levels of proinflammatory TNF associated with carriage of the A allele would be detrimental in the context of autoimmune diseases and enhanced pro-inflammatory responses in infections like malaria, leprosy and cutaneous leishmaniasis (Bayley et al., 2004). Balancing this is the requirement for TNF in the activation of macrophages to kill mycobacteria, as evidenced by reactivation of mycobacterial infections, including leprosy (Lluch et al., 2012), following treatment of autoimmune disease patients with anti-TNF antibodies. Hence, the alternative hypothesis that protection from leprosy associated with the minor A allele could be due to the requirement for TNF to control leprosy infection. A series of reporter gene studies undertaken during the 1990s to determine directly whether the −308 bp G/A (rs1800629) SNP in the *TNF* promoter region was involved in gene regulation were equivocal (Braun et al., 1996; Brinkman et al., 1995; Kroeger et al., 1997; Stuber et al., 1995; Wilson et al., 1997; Wu and McClain, 1997), leading Bayley and colleagues (Bayley et al., 2004) to conclude that the −308 bp G/A polymorphism was not functional. A more recent study using a novel reporter system purports to provide more robust evidence that the −308 bp G/A polymorphism is functional, confirming that the −308A allele expresses at a higher level compared with the −308G allele (Karimi et al., 2009). This has provided a system in which to demonstrate improved efficacy of new thalidomide analogs in specifically regulating TNF expression (Stewart et al., 2010).

In a geographically distinct population in Bahia State, Brazil, we now find that TNF mRNA expression is higher in leprosy patients compared to endemic controls, but does not differ significantly between clinical subgroups. Across leprosy patients, carriage of the minor A allele is associated with low TNF mRNA. Nevertheless, we find no evidence for either allele at this SNP as a risk factor for leprosy *per se*, or for any of the clinical subgroups of leprosy, when compared to endemic controls.

2. Materials and methods

2.1. Case patients, control subjects, and study design

The study participants were enrolled from two different hospitals in the city of Salvador, Bahia (Hospital Universitário Professor Edgard Santos and Hospital Especializado Dom Rodrigo de Menezes). Both are reference centers for the treatment of leprosy, absorbing the majority of cases from the state of Bahia. Leprosy patients were recruited after diagnostic confirmation in accordance with the guidelines of the Ministry of Health of Brazil, which provides a protocol for classification by the Ridley–Jopling score and by the WHO field classification (Ridley and Jopling, 1966; World Health Organization, 1997), as previously reported for studies of patients recruited from these hospitals in Salvador, Bahia (Machado et al., 2015). This included dermatological and neurological evaluation, skin-test sensitivity to leprosy antigen, bacilloscopy of lymph smears (Cavalcanti et al., 2012), and biopsy of one or more lesions. Individuals were classified within the leprosy clinical spectrum as having either paucibacillary (PB: TT/BT or tuberculoid/borderline tuberculoid) or multibacillary (MB: BB/BL/LL or borderline, borderline lepromatous, or lepromatous) clinical forms of the disease, with subsets of patients also being recorded clinically as having type I RR or type II ENL reactions. A total of 326 leprosy cases of both sexes, aged between 16 and 68 years, were included in the genetic association study (Table 1). The control group consisted of 331 individuals, aged between 16 and 67 years, recruited as volunteer blood bank donors in the city of Salvador. Hence, our maximum power to detect allelic association at $P = 0.05$ for a variant at minor allele frequency (MAF) of 0.1 with effect size (genotype relative risk) of 1.5 was 76%. Informed consent was obtained from all participants. The study was approved by the institutional review board of the Federal University of Bahia (CEP-50/2010) and the Brazilian National Ethical Committee (CONEP 11019).

2.2. DNA extraction and genotyping

Blood was obtained by venipuncture and collected into dodecyl citrate acid-containing Vacutainers (Becton Dickinson). Genomic DNA was prepared using the proteinase K and salting-out method as described (Sambrook et al., 1989). Validated predesigned Taqman® qPCR assays for the marker rs1800629, containing PCR primers and probes, were purchased from Life Technologies® (Thermo Fisher, Inc), and reactions carried out according to the manufacturer's protocols. This SNP corresponds to the position −308G/A in the promoter region of the *TNF* gene. To ensure the accuracy of genotyping results, three positive controls and a negative control were included in each 96-well plate. Taqman assays were performed using the 7500 standard (Life Technologies), and the ABI software v 2.0.6 was used to analyze the data.

2.3. RNA isolation, cDNA conversion and TaqMan real-time polymerase chain reaction

Total leukocytes from patients that were not under treatment with immunosuppressive drugs such as prednisone and thalidomide (in the case of type II reactions) were homogenized in TRIzol reagent (Life Technologies). RNA was extracted using the PureLink™ RNA Mini Kit (Life Technologies) according to the manufacturer's protocol. Total RNA concentration was determined from spectrophotometric optical density measurement (260 and 280 nm). For each sample tested, the