Genetics of leprosy reactions: an overview

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Type-1 (T1R) and Type-2 (T2R) leprosy reactions (LR), which affect up to 50% of leprosy patients, are aggressive inflammatory episodes of sudden onset and highly variable incidence across populations. LR are often diagnosed concurrently with leprosy, but more frequently occur several months after treatment onset. It is not uncommon for leprosy patients to develop recurring reactional episodes; however, they rarely undergo both types of LR. Today, LR are the main cause of permanent disabilities associated with leprosy and represent a major challenge in the clinical management of leprosy patients. Although progress has been made in understanding the immunopathology of LR, the factors that cause a leprosy patient to suffer from LR are largely unknown. Given the impact that ethnic background has on the risk of developing LR, host genetic factors have long been suspected of contributing to LR. Indeed, polymorphisms in seven genes [Toll-like receptors (TLR)1, TLR2, nucleotide-binding oligomerisation domain containing 2, vitamin D receptor, natural resistance-associated macrophage protein 1, C4B and interleukin-6] have been found to be associated with one or more LR outcomes. The identification of host genetic markers with predictive value for LR would have a major impact on nerve damage control in leprosy. In this review, we present the recent advances achieved through genetic studies of LR.

Key words: reversal reaction - type-1 leprosy reaction - erythema nodosum leprosum - type-2 leprosy reaction - host genetic background - leprosy

Leprosy reactions (LR) are characterised by an intense and sudden activation/reactivation of the host immune/inflammatory responses that frequently affect the peripheral nerves. Population studies indicate that during the course of leprosy 16-56% of patients develop irreversible nerve function impairment (NFI) (Britton & Lockwood 2004) that is mainly caused by LR. LR are a common cause of sensation/motor deficit and anatomical deformities in leprosy patients. In 2010, approximately 5.8% of newly detected leprosy cases worldwide presented grade-2 disabilities at diagnosis (WHO 2011), a proportion that has been oscillating between 5-8% since 2004. A substantial fraction of LR (30-40%) are diagnosed concurrently with leprosy (Scollard et al. 1994, Ranque et al. 2007) , which could partially explain the persistent detection of severe disability at the leprosy diagnosis. In fact, it is common for patients to seek medical attention in response to LR symptoms, with a subsequent diagnosis of leprosy. One current goal of leprosy control is a 35% global reduction in grade-2 disabilities (identified at the time of the leprosy diagnosis) by the end of 2015 (Pannikar 2009).

There are two major types of LR: type-1 reaction (T1R) or reversal reaction, and type-2 reaction (T2R) or erythema nodosum leprosum. Patients may also develop Lucio's phenomenon (LP), which is also known as erythema necroticans and diffuse lepromatous leprosy (LL), erythema multiform and neuritis; however, these episodes represent a small proportion of all LR cases. Even though reactional episodes are characterized as acute outcomes, patients may also present a chronic reactional state, but rarely does the same patients suffer from different types of LR (Rea & Sieling 1998, Moraes et al. 2001, Benard et al. 2009).

The clinical presentations of T1R and T2R are distinct. However, both reaction types might share certain molecular control mechanisms, as several studies have shown that the cytokine profiles presented during T1R and T2R are similar (reviewed in Scollard et al. 2006). Still, such phenotypic similarities could be caused by the generalised immune dysregulation characteristics of both reaction types. Meanwhile, the existence of a common underlying control element in LR is a subject that is under discussion. When patients with either LR type were compared with non-reactional leprosy patients, the classical mediators of the cell-mediated immune response were observed at significantly higher levels, both systemically and in the local cutaneous lesions (Modlin et al. 1984, Sreenivasan et al. 1998, Moraes et al. 2001). A pro-inflammatory cytokine of particular interest is the tumour necrosis factor (TNF) as elevations of this cytokine have been observed in the serum and cutaneous lesions of T1R and T2R and in the nerve biopsies of T1R patients (reviewed in Scollard et al. 2006).

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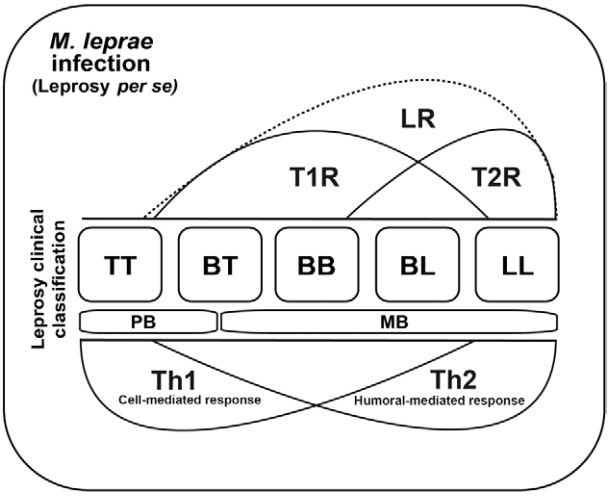
T1R - T1R LR are characterised by a delayed hypersensitivity to *Mycobacterium leprae* antigens (Gell & Coombs type-IV reaction) and a sudden, abrupt increase in the cell-immune responsiveness in lesions; this increase can be maintained for an extended period of time (Ridley & Radia 1981, Naafs 2000, Little et al. 2001). The main dermatome-pathological finding in T1R is an increase in the number of lymphocytes in the dermis with a loss of the normal granuloma structure. This breakdown of granuloma reduces the effectiveness of bacillary antigen containment. Additionally, Langhans giant cells may be observed in the later stages of T1R (Ridley & Radia 1981, Walker & Lockwood 2008). T1R affects 20-30% of leprosy patients (Roche et al. 1991, Scollard et al. 1994, Saunderson et al. 2000b, Ranque et al. 2007). The majority of these patients are classified in the borderline spectrum of the Ridley and Jopling (1966) (R & J) scheme (Figure). T1R occurs more frequently than T2R (van Brakel et al. 2005). The clinical manifestations of T1R are an acute inflammation of pre-existing lesions, which can become erythematous, oedematous and infiltrated (Saunderson et al. 2000b). The peripheral nerves are frequently affected during the acute inflammatory process. Remarkably, new leprosy lesions may become apparent, most likely caused by an inflammatory response to previously undetected bacilli in the dermis (Rose & Waters 1991). Oedema of the extremities may be present, but systemic complications are unusual (Walker & Lockwood 2008). In addition to the borderline clinical form of leprosy, important risk factors for T1R include the following: (i) age (individuals aged > 15 years in southern Vietnam and ≥ 40 years in Brazil are at increased risk) (Ranque et al. 2007, Sousa et al. 2007); (ii) a bacilloscopic index greater than 4+ (Saunderson et al. 2000b); (iii) an increased number of lesions at the leprosy diagnosis (van Brakel & Khawas 1996, Kumar et al. 2004) and (iv) *M. leprae* DNA detection by polymerase chain reaction in the lesion biopsies (Sousa et al. 2007).



Schematic distribution of leprosy reaction (LR) across clinical spectrum of leprosy. The board represents patients infected by *Mycobacterium leprae* who developed clinical manifestation of the disease. In the middle a schematic distribution of Ridley and Joplin (1996) scheme and World Health Organization clinical classes of leprosy types is presented. The upper section shows the spread of the leprosy reaction types according to the risk rates across clinical leprosy classes. The bottom section symbolizes the corresponding cytokine profile, produced according to leprosy type. BB: borderline-borderline; BL: borderline-lepromatous; BT: borderline tuberculoid; LL: lepromatous-lepromatous; MB: multibacillary; PB: paucibacillary; T1R: type-1 reaction; T2R: type-2 reaction; Th1: T-helper 1; Th2: T-helper 2; TT: tuberculoid-tuberculoid.

Epidemiological studies of T1R shows that in the vast majority of cases it is either diagnosed concurrently with leprosy or during the first two years after initiating leprosy multi-drug therapy (MDT) treatment (Scollard et al. 1994, Pocaterra et al. 2006). T1R may also occur many years after MDT completion; however, it can be difficult to distinguish between late-onset T1R and leprosy relapse (Waters 2001). The onset of T1R signs and symptoms is abrupt; therefore, immediate attention is necessary to reduce permanent NFI (van Brakel & Khawas 1996). The up-regulation of pro-inflammatory cytokines such as TNF, interleukin (IL)-6, IL-1β and the chemokine induced protein-10 (IP-10) has been repeatedly observed in T1R patients in different populations (Sarno et al. 1991, Moraes et al. 1999, Oliveira et al. 1999, Stefani et al. 2009, Lockwood et al. 2011, Scollard et al. 2011). Even with adequate and timely treatment, up to 40% of T1R patients do not achieve complete lesion recovery (van Brakel & Khawas 1996).

Initial T1R therapeutic interventions attempt to reduce the exacerbated immune response with cortisol and adrenocorticotropic hormone injections. Although initially effective, T1R relapses have been noted shortly after discontinuing such treatment (Naafs 2006). The exogenous corticoid therapy for T1R was established in the 50's (Roche et al. 1951). Today, the basic protocol is based on administering 30-40 mg/day of prednisolone, which is gradually reduced (5 mg/14 days) as the patient's condition improves (Naafs 2006). Prolonged steroid use, four-nine months (Walker & Lockwood 2008) and the accompanying adverse effects are two of the greatest challenges in managing T1R for both patients and clinicians. Interestingly, dysregulation of the cellular cortisol metabolism in leprosy lesions has been associated with T1R (Rook & Baker 1999, Andersson et al. 2007). Circulation levels of insulin-like growth factor-I (IGF-I) have also been correlated with LR (Rodrigues et al. 2011); IGF-1 interacts with endogenous glucocorticoids and cytokines. These results point to the existence of an endogenous steroid metabolic pathway that regulates the inflammatory response in LR.

T2R - The physiopathological mechanism responsible for T2R is widely unexplored. Classically, T2R has been considered an immune complex-mediated disorder (Gell & Coombs type-III reaction) that resembles serum sickness (Wemambu et al. 1969, Hussain et al. 1995, Naafs 2006). In a divergent but not mutually exclusive view, T2R has been characterised as the consequence of both a transitory shift in the CD4/CD8 T cell ratio towards T-helper (Th) lymphocytes (Modlin et al. 1983, 1985) and the increased levels of pro-inflammatory cytokines, such as interferon-γ (INF-γ), IL-1β, TNF, IL-6 and IL-

12, in patients who initially display a predominantly humoral immune response (Sarno et al. 1991, Barnes et al. 1992, Foss et al. 1993, Moraes et al. 1999, Kahawita & Lockwood 2008, Stefani et al. 2009). However, the mechanism that triggers T2R remains unclear. The histopathological findings in the skin biopsies of acute lesions demonstrate a predominance of neutrophils, eosinophils and mast cells. In chronic lesions, there is a reduction of neutrophils and an increase in the number of lymphocytes and plasmocytes (Mabalay et al. 1965). The clinical presentation of T2R includes generalised erythematous lesions, nodules and papules that may be superficial or deep, which may become ulcerative or necrotic. Some nodules reach chronicity, become painful and fibrotic and lead to scars. In contrast to T1R, the systemic effects of T2R are notorious and include high fever, oedema and a variety of complications, such nephritis, arthritis and iridocyclitis (Mabalay et al. 1965, Kahawita & Lockwood 2008).

T2R mainly affects patients with multibacillary (MB) leprosy, which are classified at the borderline-lepromatous (BL)/LL pole of the disease spectrum (Figure). Patients presenting bacterial index higher than 4+ in skin smears are at higher risk for T2R (Becx-Bleumink & Berhe 1992, Manandhar et al. 1999). There is wide variation of T2R prevalence across distinct geographical settings and ethnic boundaries. In Brazil, approximately 37% of BL and LL cases develop T2R, while in India, Nepal and Thailand, the proportion is between 19-26% (Kahawita & Lockwood 2008, Kahawita et al. 2008). The higher incidence of T2R in Brazil may reflect the relatively large proportion of MB leprosy cases in comparison with Southeast Asia. Follow-up studies have shown that the first T2R event is usually observed within the first three years after MDT implementation and that approximately one-third of all cases are diagnosed concurrently with leprosy (Manandhar et al. 1999, Saunderson et al. 2000a, Kumar et al. 2004, Feuth et al. 2008). A prospective study of BL and LL patients from India revealed that less than 10% of individuals who develop T2R presented a single episode, whereas 62% had chronic T2R (Pocaterra et al. 2006). In Ethiopia, 63% of individuals had more than one T2R episode, while 37% presented a single event (Saunderson et al. 2000a).

The reference treatment of T2R is also based on corticoid therapy, typically prednisolone at 40-60 mg/day. However, TNF-suppressive drugs seem to be more effective. For example, the use of thalidomide has been extremely effective as a treatment for severe T2R (Tadesse et al. 2006). However, the use of this medication is restricted in several countries due to its teratogenicity (Lockwood & Sinha 1999). Interestingly, thalidomide does not appear to interfere with the complement activation by *M. leprae* in vitro (Shannon et al. 2011) and its effectiveness is attributed to TNF suppression (Tadesse et al. 2006). The initial dose of thalidomide is 400 mg/day, which is reduced as soon as possible to 300 mg/day or less. Patients presenting with chronic T2R may be given 100 mg/month under clinical supervision (Walker et al. 2007). Two other TNF specific suppressors, the antibodies infliximab and etanercept, are also successful in arresting T2R in patients for whom previous drug therapies were ineffective (Faber et al. 2006, Ramien et al. 2011). Alternatively, pentoxifylline can be a drug of choice, as clinical studies have detected a reduction of INF-γ, TNF and IL-6 levels in the skin biopsies of T2R patients after pentoxifylline treatment (Sampaio et al. 1998, Moraes et al. 2000, Pocaterra et al. 2006).

LP - LP is commonly considered an extensive and aggressive subtype of T2R; it was initially described by Lúcio and Alvarado (1852) and later reviewed by Latapi and Chavez-Zamora (1948). LP primarily affects LL and BL patients, particularly those of Latin ancestry (Costa Rica and Mexico) (Donner & Shively 1967). However, cases outside Central America were reported on the West Coast of the United States of America, in South America and in Africa (Sehgal 2005).

The clinical features of LP include the development of acute, severe and necrotic lesions that are characterised by a diffuse cutaneous infiltrate and high bacillary load in the endothelium; in the advanced stages, extensive ulceration leading to secondary infection and sepsis may occur. LP differs from other LR outcomes by its frequent association with high mortality rates (Han et al. 2008). LP concomitant with other LR is rare; only a single, concomitant case of T2R and LP has been described (Benard et al. 2009). Histopathological studies show that IgG and/or C3 immunocomplex deposition in peripheral blood vessels of individuals affected by LP (Sehgal 2005). However, as LP incidence is low, systematic studies of its physiopathology are difficult. Recently, LP has been associated with a novel mycobacterium species (*Mycobacterium lepromatosis* sp.). The DNA analysis of autopsy samples from LP patients has detected sufficient variance in the sequenced regions to support the conclusion that this mycobacterial DNA is distinct from *M. leprae* (Han et al. 2008, 2009). These results are consistent with the presence of *M. lepromatosis* in the tissue biopsies of fatal LP cases from Mexico, Singapore and Ontario (Vera-Cabrera et al. 2011, Jessamine et al. 2012, Yang Han et al. 2012).

Host genetic background in LR - To date, a limited number of studies have investigated the impact of genetic variants controlling LR susceptibility. Although some results are inconclusive or suffer from the lack of replication, these studies have provided critical advances for the understanding of LR pathogenesis. Here, we present a summary of the genetic studies of LR phenotypes that were published before early 2012.

Toll-like receptors (TLR) - TLR are single-pass transmembrane proteins that mediate cell signalling during the innate immune response. TLR are key mediators of host responsiveness to a diverse range of pathogenic structures leading to the activation of transcription factors and the secretion of pro-inflammatory cytokines and chemokines (Akira & Takeda 2004, Alcais et al. 2005). Dysregulation of the TLR pathway has been associated with susceptibility to infectious disease and inflammatory disorders (Gay & Gangloff 2007). The family members TLR1, 2 and 6 form heterodimers that are involved in *M. leprae* antigen recognition (Krutzik et al. 2003).

TLR2 - TLR2 plays a role in monocyte/macrophage activation and cytokine production in leprosy (Krutzik et al. 2005). Additionally, TLR2 expression by Schwann cells, the primary target of *M. leprae*, has been associated with increased apoptosis, which may indicate a mechanism by which the activation of innate immunity could contribute to nerve injury (Oliveira et al. 2003). The over stimulation of the TLR2 signalling cascade can result in massive TNF secretions, a hallmark of reactional episodes in LR patients. A significant reduction in both gene expression and protein levels of TLR2 and TLR4 has been noted during corticoid therapy in T1R patients (Walker et al. 2012).

A single nucleotide polymorphism (SNP), rs3804099 (alias: +597 C/T), and a microsatellite located between 162-100 bp upstream of the *TLR2* start codon, were identified as genetic risk factors for T1R in an Ethiopian sample (Bochud et al. 2008). Of the 441 leprosy patients included in the study, 216 had LR data available. In a comparison of 66 T1R patients with 17 T2R and 133 neuritis patients, the rare allele T of rs3804099 was found to be a protective factor for T1R [dominant model $p = 0.002$ odds ratio (OR) = 0.34 (0.17-0.68)], while the 280 bp length microsatellite allele was associated with increased risk [recessive model $p = 0.001$ OR = 5.83 (1.98-17.15)]. Although the reported association is interesting, there is a potential bias in this study related to the selection of the control group, as it is preferable to include T1R-free patients who display borderline forms [borderline tuberculoid (BT), borderline-boderline and BL] of leprosy as controls. Epidemiological data indicate that polar form [tuberculoid-tuberculoid (TT) and LL] leprosy patients, as well as patients with generalised immune dysregulation (T2R), are less likely to develop T1R compared to borderline leprosy patients. Finally, in a stratified analysis by Ethiopian ethnicity, the association of *TLR2* markers and T1R remained significant in two of three groups ($p < 0.05$). These are potentially interesting observations that need to be replicated in larger samples over a larger range of ethnic backgrounds.

TLR1 - TLR1, 2 and 6 are phylogenetically related (Johnson et al. 2007). *TLR1* and *TLR6* show high sequence similarity and tandem arrangement in the human genome; this similarity suggests a recent gene duplication event, while *TLR2* split off earlier in the evolutionary process (Roach et al. 2005). The heterodimer TLR1/TLR2 is involved in the *M. leprae* antigen recognition (Krutzik et al. 2003, 2005), whereas TLR6 is related to *M. leprae* persistence in Schwann cells (Mattos et al. 2011). Hence, variations in these genes may variously modulate the innate immune system either negatively or positively; therefore an individual might be predisposed to mounting an ineffective or excessive inflammatory response.

*TLR1 p*olymorphisms are associated with impaired signal transduction (Johnson et al. 2007, Wurfel et al. 2008). A *TLR1* non-synonymous polymorphism (rs5743618; I602S) is of particular interest. In HEK293, cells transfected with the two *TLR1* alleles of the I602S polymorphism; the 602I allele regulated the basal level of NFκB activity and the NFκB response to *M. tuberculosis* extracts (Hawn et al. 2007). Independent studies have shown that individuals who carry *TLR1* 602I produce higher levels of the cytokines IL-6, TNF and IL-1β (Hawn et al. 2007, Johnson et al. 2007, Misch et al. 2008), a common observation in patients during reactional episodes (Sarno et al. 1991, Moraes et al. 1999, Stefani et al. 2009).

To investigate the impact of the rs5743618 *TLR1* polymorphism on LR, Misch et al. (2008) performed an association study in a Nepalese sample of 933 leprosy cases. The G allele (coding for the low response serine variant) showed a protective effect for the T1R cases when compared to a control group that comprised a mix of T2R and reaction-free patients with all forms of leprosy [$p = 0.04$, OR = 0.54 (0.30-0.96), allelic model corrected by ethnicity, gender and age]. A trend in the association of the rs5743618 G allele and LL was also noted for these individuals [$p = 0.11$ OR = 4.76 (0.58-38.87), genotypic model] (Misch et al. 2008). The association of the serine allele with T1R protection is reasonable given that this variant drives a lower production of pro-inflammatory cytokines. Interestingly, the allele frequencies of rs5743618 vary greatly across populations. While the G allele (serine) is rare, or entirely absent in Africans and Asians, it is the major allele in populations of European descendant (Wong et al. 2010). The polymorphism has also been associated with susceptibility to clinical outcomes of malaria (Leoratti et al. 2008) and an altered immune response to BCG vaccination, with the same risk allele (Randhawa et al. 2011).

An additional study showed that the rs5743618 G allele was a protective factor against leprosy *per se* in two independent samples from India [combined analysis $p = 5.7\ 10^{-8}$; OR = 0.31 (0.20-0.48)] and one from Turkey [$p = 0.004$; OR = 0.48 (0.29-0.80)] (Johnson et al. 2007, Wong et al. 2010). The role of the serine-coding allele as a protective factor for leprosy *per se* is intriguing, as the presence of the isoleucine allele supposedly guarantees an efficient translocation of TLR1 to the cell surface and the effective secretion of pro-inflammatory cytokines, thereby ensuring a better cell-mediated immune response against the infection. Alternatively, this data may suggest the TLR1/TLR2 pathway is one out of several systems potentially used by *M. leprae* for host immune evasion and macrophage invasion by the parasite. Whether the serine allele predisposes an individual to a subtype of leprosy, that increases the risk for T1R, or independently predisposes an individual to both leprosy *per se* and T1R is an unanswered question.

A second non-synonymous polymorphism of *TLR1,* rs4833095, which causes a substitution of asparagine to serine (N248S) in the external recognition site of the protein, was shown to be associated with LR susceptibility in a Bangladeshi population sample (Schuring et al. 2009). The serine-coding allele was described as a protective factor against T2R [$p = 0.04$; OR = 0.40 (0.16-0.99)]. Interestingly, the asparagine in the leucine-rich repeat motif induces a diminished response to the mycobacterial cell wall components (Omueti et al. 2007). Although the sample size was large (842 leprosy cases), the analysis of T2R focused on only 11 cases. An additional problem with this study could be that the control group

was mostly composed of paucibacillary leprosy cases, which are known not to be at risk for T2R. The authors also observed a trend for an association between the S allele and T1R. In the Bangladesh sample, the minor allele frequency for rs5743618 was close to that of the Nepalese sample (~0.05); however, no association was observed between the I602S polymorphism and T1R (p = 0.54).

Nucleotide-binding oligomerisation domain-like receptors (NLR) - NLR comprise a family of cytosolic pattern recognition receptors that are part of the innate immune system. Nucleotide-binding oligomerisation domain containing 2 (NOD2) is a recognised member of this family (Franchi et al. 2009). There is evidence that NOD2 agonists synergise with TLR ligands, particularly those for TLR4 and TLR9, to induce pathways that lead to the production of pro-inflammatory cytokines (IL-12 and INF-γ) and anti-microbial molecules (Fritz et al. 2005, Tada et al. 2005). The muramyl dipeptide NOD2 agonist mimics the bacterial peptidoglycan moiety and induces strong cell-mediated immunity that resembles delayed-type hypersensitivity (Tada et al. 2005). Importantly, delayed-type hypersensitivity to *M. leprae* antigens is a recognised event in T1R.

NOD2 is a Crohn's disease (CD) susceptibility gene (Ogura et al. 2001, Hugot et al. 2001, Noguchi et al. 2009). This finding has led to the suggestion that *Mycobacterium avium subs. paratuberculosis* (MAP) may be part of the aetiology of (at least) a subset of CD patients (Behr & Schurr 2006). The latter suggestion has received additional support by the striking overlap of susceptibility genes between CD and leprosy (Schurr & Gros 2009). In fact, *NOD2* polymorphisms have been associated with leprosy *per se* in Chinese and Nepalese patients (Zhang et al. 2009, Berrington et al. 2010).

NOD2 - Variations in the *NOD2* gene were analysed in the same Nepalese sample that had been used for the *TLR1* study (Misch et al. 2008). The SNPs selected for genotyping were: (i) tag SNPs in the European and Chinese HapMap samples, capturing the genetic variation of *NOD2* plus 50 kb up and down-stream of the gene, and (ii) known, rare, non-synonymous variants within the *NOD2* (Berrington et al. 2010). A total of 32 SNPs were analysed and multiple signals of association with leprosy *per se* (8/32), T1R (7/32) and T2R (7/32) were observed. However, strong linkage disequilibrium (LD) across the entire *NOD2* gene suggested that the signals of association may not be completely independent. Remarkably, two SNPs (rs2287195 and rs8044354) were associated with all three studied phenotypes. The best p values for these associations are as follows: leprosy *per se* [p = 0.001; OR = 2.29 (1.43-3.68) and p = 0.001; OR = 2.17 (1.36-3.46)], T1R [p = 0.013; OR = 0.74 (0.58-0.95) and p = 0.005; OR = 0.74 (0.59-0.92)] and T2R [p = 0.015; OR = 1.93 (1.14-3.30) and p = 0.001; OR = 2.83 (1.52-5.28)], respectively. These variants are in strong LD (r^2 = 0.6-0.69) and the risk allele for T2R is the protective allele for T1R and *vice versa* for both of SNPs. This finding may indicate a cryptic association between the SNPs and leprosy type, as a non-random distribution of LR is observed across the R & J scale. Surprisingly, only SNP rs1131716 was associated with an increased risk for lepromatous leprosy [p = 0.013 OR = 2.01 (1.12-3.76)]. Interestingly, rs1131716 is a non-synonymous polymorphism (P35L) in *SLIC1* exon 1. A functional study showed that SLIC1 immunoprecipitated with PSGL1 (Schaff et al. 2008), the principle glycoprotein responsible for the interaction of memory T cells with E and P-selectins. In the skin lesions of a number of skin disorders, including psoriasis, PSGL1 modulates the adhesive properties of leukocytes and T cell infiltrates in the peripheral tissues (Fuhlbrigge et al. 1997, Nestle et al. 2009).

IL6 - IL-6 is a pleiotropic cytokine first recognised for its ability to induce antibody production. However, the role of IL-6 is much broader and this cytokine is a key player in the acute phase response to infection. In addition to being produced by a wide spectrum of cell types, including T and B cells, macrophages, activated monocytes, mast cells, neutrophils and eosinophils, IL-6 can exert its effects on any of these cells. Based on IL-6 potent pro-inflammatory effect associated with its capacity to induce antibody production, several mechanisms that support a role of IL-6 in T2R pathogenesis have been proposed.

A gene expression analysis using peripheral blood mononuclear cells from reactional vs. non-reactional leprosy patients revealed that *IL-6* was over-expressed by T1R and T2R patients. When skin biopsies were analysed, an increased expression of *IL6* was detected in samples from T2R and T1R patients, whereas in non-reactional individuals, *IL-6* mRNA was only detected in the lesions from TT/BT patients (Moraes et al. 1999). Independent studies revealed that circulating levels of IL-6 were higher in the T2R patients than in the non-reactional leprosy cases (Belgaumkar et al. 2007, Stefani et al. 2009). Stefani et al. (2009) analysed 27 plasma factors and found that IL-6 was the only biomarker that was elevated in T1R and T2R when compared to the leprosy patients without reactions.

In groups matched by leprosy R & J type, our study found higher IL-6 plasma levels in T1R and T2R cases when compared to patients with no reaction. Interestingly, the IL-6 levels were strikingly higher in T2R compared to T1R cases ($p = 2.4\ 10^{-7}$) (Sousa et al. 2012). In the same study, the impact of the genetic variants of *IL6* on LR susceptibility was investigated in a population sample of 409 newly diagnosed leprosy cases. Tag SNPs were selected to cover the *IL6* gene and its promoter region. No association was observed between the *IL6* markers and T1R. However, three tag SNPs (rs2069832, rs2069840 and rs2069845) were significantly associated with T2R when the cases were compared to reaction-free patients from the lepromatous pole of the disease [p = 0.002; OR = 4.00 (1.64-9.76); p = 0.027; OR = 0.44 (0.22-0.91) and p = 0.044; OR = 1.92 (1.02-3.63), respectively]. A comprehensive search of the HapMap database revealed that SNP rs2069832 was tagging a bin containing the variant rs1800795, a known functional polymorphism localised at a negative regulatory domain in the promoter region of *IL6* (Ray et al. 1990, Fishman et al. 1998). Further analysis revealed that SNP rs1800795 was indeed associated with T2R [p = 0.005; OR = 3.71 (1.47-9.34)]. A

multivariate analysis revealed independent signals for rs1800795 and rs2069840. The haplotypic analysis that included these two SNPs showed that the presence of the risk allele "C" at both *loci* was associated with T2R susceptibility [p = 0.02; OR = 3.17 (1.21-8.29), dominant model]. Testing for an association between *IL6* genotypes and IL-6 plasma levels revealed that the presence of the "G" allele of rs2069840, which is associated with protection from T2R, correlated with lower IL-6 plasma levels in a sub-sample of 33 T2R affected individuals (p = 0.04) (Sousa et al. 2012). Together, these results support an important role of IL-6 in the T2R pathogenesis.

IL6 polymorphisms are also associated with several chronic, autoimmune and inflammatory diseases, including rheumatoid arthritis (Pascual et al. 2000), juvenile arthritis (Fishman et al. 1998), CD (Sawczenko et al. 2005, Guerreiro et al. 2009), periodontitis (Trevilatto et al. 2003, Nibali et al. 2009) and Parkinson's disease (San Luciano et al. 2012). Interestingly, *IL6* genetic variants are associated with susceptibility to leishmaniasis (Castellucci et al. 2006) and the growth of *Chlamydia pneumoniae* in macrophages (Poikonen et al. 2009).

C4B - A widely accepted assumption is that T2R is an immune complex-mediated disease. The C4b and C3b factors of the complement system are known to induce the opsonisation of microbial components for efficient uptake by macrophages (Carroll 1998). A possible role for the complement system in immune evasion by *M. leprae* was suggested by the co-localisation of C3 and PGL-1 in the lipid rafts of dendritic cell membranes (Callegaro-Filho et al. 2010). A recent study showed a possible feedback mechanism in which C3b and C4b reduced the secretion of the pro-inflammatory chemokine IP-10 in peripheral blood after being stimulated with lipopolysaccharide and IFNβ (Takeda et al. 2012). In a Brazilian sample, the C4B*Q0 allele was associated with risk to leprosy *per se* when cases were compared to healthy controls ($p = 2.7\ 10^{-5}$). Interestingly, the C4B*Q0 allele frequency was significantly higher among T2R cases (22 individuals) when compared with the LL patients who had no reaction (6) (p = 0.006) (de Messias et al. 1993). These findings suggest that the complement system may affect both the phagocytosis during the reactivation/exacerbation of the inflammatory response in T2R and the stimulation of cellular immune responses, possibly through IP-10-related pathways.

Vitamin D receptor (VDR) - The active form of vitamin D ($1,25(OH)_2D_3$) is a known regulator of the host immune response against infection (White 2008) and genetic polymorphisms of the *VDR* gene are associated with both tuberculosis and leprosy (reviewed in Mira 2006). In a Nepalese sample, the T allele of the SNP rs2228570 (VDR-*FokI*) was a statistically significant risk factor for T1R [p = 0.032; OR = 1.31 (1.01-1.68)] (Sapkota et al. 2010). Interestingly, the activation of human macrophages through TLR2/1 signalling upregulates *VDR* expression, leading to the secretion of the antimicrobial peptide cathelicidin and the killing of *M. tuberculosis* (Liu et al. 2006). Likewise, active vitamin D robustly stimulates the expression of *NOD2* in the primary human monocyte and epithelial cells from CD patients through ligation with VDR (Wang et al. 2010). These observations point to the existence of a common pathway for the host immune defence against mycobacteria (Verway et al. 2010).

Natural resistance-associated macrophage protein 1 (NRAMP1) - *NRAMP1*, also known as *SCL11A1,* is a multi-pass membrane protein that mediates the transport/transition of divalent metals (iron and manganese). In human blood, polymorphonuclear leukocytes are the most abundant site of *NRAMP1* mRNA expression (Canonne-Hergaux et al. 2002). *Nramp1* controls susceptibility to several intracellular macrophage pathogens in the mouse (Vidal et al. 1995, Govoni & Gros 1998). Studies of the human homologue *NRAMP1* have resulted in evidence of both linkage and association of the region/gene with leprosy phenotypes (Abel et al. 1998, Alcais et al. 2000, Meisner et al. 2001). A meta-analysis of 36 studies confirmed the association of *NRAMP1* polymorphism with tuberculosis susceptibility (Li et al. 2011). Studies in carefully selected patient populations showed a strong association of *NRAMP1* with primary tuberculosis (Greenwood et al. 2000, Malik et al. 2005). Susceptibility to other infectious and immune-mediated diseases, such as leishmaniasis (Mohamed et al. 2004), human immunodeficiency virus infection (Marquet et al. 1999), CD (presence of MAP DNA) (Kojima et al. 2001, Stewart et al. 2010) and systemic lupus erythematous (Pedroza et al. 2011) has also been related to *NRAMP1* variants.

A recent study in a Brazilian sample of 201 leprosy cases revealed that the SNP 274C/T of *NRAMP1*is associated with LR. An unusually high frequency of LR was observed in this population sample. However, the authors did not clarify if LR was a criterion of sample selection in the study (Teixeira et al. 2010). The presence of the "C" allele was a risk factor for T1R ($p_{fisher} = 0.03$), while being protective for T2R ($p_{fisher} = 0.04$). Interestingly, this is the same polymorphism that has been shown to be a major risk factor for primary tuberculosis in a paediatric sample of mixed ethnic background (Malik et al. 2005).

Over the past decade, technological advances in genetic research improved our understanding of the molecular basis of leprosy pathogenesis (Alcais et al. 2000, 2007, Mira et al. 2003, 2004, Ranque et al. 2005, Zhang et al. 2009, Alter et al. 2010, Orlova et al. 2011). However, only recently has the focus of the scientific community turned towards deciphering the mechanisms behind LR.

The first genetic association studies of LR led to exciting results. However, classic case-control designs raise the problem of how to properly select comparable groups when studying the LR phenotype. Defining cases and controls represents a crucial and challenging step prior to the data analysis. Only individuals at equal risk for the outcome should be selected as controls, as the inclusion of patients with T2R that rarely develop T1R or patients with polar leprosy forms (TT and LL) as controls for T1R can introduce bias. Given the striking differences in the risk for T1R across leprosy forms, it is possible that polar form patients develop T1R by different mechanisms than borderline patients. Likewise, patients with tuberculoid forms of leprosy (TT, BT) should not be included as con-

trols in a T2R study, as such individuals are not known to develop T2R. In addition, the co-variables that impact at the risk for LR (e.g., age at leprosy onset) add heterogeneity to both the case and the control group and should be accounted for in the multivariate or stratified analyses. Finally, the genetic association studies performed in LR lack replication. This issue is important, as only well-replicated results can provide the basis for functional follow-ups to the genetic findings. The functional validation of the replicated genetic associations will provide better insight into the genetic component of LR and help to unravel the mechanisms underlying this immune dysfunction. Although LR will continue to be a public health problem for many years, the knowledge of the predictive factors for LR will provide the means for the timely identification of at-risk leprosy patients. Additionally, LR can serve as a model for immune dysregulation when studying other infectious and inflammatory diseases.

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