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Toll-like receptor 1 (TLR1) N248S single nucleotide polymorphism is associated with leprosy risk and regulates immune activation during mycobacterial infection

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Abstract

TLR1 variants N248S and I602S have been described associated with leprosy, but different results were found. Here, we performed both a case-control and family-based studies followed by replication in two case-control samples from Brazil, enrolling 3,162 individuals. Results indicated risk association between *TLR1* 248S and leprosy in case-control ($OR_{SS\ genotype}$= 1.81, p=0.004) and familial study (z=2.02, p=0.05). This association was consistently replicated in other populations ($OR_{Combined}$=1.51, p-value<0.001), corroborating 248S as a susceptibility factor for leprosy. Additionally, we demonstrated that PBMCs carrying 248S produce lower log(TNF/IL-10) when stimulated with *M. leprae*, but not with LPS or PAM_3cysK_4. The same effect was observed after infection of PBMCs with BCG Moreau, but not other strains. Finally, molecular dynamics simulations indicated that 248S-TLR1 structure is different than 248N-TLR1. Our results suggest that *TLR1* 248S is associated with leprosy risk, consistent with its hypo-immune regulatory function.

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Introduction

Interaction of bacterial, fungi and viral components with Pattern Recognition Receptors (PRRs), such as Toll-like receptors (TLRs), activates the NF-kB pathway, triggers inflammation and initializes events of the adaptive immune response [1-3]. It has been suggested that subtle genetic variations in relevant genes of the innate and adaptive immune responses are regulating every step of the host-pathogen interaction [4, 5], while low genetic variability of *M. leprae* [6, 7] suggests that host genetic factors may be of major influence towards *M. leprae* infection and progression of leprosy.

In this scenario, pathways triggered by PRRs, such as TLR1 and NOD2, are key to define leprosy outcome. TLR1 and TLR2 cooperate to sense mycobacterial triacylated lipoproteins while the activation of TLR1/TLR2 dimer by *M. tuberculosis* lipoproteins triggers the TLR-mediated antimicrobial response, reducing the viability of intracellular bacilli [8, 9]. Polymorphisms at *MRC1* and *NOD2* genes have been associated with increased leprosy risk [10-12]. Likewise, polymorphisms located at *TLRs* genes were repeatedly associated with leprosy *per se* and leprosy reactions [13-18]. The S allele of I602S (T1805G) polymorphism has been associated with leprosy resistance and to the impairment of molecule expression at the cell surface, NF-κB signaling and production of pro-inflammatory cytokines [13, 15, 19]. However, I602S was not found associated with the disease in a population from Bangladesh [17]. Also, the same study described the 248SS genotype of another non-synonymous SNP, N248S (G743A), associated with leprosy risk [17]. Interestingly, allele N of TLR1 N248S has been associated with TLR1 decreased expression or lower induction of cytokines [2, 20]. Here we

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investigate whether N248S and I602S SNPs at TLR1 are associated with leprosy and the downstream activation of the TLR1 pathway.

Materials and Methods

Subjects and Study Design

We performed a case-control study in a population from Bauru, a city in São Paulo state, at Lauro de Souza Lima Institute (ILSL) and a family-based study from Almenara population, Minas Gerais state. Then, we applied a stepwise replication approach to include case-control studies from Rio de Janeiro (Rio), Fiocruz and from Rondonópolis (ROO), Mato Grosso state. In each case-control cohort, unrelated blood donors from the same endemic area as patients were recruited as controls. The familial sample included households of leprosy cases, composed originally by trios formed by the index case and their biological parents. For those in which one of parents was not present, siblings were included to infer the genotype of the absent parent. All patients were classified according to the Ridley and Jopling [22], and also according to the 1998 World Health Organization (paucibacillary or multibacillary). All subjects were ethnically classified as caucasoids, mestizoes or blacks, according to morphological characteristics of both the individual as well as his/her family. General characteristics of all samples used in these studies are presented in Supplementary table 1.

For functional studies, a group of 24 healthy female individuals with a mean age of 45 years was selected among staff of the ILSL, in order to represent individuals carrying 248NN genotype (n=9), 248NS (n=9) and 248SS (n=6). A

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second sample was enrolled among health workers of Rio/FIOCRUZ, and included 22 individuals: 5 females and 12 males, mean age of 33 years old, distributed according to the genotype as follows: 248NN (n=6), 248NS (n=13) and 248SS (n=3). All individuals enrolled in the current study signed an informed consent. The study protocol was approved after revision by local ethical boards of involved institutions.

DNA extraction and SNP genotyping

Genomic DNA was extracted from frozen blood samples by the salting out method. N248S polymorphism (rs4833095, A743G) was genotyped by real-time PCR allelic discrimination and reactions were carried out using a TaqMan Assay from Applied Biosystems (Assay ID: C__44103606_10) according to manufacturer's instructions. The I602S SNP (rs5743618, T1805G) was genotyped using nested PCR, with conventional reaction performed as described by Johnson et al., 2007 [13]. Then, the amplicons were used for real-time PCR reactions with TaqMan Design Assay provided by Applied Biosystems. All real-time reactions were conducted using the StepOne Plus Real-Time PCR System, and the genotyping was based on allelic discrimination, through the StepOne 2.1 software (Applied Biosystems).

Sequencing

In order to identify other possibly associated SNPs at the TLR1 coding region we sequenced PCR products of the *TLR1* gene (from 103 to 1925 gene position) in 114 healthy controls. Briefly, we used two pairs of overlapping primers for both PCR and sequencing: TLR1F103: 5'-GGT CTC ATC CAC GTT CCT AAA-3'/ TLR1R1100:5'-TTT TCA AAA ACC GTG TCT GTT-3';

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b)TLR1F935:5'-TCG GTT TTC CGC AAA GTT AT-3'/ TLR1R1925:5'-AAA TAA ATG CAT GAA ACT GGA GAT-3'. Sequencing reactions were performed with Big Dye® Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems), using Applied Biosystems 3730 DNA Analyzer. The sequences were analyzed by SeqScape software 2.1 (Applied Biosystems), and compared against *TLR1* reference sequence (GenBank accession number: NM_003263.3).

Peripheral blood mononuclear cells (PBMCs) culture

PBMCs were purified using Histopaque (Sigma, St. Louis, USA) and cells were cultivated in 24-well plates at 5 x 10^5 cells/well concentration in RPMI-1640 medium with supplements as described [23]. PBMCs of individuals from Bauru were stimulated with (i) 100 ng/mL of PAM3CysK4 (PAM, *N*- palmitoyl-*S*-[2,3-bis(palmitoyl-oxy)-(2*RS*)-propyl]-[*R*]-cysteinyl-[*S*]-seryl-[*S*]lysyl-[*S*]-lysyl-[*S*]-lysyl-[*S*]-lysine, Invitrogen – San Diego – USA); (ii) 100 ng/mL of *Escherichia coli* lipopolysaccharide (LPS, Sigma, St. Louis, USA); and (iii) 10 µg/mL of sonicated *M. leprae* (ML) antigen. In study from Rio population, blood cells were cultivated under the same conditions, except for cell concentration (3x10^5 cells/well) and stimuli with live *Mycobacterium bovis* BCG Danish, Moreau (Brazilian strain) and Pasteur in multiplicity of infection (MOI) 10:1, without antibiotics. Cultures were kept at 37°C in a humidified 5% CO2 atmosphere for 18h (Bauru samples), 24h or 72h (Rio samples). After this, the cell suspension was harvested and centrifuged. In all samples, the supernatant was kept at –80°C for cytokine measurements while only in Bauru samples, the cell pellet was resuspended in the residual volume and evaluated by flow cytometry.

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ELISA

The levels of TNF and IL-10 in the supernatant of PBMC cultures were evaluated by ELISA in two independent assays. For the Bauru assay, the supernatant was collected 18h after the stimuli. In PBMC samples from Rio, the supernatant was collected after 24h and 72h for TNF and IL10 quantification, respectively. The commercial kits Quantikine® HS (R&D Systems, Minneapolis, MN, USA) for IL-10, and BD OptEIA (BD Biosciences) for TNF, were used according to manufacturer instructions.

Flow Cytometry

TLR1 expression in monocytes was assessed for fifteen individuals, five from each genotype of N248S polymorphism, right after the purification or subsequently to the cell culture. Cells were incubated with PE-conjugated anti-human TLR1 (eBiosciences, San Diego, USA) and APC-conjugated anti-human CD11c (BD Biosciences, San Jose, USA). Isotype matched antibodies were used as negative controls and AB human serum was used to block non-specific binding. A total of 70,000 events were reached in a FACScalibur flow cytometer (BD Bioscience). Data were analyzed using FACS Express Version 3 software (De Novo Software, Los Angeles, CA).

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Comparative modeling and Molecular Dynamics

The 8 haplotypes formed by polymorphisms at positions N248S, H305L and P315L (NHL, NHP, NLL, NLP, SHL, SHP, SLL, and SLP) were incorporated into the crystal structure (PDB code 2Z7X), that corresponds to NHP haplotype, by using the Pymol program [24]. The simulations were run on Gromacs 4.5.4. [25]. The systems were energy-minimized using periodic boundary conditions to fit the atomic positions to the GROMOS96 force field [26]. The net charge of the complexes was held neutral by adding 4 Na+ counter ions. The equilibration phase consisted of 2 stages: 3 nanoseconds, restraining the protein coordinates to their initial positions by applying harmonic potential with spring constant K=1000 kJ/(mol nm2) to stabilize water molecules around the protein at 100K, 200K, and 300K, respectively, plus 2 ns at 310K, keeping the complexes free, totalizing 5 ns. After the equilibration period, simulations evolved freely for more 50 ns, saving trajectories and velocities every 5 picoseconds so that to analyze structural features.

Statistical Analyses

For case-control samples, all statistical analyses (genotypic, allelic and haplotypic with and without adjustment for the covariates) were performed as previously described [23, 27]. SNPs at *TLR1* were tested for deviations from Hardy-Weinberg Equilibrium (HWE) in control groups, and were in agreement in all populations studied. We evaluated if allelic dose and their OR values were directly proportional (allele-dose effects) by the Cochran-Armitage trend test. Linkage disequilibrium analysis between markers was performed through r^2 statistics in the control group. All analyses were performed using the statistical

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software R for Windows version 2.14.0 [28], using “genetics” [29], “haplo.stats” [30] and “coin” [31] packages.

In the family-based study, a Transmission Disequilibrium Test (TDT) was performed, based on counts of transmission of a marker allele from heterozygous parents to affected child [32]. We obtained the number of transmitted and non-transmitted alleles using the ‘tdthap’ package from R [33]. The analysis of the family study was conducted in software FBAT - Family Based Association Test [34].

For TRL1 expression, we considered the percentage of cells expressing the receptor among different groups or stimuli. Data about TLR1 expression and cytokines measurements were analyzed applying non-parametric Mann-Whitney U test to compare medians between S carriers (genotypes NS + SS) and non-carriers (genotype NN) of SNP N248S. Analyses were performed using GraphPad Instat version 3.00 for Windows (GraphPad Software, San Diego, CA, USA), considering p values < 0.05 as statistically significant.

Results

The N248S SNP is associated with leprosy

The frequencies of genotypes, alleles and minor allele carriers of N248S and I602S SNPs at the Bauru population are shown in Table 1. The results indicated that 248 genotypes (248NS and 248SS), 248S allele and 248S carriers were significantly associated with leprosy susceptibility. In contrast, although the

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602S variant has been more frequent in controls (suggesting protection), it was not significantly associated with leprosy.

To check the presence of other SNPs that might explain the 248S association with leprosy susceptibility, sequencing of the entire polymorphic region in 114 individuals were performed. Linkage disequilibrium (LD) analysis was conducted and N248S showed LD with a synonymous SNP S506S (r^2=0.92). Also, moderate LD was detected between N248S and I602S (r^2= 0.67). No other important LD was observed among any SNPs detected (Figure 1). Then, we performed a N248S-I602S haplotypic analysis (Table 2). Using N248/602S haplotype as baseline, the combination S248/I602 was, in the same direction, associated with leprosy susceptibility ($OR_{corrected}$=1.26; p=0.03). Furthermore, similar to that seen previously in Indians, the arrangement 248S/602S was virtually absent (frequency <0.01) in Brazilians (Table 2).

In the family-based replication study from Almenara, the TDT revealed that the S248 allele was over-transmitted to affected individuals (z=2.15, p=0.05, Supplementary table 2), confirming the association with susceptibility to leprosy per se. The familial sample was tested for I602S, and despite the 602S allele has been under-transmitted to affected individuals, the association was marginally significant (data not shown, z=2.16, p=0.06). In Almenara sample, N248S and I602S also are in moderate LD (r^2= 0.55) and consistently, the 248S/I602 haplotype was associated with risk to leprosy, replicating the case-control result (Table 2).

Considering the relevant genetic effect of N248S observed in two populations, we turned our focus to this SNP, and proceeded with a second replication

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experiment using two independent case-control studies with population samples from Rio and ROO cities. In the Rio sample, the 248SS homozygous genotype and the 248S allele were significantly more frequent in patients as compared to controls (Table 3), replicating the Bauru data in the same direction. Furthermore, the allele-dose effect was confirmed by the Cochran-Armitage trend test (χ^2 = 21.23, p< 0.001), since the OR value was more prominent for SS genotype than for NS genotype. The second case-control replication in ROO also revealed association of 248S (248SS genotype and S carriers) with susceptibility to leprosy (Table 3). A combined analysis involving all three case-control population samples (1,276 cases and 1,353 controls) increased the power of the study and corroborated the associations previously observed between 248S allele at *TLR1* and leprosy susceptibility (Table 4). Also, in combined analysis OR values shown allele-dose effect (χ^2 = 15.20, p< 0.001).

Additionally, we performed a systematic review of the literature for association studies between N248S and leprosy *per se* that could qualify for a meta-analysis, but the search resulted in only one eligible study, a case-control performed by Schuring and colleagues [17] in Bangladesh (Supplementary table 2). The meta-analysis showed a risk pooled OR (1.22,p<0.0001), supporting the association between 248S and susceptibility to leprosy *per se* (Supplementary figure 1).

The N248S SNP is associated with hypo-responsive immune activation

We then investigated the effect of the N248S variation over TLR1 function. As presented in Figure 2A, we evaluated the expression of TLR1 in monocytes

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obtained from healthy donors PBMCs non-carriers and carriers of allele “S” (NN vs NS+SS), cultured with either PAM, LPS, and *M. leprae* (ML). The monocytes were assessed immediately after isolation (ex-vivo) for TLR1 expression. There were no statistical differences in TLR1$^+$ cells between 248S carriers (248NS/SS) and non-carriers, under any stimulus (Figure 2A). The stratification for I602S (II vs SS + SI) did not result in significant differences in the expression of TLR1 either (data not shown). Next, we examined cytokine levels in the supernatant of stimulated PBMCs obtained from 248S healthy carriers and non-carriers recruited in Bauru. When evaluated individually, IL10 and TNF levels were not significantly different among carriers and non-carriers of 248S allele (data not shown). However, the log(TNF/IL10) ratio was significantly lower ($p< 0.0001$) in 248S carriers stimulated with sonicated antigen of *M. leprae* (ratio = -1.3) than non-carriers (ratio= 0.6) (Figure 2B). Yet, the log(TNF/IL10) ratio was not different when we compared 602S carriers (602IS/SS) and non-carriers (602II) (data not shown). The IL-6 levels in the supernatant were also stratified by N248S and I602S, but the differences were not significant for either (data not shown). We also performed an independent sample recruitment of healthy subjects from Rio. Similarly to the results from the Bauru sample, the log(TNF/IL10) ratio was significantly lower ($p=0.01$) in 248S carriers (ratio= 0.1) than in non-carriers (ratio= 0.6), but significance was reached only when BCG Moreau was used as stimulus (Figure 2C).

Finally, to investigate the influence of N248S over TLR1 tridimensional structure, we conducted a comparative modeling analysis using haplotypic combinations – therefore, considering different amino acids at positions N248S, H305L and P315L, selected based on their shared position an important TLR1

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site for lipopeptide recognition [38]. It was not possible to include the I602S in comparative modeling, since the X-ray crystallography of the region containing this SNP was not deposited in the protein database [39]. The molecular dynamics approach has the advantage to consider the positional information of the atoms along time, in contrast to other methods that predict interactions and properties from a static snapshot. Molecular dynamics showed that, when compared to baseline N248/H305/P315, the presence of the haplotype S248/H305/P315 resulted in a change of electrostatic potential and solvation layer around TLR1 (Figure 3A). An estimate of the electrostatic surface potential showed a redistribution of atomic partial charges, yielding a more negative environment in the presence of S248, while in the vicinity of N248S the potential is very close to neutral. The analysis of the number of hydrogen bonds formed during the molecular dynamics (MD) showed a lower number of bonds for the four 248S haplotypes indicating that the transition from asparagine (Asn) for a serine (Ser) results in a slight loss of interaction with water (Figure 3B).

Discussion

The most important finding of our study is that *TLR1* N248S SNP controls responses to mycobacteria and contributes to leprosy susceptibility. Also, N248S regulates immune responses to BCG stimulation, which is crucial towards understanding of customization of vaccines among “hypo-responsive” individuals.

We found a very reliable genetic association effect of *TLR1* 248S with leprosy susceptibility. This genetic finding was obtained through two replications

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of an initial positive association signal in which 248S variation were found at increased risk of developing leprosy in case-control and familial-based studies. The association of the SS genotype and risk of leprosy was previously reported in a Bangladesh population [17]. This paper was added to our sample for meta-analysis, after our systematic review; as a result, the study reached a total of 4,207 individuals enrolled, and a pooled OR of 1.22 (p<0.0001). Curiously, allele 248S may be involved with susceptibility control of other mycobacterial diseases like tuberculosis in African-Americans that was also associated the 248S allele [40].

The present findings implicating N248S SNP along with the absence of association observed between I602S and leprosy in our study is intriguing because other studies conducted among Turkeys [13] and Indians [18] implicated 602S as associated with leprosy protection. While few papers have provided a detailed analysis of other SNPs in the locus in leprosy studies, in TB, both the 602S allele [40] and the 602I allele were associated with risk [20]. Also, reanalysis of genome-wide study did not find I602S associated with leprosy among the Chinese [41]. Maybe the heterogeneous distribution of I602S frequencies in different regions of the world, and the changes in LD could explain differential associations in the locus. In Caucasians [2] the r^2 between N248S and I602S was around 0.80, whereas in Indians the r^2 is reported to be around 0.08 [18]. Yet, the arrangement of the 248S/602S haplotype was virtually absent in our study and in Indians, although among Euro-Americans this haplotype has a frequency of 7% [19] (Table 2).

Even though we have evaluated populations from different geographical regions of Brazil, we calculated the linkage disequilibrium between N248S and

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I602S in all of them (data not shown), and observed a moderate LD (between 0.55 and 0.67). Thus, we cannot ignore the possibility of genetic confounding by I602S in N248S effect. Nevertheless, our genetic study indicated an important association of N248S with leprosy and inflammatory profile, but only a marginal significantly association of I602S, suggesting a pronounced effect of N248S in Brazilians. Analysis involving a larger number of SNPs genotyped in the region after sequencing (Figure 1) suggested that only S506S is in high LD with N248S in a way that we could not rule out that S506S is the functional SNP since other synonymous SNPs have been associated with a myriad of diseases [42]. In this regard, it is likely that different genotypes contribute to the same phenotype, i.e leprosy outcome.

Given our results, we turned our focus to the N248S effect, since it was genetically and biologically associated with leprosy by independent results. However, additional studies still need to be conducted to understand the contribution of the genetic effect of I602S with leprosy in the Brazilian population, to evaluate the LD profile of an extended region in the locus, since more thorough study of the entire block of *TLR1* in different populations in associations with diseases is still missing.

Overall, our functional and molecular dynamics results indicates that N248S is indeed has a important biological role for a N to S aminoacid change. To advance on the understanding of the biological effect underlying the strong evidence for genetic association between N248S and leprosy, we investigated the functional profile of PBMCs obtained from carriers and non-carriers of allele “S”. Unlike previously reported [20], the expression of TLR1 was detectable in PBMCs surface of both S carriers and non-carriers. But, the investigation of

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genotype/phenotype correlation revealed that cells from 248S carriers had a lower TNF/IL10 ratio when mycobacterial stimulation was used. Interestingly, 248S were hypo-responsive for every BCG strain tested; however, the effect only reached statistical significance when BCG Moreau was used. It has been reported that host-pathogen joint effects on susceptibility to mycobacterial infections [43, 44]. Also, the observation suggests the existence of specific host-pathogen interactions impacting on vaccine response. Curiously, a specific agonist of TLR1 induced similar cytokine production between the two genotype groups. This observation raises the discussion on whether a possible approach could be to use the specific TLR1 agonists as adjuvant in addition to BCG vaccination, which could contribute to improve immune response in susceptible individuals. Recently, TLR pathway variations have been related with altered *in vivo* immune response to BCG after newborn vaccination, suggesting novel adjuvant vaccine strategies [45]. The use of TLRs ligands has already been studied to potentiate vaccine-induced responses to provide not only prophylactic, but therapeutic protection against infectious diseases [46, 47].

Polymorphism N248S, as well as H305L and P315, are located in the extracellular domain of TLR1, more specifically at a leucine-rich repeat (LRR) [48], which is responsible to define specificity towards different lipopeptides agonists [38]. Interestingly, our simulations by molecular dynamics revealed, for the first time, an influence of N248S “S” allele over electrostatic potential and water interactions; this provides a reasonable explanation for the functional effects observed associated with this polymorphism. Indeed, a subtle decrease in water interaction may reflect a diminished ability of the TLR1-248S molecule to ligate to hydrophilic components. The molecular analysis adds important

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information suggesting that lower immune response, as measured by TNF/IL-10 ratio, could be associated with molecular structure of TLR1 248S.

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Figure Legends

Figure 1: Detailed sequence analysis of *TLR1* coding region in Brazilians. A: Schematics *TLR1* exons (grey and white squares) and introns (lines). The arrows represent two PCR fragments sequenced region encompassing most important SNPs. A total of 1.823pb of *TLR1* coding region were sequenced. LRR (Leucine-Rich Repeats), trans-membrane, and TIR (Toll-Interleukin 1 Receptor) domains are exemplified. The frequency and positions of each SNP are indicated in the table (N= 114). B: Linkage disequilibrium (LD) map of polymorphisms identified at *TLR1*. The r^2 values are shown in each box (Haploview software).

Figure 2: TLR1 expression and TNF/IL10 ratio in non-carriers (NN) and carries (NS +SS) of S allele of N248S. A: Monocytes were assessed immediately after isolation (ex vivo) from healthy subjects, or after cultured with PAM3CysK4 (PAM, 100ng/mL), lipopolysaccharide (LPS, 100ng/mL), sonicated antigen of *M. leprae* (ML, 10µg/mL). Results were obtained by flow cytometry and expressed as percentage of monocytes. B: PBMCs from Bauru healthy subjects were stimulated with PAM, 100ng/mL, LPS, 100ng/mL or ML 10µg/mL. After culture for 18h, cytokines production was evaluated in supernatants by ELISA. C: PBMC were stimulated with BCG Danish, Moreau or Pasteur strains (MOI 10:1) for 24h (TNF) or 72h (IL10). Cytokines production was evaluated by ELISA. The lines represent the median value of each group, which were compared by Mann-Whitney t test. *p = 0.01, **** p < 0.0001

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Figure 3: Effect of N248S in electrostatic profile and hydrogen bonds at TLR1. The variation at 248S residue was compared using as baseline N248/H305/P315. A: The figure corresponds to the initial state (t=0 ns) for both haplotypes. The white circles encompass the N248 or S248 region. It is remarkable the electronegative profile in the S248 area, represented by the red color. B: Number of hydrogen bonds between solvent and N248S/H305L/P315L combinations in molecular dynamics. Results are shown as mean, and compared by Mann-Whitney t test. ****p<0.0001

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Tables

Table 1. Frequencies of N248S and I602S SNPs at *TLR1* in case-control study in Bauru population.

SNP	Genotype/ allele	N (frequency)[a]		Logistic Regression Model	
		Cases	Controls	OR [95% CI] p-value	OR [95% CI] p-value
N248S[c] (rs4833095)	**NN**	95 (0.21)	105 (0.28)	reference	reference
	NS	227 (0.50)	174 (0.46)	**1.44 [1.02-2.02] p= 0.03**	**1.58 [1.10-2.24] p= 0.01**
	SS	128 (0.28)	97 (0.26)	**1.46 [0.99-2.13] p=0.05**	**1.81 [1.20-2.71] p=0.004**
		450	376		
	Allele N	417 (0.46)	384 (0.51)	reference	reference
	Allele S	483 (0.54)	368 (0.49)	1.21 [0.91-1.59] p= 0.17	**1.34 [1.01-1.79] p= 0.04**
	S carriers	355 (0.78)	271 (0.72)	**1.45 [1.05-1.99] p=0.02**	**1.65 [1.18-2.30] p= 0.003**
I602S[c] (rs5743618)	**II**	171 (0.38)	133 (0.35)	reference	reference
	IS	213 (0.47)	191 (0.50)	0.87 [0.64-1.16] p=0.35	0.79 [0.58-1.08] p=0.15
	SS	68 (0.15)	55 (0.15)	0.96 [0.63-1.46] p=0.85	0.83 [0.53-1.28] p=0.40
		452	379		
	Allele I	555 (0.61)	457 (0.60)	reference	reference
	Allele S	349 (0.39)	301(0.40)	0.95 [0.72-1.26] p=0.74	0.88 [0.66-1.18] p=0.42
	S carriers	281 (0.62)	246(0.65)	0.89 [0.66-1.18] p=0.42	0.80 [0.59-1.08] p=0.15

[a] Results are shown as total counts (frequency). [b] OR, 95%CI (95% confidence intervals) and p-value obtained by logistic regression model and adjusted for covariates sex and ethnicity. [c] Global p-value=0.07 (N248S genotypes versus the reference) and 0.60 (I602S genotypes versus the reference). Bold values express statistically significant results.

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Case-Control					TDT			Previous studies	
								Euro-Americans (Hawn et al, 2007)	New Delhi/India (Wong et al, 2010)
N248S/I602S[a]	**Frequency**		**Logistic Regression Model**		**Frequency**	**T:U[c]**	**Z (p-value)**	**Frequency**	
	Case	**Control**	**OR [95% CI] p-value**	**OR [95% CI] [b] p-value**					
N248/I602	0.08	0.12	0.70 [0.49-1.00] (p=0.05)	**0.67 [0.47-0.98] (p=0.03)**	0.14	18:16	-0.25 (0.78)	*	0.42
248S/I602	0.52	0.48	1.13 [0,92-1.40] (p=0.23)	**1.26 [1.01-1.57] (p=0.03)**	0.53	49:33	**2.32 (0.03)**	0.23	0.47
N248/602S	0.38	0.39	Reference	Reference	0.32	27:45	-2.24 (0.05)	0.70	0.09
248S/602S	*	*	*	*	*	*	*	0.07	*

Table 2: Frequencies of N248S/I602S haplotype at *TLR1* in case-control and familial studies and association with leprosy.

[a] Haplotype as shown as frequency estimated by maximum likelihood (* the haplotype was absent - frequency< 0.01). [b] OR and p-value obtained by logistic regression model and adjusted for covariates sex and ethnicity. [c] Number of transmitted (T) versus untransmitted (U) by Transmission Disequilibrium Test (TDT). Bold values express statistically significant results.

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Table 3. Frequency of N248S at *TLR1* in case-control studies from Rio de Janeiro and Rondonopolis populations.

	Rio				**Rondonopolis**			
	N (frequency)[a]		**Logistic Regression Model**		**N (frequency)[a]**		**Logistic Regression Model**	
N248S[c] Genotype/allele	**Cases**	**Controls**	**OR [95% CI] p-value**	**OR [95% CI][b] p-value**	**Cases**	**Controls**	**OR [95% CI] p-value**	**OR [95% CI][b] p-value**
NN	142 (0.24)	142 (0.25)	reference	reference	76 (0.19)	107 (0.26)	reference	reference
NS	234 (0.40)	288 (0.51)	0.81 [0.60-1.08] p=0.16	0.83 [0.61-1.12] p=0.23	206 (0.51)	197 (0.48)	**1.47 [1.03-2.09] p=0.03**	**1.47 [1.04-2.29] p=0.03**
SS	208 (0.36)	134 (0.24)	**1.55 [1.12-2.13] p=0.007**	**1.59 [1.13-2.22] p=0.006**	120 (0.30)	109 (0.26)	**1.55 [1.04-2.29] p=0.03**	**1.56 [1.05-2.31] p=0.03**
	584	564			402	413		
Allele N	518 (0.44)	572 (0.51)	reference	reference	358 (0.45)	411 (0.50)	reference	reference
Allele S	650 (0.56)	556 (0.49)	**1.29 [1.02-1.62] p=0.03**	**1.28 [1.01-1.63] p=0.04**	446 (0.55)	415 (0.50)	1.23 [0.93-1.62] p=0.14	1.24 [0.94-1.63] p=0.13
S carriers	376 (0.75)	430 (0.74)	1.04 [0.80-1.36] p=0.73	1.05 [0.79-1.38] p=0.72	326 (0.81)	306 (0.74)	**1.50 [1.07-2.09] p=0.02**	**1.50 [1.07-2.09] p=0.02**

[a] Results are shown as total counts (frequency). [b] OR, 95%CI (95% confidence intervals) and p-value obtained by logistic regression model and adjusted for covariates sex and ethnicity. [c] Global p- value (N248S genotypes versus the reference): p <0.001 (Rio) and p= 0.05 (Rondonopolis). Bold values express statistically significant results.

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Table 4. Frequency of N248S at *TLR1* in case-control study combining Bauru, Rio de Janeiro and Rondonopolis populations.

	N (frequency)[a]		Logistic Regression Model	
N248S[c] Genotype/allele	**Cases**	**Controls**	**OR [95% CI] p-value**	**OR [95% CI][b] p-value**
NN	280 (0.22)	354 (0.26)	reference	reference
NS	622 (0.49)	659 (0.49)	1.19 [0.98-1.44] p=0.07	**1.22 [1.01-1.49] p=0.03**
SS	374 (0.29)	340 (0.25)	**1.39 [1.12-1.72] p=0.003**	**1.51 [1.21-1.89] p< 0.001**
	1276	1353		
N	1182 (0.46)	1367 (0.51)	reference	reference
S	1370 (0.54)	1339 (0.49)	**1.18 [1.01-1.37] p=0.03**	**1.23 [1.05-1.44] p=0.008**
S carriers	996 (0.78)	999 (0.73)	**1.26 [1.05-1.50] p=0.01**	**1.31 [1.01-1.58] p=0.003**

[a] Results are shown as total counts (frequency). [b] OR, 95%CI (95% confidence intervals) and p-value obtained by logistic regression model and adjusted for covariates sex, ethnicity and population. [c] Global p-value=0.01 (genotypes *vs.* the reference). Bold values express statistically significant results.

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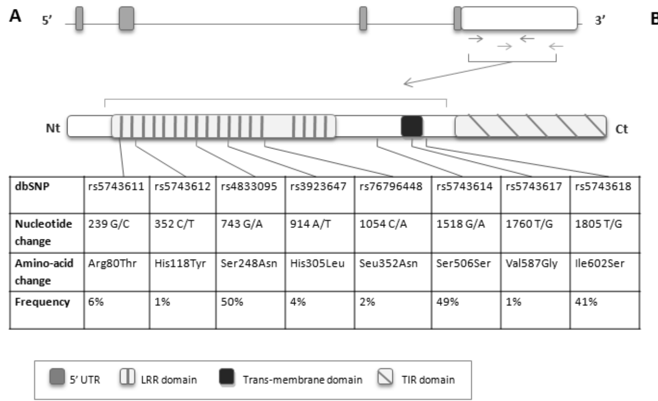
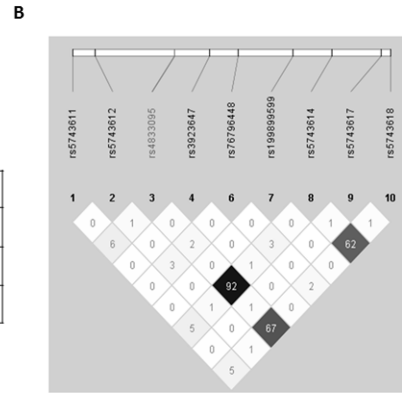


dbSNP	rs5743611	rs5743612	rs4833095	rs3923647	rs76796448	rs5743614	rs5743617	rs5743618
Nucleotide change	239 G/C	352 C/T	743 G/A	914 A/T	1054 C/A	1518 G/A	1760 T/G	1805 T/G
Amino-acid change	Arg80Thr	His118Tyr	Ser248Asn	His305Leu	Seu352Asn	Ser506Ser	Val587Gly	Ile602Ser
Frequency	6%	1%	50%	4%	2%	49%	1%	41%



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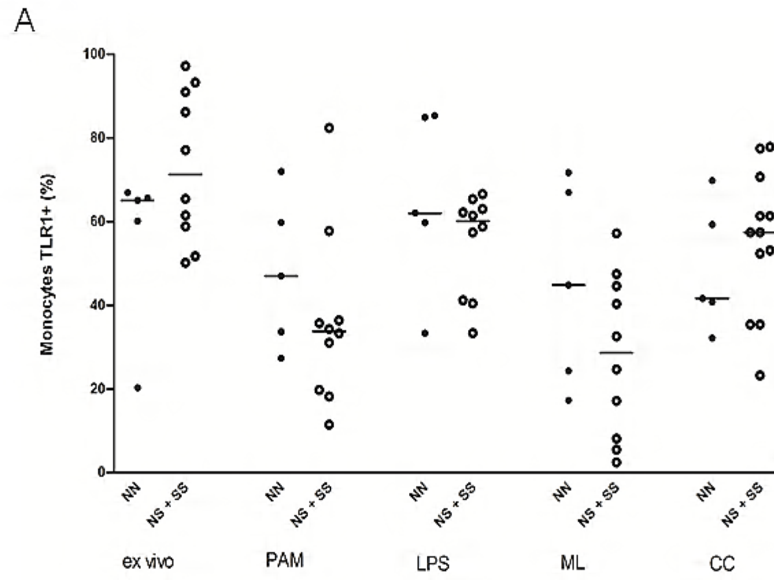

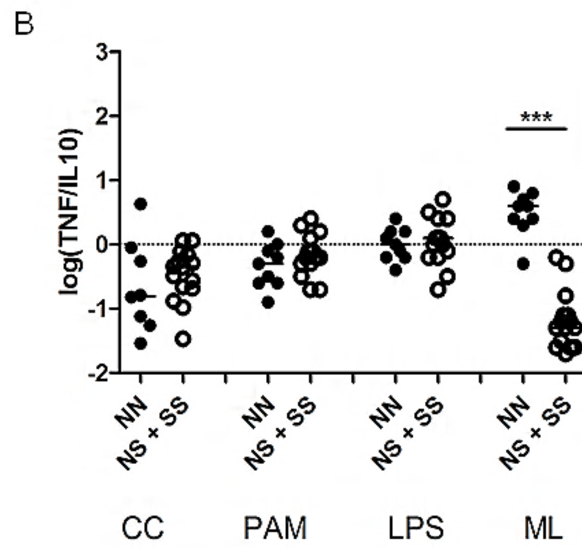

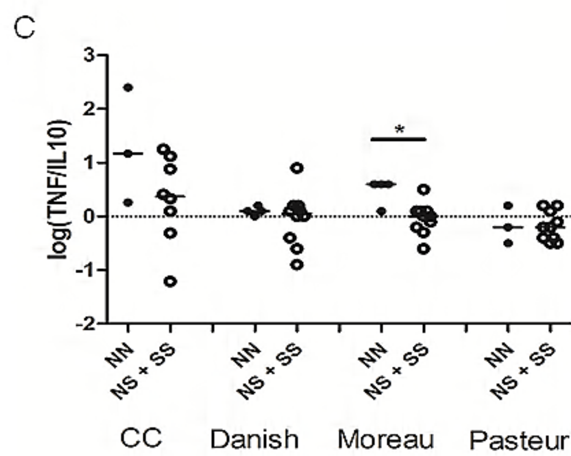

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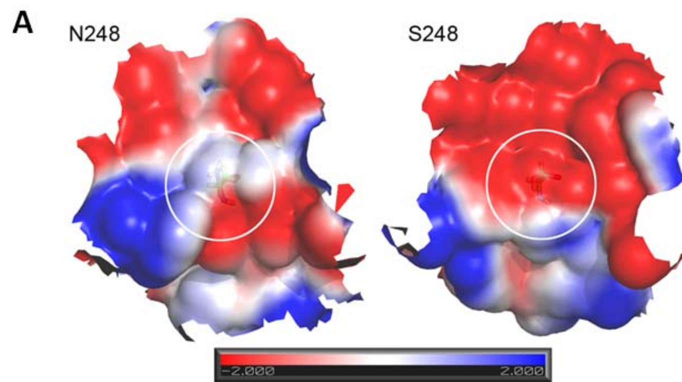

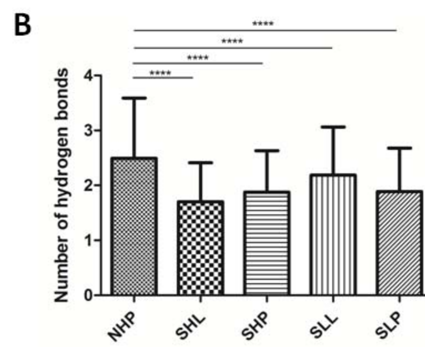

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