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Genetic ancestry effects on the distribution of toll-like receptors (TLRs) gene polymorphisms in a population of the Atlantic Forest, São Paulo, Brazil

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ABSTRACT

The innate immune system governed by toll-like receptors (TLRs) provides the first line of defense against pathogens. Surface-localized TLR1 and TLR6 are known to detect parasite components. TLR encoding genes were shown to display signatures of recent positive selection in Europeans and might be involved in local adaptation at immune-related genes. To verify the influence of Brazilian population admixture on the distribution of polymorphisms in TLRs, we analyzed the genotype frequencies of 24 polymorphisms distributed across five TLR genes in a Southeastern Brazilian population where autochthonous cases of malaria occur in small foci of transmission. The estimation of ancestry showed mainly European ancestry (63%) followed by African ancestry (22%). Mean proportions of European ancestry differed significantly between the genotypes of the TLR1 (I602S) gene and in the TLR6 (P249S) gene. The chance of having the G allele in TLR1 gene increases as European ancestry increases as well as the chance of having the T allele in the TLR6 gene. The 602S allele is related to a "hypo-responsiveness" possibly explaining the high prevalence of asymptomatic malaria cases in areas of Southeastern Brazil. Our results underline the necessity to include informative ancestry markers in genetic association studies in order to avoid biased results.

1. Introduction

The first line of defense against pathogens is provided by the innate immune system including toll-like receptors (TLRs) [1]. Ten TLR encoding genes have been described in humans dispersed all over the genome [1]. TLR1, TLR6, and TLR10 occur on the cell surface and are known to be activated by bacterial, fungal, and parasite components including flagellin and glycolipids [1]. TLRs are highly conserved, although cell-surface TLRs are less strictly constrained than intracellular TLRs [2]. In fact, the region including the TLR1/TLR6/TLR10 genes is under natural selection [3–5] and has been shown to have signatures of recent positive selection in Europeans [2,6] suggesting that they might be involved in local adaptation.

Single nucleotide polymorphisms (SNPs) are present in variable frequencies in different populations. They play an important role in different diseases to which populations are differently exposed, therefore, their analysis is important for genetic studies in humans. SNPs in TLR genes have been associated with susceptibility to various infectious diseases [1], including malaria [7–11]. Analysis of the genetic structure of human populations has become an important practical issue for the study of complex genetic disorders. Recent studies have shown that genetic ancestry and natural selection drive population differences in immune responses to pathogens [12]. Differences in frequency of alleles between ethnic groups and subgroups and admixture between different ethnic groups may result in frequent false-positive results or reduced power in genetic studies [13]. Thus, with the prospect of future genetic Association studies using SNPs in TLRs and malaria susceptibility, it is first necessary to characterize the genetic structure of the study population in order to avoid biases and falsely interpreted results.

Currently, one of the most diverse populations in the world is the Brazilian population, which resulted from five hundred years of interethnic admixture among European, African and Amerindian ancestors. Estimates of ancestry results produced by genetic studies have shown that the Brazilian population has a major contribution of European ancestry (around 75–77%) followed by African and Amerindian contributions [14,15]. The population from the southern region has

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displayed the highest proportion of European ancestry (87.7%) and a lower degree of variance of the estimates of individual admixture causing a significant genetic differentiation from the southeast, northeast, center-west and northern regions [15].

Considering the relevance of SNPs in important TLRs and their relationship with the European population coupled with the fact that the Southeastern Brazilian population has the second highest proportion of European ancestry (79.9%), we set out to analyze the connection between genomic ancestry in a population from Juquitiba region, a typical town in Southeastern Brazil in an area of Atlantic Forest with the second highest number of autochthonous malaria cases in São Paulo State [16]. For this, we used ancestry informative markers (AIMs) which are better indicators of genetic ancestry than physical appearances, such as skin color, used in self-declaration of ethnicity [17–20]. Our results analyze (i) the genotype frequencies of 24 polymorphisms distributed across five TLR genes (TLR1, TLR2, TLR4, TLR6 and TLR9) in a Brazilian population of the Atlantic Forest; (ii) the influence of Brazilian population admixture on the distribution of these polymorphisms; and (iii) the comparison of the allele and genotypic frequencies found in this population with those observed in African, European, and American populations from the 1000 Genomes Project obtained by International Genome Sample Resource (IGSR) by dbSNP database [21].

2. Materials and methods

2.1. Samples

The study was conducted in a population living along the Palestina road in Juquitiba (São Paulo State) (23°55′55″ S; 47°04′04″ W), situated in the Brazilian Atlantic Forest region (Fig. 1). Juquitiba County is located in the metropolitan area of São Paulo and covers an area of 521.6 km^2 with a population of 28 thousand inhabitants of whom 22.6% live in the rural area [22]. It has a significant area of preserved Atlantic Forest in the Metropolitan Region of São Paulo.

The sample of this study was composed of 195 individuals (96 men and 99 women) aged from fourteen to eighty-one years. All the participants signed informed consent forms. This project was approved by the Ethics Committee of Institute of Biomedical Sciences, University of São Paulo (CEPSH 1022/2011).

DNA was extracted from peripheral blood samples using the Illustra™ Blood Genomic Prep Mini Spin Kit (GE Healthcare, UK), according to the manufacturer's instructions.

Fig. 1. Origin of samples used in the study. Location of the County (Municipality) of Juquitiba (red dot) in São Paulo State, Brazil. Modified from Google Earth and Wikimedia Commons.

2.2. SNP genotyping

We analyzed the presence of 24 polymorphisms: (i) 20 non-synonymous SNPs: TLR1 I602S, TLR2 (P631H, R650Q, L658, P669L, R677W, F679I, I693T, E694D, Y715N, F722L, R723C, N729S, E738Q, P746S, R748H, R753Q), TLR4 (D299G, T399I) and TLR6 P249S; (ii) the two promoter SNPs at TLR9 in positions −1486 T.C and −1237 T.C as well as one SNP in the intron region at position 1174A.G; and (iii) a GT dinucleotide repeat that varies by approximately 12–30 repeats (GTn) that is present within the second intron, approximately 100 bp upstream of the translational start site of TLR2. PCR-restriction fragment length polymorphism approaches were used to determine genotypes of five SNPs: TLR1 I602S, TLR4 D299G, TLR6 P249S, TLR9 −1486 T.C and TLR9 −1237 T.C. The amplified PCR fragments were digested with appropriate restriction endonucleases and then resolved on 3% agarose gels. To ensure the validity of our genotyping methods, a known genotype for each SNP was used as a control. PCR primers, amplification conditions and the restriction enzymes used for each polymorphism are described in Supplementary Table 1. Eighteen SNPs were analyzed by PCR and sequencing. PCR primers and amplification conditions used for this analysis are also listed in Supplementary Table 1. TLR2 GTn genotypes were based on size discrimination of PCR products separated on a 3500 capillary sequencer (Applied Biosystems) at a 1/70 dilution. Allele sizes were measured by comparison with size standards using Gene Scan 500 software (Applied Biosystems). The lengths and relative abundance of peak heights in electropherograms were determined using the open-access STRand software [23]. The number of GTn repeats was categorized to short (S) (≤16 repeats or 96 bp), medium (M) (17–22 repeats or 98–108 bp) and large (L) (≥23 repeats or ≥110 bp) as described [24].

2.3. Ancestry informative marker analysis

The evaluation of genomic ancestry was conducted as described [25], using forty-eight biallelic ancestry informative markers (AIMs) and insertions-deletions (INDELS), from autosomal chromosomes. Estimation of the parental ancestry of the Brazilian samples was performed considering three parental populations: Africans (from Angola, Mozambique, Zaire, Cameroon, and the Ivory Coast), Europeans (mainly Portuguese), and Native Americans (individuals from indigenous tribes of the Brazilian Amazon region) [25]. The genotypes of the sample population from Juquitiba, São Paulo, Brazil, and parental populations (Europeans, Africans, and Amerindians) were analyzed together, assuming three main clusters (K = 3).

2.4. Statistical analysis

We used the Structure v.2.3.4 program for Bayesian model-based estimates of the proportion of ancestry for each subject [26–29]. The major populations of origin in Brazil are European, African and Amerindian, and hence three subpopulations (k = 3) were modeled. Quantitative genetic ancestry was estimated for individuals by including contemporary descendants of approximate populations of origin (pseudo-ancestors) [28]. The admixture model was employed assuming correlated population allele frequencies with a burn-in period of 100,000 and 1,000,000 iterations. All other analyses were conducted using packages contained in the R environment, version 3.3.2 [30].

The Genetics package was used to obtain: (i) the allele and genotype frequencies for each variant; (ii) deviations from Hardy-Weinberg equilibrium by the Chi-square test; and (iii) the linkage disequilibrium (LD) between pairs of loci using parameter D' [31]. Linkage disequilibrium was evaluated between the pairs of SNPs in the TLR1 (I602S) and TLR6 (P249S) genes located in the chromosome 4p14 region, TLR2 (P631H and R753Q) gene located in the chromosome 4q31.3 region, TLR4 (D299G and T399I) gene located in the chromosome 9q32–33 region and TLR9 (−1237, −1486 and 1174) gene

located in the chromosome 3p21.3 region.

We used the maximum likelihood method which uses the expectation-maximization algorithm (part of the haplo.stats package) to estimate the haplotype frequencies [32]. To assess whether the haplotypes are associated with differences in the proportions of ancestry the haplo.stats computer program was also used [32]. Thus, a score for each haplotype and a *p*-value for each hap.score were generated. If the haplotype is associated with an increase/decrease in ancestry, a positive/negative score for this haplotype is obtained.

To check for differences in the proportions of each of the ancestries between different genotypes we used analysis of variance (ANOVA) and Student's *t*-test in the agricolae package [33]. To graphically search the Association of polymorphisms with individually estimated ancestry, a binary logistic regression model was built using the ggplot2 package [34].

To compare the observed frequency of TLR1 I602S in Brazilians with that expected given their admixture proportions, the expected frequency of TLR1 I602S was estimated using the average of allele frequencies p in the parental populations, weighted by admixture proportions estimated with AIMs in Brazilians: p(EUR) × mean EUR ancestry + p(AFR) × mean AFR ancestry + p(AME) × mean AME ancestry. Estimates of TLR1 I602S frequency from p(EUR), p(AFR) and p (AME) were obtained in dbSNPs databank (1000 Genomes project, ss1309283628) (https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs = 5743618). Significant differences between observed frequencies and frequencies expected given admixture proportions estimated for Brazilians were tested with binom.test in R.

Variations in allelic frequencies were quantified using F_{ST}. The statistical significance of departures from zero was tested using bootstrapping over loci, using R package, diveRsity [35].

3. Results

3.1. Estimate of ancestry

As shown in Fig. 2, the major contribution in our sample was of European genomic ancestry with an average of 63% (SD = 17.2%), followed by the contribution of African genomic ancestry at 22% (SD = 15.8%) and Amerindian genomic ancestry at 15% (SD = 9.6).

3.2. Distribution of polymorphisms according to ancestry

Polymorphisms were found in nine SNPs and one microsatellite located on the following genes: TLR1 (I602S), TLR2 (P631H, R753Q), TLR4 (D299G, T399I), TLR6 (P249S), TLR9 (−1486T.C, −1237T.C, 1174A.G) and TLR2 GTn. All other SNPs analyzed here were found monomorphic.

Table 1 describes the genotype frequencies of polymorphisms found in this study, including reference SNP according dbSNP [21], its residues and alleles. The frequencies of the polymorphic SNPs from these Juquitiba samples are also available in ALFRED (The ALlele FREquency Database) [36].

All polymorphisms were in Hardy-Weinberg equilibrium expected proportions, after Bonferroni correction (alpha = 0.005).

Allele frequencies found in the current study are presented in Supplementary Table 2. No significant difference was detected between observed (0.459) and expected frequencies (0.47) in TLR1 I602S when analyzed by binom.test (*p*-value of .7429). Allele frequencies described in other geographical populations are also presented in Supplementary Table 2. According to the F_{ST} value that compared the allele frequencies found in this study with those found in other studies performed with Brazilian populations, the populations presented very weak differentiation ($F_{ST} < 0.05$) (Supplementary Table 2). However, two SNPs (TLR1 I602S and TLR9-1237T/C) presented allele frequencies that demonstrated intermediate differentiation ($0.1 < F_{ST} < 0.2$) and two (TLR1 I602S and TLR9 −1486 T/C) showed weak differentiation ($0.05 < F_{ST} < 0.1$).

Variance analysis showed that the mean proportions of European and African ancestry differed significantly between the genotypes of the TLR1 I602S gene (*p*-values = 2.59E−06 and .000149, respectively). The Tukey post hoc test indicated that the mean European ancestry was higher for the GG genotype than for the TG (*p*-value = .001) and TT genotypes (*p*-value = .001). For African ancestry, the Tukey post hoc test indicated that the mean was higher for the TT genotype than for the TG (*p*-value = .001) and GG genotypes (*p*-value = .001). In the TLR6 P249S gene and in the microsatellite in the TLR2 gene, GTn, the mean proportion of European ancestry also differed significantly between the genotypes (*p*-values = .0294 and .0146, respectively). In the case of TLR6 P249S, the Tukey post hoc test indicated that the mean European ancestry was higher for the CC genotype than for the TC (*p*-value = .001) and TT genotypes (*p*-value = .001). For GTn in TLR2, the Tukey post hoc test indicated that the mean European ancestry was higher for the SL genotype than for all other genotypes (*p*-value = .01). There were no significant differences in the proportions of ancestry for the other SNPs studied (Table 1).

The results of the binary logistic regression using the generalized linear model and implemented in the program R are presented in Fig. 3. They indicate that the chance of having at least one G allele for the TLR1 I602S gene increases as European ancestry increases (*p*-value = .0001506487) and decreases as African ancestry increases (*p*-value = .038307509).

In relation to the TLR6 P249S gene, the chance of having the T allele increases as European ancestry increases (*p*-value = .001042303). The chance of an individual having the M allele in GTn microsatellite region in the TLR2 gene increases as the European ancestry increases, however, this was a non-significant trend (*p*-value = .052551987).

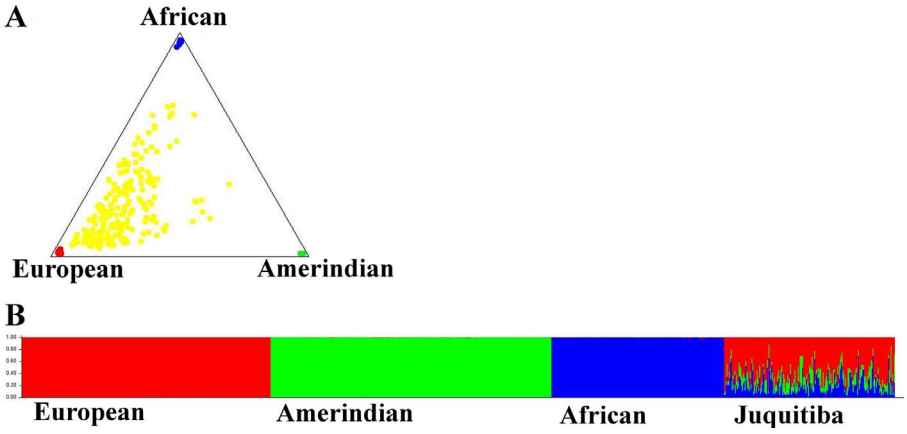

Fig. 2. Schematic representation of the individual admixture estimates (IAEs) in the Juquitiba population using STRUCTURE v.22 software for K = 3. A. Triangle plot: each individual is represented by a colored point, and the correspondent admixture proportions are indicated by the distance to the edges of the triangle. Red, blue and green colors correspond to individuals from the parental populations (labeled as below), and individuals from admixed population are plotted in yellow. B. Bar plot: each vertical line represents one individual and the correspondent European (red), Amerindian (green) and African (blue) admixture proportions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Genotypic proportion of the African, European and Amerindian ancestry according to genotypes of polymorphisms studied.

Polymorphism	Genotype	Count	Proportion	European	Amerindian	African
TLR1.I602S	T/T	58	0.297	0.538 (0.491–0.585)	0.167 (0.136–0.198)	0.295 (0.250–0.340)
(I = T; S = G)	T/G	95	0.487	0.636 (0.603–0.668)	0.158 (0.141–0.176)	0.206 (0.176–0.237)
rs5743618	G/G	42	0.215	0.723 (0.686–0.759)	0.126 (0.104–0.147)	0.152 (0.121–0.183)
p				***2.59E−06***	*.216*	***.000149***
TLR2.P631H	C/C	185	0.949	0.628 (0.604–0.652)	0.156 (0.142–0.170)	0.216 (0.194–0.238)
(P = C; H = A)	C/A	9	0.046	0.551 (0.380–0.721)	0.110 (0.067–0.153)	0.340 (0.179–0.500)
rs5743704	A/A	1	0.005	0.546 (−)	0.073 (−)	0.382 (−)
p				*.642*	*.324*	*.151*
TLR2.R753Q	G/G	189	0.969	0.625 (0.600–0.650)	0.154 (0.140–0.168)	0.221 (0.198–0.244)
(R = G; Q = A)	G/A	5	0.026	0.645 (0.537–0.752)	0.127 (0.092–0.162)	0.228 (0.134–0.322)
rs5743708	A/A	1	0.005	0.541 (−)	0.304 (−)	0.155 (−)
p				*.861*	*.243*	*.913*
TLR4.D299G	A/A	177	0.908	0.617 (0.592–0.643)	0.156 (0.142–0.171)	0.226 (0.203–0.250)
(D = A; G = G)	A/G	17	0.087	0.695 (0.620–0.770)	0.134 (0.100–0.169)	0.171 (0.111–0.231)
rs4986790	G/G	1	0.005	0.830 (−)	0.063 (−)	0.107 (−)
p				*.102*	*.421*	*.301*
TLR4.T399I	C/C	178	0.913	0.619 (0.593–0.644)	0.156 (0.141–0.170)	0.225 (0.202–0.249)
(T = C; I = T)	C/T	15	0.077	0.681 (0.600–0.762)	0.139 (0.102–0.176)	0.180 (0.112–0.247)
rs4986791	T/T	2	0.010	0.775 (0.667–0.883)	0.111 (0.017–0.205)	0.114 (0.100–0.128)
p				*.19*	*.665*	*.357*
TLR6.P249S	C/C	107	0.549	0.597 (0.562–0.631)	0.163 (0.143–0.183)	0.240 (0.209–0.272)
(P = C; S = T)	C/T	75	0.385	0.655 (0.619–0.690)	0.147 (0.128–0.166)	0.199 (0.165–0.232)
rs5743810	T/T	13	0.067	0.691 (0.607–0.775)	0.121 (0.092–0.151)	0.188 (0.122–0.253)
p				***.0294***	*.244*	*.157*
TLR9.−1237 T.C	T/T	121	0.621	0.635 (0.604–0.665)	0.156 (0.137–0.174)	0.210 (0.183–0.236)
rs5743836	T/C	65	0.333	0.620 (0.578–0.662)	0.154 (0.135–0.174)	0.225 (0.184–0.268)
	C/C	9	0.046	0.539 (0.443–0.635)	0.128 (0.082–0.173)	0.333 (0.220–0.447)
p				*.264*	*.7*	*.0728*
TLR9.−1486 T.C	T/T	91	0.467	0.634 (0.598–0.669)	0.156 (0.135–0.176)	0.211 (0.178–0.243)
rs187084	T/C	83	0.426	0.609 (0.571–0.648)	0.150 (0.131–0.169)	0.241 (0.205–0.277)
	C/C	21	0.108	0.651 (0.589–0.713)	0.163 (0.120–0.207)	0.186 (0.137–0.235)
p				*.508*	*.825*	*.26*
TLR9.1174A.G	A/A	53	0.272	0.645 (0.598–0.691)	0.160 (0.128–0.191)	0.196 (0.156–0.235)
rs352139	A/G	94	0.482	0.615 (0.580–0.650)	0.150 (0.133–0.167)	0.235 (0.203–0.267)
	G/G	48	0.246	0.624 (0.576–0.672)	0.155 (0.130–0.180)	0.221 (0.174–0.268)
p				*.606*	*.838*	*.352*
TLR2.GTn	M/M	78	0.400	0.671 (0.636–0.707)	0.137 (0.117–0.156)	0.192 (0.161–0.223)
	M/L	72	0.369	0.588 (0.551–0.626)	0.163 (0.144–0.183)	0.248 (0.210–0.287)
	M/S	18	0.092	0.585 (0.485–0.684)	0.172 (0.117–0.226)	0.244 (0.153–0.335)
	L/L	18	0.092	0.611 (0.543–0.678)	0.178 (0.127–0.229)	0.211 (0.148–0.274)
	L/S	7	0.036	0.691 (0.518–0.864)	0.127 (0.005–0.248)	0.183 (0.059–0.306)
	S/S	2	0.010	0.418 (0.177–0.658)	0.204 (0.202–0.206)	0.379 (0.138–0.620)
p				***.0146***	*.302*	*.174*

African, European and Amerindian ancestry expressed as mean (95% CI).
(−) Data to calculate the CI are missing.
The Fisher's Exact Test was used to determine differences in the genotype frequencies among the ancestries. Values are expressed in *p*. Bold value indicates $p < .05$ (a statistically significant value).

Moreover, we also observed that European and African ancestries are highly correlated in Brazilians as showed by the plot in the Supplemental figure [correlation coefficient = −0.831, *p*-value < 2.2e−16].

3.3. Linkage disequilibrium and haplotypes

Absolute linkage disequilibrium (D' = 1) was not found. The Supplementary Table 3 shows the values of D' observed for all the pairs of SNPs.

Nineteen haplotypes of the TLR1, TLR6, TLR2, TLR4, and TLR9 genes were found in the study sample with frequencies ranging from 0.00257 to 0.95385 (Table 2). TC, GT and GC haplotypes in TLR1 I602S/TLR6 P249S were significantly associated with African or European ancestry. The TC haplotype in TLR1 I602S/TLR6 P249S was the only one that presented a significant Association with Amerindian ancestry. The highest Association in this chromosome region was found with TC haplotype and both African and European ancestry. The AC haplotype in TLR4 D299G/TLR4 T399I presented a significant Association with European ancestry. The CCG haplotype in TLR9 (−1237/−1486/1174) had significant Associations with African and European ancestry. However, when the Bonferroni correction was applied [corrected p-value < .004 (4p14 and 9q32–33) or corrected p-value < .002 (3p21.3)], only the TC and GC haplotypes in TLR1 I602S/TLR6 P249S (4p14) remained significantly associated with African or European ancestry.

4. Discussion

TLRs are major players in the recognition of pathogens in the innate immune system [37,38]. TLR1, TLR6, and TLR10 occur on the cell surface and are known to detect parasite components including flagellin and glycolipids. Although genetically relatively conserved, TLR encoding genes may differ in different populations around the globe, perhaps as an adaptation to different immunologic challenges. In this regard, certain alleles were found under positive selection in Europeans [2,6]. Herein, we have analyzed the differential occurrence of polymorphisms in parasite-relevant TLRs in an admixed Brazilian population. In order to avoid erroneous interpretation of results due to a specific population admixture background, we calculated the proportions of each ancestor's participation in the study population. Polymorphisms were found in nine SNPs and one microsatellite: TLR1

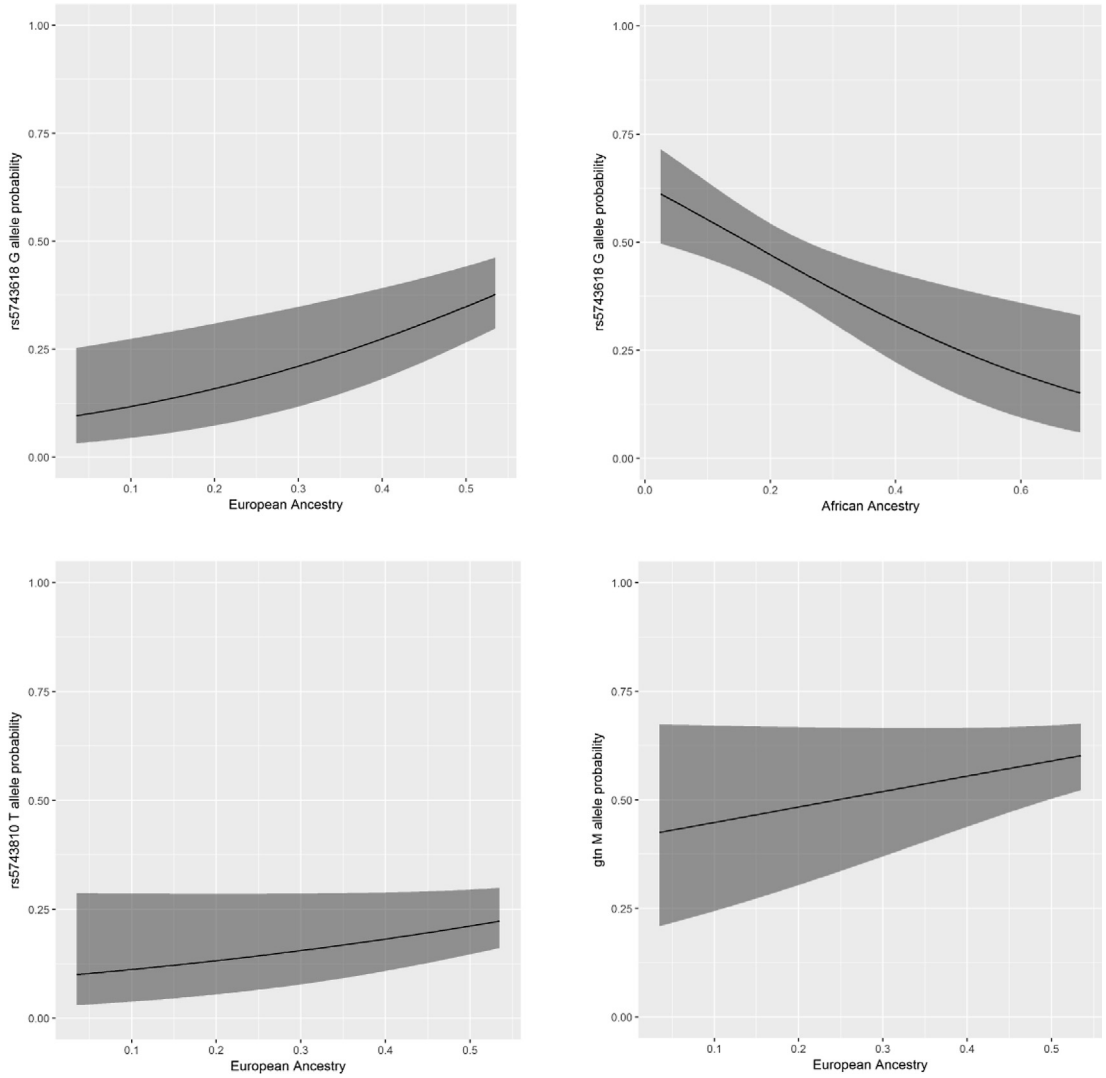

Fig. 3. Fitted logistic regression describing the association between ancestry and polymorphisms. (a) TLR1 I602S. Chance of having a G allele according to European Ancestry and (b) according to African ancestry. (c) TLR6 P249S. Chance of having a T allele according to European ancestry. (d) TLR2 GTn. Chance of having an M allele according to European ancestry. Gray shadows show 95% confidence intervals. Graphics were created using ggplot2 in R.

(I602S), TLR2 (P631H, R753Q), TLR4 (D299G, T399I), TLR6 (P249S), TLR9 (−1486T.C, −1237T.C, 1174A.G) and TLR2 (GTn).

In our population, most individuals possessed higher European contribution in their ancestry (168 individuals). Thus, our first analysis of the results compared the obtained frequencies with those of previous studies performed with European (1000 Genomes and published references) or Brazilian populations (published references), which correlated or not with diseases. We used the frequencies of the general population studied (with and without disease) since the correlation with disease is not the subject of this work.

In relation to TLR2, fourteen SNPs were found monomorphic for the ancestral allele, but, unfortunately, in dbSNP, there is only information for five of them (F679I, Y715N, R723S, N729S and E738Q) showing also a frequency of 100% for the ancestral allele in all European populations studied so far. Two SNPs that were found polymorphic in TLR2 (P631H and R753Q) did not present significant Association with ancestry. For GTn polymorphism, also located in TLR2, we found a significant Association with European ancestry, but the results of the binary logistic regression showed a p-value $> .05$ for the allele with greater Association (M allele). However, some authors speculate that the M allele is less prone to inflammation than the S allele or the L allele, possibly explaining why mid-sized GT repeats are the most abundant in some populations [39]. There are only two previous studies using European populations to compare the allele frequencies [40,41]. Theses studies analyzed either Croatian Caucasians [40] or Caucasians from North America [41]. In both of them, the M allele was also more common, with 70 and 58.3% of frequency, respectively, while we found an intermediate frequency (63.1%). There are no GTn frequencies from other European populations available for comparison but it is important to note that in Asian populations the L allele has been shown more prevalent [24,41].

In relation to TLR1 and TLR6, our results showed higher frequencies than those described in the previous Brazilian study [7] for TLR1 I602S (allele G) and TLR6 P249S (allele T), the variant alleles. In fact, the frequencies described by us in these two SNPs are intermediates between those described for the northern Brazilian population and the European populations from the 1000 Genomes Project. However, it is important to note that the Brazilian population analyzed by Leoratti et al. [7], according to the authors, "*was predominately Amerindian, with a few individuals who were white or black*" [7] and who were selected for the presence of malaria. Moreover, it has been demonstrated that there is a north-south gradient towards increased European ancestry [15] indicating that the population of the southeast analyzed in this work could be closer to the European population than to the northern Brazilian population.

Variance analysis was performed to check the difference of each

Table 2
Haplotype frequencies of SNPs found polymorphic in this study and association with African, European and Amerindian ancestry.

Gene	refSNP	Chromosome Region	Haplotype	Frequency	African	*p*	European	*p*	Amerindian	*p*
					hap.score		hap.score		hap.score	
TLR1 I602S (T/G)TLR6 P249S	rs5743618	4p14	TT	0.01609	0.49026	.62395	0.16219	.87116	−1.0769	.28152
(C/T)	rs5743810		TC	0.52494	4.57006	**0**	5.50234	**0**	2.3575	**.0184**
			GT	0.24289	−1.99296	**.04627**	2.59965	**.00933**	−1.38616	.1657
			GC	0.21609	−3.65202	**.00026**	3.96012	**7e−05**	−1.10362	.26976
TLR2P631H	rs5743704 rs5743708	4q31.3	CG	0.95385	−1.38354	.1665	0.82603	.40879	0.79003	.42951
(C/A)R753Q			CA	0.01795	−0.20439	.83805	−0.13798	.89026	0.58638	.55762
(G/A)			AG	0.02821	1.9452	.05175	−0.94278	.34579	−1.50557	.13218
			AA	–	–	–	–	–	–	–
TLR4D299G	rs4986790 rs4986791	9q32–33	AC	0.94871	1.58372	.11326	−2.08992	**.03662**	1.14339	.25288
(A/G)T399I			AT	0.00257	−0.63273	.52691	0.55167	.58117	0.05225	.95833
(C/T)			GC	0.00257	−0.84173	.39994	1.45827	.14477	−1.23388	.21725
			GT	0.04615	−1.3894	.16471	1.81863	.06897	−0.97576	.32919
TLR9	rs5743836	3p21.3	TTG	0.01909	0.74642	.45541	−0.50642	.61256	−1.45642	.14528
−1237 (T/C)	rs187084		TTA	0.48037	−0.87883	.37949	0.59037	.55495	0.38451	.7006
−1486 (T/C)1174	rs352139		CTG	0.15846	0.82238	.41086	−0.40093	.68848	−0.62554	.53161
(G/A)			CTA	0.02157	−0.10958	.91274	−0.09543	.92397	0.35157	.72516
			TCG	0.28189	−0.88426	.37655	0.56937	.56911	0.43148	.66612
			TCA	0.00583	−0.2666	.78978	1.04452	.29624	−1.45642	.14528
			CCG	0.02775	2.53924	**.01111**	−2.1585	**.03089**	−0.30558	.75993
			CCA	0.00505	0.52564	.59914	−0.40584	.68486	−0.13306	.89414

The Fisher's Exact Test was used to determine differences in the haplotype frequencies among the ancestries. Values are expressed in *p*.
Bold value indicates $p < .05$ (a statistically significant value).

ancestry between the different genotypes and showed that the mean proportions of European ancestry differed significantly between the genotypes of the TLR1 I602S gene and TLR6 P249S gene. The results of the binary logistic regression indicated that the chance of having the G allele in TLR1 gene increases as European ancestry increases as well as the chance of having the T allele in the TLR6 gene.

TC and GC haplotypes in TLR1/TLR6 were the only ones that remained significantly associated with European ancestry after the use of Bonferroni correction. Therefore, although the alleles G and T in TLR1 and TLR6 have been separately associated with European ancestry, the Association of this haplotype with ancestry was not significant. On the other hand, TC and GC haplotypes in TLR1/TLR6 were associated with European ancestry. Considering only these two SNPs among the haplotypes appearing more than once in the European population, the TC haplotype has been shown in 32% of the European population studied while GT frequency was 25% (see Fig. S5 from Barreiro et al., [2]). Moreover, the allele frequency of the variant allele G in TLR1 in European populations can reach 51% while the variant allele T in TLR6 has a frequency of 28% [2]. In fact, the Association of the variant allele G of TLR1 and European population has been much better documented than the Association of the variant allele T in TLR6 with this same population [2,42,43].

Previous studies have shown that, homozygous (GG), and to a lesser extent heterozygous (TG), individuals for the TLR1 (I602S) present impaired TLR1-mediated immune responses after whole blood stimulation [44–46]. Moreover, Barreiro et al [2] have also shown that among the two nonsynonymous variants TLR1 602S and TLR6 249S, only TLR1 602S had a functional impact leading to impaired signaling. In fact, TLR1 602S appears to result in a decreased function of the molecule, which is positively selected in Europeans but its putative selective advantage is unknown. The first hypothesis that would make relevance for innate immunity gene variations is that they provide advantages in infections but Associations of this SNP with common infectious diseases did not reveal an obvious protective effect. However, in the study of Leoratti et al. [7], where the variant 602S in TLR1 was associated with increased susceptibility to malaria, the low frequency of this variant (19%) could be associated with the study population, as mentioned above, a population with little European ancestry. A study in malaria area with a population of a strong contribution of European ancestry could add relevant information on this issue. This "hypo-responsiveness" caused by 602S could explain the high prevalence of asymptomatic malaria individuals in areas of Southeastern Brazil. Further studies integrating genetic data with clinical and epidemiological information about malaria from these individuals are necessary to better define the relevance of these host defense genes in the region.

Regarding the linkage disequilibrium, although absolute LD has not been found, there was evidence for LD ($D' > 0.7$) in three out of four chromosome regions evaluated: (i) *4p14 (TLR1.I602S and TLR6.P249S)*. Linkage disequilibrium in these SNPs has already been shown since these genes encode proteins that share a high degree of homology in their amino acid sequences and are known co-receptors [47]; (ii) *4q31.3 (TLR2.P631H and TLR2.R753Q)*. Although a high D′ value was found, its *p*-value was not statistically significant ($p = .68$). In a study conducted in Russia, these SNPs were also considered not linked although the amino acid change 753Arg > Gln has revealed Association with pneumonia [48]; (iii) *9q32–33 (TLR4.D299G and TLR4.T399I)*. This is a well-documented Association since these two polymorphisms were found co-segregating [49,50]; (iv) *3p21.3 (TLR9.−1237 and TLR9.1174)*. The TLR9 C allele at −1237 and the G allele at 1174 were demonstrated strongly linked among Ugandan children with malaria [50]. LD was also observed between TLR9.−1237 and TLR9.1174 among Ghanaian children [51], but with D′ values slightly higher than those observed by us ($D' = 0.86$ versus $D' = 0.70$). Similar values of D′ were also found for *TLR9.−1486 and TLR9.1174* in our population ($D' = 0.92$) and among Ghanaian children ($D' = 0.85$) [9]. It is important to note that, as expected, linkage disequilibrium was not found for *TLR9.−1486 and TLR9.−1237*, since this has never been demonstrated in the literature previously. A study carried out with Polish infants found no evidence for LD with any pair of these three examined TLR9 SNPs [52].

In summary, the growing interest in genetic predisposition to common diseases and the rapid advance in high-throughput genotyping technology have resulted in a remarkable amount of published epidemiological evidence on gene-disease Associations. Reported genetic Associations with common diseases have become numerous, are mostly of small magnitude and can describe true genetic Associations but also

false positive results. Convincing evidence of true Association, therefore, requires careful control over potential biases and effects by chance [53]. The population structure is responsible for biases that can lead to false results in genetic Association studies. Previous studies have demonstrated an Association of polymorphisms in immune response genes with genomic ancestry [42,54]. Thus, in mixed populations, it is essential to study the haplotype frequencies and their Associations with the levels of ancestry. In this study we describe the Association of SNPs in TLR1 and TLR6 genes with ancestry in the Brazilian population. Expected and observed frequencies were the same, and thus, the variant TLR1 I602S has evolved neutrally in Brazil and it has not been under positive selection since admixture. Farther, our results strengthen the requirement to use AIMs in genetic Association studies concerning these polymorphisms in the Brazilian population.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.humimm.2017.11.007.

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