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Association of *toll-like receptor* polymorphisms with HIV status in North Americans

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Abstract

Single nucleotide polymorphisms (SNPs) in toll-like receptor (TLR) genes *TLR2-4* and *TLR7-9*, but not in *TLR1* and *TLR6*, have been previously evaluated regarding HIV acquisition and disease progression in various populations, most of which were European. In the present study, we examined associations between a total of 41 SNPs in 8 *TLR* genes (*TLR1-4*, *TLR6-9*) and HIV status in North American subjects (total $n = 276$ [Caucasian, $n = 102$; African American, $n = 150$; other, $n = 24$]). Stratification of the data by self-identified race revealed that a total of 9 SNPs in *TLR1*, *TLR4*, *TLR6*, and *TLR8* in Caucasians, and 2 other SNPs, one each in *TLR4* and *TLR8*, in African Americans were significantly associated with HIV status at $P < 0.05$. Concordant with the odds ratios of these SNPs, significant differences were observed in the SNP allele frequencies between HIV+ and HIV− subjects. Finally, in Caucasians, certain haplotypes of single (*TLR1*, *TLR4*) and heterodimer (*TLR2_TLR6*) genes may be inferred as “susceptible” or “protective”. Our study provides in-depth insight into the associations between *TLR* variants, particularly *TLR1* and *TLR6*, and HIV status in North Americans, and suggests that these associations may be race-specific.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

Keywords

African American; Caucasian; HIV; SNP; *TLR1*; *TLR6*

INTRODUCTION

Susceptibility to the human immunodeficiency virus (HIV) infection and the rate of disease progression are variable among individuals and populations, and in part, genetically determined. Among a multitude of host genetic factors associated with susceptibility to HIV infection and/or disease progression, chemokine receptors, serving as HIV co-receptors, and their ligands have been well described.[1] Outside the chemokine receptor-ligand nexus, host genetic factors that are associated with viral load control have been identified by recent genome-wide association studies.[2] Among these, polymorphisms in innate immune response genes,[3, 4] including those encoding β-defensins[5, 6] and toll-like receptors (TLRs),[7] have been found to affect the natural history of HIV infection and disease progression.

Toll-like receptors are the most important class of pattern recognition receptors, involved in the host defense against bacteria, viruses, fungi, and protozoa.[8–10] They are the primary molecular mechanism by which the host responds to invading microbes through the recognition of conserved motifs, which are termed pathogen-associated molecular patterns (PAMPs). The molecular interaction of TLRs with PAMPs and subsequent interactions with TLR adapters, kinases, and transcription factors trigger a cascade of signaling events that induce the production of pro-inflammatory cytokines and chemokines.[11, 12]

There are 10 TLRs expressed in humans. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are expressed largely on the cell surface, whereas TLR3, TLR7, TLR8, and TLR9 are intracellular (endosomal).[13] Although the TLRs on the cell surface primarily recognize PAMPs of bacteria, fungi, and protozoa, TLR1, TLR2, TLR4, and TLR6 have also been shown to be involved in responses to viral infection.[14] Similarly, the intracellular TLRs function primarily to detect viruses, although it has been shown that they detect other microbes as well.[13] TLR2 heterodimerizes with TLR1 or TLR6.[15] A recent report describes heterodimerization of TLR4 with TLR6.[16]

Studies evaluating TLR expression and response related to HIV have provided evidence that TLR1,[17] TLR2,[18–21] TLR3,[20, 22, 23] TLR4,[18–22, 24] TLR6,[20] TLR7/8,[20, 22, 25–27] and TLR9[22] play a functional role in HIV infection and disease. Single nucleotide polymorphisms (SNPs) in *TLR2*,[28–32] *TLR3*,[28–31] *TLR4*,[28–32] *TLR7*,[28–30, 33] *TLR8*,[28–30, 34] and *TLR9*[28–32, 35] have been evaluated for their effects on HIV acquisition and disease progression in various populations under a variety of study designs. Although differences in populations, the number of markers (SNPs or combinations thereof), and outcome measures make the comparison of data difficult, 2 general conclusions may be drawn from these previous studies: (a) SNPs in *TLR1* and *TLR6* were not included in these studies and (b) only one of these studies was conducted in North America on predominantly white patients,[31] whereas most were conducted in Europe,[29, 32–35] and a few in Africa.[28, 30]

In the present study, we examined associations between a total of 41 SNPs in 8 *TLR* genes (*TLR1*, *2*, *3*, *4*, *6*, *7*, *8*, and *9*) and HIV status in North American subjects belonging to 2 major races: Caucasian and African American. Many of the SNPs included in the study were from aforementioned HIV/AIDS studies.[28–35] Other SNPs, including those in *TLR1* and *TLR6*, have been evaluated in other infectious[36–45] as well as inflammatory and immune-mediated non-infectious[46–56] diseases. In addition to the SNP-based association analyses, we performed gene as well as heterodimer haplotype-based analyses. Our study provides in-depth insight into the role of *TLR* variants, particularly *TLR1* and *TLR6* variants, in susceptibility to or protection against HIV acquisition. Furthermore, our results suggest that the associations between *TLR* variants and HIV status may be race-specific.

RESULTS

Study populations

The demographic characteristics of HIV+ patient (n = 180) and HIV− random blood donor (n = 96) populations are summarized in Table 1. A majority of the HIV+ patients were African Americans and the sex distribution reflected the demographics of the clinic with a predominance of males. HIV− donor population had an equal representation of the Caucasian and African American races, and male and female sex.

Minor allele frequency and potential batch effect

The minor allele frequency (MAF) of all 41 SNPs, according to the HIV status, as well as the HIV status stratified by race, are presented in Table 2. MAF ranged from 0.01 to 0.50. The MAF of all SNPs in HIV− Caucasians and African Americans were in agreement with those reported in the dbSNP database[57] for comparable populations (Supplementary Table A). No difference in MAF of any of the SNPs was observed between the 2 plates containing samples from HIV+ patients (t-test, $P > 0.05$ for all comparisons).

Linkage disequilibrium patterns

The pairwise linkage disequilibrium (LD) patterns of *TLR1*, *TLR4*, *TLR6*, *TLR8*, and *TLR1_TLR6*, based on $D' \geq 0.8$ and $r^2 \geq 0.5$,[58] for both HIV+ and HIV− Caucasians and African Americans are presented in Supplementary Table B. No strong LD was observed between SNPs in the *TLR7_TLR8* gene pair.

Regression analysis of SNPs

Following logistic regression using all 276 samples, 3 of the 41 SNPs were significantly associated with modestly increased odds of HIV infection, after the correction for multiple testing ($\alpha = 0.001$).[59] These were: *TLR1* rs5743551 (−7202G, odds ratio [OR] = 1.76; 95% confidence interval [CI] = 1.51, 2.18; $P = 0.0005$), *TLR1* rs5743618 (1805T, OR = 1.71; 95% CI = 1.48, 2.09; $P = 0.0001$), and *TLR6* rs5743810 (745T, OR = 1.6; 95% CI = 1.38, 2.00; $P = 0.0002$). However, after co-varying for self-identified race, no significant association was observed between these SNPs and HIV status at the $\alpha = 0.001$ level (P = 0.017, 0.006, and 0.012, respectively).

Stratification of the data by self-identified race, and adjustment for sex, revealed that none of the 41 SNPs was significantly associated with HIV status at the $\alpha = 0.001$ level in either racial group. However, considering significance at $P < 0.05$, a total of 9 SNPs in *TLR1* ($n = 2$), *TLR4* ($n = 1$), *TLR6* ($n = 4$), and *TLR8* ($n = 2$) were significantly associated with HIV status under an additive genetic model in Caucasians (Table 3). Of these, a total of 5 SNPs in *TLR1* ($n = 2$), *TLR4* ($n = 1$), and *TLR6* ($n = 2$) were also significantly associated with HIV status under a dominant genetic model (Table 3). The 5 SNPs, showing significance in both genetic models, included 3 SNPs (rs5743551, rs5743618, and rs5743810) that were significantly associated with HIV status in the regression analysis performed on all samples combined. In contrast to Caucasians, only one SNP in *TLR4* under both genetic models, and one SNP in *TLR8* under the additive genetic model were significantly associated with HIV status at $P < 0.05$ in African Americans (Table 3). These 2 SNPs were not among the 9 SNPs that were significantly associated with HIV status in Caucasians.

After adjusting for sex, we noticed that it was consistently a significant contributor to both genetic models, especially in Caucasians (*P*-values < 0.001, data not shown). However, the significance of sex as a covariate could be attributed to the uneven distribution of sex between HIV+ and HIV− subjects (Table 1).

Haplotype analysis for genes and heterodimers by HIV status

Significant global *P*-values were observed for the *TLR1* and *TLR4* genes, and for the *TLR2_TLR6* heterodimer in Caucasians ($P = 0.025$, 0.032, and 0.017, respectively; Table 4). This indicates significant differences in the overall haplotype profiles of *TLR1*, *TLR4*, and *TLR2_TLR6* between HIV+ and HIV− Caucasians. Two haplotypes in *TLR1*, one haplotype in *TLR4*, and one haplotype in *TLR2_TLR6* were significantly associated with HIV status (Table 4). The *TLR1* haplotype GTGT was significantly more frequent in HIV+ patients (hap-score 2.198, $P = 0.028$), whereas the haplotype ATGG was significantly more frequent in HIV− donors (hap-score −3.313, $P = 0.001$). The *TLR4* haplotype AGCACGG was significantly more frequent in HIV+ patients than in HIV− donors (hap-score 2.529, $P = 0.011$). The *TLR2_TLR6* heterodimer haplotype TGTTG_GTCTCATC was significantly more frequent in HIV− donors than in HIV+ patients (hap-score −2.839, $P = 0.005$). In contrast to Caucasians, no haplotype, either in genes or in heterodimers, was significantly associated with HIV status in African Americans.

Summary

For further clarity, we provide summary of all results, arranged according to significant *TLR* SNPs and haplotypes, as Supplementary Results.

DISCUSSION

In the present study, utilizing samples from North American HIV+ and HIV− subjects, we provide evidence indicating that a total of 9 SNPs in *TLR1*, *TLR4*, *TLR6*, and *TLR8* in Caucasians, and one SNP each in *TLR4* and *TLR8* in African Americans have potential roles in susceptibility to or protection against HIV infection. Although expressed on the cell

surface, TLR1, TLR4, and TLR6 have been shown to be involved in responses to viral infection,[14] including HIV (TLR4).[18–22, 24]

TLR1 SNPs and HIV

There is a paucity of information about the role of TLR1 in HIV/AIDS. In a Kenyan cohort of untreated women, the mRNA expression of *TLR1* in peripheral blood mononuclear cells (PBMCs) was equivalent between HIV-infected and uninfected subjects.[20] In a North American, predominantly male cohort, where a majority of the patients were treated, the TLR1 surface expression level was diminished on monocytes and myeloid dendritic cells from HIV-infected persons compared with the expression on cells from control donors.[17] To date, no genetic study, analyzing the role of *TLR* polymorphisms in influencing HIV infection and/or disease progression, has included *TLR1* SNPs.[28–35]

The mechanisms by which the −7202G and 1805T alleles influence HIV status is currently unknown. These 2 alleles were functionally significant in sepsis,[43, 44] tuberculosis,[37] leprosy,[39] and candidemia,[40] where they were associated with higher NF-kB activation and signaling, and elevated inflammatory cytokine production, including that of IL-6.[37, 40, 44] Elevated levels of IL-6 have been associated with HIV infection[60] and could contribute to HIV disease progression.[61] Using a human monocytic cell line, THP-1, it has been shown that glycoprotein (gp) 41 is the primary HIV-encoded protein involved in inducing IL-6 production.[62] However, in the clinical studies, there was weak or no correlation between plasma levels of IL-6 and HIV-1 RNA, but IL-6 levels were correlated with plasma levels of the lipopolysaccharide (LPS) co-receptor CD14.[63] Furthermore, macrophages stimulated with LPS or flagellin showed robust production of IL-6, but there was no increase in IL-6 production after HIV-1 infection.[63] Regardless of whether IL-6 production is driven by an HIV molecule (gp41) or HIV-associated bacterial products (LPS/flagellin), our finding that *TLR1* SNPs and haplotypes are associated with HIV status in Caucasians is noteworthy, and may be considered as a starting point in identifying the contribution of *TLR1* genetic variation to HIV infection and disease progression.

TLR4 SNPs and HIV

TLR4 has an important role in HIV/AIDS. The expression of TLR4 in PBMC subpopulations[19, 20, 24] and dendritic cells[18] from untreated HIV-infected patients is upregulated, whereas in PBMCs from chronic patients failing therapy it is reduced.[22] However, the information regarding the role of *TLR4* SNPs in influencing HIV infection and/or disease progression is mixed. In a treatment-naïve, predominantly white North American cohort, SNPs Asp299Gly (rs4986790) and Thr399Ile (rs4986791) were associated with high peak plasma viral load.[31] On the other hand, in Swiss,[29] Spanish,[32] and Kenyan cohorts,[28, 30] these and other *TLR4* SNPs were not associated with HIV infection and/or disease progression. In the present study, we did not find an association between Asp299Gly/Thr399Ile, considered singly or in haplotypes, and HIV status in either racial group. Most of the aforementioned studies[28–30, 32] did not include −1607T>C and +12186C>G. The study[31] that included these SNPs did not find an association with peak plasma viral load or disease progression.

The information regarding the functional significance of −1607T>C and +12186C>G is limited.[50, 64–66] The −1607C allele may be a risk factor for prostate cancer[47] and traffic-related air pollution-associated childhood asthma,[51] and the +12186C allele may be a risk factor for rheumatoid arthritis.[56] However, none of these studies looked into the possible mechanisms of these allelic associations. In female genital epithelial cells, TLR4 binds to HIV-1 gp120 and triggers pro-inflammatory cytokine production via activation of NF-kB.[21] Being located in the promoter and 3′-untranslated (UTR) regions, respectively, it is plausible that these SNPs affect TLR4 activity via affecting gene expression and mRNA stability. Therefore, further functional and clinical studies are needed to determine whether these SNPs influence HIV-associated TLR4-mediated activation of NF-kB and production of pro-inflammatory cytokines. Alternatively, it may be that these SNPs affect responsiveness to LPS, as has been shown with other *TLR4* SNPs (Asp299Gly and Thr399Ile),[67] and thus influence HIV-associated systemic immune activation and pathogenesis.

TLR6 SNPs and HIV

TLR6 seems to play an important role in HIV/AIDS.[20] In a Kenyan cohort of untreated women, the mRNA expression of *TLR6* was significantly increased in PBMCs from HIV-infected subjects compared with those from uninfected subjects, and the expression level of *TLR6* was positively correlated with the plasma viral load.[20] However, the role of *TLR6* SNPs in influencing HIV infection and/or disease progression has not yet been identified,[28–35] as is the case for *TLR1* SNPs.

Despite the fact that the chromosomal regions containing *TLR6* (*TLR1_TLR6_TLR2* and *TLR10_TLR1_TLR6*) have been implicated in a variety of diseases, including infectious diseases, such as tuberculosis,[8, 36] most of the information regarding the functional significance of *TLR6* SNPs is limited to non-synonymous 745T>C, which is in strong LD with a promoter SNP −502T>C. In tuberculosis studies,[41, 42] the 745T allele, compared with the 745C allele, was associated with lower NF-kB signaling, lower levels of IL-6, and higher levels of IFN-γ. Association of this allele with a decreased NF-kB activation and IL-6 production, but no effect on IL-10 production, may also play a role in protection against coronary artery disease.[48] IFN-γ plays various roles in HIV/AIDS pathogenesis, including controlling HIV-1 replication.[68, 69] Thus, it is plausible that the observed protective effect of the 745T allele in our study, 55–72% decrease in OR, is due to regulation of the IFN-γ IL-6 cytokine profile. Clinical studies are needed to confirm this hypothesis. On the other hand, the information regarding the functional significance of −673C>T[70] and 1263A>G[44] is scarce.

TLR8 SNPs and HIV

A number of studies have shown an important role of TLR8 (TLR7/8) in HIV/AIDS.[20, 25–27] It may be summarized from these studies that the mRNA expression of *TLR8* is significantly increased in HIV-infected subjects; HIV ssRNA upregulates *TLR8* expression; and stimulation of TLR8 (TLR7/8) affects HIV pathogenesis, which depends on the stage of infection as well as the cell type examined. Given the significance of TLR8 in HIV/AIDS, the role of *TLR8* SNPs, including 1A>G, in influencing HIV infection and/or disease progression has been explored.[28–30, 34] The 1G allele displayed impaired NF-kB activation

in vitro, and was associated with modulation of cytokine induction (higher TNF-α and lower IL-10) in monocytes.[34] The allele was significantly associated with reduced disease progression in a Caucasian German[34] and a Kenyan cohort.[30] However, among Kenyan female infants, the 1G allele was significantly associated with higher peak plasma viral load.[28] No significant association was observed between *TLR8* SNPs, including 1A>G, 1953G>C, and 354C>T, and disease progression in a Swiss cohort.[29] Thus, these studies, together with our finding that the 1G allele was significantly associated with HIV status in Caucasian Americans, suggest that the association between the allele and HIV infection/disease progression may be population- and/or outcome measure-specific.

Among the abovementioned studies, except one,[29] most[28, 30, 34] did not include 1953G>C and 354C>T. Both 1953G[38, 54] and 1953C[55] alleles may be associated with respiratory infections and diseases. No reference to the possible mechanisms of these allelic associations was made in these studies. To our knowledge, no report is available regarding the functional significance of 354C>T. Also, it does not appear that the SNP is in high LD with any other SNP in *TLR8*, or with any SNP in *TLR7*.[54] Given that 354C>T had a protective effect in African Americans, and that HIV/AIDS continues to disproportionately affect this population,[71] evaluating the functional and clinical effects of this SNP in further studies is important and highly relevant.

Limitations

We acknowledge that our study has some limitations. First, the SNPs in *TLR1*, *TLR4*, *TLR6* and *TLR8* were not significantly associated with HIV status at the multiple testing correction level of 0.001, but at $P < 0.05$ (Table 3). It is possible that the uneven distribution of HIV+ ($n = 180$) and HIV− ($n = 96$) subjects overall as well as within African Americans (HIV+, $n = 102$; HIV−, $n = 48$) partly contributed to the lower levels of significance. In our power analysis using CaTS,[72] we had sufficient power to detect a minor allele with OR of 2.0 to 3.0, but we were underpowered to detect a minor allele with an OR of <2.0 (Supplementary Table D). Nevertheless, it is important to note that our findings pertaining to ORs were concordant with significant differences in the SNP allele frequencies between HIV+ and HIV− subjects (Table 2) and, in Caucasians, with our haplotype analyses, by which certain haplotypes may be inferred as “susceptible” or “protective” (Table 4).

Second, the race of our HIV+ and HIV− populations is self-identified. Studies investigating the association between genetic markers and HIV/AIDS outcomes have heavily relied upon self-identified race classification. Only recently have researchers begun to consider genetic ancestry into their analyses, showing that the self-identified race and genetic ancestry could be poorly[73, 74] or highly[75] concordant. In addition, our HIV+ and HIV− population samples were collected at locations in the Midwest (East North Central) and South (South-Atlantic) regions of the United States, respectively, with a distance of approximately 400 miles. We did notice a higher overall extent of LD in HIV− donors than in HIV+ patients, despite their racial status (Supplementary Table B), which may be due to differences in demographic factors. In the continental United States, the African ancestry contribution to Caucasian populations is 1–2%, whereas the European ancestry contribution to African-American populations varies substantially (3% to >30%).[76] However, these 2 regions are similar

regarding the European ancestry contribution to African-American populations (16–20% and 13–19%, respectively).[76] These estimates were obtained using especially selected ancestry informative markers and are quite precise.[76] We also quantified admixture in the HIV+ and HIV− African-American groups by using the Duffy blood group antigen (FY) as a population-specific marker. Among the 3 most common *FY* alleles, *FY*A*, *FY*B*, and $FY*B^{ES}$ (erythroid silent), $FY*B^{ES}$ is a key marker for African ancestry.[77, 78] Furthermore, the unique utility of this marker is reflected in the fact that the allele frequencies of this marker match the African-American admixture proportions estimated using a number of autosomal markers.[77] Frequency of the $FY*B^{ES}$ allele was 0.72 (*FY*A*, 0.14; *FY*B*, 0.14) among the HIV+ African-American group, and 0.73 (*FY*A*, 0.12; *FY*B*, 0.15) among the HIV− African-American group, indicating that the admixture proportions at this genetic locus were highly similar between the 2 groups.

Third, we adjusted for self-identified race and sex in our regression analyses. We cannot exclude the fact that residual confounding may exist due to unmeasured ethnic factors (environmental, social, cultural, or behavioral). This information is not available for HIV− donors, and therefore the impact of any other potential confounder could not be considered in the study. In addition, no information is available regarding HIV exposure in HIV− donors. However, a number of studies have reported the prevalence, incidence, and residual risk of HIV in blood donor populations from the American Red Cross,[79–82] which is the source of our HIV− donor samples. These data indicate that random blood donors cannot be considered to be HIV unexposed.

Finally, to our knowledge, among the studies that have evaluated the influence of genetic variation in *TLRs* on HIV/AIDS outcomes, ours is the only other study conducted in North America. A previous study was conducted in a different, predominantly white cohort.[31] Most of the other studies were conducted in Europe,[29, 32–35] and a few in Africa.[28, 30] Because the data regarding *TLR* variants and HIV infection/disease in admixed populations are still scarce, caution is recommended in the interpretation and comparison of our study findings. Unique findings of our study are the potential roles of *TLR1* and *TLR6* SNPs in influencing HIV status. On the other hand, we did not find a role of *TLR9* 1635G>A (rs352140, Pro545Pro), which has been found significantly associated with HIV/AIDS outcomes in many studies.[28–32, 35] A number of factors, including a different outcome measure, could account for this difference.

Conclusions

Our study provides in-depth insight into the influence of genetic variation in *TLRs* on HIV status in North American subjects. To our knowledge, our study is the first to evaluate the association between SNPs in *TLR1* and *TLR6* and an HIV-related outcome. We found that SNPs in *TLR1*, *TLR4*, *TLR6* and *TLR8* are associated with HIV status, and these associations appear to be race-specific. We also identified haplotypes of *TLR1* and *TLR4*, which may be inferred as “susceptible” or “protective” haplotypes. Furthermore, by performing heterodimer haplotype-based analysis, we found that a *TLR2_TLR6* haplotype may be “protective”. The mechanisms by which the aforementioned *TLR* SNPs, singly or in haplotypes, influence HIV status need to be further elucidated. Analysis of mRNA and

protein levels of the *TLR* variants, and investigation of interactions of the variant TLRs with adaptor molecules and subsequent recruitment of downstream targets are needed to define the biological mechanisms that underlie the influence of genetic variation in *TLRs* on HIV status, infection dynamics, and disease progression.

SUBJECTS AND METHODS

Study populations

A total of 280 subjects were analyzed in this study. Among these, 184 were adults with confirmed HIV infection (HIV+), receiving care at the Special Immunology Unit of Case Western Reserve University/University Hospitals Case Medical Center, Cleveland, OH. De-identified packed blood pellets, collected from these patients, were obtained from the Case Western Reserve University Center for AIDS Research (CFAR) specimen repository. All patients provided written informed consent for de-identified clinical data and specimen collection, storage, and usage in genetic and non-genetic studies. The data and specimen collection protocol was approved by the Institutional Review Board of University Hospitals Case Medical Center. Additionally, 96 de-identified samples, collected from healthy, adult North American random blood donors (HIV−), were obtained from American Red Cross National Histocompatibility Laboratory, University of Maryland Medical System, Baltimore, MD.[78] Blood samples from these de-identified donors were collected under protocols, including the procedures for informed consent, approved by the respective institutional review boards.

TLR SNPs

A total of 45 SNPs in 8 *TLR* genes (*TLR1*, *2*, *3*, *4*, *6*, *7*, *8*, and *9*), which have been evaluated in HIV/AIDS[28–35] and other infectious[36–45] as well as inflammatory and immune-mediated non-infectious[46–56] diseases, were included in the present study. These SNPs were located in promoter regions, 5′-UTRs, exons, introns, and 3′-UTRs (Supplementary Table A). In HIV/AIDS studies, most of these SNPs were selected from the dbSNP, Innate Immunity Programs for Genomic Applications, and Genome Variation Server (University of Washington) databases,[28, 29, 31] using haplotype tagging[28, 29, 31] and candidate SNP[28, 31] approaches. Similar strategies, together with prediction of functionality using *in vitro* transfection assays and/or bioinformatics tools,[48, 50, 53, 54, 64, 65] were used in other studies.

Genotyping of SNPs

DNA was extracted from 200 μl of packed blood pellets from HIV+ patients and whole-blood samples from HIV− donors using the QIAamp 96 spin blood kit (QIAGEN, Valencia, CA). DNA concentrations were measured using Qubit® Fluorometer (Invitrogen, Carlsbad, CA). SNPs were genotyped using Illumina's GoldenGate® genotyping assay system combined with VeraCode® Technology (Illumina Inc., San Diego, CA). Allelic discrimination was performed using a BeadXpress® Reader (Illumina) according to the manufacturer's instructions.

The genotype data were uploaded and filtered using the GenomeStudio data analysis software v2011.1 (Illumina Inc., San Diego, CA). SNPs were filtered by genotype call

frequency (<0.9, $n = 1$) and replicate errors ($n = 2$). Samples with genotype call frequency <0.9 were excluded ($n = 4$). Subsequently, SNPs were excluded from analysis if genotypic distribution among HIV− donors, stratified by race, deviated from the Hardy-Weinberg equilibrium (HWE) with a significant cutoff value of $P \leq 0.001$ ($n = 1$). Thus, in the final analysis, 41 SNPs, as listed in Supplementary Table A, were examined in a total of 276 subjects (HIV+, $n = 180$; HIV−, $n = 96$).

Genotyping of Duffy (*FY*) blood group antigen

In order to quantitatively measure admixture in our African American groups, *FY* genotyping (−46T>C, 625G>A [Gly44Asp]) was performed as previously described.[78]

Statistical analysis

Minor allele frequency, potential batch effect, and HWE were calculated using PLINK v1.07.[83] An online 2 × 2 contingency table for Fisher's exact test [http://www.langsrud.com/fisher.htm] was used to calculate differences in allele frequencies between populations, and a 2-tailed $P < 0.05$ was considered to be significant. Samples from HIV+ patients ($n = 180$) were analyzed on 2 plates. Potential batch effect was assessed by comparing allele frequencies from the 2 plates using a t-test.[84] Pairwise LD between SNPs of a gene or 2 genes that are nearby (*TLR1* and *TLR6* [12 kb], and *TLR7* and *TLR8* [10 kb]) (Figure 1A and 1B) was determined for both HIV+ and HIV− Caucasians and African Americans using SHEsis.[85] Strong LD was defined by high values for both D' (≥0.8) and r^2 (≥0.5) parameters.[58]

Logistic regression analysis was performed on all 41 SNPs using PLINK v1.07.[83] Initially, all subjects were included in a single analysis, without adjusting for race or sex. A second regression analysis adjusted for race within the regression equation. Finally, the data were stratified by race, analyzing Caucasians and African Americans separately, and adjustment for sex was made in both analyses. SNPs were coded under an additive genetic model, and then under a dominant genetic model, except those in *TLR7* and *TLR8*, located on chromosome X. Under the additive model, subjects having 2, 1, or 0 copy of the minor allele were coded as a 2, 1, and 0, respectively. Under the dominant model, subjects having 2 or 1 copy of the minor allele were coded as a 1, whereas those with 0 copy of the minor allele were coded as a 0.

Multiple testing correction for all regression analyses was determined by using SNPSpDlite.[59] SNPSpDlite calculates a multiple testing correction for SNPs that are in LD with one another, by calculating the LD correlation matrix for given SNPs, then estimating the number of independent tests within the sample. This is an alternative to the more conservative Bonferroni correction, which assumes all tests are independent. Thus, the significance threshold, α, for all SNP association tests was 0.001 (effective number of independent tests = 35). The additive and dominant models were tested separately, with the same significance threshold (0.001) applied to both sets of results.

Single locus and multilocus, whose products jointly form heterodimers (*TLR1_TLR2* and *TLR2_TLR6*), haplotype analyses were performed using the haplo.stats package v1.2.2. for

R. Haplotype scores (hap-score)[86] were calculated using haplo.score within the haplo.stats package and were used to test the association between haplotypes and HIV status. Haplotype scores cannot be used as a means of interpreting the measure of the haplotype effect, but simply as an indicator of the strength of the association between the haplotype and the outcome of interest.[86] A positive hap-score indicates that the haplotype occurs more frequently in case subjects, whereas a negative hap-score indicates that the haplotype occurs more frequently in control subjects.[86–88] The analysis output includes both gene-specific (global) and haplotype-specific *P*-values, which were considered significant if < 0.05. A global *P*-value < 0.05 was inferred as a significant difference in an overall haplotype profile of a gene/heterodimer between HIV+ and HIV− subjects. If the global *P*-value was significant, only then were the haplotype-specific *P*-values considered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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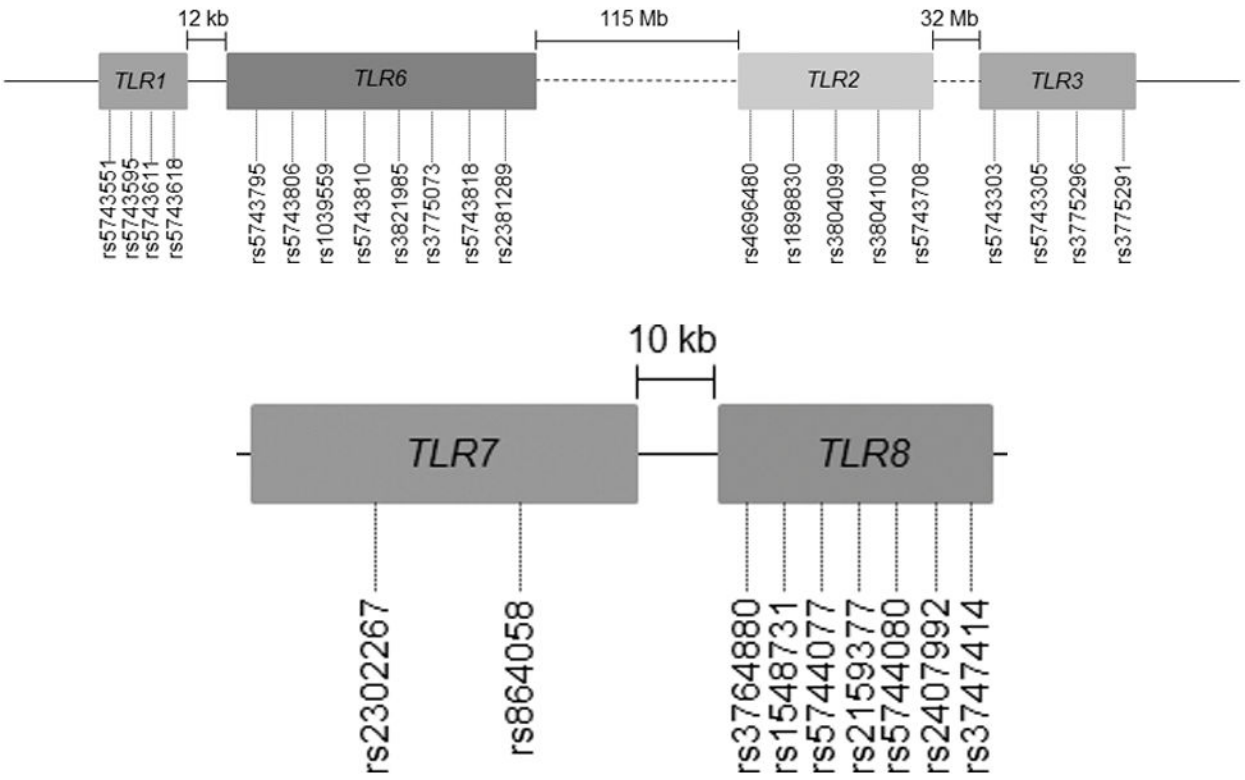

Figure 1.

Diagrammatic representation of the location of the *TLR* genes and SNPs therein on (A) chromosome 4 and (B) chromosome X.

Table 1

Demographic characteristics of study populations

Characteristic	HIV+ (n = 180)	HIV− (n = 96)
Race[†]		
Caucasian, n (%)	54 (30)	48 (50)
African American, n (%)	102 (57)	48 (50)
Other, n (%)	24 (13)	0
Sex		
Male, n (%)	135 (75)	47 (49)
Female, n (%)	41 (23)	47 (49)
Unknown, n (%)	4 (2)	2 (2)

[†]Self-identified.

HIV− subjects were random blood donors.

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Table 2

Distribution of *TLR* minor alleles and their frequencies

Gene (Chr #, ID)	rs number	SNP	MAF (allele) HIV+[a]	MAF (allele) HIV−	MAF (allele) HIV+ CA	MAF (allele) HIV− CA	MAF (allele) HIV+ AFA	MAF (allele) HIV− AFA
TLR1 (#4, 7096)	rs5743551	−7202G>A	0.38 (A)	0.44 (G)	0.37 (G)[1]	0.16 (G)[1]	0.24 (A)	0.27 (A)
	rs5743595	−2192T>C	0.11 (C)	0.09 (C)	0.20 (C)	0.14 (C)	0.05 (C)	0.05 (C)
	rs5743611	239G>C	0.05 (C)	0.05 (C)	0.09 (C)	0.09 (C)	0.03 (C)	0.01 (C)
	rs5743618	1805G>T	0.31 (G)	0.49 (T)	0.44 (T)[1]	0.21 (T)[1]	0.16 (G)	0.23 (G)
TLR2 (#4, 7097)	rs4696480	−16934T>A	0.46 (A)	0.39 (A)	0.49 (T)	0.40 (A)	0.44 (A)	0.39 (A)
	rs1898830	−15607A>G	0.22 (G)	0.26 (G)	0.27 (G)	0.36 (G)	0.18 (G)	0.14 (G)
	rs3804099	597T>C	0.44 (T)	0.48 (T)	0.45 (T)	0.46 (C)	0.42 (T)	0.42 (T)
	rs3804100	1350T>C	0.05 (C)	0.05 (C)	0.04 (C)	0.03 (C)	0.04 (C)	0.06 (C)
	rs5743708	2258G>A	0.01 (A)	0.02 (A)	0.01 (A)	0.03 (A)	0.01 (A)	0.01 (A)
TLR3 (#4, 7098)	rs5743303	−8921A>T	0.16 (T)	0.18 (T)	0.20 (T)	0.22 (T)	0.15 (T)	0.15 (T)
	rs5743305	−8441T>A	0.38 (A)	0.37 (A)	0.43 (A)	0.45 (A)	0.37 (A)	0.29 (A)
	rs3775296	−299698G>T	0.14 (T)	0.19 (T)	0.18 (T)	0.22 (T)	0.13 (T)	0.16 (T)
	rs3775291	1234C>T	0.16 (T)	0.19 (T)	0.29 (T)	0.27 (T)	0.07 (T)	0.11 (T)
TLR4 (#9, 7099)	rs2770150	−3612A>G	0.23 (G)	0.22 (G)	0.32 (G)	0.31 (G)	0.18 (G)	0.14 (G)
	rs2737190	−2604G>A	0.40 (A)	0.50 (A)	0.43 (G)	0.26 (G)	0.28 (A)	0.26 (A)
	rs10759932	−1607T>C	0.23 (C)	0.14 (C)	0.21 (C)[1]	0.05 (C)[1]	0.24 (C)	0.23 (C)
	rs4986790	896A>G	0.06 (G)	0.08 (G)	0.06 (G)	0.08 (G)	0.06 (G)	0.08 (G)
	rs4986791	1196C>T	0.02 (T)	0.05 (T)	0.03 (T)	0.08 (T)	0.01 (T)	0.01 (T)
	rs11536889	+11381G>C	0.06 (C)	0.11 (C)	0.08 (C)	0.19 (C)	0.03 (C)	0.04 (C)
	rs7873784	+12186C>G	0.22 (C)	0.14 (C)	0.18 (C)	0.15 (C)	0.25 (C)[2]	0.13 (C)[2]
TLR6 (#4, 10333)	rs5743795	−1401G>A	0.11 (A)	0.10 (A)	0.19 (A)	0.16 (A)	0.05 (A)	0.04 (A)
	rs5743806	−673C>T	0.50 (C)	0.42 (C)	0.38 (C)[3]	0.24 (C)[3]	0.43 (T)	0.40 (T)
	rs1039559	−502T>C	0.30 (C)	0.41 (C)	0.40 (C)[4]	0.41 (T)[4]	0.23 (C)	0.23 (C)
	rs5743810	745T>C	0.17 (T)	0.33 (T)	0.32 (T)[4]	0.49 (C)[4]	0.09 (T)	0.15 (T)
	rs3821985	1083C>G	0.48 (C)	0.44 (G)	0.39 (G)	0.25 (G)	0.39 (C)	0.38 (C)

Gene (Chr #, ID)	rs number	SNP	MAF (allele)	MAF (allele)	MAF (allele)		MAF (allele)	
			HIV+[a]	HIV−	HIV+ CA	HIV− CA	HIV+ AFA	HIV− AFA
	rs3775073	1263A>G	0.47 (A)	0.45 (G)	0.40 (G)[3]	0.25 (G)[3]	0.38 (A)	0.35 (A)
	rs5743818	1932T>G	0.17 (G)	0.17 (G)	0.28 (G)	0.22 (G)	0.10 (G)	0.11 (G)
	rs2381289	4224C>T	0.34 (T)	0.33 (T)	0.50 (T)	0.40 (T)	0.24 (T)	0.26 (T)
TLR7 (X, 51284)	rs2302267	1–120T>G	0.03 (G)	0.07 (G)	0.05 (G)	0.10 (G)	0.02 (G)	0.04 (G)
	rs864058	2403G>A	0.12 (A)	0.17 (A)	0.09 (A)	0.14 (A)	0.16 (A)	0.19 (A)
TLR8 (X, 51311)	rs3764880	1A>G	0.28 (G)	0.26 (G)	0.39 (G)[4]	0.18 (G)[4]	0.24 (G)	0.34 (G)
	rs1548731	+3121T>C	0.50 (T)	0.41 (T)	0.30 (T)	0.30 (T)	0.39 (C)	0.48 (C)
	rs5744077	28A>G	0.06 (G)	0.06 (G)	0.00 (G)	0.00 (G)	0.10 (G)	0.12 (G)
	rs2159377	354C>T	0.11 (T)	0.18 (T)	0.16 (T)	0.16 (T)	0.09 (T)[5]	0.21 (T)[5]
	rs5744080	645C>T	0.38 (C)	0.46 (C)	0.45 (C)	0.35 (T)	0.30 (C)	0.27 (C)
	rs2407992	1953G>C	0.28 (G)	0.41 (G)	0.39 (G)[4]	0.35 (C)[4]	0.17 (G)	0.16 (G)
	rs3747414	2253C>A	0.33 (A)	0.30 (A)	0.44 (A)	0.25 (A)	0.30 (A)	0.36 (A)
TLR9 (#3, 54106)	rs187084	−1486C>T	0.38 (C)	0.36 (C)	0.37 (C)	0.35 (C)	0.39 (C)	0.38 (C)
	rs5743836	−1237C>T	0.26 (C)	0.23 (C)	0.17 (C)	0.11 (C)	0.33 (C)	0.34 (C)
	rs352139	+1174G>A	0.44 (A)	0.44 (A)	0.49 (A)	0.47 (G)	0.39 (A)	0.35 (A)
	rs352140	1635G>A	0.43 (A)	0.43 (A)	0.47 (A)	0.47 (A)	0.43 (A)	0.40 (A)

Abbreviations: Chr, chromosome; MAF, minor allele frequency; CA, Caucasian; AFA, African American.

[a] Includes individuals from other race (see Table 1).

Minor allele frequencies of the SNPs presented in Table 3 show significant differences:

[1] $P < 0.001$;

[2] $P = 0.015$;

[3] $P = 0.03$;

[4] $P \leq 0.01$;

[5] $P = 0.029$.

Table 3

Regression analysis of *TLR* SNPs

Racial group	Gene	rs number	SNP	Amino acid	Allele	Test	OR	95% CI	*P*-value
Caucasian	*TLR1*	rs5743551	−7202G>A	-	G	Add	2.69	1.31, 5.53	0.007
		rs5743618	1805G>T	Ser602Ile	T		2.51	1.29, 4.88	0.007
	TLR4	rs10759932	−1607T>C	-	C		4.03	1.4, 11.59	0.010
	TLR6	rs5743806	−673C>T	-	C		2.09	1.03, 4.23	0.040
		rs1039559	−502T>C	-	C		0.41	0.22, 0.79	0.007
		rs5743810	745T>C	Ser249Pro	T		0.45	0.24, 0.83	0.010
		rs3775073	1263A>G	Lys421Lys	G		2.05	1.02, 4.09	0.043
	TLR8	rs3764880	1A>G	Met1Val	G		3.01	1.16, 7.83	0.024
		rs2407992	1953G>C	Leu651Leu	C		2.43	1.1, 5.36	0.028
	TLR1	rs5743551	−7202G>A	-	G	Dom	2.75	1.11, 6.82	0.028
		rs5743618	1805G>T	Ser602Ile	T		2.52	1.05, 6.1	0.040
	TLR4	rs10759932	−1607T>C	-	C		4.23	1.31, 13.68	0.016
	TLR6	rs1039559	−502T>C	-	C		0.31	0.11, 0.88	0.028
		rs5743810	745T>C	Ser249Pro	T		0.28	0.11, 0.73	0.010
African American	*TLR4*	rs7873784	+12186C>G	-	C	Add	2.37	1.16, 4.84	0.018
	TLR8	rs2159377	354C>T	Asp118Asp	T		0.39	0.16, 0.92	0.031
	TLR4	rs7873784	+12186C>G	-	C	Dom	2.31	1.06, 5.01	0.035

Abbreviations: Add, additive genetic model; Dom, dominant genetic model; OR, odds ratio; CI, confidence interval.

Table 4

Haplotype analysis of *TLR* SNPs by HIV status in Caucasians

Haplotype[†‡]	Hap-Freq (total)	Hap-Freq HIV+	Hap-Freq HIV−	Hap-score	*P*-value Global	*P*-value Haplotype-specific
TLR1					0.025	
GTGT**T**	0.093	0.157	0.021	2.198		0.028
ATG**G**	0.575	0.467	0.698	−3.313		0.001
TLR4					0.032	
AG**C**ACGG	0.098	0.146	0.042	2.529		0.011
TLR2_TLR6					0.017	
TGTTG_G**TCT**C**A**TC	0.139	0.080	0.206	−2.839		0.005

Abbreviations: Hap-Freq, haplotype frequency; Hap-score, haplotype score.

[†]Nucleotide positions are in the same order as described in Table 2.

[‡]All haplotypes of *TLR1*, *TLR4*, and *TLR2_TLR6* are presented in Supplementary Table C.

The SNPs presented in Table 3 are shown in bold.