ORIGINAL ARTICLE

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

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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 human immunodeficiency virus (HIV) acquisition and disease progression in various populations, most of which were European. In this 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 nine SNPs in TLR1, TLR4, TLR6 and TLR8 in Caucasians, and two 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 and 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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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-defensins5,6 and Toll-like receptors (TLRs),7 have been found to affect the natural history of HIV infection and disease progression.

TLRs 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. The molecular interaction of TLRs with pathogen-associated molecular patterns 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 pathogen-associated molecular patterns 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.15 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 TLR3,22 TLR4,18–22,26 TLR6,20 TLR7/820,22,25–27 and TLR922 have a functional role in HIV infection and disease. Single-nucleotide polymorphisms (SNPs) in TLR2,28–32 TLR3,28–31 TLR4,28–32 TLR7,28–30,53 TLR8,28–30,34 and TLR928–32,35 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, two 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.26,30

In this 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 two 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 infectious6–47 as well as inflammatory and immune-mediated non-infectious46–56 diseases. In addition to the SNP-based

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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 all SNPs in HIV− Caucasians and African Americans (Table 3). These two SNPs were not among the nine SNPs that were significantly associated with HIV status in African Americans (Table 3). These two SNPs were not among the nine 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 (\( P < 0.001 \), 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).

Linkage disequilibrium patterns
The pairwise linkage disequilibrium (LD) patterns of TLR1, TLR4, TLR6, TLR8 and TLR1_TLR6, based on \( D^r \geq 0.8 \) and \( r^2 \geq 0.5 \),\(^ {30} \) 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 \( (\alpha = 0.001) \).\(^ {39} \) These were: TLR1 rs5743551 (−2702G, 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, \( OR = 1.38 \); 2.00; \( P = 0.0002 \)). However, after co-variating 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 nine 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 five 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 five SNPs, showing significance in both genetic models, included three 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 two SNPs were not among the nine SNPs that were significantly associated with HIV status in Caucasians.

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 AGCCACGG was significantly more frequent in HIV+ patients than in HIV− donors (hap-score 2.529, \( P = 0.011 \)). The TLR2_TLR6 heterodimer haplotype TTGTGG_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 this study, utilizing samples from North American HIV+ and HIV− subjects, we provide evidence indicating that a total of nine 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,\(^ {22} \) including HIV (TLR4).\(^ {18} 22,24 \)

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**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) |

Abbreviation: HIV, human immunodeficiency virus. HIV− subjects were random blood donors.

*Self-identified.
TLR variants and HIV status

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Table 2. Distribution of TLR minor alleles and their frequencies

| Gene (Chr #, ID) | rs number | SNP | MAF (allele) |
|-----------------|-----------|-----|-------------|
| TLR1 (#4, 7096) | rs743551  | −7202 G>A | 0.38 (A) 0.44 (G) 0.37 (G) | HIV+ A |
| TLR1 (#4, 7096) | rs743595  | −2192 T>C | 0.11 (C) 0.09 (C) 0.20 (C) | HIV− |
| TLR1 (#4, 7096) | rs743611  | 239G>C | 0.05 (C) 0.05 (C) 0.09 (C) | HIV+ CA |
| TLR2 (#4, 7097) | rs743618  | 1805G>T | 0.31 (G) 0.49 (T) 0.44 (T) | HIV− |
| TLR2 (#4, 7097) | rs1988830 | −1507 A>G | 0.22 (G) 0.26 (G) 0.27 (G) | HIV− |
| TLR3 (#4, 7098) | rs3804099 | 597T>C | 0.44 (T) 0.48 (T) 0.45 (T) | HIV− |
| TLR3 (#4, 7098) | rs3804100 | 1305T>C | 0.05 (C) 0.05 (C) 0.04 (C) | HIV+ A |
| TLR4 (#9, 7099) | rs277291  | 1234C>T | 0.14 (T) 0.19 (T) 0.18 (T) | HIV+ A |
| TLR4 (#9, 7099) | rs277291  | 1234C>T | 0.14 (T) 0.19 (T) 0.18 (T) | HIV+ A |
| TLR6 (#4, 10333) | rs743795  | −1401 G>A | 0.11 (A) 0.10 (A) 0.19 (A) | HIV+ A |
| TLR8 (X, 51284) | rs396080  | −673 C>T | 0.17 (T) 0.33 (T) 0.33 (T) | HIV+ A |
| TLR8 (X, 51311) | rs3764880 | 1A>G | 0.28 (G) 0.26 (G) 0.39 (G) | HIV+ A |
| TLR9 (#3, 54106) | rs1548731 | +3121 T>C | 0.50 (T) 0.41 (T) 0.30 (T) | HIV+ A |
| TLR9 (#3, 54106) | rs4740077 | 28 A>G | 0.06 (G) 0.06 (G) 0.00 (G) | HIV+ A |
| TLR9 (#3, 54106) | rs2159377 | 354 C>T | 0.11 (T) 0.18 (T) 0.16 (T) | HIV+ A |
| TLR9 (#3, 54106) | rs4740080 | 645 C>T | 0.38 (C) 0.46 (C) 0.45 (C) | HIV+ A |
| TLR9 (#3, 54106) | rs2407992 | 1953 G>C | 0.28 (G) 0.41 (G) 0.39 (G) | HIV+ A |
| TLR9 (#3, 54106) | rs3747414 | 2253 A>G | 0.33 (A) 0.30 (A) 0.44 (A) | HIV+ A |
| TLR9 (#3, 54106) | rs187804 | −1468 C>T | 0.38 (C) 0.36 (C) 0.37 (C) | HIV+ A |
| TLR9 (#3, 54106) | rs743836 | −1237 C>T | 0.26 (C) 0.23 (C) 0.17 (C) | HIV+ A |
| TLR9 (#3, 54106) | rs352139 | +1174 G>A | 0.44 (A) 0.44 (A) 0.49 (A) | HIV+ A |
| TLR9 (#3, 54106) | rs352140 | 1635 G>A | 0.43 (A) 0.43 (A) 0.47 (A) | HIV+ A |

Abbreviations: AFA, African American; CA, Caucasian; Chr, chromosome; HIV, human immunodeficiency virus; MAF, minor allele frequency; SNP, single-nucleotide polymorphism; TLR, Toll-like receptor. MAFs of the SNPs presented in Table 3 show significant differences: *P < 0.001; **P = 0.015; ***P < 0.03; ****P < 0.01; ϕP = 0.029.

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 was equivalent between HIV-infected and HIV-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.21 To date, no genetic study, analyzing the role of TLR polymorphisms in influencing HIV infection and/or disease progression, has included TLR1 SNPs.22–28

The mechanisms by which the −7202G and 1805T alleles influence HIV status are currently unknown. These two alleles were functionally significant in sepsis,37,38 tuberculosis,37 leprosy37 and candidemia,40 where they were associated with higher nuclear factor-kB (NF-kB) activation and signaling, and elevated inflammatory cytokine production, including that of interleukin-6 (IL-6).61,62 Elevated levels of IL-6 have been associated with HIV infection62 and could contribute to HIV disease progression.61 Using a human monocytic cell line, THP-1, it has been shown that glycoprotein 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 co-receptor CD14.42 Furthermore, macrophages stimulated with lipopolysaccharide 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 (glycoprotein 41) or HIV-associated bacterial products (lipopolysaccharide/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 peripheral blood mononuclear cell sub-populations and dendritic cells from untreated HIV-infected patients is upregulated, whereas in peripheral blood mononuclear cells from chronic patients failing therapy it is reduced. However, the information regarding the role of TLR4 SNPs in influencing HIV infection and/or disease progression is mixed. In a treatment-naive, predominantly white North American cohort, SNPs Asp299Gly (rs4986790) and Thr399Ile (rs4986791) were associated with high peak plasma viral load. On the other hand, in Swiss, Spanish and Kenyan cohorts, these and other TLR4 SNPs were not associated with HIV infection and/or disease progression. In this 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 did not include −1607T>C and +12186C>G. The study included these SNPs but 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. The −1607C allele may be a risk factor for prostate cancer and traffic-related air pollution-associated childhood asthma, and the +12186C allele may be a risk factor for rheumatoid arthritis. However, none of these studies looked into the possible mechanisms of these allelic associations. In female genital epithelial cells, TLR4 binds to HIV-1 glycoprotein 120 and triggers pro-inflammatory cytokine production via activation of NF-κB. Being located in the promoter and 3′-untranslated 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-κB and production of pro-inflammatory cytokines. Alternatively, it may be that these SNPs affect responsiveness to lipopolysaccharide, as has been shown with other TLR4 SNPs (Asp299Gly and Thr399Ile), and thus influence HIV-associated systemic immune activation and pathogenesis.

TLR6 SNPs and HIV

TLR6 seems to have an important role in HIV/AIDS. In a Kenyan cohort of untreated women, the mRNA expression of TLR6 was significantly increased in peripheral blood mononuclear cells from HIV-infected subjects compared with those from uninfected subjects, and the expression level of TLR6 was positively correlated with the plasma viral load. However, the role of TLR6 SNPs in influencing HIV infection and/or disease progression has not yet been identified, 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

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**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 |
|              |      | rs7873784 | +12186C>G | — | C | Dom | 2.31 | 1.06, 5.01 | 0.035 |

Abbreviations: Add, additive genetic model; CI, confidence interval; Dom, dominant genetic model; OR, odds ratio; SNP, single-nucleotide polymorphism; TLR, Toll-like receptor.

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**Table 4. Haplotype analysis of TLR SNPs by HIV status in Caucasians**

| Haplotypea | Hap-Freq (total) | Hap-Freq HIV+ | Hap-Freq HIV− | Hap-score | P-value global | P-value haplotype specific |
|------------|-----------------|--------------|--------------|-----------|----------------|---------------------------|
| TLR1 GTGT  | 0.093           | 0.157        | 0.021        | 2.198     | 0.025          | 0.028                     |
|            | 0.575           | 0.001        | 0.698        | −3.313    | 0.001          |                           |
| TLR4 AGCGAG | 0.098 | 0.146 | 0.042 | 2.529 | 0.017 | 0.011 |
| TLR1–TLR6 TGTTG_GTCTCATC | 0.139 | 0.080 | 0.206 | −2.839 | 0.005 |                |

Abbreviations: Hap-Freq, haplotype frequency; Hap-score, haplotype score; HIV, human immunodeficiency virus; SNP, single-nucleotide polymorphism; TLR, Toll-like receptor. The SNPs presented in Table 3 are shown in bold. All haplotypes of TLR1, TLR4 and TLR2, TLR6 are presented in Supplementary Table C.

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variety of diseases, including infectious diseases, such as tuberculosis, most of the information regarding the functional significance of TLR6 SNPs is limited to non-synonymous 74ST>C, which is in strong LD with a promoter SNP −502T>C. In tuberculosis studies, the 74ST allele, compared with the 745C allele, was associated with lower NF-κB signaling, lower levels of IL-6 and higher levels of interferon-γ. Association of this allele with a decreased NF-κB activation and IL-6 production, but no effect on IL-10 production, may also have a role in protection against coronary artery disease. Interferon-γ has various roles in HIV/AIDS pathogenesis, including controlling HIV-1 replication. Thus, it is plausible that the observed protective effect of the 74ST allele in our study, 55–72% decrease in OR, is due to regulation of the interferon-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 and 1263A>G is scarce.

**TLR8 SNPs and HIV**

A number of studies have shown an important role of TLR8 (TLR7/8) in HIV/AIDS. It may be summarized from these studies that the mRNA expression of TLR8 is significantly increased in HIV-infected subjects; HIV sRNA 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. The 1G allele displayed impaired NF-κB activation in vitro, and was associated with modulation of cytokine induction (higher tumor necrosis factor-α and lower IL-10) in monocytes. The allele was significantly associated with reduced disease progression in a Caucasian German and a Kenyan cohort. However, among Kenyan female infants, the 1G allele was significantly associated with higher peak plasma viral load. No significant association was observed between TLR8 SNPs, including 1A>G, 1953G>C and 354C>T, and disease progression in a Swiss cohort. 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 specific and/or outcome measure specific.

Among the above-mentioned studies, except one, most did not include 1953G>C and 354C>T. Both 1953G>C and 1953C> T alleles may be associated with respiratory infections and diseases. No reference to the possible mechanisms of these allelic variations 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. Given that 354C>T had a protective effect in African Americans, and that HIV/AIDS continues to disproportionately affect this population, 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 Cat5, 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 or highly 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%). However, these two regions are similar regarding the European ancestry contribution to African-American populations (16–20% and 13–19%, respectively). These estimates were obtained using especially selected ancestry informative markers and are quite precise. 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 three most common FY alleles, FY*A, FY*B and FY*B2 (erythroid silent), FY*B2 is a key marker for African ancestry. 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. Frequency of the FY*B2 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 two groups.

Third, we adjusted for self-identified race and sex in our regression analyses. We cannot exclude the fact that residual confounding may exist because of 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, 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 TLR3 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. Most of the other studies were conducted in Europe, and a few in Africa. As 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. 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 needed to be further elucidated. Analysis of mRNA and protein levels of the TLR variants, and investigation of interactions of the variant TLRs with adapter 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.

MATERIALS 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, USA. 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. In addition, 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, USA. 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/AIDS8-35 and other infectious36-45 as well as inflammatory and immune-mediated non-infectious46-56 diseases, were included in this study. These SNPs were located in promoter regions, 5′-untranslated regions, exons, introns and 3′-untranslated regions (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) databases28,29,31 using haplotype tagging28,29,31 and candidate SNP28,31 approaches. Similar strategies, together with prediction of functionality using in vitro transfection assays and/or bioinformatics tools,38,50,53,34,64,65 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, USA). DNA concentrations were measured using Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA). SNPs were genotyped using Illumina’s GoldenGate genotyping assay system combined with VeraCode Technology (Illumina Inc., San Diego, CA, USA). Allelic discrimination was performed using a BeadXpress Reader (Illumina Inc.) according to the manufacturer’s instructions.

The genotype data were uploaded and filtered using the GenomeStudio data analysis software v2011.1 (Illumina Inc.). 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 with a significant cutoff value of P=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.28

Statistical analysis

MAF, potential batch effect and Hardy–Weinberg equilibrium were calculated using PLINK v1.07.81 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 two-tailed P=0.05 was considered to be significant. Samples from HIV+ patients (n=180) were analyzed on two plates. Potential batch effect was assessed by comparing allele frequencies from the two plates using a t-test.84 Pairwise LD between SNPs of a gene or two genes that are nearby (TLR1 and TLR6 (12 kb), and TLR7 and TLR8 (10 kb)) (Figures 1a and b) 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 r2(≥0.5) parameters.85

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 Figure 1. Diagrammatic representation of the location of the TLR genes and SNPs therein on (a) chromosome 4 and (b) chromosome X.
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, \( \alpha \), 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 (http://www.r-project.org/). Haplotype indicator of the strength of the association between the haplotype and the outcome of interest. A positive hapl-score indicates that the haplotype occurs more frequently in control subjects having 2, 1 or 0 copy of the minor allele were coded as a 2, 1 and 0. 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.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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