

Polymorphisms in toll-like receptor 4 and toll-like receptor 9 influence viral load in a seroincident cohort of HIV-1-infected individuals

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Objectives: Toll-like receptors (TLRs) are innate immune sensors that are integral to resisting chronic and opportunistic infections. Mounting evidence implicates TLR polymorphisms in susceptibilities to various infectious diseases, including HIV-1. We investigated the impact of TLR single nucleotide polymorphisms (SNPs) on clinical outcome in a seroincident cohort of HIV-1-infected volunteers.

Design: We analyzed *TLR* SNPs in 201 antiretroviral treatment-naïve HIV-1-infected volunteers from a longitudinal seroincident cohort with regular follow-up intervals (median follow-up 4.2 years, interquartile range 4.4). Participants were stratified into two groups according to either disease progression, defined as peripheral blood CD4⁺ T-cell decline over time, or peak and setpoint viral load.

Methods: Haplotype tagging SNPs from *TLR2*, *TLR3*, *TLR4*, and *TLR9* were detected by mass array genotyping, and CD4⁺ T-cell counts and viral load measurements were determined prior to antiretroviral therapy initiation. The association of TLR haplotypes with viral load and rapid progression was assessed by multivariate regression models using age and sex as covariates.

Results: Two *TLR4* SNPs in strong linkage disequilibrium [1063 A/G (D299G) and 1363 C/T (T399I)] were more frequent among individuals with high peak viral load compared with low/moderate peak viral load (odds ratio 6.65, 95% confidence interval 2.19–20.46, $P < 0.001$; adjusted $P = 0.002$ for 1063 A/G). In addition, a *TLR9* SNP previously associated with slow progression was found less frequently among individuals with high viral setpoint compared with low/moderate setpoint (odds ratio 0.29, 95% confidence interval 0.13–0.65, $P = 0.003$, adjusted $P = 0.04$).

Conclusion: This study suggests a potentially new role for *TLR4* polymorphisms in HIV-1 peak viral load and confirms a role for *TLR9* polymorphisms in disease progression.

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Introduction

Persons with HIV-1 infection exhibit a high degree of variability in their rate of progression to immunodeficiency and associated complications. In the absence of

antiretroviral treatment, some infected persons progress to AIDS within 1–5 years, whereas others may remain asymptomatic for over 20 years [1–4]. A large accumulation of data has demonstrated a significant contribution of human leukocyte antigen (HLA)

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haplotypes to this variability of disease progression rates. For example, certain HLA alleles lend remarkable protection from viral escape mutations [5,6], whereas others are associated with more prolific viral escape mutation [7,8]. Heightened expression levels of β -chemokines and lower expression of the inhibitory receptors of cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed death-1 (PD-1) are examples of other mechanisms that can slow disease progression [9–12]. Although some of these mechanisms of resistance to disease progression have been associated with genetic polymorphisms, such as possessing certain HLA-B alleles [13–15], HLA heterozygosity [7,16], β -chemokines, and their receptors (chemokine (C-C motif) receptor 5 (CCR5) and chemokine (C-X-C motif) receptor 4 (CXCR4)) [17,18], it is likely that other genes are also associated with these differences.

Toll-like receptors (TLRs) are transmembrane proteins expressed on most immune cells that detect specific molecular patterns on bacteria, viruses, fungi, and parasites and stimulate the production of innate immune effectors such as inflammatory and chemotactic cytokines [19]. Support for the central role of TLR activation in both the innate and adaptive immune responses comes partially from *TLR* polymorphisms that have been shown to influence susceptibility to a number of infectious human pathogens [20–27]. For example, HIV-1 virions can activate TLR7/TLR8 directly with the single-stranded RNA genome, and the HIV-1 envelope protein, gp120, has been shown to inhibit TLR9-triggered plasmacytoid dendritic cell (pDC) activation and interferon- α (IFN α) production [28–30]. TLRs also have indirect effects on HIV-1 pathogenesis, such as when B cells from viremic HIV-infected individuals lose their ability to proliferate in response to TLR9 stimulation [31]. Finally, the lower levels of IFN α in response to TLR7 and TLR9 agonists in sooty mangabeys are characteristic of the naturally occurring nonpathogenic simian immunodeficiency virus (SIV) infection in these primates [32].

Recently, two studies [24,33] have highlighted a role for *TLR9* polymorphisms in the clinical manifestations of HIV-1 infection. Although these findings have begun to shed light on the role of innate immunity in HIV-1 infection, both studies were limited by several biases and differing definitions of disease progression. The Swiss HIV Cohort Study is a seroprevalent cohort established before HAART was widely available; therefore, rapid progressors may not have been included due to higher mortality rates, and the analyses may have been influenced by onset confounding or differential length biases. In contrast, Soriano-Sarabia *et al.* [33] analyzed a therapeutic cohort comprised of patients who entered at treatment initiation. This clinical time point can be influenced by contemporaneous clinical practices and drug availability can change over time [33], and could thus bias towards less enrollment of rapid progressors as treatment initiation

criteria became more stringent. Furthermore, although one *TLR* gene was examined by tagging single nucleotide polymorphisms (SNPs) in the Swiss HIV Cohort Study, both studies primarily examined only those SNPs that had previously reported associations with other diseases. Haplotype analyses using tagging SNPs take into account the shared evolutionary history of many correlated SNPs (i.e., linkage disequilibrium) and allow for an optimized approach to haplotype association analyses [34,35], and neither of these two HIV-1 disease progression studies utilized an objective sampling of *TLR* haplotype tagging SNPs. In an attempt to overcome these limitations, we examined the role of haplotype tagging SNPs in several *TLR* genes in disease progression using a seroincident cohort of individuals with an extensive longitudinal clinical and virological database.

Methods

Study populations

The Seattle Primary Infection Cohort (PIC) is a longitudinal seroincident cohort of HIV-infected individuals enrolling volunteers during the acute stages of disease. Enrollment criteria have been previously reported in detail elsewhere [36]. Briefly, individuals were enrolled within 90 days of HIV-1 infection, confirmed by viral RNA and antibody tests, and followed-up for sample and clinical data collection bi-weekly for 1 month, monthly for a quarter, and quarterly thereafter. The PIC is over 90% white; therefore, we restricted the analysis to white enrollees only to reduce the risk of population stratification. All individuals gave written informed consent for these studies, and the study was approved by the Institutional Review Boards of the University of Washington and the Fred Hutchinson Cancer Research Center, as well as the Western Institutional Review Board.

HIV-1 plasma viral load in volunteers was determined by branched DNA (bDNA) assay (Bayer, Terrytown, New York, USA) and/or quantitative reverse transcription-PCR (Roche, Pleasanton, California, USA) [37–39]. The PIC includes individuals who were enrolled during the early stages of HIV-1 infection, allowing assessment of peak viral loads before a setpoint viral load is established. The peak viral load was defined by the maximum amount of HIV-1 plasma RNA observed during the first 100 days following the determined date of HIV-1 infection and before antiretroviral therapy (ART)/HAART has been started. The setpoint was defined as the mean viral load during days 100–730 after infection and before initiation of ART/HAART [40]. CD4⁺ T cells in peripheral blood were measured at each follow-up visit by routine flow cytometry methods (BD Biosciences, San Jose, California, USA) [39], and disease progression was defined as the rate of CD4⁺ T-cell count decline over time in the absence of ART [41–43]. Quantitation of CD4

cell slope measurement was determined using a linear regression of time versus the square root of the CD4⁺ T-cell count, as described previously [24,42]. Values of CD4 cell slopes and viral loads were categorized into tertiles to define test groups of rapid progressors or high viral load individuals, respectively. Rapid progressors were defined as those participants with CD4 cell slopes below the lower tertile (steepest slopes), and all remaining participants with CD4 cell slopes above this value were defined as slow/moderate progressors. High and low/moderate viral load participants were similarly defined using the higher tertile of log₁₀-transformed viral load values. Sex-specific tertiles were defined separately to account for the high proportion of males present in the cohort. To check for potential biases in our cohort definitions, we reversed the designation of cases from rapid progressors/high viral loads to slow progressors/low viral loads and obtained the converse results of our original analyses.

Genotyping

Fourteen haplotype tagging SNPs were retrieved for *TLR2*, *TLR3*, *TLR4*, and *TLR9* from the Innate Immunity Program in Genomic Applications (<http://innateimmunity.net>) and cross-referenced for function and location in the SNP public database (dbSNP) (<http://ncbi.nlm.nih.gov>). Five additional SNPs that had previously been associated with a human disease and/or predicted to induce an amino acid change in the protein were also genotyped [20]. DNA was extracted from cryopreserved peripheral blood mononuclear cells or Epstein-Barr virus (EBV)-transformed B-cell lines with QIAamp DNA Blood Mini kits (Qiagen, Valencia, California, USA), and genotypes were ascertained by a mass spectrometry-based genotyping method as previously described [44] (Sequenom, San Diego, California, USA). SNPs were designated by nucleotide position relative to the translational start site on the mRNA or genomic DNA sequence, and a plus (+) or minus (-) sign

indicates a nontranslated nucleotide upstream or downstream from the first translated base pair, respectively.

Statistical analysis

Testing for Hardy-Weinberg equilibrium and pairwise linkage disequilibrium calculations were performed with the genhw and pwld packages in Stata, respectively (version 9.1; StataCorp, College Station, Texas, USA). Haplotypes were inferred by using an expectation-maximization algorithm within the DECIPHER program as implemented in Statistical Analysis for Genetic Epidemiology (SAGE, version 5.4.1, <http://darwin.cwru.edu>).

The risk of having a high viral load and the risk of having a rapid CD4⁺ T-cell count decline were assessed in logistic regression models by TLR polymorphisms, using age and sex as covariates in all cases [45]. For SNPs analyses, initial testing was performed using the additive model, in which SNPs were coded by the presence of zero, one, or two copies of a given allele. SNPs found to have significant associations were further tested under all possible models against a neutral model that did not assume any mode of inheritance in order to select the most likely model. For haplotype analyses, each haplotype within a gene was coded by the presence of zero, one, or two copies of the haplotypes and entered into a single model using the most frequent haplotype as a reference. Multiple comparisons were accounted for by classical Bonferroni correction using 16 independent tests for SNP analyses (one for each independent SNP, i.e., those with R² < 0.8) and four tests in the haplotype analyses (one for each gene).

Results

The demographic characteristics of the HIV-infected individuals are shown in Table 1. The cohort included

Table 1. Demographic characteristics of HIV-1-infected individuals.

	Peak viral load analysis (days 0–100)		Viral setpoint analysis (days 100–730)		CD4 ⁺ T-cell progression analysis		Total n (%)
	High n (%)	Low/moderate n (%)	High n (%)	Low/moderate n (%)	Rapid n (%)	Slow/moderate n (%)	
Assessable cases	50 (100)	99 (100)	42 (100)	80 (100)	27 (100)	50 (100)	201 (100)
Age, median (IQR)	34.0 (9)	33.0 (9) ^b	32.5 (7)	31.0 (10)	32.5 (8)	35.0 (10)	33.0 (9)
Sex							
Male	49 (98)	97 (98)	40 (95)	78 (97)	25 (93)	48 (96)	196 (98)
Female	1 (2)	2 (2)	2 (5)	2 (3)	2 (7)	2 (4)	5 (2)
Risk ^a							
MSM	48 (96)	95 (95)	39 (93)	78 (97)	25 (97)	47 (94)	193 (96)
Heterosexual	2 (4)	5 (5)	2 (5)	5 (6)	3 (11)	3 ((6)	10 (5)
IDU	2 (4)	3 (3)	2 (5)	4 (5)	1 (4)	1 (2)	9 (4)
Peak viral load, median (IQR)	5.92 (1.03)	4.57 (0.69)	5.08 (0.74)	4.26 (1.03)	4.76 (1.09)	4.54 (1.08)	4.85 (1.19)
Viral setpoint, median (IQR)	4.70 (0.43)	4.45 (0.88)	4.94 (0.22)	4.07 (0.83)	4.46 (0.88)	4.17 (1.09)	4.71 ((1.02)
CD4 cell slope, median (IQR)	-1.21 (2.26)	-1.93 (4.10)	-3.99 (4.11)	-1.42 (2.13)	-4.91 (2.46)	-0.98 (1.43)	-1.76 (3.20)

IQR, interquartile range.

^aParticipants may have reported more than one risk factor.

^bP=0.03 in a linear regression model comparing age between individuals with high versus moderate/low peak viral load.

Table 2. Toll-like receptor single nucleotide polymorphisms in HIV-1-infected individuals.

Gene (location)	Region	SNP	Amino acid change	SNP reference number ^a	MAF (n = 201)	HWE (n = 201)	LD (R ²) ^b (n = 201)
TLR2 (4q32)	Intron 1	-16934 T/A		rs4696480	0.49	0.71	
	Exon 3	597 C/T	N199N	rs3804099	0.42	0.31	
	Exon 3	1350 T/C	S450S	rs3804100	0.06	0.98	
TLR3 (4q35)	5'	-8921 A/T		rs5743303	0.23	0.59	
	5'	-8441 T/A		rs5743305	0.35	0.26	
	Intron 3	2602 G/C		rs5743314	0.19	0.31	
TLR4 (9q32)	Exon 4	1234 C/T	L412F	rs3775291	0.30	0.68	
	5'	-3612 A/G		rs2770150	0.26	1.00	
	5'	-2604 A/G		rs273190	0.27	<0.01	
	5'	-1607 T/C		rs10759932	0.12	1.00	
	Exon 4	1063 A/G	D299G	rs4986790	0.07	0.48	1.0
	Exon 4	1363 C/T	T399I	rs4986791	0.07	0.48	
	3'	+11381 G/C		rs11536889	0.20	0.52	
3'	+12186 G/C		rs7873784	0.14	0.37		
TLR9 (3p21.3)	5'	-1486 T/C		rs187084	0.38	0.65	
	5'	-1237 T/C		rs5743836	0.16	0.88	
	Intron 1	+1174 G/A		rs352139	0.47	0.14	0.99
	Exon 2	1635 A/G	P545P	rs352140	0.47	0.30	

HWE, Hardy-Weinberg equilibrium; LD, linkage disequilibrium; MAF, minor allele frequency; SNP, single nucleotide polymorphism; TLR, toll-like receptor.

^aPublic database (dbSNP) reference number.

^bSNPs in strong LD ($R^2 > 0.8$) are indicated.

201 participants, of whom 196 were men (98%), 193 reported a repeated history of MSM as their HIV-1 risk factor (96%), and the median age was 33 years (interquartile range 9). Data were available to assess peak viral load, setpoint viral load, and CD4⁺ T-cell depletion slopes in 149, 122, and 77 participants, respectively (Table 1). The demographics of participants in the comparison groups in each arm of the study (high versus low/moderate peak and setpoint viral load and rapid versus slow/moderate CD4⁺ T-cell decline) were not different, except for age in the peak viral load comparison ($P = 0.03$).

The characteristics of SNPs in the four TLRs (2, 3, 4, and 9) detected in the study population are shown in Table 2. All tested SNPs except one (*TLR4* -2604 A/G) were at Hardy-Weinberg equilibrium. The primers used to genotype *TLR4* -2604 A/G were used successfully to genotype other cohorts during the same study period, suggesting that the absence of equilibrium was not due to a technical issue but rather to a random effect and/or the small sample size. One SNP was present at a frequency of less than 5%, and therefore was not included in the analyses (*TLR2* 2258 G/A, rs5743708, minor allele frequency 4%). As expected from previous observations, two SNP pairs were in strong linkage disequilibrium (*TLR4* 1063 A/G and 1363 C/T, $R^2 = 1$; and *TLR9* +1174 G/A and 1635 A/G, $R^2 = 0.97$) [20,24].

The risk of having a high viral load by *TLR* polymorphisms was assessed in logistic regression models after adjustment for age and sex (Table 3). We observed that two *TLR4* SNPs in strong linkage disequilibrium [1063 A/G (D299G) and 1363 C/T (T399I)] were associated with the high peak viral load phenotype; 74,

26, and 0% of individuals with high peak viral load had zero, one, and two copies of the minor allele, respectively, versus 95, 4, and 1% of low/moderate peak viral load individuals [odds ratio (OR) 4.70, 95% confidence interval (CI) 1.65-13.36, adjusted $P = 0.004$ under the additive model; OR 6.65, 95% CI 2.16-20.46, adjusted $P < 0.001$ under the dominant model, Tables 3 and 4]. This difference was still significant after correction for multiple testing ($P = 0.002$, dominant model). We also observed that two *TLR3* SNPs (-8921 A/T and 1234 C/T) tended to be associated with peak viral load, but these associations were not significant after correction for multiple testing ($P = 0.8$ and $P = 1.0$, respectively).

The SNPs associated with high peak viral load were not associated with high viral load setpoint or rapid HIV-1 disease progression as measured by CD4 cell slope. However, we observed that two SNPs in *TLR9* were associated with these endpoints (Tables 3 and 4). A *TLR9* SNP (1635 A/G) was protective against high viral load setpoint (OR 0.29, 95% CI 0.13-0.65, $P = 0.003$, dominant model) and rapid progression (OR 0.45, 95% CI 0.24-0.97, $P = 0.04$, additive model); this difference remained significant after correction for multiple testing for the viral load setpoint ($P = 0.04$) but not the disease progression phenotype ($P = 0.4$). Another *TLR9* SNP (-1237 T/C) tended to be associated with high viral load setpoint (OR 2.12, 95% CI 0.99-4.54, $P = 0.05$) and rapid progression (OR 3.21, 95% CI 1.16-8.90, $P = 0.03$), but neither of these associations was significant after correction for multiple testing ($P = 0.8$ and $P = 0.4$, respectively). We also observed that a *TLR2* SNP (-16934 T/A) was associated with protection against rapid progression (OR 0.43, 95% CI 0.20-0.92, $P = 0.03$, under the additive model), but again, the

Table 3. Frequencies of toll-like receptor alleles in individuals with high versus low/moderate viral load/setpoint and rapid versus slow/moderate progression by CD4⁺ T-cell decline.

Gene	Alleles	Viral peak (Maximum days 0–100)			Viral setpoint (Mean days 100–730)			CD4 ⁺ T-cell decline		
		High (n = 50)	Low/moderate (n = 99)	P ^a	High (n = 42)	Low/moderate (n = 80)	P ^a	Rapid (n = 27)	Slow/moderate (n = 50)	P ^a
TLR2	–16934A	0.74	0.77	0.8	0.66	0.70	0.9	0.63	0.80	0.03 ^b
	597T	0.62	0.71	0.3	0.57	0.60	0.7	0.59	0.66	0.9
	1350C	0.12	0.10	0.6	0.07	0.10	0.7	0.11	0.10	0.7
TLR3	–8921T	0.52	0.5	0.06 ^b	0.29	0.44	0.1	0.30	0.45	0.1
	–8441A	0.56	0.52	0.5	0.67	0.47	0.1	0.67	0.48	0.2
	2602C	0.30	0.32	0.9	0.40	0.29	0.5	0.44	0.28	0.2
TLR4	1234T	0.40	0.54	0.05 ^b	0.55	0.49	0.2	0.56	0.51	1.0
	–3612G	0.44	0.44	0.5	0.34	0.45	0.1	0.44	0.43	0.9
	–2604G	0.38	0.44	0.9	0.46	0.41	1.0	0.37	0.45	0.7
	–1607C	0.22	0.24	0.7	0.29	0.18	0.2	0.22	0.16	0.4
	1063G ^c	0.26	0.05	0.004 ^b	0.20	0.13	0.3	0.19	0.17	0.6
	+11381C	0.36	0.36	0.7	0.38	0.30	0.4	0.26	0.40	0.3
TLR9	+12186C	0.24	0.29	0.9	0.28	0.25	0.9	0.22	0.31	0.5
	–1486C	0.60	0.61	1.0	0.69	0.57	0.4	0.63	0.54	0.6
	–1237C	0.26	0.34	0.3	0.44	0.28	0.05 ^b	0.48	0.22	0.06 ^b
	1635G ^d	0.72	0.67	0.4	0.49	0.76	0.06 ^b	0.59	0.76	0.04 ^b

LD, linkage disequilibrium; SNP, single nucleotide polymorphism; TLR, toll-like receptor.

^aAdjusted for age and sex.

^bFor consistency, all comparisons are shown for the additive model only. For associations with a P value less than 0.1, all possible models (dominant, additive, and recessive) were tested against a neutral model (that did not assume any mode of inheritance), and the most likely model was selected (see Table 4).

^cAs TLR4 1063 A/G was in strong LD with TLR4 1363 C/T (R² = 1.0), only one of the SNPs is shown.

^dAs TLR9 +1174 G/A was in strong LD with TLR9 1635 A/G (R² = 0.99), only one of the SNPs is shown.

Table 4. Comparison of toll-like receptor genotype frequencies in individuals with high versus low/moderate viral load/setpoint and rapid versus slow/moderate progression by CD4⁺ T-cell decline.

Gene	Location	Genotype	High peak viral load (n = 50)	Low/moderate peak viral load (n = 99)	OR (95% CI)	P ^a	Model ^b			
TLR3	–8921	A/A	0.48	0.65	1.69 (0.97–2.94)	0.06	Additive			
		A/T	0.42	0.30						
		T/T	0.10	0.05						
	1234	C/C	0.60	0.46						
		C/T	0.36	0.42						
		T/T	0.04	0.11						
TLR4	1063	A/A	0.74	0.95	0.57 (0.32–1.01)	0.05	Additive			
		A/G	0.26	0.04						
		G/G	0.00	0.01						
TLR9	–1237	T/T	0.56	0.72	2.12 (0.99–4.54)	0.05	Additive			
		T/C	0.41	0.28						
		C/C	0.02	0.00						
	1635	A/A	0.51	0.24				0.29 (0.13–0.65)	0.003 ^d	Dominant
		A/G	0.27	0.55						
		G/G	0.22	0.21						
Gene	Location	Genotype	Rapid progressors (n = 27)	Slow/moderate progressors (n = 50)	OR (95% CI)	P ^a	Model ^b			
TLR2	–16934	T/T	0.37	0.20	0.43 (0.20–0.92)	0.03	Additive			
		T/A	0.56	0.51						
		A/A	0.07	0.29						
TLR9	–1237	T/T	0.52	0.78				3.21 (1.16–8.90)	0.03	Dominant
		T/C	0.48	0.20						
		C/C	0.02	0.02						
1635	A/A	0.41	0.24	0.48 (0.24–0.97)	0.04	Additive				
	A/G	0.48	0.45							
	G/G	0.11	0.31							

CI, confidence interval; OR, odds ratio; TLR, toll-like receptor.

^aAdjusted for age and sex.

^bAll possible models (additive, dominant, and recessive) were tested against a neutral model (that did not assume any mode of inheritance), and the most likely model was selected.

^cP = 0.002 after correction for multiple testing.

^dP = 0.04 after correction for multiple testing.

Table 5. Association of toll-like receptor haplotypes with viral load/setpoint and rapid progression by CD4⁺ T-cell decline in HIV-positive individuals.

Gene	Haplotype ^a	High (n = 50) versus moderate/low (n = 99) viral peak (days 0–100)		High (n = 42) versus moderate/low (n = 80) viral setpoint (days 100–730)		Rapid progressors (n = 27) versus slow/moderate progressors (n = 50)	
		OR (95% CI)	Adjusted P ^b	OR (95% CI)	Adjusted P ^b	OR (95% CI)	Adjusted P ^b
<i>TLR2</i>	TTT	Reference		Reference		Reference	
	ACT	0.76 (0.38–1.49)	0.4	0.91 (0.44–1.86)	0.8	0.45 (0.16–1.26)	0.13
	ATT	1.28 (0.60–2.72)	0.5	1.60 (0.78–3.31)	0.2	0.78 (0.27–2.22)	0.64
	TCT	1.03 (0.44–2.40)	1.0	1.61 (0.65–3.98)	0.3	2.89 (0.79–10.62)	0.11
	ACC	1.01 (0.33–3.11)	1.0	1.15 (0.33–4.01)	0.8	1.19 (0.21–6.85)	0.84
<i>TLR3</i>	ATGC	Reference		Reference		Reference	
	AACC	1.08 (0.49–2.36)	0.8	1.38 (0.62–3.07)	0.4	1.53 (0.56–4.22)	0.41
	TTGC	1.61 (0.80–3.23)	0.2	0.67 (0.29–1.54)	0.3	0.46 (0.16–1.31)	0.15
	ATGT	0.59 (0.25–1.36)	0.2	1.13 (0.48–2.64)	0.8	0.65 (0.21–1.96)	0.44
	AAGT	1.06 (0.39–2.83)	0.9	2.18 (0.82–5.81)	0.1	1.92 (0.55–6.70)	0.30
	AAGC	1.57 (0.52–4.73)	0.4	0.74 (0.15–3.69)	0.7	0.20 (0.02–2.30)	0.19
<i>TLR4</i>	GATAGG	Reference		Reference		Reference	
	AATAGG	0.76 (0.38–1.49)	0.4	1.66 (0.74–3.74)	0.2	1.44 (0.53–3.88)	0.48
	AATACG	1.28 (0.60–2.72)	0.5	2.21 (0.92–5.32)	0.08	0.78 (0.27–2.25)	0.65
	AGTAGC	1.03 (0.44–2.40)	1.0	0.98 (0.31–3.07)	1.0	0.47 (0.11–2.07)	0.32
	AGCAGG	1.01 (0.33–3.11)	1.0	2.34 (0.79–6.92)	0.1	1.49 (0.35–6.32)	0.59
	AGCAGC	1.14 (0.25–5.25)	0.9	7.21 (1.02–50.84)	0.05	1.48 (0.16–13.38)	0.72
	AATGGG	5.71 (1.34–24.45)	0.02	3.07 (0.71–13.21)	0.1	1.28 (0.26–6.32)	0.76
<i>TLR9</i>	TTG	Reference		Reference		Reference	
	CTA	0.89 (0.53–1.48)	0.6	1.56 (0.88–2.79)	0.13	1.70 (0.82–3.53)	0.16
	TCA	0.56 (0.26–1.21)	0.1	2.54 (1.12–5.78)	0.03	3.56 (1.21–10.49)	0.02

CI, confidence interval; OR, odds ratio; TLR, toll-like receptor.

^aIndividuals with rare haplotypes (<3%) for the specific TLR are not included in the analyses.

^bAdjusted for age and sex.

association was not significant after adjustment for multiple testing ($P=0.5$).

In the analyses by haplotype, we observed that *TLR4* haplotype 7, present in *TLR4* 1063G and 1363T carriers, was associated with an increased risk of having a high viral load compared with the reference haplotype (OR 5.71, 95% CI 1.34–24.45, adjusted $P=0.02$, Table 5), but the association was lost after correction for multiple testing ($P=0.08$). We also found that *TLR9* haplotype 3, present in *TLR9* –1237C and 1635G carriers, was associated with an increased risk of having a high viral load setpoint (OR 2.54, 95% CI 1.12–5.78) as well as rapid progression measured by CD4⁺ T-cell decline (OR 3.56, 95% CI 1.21–10.49, adjusted $P=0.02$); however, this difference was not significant after correction for multiple testing ($P=0.1$ and $P=0.08$, respectively).

Discussion

To our knowledge, this is the first study of an association between *TLR4* polymorphisms and elevated viral load in HIV-infected individuals. The two SNPs associated with the high viral load phenotype (*TLR4* D299G and T399I) are in strong linkage disequilibrium in white populations and have been previously associated with a reduced responsiveness to lipopolysaccharide (LPS) in humans [46]. In addition, carriers of these SNP alleles have greater

susceptibility to infections, including respiratory syncytial virus in newborns [47,48], severe malaria [48], and invasive aspergillosis in hematopoietic cell transplant recipients [20,49–51].

The mechanism by which D299G may influence HIV viral load is currently unknown. LPS in the bloodstream is indicative of microbial translocation across the gut mucosa [52–54], and levels of serum LPS and systemic immune activation are correlatively lower in HIV-1 nonprogressors compared with control populations, as measured by circulating IFN α and activated CD8⁺ T cells [55]. Additionally, LPS activation of TLR4 stimulates programmed death of central and effector memory CD4⁺ T cells [56], but a clear link of these observations to the D299G polymorphism is still lacking. Alternatively, the possibility exists that the effects of TLR4 on HIV-1 viral load stem not from direct interaction with the incoming pathogen but from an interplay between the innate responses at colonized mucosal surfaces and the host's ability to fight invasive infections [57,58]. Such a proposed ability to set an individual's 'innate immunity tone' would position both TLR4 and TLR9 as key systemic regulators in the mucosa [57–59], and polymorphisms in these genes could thus impact the response to a broad range of mucosal commensals and pathogens.

To our knowledge, our study is the first to focus on the association of HIV disease progression and *TLR9* polymorphisms in a seroincident cohort. Our results

showed that the 1635G allele was associated with lower viral load setpoint and slower progression. The results are consistent with the observations reported by Soriano-Sarabia *et al.* [33], who also found that the 1635G allele is associated with slow progression in a seroprevalent, dynamic therapeutic cohort. However, these results are not consistent with the observations made in the Swiss HIV Cohort Study, in which the 1635G allele was associated with rapid progression. A possible explanation for this discrepancy may be that the latter cohort included individuals enrolled before the era of HAART, and thus may have been influenced by onset confounding or differential-length biases [60,61]. The Seattle PIC may provide a more accurate picture of primary disease progression on the basis of common clinical parameters, as it is a seroincident cohort with viral load and CD4⁺ T-cell measurements at close intervals during the initial stages of HIV disease prior to ART initiation.

Although evidence that TLR9 may be a critical factor in HIV pathogenesis continues to accumulate, the mechanisms by which TLR9 interferes with HIV pathogenesis remain unknown. Although the 1635G allele does not induce an amino acid change, it has been associated with lower TLR9 expression [62], and lower levels of TLR9 are found in the memory B cells of viremic compared with aviremic HIV-infected patients [31]. Additionally, a recent study showed that sooty mangabeys have reduced levels of innate immune activation during apathogenic SIV infection. Moreover, pDCs from these animals produced substantially reduced levels of IFN α in response to TLR7 and TLR9 activators [32]. The TLR9 signaling pathway, in particular, will be important to examine in a more comprehensive manner, as isolated genetic or protein analyses are unlikely to fully describe the interactions between HIV-1 and TLR9.

Determining the causes of heterogeneity in disease outcome following HIV-1 infection is an ongoing challenge. By using a closely monitored seroincident cohort in this study, we show associations between *TLR4* polymorphisms and increased peak viral loads, as well as between *TLR9* polymorphisms decreased viral load setpoint and CD4⁺ T-cell decline. These findings add to the current understanding of HIV pathogenesis by providing a basis for future studies on mechanisms of HIV-1 disease progression. However, more research is needed to establish reliable predictive measures that can improve HIV-1 treatment outcomes.

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All of the authors contributed to the study design and analyses.

S.O.P. wrote the manuscript, collated and analyzed clinical data, and performed genomic DNA extractions and sample preparations.

M.J.M. provided linkage to the study cohort, financial support for the study, and critical review of the manuscript.

P.Y.B. oversaw the genotypic analyses, performed clinical data calculations and statistical analyses, and provided critical review of the manuscript.

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