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Polymorphisms in *TLR4* and *TLR9* influence viral load in a sero-incident cohort of HIV-1-infected individuals

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Abstract

OBJECTIVES—Toll-like receptors (TLR) 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 sero-incident cohort of HIV-1-infected volunteers.

DESIGN—We analyzed *TLR* SNPs in 201 antiretroviral treatment-naïve HIV-1 infected subjects from a longitudinal sero-incident cohort with regular follow-up intervals (median follow-up 4.2 years, interquartile range 4.4). Subjects 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 (1063A/G [D299G] and 1363C/T [T399I]) were more frequent among subjects with high peak viral load compared to low/moderate peak viral load (OR=6.65, 95% CI 2.19–20.46, P<0.001; adjusted P=0.002 for 1063A/G). In addition, a *TLR9* SNP previously associated with slow progression was found less frequently among subjects with high viral setpoint compared to low/moderate setpoint (OR=0.29, 95% CI 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.

Keywords

HIV-1; Innate Immunity; HIV genetics; HIV immunology; Single Nucleotide Polymorphism; Toll-Like Receptor 9; Toll-Like Receptor 4

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 anti-retroviral treatment, some infected persons progress to AIDS within 1–5 years, while others may remain asymptomatic for over twenty years [1–4]. A large accumulation of data has demonstrated a significant contribution of human leukocyte antigen (HLA) haplotypes to this variability of disease progression rates. For example, certain HLA alleles lend remarkable protection from viral escape mutations [5,6], while 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 CTLA-4 and 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 CCR5 and 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 TLR7/8 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 alpha (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 non-pathogenic SIV infection in these primates [32].

Recently two studies have highlighted a role for *TLR9* polymorphisms in the clinical manifestations of HIV-1 infection [24,33]. 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 sero-prevalent cohort established before highly-active anti-retroviral therapy (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 and colleagues analyzed a therapeutic cohort comprised of patients who entered at treatment initiation. This clinical timepoint can be influenced by contemporaneous clinical practices and drug availability can change over time [33], and thus could bias towards less enrollment of rapid progressors as treatment initiation criteria became more stringent. Furthermore, although one TLR gene was examined by tagging SNPs in the Swiss HIV Cohort Study, both studies primarily examined only those SNPs that had previously reported associations with other pathologies. 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 sero-incident cohort of subjects with an extensive longitudinal clinical and virological database.

METHODS

Study Populations

The Seattle Primary Infection Cohort (PIC) is a longitudinal sero-incident 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 one month, monthly for a quarter, and quarterly thereafter. The PIC is over 90% Caucasian; 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 bDNA assay (Bayer, Terrytown, NY) and/or quantitative reverse-transcription PCR (Roche, Pleasanton, CA) [37–39]. The PIC includes subjects who were enrolled during the early stages of HIV-1 infection, allowing assessment of peak viral loads before a set-point 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 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, CA) [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 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 slopes and viral loads were categorized into thirds to define test groups of rapid progressors or high viral load subjects, respectively. Rapid progressors were defined as those participants with CD4 slopes below the lower tertile (steepest slopes), and all remaining subjects with CD4 slopes above this value were defined as slow/moderate progressors. High and low/moderate viral load test subjects were similarly defined using the higher tertile of log_{10}-transformed viral load values. Gender-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 dbSNP public database (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 EBV-transformed B

cell lines with QIAamp DNA Blood Mini kits (Qiagen, Valencia, CA), and genotypes were ascertained by a mass spectrometry-based genotyping method as previously described [44] (Sequenom, San Diego, CA). 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 non-translated nucleotide upstream or downstream from the first translated base pair, respectively.

Statistical Analysis

Testing for Hardy-Weinberg equilibrium and pairwise linkage disequilibrium (LD) calculations were performed with the genhw and pwld packages in Stata, respectively (version 9.1, StataCorp, College Station, TX). Haplotypes were inferred by using an expectation-maximization algorithm within the DECIPHER program as implemented in SAGE (Statistical Analysis for Genetic Epidemiology, v5.4.1, http://darwin.cwru.edu).

The risk of having a high viral load and the risk of having a rapid CD4$^+$ T-cell count decline was 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, where SNPs were coded by the presence of 0, 1, or 2 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 0, 1, or 2 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 $R^2<0.8$) and four tests in the haplotype analyses (one for each gene).

RESULTS

The demographic characteristics of the HIV-infected subjects are shown in Table 1. The cohort included 201 participants, of whom 196 were males (98%), 193 reported a repeated history of men having sex with men as their HIV-1 risk factor (96%), and the median age was 33 (interquartile range 9). Data was available to assess peak viral load, setpoint viral load and CD4$^+$ T-cell depletion slopes in 149, 122 and 77 subjects, respectively (Table 1). The demographics of subjects 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 LD (*TLR4* 1063 A/G and 1363 C/T, $R^2=1$; and *TLR9* +1174 G/A and 1635A/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 2 *TLR4* SNPs in strong LD (1063 A/G [D299G] and 1363 C/T [T399I]) were associated with the high peak viral load phenotype; 74%, 26% and 0% of subjects with high peak viral load had 0, 1 and 2 copies of the minor allele, respectively, versus 95%, 4% and 1% of low/moderate

peak viral load subjects (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* SNP (−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 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, 9%% CI 0.20–0.92, P=0.03, under the additive model), but again, the 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 to 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 report 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 LD in Caucasian 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 non-progressors compared to control populations, as measured by circulating interferon-alpha 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 sero-incident 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., who also found that the 1635G allele is associated with slow progression in a sero-prevalent, dynamic therapeutic cohort [33]. However, these results are not consistent with the observations made in the Swiss HIV Cohort Study, where the 1635G allele was associated with rapid progression. A possible explanation for this discrepancy may be that the latter cohort included subjects 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 based on common clinical parameters, since it is a sero-incident cohort with viral load and CD4^{+} T cell measurements at close intervals during the initial stages of HIV disease prior to ART initiation.

While 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 individuals [31]. Additionally, a recent study showed that sooty mangabeys have reduced levels of innate immune activation during apathogenic SIV infection. Moreover, plasmacytoid dendritic cells from these animals produced substantially reduced levels of interferon alpha in response to TLR7 and TLR9 activators [32]. The TLR9 signaling pathway, in particular, will be important to examine in a more comprehensive manner, since 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 sero-incident 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.

Acknowledgments

REFERENCES

1. Buchbinder S, Vittinghoff E. HIV-infected long-term nonprogressors: epidemiology, mechanisms of delayed progression, and clinical and research implications. Microbes Infect 1999;1:1113–1120. [PubMed: 10572315]

2. Buchbinder SP, Katz MH, Hessol NA, O'Malley PM, Holmberg SD. Long-term HIV-1 infection without immunologic progression. Aids 1994;8:1123–1128. [PubMed: 7986410]

3. Cao Y, Qin L, Zhang L, Safrit J, Ho DD. Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. N Engl J Med 1995;332:201–208. [PubMed: 7808485]

4. Pereyra F, Addo MM, Kaufmann DE, Liu Y, Miura T, Rathod A, et al. Genetic and immunologic heterogeneity among persons who control HIV infection in the absence of therapy. J Infect Dis 2008;197:563–571. [PubMed: 18275276]

5. Navis M, Schellens I, van Baarle D, Borghans J, van Swieten P, Miedema F, et al. Viral replication capacity as a correlate of HLA B57/B5801-associated nonprogressive HIV-1 infection. J Immunol 2007;179:3133–3143. [PubMed: 17709528]

6. Bailey JR, Brennan TP, O'Connell KA, Siliciano RF, Blankson JN. Evidence of CD8+ T-cell-mediated selective pressure on human immunodeficiency virus type 1 nef in HLA-B*57+ elite suppressors. J Virol 2009;83:88–97. [PubMed: 18945771]

7. Carrington M, Nelson GW, Martin MP, Kissner T, Vlahov D, Goedert JJ, et al. HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage. Science 1999;283:1748–1752. [PubMed: 10073943]

8. Itescu S, Mathur-Wagh U, Skovron ML, Brancato LJ, Marmor M, Zeleniuch-Jacquotte A, Winchester R. HLA-B35 is associated with accelerated progression to AIDS. J Acquir Immune Defic Syndr 1992;5:37–45. [PubMed: 1738086]

9. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, et al. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. Nature 2006;443:350–354. [PubMed: 16921384]

10. Kaufmann DE, Kavanagh DG, Pereyra F, Zaunders JJ, Mackey EW, Miura T, et al. Upregulation of CTLA-4 by HIV-specific CD4+ T cells correlates with disease progression and defines a reversible immune dysfunction. Nat Immunol 2007;8:1246–1254. [PubMed: 17906628]

11. Zhang JY, Zhang Z, Wang X, Fu JL, Yao J, Jiao Y, et al. PD-1 up-regulation is correlated with HIV-specific memory CD8+ T-cell exhaustion in typical progressors but not in long-term nonprogressors. Blood 2007;109:4671–4678. [PubMed: 17272504]

12. Saha K, Bentsman G, Chess L, Volsky DJ. Endogenous production of beta-chemokines by CD4+, but not CD8+, T-cell clones correlates with the clinical state of human immunodeficiency virus type 1 (HIV-1)-infected individuals and may be responsible for blocking infection with non-syncytium-inducing HIV-1 in vitro. J Virol 1998;72:876–881. [PubMed: 9420304]

13. Flores-Villanueva PO, Hendel H, Caillat-Zucman S, Rappaport J, Burgos-Tiburcio A, Bertin-Maghit S, et al. Associations of MHC ancestral haplotypes with resistance/susceptibility to AIDS disease development. J Immunol 2003;170:1925–1929. [PubMed: 12574360]

14. Kiepiela P, Leslie AJ, Honeyborne I, Ramduth D, Thobakgale C, Chetty S, et al. Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. Nature 2004;432:769–775. [PubMed: 15592417]

15. Migueles SA, Sabbaghian MS, Shupert WL, Bettinotti MP, Marincola FM, Martino L, et al. HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. Proc Natl Acad Sci U S A 2000;97:2709–2714. [PubMed: 10694578]

16. Tang J, Costello C, Keet IP, Rivers C, Leblanc S, Karita E, et al. HLA class I homozygosity accelerates disease progression in human immunodeficiency virus type 1 infection. AIDS Res Hum Retroviruses 1999;15:317–324. [PubMed: 10082114]

17. Gonzalez E, Dhanda R, Bamshad M, Mummidi S, Geevarghese R, Catano G, et al. Global survey of genetic variation in CCR5, RANTES, and MIP-1alpha: impact on the epidemiology of the HIV-1 pandemic. Proc Natl Acad Sci U S A 2001;98:5199–5204. [PubMed: 11320252]

18. Dean M, Carrington M, Winkler C, Huttley GA, Smith MW, Allikmets R, et al. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. Science 1996;273:1856–1862. [PubMed: 8791590]

19. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006;124:783–801. [PubMed: 16497588]

20. Bochud PY, Bochud M, Telenti A, Calandra T. Innate immunogenetics: a tool for exploring new frontiers of host defence. Lancet Infect Dis 2007;7:531–542. [PubMed: 17646026]

21. Misch EA, Hawn TR. Toll-like receptor polymorphisms and susceptibility to human disease. Clin Sci (Lond) 2008;114:347–360. [PubMed: 18230059]

22. Tulic MK, Hurrelbrink RJ, Prele CM, Laing IA, Upham JW, Le Souef P, et al. TLR4 polymorphisms mediate impaired responses to respiratory syncytial virus and lipopolysaccharide. J Immunol 2007;179:132–140. [PubMed: 17579031]

23. Tal G, Mandelberg A, Dalal I, Cesar K, Somekh E, Tal A, et al. Association between common Toll-like receptor 4 mutations and severe respiratory syncytial virus disease. J Infect Dis 2004;189:2057–2063. [PubMed: 15143473]

24. Bochud PY, Hersberger M, Taffe P, Bochud M, Stein CM, Rodrigues SD, et al. Polymorphisms in Toll-like receptor 9 influence the clinical course of HIV-1 infection. Aids 2007;21:441–446. [PubMed: 17301562]

25. Hawn TR, Wu H, Grossman JM, Hahn BH, Tsao BP, Aderem A. A stop codon polymorphism of Toll-like receptor 5 is associated with resistance to systemic lupus erythematosus. Proc Natl Acad Sci U S A 2005;102:10593–10597. [PubMed: 16027372]

26. Wurfel MM, Gordon AC, Holden TD, Radella F, Strout J, Kajikawa O, et al. Toll-like Receptor 1 Polymorphisms Affect Innate Immune Responses and Outcomes in Sepsis. Am J Respir Crit Care Med. 2008

27. Oh DY, Taube S, Hamouda O, Kucherer C, Poggensee G, Jessen H, et al. A Functional Toll-Like Receptor 8 Variant Is Associated with HIV Disease Restriction. J Infect Dis 2008;198:701–709. [PubMed: 18605904]

28. Martinelli E, Cicala C, Van Ryk D, Goode DJ, Macleod K, Arthos J, Fauci AS. HIV-1 gp120 inhibits TLR9-mediated activation and IFN-{alpha} secretion in plasmacytoid dendritic cells. Proc Natl Acad Sci U S A 2007;104:3396–3401. [PubMed: 17360657]

29. Heil F, Ahmad-Nejad P, Hemmi H, Hochrein H, Ampenberger F, Gellert T, et al. The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. Eur J Immunol 2003;33:2987–2997. [PubMed: 14579267]

30. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 2004;303:1526–1529. [PubMed: 14976262]

31. Jiang W, Lederman MM, Mohner RJ, Rodriguez B, Nedrich TM, Harding CV, Sieg SF. Impaired naive and memory B-cell responsiveness to TLR9 stimulation in human immunodeficiency virus infection. J Virol 2008;82:7837–7845. [PubMed: 18524824]

32. Mandl JN, Barry AP, Vanderford TH, Kozyr N, Chavan R, Klucking S, et al. Divergent TLR7 and TLR9 signaling and type I interferon production distinguish pathogenic and nonpathogenic AIDS virus infections. Nat Med 2008;14:1077–1087. [PubMed: 18806803]

33. Soriano-Sarabia N, Vallejo A, Ramirez-Lorca R, Rodriguez MD, Salinas A, Pulido I, et al. Influence of the Toll-Like Receptor 9 1635A/G Polymorphism on the CD4 Count, HIV Viral Load, and Clinical Progression. J Acquir Immune Defic Syndr 2008;49:128–135. [PubMed: 18769358]

34. Sebastiani P, Lazarus R, Weiss ST, Kunkel LM, Kohane IS, Ramoni MF. Minimal haplotype tagging. Proc Natl Acad Sci U S A 2003;100:9900–9905. [PubMed: 12900503]

35. Halldorsson BV, Istrail S, De La Vega FM. Optimal selection of SNP markers for disease association studies. Hum Hered 2004;58:190–202. [PubMed: 15812176]

36. Schacker T, Collier AC, Hughes J, Shea T, Corey L. Clinical and epidemiologic features of primary HIV infection. Ann Intern Med 1996;125:257–264. [PubMed: 8678387]

37. Stekler J, Sycks BJ, Holte S, Maenza J, Stevens CE, Dragavon J, et al. HIV dynamics in seminal plasma during primary HIV infection. AIDS Res Hum Retroviruses 2008;24:1269–1274. [PubMed: 18844461]

38. Mulder J, McKinney N, Christopherson C, Sninsky J, Greenfield L, Kwok S. Rapid and simple PCR assay for quantitation of human immunodeficiency virus type 1 RNA in plasma: application to acute retroviral infection. J Clin Microbiol 1994;32:292–300. [PubMed: 8150937]

39. Schacker TW, Hughes JP, Shea T, Coombs RW, Corey L. Biological and virologic characteristics of primary HIV infection. Ann Intern Med 1998;128:613–620. [PubMed: 9537934]

40. Patke DS, Langan SJ, Carruth LM, Keating SM, Sabundayo BP, Margolick JB, et al. Association of Gag-specific T lymphocyte responses during the early phase of human immunodeficiency virus type 1 infection and lower virus load set point. J Infect Dis 2002;186:1177–1180. [PubMed: 12355372]

41. Gilbert PB. Some statistical issues in the design of HIV-1 vaccine and treatment trials. Stat Methods Med Res 2000;9:207–229. [PubMed: 11084705]

42. Speelmon EC, Livingston-Rosanoff D, Li SS, Vu Q, Bui J, Geraghty DE, et al. Genetic association of the antiviral restriction factor TRIM5alpha with human immunodeficiency virus type 1 infection. J Virol 2006;80:2463–2471. [PubMed: 16474153]

43. Urassa W, Bakari M, Sandstrom E, Swai A, Pallangyo K, Mbena E, et al. Rate of decline of absolute number and percentage of CD4 T lymphocytes among HIV-1-infected adults in Dar es Salaam, Tanzania. Aids 2004;18:433–438. [PubMed: 15090795]

44. Storm N, Darnhofer-Patel B, van den Boom D, Rodi CP. MALDI-TOF mass spectrometry-based SNP genotyping. Methods Mol Biol 2003;212:241–262. [PubMed: 12491915]

45. Zhao LP, Li SS, Khalid N. A method for the assessment of disease associations with single-nucleotide polymorphism haplotypes and environmental variables in case-control studies. Am J Hum Genet 2003;72:1231–1250. [PubMed: 12704570]

46. Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet 2000;25:187–191. [PubMed: 10835634]

47. Awomoyi AA, Rallabhandi P, Pollin TI, Lorenz E, Sztein MB, Boukhvalova MS, et al. Association of TLR4 polymorphisms with symptomatic respiratory syncytial virus infection in high-risk infants and young children. J Immunol 2007;179:3171–3177. [PubMed: 17709532]

48. Mockenhaupt FP, Cramer JP, Hamann L, Stegemann MS, Eckert J, Oh NR, et al. Toll-like receptor (TLR) polymorphisms in African children: Common TLR-4 variants predispose to severe malaria. Proc Natl Acad Sci U S A 2006;103:177–182. [PubMed: 16371473]

49. Bochud PY, Chien JW, Marr KA, Leisenring WM, Upton A, Janer M, et al. Tolllike receptor 4 polymorphisms and aspergillosis in stem-cell transplantation. N Engl J Med 2008;359:1766–1777. [PubMed: 18946062]

50. Ferwerda B, McCall MB, Verheijen K, Kullberg BJ, van der Ven AJ, Van der Meer JW, Netea MG. Functional consequences of toll-like receptor 4 polymorphisms. Mol Med 2008;14:346–352. [PubMed: 18231573]

51. Ferwerda B, Kibiki GS, Netea MG, Dolmans WM, van der Ven AJ. The toll-like receptor 4 Asp299Gly variant and tuberculosis susceptibility in HIV-infected patients in Tanzania. Aids 2007;21:1375–1377. [PubMed: 17545720]

52. Cooke KR, Olkiewicz K, Erickson N, Ferrara JL. The role of endotoxin and the innate immune response in the pathophysiology of acute graft versus host disease. J Endotoxin Res 2002;8:441–448. [PubMed: 12697087]

53. Schietroma M, Cappelli S, Carlei F, Di Giuro G, Agnifili A, Recchia CL, et al. [Intestinal and systemic endotoxaemia after laparotomic or laparoscopic cholecystectomy]. Chir Ital 2006;58:171–177. [PubMed: 16734165]

54. Wellmann W, Fink PC, Benner F, Schmidt FW. Endotoxaemia in active Crohn's disease. Treatment with whole gut irrigation and 5-aminosalicylic acid. Gut 1986;27:814–820. [PubMed: 3732891]

55. Brenchley JM, Price DA, Schacker TW, Asher TE, Silvestri G, Rao S, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. Nat Med 2006;12:1365–1371. [PubMed: 17115046]

56. Funderburg N, Luciano AA, Jiang W, Rodriguez B, Sieg SF, Lederman MM. Toll-like receptor ligands induce human T cell activation and death, a model for HIV pathogenesis. PLoS ONE 2008;3:e1915. [PubMed: 18382686]

57. Brandl K, Plitas G, Schnabl B, DeMatteo RP, Pamer EG. MyD88-mediated signals induce the bactericidal lectin RegIII gamma and protect mice against intestinal Listeria monocytogenes infection. J Exp Med 2007;204:1891–1900. [PubMed: 17635956]

58. Cash HL, Whitham CV, Behrendt CL, Hooper LV. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. Science 2006;313:1126–1130. [PubMed: 16931762]

59. Pamer EG. TLR polymorphisms and the risk of invasive fungal infections. N Engl J Med 2008;359:1836–1838. [PubMed: 18946070]

60. Alcabes P, Pezzotti P, Phillips AN, Rezza G, Vlahov D. Long-term perspective on the prevalent-cohort biases in studies of human immunodeficiency virus progression. Am J Epidemiol 1997;146:543–551. [PubMed: 9326431]

61. Brookmeyer R, Gail MH, Polk BF. The prevalent cohort study and the acquired immunodeficiency syndrome. Am J Epidemiol 1987;126:14–24. [PubMed: 3647718]

62. Tao K, Fujii M, Tsukumo S, Maekawa Y, Kishihara K, Kimoto Y, et al. Genetic variations of Toll-like receptor 9 predispose to systemic lupus erythematosus in Japanese population. Ann Rheum Dis 2007;66:905–909. [PubMed: 17344245]

Table 1

Demographic Characteristics of HIV-1 Infected Subjects

	Peak Viral Load Analysis (days 0–100)		Viral Setpoint Analysis (days 100–730)		CD4+ T cell Progression Analysis		All Subjects
	High N (%)	Low/Moderate N (%)	High N (%)	Low/Moderate N (%)	Rapid N (%)	Slow/Moderate N (%)	Total 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)[2]	32.5 (7)	31.0 (10)	32.5 (8)	35.0 (10)	33.0 (9)
Gender							
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[1]							
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 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)

Note: MSM, men having sex with men; IDU, injection drug use.

[1] Subjects may have reported more than one risk factor.

[2] P=0.03 in a linear regression model comparing age between subjects with high versus moderate/low peak viral load.

Table 2

TLR Single Nucleotide Polymorphisms in HIV-1 Infected Subjects

Gene (location)	Region	SNP	Amino acid change	SNP reference number[1]	MAF (N=202)	HWE (N=202)	LD (R²)[2] (N=202)
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	
	Exon 4	1234 C/T	L412F	rs3775291	0.30	0.68	
TLR4 (9q32)	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	
	Exon 2	1635 A/G	P545P	rs352140	0.47	0.30	0.99

Note: SNP, single nucleotide polymorphism; MAF, minor allele frequency; HWE, Hardy–Weinberg equilibrium; LD, linkage disequilibrium.

[1] Public database (dbSNP) reference number.

[2] SNPs in strong linkage disequilibrium (R²>0.8) are indicated.

Table 3

Frequencies of *TLR* Alleles in Subjects with High versus Low/Moderate Viral Load/Setpoint and Rapid versus Slow/Moderate Progression by CD4^{+} T-Cell Decline

Gene	Alleles	Viral Peak (Max. days 0–100)			Viral Setpoint (Mean days 100–730)			CD4+ T Cell Decline		
		High (N=50)	Low/ Moderate (N=99)	P[1]	High (N=42)	Low/ Moderate (N=80)	P[1]	Rapid (N=27)	Slow/ Moderate (N=50)	P[1]
TLR2	−16934A	0.74	0.77	0.8	0.66	0.70	0.9	0.63	0.80	**0.03**[2]
	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**[2]	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
	1234T	0.40	0.54	**0.05**[2]	0.55	0.49	0.2	0.56	0.51	1.0
TLR4	−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[3]	0.26	0.05	**0.004**[2]	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
	+12186C	0.24	0.29	0.9	0.28	0.25	0.9	0.22	0.31	0.5
TLR9	−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**[2]	0.48	0.22	**0.06**[2]
	1635G[4]	0.72	0.67	0.4	0.49	0.76	**0.06**[2]	0.59	0.76	**0.04**[2]

[1] Adjusted for age and sex.

[2] For consistency, all comparisons are shown for the additive model only. For associations with a P value <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).

[3] Since *TLR4* 1063 A/G was in strong LD with *TLR4* 1363 C/T (R[2]=1.0), only one of the SNPs is shown.

[4] Since *TLR9* +1174 G/A was in strong LD with *TLR9* 1635 A/G (R[2]=0.99), only one of the SNPs is shown.

Table 4

Comparison of *TLR* Genotype Frequencies in Subjects with High versus Low/Moderate Viral Load/Setpoint and Rapid versus Slow/Moderate Progression by CD4^{+} T-Cell Decline

Gene	Location	Geno-type	High Peak Viral Load (N=50)	Low/ Moderate Peak Viral Load (N=99)	OR (95%CI)	P[1]	Model[2]
TLR3	−8921	A/A	0.48	0.65			
		A/T	0.42	0.30			
		T/T	0.10	0.05	1.69 (0.97–2.94)	0.06	additive
	1234	C/C	0.60	0.46			
		C/T	0.36	0.42			
		T/T	0.04	0.11	0.57 (0.32–1.01)	**0.05**	additive
TLR4	1063	A/A	0.74	0.95			
		A/G	0.26	0.04			
		G/G	0.00	0.01	6.65 (2.16–20.46)	**<0.001**[3]	dominant

Gene	Location	Geno-type	High Viral Setpoint (N=42)	Low/ Moderate Viral Setpoint (N=80)	OR (95%CI)	P[1]	Model[2]
TLR9	−1237	T/T	0.56	0.72			
		T/C	0.41	0.28			
		C/C	0.02	0.00			
	1635	A/A	0.51	0.24	2.12 (0.99–4.54)	**0.05**	additive
		A/G	0.27	0.55			
		G/G	0.22	0.21	0.29 (0.13–0.65)	**0.003**[4]	dominant

Gene	Location	Geno-type	Rapid Progressors (N=27)	Slow/ Moderate Progressors (N=50)	OR (95%CI)	P[1]	Model[2]
TLR2	−16934	T/T	0.37	0.20			
		T/A	0.56	0.51			
		A/A	0.07	0.29	0.43 (0.20–0.92)	**0.03**	additive

Gene	Location	Genotype	High Peak Viral Load (N=50)	Low/ Moderate Peak Viral Load (N=99)	OR (95%CI)	P[1]	Model[2]
TLR9	−1237	T/T	0.52	0.78			
		T/C	0.48	0.20			
		C/C		0.02	3.21 (1.16–8.90)	**0.03**	dominant
	1635	A/A	0.41	0.24			
		A/G	0.48	0.45			
		G/G	0.11	0.31	0.48 (0.24–0.97)	**0.04**	additive

[1] Adjusted for age and sex.

[2] All 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.

[3] P=0.002 after correction for multiple testing.

[4] P=0.04 after correction for multiple testing.

Table 5

Association of *TLR* Haplotypes with Viral Load/Setpoint and Rapid Progression by CD4^{+} T-Cell Decline in HIV^{+} Subjects.

Gene	Haplotype[1]	High (N=50) versus Moderate/Low (N=99) Viral Peak (Max. days 0–100)		High (N=42) versus Moderate/Low (N=80) Viral Setpoint (Mean days 100–730)		Rapid Progressors (N=27) vs. Slow/Moderate Progressors (N=50)	
		OR (95%CI)	Adj. P[2]	OR (95%CI)	Adj. P[2]	OR (95%CI)	Adj. P[2]
TLR2	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
TLR3	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
TLR4	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
TLR9	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**

[1] Subjects with rare haplotypes (<3%) for the specific TLR are not included in the analyses.

[2] Adjusted for age and sex.