HHS Public Access

Author manuscript

J Acquir Immune Defic Syndr. Author manuscript; available in PMC 2023 March 16.

Published in final edited form as:

J Acquir Immune Defic Syndr. 2020 October 01; 85(2): 252–256. doi:10.1097/QAI.0000000000002437.

Toll-like receptor 9 1635A/G polymorphism is associated with HIV-1 rebound after four weeks of interruption of antiretroviral therapy

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Abstract

Objectives: This study aims to analyze the association of the presence of common polymorphisms (SNPs) on Toll-like receptors (TLR), such as TLR9-1635A/G, TLR2-1892A/C, TLR2-2258G/A, TLR4-899A/G, TLR4-1196C/T, with the viral rebound after stopping antiretroviral treatment (ART). CCR5-Δ32 deletion and HLA-A/HLA-B alleles were also analyzed.

Design: Interruption of ART may be required to investigate the outcome of strategies aimed to achieve drug-free HIV remission or cure. However, interruption of ART is currently not indicated. This was a retrospective longitudinal study that included 57 long-term suppressed HIV-1-infected individuals.

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Conflict of interests: The authors declare that they have no conflict of interests.

Methods: TLR SNPs were detected by RT-PCR. CCR5-Δ32 was analyzed by conventional PCR and HLA-A and HLA-B alleles by PCR-SSOP Luminex.

Results: HIV-1 RNA rebound at week 4 after treatment interruption positively correlated with pre-ART HIV-1 load (p=0.025). The TLR9-1635AA genotype was independently associated with a higher HIV-1 rebound compared to those with AG+GG genotype (multivariate stepwise regression analysis, p=0.004). Women had lower HIV-1 RNA load both at rebound and during the 72 weeks of follow-up compared to men (p<0.05 at all time-points), while CD4 nadir and CD4 count set-point were similar according to sex. Pre-ART viral load was independently associated with the viral set-point (p=0.001), while the presence of the HLA-A01 allele (p=0.027) and the CD4 nadir (p=0.001) were associated with the CD4 count set-point.

Conclusions: The association of the TLR9-1635AA genotype with a higher HIV-1 rebound suggests that this SNP may affect the results from strategies requiring interruption of ART aimed to cure HIV-1 infection.

Keywords

TLR9 polymorphism; SNP; HIV; HIV latency; viral rebound; interruption of antiretroviral therapy

INTRODUCTION

Over a decade ago, antiretroviral treatment (ART) interruptions were assayed in an attempt to reduce the negative effects associated with the prolonged exposure to ART and also to induce stronger cellular and humoral immune responses.[1,2] Unfortunately, after interruption of therapy, plasma HIV-1 RNA often reached levels similar to those found during the pre-treatment period.[3,4] This practice became inadvisable due to the evidence demonstrating an association of the interruption with an increased risk of clinical progression.[5,6] However, treatment discontinuation has re-gained attention due to current approaches aimed at reducing the persistent viral reservoir as part of the HIV-1 cure agenda.[7,8]

After ART discontinuation, plasma HIV-1 load rebound, and consequent set-point viremia levels are shown to be highly variable among HIV-infected individuals.[6,9,10] Mechanisms involved in heterogenic viral kinetics include, but may not be limited to, intact HIV-1 proviruses in circulating CD4 T cells,[11] potent HIV-1-specific or innate immune responses,[12–14] greater thymic volume and function,[9] CCR5 co-receptor expression,[15] or established HIV-1 reservoir size.[16,17]

Toll-Like Receptors (TLRs) are critical proteins involved in the establishment of effective immune responses.[18,19] TLR2 and TLR7 SNPs have been associated with HIV-1 set-point, explaining up to 6% of the variation.[13] We and others also reported the association of TLR2 (1892A/C and 2258G/A) and TLR4 (899A/G and 1196C/T) with HIV-1 RNA load, CD4 count and disease progression.[20–22] In addition, the combination of TLR7 (AA) and TLR9 (GG) genotypes might be linked to higher CD4 counts during the viremic period.[23] Finally, we reported that the AA genotype of the TLR9-1635A/G SNP was associated with higher HIV-1 RNA load in ART-naïve chronically HIV-1-infected individuals.[20]

Since the knowledge about the influence of SNPs in TLRs on the viral rebound and/or the subsequent set-point achieved after ART interruption is limited, we hypothesized that the plasma HIV-1 RNA rebound variability after ART interruption may be influenced by the TLR9-1635A/G SNP. We also investigated the potential implication of other SNPs in TLR2 and TLR4, the CCR5-Δ32 deletion, and the presence of HLA-A and HLA-B alleles on the viral rebound and set-point upon ART interruption.

MATERIALS AND METHODS

This was a retrospective longitudinal study performed from July 2003 to March 2008 aimed to analyze the potential association between common genetic variants in TLR2, TLR4 and TLR9 and the HIV-1 RNA load rebound and set-point upon ART interruption, and the establishment of a CD4 count set-point. This study included 57 Caucasian HIV-1-infected individuals who had undetectable plasma viremia (<50 copies/mL) for at least 12 months before ART interruption and CD4 count above 500 cells/mm^3. Clinical and immuno-virological determinations were recorded at baseline and monthly subsequently. HIV-1 RNA load rebound was considered at week four upon interruption.[9,15] All individuals provided written informed consent and the Ethical Committee of the Virgen del Rocio Hospital approved the study which complied with the stipulations of the Declaration of Helsinki.

Total CD4 count were obtained from the individual's routine laboratory follow up visits provided by the Hospital. Plasma HIV-1 RNA was measured by quantitative RT-PCR (HIV Monitor™ Test Kit, Roche Molecular System, Basel, Switzerland), according to the manufacturer's instructions (with detection limit of 50 HIV-1 RNA copies/mL). HCV infection was diagnosed by the detection of anti-HCV antibodies by ELISA (HCV-specific ELISA, Siemens Healthcare Diagnosis, Malvern, Pennsylvania), and the presence of plasma HCV RNA by RT-PCR (COBAS Amplicor, Roche Diagnostics, Barcelona, Spain).

Genomic DNA was extracted from PBMC using the QIAmp DNA Mini Kit (Qiagen, Barcelona, Spain), and stored at −20°C. The purity of the DNA was determined using Nanodrop® spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware). TLR9-1635A/G, TLR2-1892A/C and 2258G/A, and TLR4-899A/G and 1196C/T single nucleotide polymorphisms (SNPs) were determined by real-time PCR and melting curve technology, as previously reported.[19,21] Briefly, PCR amplification was performed with initial denaturation of 95°C/5 min, 45 cycles of denaturation (95°C/30 s), annealing (55°C/30 s), and elongation (72°C/30 s) using the LightCycler® 480 System (Roche Diagnostics, Mannheim, Germany). Melting curves analyses were performed using 0.2 μM of each detection probe. The Lightcycler 480 control kit to detect the SNPs, was run in parallel. After initial denaturation at 95°C/2 min at ramp rate of 4.4°C/s, temperature was dropped to 45°C at ramp rate of 1°C/s and finally led to 80°C with one acquisition per degree centigrade. CCR5 alleles were amplified using primers flanking the 32-bp deletion in the CCR5 gene (Δ32F, ggaatcatctttaccagatctcaaaaa-3´ and Δ32R catgatggtgaagataagcctcaca-3´). PCR consisted in 35 cycles, including denaturation step at 94°C/20 s, hybridization step at 56°C/30 s, and extension step at 72°C/30 s. The fragment products were run and analyzed in 2% agarose gel. HLA-A and HLA-B low

resolution typing were carried out by PCR-SSOP Luminex method using LABType SSO using group-specific primers (One Lambda Inc., Canoga Park, California). The biotinylated PCR products were denatured and hybridized with specific probes bound to colored-coded microspheres. Reactions were analyzed through LABScanTM 100 flow analyzer and typed with HLA Fusion 2.0 software (One Lambda Inc).

Statistical Analysis

Continuous variables are expressed as median and interquartile range [$IQ_{25–75}$], and categorical as number and percentages. The Mann Whitney *U*-test was used to analyze differences between continuous variables with two different levels. Two-tailed Spearman's correlation test was used to associate quantitative variables, and the Chi-square test for comparison of qualitative variables. Models for the likelihood were tested to analyze differences according to TLR's SNPs and recessive model for the wild type allele was used for the comparison between the different genotypes in both cohorts.[20,22] Variance Inflation Factor (VIF) was used to detect multicollinearity. Variables with $p<0.1$ in the univariate analysis were introduced in the forward stepwise multivariate linear regression analysis to assess independent factors associated with HIV rebound and set-point, and the CD4 T count set-point. All statistical analyses were conducted using SPSS v.24 and graphs were generated using GraphPad Prism v.8.

RESULTS

The characteristics of the 57 HIV-1-infected individuals are summarized in Supplemental Digital Content (SDC) 1. TLR9-1635A/G-AA genotype was present in 13 individuals (23%), AG genotype in 35 (61%), and GG genotype in nine (16%) (AG+GG genotypes in 44 individuals, 79%), as shown in SDC-2, showing a similar distribution to that found in our chronically naïve HIV-1-infected population, as previously reported.[20] All frequencies were in accordance with the Hardy-Weinberg equilibrium (data not shown). Frequencies of SNPs in TLR2-2258G/A were lower in this study than those previously reported for our general HIV-1-infected population.[12] Three individuals (6%) harbored the TLR2-1892A/C SNP, while only one individual (2%) harbored the TLR2-2258G/A SNP. Finally, 13 individuals (25%) harbored the TLR4-896 A/G SNP, while 14 individuals (26%) harbored the TLR4-1296C/T SNP. In line with previous studies[25], four individuals (7%) were heterozygous for the CCR5-Δ32 deletion, while homozygosity was not detected (SDC-2). HLA-A and HLA-B allele frequencies in our study population are shown in SDC-3A and B.

HIV-1 RNA rebound positively correlated with the pre-ART viral load ($R=0.34$, $p=0.025$, Figure 1A). Consistent with a previous report[5], women had lower HIV-1 RNA rebound compared to men (2.6 [1.7–3.8] and 4.1 [2.5–5.2] $\log_{10}$ HIV-1 RNA copies/mL, respectively, Figure 1B). Interestingly, individuals with the TLR9-1635AA genotype had higher levels of HIV-1 RNA rebound compared to those with AG+GG genotype ($p=0.007$, Figure 1C). Finally, individuals harboring the HLA-A26 allele had lower HIV-1 RNA rebound ($p=0.026$, Figure 1D). The analysis of HIV-1 RNA levels during the 72 weeks of follow-up showed that women consistently had a lower viral load compared to men ($p<0.05$ in all time-points,

SDC-7). However, TLR9-1635A/G SNP and HLA-A26 allele were not associated with HIV-1 RNA levels during the follow up (SDC-7B and C, respectively).

To determine factors involved in the HIV-1 RNA rebound, a stepwise linear regression analysis was performed. Since HIV-1 RNA load was lower in women both at rebound and during the 72 weeks of follow-up (Figures 1B and SDC-7A), the statistical analysis was restricted to male individuals. Men and women had comparable baseline characteristics ($p>0.05$, not sown). HLA-A and B allelic distribution in males is shown in SDC-5. We initially assessed that variables were not collinear (VIF>1 in all cases). Variables associated with HIV-1 RNA rebound included TLR9-1635AA, HLA-A26, and HLA-B18 and were included in a stepwise multivariate analysis. Only the TLR9-1635AA genotype was independently associated with higher HIV-1 RNA rebound ($p=0.004$, Table 1).

Both HIV-1 RNA load and CD4 count set-points were estimated as the median value of all available quantifications from week 8 to week 72 of follow-up after treatment interruption (4.4 [3.9–4.9] $\log_{10}$ copies/mL and 577 [425–650] cells/mm^3, respectively). As expected, the HIV-1 RNA load set-point after ART interruption positively correlated with pre-ART HIV-1 RNA load ($R=0.69$, $p<0.001$, SDC-8A), and with the HIV-1 RNA rebound ($R=0.52$, $p<0.001$, SDC-8B). In addition, CD4 count set-point correlated positively with nadir CD4 count ($R=0.52$, $p<0.001$, SDC-8C). While pre-ART HIV-1 RNA load was similar according to sex, men had higher HIV-1 load set-point (SDC-9A). However, both CD4 nadir and CD4 count set-points were similar between men and women (SDC-9B).

Stepwise linear regression analysis showed that both pre-ART HIV-1 RNA load and time with undetectable HIV-1 RNA load were independently associated with the establishment of the HIV-1 RNA load set-point ($p=0.001$ and $p=0.002$, respectively, SDC-6). In addition, HLA-01 allele and nadir CD4 count was independently associated with the establishment of the CD4 count set-point ($p=0.027$ and $p=0.001$, respectively, SDC-6).

DISCUSSION

Our results show an association between the TLR9-1635A/G SNP and the HIV-1 RNA load rebound after interruption of ART. Specifically, patients with the TLR9-1635AA genotype showed a greater risk of higher viral load at rebound. We previously reported that chronically HIV-1-infected individuals, naïve for ART who harbored the TLR9-1635AA genotype were more likely to have both lower CD4 count and higher HIV-1 RNA load, and a higher risk of clinical disease progression.[20] We hypothesized that in the setting of ART interruption, the TLR9-1635A/G SNP could be involved in both the magnitude of the viral load rebound and/or the subsequent establishment of the HIV-1 RNA load set-point.

TLR9 contributes to HIV-induced immune activation, and TLR9 SNPs are likely to contribute to the individual variability in the clinical course of HIV disease.[19] Despite the limited size, this valuable interruption cohort allowed us to find that the TLR9 SNP was associated with the viral rebound upon treatment discontinuation. In this study, individuals with the TLR9-1635AA genotype showed higher HIV-1 RNA rebound after ART interruption, which was in turn associated with the establishment of the viral set-

point. These results may help understand the individual variation response to current TLR agonist's studies aimed to reactivate the latent virus as part of the effort to cure HIV-1 infection.[26–29] TLR9 expression is directly correlated with the rate of immune activation, but in chronic infections shows reduced up-regulation and diminished responsiveness after CpG-DNA stimulation.[30] In the present study, plasma levels of β−2 microglobulin and TNF-α were not associated with HIV-1 plasma load rebound or HIV-1 load and CD4 count set-points (data not shown). However, we cannot exclude that the higher rebound in patients with the AA genotype was the consequence of higher immune activation.

After the initial viral rebound, both plasma HIV-1 RNA levels, and CD4 count reached set-points that were maintained through the follow-up. Interestingly, pre-ART viral load and time with undetectable plasma HIV-1 RNA load were associated with the viral load set-point. Our results also show a marginal effect of the presence of the HLA-B27/B57 protective alleles.[31,32] However, we did not find a protective role of either of them separately likely due to the limited sample size. Although the presence CCR5-Δ32 deletion in heterozygosity has been associated with lower viral loads,[33,34] in treatment interruption it may not be involved in the viral or the CD4 count set-point, in line with a previous report.[35] Finally, our results showed that the presence of the HLA-01 allele was associated with a higher CD4 count set-point, confirming a previous report that identified the presence of this class I allele as protective.[36]

Our work highlights the importance of analyzing the presence of common SNPs on TLR9, and potentially on others, such as TLR7, when performing studies aimed to interrupt treatment in the clinical setting of HIV-1 eradication. These common SNPs may help explain variations and may constitute a tool to predict time to rebound after treatment discontinuation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

Authors would like to thank the participants who made the study possible. Statistical analysis was supervised by Eloisa Rubio-Perez, from the Andalusian Public Foundation for the Management of Health Research in Seville (FISEVI), Seville, Spain. NSS designed the study, AV, SMP, ML, NSS analyzed results and wrote the manuscript; BdF, MA and MFGE performed experiments and wrote the manuscript.

Funding:

NSS is supported by NIH R01 AI125097. SMP is currently funded by the Ministry of Health and Social Welfare of Junta de Andalucia (PI-0046-2012, Nicolas Monardes Program C-0040-2016).

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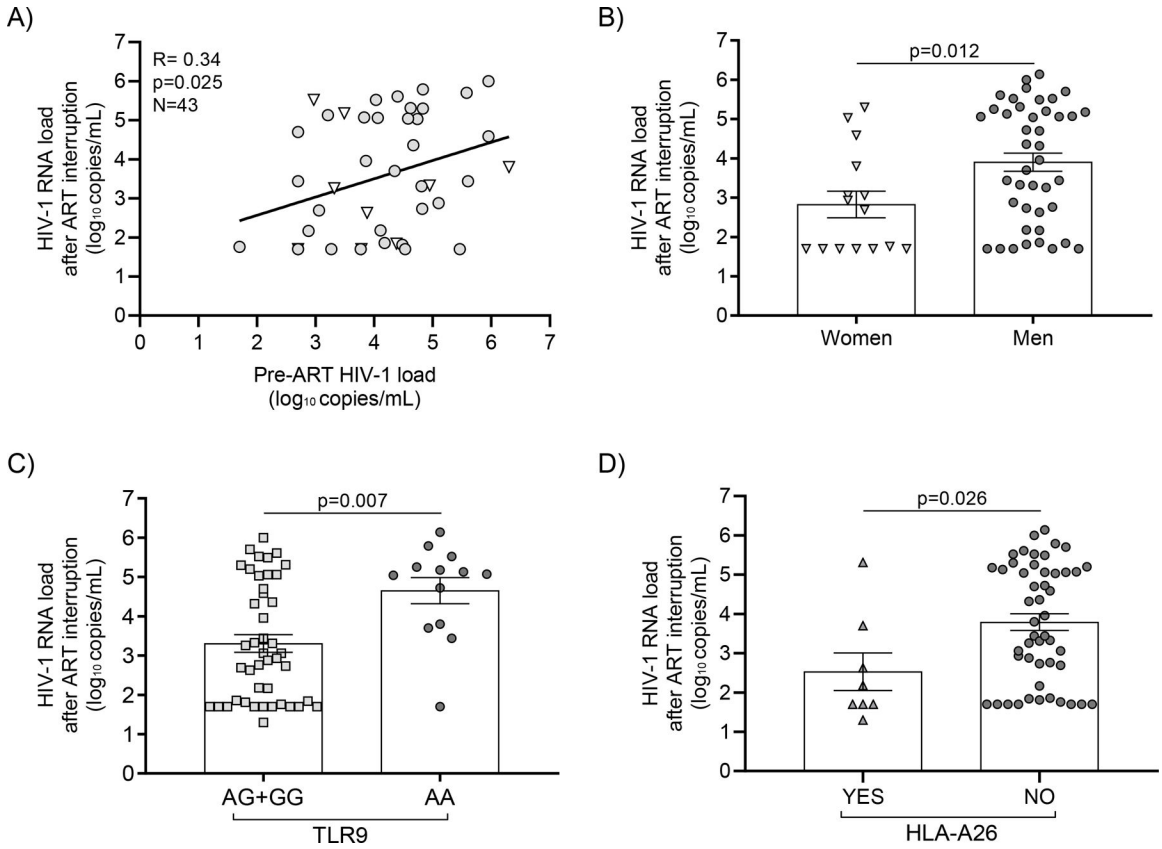

Figure 1. Variables associated with the HIV-1 RNA rebound after four weeks of ART interruption.

A) Pre-ART HIV-1 plasma load positively correlated with post-interruption HIV-1 load. Pre-ART viral load was available in 43 individuals. Women are represented with open triangles and men with filled circles. B) Women had lower viral rebound compared to men. C) Individuals harboring the TLR9 1635AA genotype had lower HIV-1 rebound after ART interruption. D) Individuals with the HLA-A26 allele showed lower viral rebound compared to individuals who did not harbor the allele.

Table 1:

Univariate and multivariate linear regression analyses to assess factors independently associated with the HIV-1 RNA load rebound after ART interruption.

	Univariate		Multivariate	
	p	**Regression coefficient [95% Confidence Interval]**	**p**	**Regression coefficient [95% Confidence Interval]**
TLR9 1635AA	**0.004**	1.49 [0.51–2.47]	**0.004**	1.49 [0.51–2.47]
HLA-A26	0.056	−1.27 [−2.58-−0.03]	ns	
HLA-B18	0.057	1.37 [−0.04–2.78]	ns	

ns; not significant.