Toll Like Receptor 2 promoter -196 to -174 deletion affects CD4 levels along HIV infection progression.

Laplana M[1,2*], Bravo MJ[3*], Fernández-Fuertes M[4*], Ruiz-Garcia C. [5], Alarcón-Martin E[3], Colmenero JD[6], Caruz A. [5+], Fibla J [1+], Real LM[3,4,+], Royo JL[3+].

1. Unitat de Genètica Humana, Departament de Ciències Mèdiques Bàsiques, IRBLleida, Universitat de Lleida, 25198 Lleida, Spain

2. Departament de Ciència Animal, Universitat de Lleida, 25198 Lleida, Spain

3. Departamento de Cirugía, Bioquimica e Inmunología. Facultad de Medicina. Universidad de Málaga. 29071 Málaga, Spain.

4. Unidad Clínica de Enfermedades Infecciosas y Microbiología, Hospital Universitario de Valme, 41001 Sevilla, Spain

5. Unidad de Inmunogenética, Universidad de Jaén, 23071, Jaén, Spain.

6. Servicio de Enfermedades Infecciosas. Hospital Regional de Málaga. 29010 Málaga, Spain.

*These authors contributed equally to this work

+ To whom correspondence should be addressed

Dr. Jose Luis Royo: jlroyo@uma.es; Dr. Luis Miguel Real: lmreal67b@gmail.com; Dr. Joan Fibla: joan.fibla@udl.cat; Dr. Antonio Caruz: caruz@ujaen.es

Summary of the article's main point: Our results suggest a role of TLR2 germline variants in HIV-1 disease progression rate. Toll Like Receptor 2 promoter -196 to -174 deletion analysis link lower levels of TLR2 and a diminished CD4+ T-cell counts and a worse prognosis, probably due to a lower activation of the immune system.

Abstract

TLR2 plays a key role in innate immune response recognizing molecular patterns expressed by pathogens. rs111200466 is a *TLR2* promoter Insertion/Deletion polymorphism with contradictory data about its role in HIV-1 infection. We analyzed rs111200466 in HIV-1 disease progression and showed a correlation with a faster progression to the CD4+<200cells/µL outcome for Deletion allele carriers (Cox regression analysis: Hazard Ratio=2.4; 95%CI:1.4-4, P=0.001). When naïve patients with CD4+<200cells/µL start antiretroviral treatment, rs111200466-Deletion carriers showed a trend towards a slower, recovery rate (time required to reach CD4+>350cells/µL, Cox P=0.36). Our data suggests rs111200466 as a prognosis factor for HIV-1 disease progression.

Keywords: *TLR2*; *Polymorphism*; HIV infection; AIDS; HIV progression; rs111200466

Background

Genetic differences in immune responses can be explained by the polymorphic nature of immune system genes. The ability of the immune system to distinguish pathogens is prompted via their binding to a family of pathogen recognition receptors (PRRs). Since their discovery, the focus has been on the recognition of pathogen-associated molecular patterns (PAMPs) that trigger innate immunity and enhance the adaptive immune response. The functions of several different classes of PRRs have been identified. In particular, Toll-like receptors (TLRs) family comprised by 10 genes that encode non-catalytic membrane receptors is the most characterized.

A number of viral proteins have been identified as PAMPs for TLR2. Specific HIV structural proteins such as p17, p24, and gp41 interact with TLR2 leading to NFκB activation and the production of proinflammatory cytokines [1]. The primary consequence of viral recognition to the immune system is the production of proinflammatory cytokines and the subsequent recruitment of additional target cells. However, virally induced TLR2-dependent cellular activation has been shown to contribute to viral spread and pathogenesis due to enhanced expression of various viral entry receptors [2], thereby increasing viral infection [1]. These unique viral-PAMP specific alterations in receptor expression suggest a novel mechanism by which viruses can manipulate innate sensing with specific viral proteins. Indeed, different authors reported a significant increase in *CCR5* expression in macrophages exposed to HIV-1 PAMPs that led to significantly increased *in vitro* cell-free R5-HIV infection [1]. Also, previous publications have shown that there is a TLR2-dependent increase in *CCR5* expression on permissible cells, resulting in significantly increased HIV infection[3]. Taken together, these studies highlight the role of TLR2 as an important extracellular PRR for viral PAMP recognition, resulting in increased cellular activation and facilitating viral infection. Under this scenario, we proposed that *TLR2* variants might compromise host immune response to HIV-1 infection. One of the mostly studied variant of *TLR2* comprises a proximal promoter deletion (-196 to -174 or rs111200466) which has been associated to HIV-1 infection progression with controversial results [4,5]. Thus, the objective of this work was to explore the impact of TLR2 insertion/deletion polymorphism on the immune impairment progression, measured as CD4+ T-cell count along the

HIV-1 infection progression. To this end we designed a multicentric study where *TLR2* rs111200466 was determined in series of HIV-1 infected subjects and correlated with parameters related to HIV-1 disease progression.

Methods

Patients

In this multicentric study samples from 3 different series of HIV-infected individuals were analysed. The Malaga series was comprised by patients recruited between 2011 and 2013 at the Regional University Hospital of Malaga (Spain). The Lleida prospective cohort is comprised by HIV-1–seroprevalent injection drug users recruited between 1982 and 1991 from the AIDS Service of the Hospital Arnau de Vilanova of Lleida (Spain). These patients had been followed for a median of 127.7 months (interquartile range: 84–198) [6,7]. The Valme series was comprised by HIV-seroprevalent individuals who attended the Hospital Universitario de Valme, Seville (Spain). Clinical data from patients with available DNA were retrospectively digitalized. Valme series recruitment started in 1999 with a median follow-up of 59.5 months (interquartile range: 18–135). Antiretroviral therapy was provided according to the standardized medical guidelines in force at the moment of the visit in each hospital (Supplementary Table 1). This study was in compliance with the national legislation and it was performed according to the ethical guidelines of the Declaration of Helsinki. The Hospital Arnau de Vilanova Ethic's committee approved the study for the Lleida cohort while the Malaga's Hospital Carlos Haya committee did it for the Valme and Malaga series. Informed consent was obtained from all individuals before sampling. Biochemical and genetic analysis are described in the Supplementary methods.

Statistical Analysis

Statistical analyses were performed with R (v3.6.0) and IBM SPSS 21 software (IBM corporation, NY, USA). Non-parametric tests were applied for the analysis of CD4+ T-cell counts after verifying a non-normal distribution using Shapiro-Wilk's method in the Lleida (P=1.28e-07), Malaga (P=2.7e-03) and Valme (P=5.02e-08) series. Thus, distribution of CD4+ T-cell counts was evaluated according to *TLR2* rs111200466 genotype for each series by means of Kruskal-Wallis test. A meta-analysis combining all patients was performed using random effects model with meta package in R environment. HIV-1 disease progression was evaluated by Kaplan–Meier survival analysis. The first decrease in the CD4+ T-cell count below 200cells/μL was considered the outcome for disease progression. Time to the outcome ranged from date of the first HIV-1–positive test to the outcome date or censoring date (defined as the last clinic examination date or the date of death, if not caused by HIV-1 infection). Differences in profiles of time to the outcome between groups were compared by the Cox regression analysis. Hazard ratios were estimated using a Cox proportional hazard model that was further adjusted for sex and *CCR5Δ32* genotype when appropriate[6,7]. In the same line, recovery of naïve patients with CD4+<200 cells/μL from the Valme series (n=51) was evaluated by Kaplan–Meier survival analysis as the time to reach CD4+>350 cells/μL after treatment setup. Differences in recovery profiles between rs111200466 genotypes of naïve patients were compared by the Cox regression analysis.

Results

The Lleida, Valme and Malaga series were constituted by 151, 133 and 223 HIV-infected individuals (Supplementary Table 1). All of them were genotyped for the *TLR2* rs111200466 and *CCR5Δ32* genetic variants. The genotype distribution fitted the Hardy-Weinberg equilibrium in all series (Supplementary Table 2).

In a first approach, we retrospectively analyzed patient CD4+ T-cell count levels at the recruitment timepoint. We observed that CD4+ levels (median, Q1-Q3) were lower among the Del allele carriers in the Malaga (439.9, 336.6-583.7 vs 500.1, 347.1-678.4, P=0.21) and Lleida series (286, 152-491.5

vs 385.5, 225.8-537.8, P=0.11) (Figure 1A, B), however the same trend was not found in the Valme series (307.5, 164-390.8 vs 254, 139.5-379.5, P=0.43) (Figure 1C). An overall analysis with data from all series combined showed significant lower CD4+ levels among the rs111200466 Del carriers (339, 196-510 vs 397.8, 240.3-605.5, P=0.02). However, these differences were lost when a meta-analysis using random effects model was performed in order to account for inter-population variability (P=0.16).

CD4+ T-cell count evolution was analyzed in the Lleida cohort as an outcome of disease progression. Of the 151 HIV-1–positive patients, the CD4+ T-cell count during follow-up remained at >200cells/μL in 88 (58.3%). Figure 2A shows the corresponding Kaplan-Meier plot for disease progression in the Lleida series grouping patients according to genotype under a dominant model. Of mention, those patients harboring the Ins/Ins genotype exhibited a significantly slower progression rate (median time to the outcome 171.3 months) than those with the Del allele (median time to the outcome 105.3 months; Cox regression analysis Hazard Ratio (HR)= 2.1; 95% confidence interval (CI): 1.27-3.5; P=0.004 Figure 2A) that remained significant after adjusting Cox regression analysis by sex and *CCR5Δ32* (HR=2.4; 95%CI: 1.4-4; P=0.001). Moreover, this effect was independent of those observed for sex (P=0.4) and *CCR5Δ32* genotype (P=0.07) (Supplementary Table 3). Next, we analyzed the CD4+ T-cell recovery of naïve patients with CD4+<200 cells/μL from the Valme series (n=51), measured as the time to reach CD4+>350 cells/μL after treatment setup. Although patients with the Ins/Ins genotype (n=38) reached the recovery goal ≈50% earlier than the Del carriers (average±standard error: 111±18.5 vs 207.8±53.9 weeks, respectively), no statistically significant differences were found (HR=0.67; 95%CI: 0.29-1.56; P=0.36) (Figure 2B, Supplementary Table 3).

The role of rs111200466 polymorphism in the expression of *TLR2* gene has been investigated through the GTEx portal (ReleaseV7, https://www.gtexportal.org/home/) [8]. *TLR2* was found to be generally expressed along the different tissues (Supplementary Figure 1A). When expression-quantitative trait locus (eQTL) analysis was performed, we observed that the Del allele correlated with a lower *TLR2* expression (Supplementary Figure 1B). As an illustrative example is illustrated in Supplementary

Figure 1C. Average expression of the Ins/Ins subjects is significantly higher (Normalized Effect Size= -0.72, P=4.2e-34). In the same line, polymorphisms in strong linkage disequilibrium with rs111200466 such as rs1898832, rs62323831 and rs17270673 have also been reported as eQTLs for *TLR2* (Data not shown).

Discussion

In the present study we evaluated the role of *TLR2* rs111200466 in HIV-1 infection progression. We conducted a survival analysis taking advantage of the longitudinal CD4+ T-cell count data available at the Lleida cohort. We evaluated profiles of HIV-positive patients according to rs111200466 variants using the first decrease in CD4+ T-cell count <200cells/μL as a primary outcome for progression. Patients harboring the rs111200466 deletion allele exhibited a more rapid progression. The significance was independent of the *CCR5Δ32* genotype suggesting that the mechanism underlying disease progression might not involve virus entry. Next, although results were not statistically different, we observed at the Valme series that naïve patients with low CD4+ T-cell count and rs111200466 deletion allele showed a worse response to the antiretroviral treatment, and required nearly twice as much time to recover CD4+>350 cells/μL. All these data together seems to indicate that rs111200466 deletion might be compromising the immune response of the patients.

TLR2 is expressed in sentinel cells, skin and epithelial cells such as the lung, and renal tubules and has the ability to act forming heterodimers with TLR1, 6 and 10 what further expands the number of PAMPs that it can interact with [9,10]. It has been also shown that *TLR2* is overexpressed in monocytes from HIV-1 infected patients [11] and it has been postulated that the increase in *TLR2* expression may favour HIV-1 cell-entry. TLR2 is found in different forms in the human body and their role in HIV-1 infection has been shown to be different. For example, some HIV-1 structural proteins act as PAMPS activating membrane bound TLR2 heterodimers and the subsequent pathways modulating NFκB or CCR5 expression, while others block TLR2 activation [1]. Our results, together with those from the eQTL analysis point out that a decrease of *TLR2* expression mediated by the presence of the Del allele will have a risk effect on disease progression rates. We can only speculate

to which extend *TLR2* rs111200466 deletion allele might compromise the production of proinflammatory cytokines and different transcription factors and could affect the sensing of different PAMPS. Previous studies evaluated the role of this polymorphism and the risk of HIV-1 infection with contradictory results [5,6]. Initially, Vidyant et al. proposed the *TLR2* rs111200466 deletion as a susceptibility allele, however in a later study Royo et al. reported a Hardy-Weinberg disturbance in Vidyant`s data and found a protective effect of *TLR2* rs111200466 deletion to HIV-1 infection. This is not the first polymorphism with a dual effect, affecting susceptibility and infection progression simultaneously[12,13]. TLR2 involved in the response of opportunistic pathogens such as tuberculosis, what is associated to NFκB activation and a higher HIV replication rate. This might explain the influence of TLR2 in both susceptibility and progression. Additional *TLR2* polymorphisms have been correlated with viral load with different results[14,15].

We acknowledge a number of limitations in our study. First, evidence of CD4+ T-cell counts affected by *TLR2* genotype is only described in HIV-1 infected patients and the role in healthy controls remains unclear. Survival analyses are significant in a unique cohort and will require replication. Second, the discrepancies in the initial CD4+ counts observed in the Valme series might reflect a differential time of infection prior to attending to the Hospital, what might be explained by prospective vs retrospective nature of the Lleida and Valme series. Finally, we should state that the Valme analysis was conditioned by the availability of DNA samples. Thus, a selection bias cannot be excluded.

In conclusion, our results suggest a role of TLR2 in HIV-1 disease progression rates that may be independent of the effect of this gene in the susceptibility to infection. Our analysis together with GTEx data link lower levels of *TLR2* and a diminished CD4+ T-cell counts and a worse prognosis, probably due to a lower activation of the immune. Altogether, these findings reveal novel mechanisms of viral infection control that should be further studied to fully understand the complex regulatory network orchestrating immune response upon HIV-1 infection.

Acknowledgments

We would like to thank all the patients who participated in this study. We are also grateful to the clinicians and allied health care professions that contributed to the study.

Funding

This work was supported by grant SAF2016-80125-R (Ministerio de Economía, Industria y Competitividad, Spain) to AC., grant PI-0001-2017 (Consejería de salud, Spain) to L.M. Real and by grant SAS111229 supported by the Junta de Andalucía to M.J. Bravo.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Previous report of the data

The data presented in this paper has not been presented elsewhere.

References

1. Henrick BM, Yao X-D, Rosenthal KL, INFANT study team. HIV-1 Structural Proteins Serve as PAMPs for TLR2 Heterodimers Significantly Increasing Infection and Innate Immune Activation. Front Immunol; 2015;6:426.

2. Compton T, Kurt-Jones EA, Boehme KW, Belko J, Latz E, Golenbock DT, et al. Human cytomegalovirus activates inflammatory cytokine responses via CD14 and Toll-like receptor 2. J Virol; 2003;77(8):4588–96.

3. Heggelund L, Damås JK, Yndestad A, Holm AM, Müller F, Lien E, et al. Stimulation of toll-like receptor 2 in mononuclear cells from HIV-infected patients induces chemokine responses: possible pathogenic consequences. Clin Exp Immunol; 2004;138(1):116–21.

4. Vidyant S, Chatterjee A, Agarwal V, Dhole TN. Susceptibility to HIV-1 infection is influenced by toll like receptor-2 (-196 to -174) polymorphism in a north Indian population. J Gene Med; 2017;19(8):e2971.

5. Royo JL, Alarcón-Martín E, Díaz-Fuentes J, Colmenero JD, Bravo MJ. Discordance in TLR2 (-196 to -174) polymorphism effect on HIV infection risk. J Gene Med; 2018;20(10-11):e3051.

6. Laplana M, Caruz A, Pineda JA, Puig T, Fibla J. Association of BST-2 gene variants with HIV disease progression underscores the BST-2 role in HIV-1 infection. J Infect Dis; 2013;207(3):411–9.

7. Laplana M, Sanchez-de-la-Torre M, Puig T, Caruz A, Fibla J. Vitamin-D pathway genes and HIV-1 disease progression in injection drug users. Gene; 2014;545(1):163–9.

8. GTEx Consortium. Human genomics. The Genotype-Tissue Expression (GTEx) pilot analysis: multitissue gene regulation in humans. Science; 2015;348(6235):648–60.

9. Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, et al. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. PNAS; 2000;97(25):13766–71.

10. Guan Y, Ranoa DRE, Jiang S, Mutha SK, Li X, Baudry J, et al. Human TLRs 10 and 1 share common mechanisms of innate immune sensing but not signaling. J Immunol; 2010;184(9):5094–103.

11. Heggelund L, Müller F, Lien E, Yndestad A, Ueland T, Kristiansen KI, et al. Increased expression of toll-like receptor 2 on monocytes in HIV infection: possible roles in inflammation and viral replication. Clin Infect Dis; 2004;39(2):264–9.

12. Barreiro P, Pineda JA, Rallón N, Naggie S, Martín-Carbonero L, Neukam K, et al. Influence of interleukin-28B single-nucleotide polymorphisms on progression to liver cirrhosis in human immunodeficiency virus-hepatitis C virus-coinfected patients receiving antiretroviral therapy. J Infect Dis; 2011;203(11):1629–36.

13. Labarga P, Soriano V, Caruz A, Poveda E, Di Lello FA, Di Lello F, et al. Association between IL28B gene polymorphisms and plasma HCV-RNA levels in HIV/HCV-co-infected patients. AIDS. 2011 Mar 27;25(6):761–6.

14. Mackelprang RD, Carrington M, Thomas KK, Hughes JP, Baeten JM, Wald A, et al. Host genetic and viral determinants of HIV-1 RNA set point among HIV-1 seroconverters from sub-saharan Africa. Hahn BH, editor. J Virol; 2015;89(4):2104–11.

15. Pine SO, McElrath MJ, Bochud P-Y. Polymorphisms in toll-like receptor 4 and toll-like receptor 9 influence viral load in a seroincident cohort of HIV-1-infected individuals. AIDS. 2009;23(18):2387–95.

Figure captions

Figure 1. Boxplot depicting CD4+ T-cell counts distribution including the number of patients per group. Line depicts median CD4+ T-cell count, box lower and upper hinges correspond to the first and third quartiles, whiskers extend from the hinge to the largest or lower value (no further than 1.5 times the inter quartile range) and the numbers above each boxplot represent the number of individuals represented.

Figure 2. Role of rs111200466 genotype on CD4+ level progression under the Del allele dominant model by means of Kaplan–Meier analysis. A) Lleida patients reaching the outcome of CD4+ T-cell count below 200 cells/μL for disease progression during follow-up. P-Value from adjusted Cox regression analysis, B) Time to recovery (time to CD4+ >350 cells/μL) of Valme naïve patients. P-Value from univariate Cox regression analysis.

Figure 1

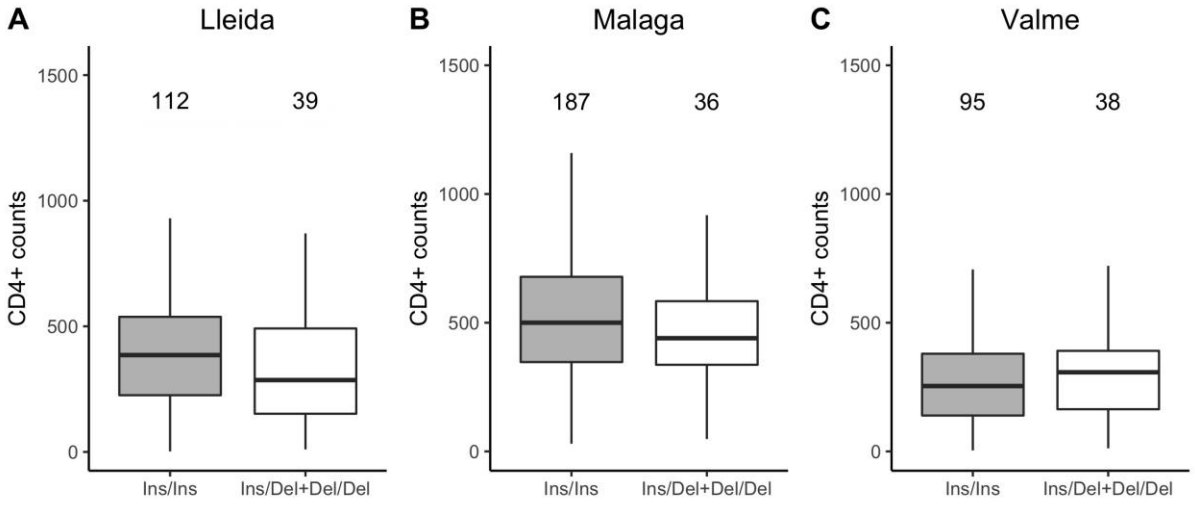

Figure 2