Author’s response to reviews

Title: TLR9 polymorphism correlates with immune activation, CD4 decline and plasma IP10 levels in HIV patients.

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Reviewer 1:

There was no mention of the type of drugs used for the HAART. Were all the HIV positive subjects on exactly same drug combinations? If different combinations were used for different individuals, this could be a confounding factor especially with respect to inflammatory markers.

A note should also be made on the HIV disease stage of the study group as different disease stages can affect the studied parameters.

We agree with all the comments made by the reviewer. Our study was largely cross sectional and the patients were recruited at the time they visited the TTUHSC HIV clinic. This included some patients that were on HAART and others that were not on HAART. Those not on HAART was primarily because it was their first visit to the clinic after diagnosis. We have previously reported the HAART status of the patients and correlation with viremia and CD4 counts. We have made a note of this in the manuscript as well. We agree with the reviewer that differences in HAART can be a confounding factor in inflammation. We have included this information in the discussion as well.

As far as the disease stage is concerned, at the TTUHSC clinic, hematological parameters like CD4 counts and viremia are the standard for disease monitoring and hence we have used those parameters to define our population. However, as suggested by the reviewer we have added in the discussion that disease stage can affect inflammatory markers.

Reviewer 2:
Joshi et al. studied in a cohort of 50 HIV infected individuals and corresponding controls a possible relationship of two TLR9 polymorphisms (1635A/G and 1486C/T) with HIV disease progression and/or immune activation. It is stated that the presented findings provide several new insights into HIV mediated immune activation and the underlying mechanism. Although the authors demonstrate some correlations of the SNP and immune activation, mainly the IP-10 production, the overall conclusions drawn from these experiments might be overestimated which is my major concern.

We agree with the reviewer that the conclusions from our data may have been overestimated. We also acknowledge that TLR9 1635A/G has been studied by others in the context of HIV disease. However, our study has looked at multiple immunopathological markers of HIV disease and found that TLR91635A/G correlates with CD4 counts as well as with immune activation markers that has not been shown previously. We have made modifications in the manuscript to both acknowledge the previous findings related to TLR91635A/G in terms of HIV pathogenesis and limited our findings to correlation with immune activation and IP10 levels. The discussion and conclusion sections have been modified accordingly.

Furthermore, it might be really difficult to analyze the two SNPs independently in this cohort, since 6 out of seven HIV+ patients with 1486 CC also carries the 1635AA SNP which was already described before to correlate with HIV infections, disease progression, CD4 counts and viral load. These studies were all correctly cited by the authors, but also demonstrate that most of the findings are not fundamentally new.

Once again we agree with the reviewer that analysis of the SNPs may be difficult because they are in linkage disequilibrium. However, as the reviewer has pointed out, the 1635A/G polymorphism has only been shown to associate with CD4 counts and viral load. In previous study by Soriano-Sarabia et al (2008) the authors have speculated that TLR9 1635AA genotype may have higher immune activation in the discussion section of their manuscript. In this context we have looked at multiple immune pathological markers like LPS, sCD14, IL6, IP10 as well as CD8 and CD4 activation and their association with 1635A/G polymorphism. Our study is the first to demonstrate immune activation as the missing link between TLR9 polymorphism and HIV disease.

Main findings:

1635AA patients have reduced CD4 counts, increased frequencies of activated CD4 and CD8 cells, but no significant differences in VL (all compared to AG/GG mixed population).Using further separation into the three possible allele combinations, only significant differences were shown for CD4 counts and CD8 activation. In my opinion, the separation into these three categories would be much more meaningful and should be presented for all results.

We agree with the reviewer that separation into different allele combination can be meaningful in some cases. In this particular case the 1635 A/G polymorphism has been studied by other researchers (Mackelprang et al, 2014; Soriano-Serbia et al, 2008) and they have used the same stratification AA vs AG/GG based on a dominant model. We have separated the alleles for some
of the most relevant associations like CD8 activation and CD4 counts and the data shows that AA is different from AG/GG groups.

In fig.3, it is shown that HIV+ individuals have higher levels LPS, sCD14, IP-10 in the sera compared to HIV- patients (by the way "normal" might be not best choice to indicate the controls). This also confirmed already existing data. IP-10 levels are also higher in 1635AA patients, but not the other ones. Fig 5 demonstrates that IP-10 also correlates with VL. By this finding, the authors draw the conclusion that the lower IP-10 correlates with SNP and VL and thereby the SNP with VL. But a direct correlation has not been shown in Fig 2.

We agree with all the points made by the reviewer. We have changed the labeling to HIV-subjects in Figures 3a-d. Regarding the correlation of the SNP with viral load, as correctly pointed out by the reviewer, our data does not show significant difference between the 1635AA vs AG/GG groups in terms of VL. At the same time IP10 levels and immune activation are different. This suggests that with the same level of virus replication, 1635AA genotype shows higher immune activation along with higher IP10 levels. It has been shown via in vitro studies by Biegnon et al (2016) that endocytosis of HIV virions by plasmacytoid dendritic cells activates Toll Like Receptors. Hence immune activation and IP10 levels are expected to be higher in viremic patients. One could infer that viral load is the cause and TLR9 polymorphism is the cofactor in HIV mediated immune activation. Further studies will be needed to determine whether stimulation of cells with TLR91635AA genotype with HIV will lead to enhanced immune activation or higher IP10 secretion. We have modified the discussion section to reflect this more clearly.

In Fig. 7+8, they add the second SNP to the analyses with the above mentioned limitation and demonstrates that the AA-CC genotype seems to be the best correlate for the IP-10 levels and not AA alone, which in combination with CT is not substantially different from the other genotypes. The combination of the genotypes should be also included in the table 1.

Excellent point by the reviewer. We have now included this additional information in the Table

Further major concerns:

- using a consecutive T-test as the statistical analyses for comparisons of more than two groups is not correct. ANOVA with post-test for multiple comparisons should be used. Therefore some of the significances might be questionable

We agree with the reviewer that ANOVA is a more appropriate analysis of variance when more than two groups are involved. As the data is non parametric we used the Kruskal-Wallis test with a Dunns multiple comparison post-test. Using this method we do loose significance in some of the analysis but the significant difference between AA-CC vs AG-CT in terms of IP10 is retained in figure 8.

We also agree with the limited statistical significance in our analysis. We would like to point out that HIV disease is multifactorial with a number of host and viral factors collectively
determining disease progression and TLR9 being one of several factors. Hence limited significance in these complex diseases warrants further investigation and perhaps complex disease modeling in the future. These caveats have been added to the discussion.

- there are no mechanistic insights as it is suggested in the text. The source of IP-10 is not known and also the consequence for the T-cell activation Therefore the study provides only very little new information to the field and mainly reproduced some previous published data in their cohort. The major contribution was the additional analyses of the 1486 SNP which has not been reported in the context of HIV disease progression. Unfortunately, the linkage disequilibrium between the two SNPs makes independent analyses very difficult. The proposed fundamental mechanistic insights are unfortunately not really analyzed and rather of theoretical nature.

We agree with the reviewer that some of the mechanistic insights are circumstantial at best. We agree that the specific mechanism behind the role of TLR9 polymorphism in HIV disease will require careful in vitro assays to tease out the mechanism, like which cells produce IP10 and the role it plays in T cell activation, if any. Whether plasmacytoid dendritic cells from TLR9 1635 AA genotype are more prone to activation or produce higher levels of IP10 when stimulated with HIV virions would be worth testing. These are ongoing studies in our lab which we feel are beyond the scope of this manuscript. We hope to publish these mechanistic studies as a separate stand-alone study when complete.