Author's response to reviews

Title: KIR3DS1/L1 and HLA-Bw4-80I are associated with HIV disease progression among HIV typical progressors and long-term nonprogressors

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Author's response to reviews: see over
Dear editor:

We greatly appreciate the reviewers’ efforts and helpful review of our manuscript (MS: 5384449779662493), “KIR3DS1/L1 and HLA-Bw4-80I affect HIV disease progression among HIV typical progressors and long-term nonprogressors” (we have changed the title as “KIR3DS1/L1 and HLA-Bw4-80I are associated with affect HIV disease progression among HIV typical progressors and long-term nonprogressors”). We have significantly revised the manuscript to accommodate all the reviewers’ comments. The major changes in the manuscript have been highlighted with yellow color. Please find our point-by-point responses listed below. We thank you for your consideration of our revised manuscript.

Reviewer 1 (Dr. Brian Long)’s comments and our responses (underlined):

In this study, Jiang et. al. report on natural killer cell immunoglobulin receptor (KIR) and HLA genotype and relate KIR gene frequency and expression levels with HIV viral load, CD4+ T cell count and disease progression in a cohort of 132 HIV seropositive ethnic Han subjects. The authors first determine the KIR and HLA genotype of the infected subjects, and analyze the HLA-B locus for the presence of isoleucine at position 80 (Bw4-80I) that has been previously associated with delayed HIV disease progression in the presence of particular KIR ligands, namely KIR3DS1. This information is then related back to categorized disease status parameters within the study group. Next, the mRNA expression levels of 2 pertinent KIR genes, KIR3DS1 and KIR3DL1, which segregate as alleles, were determined by quantitative PCR analysis. Gene expression level was then related back to disease parameters to reinforce the notion that particular KIR receptors are associated with improved outcomes in HIV disease. The results of this study are in agreement with a multitude of previously published reports on the effect of the KIR3DS1 and HLA-Bw4-80I compound genotype on HIV disease progression initiated by Martin et al. Nature Genetics 31 (2002). The novelty of this study is restricted to the fact the study subjects are all Chinese and ethnic Han, but as such represents a valuable contribution to the literature on this subject.

Major Compulsory Revisions

1. The KIR gene frequency and HLA genotyping appear to be well done and provide for compelling data. However, there are some serious concerns regarding the methodology for the quantitative PCR. Most importantly, it appears the data are not normalized for the number of input cells, as RNA was isolated from an unspecified number of PBMC (Materials and Methods, pg. 9-10). This is problematic in that some subjects may have more or less PBMC per unit volume of whole blood. More importantly, irrespective of the PBMC number, the frequency of NK cells within the PBMC population can be highly variable, especially in HIV infected subjects. For example, you will find a larger quantity of KIR mRNA in a sample that has twice the number of NK cells per unit volume of whole blood relative to a subject that has half the number. The data in this case needs to be normalized to the number of NK cells that the RNA was isolated from, or at a minimum to the number of PBMCs. Perhaps
the authors have some CBC data from the time of collection they could refer back to.

Response: We agree with the reviewer that the frequency of NK cells within the PBMC population can be highly variable, especially in HIV infected subjects. The reviewer also suggested that the quantification of the KIR mRNA may need to be normalized to the number of NK cells, or to the number of PBMCs. Nevertheless, we used GAPDH as the reference to control for different input RNA. The reason is that we need an internal control to ensure that the mRNA purification procedure did not contribute to the variability. We frequently observe that even equal number of cells is used, the amount of mRNA recovered varies, and we can not guarantee that there is no loss of mRNA during purification. The use of equal internal GAPDH mRNA would reflect equal number of cells used. Such method has been published by others, such as Ravet et al (Ref. 23 in the revised manuscript) who have also used GAPDH mRNA as an internal reference, similar to ours. In addition, we found that the KIR3DS1 mRNA was higher but the KIR3DL1 mRNA was lower in LTNPs, when GAPDH mRNA was used as a control. This cannot be explained by possibly higher number of NK cells used in the comparison.

2. The authors list several primers in the materials and methods used to amplify the KIR3DS1 and KIR3DL1 gene products. However upon performing a BLAST search of the sequences (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), it appears each is specific for several different KIR genes. For example, the KIR3DS1 primer set listed will also amplify KIR3DL1 and KIR3DL2. The authors will need to provide evidence that the primers they’ve designed have specificity for the intended gene products.

Response: The primer sets have been used by others (Koehler RN, et al, ref. 24, and Ravet S, et al. ref, 23 in the revised manuscript). We also confirmed these primer sets by PCR and only saw a single band. DNA sequencing analyses (done by BGI China) confirmed that the PCR product has the expected sequence.

The reviewer has noticed that our primer sets such as KIR3DS1 may also amplify KIR3DL1 and KIR3DL2. However, we did not observe the unspecific amplification in PCR. The likely reason is that the primer set did not perfectly match with the other two non-target genes, and our PCR condition is stringent enough to discriminate them.

3. There is no mention in the methods as to whether the isolated RNA was assayed for quality and quantification. Were the starting concentrations of RNA normalized? I would assume this to be the case, but it needs to be described.

Response: We apologize for not providing details of the methods, and now we have provided these details in the revised manuscript. The RNA was isolated using a standard Qiagen kit and was assayed for purity and concentration before experiment.

4. Ideally, qPCR should be normalized to more than one housekeeping gene (GAPDH in this case) as it has been demonstrated that a single housekeeping gene can have varying levels of expression in different cell types. I would suggest the authors refer to J. Huggett et.al, Genes and Immunity (2005) 6, 279-284 for clarification. It is now
standard procedure for qPCR to normalize to at least 3 to 6 internal control genes with an algorithmic determination of the most suitable candidate (i.e. GeNorm, http://medgen.ugent.be/~jvdesomp/genorm/).

Response: Ideally, we would like to use several housekeep genes in addition to GAPDH, as suggested by the reviewer. However, previously published papers have shown GAPDH alone is sufficient as a control (e.g. ref. 23 in the revised manuscript). The others gene such as β-actin, HPRT, 18S ribosomal RNA described by J. Huggett et.al (Genes and Immunity (2005) 6, 279-284) have not been used for similar studies. Currently, we did know the express patterns of these genes in PBMC and its cell subpopulation.

Minor Essential Revisions
1. As the paper in currently configured, Table 1 is supplementary data, and the manuscript begins with Table 2 (i.e., there is no Table 1 in the manuscript itself). Table 2 should be relabeled as Table 1, and Table 3 should be relabeled as Table 2.

Response: Table 2 has been relabeled as Table 1. We also added a new Table 2.

2. The table formatting needs revision. The column headings especially are difficult to discern, and the tables themselves spread over more than one page.

Response: We have revised our tables to make the column headings easy to discern and understand. We have reformatted our tables and added notes below the table for clarification.

3. Under Methods for HIV viral load measurement, the authors misstate the detection limit as between 20 and 10^7 copies per ml, however the manufacturer states the detection limit is from 50 to 7.5 x 10^5 copies per ml. (http://molecular.roche.com/assays/Pages/COBASAMPLICORHIV-1MONITORTestv15.aspx). The authors should further specify that they are measuring RNA copies.

Response: We have corrected the detection limit in the revised manuscript.

In this kit, there are two different specimen preparations: UltraSensitive and Standard. In the UltraSensitive, the quantification of HIV RNA is from 50 to 7.5×10^4 copies/ml. In the Standard, the quantification of HIV RNA is from 400 to 7.5×10^5 copies/ml. In our study, we used the standard, so the detection limit was from 400 to 7.5×10^5 copies/ml.

4. It would be interesting to know the viral loads of the long term nonprogressors relative to the typical progressors. Do any of their subjects meet the definition of viral controllers? (i.e. viral loads below the limit of detection in the absence of therapy and normal CD4 counts). If so, are there correlations with KIR and HLA?

Response: None of the long term nonprogressors in our study meets the definition of viral controllers.

Discretionary Revisions
1. On page 12, second paragraph, first sentence, should add the word ‘maintain’ in front of CD4+ T cell counts.
Response: We have rephrased the sentence to ‘the maintenance of CD4+ T cell counts.

2. Page 5, reference 8 refers to Rhesus macaques. Is there similar data or references specific to human KIR and HLA?
Response: The original ref. 8 has been replaced with a new reference: “Parham P: MHC class I molecules and KIRs in human history, health and survival. Nat Rev Immunol 2005, 5(3):201-214.”.

3. On page 16, last sentence. Remove the word DNA following KIR3DS1/3DL1. Should read “KIR3DS1/3DL1 gene is associated”. That a gene is DNA is a given.
Response: We have removed the word “DNA” on page 16, the last sentence.

4. On Page 17, second paragraph, last sentence. I wouldn’t say mRNA levels of KIR3DL1 promote disease progression. There’s no evidence for that. I would say it is associated, but promoting implies a more active participation than has been demonstrated.
Response: We thank the reviewer for the suggestion and have revised the sentence, which now states, “Together, these results indicate that mRNA levels of the KIR3DL1 gene in HIV-infected individuals may be associated with disease progression”.

5. Page 17 to page 18. Remove the word ‘might’. The data does demonstrate this, not that it might demonstrate this.
Response: We have removed the word ‘might’.

6-7. Top of page 21, the word autologous seems to have a capital ‘F’ placed in the middle of it. Also, the next sentence should be ‘At the DNA level’, not ‘On the DNA level’. Please review the figure legends for typos. For instance, in Figure legend 1, there is a sentence that starts with ‘And’
Response: We apologize for the typos and have asked a native speaker to edit the revised manuscript.

Reviewer 2 (Dr. Nicole Bernard)’s comments and our responses (underlined):
This article by Y Jiang et al. describes an analysis of the frequency of KIR genes, HLA-B antigen subsets and combinations of KIR3DL1/S1 and HLA-Bw4*80I combined genotypes in 132 HIV infected individuals, which include 40 long term non progressors (LTNP). These individuals are classified as having either high or low CD4 counts, high or low viral load or being LTNP versus typical progressors (TP). Overall they find that carriage of the KIR3DL1+KIR3DS1+HLA-Bw4*80I+ is more frequent in LTNP than TP, that Bw6 homozygotes are more frequent in TP and that carriage of the KIR3DL1+KIR3DS1-HLA-Bw4*80I- genotype is associated with higher viral load and lower CD4 counts. They also observe higher levels of mRNA for KIR3DS1 in LTNP and low levels of KIR3DL1 mRNA in TP.
The question posed by the authors could be better defined. The methods are in general appropriate but should be better described. There are some questions regarding the data as discussed below in the review. The discussion and conclusions are in general well balanced and supported by the data. The limitations of the work are not clearly stated. The authors acknowledge work upon which they are building but because they have categorized study subjects in a manner that is different from what has been done by other it is difficult to place this article in the context of the field as it stand at present. The title needs to be changed. The abstract accurately conveys what has been found. The writing is acceptable but needs to be gone over again to make language usage more rigorously what is being reported and to make additional corrections.

Overall, this manuscript needs to be reorganized and refocused in order to build on what has already been published on KIR3DL1/S1 HLA-B and HIV infection. In its present state it is difficult to appreciate how the information presented relates to previous work because the alleles and genotypes studies are not categorized as others have done and because no rationale is provided for why they are categorized differently in this manuscript. These points should be addressed before this manuscript is accepted for publication.

Major Compulsory Revisions
1) The main critique I have with this manuscript is that it uses language that pushes their interpretation of the results that certain genotypes are the cause of a phenotype (whether that be low or high CD4 count, low of high VL or LTNP versus TP status). Their data only show associations between certain genotypes and HIV outcomes and it is an over interpretation of their results to assume a causal relationship between the 2. The title is an example of this: “KIR3DS1/L1 and HLA-Bw4-80I affect HIV disease progression among HIV typical progressors and long-term nonprogressors” should be changed to soften the idea that these genotype “affect” disease progression. The authors need to change the text to remove language that reflects a causal relationship between their data and HIV outcome.

Response: We have softened our language as suggested. We agree that our study, although indicative, did not reflect a causal relationship. In addition, we have changed the title to “KIR3DS1/L1 and HLA-Bw4-80I are associated with HIV disease progression among HIV typical progressors and long-term nonprogressors”.

2) KIR3DL1 and KIR3DS1 have a high degree of homology. The description of the relative quantitative analysis for KIR mRNA expression section on page 9-11 was difficult to follow. Please clarify this section by adding additional detail linking the data generated with the data shown in Figure 2. The authors should include in this manuscript examples of the raw data that was used to generate Figure 2 that demonstrate that the reactions are specific for KIR3DL1 and KIR3DS1. Does this methodology pick up the copy number variation for KIR3DS1 and KIR3DL1 reported by Pelak et al? If so this should be mentioned and reported.

Response: We have revised the manuscript to add more detailed for the.
procedure. In addition, we have added an example of raw data in Figure 2 to demonstrate the PCR specificity. The PCR reaction only generated a single band, and sequence analysis confirmed that they are indeed specific for KIR3DL1 and KIR3DS1.

Our method is a relative quantification of KIR mRNA and does not distinguish the source of differences; it remains unknown whether these differences are derived from transcriptional control or from differences in the gene copy number, as described by Pelak.

3) On page 12 1st and 2nd paragraphs the gene frequency of KIR3DS1 and KIR3DL1 in subjects having different outcomes is reported. I do not think that the terminology used here is correct. KIR3DL1/S1 is a locus (or a gene) within the KIR region that encodes both KIR3DL1 and KIR3DS1 alleles. When reporting that the KIR3DS1 gene is more frequent in those with high versus low CD4 counts the authors apparently mean alleles since they consider the 5 individuals who only have KIR3DS1 alleles as being KIR3DS1 homozygotes and having 2 allele copies (Table 2 for KIR3DS1 consider 44 alleles from 34 heterozygotes and 10 homozygotes). What is being analyzed for the KIR3DL1 “gene” in the text and Table 2 is confusing. The sum of KIR3DL1+ observations adds up to 127 for CD4 counts and viral load and 118 for LTNP+TP. This does not correspond to the number of KIR3DL1 alleles in homozygotes + heterozygotes. One would expect that this is what should be analyzed given that this is what was analyzed for KIR3DS1. What do these KIR3DL1 “gene” numbers correspond to? This section and Table 2 either need to be clarified to justify the use of the word gene to describe KIR3DL1 and KIR3DS1, which I think is incorrect. The results should be reanalyzed by considering alleles (usually 2 copies per locus) in which case the denominator for their analyses would be 132 subjects X 2 alleles per subject = 264 alleles (unless they have access to copy number variation information) or analyze between group differences in genotypes (KIR3DS1 homozygotes, KIR3DL1/S1 heterozygotes and KIR3DL1 homozygotes). Also Table 1 line 1 “KIR allele” should be “KIR gene” for most of the data presented in this table excluding that for the KIR3DL1/S1 locus is reporting the frequency with which each gene is present.

Response: We apologize for the confusion and now have revised all the tables. We also added a new table (new table 2) for the allele frequency. In addition, in our study, although all individuals were used for the analysis based on CD4+ T cell counts and viral loads, 9 individuals were excluded for the analysis based on LTNP or TPs. These 9 individuals didn’t meet the definitions of LTNP or TPs. They were infected for less than 10 years but their CD4+ T cell counts were still higher than 500 cells/µl. We were unable to determine their disease status for either TPs or LTNP. In the revised manuscript, all the information has now been given in the tables.

4) On page 13 paragraph 3 states that persons who are HLA-Bw4*801 have higher CD4 counts. HLA-B*57 belongs to this group, which is an allele well recognized to be associated with LTNP status and viral load control. Since 40 LTNP are included in
this study a high frequency of HLA-B*57 would be expected and may be driving the results the authors attribute to HLA-Bw4*80I. Do the authors have information on the HLA-B locus allotype? If so please report whether the frequency of HLA-B*57 explains these observations. This point should be discussed in the discussion section as well.

Response: We thank the reviewer for the good advice. We did not find any subject with HLA-B*57 in our study. Our data suggest that KIR3DS1 and HLA-Bw4-80I are associated with HIV disease without the influence of HLA-B*57 in our study. We have also discussed this in the revised manuscript. In addition, we have no information on the HLA-B locus allotype.

5) On page 14 paragraph 3 this section would be much clearer if the genotypes were better described. Lines 1/2 and 4 KIR3DS1/3DL1 “gene” should be “genotype”. On line 5 does KIR3DS1+HLA-Bw4-80I+ genotype describe genotypes with at least 1 copy of KIR3DS1 (i.e. heterozygotes and homozygotes?) Who is included in the KIR3DS1-HLA-Bw4*80I- genotype group (Only KIR3DL1 homozygotes?). On page 15 par 1 line 5/6 are not KIR3DS1-HLA-Bw4*80I- and KIR3DL1+KIR3DS1-HLA-Bw4*80I- the same group? And so on…. These paragraphs would benefit from better organization and synthesis and this would follow from a better definition of groups in the methods section or by reporting on the 3 generic genotypes with and without co carriage of Bw4*80I. Another issue not addressed is that HLA-Bw4 alleles that are not *80I, i.e. those that have a threonine at position 80 of the HLA heavy chain are ligands for KIR3DL1 and can educate NK cells through the KIR3DL1 receptor for activity upon encountering targets with reduced HLA-B expression. This is not considered by in this manuscript at all. Why?

Response: We apologize for the confusion and now have revised all the tables. We also added a new table (new table 2) for the allele frequency. We also considered the reviewer’s suggestion about 80T and the new results have been added to the new Table 3.

6) For the data appearing in Figure 2 the number of datapoints shown indicates that this represents a subset of the study population with approximately 30 data points shown. How were these selected? Are all those tested KIR3DL1/S1 in this figure heterozygotes? Add this information to the text.

Response: The 30 data points were selected based on the availability of the blood sample. We could not acquire sufficient amounts of peripheral blood for both DNA and mRNA analyses for most of the donors. Only some individuals in Figure 2 are KIR3DL1/S1 heterozygotes. We did not determin the zygosity of every individual.

7) For the data shown in Figure 3A there is one data point that is an outlier with relative KIR3DS1 mRNA levels between 9 and 12. Does the statistical significance of the correlation between CD4 counts and KIR3DS1 mRNA levels survive when this point is removed? If not this should be mentioned in reported.
Response: The statistical significance ($p=0.022$) was achieved by using all data points. If the data point between 9 and 12 was removed, we could not research a statistically meaningful conclusion. As suggested, we have mentioned this in our discussion.

Minor Essential Revisions
1) There are several instances where references have been incorrectly cited. Reference 4 by Pelak et al is not a correct reference for the statement on page 4 that “…the polygenic KIR gene complex codes for varying numbers of inhibitory and activating receptors.” It may be if the author mean the KIR3DL1 locus specifically. The statement one on page 5 regarding NK activation status being regulated by interactions between KIR and HLA class I gene products. I do not think that reference 8 is appropriate for the statement that KIR and HLA exhibit remarkable diversity and rapid evolution as this reference describes work done in non human primates.


2) On page 5 and later in the text the authors need to be cautious regarding whether HLA-Bw4*80I is a ligand for KIR3DS1 as there is no direct evidence for this statement and much against this possibility. I would refer then to JP Vivien et al in Nature 2011.

Response: We thank the reviewer for the suggestion and have removed any reference of HLA-Bw4*80I as the ligand for KIR3DS1.

3) The statement made on page 6 that KIR2DL3 has a strong protective effect in HIV infected individuals preventing them from becoming immunological non responders is too strong a statement for an observation that found an association between presence of a KIR/HLA genotype and an outcome after starting HAART. The authors did not show that carrying the genotype was the cause of the outcome measured.

Response: We have rephrased our sentences to “In addition, Soria, A. et al., found that the functional compound genotype HLA-C1(+)/KIR2DL3(+), was associated with reduced risk of immunological no responder status in treated HIV-infected individuals.”

4) LTNPs are HIV infected and therefore not resistant to HIV infection. Rather they exhibit a slower rate of disease progression. The statement to this effect on page 6 par 2 line 4 should be corrected.

Response: We have rephrased our sentences.

5) Also on page 6 the authors reference a manuscript by Bostik at al. The mRNA levels that are increased and reported to be associated with high SIV viral load is KIR3DL and not KIR3DL1.

Response: We have corrected the mistake.
6) The authors study a cohort of 132 HIV positive subjects of which 40 were LTNP. This is a very high frequency of LTNP since one would expect that only approximately 5% of HIV infected individuals would fit the criteria for being LTNP, i.e. only 6 or 7 among 132 subjects. If this is a selected population it should be mentioned in the manuscript. The definitions for high versus low viral load and high versus low T cell counts are arbitrary. Is there a rationale for setting the cut offs where they did for these groups?

Response: The LTNPs are selected for this study, and thus, the frequency is higher than the natural occurrence of LTNPs in China.

The settings for the cutoff on CD4+ T cell counts and viral load are based on our clinical experience in treating HIV patients. Patients with 500 CD4+ T cell counts are generally healthy. In the field of present HIV research, there is no single definition of cut offs on viral load. Researchers used many different cut offs on viral load, such as 2000, 10^5, 10^4 or 10^{4.5} as cutoffs. In our study, we used viral load 10^4 as a cutoff. The viral load (10^4 copies/ml) is in the proximity of the median viral load in our subjects and this cutoff has also been used by others. (Thomas R, Apps R, Qi Y, Gao X, Maley C, O'HUigin C, O'Connor G, Ge D, Fellay J, Martin JN et al: HLA-C cell surface expression and control of HIV/AIDS correlate with a variant upstream of HLA-C. Nat Genet 2009, 41(12):1290-1294.).

7) Regarding KIR and HLA genotyping please state what information the KIR Genotyping SSP kit provides. Is it KIR3DL1/S1 generic genotyping? Does the kit also give information on KIR3DL1 allotyping? Is the HLA typing done to a 2 digit or 4 digit resolution? Is HLA-A considered in assigning whether an individual has a Bw4*80I allele?

Response: We have added more descriptions on the KIR Genotyping SSP kit. The kit doesn’t provide information on KIR3DL1 allotyping. In our study, the HLA typing was done to a 2 digit resolution. HLA-A was not considered in our study. It is very complicated and quite different from HLA-B. We may consider KIR3DL1 interactions with HLA-A Bw4 in our future study.

8) On page 11 paragraph 3 line 1 the word “detected” should be changed to “screened” since it is unlikely that that they detected all the KIR genes and pseudogenes in each person tested.

Response: We have made a change as suggested.

9) On page 18 the authors mention that others have shown that KIR3DS1 independently associates with higher CD4+ T cell counts but does not have an effect on viral load. Please add the reference for this statement.

Response: We have added a new reference (Ref. 12 in the revised manuscript).

10) Multiple analyses were done that should be subjected to Bonferomni’s correction for multiple comparisons. This has not been done in this manuscript. This should be
addressed in the discussion as a limitation of the data or the correction should be applied in order to see which comparisons remain statistically significant.

Response: we have added a discussion to acknowledge this limitation of our study.

Reviewer 3 (Dr. Francesco Trapasso)’s comments and our responses (underlined):

In their manuscript, Jiang and colleagues have investigated the role of KIR3DS1/L1 and HLA-Bw4-80I in HIV disease progression. To this purpose, the authors have investigated the KIR-HLA genotypes in a cohort of Chinese patients infected by HIV; as a result they found that the KIR3DL1+KIR3DS1+HLA-Bw4-80I+ genotypes was mainly present in long-term nonprogressors versus typical progressors, whereas KIR3DL1+KIR3DS1-HLA-Bw4-80I- was mainly associated to higher viral loads and lower CD4+ T cell counts, proposing KIR-HLA genotypes as candidate genetic markers of disease progression in HIV patients.

Even though the study was performed in a relatively small group of patients of restricted ethnicity, this investigation is of great interest because it proposes the possibility of candidate predictors in the outcome of HIV-infected patients. The manuscript is well written and both experiments and results are enough to support the authors’ conclusions.

Response: We thank the reviewer for the positive comments.

Dr. Tao Dong’s comments and our responses (underlined):

This manuscript by Jiang et al performed KIR and HLA genotype analysis and determined their association with HIV disease outcome by comparing two relative large cohorts (long term none progressor and Typical Progressor) in China. Firstly, the authors confirmed strong KIR3DS1/HLABw4-80I association with slow disease progression, previously observed in Western cohorts. Secondly, they showed KIR3DS1 expression at mRNA level was also associated with a protective effect while KIR3DL1 mRNA expression was associated with rapid disease progression. Overall, the study was well performed and the data analysis was appropriately done. The association between level of KIR3DS1 and 3DL1 expression at mRNA level and outcome of disease in chronic HIV infection is particular interesting, meriting further investigation.

Response: We thank the reviewer for the positive comments.