Author's response to reviews

Title: Response to M. tuberculosis selected RD1 peptides in Ugandan HIV-infected patients with smear positive pulmonary tuberculosis: a pilot study

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Author's response to reviews: see over
Editors-in-Chief of BMC Infectious Diseases

Dear Editors, attached please find the revised manuscript “Response to M. tuberculosis selected RD1 peptides in Ugandan HIV-infected patients with smear positive pulmonary tuberculosis: a pilot study” by Goletti Delia et al., submitted for publication on BMC Infectious Diseases as research article. As requested we have modified the manuscript according the reviewers’ comments. Below are the answers to the questions asked.

Reviewer's report 1:
General
The paper from D. Goletti et al. reported on the response to RD1 selected peptides in HIV/TB co-infected patients from a TB endemic area. This is a pilot study performed on a very small number of individuals. Notwithstanding this, the results are interesting and suggestive of the potential role of RD1-based assays for the diagnosis of active TB in HIV-infected subjects.

Patients and Methods:
- It is not clear where the tests have been performed (in the laboratory of Kampala, Cleveland or Rome) and whether fresh or frozen PBMC have been used (using frozen cells the sensitivity of the ELISPOT assay is reduced).
  
  **ANSWER:** the tests were performed in the laboratory of Kampala with fresh samples whereas the spots of the ELISPOT plates were counted in Italy. This information is now included in the text on page 7 (First rows of the “ELISPOT assay” section).

- It is not clear whether a positive control, like a mitogen, has been used in the ELISPOT assay.
  
  **ANSWER:** no mitogen was used as positive control. We were interested in evaluating the M. tuberculosis-specific cellular responses.

- The two cohorts studied are not homogeneous in term of CD4+ T cells/ml (55% of active TB subjects have <200 CD4/ml while 80% of subjects with no active TB have >300 CD4/ml).
  
  **ANSWER:** it is true that the 2 cohorts are not homogeneous in terms of CD4+ T cell counts. However, based on the fact that RD1 responses are mainly mediated by CD4+ T cells (Goletti et al, JID 2006) the control group had high potentiality to respond to the RD1 selected peptide assay; however in this group, differently from that with active TB, few positive responses to the RD1 selected peptide test were found indicating that the test has good chances to identify active TB. This is now discussed on page 14 (second paragraph).

- No data on antiretroviral therapy are reported.
  
  **ANSWER:** patients were not under antiretroviral therapy. It is now enclosed in the manuscript on page 6 (20th rows).

Results:
- page 8, line 8, “one patient was deemed anergic to M. tuberculosis…”: to be sure the patient was really anergic should be better to have a positive control (like a mitogen) in the ELISPOT assay.
ANSWER: Based on the fact that we did not have a mitogen as control, we modified the sentence as “the patient deemed unresponsive to *M. tuberculosis* antigens”, not that he was “anergic” at page 9 (second row of the section “Response to selected RD1 peptides is associated with active TB”).

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

- Page 6, line 20: the sentence “subjects without TB” should be corrected in “subjects without active TB” (in an area endemic for TB most controls could be latently infected).

ANSWER: Thank you for the comment and we corrected as suggested.

- It is not reported where Figure 4A/B should be located in the manuscript.

ANSWER: Thank you for the comment and we corrected as suggested. See page 12 (second paragraph).

Reviewer's report 2

General

This preliminary report deals with the TB diagnosis accuracy of the ex vivo immune response to RD1 selected peptides, measured with an ELISPOT technique, in 20 HIV infected patients with sputum positive TB pulmonary disease. It compared this accuracy with an other ELISPOT test using 2 intact RD1 proteins. These immunological data are also evaluated in relation to the CD4+ counts and the HIV viral load in the patients at the time of TB diagnosis. Moreover, a part of these patients (9 and 12, respectively) were retested at 3 and 6 months after starting specific anti-TB treatment. At the time of TB diagnosis, similar accuracy was found using the 2 groups of antigens, even if statistics showed some differences when applied to absolute sum ESAT-/CFP10 spot counts. However, due to the fact that these 2 responses were shown to be CD4+ related, the AUC of the spots/CD4+ counts did not differ significantly. The same is also indicated in Table 2. Thus, the statement that the RD1 selected peptides response is associated with active TB disease in HIV-infected individuals, but not the RD1 proteins (in the discussion) has to be changed. Since, this preliminary study, as discussed by the authors, presents limitations, the principal being the small number of patients and mostly the controls, the statements comparing the 2 tests (in the abstract and in the conclusion) should to be more accurate. Individual small or major discordance between the 2 tests should be indicated.

ANSWER: According to the referees’ suggestions, the statement that the RD1 selected peptides response is associated with active TB disease in HIV-infected individuals, but not the RD1 proteins (in the discussion page 14, first paragraph) has been changed. Similarly in the abstract we modified the text in the conclusions (page 3).

Specific comments:

- Title: it should be more specific indicating that the HIV-infected patients are patients with smear positive pulmonary tuberculosis, as described in M&M and results. Such HIV-TB coinfected patients are not really difficult to diagnose as compared to smear negative HIV-TB coinfected patients that represent the most difficult group to be diagnosed as early as possible.

ANSWER: According to the referee we modified the title as “Response to *M. tuberculosis* selected RD1 peptides in Ugandan HIV-infected patients with smear positive pulmonary tuberculosis: a pilot study”.
- Abstract: the exact number of evaluated HIV-TB coinfected patients needs to be indicated.

ANSWER: According to the referee we now enclosed in the abstract the exact number of evaluated HIV-TB coinfected patients (page 2, see result section).

- Results: The correlation between the RD1 selected responses and the clinical, microbiological radiological data, being indicated in the subtitle (page 9) are not given in the text, apart the fact that the culture were negative after 6 months of therapy. Since, the authors did wrote in the M&M that the sputum examination was performed monthly, if would be interesting to describe the microbiological follow-up (either as the semiquantitative smear test or the time of culture positivity) at different time points. It would be also interesting to evaluate such individual microbiological follow up with the individual spots follow up (at 3 and 6 months). Relative to the significant, decrease of the RD1 selected peptides spots, it is shown that the levels reached at 6 months of therapy was not complete for several patients. The same seems to be occur when the authors used the RD1 proteins. This is not discussed later. Why? Did these patients being followed after stopping the treatment? Are they more prone to develop relapse? The Figure 4AB is not indicated in the text.

ANSWER: we have tried to find a correlation between microbiological load and response to RD1 antigens in terms of spot forming cells per million PBMC. Unfortunately, no clear correlation was found between M. tuberculosis load in sputum and the response to selected RD1 peptides and radiological lesions over time.

Data comparison of the responses to RD1 proteins and peptides are reported in the result section at page 10 (last paragraph) and page 11 (first paragraph) and discussed at page 15 (first paragraph).

Moreover both groups, the treated and untreated patients, are currently followed over time in the clinic in Kampala. At the moment no relapse occurred in those treated, nor active TB occurred in those without disease used as control group (see page 16, third paragraph).

ANSWER: figure 4AB is now indicated in the text at page 12.

- Table: in the table 2 the reference to Rangaka should be indicated as a reference number.

ANSWER: thank you for the comment. We corrected as suggested.

- Discussion: The inverse correlation between the individual HIV load and the number of spots could be associated with the same inverse correlation with the individual CD4+ counts. The reference (28) indicated in page 12 (line 17 and 20) is not appropriate.

ANSWER: thank you for the comments: We have tried to perform the statistical analysis suggested by the referee: however no significant correlations were found. Moreover, we eliminated the reference as suggested.

- References: There are two references 29. All references beyond the number 29 need to be adjusted in the text and in the reference list.

ANSWER: thank you for the comment. We corrected as suggested.
Reviewer's report 3
Title: Response to M. tuberculosis RD1 selected peptides in Ugandan HIV-infected patients with tuberculosis
Version: 1 Date: 17 September 2007
Reviewer's report 3:
Major Compulsory Revisions
1. This is an interesting study of relevance to working in this field. However, as pointed out by the authors, a major limitation is the small sample size. The authors acknowledge that this is pilot work that needs to be confirmed. So, I think the title and the abstract should explicitly state that this is a pilot study.
ANSWER: We corrected the title as suggested and we stated that this is a pilot study in the abstract (in the conclusion section, page 3)

2. I think the authors overstate the issue of change in responses over time. The authors claim (Page 10) that a successful therapy for TB causes a significant decrease of the sum of ESAT6 and CFP10 peptide responses. However, even after successful therapy, the median SFC count was as high as 100! Surely, if a dichotomous result were to be used, then all patients would be scored ELISPOT positive, even after completion of treatment. So, although treatment may lead to a decline in ELISPOT responses, all patients still had high residual responses at the end of therapy. None probably reverted to negativity. The authors need to address this issue and provide some explanation as to why all treated patients had fairly high responses at the end of therapy. At least 3 studies from India have shown high T cell responses despite therapy (Infection. 2007 Apr;35(2):98-103; J Occup Med Toxicol. 2006 May 23;1:7; and Am J Respir Crit Care Med. 2006 Aug 1;174(3):349-55.). I suspect TB patients in TB endemic countries often have strong baseline T cell responses. So, the responses have to drastically drop for the assays to revert to negativity. Alternatively, there may be other factors that keep the T cells partially stimulated in high burden countries – exposure to NTM, repeat exposures to TB, etc. These issues deserve some discussion.
ANSWER: Thank you for the comments. We added a sentence in the discussion as suggested, on page 15, first paragraph. The trend of decrease of the RD1 selected peptides response over time is in line with previous reports using the ELISPOT technology indicating that these responses better correlate with bacterial burden than whole blood tests. Here conversion to negative response to RD1 selected peptides was found only in 30% of the patients analyzed, differently from our previous study in which this was found in 100% of the patients after successful therapy. We agree, as you stated in your comments, that the different setting may have influenced the results. In particular a potential re-exposure to M. tuberculosis, a likely scenario in endemic countries, after the completion of treatment or to environmental mycobacteria, commonly encountered in the tropics, which share the esat-6 and cfp-10 genes, may account for the diverse finding.

3. Figures 3A and 3B show correlation between CD4 counts and responses to ESAT6/CFP10. Although the correlation coefficients are statistically significant, I find the plot unimpressive. Visually, it is hard to make out a linear trend, and I suspect the R is significant because of one outlier (the person with a very high CD4 (>500) and SFC of nearly 1000. If this outlier were to be dropped, I am sure the correlation coefficient will not look impressive. I suggest the authors repeat the analyses after excluding the outlier and report these results as well.
ANSWER: We performed the analysis as suggested, and as foreseen, coefficients are not statistically significant if the data from the patient with high CD4+ T cells and SFC are removed. This is enclosed in the result section on page 12, first paragraph.

4. The authors have done a ROC analysis, but because their controls are from a high endemic area, they have no way of excluding LTBI among the controls. Figure 1 clearly shows that controls are demonstrating responses to ESAT6 and CFP10, and that tells me that at least some were latently infected. In fact, this is confirmed by the lower specificity reported by the authors. I would recommend adding the ROC plots to the paper, and to discuss the impact of including controls from a high TB endemic country.

ANSWER: The figure with the ROC analysis has now been enclosed in the result section on page 12, as figure 5A-B. Also few words on the impact of including controls from a high TB endemic country have been included on page 16 (third paragraph).

Minor Essential Revisions
1. The authors use the word “overtime.” It should be “over time”.
ANSWER: We modified it as suggested.

2. In the background, the authors state that IGRAs should be shown to be useful in TB-endemic settings. I agree with this assertion. In fact, FIND, TDR and Stop TB recently published a research agenda on IGRAs and specifically called for more studies in TB endemic countries (Lancet Infect Dis. 2007 Jun;7(6):428-38).

ANSWER: Thank you for the comment. We now enclosed the suggested reference (number 10).

3. Page 5, first line: please substitute the word “circulation” with “transmission.”
ANSWER: We modified it as suggested.

4. Page 7: because an in-house ELISPOT was used, it will be helpful to add more text on how exactly the assay was performed. Readers should be able to reproduce the assay, if they so wished.
ANSWER: We enclosed it as suggested, at page 7 on the “ELISPOT assays” section.

5. Page 8: Table 1 shows that controls were less immunosuppressed than TB cases. What implications can this have for the comparison between cases and controls?
ANSWER: it is true that the 2 cohorts are not homogeneous in terms of CD4+ T cell counts. However, based on the fact that RD1 responses are mainly mediated by CD4+ T cells (Goletti et al, JID 2006) the control group had high potentiality to respond to the RD1 selected peptide assay; however in this group, differently from that with active TB, few positive responses to the RD1 selected peptide test were found indicating that the test has good chances to identify active TB disease. This is now discussed on page 14, second paragraph.

6. Page 8: The authors found one patient with “anergy.” Why was mitogen not used as the positive control? If it was used, what was the mitogen response for this individual?
ANSWER: We did not have a mitogen as control, and consequently we modified the sentence as “the patient deemed unresponsive to M. tuberculosis antigens” not that he
was “anergic per se” (page 9, second paragraph). Based on these results I can not predict if that subject would have responded to the mitogen.

7. Figure 1B: p-value not included.
ANSWER: Thank you for the comment. We corrected as suggested.

8. Figure 2B: p-value not included. Also, please only include 1, 3 and 6 months on the x-axis.
ANSWER: Thank you for the comment. We corrected as suggested.

Reviewer's report 4

General
This interesting pilot study describes the response to selected RD1 region peptides from M. tuberculosis in Ugandan HIV patients coinfected with HIV. The results indicate that obtaining a ratio of spot forming cells to the CD4 count may provide a sensitive and specific test for tuberculosis.

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Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct) 1. Methods. The HIV infected subjects without TB were recruited as control subjects “to assess changes in spot forming cells over time”. This statement is confusing as the longitudinal data shown is only from the TB – HIV coinfected group.
ANSWER: Thank you for the comment. We agree and we removed that sentence at page 6.

2. There is no statement concerning whether the HIV infected subjects (± active TB) were receiving anti retroviral therapy. This information is necessary in order to interpret the changes in responses over time. How did the CD4 counts change over this six month period?
ANSWER: No antiretroviral therapy was performed and it is stated at page 6 (20th row). Moreover CD4+ T cell counts were not monitored over the 6 months of observation.

3. Two of the “non TB” controls had high ESAT6 / CFP10 peptide responses, and three gave good responses to the ESAT6 and CFP10 proteins. Have any of these subjects subsequently developed TB?
ANSWER: We are following the controls tested RD1 positive either to proteins and peptides. No one developed TB at the moment but they are currently studied over time as already stated at page 16 (third paragraph).

4. Some statement could be added about whether combined ELISPOT/CD4 count ratios are likely to be used as a research tool, or as a routine diagnostic test in the settings where coinfection with TB and HIV are common.
ANSWER: At page 16 we already stated “According to the literature (24), our analysis of these responses suggests that the ELISPOT test could be incorporated into practice in the context of HIV infection” (second paragraph).

5. I do not think a lack of response to two RD1 proteins/peptides can really be used to define anergy, in the absence of an antigen such as PPD or a non-mycobacterial antigen such as Candida. (p8).
ANSWER: We modified the sentence writing that the absence of response to RD1 proteins/peptides in the presence of active TB can be defined as “unresponsiveness to M. tuberculosis” (page 9, second paragraph).

6. Discussion, lines 1 – 4. The results presented show a better association of ELISPOT responses to the RD1 peptides with active disease than of responses to the RD1 antigens, but the results on pages 8 – 9 and in Figure 1 seem to show a similar trend for the protein responses, and it would be better to say these responses did not change significantly (p9). The exact p values should be shown for the RD1 protein differences in Figures 1b and 2b.
ANSWER: We enclosed the p values at page 9 and in figure 1B and 2B.

7. Table 1. A statement should be included that the groups with/without active TB were not matched for extent of HIV infection.
ANSWER: We enclosed at page 9 (first paragraph) that the two cohorts studied were not homogeneous in terms of CD4+ T cells counts. This is now discussed on page 14 (second paragraph).

8. Table 2. This needs to be labelled more clearly as a ratio rather than as a frequency of SFCs.
ANSWER: We corrected as suggested.

9. The control group is inconsistently labelled as no active TB, no TB patients, and referred to as the group without TB; also the Figure legends say 1 A-B but 2AB etc.
ANSWER: We corrected as suggested.

10. The English requires some editing; for example, I would prefer “selected RD1 peptides”; overtime should be “over time” throughout, p12; line 3 of second paragraph, “only one patient gave and indeterminate result”; lines 6 – 7, “thus an impairment is expected in the presence of severe immunosuppression”.
ANSWER: We corrected as suggested. The manuscript has now been edited by a English colleague.

I thank you for your attention and I hope that this manuscript is now suitable for publication on BMC Infectious Diseases

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Best regards

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