Dear Editor

We were very pleased that you found our manuscript entitled: "T-cell and serological responses to Erp, an exported Mycobacterium tuberculosis protein, in tuberculosis patients and healthy individuals", potentially acceptable for publication in your journal.

We enclose the revised manuscript, modified in accordance with your comments and those of the reviewers and a point-by-point response:

1. Reviewer: Pascal Meylan

In the present paper, the authors characterize in quite exquisite details the
immune response against a recently described protein, with the awowed goal of developing a test able to differentiate latent from active Tb.

The major problem in this respect is that neither the patient number (especially as not all patients had samples available for each test modality; see for instance patient number in table 1) in the different groups, nor the statistical evaluation are appropriate to accurately determine the potential of anti-Erp reponses to differentially diagnose latent from active Tb.

For instance, small groups of patients with results which are not obviously normally distributed require non parametric tests and descriptors (e.g. median instead of means). In addition, the question is not so much as to whether groups have statistically different results, but rather whether the different groups are recognized by the test, i.e. given a chosen threshold, the test will accurately classify the patient as sick with Tb or latently infected, defining a sensibility and specificity indeed subjectable to a ROC analysis.

We agree with these remarks consequently, in the revised version, we use median instead of mean, and have removed the statistical analysis for the lymphoproliferation assay and for all methods when the number of patients was less than 4. We maintain the statistical analysis for the Elispot assay which included 22 TB patients, 16 BCG+ and 7 BCG- (sufficiently large numbers) and because we compared the magnitude of the responses and not the number of positive responses. Moreover, with all assays, we observed the same trend for antigen responses according to the different groups of patients.

The study falls short of this and thus can only be considered as a pilot study suggesting that anti-Erp reponses should be studied in an appropriate fashion to this end. The definition of the patients groups is also debatable. Indeed, a group of BCG vaccinated individuals were divided, according to ESAT tests into "healthy BCG+" and "latently Tb BCG+". This is confusing. On one hand, BCG vaccination is confounding groups that should be formed with Tb-exposed individuals with any testing supporting infection, but with a negative work up for tb disease. I feel like the current definition by ESAT-6 response is not the state of the art of defining patients categories. In fact the proper denomination of the current groups should rather be "BCG vaccinated, M. tb-non infected" and "BCG-vaccinated, M. tb-infected":

ESAT-6 was included to define latent TB-infected individuals as proposed by the scientific community. We agree that this definition is not the best but it is the only biological definition available currently. We renamed the groups of patients, in line with the suggestions of the two reviewers, as follows: "BCG+ without infection" group and "latently TB infected BCG+" (LTBI) group.

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

The two tables are very difficult to grasp; there should be a way of expressing these results in a way that makes them easier to understand. The text is of little
help to this end.
We accept this comment and have removed the 2 tables. The results are now presented in figures 3 and 4 in our revised manuscript as illustrating for lymphoproliferation and Elispot assays.

Minor Essential Revisions
- The manuscript should have the pages numbered!
We have corrected this oversight.
- There are numerous locutions that are inappropriate or indeed francicisms. I propose to have the text edited by a English native or at least someone with a good command of English: some examples:
The manuscript had been revised by English scientific editor.
- Abstract, end of conclusions: should read "do not":
We have made the correction.
- Introduction: a gene encodes a protein, but a protein is a cell surface component:
We have modified the manuscript appropriately.
- p4 top: This antigen might offer a useful approach in the diagnosis (not management) of tuberculosis disease versus latent infection:
We have made the appropriate correction.

Subjects and methods
- p5: patients who (not that, they are not things):
We have made the correction.
- next paragraph: this group was further divided (not divided) into 2 ...:
We have modified the manuscript.
- non infected (instead of no infected):
We have modified the manuscript.
- Antigens: were dilutions tested for each assay? " and the optimal concentration was chosen for each antigen", but what are the criteria for optimal?:
Using different concentrations, we observed an exponential curve with a plateau. The optimal concentration is the second concentration on this plateau. All concentrations chosen correspond to the optimal concentration obtained with each antigen.
- Lymphoproliferation, either quote a paper for that standard method, or if describing, mention the incorporated label:
We indicate the incorporated label in the methods section, page 7.
- Elispot: the fact that the cell preparation does not matter might arguably be moved into a first paragraph of the results.
We agree and these data have been moved to the results section.

- Methods last paragraph: the title should mention that this is an ELISA:
We have modified the manuscript accordingly, page 9.

Results.
- If there were disseminated infections, those may have to be analysed separately as they may be anergic third paragraph: according (not regarding) to their responses to ESAT-6:
No patient was anergic in our study. All of them responded to the positive control (mitogen) used in the different tests, page 11.

- Lymphoproliferation: there are no negative controls. To give percents in a group of three is somewhat misleading.
We included in all assays and for all patients a negative (cells alone) and a positive (cells and mitogen) control, as stated in the Methods section.

- As there are many statistical comparisons between groups, I wonder whether a Bonferonni's adjustment should be applied, which may change the conclusions. T cell responses: here the argument of overlap between the groups particularly applies. See figure Erp for Instance
We agree with these two remarks. In our revised version, we modified our analyse as precised above (we use median instead of mean). We maintain the statistical analysis for Elispot assay which included 22 TB patients, 16 BCG+ and 7 BCG- (a sufficiently large number of patients) and because we compared the magnitude of the responses and not the number of positive responses. Moreover, all assays revealed the same trend for antigen responses according to the patient groups.

2. Reviewer: Katalin Andrea A Wilkinson
T cell based interferon-gamma release assays based on RD1 encoded proteins represent a potentially significant advance on the tuberculin skin test for the diagnosis of tuberculosis. However, a major obstacle to the widespread introduction of these tests in endemic areas is their inability to differentiate between active and latent disease. The present article is aiming to address this issue by exploring the T cell and serological responses to Erp (exported repeated protein) of M. tuberculosis, in tuberculosis patients and controls from a non-endemic area.

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Major Compulsory Revisions
1. The text in many parts of the manuscript is scrambled and it is very difficult to read. 2. The manuscript would also greatly benefit from editing by a native
The manuscript had been revised by an English scientific editor.

3. The term 'latently TB BCG+ individuals' should be changed to individuals with latent TB infection (LTBI):
We have modified the manuscript accordingly.

4. The antigen called '85B' in the manuscript is generally known as 'antigen 85B' or 'Ag85B':
We have modified the manuscript accordingly.

5. The introduction states that Erp has 'not yet been investigated in humans'. This is immediately followed by the sentence that 'this antigen could offer an interesting way in the management of tuberculosis disease or infection'. These two statements are contradictory and should either be expanded with further explanation and references, or the second speculatory statement moved to the discussion.
We have made appropriate modifications, page 3.

6. In the Methods: Detection of specific human antibodies against recombinant M. tuberculosis proteins: the plates were coated with 3μg/ml recombinant protein in PBS, and positive values were arbitrarily decided to be >0.1 (I assume OD). The usual control experiment to perform in order to define positive values is coating a plate with PBS alone.
We indeed performed plate coating with PBS alone and this was used to define positive values. We subtracted the values obtained with PBS for all antigens and the value was considered positive if > 0.1 optical density units.

Minor Essential Revisions

1. The median age in the abstract is not relevant. The median number of IFN-g spot forming cells in response to Erp in the LTBI group compared to the other groups, leading to the p value of 0.019 mentioned in the abstract would be a more relevant piece of information here.
We have modified the manuscript accordingly.

2. 85B in the abstract should be changed to Ag85B:
We have made this change.

3. Define Erp the first time it is used:
We have now done so.

4. ESAT-6 was clearly included to define LTBI people, however, this is not explained. It would make the manuscript clearer if this was explained, together with the reasons why Ag85B was included in the comparison.
We have added informations about these 2 antigens used as comparison to Erp
in the introduction and the rational for their utilisation is now explained: "We studied and compared specific cellular and humoral immune responses to Erp, an antigen of latency, with those to ESAT-6, Ag85B, 2 antigens involved in the virulence of the mycobacteria and PPD as comparison in TB patients and in individuals vaccinated (healthy and latent TB) or not vaccinated with BCG.

" page 4.

5. In the introduction: 'Erp is a protein encoding a cell surface component' should be deleted. Since proteins don't encode, only genes do, a statement like 'The erp gene encodes a cell surface protein with repetitive structure' would be more appropriate.

We have made this correction.

6. In Subjects and methods: the description of the 16 BCG-vaccinated individuals is very confusing and would benefit from a major rewrite and clearing up. For example 'no infected' change to 'uninfected'; 'divised' change to 'divided'; '7 displayed positive responses' -to ESAT-6 I assume, this should be clarified; 'were considered with probable latent TB' change to 'were considered to have LTBI' etc.

We now use terms as proposed by both reviewers, as follows: "BCG+ without infection" group and "latently TB infected BCG+" individuals (LTBI).

7. CD4 T cell purification: what method was used to determine efficiency?

The method used to determine the CD4 T cell purity is flow cytometry and is now described in the Methods section, page 6.

8. Antigens: it is mentioned that they were tested for LPS contamination but no figures are given. This is very important when recombinant antigens are tested as high LPS leads to high TNF production and non-specific responses.

We agree and now state the quantity of LPS measured in our antigens in the Materials and Methods section as provided by the manufacturer. Of course, the quantity was very low for all antigens (none or <100 EU/mg), page 6.

9. Why was the ELISPOT assay incubated for 40 hours? the standard assay is based on overnight incubation (about 16 hours), and detects circulating effector cells. The detection of effector cells does not increase by extending the incubation time, and 40 hours are too short for detecting memory cells. An explanation as to why a 40 hour incubation was chosen would be helpful.

We developed the CD4 ELISPOT IFN- assay long time ago in our laboratory, and we have since published numerous papers with this method, in particular to evaluate responses to PPD in patients coinfected with HIV and TB (Bourgarit A, Carcelain G, Martinez V, Lascoux C, Delcey V, Gicquel B, et al. Explosion of tuberculin-specific Th1-responses induces immune restoration syndrome in tuberculosis and HIV co-infected patients. Aids. 2006 Jan 9;20:F1-7.), and added it in the reference bibliography. In our experience, 40 hours is the best duration to
quantify memory effective cells in particular in patients with very low frequencies of specific T cells.

10. Results: were the patients counselled for HIV testing?
Patients have indeed been counselled. There is mentioned in page 11.

11. Proliferation assays: why were only 20/22 patients tested for PPD proliferation?
Cells were available for only 20 patients, and this is now stated in the manuscript, page 12.

12. No meaningful statistical comparisons can be performed with groups of n=3.
We agree and as stated in the replies to reviewer 1, these analyses have been removed.

13. Cytokine production by CBA: a brief description of the cytometric bead assay in the methods would have been appropriate.
We added a brief description of this assay to the Methods section.

14. 'After Erp stimulation, high level of TNF' was detected: in order to define whether this is real, the LPS content of Erp should be stated.
We have added results of the test for LPS contamination (provided by the manufacturer) to the Material and Methods section as mentioned above (point 8), page 6.

15. 'In the latent TB BCG+ group, high levels of IFN-g and TNF were detected after stimulation with Erp'. This is based on n=2, and values for IFN-g 237 +/- 254, and for TNF 280 +/- 245. This obviously means that one value was very high and the other was very low. Unless numbers are significantly expanded, such comparisons are not meaningful.
We agree and have mentioned above (see also the reply to point 12). We have removed the comparison. In addition, the table has been withdrawn and the results are now expressed in a figure (figure 3) to facilitate comprehension of the manuscript.

16. Humoral responses: as no control experiment was performed in order to determine positive responses and non-specific binding, it is very difficult to judge the meaning of these results.
We indeed performed plate coating with PBS alone and this was used to define positive values. We subtracted the values obtained with PBS for all antigens and the value was considered positive if > 0.1 optical density units. This was added, page, line.

We thank the Editor and the Reviewers for their particular interest in our work and their accurate and constructive comments.
We are sure that these modifications have improved the manuscript. We hope that you will be satisfied with them and that you find this revised paper merits
publication in your journal.

Very truly yours

Valerie MARTINEZ
Guislaine CARCELAIN