Author's response to reviews

Title: Identification of mimotopes from Mycobacterium leprae as potential diagnostic reagents

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Author's response to reviews: see over
Dear Editor

would like to thank the opportunity to resubmit the manuscript MS: 4449869167408621 now nominated Identification of mimotopes from Mycobacterium leprae as potential diagnostic reagents. Below are the answers point I point requested by the reviewers.

Reviewer's report 1
Title: Delayed-type hypersensitivity responses to mimotopes from Mycobacterium leprae in guinea pigs
Version: 2 Date: 10 June 2012
Reviewer: Annemieke Geluk
Reviewer's report:

Major Compulsory Revisions

• Figure 3: it would be more informative if the authors would link the responses for each peptide and the M, ML and C to each other so it would e clear what the responses in each guinea pig are. Now the figure 3 was modified as suggested.

• Why is the response to the pool completely negative for all guinea pigs?
Probably the negative response is due the concentration of antigen used in the pool. The concentration used when for the peptides used alone was 10 μg for each one.

• Legend: indicate the number of guinea pigs tested.
The numbers of guinea pigs were included in the figure.

• Indicate in the legend what exactly is meant with: pool.
The explanation of its meaning is indicated at the legend of the figure.

Minor Essential Revisions:

Use line numbering everywhere in the text.
The lines were numerated.

Abstract:
Researched line 5: suggested change in to: “investigated “or “analyzed”.
The word was modified as your suggestion.

Background:
• Page 4, line 8: should be: lepromin can not be used. Only MB sera were used whereas also sera of PB can be used for Ab screening (e.g. Immunologically reactive M. leprae antigens with relevance to diagnosis and vaccine development. Sampaio LH, Stefani MM, Oliveira RM, Sousa AL, Ireton GC, Reed SG, Duthie MS. 2011).
• Why were PB sera not addressed as well? This would have broadened the use of the peptides.
In this work we chose to use only MB sera since these patients have strong antibody response and therefore more likely to identify candidate antigens. The results encourage the group to investigate the use of PB serum by similar methods aiming to seek other antigens. Preliminary data obtained from this work opens possibilities to further studies.

Methods/ materials:
Page 7, the colonies were picked at random: explain why and how exactly this was performed?
There are no parameters defined to determinate the number of colonies to be collected. The criterion established to this work was to limit the number of colonies to a number around 400.
It is known that the more colonies are tested the bigger are the chances to select different sequences.

Page 8: could the number of bacteria be enumerated from the sensitized guinea pigs: The authors should elaborate on how this sensitization does reflect infection in vivo as M. leprae infection probably does not proceed through the skin. How sure is it that the same DTH occurs after aerosol infection.
The animals were sensitized by inoculation of irradiated and sonicated cells of M. leprae, therefore in this case there is no possibility of development of infection. The sensitization of guine pigs was made as previously described (Melancon-Kaplan J, Hunter SW, McNeil M, Stewart C, Modlin RL, Rea TH, Convit J, Salgame P, Mehra V, Bloom BR, Brennan PJ:

Can the peptides be tested in e.g. naturally infected armadillo’s?
Certainly, could be tested based at the fact that the armadillo’s are the closest condition happened with human beings. The group will consider this fact to future works.

Using *M. tuberculosis* (aerosol) infected mice as second check for specificity is very important and should be performed, since this will also be a control on the procedure of this study using the phage libraries.
This work presents preliminary data over the capacity of peptides inducting cellular response through DTH tests. The principal gold at the point is the identification of other peptides that combined to the described at this work is able to induce responses in a bigger number of animals, in other words, increase the sensibility. Studies must be performed to determine the ideal doses of each peptide at the mixture that composes the skin testing reagent. Based on that, the evaluation of the specificity will be considered not only to *M. tuberculosis* as to other environmental mycobacteria.

Results and discussion:
Page 10: humoral response also depends on T helper cells  
The word was replaced.

Page 10, 2nd paragraph, line 9 and 16: are the animals immunized or sensitized?
The word was replaced.

Genral comment: what are the equences of the peptides and from which *M. leprae* antigens are they derived: this should be included in the paper. Are the *M. leprae* antigens specific or is only the induced Ab response specific?  
Data’s were included at the manuscript, in a manner to inform the reader about the antigens *M. leprae* that may be mimicked by the peptides. The data that were not published yet direct us to an antigen of *M. leprae*, but since they are data’s involving other aspects of our studies, we considered inappropriate to describe them at this work.
Page 11, 2nd paragraph, line 2: HLA should here be MHC.
The word was changed as your suggestion.

Page 11, 2nd paragraph, line 9: or can the peptides be applied as mix of peptides? data for this can and should be easily generated using the available sera.
The identification of mimotopes, was based on the use of antibodies from patients and not necessarily the response based on the sero-reactivity reflects at the response observed in DTH tests. Peptides described were evaluated in serologic analysis (data not published), as also are being tested in combination with new peptides already identified. The proposition of the study presented is to evaluate the ability of the peptide selected to induce a cellular response.

Page 12, line 1: it is not always the case that the detection of IFN-gamma levels cannot be assessed in the field. In fact, the authors should include a field friendly assay for measuring cytokines (e.g. Corstjens, PLAM, et al. 2011. Lateral flow assay for simultaneous detection of cellular- and humoral immune responses. Clinical Biochemistry 44: 1241-1246.) Field friendly assay for measuring cytokines was included.

**Level of interest:** An article of importance in its field

**Quality of written English:** Needs some language corrections before being published

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**
I declare that I have no competing interests.

**Reviewer's report 2**

**Title:** Delayed-type hypersensitivity responses to mimotopes from *Mycobacterium leprae* in guinea pigs

**Version:** 2  **Date:** 31 May 2012

**Reviewer:** Varalakshmi Vissa

**Reviewer's report:**
Comments are included in the attached document.

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests:
'I declare that I have no competing interests'

Reviewer's report:

The strategy for discovering new M. leprae ‘like’ peptide antigens (mimetopes) as lepromin substitutes for skin test Fernandez reaction in leprosy diagnosis is interesting and innovative.

The work and concept is appreciated, however, overall the reviewer’s impression is that the efforts were not completely successful. It is suggested that the title, abstract and conclusions stop at the description of the phage clones and peptides identified. The subsequent phase of testing for DTH in guinea pigs and finding 60% % positivity and suggesting that the 2 peptides (5A and 1B) have the diagnostic value equivalent to ‘lepromin’ is somewhat of a data crunching effect. The results are marginally supportive of the possibility that peptides 5A and 1B are suitable for DTH based diagnostic tests (even if other considerations such as altered conformation of peptides, dose response curves, more animals etc. are tested). This DTH study can definitely be stated in the results, methods and discussion, but for now it appears a little premature to be included in the title of the manuscript and as the thrust of conclusions of the abstract.

Thanks for your comments. Now the title, abstract and conclusions were modified to include the description of phage clones data and peptides identified.

DTH tests were only described in methods, results and discussion.

Some of the comments and reservations in accepting the manuscript as such, particularly with regard to the conclusions drawn are as follows:

1) The entry point for this study is based on the strategy that a pool of IgGs within (a pool of) paucibacillary (PB) and multibacillary (MB) patient sera will be the hook to find the ‘peptide’ antigens in the phage library. The pool of leprosy patient IgGs was eluted after batch binding to (SDS PAGE) resolved M. leprae extract immobilized on a membrane. The membrane based binding and elution of IgGs is interesting, but inadequately described. The sero-reactivity to M. leprae fractions is well described over the last few decades. There are dominant proteins and non protein components that can contribute to the majority of the binding. The authors do not
describe what they found. The PB patient sera may have contributed little to the IgG pool although some new unknown IgGs may have been enriched by this ‘blind’ batch binding approach. But since the data were not shown, it is difficult to see exactly if there was any binding or not, and to what components. Positive and negative controls were not described.

Thank you for the criticism and suggestions. This information was included in the sessions of results and discussion. In this work, only IgG from serum MB patients were used for the selection of mimotopes.

After the multiple rounds of panning, and considering two peptides (5A and 1B) were deemed to be promising as diagnostic tests, these were the results (Figure 2): Peptide 5A phage clone was largely non reactive to ‘bonafide’ leprosy patients: the serum from 9/10 PB patients and 18/23 MB patients did not recognize these clones. Peptide 1B performed better, but only in MB cases (11/23 were negative) while 8/10 were negative in PB cases. The number of individuals reactive to the identified phage clones was not very different for PB, control and TB groups.

The seven phage clones showed low sensitivity for detection of leprosy patients. But among them, the clones 5A and 6A 1B, which were detected more patients. The remaining clones detected two, three or four patients. In this paper, as a criterion, we chose to select only phage clones that recognized a greater number of patients. The other identified phage clones will be studies and have not been discarded. The reactivity of the sequence can be changed when the same sequence is used as a synthetic peptide. As example we can cite the study made by Van Nieuwenhove LC et al (Identification of peptide Mimotopes of Trypanosoma brucei gambiense variant surface glycoproteins. Trop Dis PLoS Negl 2011, 5: e1189). Results obtained by our group, but not yet published, show this. The non-specific reaction of phage with controls serum samples was eliminated when peptide was used free, that is, not associated with phage. While the reactivity of the peptide with sera from patients with leprosy was not significantly different from that observed with the phage clone and serum sample.

The limiting factor in DTH analysis was the availability of M. leprae to sensitize animals. The results were inconclusive, but suggestive enough to advance the studies, involving not only DTH testing as well as other methodologies for assessing cellular response.

2) Though it is not clearly specified, it is construed that 1B was the peptide identified from the direct screening of phage library with pooled leprosy patient serum, while 5A came from the ‘phage library depleted of TB patient serum binding clones. 1B was found as a common sequence in all sequenced clones of strategy 2. Yet, unfortunately, 1B did not perform well in
DTH tests in guinea pigs. Only 1 in 5 animals was positive. Similarly only 2 out of 5 were positive for 5A. Cumulative reporting has elevated the DTH ‘positivity’ to 60%. The pool of peptides negated the effect of 5B or 1A (none of the 5 animals were DTH positive). Thus while there may be some level of specificity in the DTH tests, the sensitivity is low.

Our hypothesis in this study was use IgGs from MB patients to identify peptides and test these peptides when the ability to induce a cellular response. The peptides identified may only be B cell epitopes which determines a low sensitivity in DTH tests. Or these peptides may include T cell epitopes as well, which would meet our expectations. In addition to test other identified peptides and seek new peptides, our future goals include to evaluate dose and composition of the mixture used in the tests of DTH. The interest will increase sensitivity but also evaluate the specificity.

3) In general DTH is useful in predicting the ‘tuberculoid’ forms of leprosy, where in the cell immunity contributes to the reaction. What does the literature report for Fernandez tests in Brazil and other populations of leprosy patients? Are the multibacillary patients usually positive or negative? The authors do not adequately defend or describe the logic of whether *M. leprae* reactive IgGs in PB and MB patients will successfully lead to finding those antigens that will generate DTH responses, and whether this MB population will be benefited or detected by the DTH test (where a simple serology is suitable). Thus, while the experiment started with a design to screen for antigens (mimetopes) based on sero-reactivity of PB and MB patient serum to the *M. leprae* crude fractions, the final DTH test in guinea pigs may not necessarily be compatible with sero-reactivity.

In general, positive Fernandez reactions occur only in individuals who are also Mitsuda positive. In leprosy endemic countries, frequency of positive Fernandez responses increases with closeness of contact with leprosy patients. Tuberculoid leprosy patients are usually Fernandez positive and lepromatous patients are Fernandez negative.

Due to the wide spectrum of disease manifestations, serological analyzes and analyzes based on T cell response is required for the diagnosis of leprosy. Our strategy in this work was to use IgGs from patients to identify peptides and testing them when the ability to induce a cellular response through DTH tests. Results show evidence that peptides are capable of inducing cellular response. This brings us to continue investigating using the methodology described and its variants in an attempt to find other antigens for cellular assays.

The antigens identified (and new antigens that will come with continued studies) could be evaluated whether constitute T cell antigens and/or humoral response antigens.
Unpublished data show the possibility of using peptides for serological analysis, however, as to DTH testing, the sensitivity is low. Right now, other peptides are being identified by the group and may soon be evaluated in combination with those described in this work in order to increase the sensitivity in serological tests.

Through both serological and T cell tests will possible determine whether the peptide behaves better as component in one or another analysis.

4) The peptide sequences have not been shown (which may be OK), but some discussion on what the natural targets are (protein or non protein), and if these then can be related to previously described antigens or not could be added. Next, if these are known antigens, this may be useful to improve the design of peptides (sensitivity and specificity).

Data were included in the manuscript in order to inform the reader about the antigens in *M. leprae* which can be mimicked by peptides. Unpublished data already on the drive to one of the antigens of *M. leprae*, but how are data involving another aspect of our studies, we found more appropriate not to describe them in this work.