Author's response to reviews

Title: Immunologically reactive M. leprae antigens with relevance to diagnosis and vaccine development.

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Version: 4 Date: 9 December 2010

Author's response to reviews: see over
Reviewer 1's report:

This work examines the serological and cell mediated responses of leprosy patients at the two ends of the clinical spectrum to determine whether one can predict if any of 33 different M. leprae recombinant proteins will stimulate a response based on programs that identify potential T cell and B cell epitopes in these proteins. The study is comprehensive, well written, well executed and clearly described. Several minor points for consideration:

We thank the reviewer for these comments and understanding the extensive nature of these analyses. Point by point responses are inserted below.

Minor essential revisions:

1. The study that first identified ML0405, ML0331 and ML2055 as good antigens (Ref 28) used an expression cloning system to screen reactivity of pooled LL/BL patient sera to identify potential M. leprae reactive antigens. Three members of the Ag85 complex (ML0097, ML2028, and ML2655) were reactive, some very strongly by ELISA, so it was surprising that none of these (ML0098, ML2028, and ML2655) showed any reactivity in cell mediated or serological responses in this study. Is this due to batch to batch variation or folding issues with the recombinant proteins?

Although we have not stringently analyzed batch-to-batch variation across all proteins, we have observed similar responses (more extensively in serological analyses but also in WBA) with the proteins that have had various batches tested. We have not analyzed the effect of different expression systems that would allow different folding (e.g. glycosylation as might occur after recombinant expression in *M. smegmatis* but does not occur in *E. coli*). However we believe that publication of our positive (and even negative) data will permit other research groups to explore this possibility if they deem fit. We have added additional wording within lines 331-335 (page 12) to highlight this possibility - “Several other possibilities could explain the lack of reactivity. “Although we consider it unlikely given the recognition of positive responses to many proteins, the simplest explanation would be that recombinant expression in *E. coli* leads to significantly different folding and processing of proteins than occurs during native expression in *M. leprae*”. A more likely possibility is that these proteins, although present in the genome of *M. leprae*, may not be translated [16].

2. It was mentioned in the WBA methods section that whole M. leprae cell sonicate (MLCS) was used, assumingly for a positive control, but no results were
shown for this antigen (cell mediated or serological) or a T cell mitogen, such as PHA. Were such positive controls included in WBA experiments to ensure that each blood sample was capable of a good response, and if so what kinds of IFN-γ levels were elicited?

These control antigens were indeed included during the WBA evaluations and results are now shown in the revised manuscript as Supplementary figure 1.

3. The statement in the Abstract that “Relatively little is known about the immune responses to individual proteins of M. leprae recognized during leprosy.” is not exactly true based on a lot of earlier studies and more recent post-genomic studies cited in Refs 3, 4, 19-26, and 28.

We agree with the reviewer and have changed the text to read - “Until recently, relatively little has been known about the immune responses to individual proteins of M. leprae recognized during leprosy”. Abstract lines 34-35.

4. Table 2, ML1685 listed as “malato” should be malate; there is an extra “y” in immunogenicity in the far right column.

These typographical errors have been corrected.

Discretionary revisions:

5. Previous studies by this group examined either serological responses or T cell responses against M. leprae recombinant proteins, whereas this study links both, which is an improvement. In a previous study, 36% of the recombinant proteins (5/14) were recognized, while in this study, 27% (9/33) were recognized as T cell antigens, so the numbers are fairly consistent. It is disappointing that some of the antigens, particularly ML2346 which is unique to M. leprae, shows good responses in TT/BT and HHC, but lacks specificity. There must be regions within these proteins that elicit this cross-reactive or nonspecific response in the other groups. Is it possible to screen the 5 potential peptides that are predicted by PROPRED for ML2346 to see if one can clean up the response in the control groups? Ditto for some of the other proteins such as ML2358 (3 predicted T epitopes), ML0276 (5 epitopes), ML2541 (5 epitopes), ML2603 (5 epitopes), and
ML2380 (3 epitopes).

We thank the reviewer for this helpful comment. While peptides representing the predicted epitopes may unveil some degree of specificity, the counter-argument could be made that these peptides may actually represent the regions that are cross-reactive. At this time, given the positive and leprosy-specific nature of the responses we observed with several other full proteins, we are not considering further exploration of these cross-reactive proteins/peptides. The reviewer’s comment does, however, highlight an important reason for publication of our results, alerting other investigators to such possibilities and allowing them to pursue this line of investigation.

**Level of interest:** An article of importance in its field
Reviewer 2’s report:

General remarks:
The authors have investigated both HMI (IgG) and CMI (IFN-g) directed against M. leprae recombinant proteins that were selected based on their sequence specificity and epitope prediction pattern. The set-up of this study is straightforward and is performed adequately. The research groups have ample experience with recruitment and diagnosis of leprosy patients and in the production of purified recombinant M. leprae proteins. However, in view of the work already performed by these groups and others the study is not really innovative. In addition, the major conclusion of the manuscript is only slightly different from the work described by these groups previously, e.g. the authors describe data on several Ag including ML0405 and ML2311 that were described previously by these authors. A weak point of the study is that the authors have only tested individuals from one leprosy endemic region. Thus, the authors should restrict their conclusions regarding their proteins to the application of the Ag as vaccines or diagnostic tools for Brazil.

We agree to some extent with the reviewer that the work in not particularly innovative, but considering that the “antigen discovery phase” in leprosy is not over yet analyzing cellular and humoral immune responses to 33 proteins in parallel represents the largest study of defined antigens to date. We also agree with the reviewer’s inference that similar studies in other leprosy regions are merited, and believe that reporting of these data could provide impetus for such analyses. Although the reviewer’s request to restrict our conclusions and discussion points to Brazil is literally valid, until the time that such studies are conducted to refute our tentative claims and definitively demonstrate that these antigens are not of utility in other regions, we prefer to maintain the text. Point by point responses are inserted below.

Major Compulsory Revisions
• Line 38: since 45 individuals per group were tested for Ab and 20 individuals per group for IFN-g it is not clear whether the same individuals were tested for both groups or whether separate (previous) studies on T cell assays and serology were combined. If all 20 individuals tested for IFN-g were also included in the serology tests. The ms would benefit from an addition of such data per individual for both assays in order to strengthen the hypothesis that one individual is recognized by CMI and HMI and thus can be used for diagnosis of both TT and LL leprosy.
We have clarified this issue on page 5 lines 132-134 “The serology study included 45 participants per group and the T cell study included 20 participants per group and results from these two separate studies were combined in this manuscript.”

The serology and T cell studies were not performed at the same time as funding for these were not obtained simultaneously. So each study had a specific and distinct Informed Consent Form allowing either serology or T cell evaluation.

• The authors do not provide sufficient information on the social-economic background of the EC that are used in the study, which may be crucial to the specificity for M.leprae.

More information about EC group is provided in page 5, lines 127-129. “EC were healthy individuals with a BCG scar, recruited among non leprosy patients at a public health center from the same endemic setting. EC had neither tuberculosis nor history of leprosy in their families.”

Besides age, sex, address, previous history/exposure to leprosy and/or TB, no other information was collected due to ethical considerations since the Informed Consent they signed referred a blood collection and responses to a short questionnaire which was attached.

• Line 355: useful for diagnosis in Brazil, as only Brazilians were investigated in the authors’ study and responses in other endemic areas need yet to be studied for these Ag.

As mentioned above, although the reviewer’s request to restrict our conclusions and discussion points to Brazil is literally correct, until the time that such studies are conducted to refute the tentative claims and definitively demonstrate that these antigens are not of utility in other regions, we believe the sentence can be maintained.

• Line 262: the authors state that ML0840 is unique for M.leprae, this is, however, not the case as it contains 59% homology with Mycobacterium avium subspecies paratuberculosis (MAP).

We have corrected this sentence page 11, lines 284-286: “Only the ML2346 protein could be considered M. leprae unique, with no homologues found in the other mycobacteria species examined (Table 2)”.

Minor Essential Revisions
• Line 35: the authors themselves and several other groups have studied the IR
against M. leprae antigens even before the genome was sequenced so it is not
correct to state that: “relatively little is know… during leprosy.”

As also suggested by reviewer 1, we have amended the statement in the abstract to
read “Until recently, relatively little has been known about the immune responses to
individual proteins of M. leprae recognized during leprosy”.

• Line 37: high endemic: please provide the prevalence rate of the region of all
individuals that were included in the study.

Leprosy prevalence rate in Goias state (6.02/10000 habitants) has been added to
Materials and Methods section (page 5, lines 131-132).

• Line 46: 16 single proteins (Table 2 idem) vs. line 49-50: n=9 + n= 3 + 3; what is
the correct number meant? of these proteins.

We have clarified the sentence in the abstract (lines 48-52) to: “Among the 16
immunogenic proteins, 9 were considered leprosy specific in WBA inducing cell-
mediated IFNγ secretion from TT/BT patients. Three of these proteins were also leprosy
specific in serology being recognized by serum IgG from LL/BL patients. Seven of the
immunogenic proteins were not leprosy specific”.

Is this exactly similar to the social-economic background of the leprosy patients and
HHC?

As mentioned in Material and Methods (line 126-127), the HHC and patients lived in the
same dwellings as leprosy patients, so their socio-economic and other environmental
factors were essentially identical.

Methods/ materials

• Line 111: please provide the number of the ethical permission obtained locally.

The ethical approval numbers have been inserted (page 5 lines 111-112). “This study
was approved by local (Comitê de Ética em Pesquisa Humana e Animal do Hospital
das Clínicas da Universidade Federal de Goias) and national ethics commission
(Comissão Nacional de Ética Pesquisa/ CONEP/Brazil protocols #4862 and #12962).”

• Why was LID-1 (as previously described as a hybrid of these Ag) not used
instead of using both ML0405 and ML2311?

In this study we wanted to delineate responses to individual proteins, hence any
combinations of antigens within fusion constructs were not included.
• Line 125: how long after initiation of treatment were the TB recruited? In order for TB patients to be good controls their responses to PPD or Mtb Ag should also be shown in comparison to leprosy patients’ responses to the same Ag.

This information is already part of description of TB patients (page 5 lines 124-126) “TB patients were HIV-negative individuals with clinically confirmed pulmonary tuberculosis (M. tuberculosis sputum-positive) in their final 3 months of chemotherapy”. Neither PPD nor Mtb Ag was tested in this study.

• Please also show the medium values for all groups tested in a separate graph or supplemental data.

A table with medium values (WBA and Serology) for all groups tested was added as supplementary data (Supplementary Table 1).

• Line 166: please provide city, state of company.

(Molecular Devices, Orleans Drive, Sunnyvale, CA) is now listed in Methods.

• Line 193-194: M.leprae specific…proteins: sentence is not correct.

This sentence does not refer to genetic data comparing strains of mycobacteria, which would indicate that such proteins are not M. leprae-specific. Our definition was guided by the actual, observed immune responses based upon our data, so is factually correct.

• Line 272-283: The method can identify and predict HLA binding regions from antigen sequence. So it is not that surprising that it is not predictive for T cell epitopes as it does not predict interactions with the TCR. Instead the authors could use SYFPEITHI which also includes probability of being processed in addition to presentation in the context of a certain HLA allele. However, it remains a prediction and the authors’ conclusion is justified in that it does not imply a prediction of T cell epitopes if one uses a program that applies HLA-peptide binding motifs only.

We have added a comment about this limitation of the insilico predictions used: “We are aware that the softwares employed identify and predict HLA binding regions from antigen sequence without predicting interactions with the T cell receptor (TCR). Therefore the use of other in silico prediction softwares that also include the probability of the antigen being processed, presented in the context of a certain HLA allele and recognized by TCR could lead to different conclusions”. (Page 13, lines 340-345).
• Table 1: please provide also PGL-I data.

PGL-I data was added to Table 1.

• Table 2: the authors should include sequence comparisons of more sequenced (myco)bacteria like M. smegmatis, M. microti and M. paratuberculosis.

In order to keep the information inserted within the table at a manageable level, we have maintained Table 2 with mycobacteria species that cause disease in humans. We have inserted an additional supplementary table showing sequence comparisons with M. smegmatis, M. microti and M. paratuberculosis (Supplementary table 2).

• Line 321: cross-reactivity and lack of specificity indicate similar observations, please leave one out.

We respectfully disagree with the reviewer’s comment, believing that a lack of specificity is demonstrated by responses within all groups (including EC) whereas cross-reactivity is demonstrated by responses within groups with the exception of EC.

Discretionary Revisions
• Line 155: cut off of 50pg/ for positive responses in a WBA that also has a detection limit of 20pg/ml is rather low.

Although 50pg/ml is a relatively low cytokine concentration, we do not consider the cut-off as low because, as acknowledged by the reviewer, this concentration is clearly above the threshold (and within the doubling-dilution of the standard curve, also above the second lowest concentration within the standard curve).

Level of interest: An article whose findings are important to those with closely related research interests