Author's response to reviews

Title: Cytokine profile, proliferation and phosphorylation of ERK1/2 and Akt in circulating mononuclear cells from individuals during the chronic intestinal phase of Schistosomiasis mansoni infection

Authors:

Roberta O Prado (robertaprado@cpqrr.fiocruz.br)
Iramaya R Caldas (iramayacaldas@fiocruz.br)
Andréa T Carvalho (andreat@cpqrr.fiocruz.br)
Marcus V Andrade (andradem@medicina.ufmg.br)
Rafaelle CG Fares (rafaelle@cpqrr.fiocruz.br)
Lais M Portugal (laismaroni@cpqrr.fiocruz.br)
Andréa Gazzinelli (andreag@enf.ufmg.br)
Rodrigo C Oliveira (correa@cpqrr.fiocruz.br)
José Renan C Melo (jrcmelo@medicina.ufmg.br)

Version: 2 Date: 28 July 2012

Author's response to reviews: see over
Belo Horizonte, July 29, 2012

To
Prof Janette Bradley
Editor
BMC Infectious Diseases

Thank you for your e-mail with the Reviewers comments regarding our manuscript, MS: 1149125478716652, entitled “Cytokine profile, proliferation and phosphorylation of ERK1/2 and Akt in circulating mononuclear cells from individuals during the chronic intestinal phase of Schistosomiasis mansoni infection” by Oliveira-Prado and colleagues submitted to the BMC Infectious Diseases. We have worked thoroughly to answer all queries raised by the Reviewers, in order to improve the quality of our manuscript and make it suitable to be resubmitted to the BMC Infectious Diseases.

The changes were clearly outlined in the revised manuscript, marked in yellow to make it easier for the Reviewers/Editor. Below we present, in bold, your queries and our answers in regular type.

We thank the BMC reviewers for their careful attention in revising this article and we do hope that it is now suitable for publication in the BMC Infectious Diseases.

Looking forward to hearing from you soon,

Yours sincerely,

José Renan Cunha-Melo
Full Professor
Department of Surgery, School of Medicine
Federal University of Minas Gerais
REVIEWER’S COMMENTS

Reviewer #1: Fernanda de Araujo

General Comments:
The authors evaluated the effects of *Schistosoma mansoni* antigens on cellular proliferation, cytokine production and ERK1/2 and Akt phosphorylation in PBMC from infected individuals from endemic area and control groups. The main result obtained indicates that CD4+ and CD8+ T cells from infected patients (XTO) have lower proliferation than BD group after SEA stimulation. SWAP antigens induced ERK1/2 phosphorylation but not SEA in XTO group and the culture supernatants showed a mix profile of cytokines production type 1 and type 2 after SEA and SWAP stimulation. The proposal is relevant in *S. mansoni* infection and the authors follow an important research line.

Abstract
Background: The sentence “To study the effects...” do not have a final conclusion. What is the objective? The sentence must start as: The aim of this project is to study.....

We acknowledge reviewer for this comment. The sentence was changed and corrected by American Journal Experts (AJE) (line 6).

Conclusions: CD4+ PBMC? I assume that the authors meant: CD4+ T cells from PBMC ...

We acknowledge reviewer for this comment. The sentence was changed.

The new sentence: “The data indicate that SEA-stimulated CD4+ T cells from infected patients have a lower proliferation rate than the same cells from the NI group” (line 20).

Results Section
First paragraph: The authors add in the results section figure 1 and do not discuss about that. They add the results in one sentence add the figure and do not justify the results during the discussion section. The discussion is necessary mainly because previous paper from the group showed differences in the Lymphocyte phenotyping. The previous work shows that “the HLA-DR expression on CD8+ was higher in PBMC from infected and non-infected individuals than from healthy donors”. The authors must explain better the difference between the studies and find some support in the literature about that.
The figure 1 was discussed. In the previous work the assay was *ex vivo* and in this study we evaluated the activation markers expression after SEA and SWAP stimulation. For this reason the results are different.

**Discussion Section**

The authors should add the figures that they are referring next to the text. We acknowledge reviewer for this comment. The figures number was referred in text.

Third paragraph: “fitting a typically Th0 profile.” The production of cytokine in the present work was measured by CBA using supernatant of cultures. So, the authors should be careful when they said Th response. If the experiment have been done by intracitoplasmatic staining by flow cytomter (where do you separate the CD4 population) they could have said that, but the supernatant from culture includes all PBMC so it is hard to admit Th profile. It will be more reasonable said profile type 1 or 2. We acknowledge reviewer for this comment and we agree that is more reasonable said profile type 1 or 2.

The sentence “seems to fit a Th0 variation profile. What the authors meant by that? The sentence needs to be better explained. All this part of the text must be more explored. The authors did not discuss about the meaning of having a mixture profile of cytokines during the chronic intestinal phase of *S. mansoni* infection. The authors must improve this section and find support in the literature to explain the importance of this pattern of cytokines for the evolution and pathology of the disease. We acknowledge reviewer for this comment. The sentence “… fit a Th0 variation profile” was replaced by a better description of cytokines profile. All section “Cytokine response to *S. mansoni* antigens” in Discussion was rewritten and this and the below comments was better explained.

Fourth paragraph: I agree with the authors that IL-10 secretion was higher in SEA and SWAP from XTO cultures when compared with BD and that this cytokine can have a inhibitor role, but the text needs to be clarify, because the authors said that IL-10 appears to be blocking some cytokines such as IFN-#, IL-2 and TNF-a. However, when we go to the figure 3 the results showed that IFN-γ is maintained after SWAP stimulation among the groups. IL-2 and TNF-a is increased after SWAP stimulation among the groups. So, I suggest that the
authors should clarify better since this inhibition seems to be only after SEA stimulation when comparing XTO and BD. We thank the reviewer for the comment and these questions were clarified (page 10).

Fifth paragraph: The authors suggest that CD8+T cells might be associated with increased synthesis of IL-4, IL-5 and IL-10, but again the experiment have been done in supernatant of cultures, not intracitoplasmatic staining. The authors should be careful when doing this association. How to explain the increase of TNF-a and IL-2 after SWAP stimulation? The reviewer comment was very appropriate and all section “Cytokine response to S. mansoni antigens” in Discussion was rewritten and these comments were better explained (page 12 and 13).

Ninth paragraph: The authors during this section said that 120 hours time might have influenced the lack of results. Why the authors used this time must be better clarified in the text. Also the authors should be concern about the limitation of the results due to the number of patients per group in the phosphorylation assay. The culture time was standardized at 120 hours of culture. This is the necessary time to observe the antigens effect in PBMC (page 14). The phosphorylation assay we use is a sensitive and specific method, so we believed that the number would be enough to get results.

Minor Essential Revisions
Background Section
Second paragraph: “….in infected patients (needs dot and also the references here). A typical PBMC…” The references for that sentence are 2 and 3.

Fifth paragraph: The authors discuss about MAPK and during the objective said ERK1/2. They must use the same name and also explain better what is ½. We acknowledge reviewer for this comment. The fifth paragraph was rewritten and we give the correct description of MAPK and ERK1/2 (page 4).

Results Section
Second paragraph: Figure 2B should be 2B and F. Figure 2D and 2F should be Figure 2B,D and F.
We thank the reviewer for the comment and the figure identification was changed (page 5).

Discussion Section

First Paragraph: The authors throughout the text said reactivity of CD8 or CD4. However they measured the proliferation and they did not show functional experiments to show the reactivity and also the activation markers are the same between the groups, so it is better instead said reactivity use proliferation.
We acknowledge reviewer for this comment and we replace the word “reactivity” for “proliferation” in the text.

Figure Legends: All the figure legends must contain the number of patients used in the experiment and the groups. Example: BD = blood donors volunteers; XTO
The reviewer comment was very appropriate and we add the number of patients in each group in the figure legends.

Methods Section

First paragraph: The authors should standardize the text. Or the number should be written or add as numbers.
We thank the reviewer for the comment and the text was standardized.

Eighth paragraph: the way that the authors describe the methodology is not well understood. For example the sentence: “Simultaneous quantification of six cytokines in a single sample using microparticle-based flow cytometry technology.”
We thank the reviewer for the comment and the sentence was rewritten. The new sentence: “The assay was performed by a flow cytometry application that allows us to quantify multiple cytokines simultaneously” (page 19).

The paper needs improvement in English writing and usage, to improve understanding.
The quality of written English was improved and edited by American Journal Experts (AJE).

Reviewer # 2: Quentin Q Bickle
General Comments:
This is a well conducted study which contains a significant body of work. The main original component is measurement of phosphorylation of ERK1/2 and Akt in PBMC from *S. mansoni* infected donors, non-infected endemic area donors and naive humans in response to recall stimulation with SWAP and SEA. This showed essentially no difference between the groups. The remaining studies represent valuable information necessary to provide the context of phosphorylation experiment but these observations are not novel. Nevertheless, the work is solid and worthy of publication. However, there are a few issues which the authors should address before the manuscript is considered further.

The script has not been carefully read. The English needs attention in terms of meaning, spelling and grammar. Below are a few examples from the Abstract but there are many other in the manuscript.
line 2 “...host liver and is modulated...”
line 5 “…response than in acute infection....”
Lines 5-8: “To study..... endemic area”. This is not a sentence, it doesn’t have a verb.
Line 10-13: I suggest: “When compared with the BD group, CD4+ T lymphocytes (remove the “s”) proliferation was lower in the XTO group but not in the NI group in both unstimulated and antigen (SWAP and SEA) stimulated cultures.”. NOTE: there are many places in the manuscript where the definite article “the” is omitted. This should be checked and corrected. “Individuals” could be used sometimes in place of “group” to avoid over use of “group”.

We thank the reviewer for the comment. The quality of written English was improved and edited by American Journal Experts (AJE).

Background:
Lines 1-4. The references given are for the mouse work on modulation of granuloma size and so the, admittedly scanty, evidence for changes to granuloma size in chronic human disease should be cited and described. Thank you for the comment and a new reference were included (reference number 5).

Line 5 (and other places). *Schistosoma* is a genus name and should be in italics.
We thank the reviewer for the comment and the mistakes were corrected.

Line 13 – references cited – in fact ref 2 was perhaps the first to demonstrate this
and so should be included here.
We acknowledge reviewer for this comment. The reference number 2 was included.

Line 16: “.... lower anti-SEA ... response relative to acute patients... “. Line 15-18: This statement is not entirely correct since in ref 9 responses to both SEA and SWAP were lower in the chronic patients.
We thank the reviewer for the comment and the references 9 and 10 were excluded in that paragraph. A typical PBMC response in patients during the chronic intestinal stage is characterized by lower anti-SEA responsiveness in contrast to higher anti-SWAP. This affirmation is truth and refers to the proliferation assayed by tritiated thymidine incorporation (3H-thymidine). On other hand the author number 9 for example, showed that the reduced PBMC proliferative response to SEA and SWAP antigens in acute, chronic intestinal and hepatosplenic patients was influenced by the blockage of IL-4 and IL-5. Therefore, the number 9 author did not assess simply proliferative response in the presence of antigens but the proliferation in response to blocking of cytokines.

Page 4, Lines 14-16 “The two signal.....”. A reference for this statement would be helpful and it seems more logical to me for this sentence to be moved earlier in the Background section– to before Page 3 line 25 i.e. before “One of the phenomena......”
We acknowledge reviewer for this comment. However to be more didactic, we would like to convey the idea of the signal 1 seems to be more involved in the MAPK phosphorylation as signal 2 seems to be involved with PI-3 kinase phosphorylation.

Page 4, Lines 21-22: “Such as....” this sentence does not make sense.
We thank the reviewer for the comment. The sentence was rewritten (page 5, line 4).

Methods:
Page12: It would be helpful to report the numbers of infected people found in these two communities so that the reader has an idea of the prevalence of infection. Also state the numbers of XTO and NI individuals selected from these two areas. The origin of the BDs is not clear. The Abstract states “... individuals with no prior parasite contact”. If they were simply from an area not endemic for schisto this should be stated. If they are not is should be explained how they were deemed not to have had exposure.
The reviewer comment was very appropriate. The schistosomiasis prevalence was 26% in Virgem das Graças. In São Pedro Jequitinhonha the schistosomiasis
prevalence was 47%. The BD group consisted of individuals who were born and live in the capital of Minas Gerais (urban area) and who reported not having schistosomiasis and with age ranging from 18 to 50 years old. These individuals were volunteers and their feces were not analyzed for the presence of S. mansoni and other parasites (page 16, line 7).

Page 13: lines 2-3: the organs used for recovery of eggs should be stated.
The eggs were collected from the livers of a laboratory population of out-bred Swiss mice infected with the LE strain of S. mansoni (page 17).

Page 13: Particularly ign view of the relatively high production of IFN-g in the BD with SEA and SWAP compared with medium alone (Figure 3c) it would be helpful if endotoxin levels had been established for these antigens and found to be acceptably low for use in these cultures. Also the authors should comment in the Discussion on the possible reasons for this IFN production to SWAP and SEA in the BD group.
The reviewer comment was very appropriate and all section “Cytokine response to S. mansoni antigens” in Discussion was rewritten and this comment was better explained (page 10, third paragraph).

Results:
Lymphocyte phenotyping: Although the gating is described briefly in the methods, it would be helpful to show an example of the FACS plots for this data to illustrate the gating. Can the authors comment on whether MFI showed any differences? We thank the reviewer for the comment. A representative figure to lymphocyte gating was included in Figure 1. The analysis by MFI was not performed and we prefer to show only the results in pg/mL.

Although the lack of proliferation is consistent with earlier work (Figure 2) were mitogen controls included in the study just to confirm the viability of the different cell populations? If so some reference to this would be helpful. The mitogen controls (Phytohaemagglutinin - PHA) was performed and were represented in Figure 2G and 2H to CD4+ and CD8+ cells respectively.
Figures: The use of asterisks and letters to indicate P values is cumbersome. The authors should consider using lines between groups on the figures themselves.
We thank the reviewer for the comment. The figure 3 was changed. The differences between stimuli were showed by lines. Only the differences between groups were represented by asterisks.

The only significance level reported was <0.05. Lower P values should be reported if observed.
The kind of statistical analysis provides only if the result is significant (p <0.05) or not.

Discussion:
Page 8 Lines 14-16. Can this be said to be a Th0 profile when the IFN response was not reported as significantly increased and furthermore the increase in IFN production with SWAP in BD was even greater?
The reviewer comment was very appropriate and all section “Cytokine response to S. mansoni antigens” in Discussion was rewritten and this comment was better explained (page 10).

We thank the BMC reviewers for their attention in revising this article.
Looking forward to hearing from you soon,
Yours sincerely,
José Renan Cunha-Melo
Full Professor
Department of Surgery, School of Medicine
Federal University of Minas Gerais