Author’s response to reviews

Title: Trafficking of phagocytic peritoneal cells in hypoinsulinemic-hyperglycemic mice with systemic candidiasis.

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Author’s response to reviews: see over
Trafficking of phagocytic peritoneal cells in hypoinsulinemic-hyperglycemic mice during systemic candidiasis.

Reviewer: F M Muller

Major Compulsory Revisions

1a. The authors should explain more in detail why they have addressed two hypotheses in one experiment with a limited number of mice in each group.

Authors. In general, our research focus has as target the control of the infection in diabetes mellitus. Considering the idea that the peritoneal cavity could be an interesting option for this approach, the present study brings to a point the ability of the HH condition to affect (or not) the migration of stimulated peritoneal phagocytes using the Candida infection as a tool, i.e. stimulating the recruitment of peritoneal phagocytes to the infected-organs. As to the number of mice used, it is important to highlight that in each experimental moment 18 animals have been evaluated: six of the HHCa group, six of the Ca group and six of the HH group. The number of 18 mice per experimental moment is in accordance with the ethical principles for animal research and it is accepted by several ethical committees. Furthermore, numerous studies have used this number of mice [1,2] or even less [3,4,5].

1b. What is the rationale why they did use hypoinsulinemic-hyperglycemic mice?

Authors. The choice of alloxan-induced HH mice has two rationales. The first one is due to diabetes represent an important import underlying disease in episodes of candidemia (13-21%) [6-8], and the second is because this condition interferes in the animal defense mechanisms, therefore making possible the study of the immunological alterations involved in the infections which undertake the diabetic patients. In addiction, the alloxan model used in the first hours after alloxan inoculation mimics only the hypoinsulinemia-hyperglicemia (HH) condition of diabetes, i.e., with no chronic disturbs. As consequence, the data interpretation could be more specific to this abnormality. According to the reviewer’s suggestion, the information was added to the manuscript (page 9, line 24).
1c. How this condition may potentially affect the spread of migratory peritoneal phagocytes during candidemia/invasive candidiasis?

Authors. Several studies have shown that macrophages in a microenvironment rich in glucose exhibit diverse functional alteration, such as increased oxidative stress and enhanced transcription genes encoding for cytokines, growth factors and adhesive molecules [9]. These alterations have been triggered by increased formation of the advanced glycation end products (AGEs) [10]. Considering that the hyperglycemia affects the metabolism of these cells, we have come to the idea that other cellular alterations could be occurring and that they could be affecting the migration of these cells. According to the reviewer´s suggestion, the information was added to the manuscript (page 10, line 11).

2a. Please discuss more in detail the rationale for the adoptive transfer assay.

Authors. The adoptive cell transfer assay was performed as a retest to confirm the data obtained from the direct inoculation of PKH-26 dye into the peritoneal cavity. This method has shown that the labeled cells in the tissue were indeed originated from the peritoneal cavity and not from the escaping of the dye into circulation. As Swiss mice are outbred strain, we have employed inbred mice (C57BL/6) to perform the experiments as cell donor and recipient mice. According to the reviewer´s suggestion, this aspect was added to the manuscript as following (page 6, line 24):

“In order to confirm the peritoneal cells migration, we have used the adoptive cell transfer assay. As the Swiss mice are outbred strain, we have employed C57BL/6 inbred mice to perform the experiments as cell donor and recipient mice”.

2b. Discuss potential other methods.

Authors. The analyses of peritoneal cell migration certainly are not limited to these two methods. The PKH is an extensively used supply for cell tracking protocols [11-13] that could be used associated to other methodologies such as flow cytometry and two-photon excitation microscopy. The use of transgenic mouse line with fluorescent protein-labeled is another sophisticated methodology [14]. In the present study, we have employed a simple methodology that is routinely used for screening in others situations, as in the evaluation of sentinel lymph nodes for lobular carcinoma of the breast cancer [15]. Here, we have used
these methods for identifying migrant cells in different tissues. Once these cells are identified, other methods could be employed for detailed studies. According to the reviewer’s suggestion, this aspect was added to the manuscript (page 10, line 19).

3. Mice were euthanized 1, 3 or 7 days after the fungal inoculum. The authors may discuss, whether the sample size in each group is sufficient for statistical analysis, and whether the results of a single mice was affecting the mean.

Authors. As showed in the first question, each experimental moment was performed using 18 animals, six of the HHCa group, six of the Ca group and six of the HH group. Thus, the study as a whole, have employed 60 mice. In the revised manuscript we have remade the statistical analysis in order to fit the sample according to the normality curve using Shapiro-Wilk test (page 8, line 11). As our data had showed a normal distribution, we performed a parametric test. Being a parametric test, points outside the normal ranges do not interfere with results. Thus, the results of a single animal do not affect the mean. It is important to highlight that, after the parametric analyses, the results have not changed.

4a. In the result section the authors report that the HH condition leads to an increase of fungal load on day 7 in the liver. However, why was the fungal load not elevated in the other organs.

Authors. The recovery of high viable fungi in the liver in the HHCa mice is not unique; Mosci et al. [16] observed similar results. Also in human disease, the Candida displayed a tropism for liver, particularly in the hepatosplenic clinical form, which affects mainly patients with neutropenia, a common feature in the hematopoietic malignances [17]. However, in diabetes mellitus this situation is not usual. Although the mechanisms involved in this phenomenon are not clear, the presence of substances with tropism for this organ, such as uricase, could be considered [18]. As the literature has showed no consistent data regarding this aspect, we have carefully chosen to only show our findings instead of making an over interpretation. In addition, we believe that our results could incite other researchers to address this issue.

4b. Was the fungal load elevated in the liver of all individual mice, please clarify.
Authors. Yes, it was. As shown in the figure 1, there was an increase in the individual fungal burden in the liver of HHCa mice.

Figure 1. Determination of fungal load in the liver in the Ca and HH-Ca groups at 7 days p.i.. The results are expressed as CFU (log_{10}) per gram of tissue (t test; p < 0.05).

<table>
<thead>
<tr>
<th>Animal/Group</th>
<th>Ca (n=6)</th>
<th>HHCa (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal 1</td>
<td>1,610</td>
<td>1,730</td>
</tr>
<tr>
<td>Animal 2</td>
<td>0,000</td>
<td>2,050</td>
</tr>
<tr>
<td>Animal 3</td>
<td>1,600</td>
<td>2,850</td>
</tr>
<tr>
<td>Animal 4</td>
<td>0,000</td>
<td>2,180</td>
</tr>
<tr>
<td>Animal 5</td>
<td>0,860</td>
<td>1,950</td>
</tr>
<tr>
<td>Animal 6</td>
<td>0,000</td>
<td>-</td>
</tr>
</tbody>
</table>

Minor Essential Revisions

1. Title: please change title, abstract, and in the body text from candidemia to invasive candidiasis as the infection was spread to different organs.

Authors. According to the reviewer´s note, we have changed “candidemia” to “systemic candidiasis”

2. Abstract: The conclusion indicates what was already stated in the background section "experimental studies have showed that the candidemia could be controlled by activated peritoneal macrophages". Instead the authors may stress in the abstract and in particular in the conclusion the interesting observation that peritoneal phagocytes migrate to tissues infected by fungi.

Authors. According to the reviewer´s note, the conclusion in the abstract has been re-edited (page 2, line 23): “In the present study we have observed that peritoneal phagocytes migrate to tissues infected by C. albicans and the HH condition did not interfere in this process”.

6. Please clarify the C. albicans suspension was inoculated in which vein (lateral tail vein?) at what time-point after HH induction?
Authors. According to the reviewer’s suggestion, this aspect has been included in the manuscript (page 6, line 10):

“The C. albicans suspension has been inoculated into the lateral tail vein”

7. In HH-Ca and Ca groups, PKH+ cells were also found both in the lung and brain. Please discuss the limitations by the number of mice studies.

Authors. As showed in the first question, in each experimental moment 18 animals have been evaluated. The numbers of mice per experimental moment is in accordance with the ethical principles for animal research and it is accepted by several ethical committees and several studies have used this number of mice [1,2] or even less [3,4,5].

8. Please delete the following sentence: "similar studies are being conducted in our laboratory and are subject of future publications"

Authors: This sentence has been deleted.


Authors. By “imprinting toll”, we mean “imprinting technique” - used as methodology to identify the fresh stained cells. We have changed “imprint toll” for “imprinting technique”.

10. last sentence - please stress clinical impact of the findings and future study Tasks.

Authors. As our findings have confirmed the migration of peritoneal cells to infected tissues and that the HH condition does not interfere in this phenomenon, it is our purpose now to use the peritoneal route for investigating new therapeutic strategies to control the infections which affect diabetic patients. Immunotherapy from peritoneal cavity is considered a viable and promising alternative. This route has been used to immunize mice with ex vivo-treated dendritic cells for prostate cancer intervention [19]. Experimentally, Concanavalin-A has been used to stimulate the peritoneal cell [20]; they have observed increased survival rate during lethal C. albicans hematogenous infection. Our group has addressed this option for immunotherapy employing crude Brazilian native herbs.


