Author's response to reviews

Title: Modulation of macrophage cytokine profile during evolution of solid tumors: susceptibly to Candida albicans infection

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Responses to reviewers

Reviewer 1

1. Both the writing style and proof-reading needs significant improvement. It is very hard to read and understand the manuscript. Both the table and figure legends need a lot of improvement.

Authors. Respecting the reviewer´s suggestion, the manuscript was improved and reviewed by the BioMedES English editing service.

2. In the figure legend the authors should mention the number of mice that had been used and/or how many times an (replicate) experiment has been performed. Without this information it is difficult for a reviewer to assess the statistical information provided in the paper.

Authors. The experiments were performed in triplicate. For each experiment, four animals per group were used. In accordance with the reviewer´s suggestion, this information has been added to the figure legends.

3. The data shown in Table 1 is not clearly presented. The authors should provide statistical details (mean+/SD) and significance level. Since this is a critical data for the manuscript, both the style of presentation and the quality of data should be improved.

Authors. The style of presentation of Table 1 has been modified as per the reviewer´s suggestion. The mean±SD and significance level are not included since the data shown refer to the frequency of Candida-positive mice.

4. In Fig. 1, why no significant increase in production of TNF alpha is observed in CTL cultures following LPS stimulation. Macrophages are known to produce TNF upon stimulation with LPS and therefore, this should be a positive control for the assay.
Authors: In fact, the macrophages from our CTL mice, when provoked with LPS, did release significantly more than the non-stimulated macrophages. {naive animals: supernatant of culture with no stimulus (46.57 +/- 11.06); with LPS (111.13 +/- 14.36); p = 0.0456 (paired t test)} As this was done in the usual way, it was used as a positive control for the assay. Unfortunately, because of a review flaw, the manuscript was sent out without this information and certainly generated this doubt. Thus, to make the matter clear, we have included this information plainly in the revised manuscript text {“The macrophages were cultured at 37°C, 5% CO2, in RPMI-1640 with or without 10 µg/ml LPS (Sigma) as an internal control of macrophage activity (data not shown).”} and we have left it out of the figures.

5. How does spontaneous cytokine production (particularly TNF) following tumor development compare with non tumor bearing mice? Please also provide the statistical significance. This is important since from Fig. 1 it appears that spontaneous TNF production is higher in macrophages from tumor bearing mice (7, 14 and 21 days) and as yet candida clearance is impaired on 14 and 21 day tumor bearing mice.

Authors: Respecting the reviewer´s suggestion, we have improved the figures; as observed in figure 1, tumor-bearing animals show an initial increase of TNF production but with the tumor progression, this production decreases. We observed a similar picture with IFN-y production. These results associated with the high levels of IL-10, as seen in this period, lead to impaired Candida clearance.

6. It is unclear why spontaneous production of TNF is reduced at 24 h pi in day 14 tumor bearing mice and yet the macrophages (24 h pi in day 14) respond very well (comparable to 24h day7) in response to LPS.

Authors: The spontaneous production of TNF mentioned by the referee was obtained with macrophages stimulated by C. albicans inoculated into the peritoneal cavity, i.e. stimulated by fungus; in this way, provocation by LPS (in vitro) constituted a second stimulus [1]. Thus, the response to LPS did not necessarily have to be the same as that observed in spontaneous culture. However, this reduced response may be specific to the fungus, since the literature indicates that Candida shows this property [2]. It is also important to remember that the data obtained during tumor progression (dissemination and cytokine profile) indicate the 14-day stage, a critical moment in the host´s response to tumor progression - a moment of transition of the immune response. Regarding the cytokines, we verified a mixed profile –M1 and M2 to the Candida and LPS stimuli. In the manuscript now reads: “Thus, either they exhibited a functionally mixed population or adopted a rather promiscuous activation state, which could however be ideally suited for diminishing tumor immunosurveillance”. In any case, LPS was used as an internal control, and future studies involving these receptors are being developed in our laboratories.

7. Candida infection is mostly associated with neutropenia. The authors should speculate in the Discussion section of the manuscript about whether their
findings (cytokine profile) on neutrophil function/number.

Authors: The present study was designed to acquire further evidence about the functional activity of macrophages outside the focus of tumor development during the progression of neoplastic tissue. Our objective was to elucidate the behavior of macrophages distant from the tumoral focus. To include discussions about the role of neutrophils in this context, although interesting, would be complex. More specific assays that could associate the data obtained in the present study with neutrophils would be required. Nevertheless, the point mentioned by the referee is relevant and it is being studied in our laboratories, concomitantly with the present study.

Reviewer 2

1. The authors have investigated the effect of tumour growth on the course of candidal infection. They describe changes in cytokine profiles. Although the research question is well defined, it should be realised that neoplastic disease per se clinically does not predispose to systemic fungal infection, rather does the use of chemotherapeutic agents, glucocorticosteroid treatment and indwelling catheters with parenteral nutrition.

Authors: Several studies show a direct causal relationship between the presence of a tumor and immune dysfunction [review in 3]. It has been shown, for example, that tumor cell-derived factors such as interleukin (IL)-10 and transforming growth factor (TGF) beta induce both local and systemic immunosuppression. It has been suggested that these cytokines selectively alter the expression of pro-inflammatory cytokines and other potential mediators of immunosuppression by macrophages. Thus, tumors may subvert macrophage function and consequently suppress T cell function.

2. The investigations only show associations and no mechanisms. There are no experiments to explain the changes in cytokine patterns; neither is clear whether these changes have consequences for host defence against Candida albicans.

Authors: The objective of this article was to investigate whether those associations, detected in human conditions, are also present in the experimental model used. Since this work was completed, we have formulated hypotheses that are currently being investigated in our laboratories. We considered that studies of this nature leave possibilities open to other researchers who may also be interested in the theme. The consequences of tumor-induced alterations on C. albicans infection are described in the section “Fungal colonization and dissemination”. Also, we used control groups to explain the changes in cytokine patterns as consequences for host defence against C. albicans.

3: As is clear from table 1, the experiments were performed with groups of 4 mice. There is no mention of numbers of mice for the other experiments. I am worried that the results presented represent only one big experiment. Given the inherent variation in this kind of experiments, this would not be acceptable. To establish whether the changes in Candida outgrowth over time (and the cytokine
patterns) as presented are solid and reproducible, one would need triplicate experiments.

Authors: Certainly all experiments were performed in triplicate.

4. To try and explain the reasons for outgrowth of Candida, I would like to see data on influx of granulocytes (the key players in these acute candidal infections) and monocyte/exsudate macrophages. In addition, phagocyte function would be an important parameter.

Authors: As the target cells in the present study were macrophages, the assay protocol did not lend itself to the kind of analysis required. However, we consider this question relevant, so we are performing another study aimed mainly at this cell population. Similarly, we also consider phagocyte function an important parameter and we are performing appropriate studies. In vitro assays, we verified that, although the presence of the tumor does not affect the opsonin-dependent phagocytic activity of macrophages, it decreases their opsonin-independent phagocytic activity, particularly at 14 and 21 days of neoplastic progression. This alteration, however, did not interfere with fungus internalization in vivo, i.e. the phagocytic index of tumor-bearing animals inoculated with Candida was similar to that of tumor-free animals. On the other hand, macrophages could not eliminate the fungus, indicating the possible participation of opsonins in this process. Investigating the death of C. albicans, opsonized and non-opsonized by peritoneal macrophages, other researchers have verified that 30-60% yeasts are killed; opsonization increases this percentage to around 88% [4,5,6]. This phenomenon has also been observed in bacteria; Gordon et al. [7], using S. epidermidis, verified that, although not crucial for phagocytosis of these bacteria, opsonization was needed to generate a maximal oxidative burst and consequently kill the pathogen. Similarly, in candidiasis, ingestion of the fungus via opsonins probably makes it unable to evade intracellular death mechanisms. We are now continuing the investigation by evaluating the receptors involved in this process.

5. The discussion is much too long and speculative.

Authors: We have condensed the discussion, having accepted the referee´s suggestion.

Minor essential revisions

1. The table and the figures should be more self explanatory. It took me quite a while to understand what was presented. The legends are hard to digest, and the abbreviations are not very helpful.

Authors: Table 1 has been modified according to the reviewer´s suggestion.

2. The language needs correction.

Authors: Respecting the reviewer´s suggestion, the manuscript has been improved and reviewed by the BioMedES English editing service.

Discretionary revisions
1. As has been demonstrated in the literature, IL-18 is a key inducer of IFN gamma in candidiasis (see Netea’s work). This cytokine might also be more important than IL-12 in the experimental setting.

Authors: Respecting the reviewer’s suggestion, we are going to include IL-18 in our future studies.

References


