ANSWRING REVIEWERS-29780

COMMENTS TO AUTHORS - Reviewer's code: 02618027

In the manuscript entitled "B-1 cells modulate the innate response of murine macrophages to Leishmania major infection" by Arcanjo AF and Nanes MP et al., the authors investigate the immunomodulatory effect of B-1 cells in L. major infected macrophages. Results suggest that PGE-2 and IL-10 released from infected B-1 cells increase intracellular parasite replication. The manuscript is generally well-written; however, the text should be carefully checked for spelling and other minor errors throughout. Although the findings are interesting, the following concerns should be addressed prior to publication:

Major Comments:

1) What is it about B-1 cells that triggers IL-10 productin?

It is well described in the literature that B-1 cells produce IL-10 and use this cytokine as an autocrine growth factor [O'Garra A, Chang R, Go N, Hastings R, Haughton G, Howard M. Ly-1 B (B-1) cells are the main source of B cell-derived interleukin 10. Eur J Immunol. 1992;22(3):711–7. pmid:1547817]. Also this cytokine is an important regulator of macrophages [Moore KW, O'Garra A, de Waal Malefyt R, Vieira P, Mosmann TR. Interleukin-10. Annu Rev Immunol. 1993;11:165–90. pmid:8386517]

2) What is it about B-1 cells that favors lipid production?

Lipid bodies are organelles consisting cellular sites devoted to the synthesis of PGE_2 with a core of rich neutral lipids and wrapped with a monolayer of phospholipids, cholesterol, lipids, which contain a variety of proteins associated with different functions in cell metabolism, signaling, and inflammation. Several groups have reported that host cells have their lipid metabolism altered and increase their number of lipid bodies following infection with intracellular pathogens. Furthermore, our group has shown an increase of lipid bodies in macrophages infected

with *Trypanosoma cruzi*, and their role in disease development [Freire-de-Lima et al, 2000 Nature].

3) It is interesting that the B-1 CDP cells produce anti-inflammatory mediators yet they can be infected with L. major. Besides inflammation, there are other mechanisms involved in macrophage immune response, such as respiratory burst, etc., which should be adressed in the Discussion section.

We recently published a manuscript describing the susceptibility of B-1CDP cells in Leishmania major infection. In the current manuscript, we focused our studies on the interaction of *L. major*-infected murine macrophages with precursor B-1 cells. About the others mechanisms involved in the leishmanicidal activity (NO and ROS), we added some passages in the discussion section as suggested by the referee.

4) Although the authors pruport that B-1 CDP cells produce anti-inflammatory mediators, they also suggest that phagocytic ability of these cells to ingest promastigotes are not compromised. This dichotomy in macrophage activation should be addressed in the Discussion section.

This subject was not addressed in the manuscript by the fact that we did not use the B-1CDP phagocytes in this work. Here we propose to evaluate the modulation exerted by B-1 cells on the cellular activity of macrophages infected with *Leishmania major*. We are currently developing a new work, where we will evaluate the functional difference of macrophages and B-1CDP phagocytes in the course of infection by intracellular parasites (*Leishmania major* and *Trypanosoma cruzi*). These studies will demand systematic experiments and will be subject of further publications.

5) The authors show that B-1 cells induce parasite replication when co-cultured with L. major infected macrophages, but the study lacks any

mechanistic studies to elucidate the role of B-1 cells themselves in parasite replication. Therefore, the results, with regard to B-1 cells, just become interesting observations.

Respectfully we disagree with the referee's position, as our data strongly suggest that B-1 cells are able to modulate macrophages infection by the intracellular Leishmania major parasite by an action dependent on IL-10 and PGE-2. These findings show an interesting mechanism of modulation. We used COX-2 inhibitory drugs affecting the production of PGE2, as well as neutralizing doses of anti-IL-10 antibody to block the B-1 cell inhibitory effect. In our approach, we also used IL10- deficient mice to investigate the role of this cytokine in the model.

6) The x-axis labels in Figure 3 are very difficult to read since the labeles seem to be cut off. Changing the labeles to be angled rather than horizontal would be clearer.

We agree with the referee's observation and, as requested, we have made the appropriate changes.

7) In all figure legends, it is stated that graphs are "representative result of three similar experiments". It is unclear what is meant by this statement. Did each experiment have n=3? It does nto make sense that means are presented in the bar graphs snd yet they are denoted as "representative" findings.

As indicated in the revised version of the manuscript, each experiment has been done in triplicate as three independent assays.

8) In experiments containing more than two experimental groups (Figure 3 in particular), Students' t-test is inappropriate. ANOVA followed by posthoc tests of statistical significance are more appropriate.

We used t-test because this statistical analysis was more adequate to analyze experiments with 2 groups as a paired comparison.

9) Representative images should be provided for each experimental condition shown in Figure 4.

We agree with the referee's observation and, as requested, we have added the requested experiments to the manuscript.

10) In Figure 8, were the B-1 KO groups statistically significantly different compared to the B-1 wt groups in both C57BL/6 and KO IL-10 animals?

As shown in the plot we did not observe any statistical difference between the groups.

Minor Comments:

1) BALB/c XID mice should be defined in the Methods section on page 2.

We agree with the referee's observation and, as requested, we have made the change in the manuscript.

2) I think the word "Exposition" on page 2 should be "Exposure".

We agree with the referee's observation and, as requested, we have made the change

3) The word "confirm" on page 2 should be in past tense, i.e. "confirmed".

We agree with the referee's observation and, as requested, we have made the changes accordingly.

4) Please check the manuscript carefully for errors. For example, the title for the legend of Figure 2 contains the mispelled word "independent" and the end of the sentence is missing a period.

We agree with the referee's observation and, as requested, we have made the changes accordingly.

5) Figure 3 legend has the word "factor" mispelled.

We agree with the referee's observation and, as requested, we have made the changes accordingly.

6) “Student’s t-test” is mispelled throughout as “Student’”.
We agree with the referee's observation and, as requested, we have made the changes accordingly.

7) In Figure 6, the “AAS” group should be defined in the figure legend.
We agree with the referee's observation and, as requested, we have made the changes accordingly.

COMMENTS TO AUTHORS - Reviewer’s code: 00055041

The paper is interesting. My comments are: The manuscript would benefit from inclusion of introducing/bridging sentences between the individual parts of the "Results" that explain the logical order and rationale for the experiments. In the Discussion, the Authors should highlight the possible clinical significance of their findings.
We agreed with the referee and added to the text the points requested.

COMMENTS TO AUTHORS - Reviewer’s code: 00736297

The paper is interesting. My comments are:

1) How many mice were used in experiment?
As indicated in the new text, we have used 5 animals for each experiment.

2) Clarify how many experiments n=3? or replicates =3 were done for each measurement?

As indicated in the revised version of the manuscript, each experiment has been done in triplicate as three independent assays.

3) Why authors used Student's t test? probably the number of replicates was not to high, so non-parametric test seems to be better. If the number of experiments was, high enough to checked the distribution or variables, it shall be done and after that, decision on using parametric or non-parametric test shall be made.

We used t-test because this statistical analysis was more adequate to analyze experiments with 2 groups as a paired comparison.

4) The function of B-1 cells directly linked with paper topic shall be described e.g. triggering IL-10 production and lipid mediators production - description on Figures' axis and Figure legends.

We agreed with the referee and changed in the text the point requested.

COMMENTS TO AUTHORS

The manuscript by Arcanjo et al have shown that B-1 cells contribute to the Leishmania major infection of murine macrophages. Their studies have demonstrated that Leishmania major infection first increases PGE-2 levels in the infected cells, which subsequently induce IL-10 expression in B-1 cells and further facilitate the proliferation of Leishmania major inside infected cells. Here are some issues that are needed to be clarify or corrected.

1. It is not clear if B-1 cells are much prone to Leishmania major infection? The authors only used B-1 cells as co-cultured cells. The authors had no data regarding the direct infection of Leishmania major on B-1 cells. In the experiment, the authors use a very high B-1: macrophage ratio of 10:1. What is the usual frequency of B-1 cells in macrophages?

The Infection of lymphocytes by trypanosomatids has been described only once (Trypanosoma cruzi: infection of T lymphocytes and their destruction by antibody-dependent cell-mediated cytotoxicity. Velge P, Kusnierz JP, Ouaissi A, Marty B, Pham BN, Capron A. Eur J Immunol. 1991 Sep;21(9):2145-52.). Based on the information described in the literature, in which B-1 cells are potent producers of IL-10, the objective of this work was to evaluate the ability of B-1 cells to modulate murine macrophages infection by L. major. That is exactly the purpose of our studies, using available tools (drugs NSAID, monoclonal antibodies and KO mice). The question about the frequency of B-1 and macrophages is very interesting. B-1 cells are predominant in the cavities (peritoneum and pleura for example). The number of macrophages is higher than the number of B-1 cells. However, in an infectious process this relationship changes drastically. Upon inflammatory or infectious stimulus, the B-1 cells proliferate extensively and thus the B-1: macrophages relationship changes.

2. The authors tried to point out that B-1 cells contribute to Leishmania major infection by providing IL-10. Although the anti-IL-10 antibody was used to in the study to support this hypothesis, it would be better for the authors to examine if simply providing macrophage cells with IL-10 instead of B-1 cells will also increase the infection. If IL-10 can be confirmed as the most critical factor, it can explain why macrophages from Balb/c XID mice (B-1 cells null) were still infected by Leishmania major, although at a lower infection rate than macrophages from Balb/c.

We believe that IL-10 is the main inhibitory factor released by B-1 cells in the modulation of infected macrophages. The use of antibodies and B-1 cells from IL-10 KO mice shows that the total number of infected macrophages as well as the release of promastigote forms in the supernatant from the infected cultures was statistically reduced. In our experience, we have never been able to stop the intracellular infection with the use of antibodies or KO cells, as the infection always progress from even fewer numbers of pasrasites. An aseptic cure is very difficult to

achieve in this model.

3. Fig 8, the authors stated that B-1 cells from IL-10 deficient mice were more competent to control L. major infection. This statement is misleading, as "control" means that the infection should be reduced. As shown in the figure, with or without IL-10 deficient B1 cells, infection of macrophages by L. major was not changed. Maybe it is much more proper to say that the IL-10 deficient mice failed to promote L. major infection.

We agreed with the referee and changed in the text the point requested.

4. Some references were missing. For example, in the section of "B1 cells" of Materials and Methods, the reference by Almeida et al is missing.

We agreed with the referee and changed in the text the point requested.