nature portfolio

Peer Review File

Brucella abortus impairs T lymphocyte responsiveness by mobilizing IL-1RA-secreting omental neutrophils

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Key results: The authors demonstrate the omentum as a new reservoir for for Brucella, and more specifically, that it replicates inside omental macrophages. They show a robust influx of innate immune cells to the omentum thought the infection and that these cells express PD-L1 and Sca-1, induces by a specific LPS part of Brucella. Additionally, they show that IL1-RA produced by neutrophils results in less proliferation of T cells.

General evaluation: Overall this topic is of interest and the fact, that the omentum is a reservoir for Brucella is novel. Also, the description of PD-L1 and Sca-1 expression on myeloid cells over infection time in the peritoneal cavity and the omentum is of interest. That Brucella infection can lead to less proliferation of T cells is known, however here the authors now demonstrate that IL-1Ra from Brucella stimulated neutrophils leads to T cell hyporesponsiveness. Overall, it is an interesting study with some novelty about Brucella infection and immune cell reaction to it in the peritoneum. However, I think some parts should be improved before publication.

Comments:

1: The only proof that Brucella is inside macrophages in the omentum comes from Fig. 1 c, which is not very convincing. A very small field of view is shown only. I can imagen that it is not easy to preform IF on the omentum, however, a big tile scan overview and a nice 3D reconstitution with Brucella inside the cell would be more convincing. Additionally, some Flow cytometry/ Image stream data could confirm the specific intracellular location only in macrophages in the omentum and exclude existence in other cells and this could be quantified.

2: page5/6 line 123-128: You just assume that the macrophages migrate from the peritoneal cavity to the omentum. There is no proof for that. I would also be that the macrophages in the cavity dye and therefore no appear in your analysis.
You could e.g. transfer macrophages from CD45.1 mice into the cavity of a CD45.2 mouse (or label with a dye like PKH) and infect this one. Then you could tract by Flow cytometry to which extend the adoptively transferred macrophages migrate to the omentum.

3: page6 line 149: it is unclear why you now come with patient data and why you exactly only focus on these markers. A bit more of background would be good here. I also wondered what you mean with “this public database” (line154)? Is it samples from you or were you using samples that are published and available already?

4: Figure S2c and page 8/9 line 223-230: You do not comment on the 72hrs time points. It looks like that the number of PD-L1 and Sca-1 expressing macrophages and neutrophils stimulated with Ba-wt LPS is already going down and not “accumulated from 48 hrs onwards”. Can you speculate on that or include more data on later time points?

5: page 10 line 271-277: I think the microscope pictures are overinterpreted. While it is seen that CXCL1 only is there at 48 hrs and to a lesser extend at 72hrs, to me it is not visible that the green stomal cells produce it. I barely see yellow signal. Either you provide an analysis of the picture for the colocalization for at least 5 field of views or you rephase that paragraph. Again, you could also simply include stromal cells in your Flow cytometry analysis.

Minor comments:

1: In Figure 1a it would be better to show a single dot for each mouse (as in b) and time point as this is standard for CFUs.

2: Please check you Figure legends. The number of animals is sometimes higher (each dot one mouse) than indicates in the legend (e.g. Fig. 2 c, Fig S1d).

3: page 6 line 143. You write that neutrophils peak at 8 days p.i.. Better rephrase that as you don´t check earlier time points or in between 8 and 30 days, which is fine, but is not letting you conclude that they peak at day 8.

4: Figure S2 b: Why do you include CD64 maker only for gating the peritoneal fluid and not for the omentum?

5: Page 10 line 272: Please right milky spots instead of MS and introduce the abbreviation again for a broader audience.

Reviewer #2

(Remarks to the Author)
The studies described in the manuscript by G. González-Espinoza et al. show that peritoneal and omental macrophages are a possible source of wild-type (wt) *Brucella abortus* (BA) retention to promote chronic disease. While peritoneal macrophages (pMφs) and omental (oMφs) were found to express inhibitory molecules, PD-L1 and Sca-1, inhibition appeared to be IL-1RA-dependent with the investigators promoting as omental neutrophils (oNeuts) being the primary source. The unique strength of this work is characterization of BA presence in the omentum, but enthusiasm is dampened when much of the work continued analyses of pMφs at the exclusion of oMφs. Similarly, the authors conclude that oNeuts, but at the exclusion of pNeuts, are the primary source of IL-1RA. There are additional concerns that BA LPS may not mimic all of the stealth qualities of wt BA including the capacity to suppress MHC class I, MHC class II expression, and TNF-α, naming a few. Hence, the conclusion that wt BA LPS can supplement "wt bacteria" activities may prove insufficient. Nonetheless, the findings that the omentum can serve as a reservoir for *Brucella* is a major discovery as the involvement of IL-1RA.

MAJOR CONCERNS
1) Figure 2C: While pMfs and pNeuts were characterized for their expression of inhibitory molecules, PD-L1, Sca-1, and both, no data were provided for similar analysis of oMφs and oNeuts. Such analyses are needed to validate the source of PD-L1 and Sca-1, since the authors make much of their contribution to wt BA's capacity to suppress T cell responses.

2) Figure 3: The investigators make a point of noting TIM4 induction by F4/80^{int} MHCII+ oMφs and pMφs (also referred to as small Mφs) following treatment with wt BA LPS or the LPS mutant from ΔwadC BA, but F4/80^{hi} Mφ subsets (also referred to as large Mφs) and Ly6Chi Mφs were not assessed. Analysis of F4/80^{hi} Mφs and Ly6C^{hi} Mφs is needed to be included either here or in the Supplemental Data. TIM4 was not analyzed when using wt BA - why not? Analysis of TIM4 expression is needed by F4/80^{int} MHCII+ Mφs, F4/80^{hi} Mφs, and Ly6C^{hi} Mφs is needed. Is TIM4 expression only linked to wt BA LPS or also elicited with wt BA? Data are needed showing if TIM4 is induced wt BA infection.

3) Figure 4: Similar concerns expressed above apply here as well. CXCL1 expression needs to be confirmed with wt BA infections to learn their distributions, and confirm that the induced CXCL1 is not solely a BA LPS-mediated phenomenon.

4) Figure 5: The data presented in Fig. 5a is not very convincing in showing that activated pNeuts suppress PMA + ionomycin-stimulated T cell proliferation unlike the reduced T cell proliferation observed in Figure S4b, where co-culture with pNeuts at a 1:1 ratio did obtain suppression. This study needs to be repeated since the investigators present conflicting results, and it is unclear which data are truly representative of pNeuts' capacity. In fact, the activated oNeuts seem to show increased T cell proliferation as noted in Figure 5a, but not analyzed in Figure 5b or c. Why are oNeuts stimulatory?

5) Figure S4c: Why were not the oNeuts evaluated for their suppressive capacity?

6) Since depletion of PD-L1 macrophages had little impact on interrupting the immunosuppression, what is the impact of depleting neutrophils as the source of IL-1RA? If IL-1RA is mediating the observed immunosuppression by wt BA, what is the impact of wt BA infection of IL-1RA-deficient mice? Such analysis is needed to complete the study.

Reviewer #3

(Remarks to the Author)
In this manuscript, González-Espinoza and colleagues reported that omentum as a new Brucella reservoir. They showed that B. abortus infects omental macrophages and persists during the chronic phase of infection. After B.abortus infection macrophages, monocytes and neutrophils are recruited to the omentum during the acute and chronic stages. RNA-seq transcriptional profiles demonstrate PDL1 and LY6E genes are upregulated in blood samples from acute phase brucellosis patients. In B.abortus infected mice, mobilized macrophages, monocytes and neutrophils upregulate PD-L1+ and Sca-1+ during acute and chronic phases. Mechanistically, Brucella LPS stimulation mimics that of infection and is core LPS-mediated. PD-L1+Sca-1+ macrophage, monocyte and neutrophil maintenance in the omentum depend on Brucella LPS core stimulation rather than PD-L1. CXCL1 secretion parallels the late omental recruitment of neutrophils elicited by Ba wt LPS. PD-L1+Sca1+ neutrophils impair T cell lymphocyte responsiveness. And finally, IL-1RA secreted by PD-L1+Sca1+

neutrophils dampens the response of T lymphocytes. The study represents a new development in the field as there are currently no published studies exploring omentum as Brucella reservoir. While most of the data presented is of high quality, some aspects of experimental design and rationale were lacking and interpretation of select data lacked controls, which were not entirely convincing. Specific concerns are noted.

1. Fig 1b lacks the detection of CFU/organ in peritoneal fluid as control.
2. In Fig S3b, the left column is not marked above.
3. In Fig S3c, IL-7 stimulation ensures T cell viability in culture in absence of a proliferation signal.
4. Line 158, “CD272/PDL1”in the context, while CD274/PDL1 in Fig 2b.
5. Line 187, how and why Ba-wad C was selected?
6. Fig S2e, how does Ba-wt LPS orchestrates the increase of PD-1+CD 4+ cells and Sca-1+CD69+CD8+ T cells?
7. What is difference of LPS core between Brucella and E.coli?
8. In Fig 3a, given that both Ba-wt and Ba-wadC LPS administration generated an early accumulation of Ly6G+ neutrophils in the omentum of mice, is deletion Ba-wadC able to establish chronic infection in omentum?
9. In Fig 6b, how long does IL-1RA exist after Brucella infection? Does it help brucella establish chronic infection?
10. In Fig.7, the influx of macrophage from peritoneal fluid to omentum was not demonstrated.
11. Vascular and lymphatic connections are important part of omental, however, lymphatic vessel structure is not depicted in Fig. 7.
12. Is IL-1RA secreted only by PD-L1+Sca-1+ omental neutrophils?
13. There are no evidence that LPS core moiety directly contributed to neutrophils up-regulation of IL-1RA expression.
14. What is special about the omentum in terms of its structure, biochemistry and immunity that makes it a long-term storage site for Brucellae?
15. Why did Milky spots become portals for Mycosis to invade the omentum?
16. LPS should be extracted and the difference between LPS of wad C knockout bacteria and wild type bacteria should be compared by silver staining, which could be a challenge.
17. Is the omentum, as a storage organ, specific to B. abortus or universal to Brucella species? More evidence are needed.
18. Will the proliferation titer of Brucella in the omentum be enhanced by direct injection of IL-1RA into mice? Will chronic infection be established after injection?
19. The omentum is rich in adipocytes. Do adipocytes in omentum play a role in Brucella infection of the omentum?
20. What is the accurate function of Brucella LPS core? Does it regulate the recruitment of macrophages, monocytes and neutrophils to the omentum, or the secretion of IL-1RA by neutrophils?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I appreciate the clarifications and additional data that the authors have provided. All of my concerns have been adequately addressed by either adding additional information or performing additional experiments.

Reviewer #2

(Remarks to the Author)
The authors were responsive to all of my prior concerns. The new data clearly strengthen the role for omentum as a possible brucellae reservoir following infection.

However, several minor concerns need to be addressed:
1) Figure legend 1 needs to be modified showing that *B. abortus* strain 2308 is being used. Outside of the Materials & Methods, the authors failed to define which *B. abortus* strain is being used.

2) Figure legend 1 needs to be modified to include the doses used for IP and oral gavage infections. Again, these are not mentioned in the Results nor figure legend.

3) Line 373: No where in the manuscript was Tregs defined. No mention of intracellular staining for Foxp3 was described nor antibody used for detection listed in Table S1. Figure S4h failed to define how Tregs were defined. Hence, the text and Figure S4 must be amended describing how Tregs were defined. The absence of intracellular Foxp3 staining would negate any conclusions regarding the presence of Tregs. Staining for CD25 alone is insufficient since CD25 is also increased on activated $CD4^+$ T cells.

Reviewer #3

(Remarks to the Author)
The authors carried out all necessary experiments suggested by this reviewer,and addressed all my concerns for the primary manuscripts. The current paper is acceptable for publication in Nature Communications.

Point-by-point rebuttal to Reviewers' comments

We thank the Editor and the Reviewers for highlighting the innovative and important impact of our work, for their comments and willingness to consider a revised version of our manuscript now entitled "*Brucella abortus* impairs T lymphocyte responsiveness by mobilizing IL-1RA-secreting omental neutrophils" (NCOMMS-23-35030). Reviewers' comments clearly helped us to improve our manuscript and we are grateful to them for this positive push. Given the extent of experiments done as well as technology involved to achieve our goals (spectral cytometry) more authors were added and the order of the first co-authors has been changed to be fair. We performed additional experiments as requested, and our new discoveries incorporated in the text made us change a bit the abstract (see line 56: "…the omentum, a reservoir for *B. abortus*, promotes bacterial persistence and causes $CD4^+$ and $CD8^+$ T cell immunosuppression by IL-1RA secreted by PD-$L1^+$Sca-1^+ neutrophils". Please find below a point-by-point rebuttal (line numbering of our revised manuscript refers here to the highlighted version).

Answers to Reviewer #1's comments:

Key results: The authors demonstrate the omentum as a new reservoir for for Brucella, and more specifically, that it replicates inside omental macrophages. They show a robust influx of innate immune cells to the omentum thought the infection and that these cells express PD-L1 and Sca-1, induces by a specific LPS part of Brucella. Additionally, they show that IL1-RA produced by neutrophils results in less proliferation of T cells.

General evaluation: Overall this topic is of interest and the fact, that the omentum is a reservoir for Brucella is novel. Also, the description of PD-L1 and Sca-1 expression on myeloid cells over infection time in the peritoneal cavity and the omentum is of interest. That Brucella infection can lead to less proliferation of T cells is known, however here the authors now demonstrate that IL-1Ra from Brucella stimulated neutrophils leads to T cell hyporesponsiveness. Overall, it is an interesting study with some novelty about Brucella infection and immune cell reaction to it in the peritoneum. However, I think some parts should be improved before publication.

We are pleased to see that Reviewer #1 has caught the novelty and importance of our study and in particular the role of the omentum as a reservoir for *Brucella abortus*, the first description of PD-$L1^+$Sca-1^+ suppressive myeloid cells along the course of the infection that persist in a *B. abortus* LPS core-dependent manner, as well as the discovery of IL-1RA as a T cell immunosuppressive cytokine produced notably by neutrophils.

Comments:

1: The only proof that Brucella is inside macrophages in the omentum comes from Fig. 1 c, which is not very convincing. A very small field of view is shown only. I can imagen that it is not easy to preform IF on the omentum, however, a big tile scan overview and a nice 3D reconstitution with Brucella inside the cell would be more convincing. Additionally, some Flow cytometry/ Image stream data could confirm the specific intracellular location only in macrophages in the omentum and exclude existence in other cells and this could be quantified.

To comply with Reviewer #1's request, additional infection experiments with *DsRed B. abortus* have been undergone. Fig. 1c now presents a clearer representative confocal microscopy image of wholemount staining of the omentum together with a 3D reconstituted images in Fig. 1d, showing an unambiguous colocalization of the DsRed *Brucella* with macrophages. Text and figure legends have been modified accordingly. We also precise that we tried to establish light-sheet microscopy on this organ without any success. Regarding the second option proposed by reviewer #1, i. e. flow cytometry analysis, only a small proportion of macrophages are infected by *Brucella*, meaning that they can't be detected by flow cytometry, as confirmed by our preliminary cytometry analyses of Ds-red *Brucella* within omental cells (unpublished). Anyway, this experiment is unnecessary, since we got nice immunofluorescence data.

2: page5/6 line 123-128: You just assume that the macrophages migrate from the peritoneal cavity to the omentum. There is no proof for that. I would also be that the macrophages in the cavity dye and therefore no appear in your analysis.

You could e.g. transfer macrophages from CD45.1 mice into the cavity of a CD45.2 mouse (or label with a dye like PKH) and infect this one. Then you could tract by Flow cytometry to which extend the adoptively transferred macrophages migrate to the omentum.

Reviewer #1 is referring to our sentence "A significant decrease in the numbers of F4/80^{hi} (large peritoneal macrophage, LPM) and F4/80^{int}MHCII^{hi} (small peritoneal macrophage, SPM) subsets was observed in the peritoneal fluid at 8 days p.i. (Fig. 2a, bottom) that coincided with a significant increase in the omentum (Fig. 2a, top), inferring migration of macrophages from the peritoneal fluid to the omentum during the acute phase of infection."

Migration of macrophages from the peritoneal cavity to the omentum is a well-described phenomenon in various experimental settings, although questioned recently (Salm et al, 2023, Trends in immunology for a review). It is true that to formally demonstrate such migration, we could perform the adoptive transfer experiments suggested by Reviewer #1. This type of experiment is not possible at the present time in our collaborators' BSL3. Therefore, our sentence is reformulated line 131 as "… that coincided with a significant increase in the omentum (Fig. 2a, top), inferring a possible migration of macrophages from the peritoneal fluid to the omentum during the acute phase of infection."

Similarly, we dampened our conclusion line 150 "Altogether, *B. abortus* causes a decrease in LPM and SPM in the peritoneal fluid (at the acute phase only for the latter), and at the same time elicits an increase of both populations in the omentum at the acute and chronic phases of infection."

3: page6 line 149: I was irritated, why you now come with patient data and why you exactly only focus on these markers. A bit more of background would be good here. I also wondered what you mean with "this public database" (line154)? Is it samples from you or were you using samples that are published and available already?

We agree with Reviewer #1 that the transition between the mouse infection data and the human patients' ones was a bit blunt, because of the manuscript length restriction. The paragraph now starts line 161 by: "To get some clues about the immune responses associated with *Brucella* and disease stage in humans, RNA-seq transcriptional profiles obtained from whole blood samples of brucellosis patients … were previously determined by an international consortium comprising our laboratory (reference #37, 38). As explained in the M & M, the original RNA seq data generated by an international consortium comprising our laboratory have been deposited on gene expression omnibus, for which the link is provided in reference #37. A published abstract based on these genome-wide transcriptome data is now added as reference #38, as no article is published yet. This is what "public database" referred to; now, please read line 165 "using this database…". Details about this database are in the Materials and Methods section, Human RNA-seq analysis paragraph. Furthermore, our text line 165 has been rephrased, and now clearly explains, with the help of references cited in the introduction but added again here and another one from the discussion, why we chose those genes; see line 165: "Using this database, we looked for genes that might help *Brucella* persistence and immune escape, and analyzed the blood expression of genes encoding immune inhibitory molecules (references #30, 31 and 39), like PD-L1…".

4: Figure S2c and page 8/9 line 223-230: You do not comment on the 72hrs time points. It looks like that the number of PD-L1 and Sca-1 expressing macrophages and neutrophils stimulated with Ba-wt LPS is already going down and not "accumulated from 48 hrs onwards". Can you speculate on that or include more data on later time points?

As suggested by Reviewer #1, additional experiments have been undergone to extend the kinetics to another time-point i. e. 96h post-LPS injection. The recruitment in the omentum of all myeloid cells

analyzed is decreased at this time-point, even though it is still significantly higher for macrophages and neutrophils after Ba-wt challenge compared to the other conditions. The observed decreased numbers of neutrophils and monocytes most probably reflect the shorter half-life of these cells compared to macrophages. We decided not to include these data because 1- the manuscript is already long and 2- we have now included analysis of these cells in the omentum upon *B. abortus* infection (Fig. 2f), which is more meaningful and demonstrates the importance of these cells during *B. abortus* infection in a full organism.

We therefore changed the sentences regarding this point to: line 247 "at 48h and 78h, this accumulation of PD-L1^{+}Sca-1^{+} macrophages (LPM and SPM, monocytes and neutrophils..."

5: page 10 line 271-277: I think the microscope pictures are overinterpreted. While it is seen that CXCL1 only is there at 48 hrs and to a lesser extend at 72hrs, to me it is not visible that the green stomal cells produce it. I barely see yellow signal. Either you provide an analysis of the picture for the colocalization for at least 5 field of views or you rephase that paragraph. Again, you could also simply include stromal cells in your Flow cytometry analysis.

Reviewer #1 is right; few cells were double positive for CXCL1 and gp38 upon Ba LPS treatment. Given that 1- in our additional infection experiments, we couldn't get any positive staining with these two markers in *B. abortus* infected samples and 2- we have to isolate individual cells from the omentum for cytometry without any guarantee to leave intact these cells and 3- we face a limitation of markers/ fluorochromes to be used in cytometry analysis (even by spectral cytometry), we could not add a stromal marker to our flow cytometry experiments. However, our new spectral cytometry experiments disclosed a clear production of CXCL1 upon infection in the omentum by several myeloid cells.

Therefore, we have reorganized the panels of this figure. Text is reformulated accordingly line 279 as "When comparing by confocal microscopy CXCL1 secretion in the omentum at 4h and 72h after Ba-wt LPS or PBS injection, CXCL1^{+} cells were detected in Ba-LPS challenged omentum only at 72h, with no colocalization with mesothelial cells (gp38), while Ly6G^{+} neutrophils were visible at both time-points (Fig. 4b). The high influx of neutrophils triggered by Ba-wt LPS at 48h post-Ba-wt LPS stimulation (Fig. 4c) occurred in the vicinity of the enlarged milky spot (MS), defined by CD3^{+} T cells with a nearby production of CXCL1."

Minor comments:

1: In Figure 1a it would be better to show a single dot for each mouse (as in b) and time point as this is standard for CFUs.

We agree with Reviewer #1 that a panel with a single dot for each mouse and time-point and organ was missing in Fig. 1a.

Since it seemed to us that the comparative kinetics in the three different organs was clearer to get a global view of the CFU during the course of infection, we kept it and used it to compare each organ with the other one at a given time-point (Fig. 1a top). In addition, to comply with Reviewer #1's suggestion, we added the singled dot representation of CFU for each organ along the course of infection and used it to compare within a given organ the CFU at the different time-points (Fig. S1a bottom). Figure legends have been modified accordingly.

Thanks to Reviewer #1's comment, we rechecked our original data and discovered a mistake in the CFU calculation of Fig.1a and realized that the data submitted originally were expressed as CFU/g and not CFU/organ. We thus corrected this mistake and decided to change the counts in the whole manuscript for CFU/g (but supplementary Fig. 1g, for which CFU/organ is easier for comparative strain virulence in different organs).

2: Please check you Figure legends. The number of animals is sometimes higher (each dot one mouse) than indicates in the legend (e.g. Fig. 2 c, Fig S1d).

We thank Reviewer #1 for pointing out these discrepancies. We have corrected these mistakes all along the manuscript and modified the text of the figure legends to fit the genuine number of animals used and shown in the figure. Changes are highlighted in yellow in the highlighted version of the manuscript.

3: page 6 line 143. You write that neutrophils peak at 8 days p.i.. Better rephrase that as you don´t check earlier time points or in between 8 and 30 days, which is fine, but is not letting you conclude that they peak at day 8.

This is done line now 142, you can read:" their numbers were elevated at day 8…". The same comment although not mentioned by reviewer #1 seemed to apply to line 142 in the original submitted version of our manuscript. So, we rephrased this part too. Please now read line 143 "high blood neutrophil counts were found in infected mice at 8 days p.i. which…".

4: Figure S2 b: Why do you include CD64 maker only for gating the peritoneal fluid and not for the omentum?

For one mouse, given the size of the omentum, we had only one antibody mix and a limited number of markers for flow cytometry analysis. This explains why some markers used for the peritoneal fluid analysis could not be included; but we managed a way to discriminate cells easily with a gating strategy adapted to each organ by flow cytometry. Moreover, we have now performed spectral cytometry on infected samples with an identical strategy for both the omentum and the peritoneal fluid, again with an adapted strict gating strategy, which includes CD64 as well as the LPM TIM4 marker, and gives similar results (please see Fig. 2e, f and S1c, f and S2b as well as their legends).

5: Page 10 line 272: Please right milky spots instead of MS and introduce the abbreviation again for a broader audience.

Although the text corresponding to the figure has been modified to fit the changes in the order of the rearranged figure panels, we took into consideration Reviewer's #1's comment, which has been applied to the whole manuscript. You can now read line 156 'milky spots (MS) until", line 284, "the enlarged milky spot (MS),… ", and at the beginning of the second paragraph of the discussion, line 392 "of the enlarged milky spot (MS)".

Answer to Reviewer #2's comments:

The studies described in the manuscript by G. González-Espinoza et al. show that peritoneal and omental macrophages are a possible source of wild-type (wt) Brucella abortus (BA) retention to promote chronic disease. While peritoneal macrophages (pMφs) and omental (oMφs) were found to express inhibitory molecules, PD-L1 and Sca-1, inhibition appeared to be IL-1RA-dependent with the investigators promoting as omental neutrophils (oNeuts) being the primary source. The unique strength of this work is characterization of BA presence in the omentum, but enthusiasm is dampened when much of the work continued analyses of pMφs at the exclusion of oMφs. Similarly, the authors conclude that oNeuts, but at the exclusion of pNeuts, are the primary source of IL-1RA. There are additional concerns that BA LPS may not mimic all of the stealth qualities of wt BA including the capacity to suppress MHC class I, MHC class II expression, and TNF-α, naming a few. Hence, the conclusion that wt BA LPS can supplement "wt bacteria" activities may prove insufficient. Nonetheless, the findings that the omentum can serve as a reservoir for Brucella is a major discovery as the involvement of IL-1RA.

We sincerely thank Reviewer #2, who considers major discoveries the fact that the omentum may act as a *Brucella* reservoir, and the involvement of IL-1RA as a suppressive cytokine in brucellosis. Concerning his concerns about the suppressive status of omental macrophages and neutrophils as well as IL1-RA production by omental neutrophils, they are now fully addressed; indeed, by doing additional infection

experiments and using the newly established spectral cytometry technology, we were able to demonstrate that omental macrophages, monocytes and neutrophils share with their peritoneal counterparts the same PD-L1^{+}Sca-1^{+} phenotype upon *B. abortus* infection (Fig. 2d-f, 2f). Furthermore, we show that IL1-RA is secreted by the omentum at similar levels all along the infection from the acute to the chronic stages and at levels in the range of those detected after Ba LPS administration (Fig. 6g).

Reviewer #2 in his conclusion above mentions that "*Similarly, the authors conclude that oNeuts, but at the exclusion of pNeuts, are the primary source of IL-1RA*". We'd like to emphasize that we did not mention anywhere in the original submitted version that omental neutrophils were the sole source of IL-RA and did not exclude peritoneal neutrophils. Indeed, they were already shown to produce IL1-RA upon Ba-wt LPS challenge (Fig.6d and corresponding text). We now formally show that omental neutrophils together with monocytes and SPM produce IL-1RA upon *B. abortus* infection (Fig. 6h, i). In addition, we found a preferential association between IL-1RA producing neutrophils and CD4^{+} and CD8^{+} T cells expressing the inhibitory molecules PD-1 and LAG-3, while IL-1RA producing monocytes are more linked to CD4^{+} T cells and Treg, and macrophages show little association with these cells. These new findings bring about a new light on the distinctive roles of these various IL-1RA producing cells during *B. abortus* infection (please see text added line 462 "Yet, IL1-RA is additionally produced by monocytes; the privileged link of IL1-RA-producing monocytes with PD-1^{+}LAG-3^{+} CD4^{+} T cells and Treg during *B. abortus* infection opens up a complementary and specific role for these cells in immunosuppression, which needs future investigations.". Our conclusion, it is true, is more focused on the omentum as our article shows that this organ is a new reservoir *for B. abortus* as well as neutrophils, for which we demonstrate a yet undiscovered inhibitory role on T cell responsiveness.

Lastly, Reviewer #2 wrote in his conclusion above that "*There are additional concerns that BA LPS may not mimic all of the stealth qualities of wt BA including the capacity to suppress MHC class I, MHC class II expression, and TNF-α, naming a few. Hence, the conclusion that wt BA LPS can supplement "wt bacteria*" activities may prove insufficient." We totally agree with this comment and also consider that Ba LPS mimics partially only the full bacterium. We just propose that line 235 "the influx of macrophages, monocytes and neutrophils observed after *B. abortus* LPS stimulation in the omentum and peritoneal fluid was similar to that following infection and relies at late time-points on the LPS core lateral determinant, added by the wadC mannosyl transferase." and line 249 "*B. abortus* LPS drives a fast mobilization to the omentum of macrophages, monocytes and neutrophils exhibiting a PD-L1^{+}Sca-1^{+} macrophage, the persistence of which depends on the LPS core lateral pentasaccharide moiety.".

MAJOR CONCERNS

1) Figure 2C: While pMfs and pNeuts were characterized for their expression of inhibitory molecules, PD-L1, Sca-1, and both, no data were provided for similar analysis of oMφs and oNeuts. Such analyses are needed to validate the source of PD-L1 and Sca-1, since the authors make much of their contribution to wt BA's capacity to suppress T cell responses.

Reviewer #2 raised a concern about the similar behavior of peritoneal and omental neutrophils that no longer exists. Our additional spectral cytometry experiments (presented in Fig. 2e and 2f; text added line 188-196: "When analyzing by spectral cytometry… Fig.2f).") prove that the suppressive immune status (i.e. expression of PD-L1, Sca-1 alone or together as well as of LAG-3) of macrophages/monocytes and neutrophils in the omentum during infection is similar to that observed in the peritoneal fluid. Moreover, we now show (Fig. 5c, text added line 312, "This ability to suppress T cell responses is shared by both Ba-wt or Ba-*wadC* challenged omental neutrophils (Fig.5c).") that omental neutrophils trigger hypo-proliferation and activation of T cells like the peritoneal neutrophils.

2) Figure 3: The investigators make a point of noting TIM4 induction by F4/8^{int}MHCII+ oMφs and pMφs (also referred to as small Mφs) following treatment with wt BA LPS or the LPS mutant from ΔwadC BA, but F4/80^{hi} Mφ subsets (also referred to as large Mφs) and Ly6C^{hi} Mφs were not assessed. Analysis of F4/80^{hi} Mφs and Ly6C^{hi} Mφs is needed to be included either here or in the Supplemental Data. TIM4 was not analyzed when using wt BA - why not? Analysis of TIM4 expression is needed by F4/80^{int} MHCII+ Mφs, F4/80^{hi} Mφs, and Ly6C^{hi} Mφs is needed. Is TIM4 expression only linked to wt BA LPS or also elicited with wt BA? Data are needed showing if TIM4 is induced wt BA infection.

Tim4 is a classical marker of F4/80^{hi}MHCII^{+} large peritoneal macrophage (LPM) and is not expressed in F4/80^{int}MHCII^{hi} small peritoneal macrophage (SPM) (reference #41, added). This marker was not used during our original *in vivo* infection experiments for the reasons aforementioned (limitations of the number of markers for omentum analyses, which allows given its size only one sample mix for flow cytometry, as well as available FACS machine in our BSL3). So, we used the first gating strategy described in Fig.1c for our original *in vivo* flow cytometry experiments and the gating strategy in Fig. 2b for the analysis of the omentum and peritoneal samples after LPS stimulation. For these latter ones, all populations of macrophages analyzed are now included in Fig.3a and 3b, i. e. LPM, F4/80^{int}MHCII^{hi} SPM, as well as monocytes and neutrophils. We'd like to precise that 1- as stated line 135, SPM derive from Ly6^{hi} monocytes (reference # 36), 2- F4/80^{int}MHCII^{hi} SPM and LPM represent the extreme cell phenotypes of all macrophages of the peritoneal cavity that exist in a continuum of states, which are, but LPM, Ly6C positive at various degrees. Using the Ly6C marker, we could distinguish in the F4/80^{int}MHCII^{hi} SPM population four subgroups according to its fluorescence intensity and two subgroups in the F4/80^{int}MHCII^{+} cells. This complexity explains why we decided to restrict our analysis to the generic LPM and F4/80^{int}MHCII^{h} SPM populations keeping in mind that our manuscript is not about deciphering all subtypes of macrophages that are recruited in the omentum during *B. abortus* infection. This could be the scope of another manuscript. We just illustrate the known populations, LPM and F4/80^{int}MHCII^{hi} SPM, described in the peritoneal cavity. Anyway, to address the main issues of our manuscript, further analyses by spectral flow cytometry (Fig. 2e and 2f), with a strict gating strategy common to omentum and peritoneal lavage that includes both TIM4 and CD64 markers (Fig. S1f), have been undertaken and give results comparable to our preceding analyses. Interestingly, this new cytometry technology led us discover that macrophages do not have a major role in IL-1RA-mediated T cell immunosuppression (Fig. 6j and text line 370 "linked to PD-1^{+}LAG-3^{+} CD4^{+} T cells and Treg, while no significant association was seen with IL-1RA+ macrophages"), which is one of among multiple novelties of our study.

Reviewer #2 made us understand that clarification was needed in our manuscript and we thank him for that. In the text, we now added: 1- at the beginning of this paragraph line now 125 "We first looked at the F4/80^{hi} (large peritoneal macrophage, LPM) resident macrophages and F4/80^{int}MHCII^{hi} (small peritoneal macrophage, SPM) subsets, well-known macrophage cells populating the peritoneal cavity at resting state and upon inflammation, respectively"; 2- line 188 "When analyzing by spectral cytometry the number of peritoneal cells expressing LAG-3 at day 8 p.i. (undetected in our settings by flow cytometry and using TIM4 as an additional marker for LPM), a pattern evoking that of the PD-L1^{+}Sca-1^{+} cells was obtained (Fig. 2e, Fig. S1f)". 3- in the Supplementary Materials, legend of Fig. S1f "Spectral cytometry gating strategy for the analysis of F4/80^{hi} TIM4^{+} (LPM) and F4/80^{int}MHCII^{hi} (SPM) macrophages, Ly6C^{hi} monocytes, Ly6G^{+} neutrophils with FACS profiles from the omentum of a C57BL/6 mouse 8 days post-infection with B. abortus. Cells were gated based on SSC/FSC, singlets and viable cells after UV fixable blue dead staining. Here, in contrast to flow cytometry, discrimination of macrophage populations absolutely requires the TIM4 marker as F4/80^{hi} cells are not well discriminated by the F4/80 and CD11b markers; gating for LPM is based on PBS control for which LPM are abundant. This means that this gating is strict and may

underestimate cell numbers especially for F4/80^{int}MHCII^{hi} SPM. Values obtained follow nevertheless the trend of cell absolute numbers obtained by flow cytometry".

Please see also our answer to the minor point #4 raised by Reviewer #1.

3) Figure 4: Similar concerns expressed above apply here as well. CXCL1 expression needs to be confirmed with wt BA infections to learn their distributions, and confirm that the induced CXCL1 is not solely a BA LPS-mediated phenomenon.

We agree with Reviewer #2 that this is an important point; new infection experiments have been performed. We now show by spectral cytometry (Fig. 4f) as stated line 292 that "Upon *B. abortus* infection, CXCL1 was also secreted by Ly6G^{+} neutrophils, Ly6C^{hi} CCR2^{+} monocytes as well as by F4/80^{+}MHCII^{hi} SPM, in contrast to Ba-wt LPS stimulation". Spatial localization of these myeloid cells recruited in the omentum during infection cells is shown in Fig. 2b. This means that CXCL1 secretion in omentum is triggered by *B. abortus* in a wider way than with Ba-wt LPS alone.

4) Figure 5: The data presented in Fig. 5a is not very convincing in showing that activated pNeuts suppress PMA + ionomycin-stimulated T cell proliferation unlike the reduced T cell proliferation observed in Figure S4b, where co-culture with pNeuts at a 1:1 ratio did obtain suppression. This study needs to be repeated since the investigators present conflicting results, and it is unclear which data are truly representative of pNeuts' capacity. In fact, the activated oNeuts seem to show increased T cell proliferation as noted in Figure 5a, but not analyzed in Figure 5b or c. Why are oNeuts stimulatory?

It seems that as regards Fig.5a, Reviewer #2 mixed up bone marrow neutrophils, which do not affect T cell proliferation, with omental neutrophils, which we did not study in our submitted version at the time (see below our answer to point 5). Data from four independent experiments with the bone marrow neutrophils used in proliferation assays have been pooled, and mean is now presented in (Fig. 5a, S4b and accompanying legends). They clearly show that bone marrow neutrophils have no significant impact on T cell proliferation unlike peritoneal neutrophils. These data provide with some negative control and reduce the number of animals used. Indeed, at the resting state, i. e. PBS-treated animals, very few neutrophils are present in the peritoneal cavity and omentum, meaning that it would require 5-10 mice per co-culture well to get enough cells for a control. We had to use 6-8 (peritoneal) mice and 10-12 mice (omental) for each preparation of LPS-challenged neutrophils per T cell- coculture experiment. Furthermore, we have in this revised version in Fig. 5c and S4f data showing a significant inhibition of T cell proliferation and activation by omental Ba-wt (n=5) or Ba-*wadC* (n=4) LPS-*in vivo* challenged neutrophils in a comparable fashion to peritoneal Ba-wt-LPS challenged neutrophils (Fig. 5b, S4c-e). Please see added text line 312 "This ability to suppress T cell responses is shared by both Ba-wt or Ba-*wadC* challenged omental neutrophils (Fig.5c). Hindrance of T cell responsiveness operated also at the T cell receptor (TCR) engagement level (Fig. S4b-f).".

5) Figure S4c: Why were not the oNeuts evaluated for their suppressive capacity?

Given the fragility of neutrophils, we initially focused on peritoneal neutrophils, which are more easily accessible. Peritoneal neutrophils are recruited in the peritoneal cavity via the HEV of the omentum upon *E. coli* infection (reference #11). It is most likely that the same route is used during *Brucella* infection (see the Ba LPS-triggered elevated CXCL1 levels in the serum and omentum and their stability in the peritoneal fluid). Moreover, preliminary whole omentum RNAseq data obtained after *in vivo* treatment with Ba LPS did not allow us to detect any neutrophil in the omentum in contrast to the peritoneal fluid, while we detect them by IF and cytometry analyses. This means that they probably didn't survive the treatments to process the organ and sort out the viable cells. Additional experiments done on infected samples

confirmed such fragility. Out of 10 infected omenta processed per experiment (n=3), we managed to get only one or two with viable cells after intracellular staining and spectral flow analysis. This is why to answer Reviewer #2's question, we purified omental neutrophils 48h after *in vivo* Ba-wt or Ba-*wadC* LPS-administration only, and tested them in T cell co-culture for their immunosuppressive capacity. We confirmed in Fig. 5c (see accompanying text line 312) that both types of Ba challenged neutrophils are able to inhibit T cell proliferation and activation similarly to peritoneal neutrophils, thus demonstrating the T cell suppressive ability of both peritoneal and omental neutrophils as well as the selective role of the *wadC*-encoded determinant in the maintenance of the suppressive myeloid cells in the peritoneal fluid and omentum.

6) Since depletion of PD-L1 macrophages had little impact on interrupting the immunosuppression, what is the impact of depleting neutrophils as the source of IL-1RA? If IL-1RA is mediating the observed immunosuppression by wt BA, what is the impact of wt BA infection of IL-1RA-deficient mice? Such analysis is needed to complete the study.

Answering the question raised by Reviewer #2 regarding the effect of neutrophil depletion on IL-1RA is not trivial. Neutrophils are produced very early on upon infection (in a Ba LPS-dependent manner). This means that depletion should be done during the chronic phase for at least 15 days of injection with at least 200ug of neutralizing antibodies per mouse, specific for Ly6G that are available from BioXcell. The cost of the experiment will be very high for a hazardous result. Indeed, preliminary RNAseq exp showed a decrease of Ly6G expression in peritoneal neutrophils at 48h after Ba LPS treatment; moreover, we showed that monocytes and SPM also produce IL-1RA during infection (Fig. 6h). Alternatively, using an antibody against GR1 will deplete both the neutrophil and the monocyte populations but not the SPM; it has already been reported to lead to an enhanced bacterial clearance and T cell activation (Reference #27).

We agree with Reviewer #2 that the demonstration of the direct involvement of IL-RA in the persistence of the pathogen would be ideal. However, undergoing infection of IL-1RA deficient mice with *B. abortus* is not a good option. An article recently published in Nature communications (Villatoro et al., vol. 14, 3874, 2023) shows that deletion of the IL1-RA gene in the mouse increases at the steady state the cellularity of the bone marrow and the numbers of circulating neutrophils. Moreover, the granulocyte-monocyte progenitors are reduced in the bone marrow of these deficient mice due to increased apoptosis. This means that with such perturbed environment that affects directly neutrophils, the response of this mutant mice to *B. abortus* is expected to be altered and unconclusive data obtained as regards the role of IL-1RA in the chronic stage of infection.

To try to answer Reviewer #2's important issue, we performed the reverse experiment, i.e. injected subcutaneously and daily at 300ug/Mouse ANAKINRA (purified human IL-1RA) from Day 8 onward and analyzed the omentum and spleen at day 15, the beginning of the chronic phase in our mouse model. No differences between saline- and ANAKINRA-injected animals were observed. This could be due to several factors: 1- half-life of AAKINRA in the mouse blood is 8 hours (Powers et al. JBC vol 295(3), 868, 2020) and 4-6h in humans); however due to limited access to BSL3, we could not inject twice or three times per day the molecule, which was probably not present at high enough quantity in the blood; 2- Although elevated dose administered subcutaneously, we have no guarantee that this dose was sufficient for the drug to reach and neutralize omental IL-1RA ; 3- Administration of ANAKINRA in humans drives neutropenia; if the case in the mouse, results are expected to be skewed. All these reasons may explain our unconclusive results.

We finally tried another approach and decided to prepare ourselves some anti-mouse IL-1RA neutralizing antibodies from an hybridoma. After several attempts, we never received a viable vial of

hybridoma cells from the only lab, where it is still stored. This means that this question is pending and will have to be answered in the future with maybe targeted and conditional, still to be produced, mutant mouse models.

Answer to Reviewer #3's comments:

In this manuscript, González-Espinoza and colleagues reported that omentum as a new Brucella reservoir. They showed that B. abortus infects omental macrophages and persists during the chronic phase of infection. After B.abortus infection macrophages, monocytes and neutrophils are recruited to the omentum during the acute and chronic stages. RNA-seq transcriptional profiles demonstrate PDL1 and LY6E genes are upregulated in blood samples from acute phase brucellosis patients. In B.abortus infected mice, mobilized macrophages, monocytes and neutrophils upregulate PD-L1+ and Sca-1+ during acute and chronic phases. Mechanistically, Brucella LPS stimulation mimics that of infection and is core LPS-mediated. PD-L1+Sca-1+ macrophage, monocyte and neutrophil maintenance in the omentum depend on Brucella LPS core stimulation rather than PD-L1. CXCL1 secretion parallels the late omental recruitment of neutrophils elicited by Ba wt LPS. PD-L1+Sca1+ neutrophils impair T cell lymphocyte responsiveness. And finally, IL-1RA secreted by PD-L1+Sca1+ neutrophils dampens the response of T lymphocytes. The study represents a new development in the field as there are currently no published studies exploring omentum as Brucella reservoir. While most of the data presented is of high quality, some aspects of experimental design and rationale were lacking and interpretation of select data lacked controls, which were not entirely convincing. Specific concerns are noted.

We thank Reviewer #3 for highlighting the high quality and originality of our work.

Concerns:

1. Fig 1b lacks the detection of CFU/organ in *peritoneal fluid as control*.

In Fig. 1b, mice have been infected by gavage. Proximal lymph nodes, i. e. cervical lymph nodes, as well as spleen are the reference organs here. For this infection route, peritoneal fluid CFU examination is not appropriate and gives no CFU at all, explaining why it wasn't included in the graph. You have to consider that in the case of intraperitoneal infection, total CFU in the peritoneal fluid are three Log below the CFU observed in the omentum or spleen, the reference organ (Fig. 1a). Given that upon gavage infection, at day 30 p.i., CFU in omentum and lymph nodes are in the range of three Log, it means that if the same ratio applies, which is unlikely because of the different route of infection and which would be expected to be more here, no CFU can be found in the peritoneal fluid.

2. In Fig S3b, the left column is not marked above.

We thank Reviewer #3 for pointing out this oversight. It is now written "Total cells" above this left column in Fig. S3b.

3. In Fig S3c, IL-7 stimulation ensures T cell viability in culture in absence of a proliferation signal.

We thank Reviewer #3 for pointing out this slip mistake. It is now written in the Figure legend of S3c "in absence of a proliferative signal". Indeed, without this IL-7 stimulation, no T cell would survive the three days of culture and no control would be available.

4. Line 158, "CD272/PDL1"in the context, while CD274/PDL1 in Fig 2b.

We thank Reviewer #3 for pointing out this typo, now corrected in line 168 "*CD274/PDL1*".

5. Line 187, how and why Ba-wad C was selected?

Introductory paragraph line 203 explains why we got interested in the Ba-*wadC Brucella* mutant strain, and text has been a little bit modified to make it clearer. Now, please read Line 206 "This mutation causes a lack of a characteristic core lateral pentasaccharide in *Brucella* smooth LPS that eases its detection by TLR4 and therefore makes the mutant strain less virulent". In the three references (#21 and added here now #22 and #23), all information regarding the selection of the mutant and its features upon infection as well as the effect of its purified LPS is provided. Since total reference number is limited to 70, we could not add anymore. However, our review (reference #23) presents the latest and most complete survey on the matter.

6. Fig S2e, how does Ba-wt LPS orchestrates the increase of PD-1+CD 4+ cells and Sca-1+CD69+CD8+ T cells?

Because of the lack of space in the manuscript, we have now eliminated this piece of anecdotic information regarding Ba-wt LPS driven PD-1 and CD69 expression in T cells. We preferred to add the correspondence between IL1-RA-producing cells and the expression of PD-1 and LAG-3 in $CD4^+$, $CD8^+$ and Treg, during infection (Fig. 6j and S4h), which is by far more meaningful as regards the impact on immunosuppression. Anyway, further experiments are required to answer Reviewer #3's question, which findings may be the scope of another publication.

7. What is difference of LPS core between Brucella and E. coli?

As mentioned above (answer to concern 5) *Brucella* LPS differs in his whole structure from the canonical *E. coli* LPS. To make things more understandable, we now added in the text, line 217 "canonical LPS form *E. coli* (Ec), which differs from that of *Brucella* LPS by its overall structure", together with reference #23, our review which recaps all features of *Brucella* LPS. It states: "In contrast to the LPSs of enterobacteria, such as *Escherichia coli* or *Salmonella* spp., *Brucella* LPS displays different physicochemical properties, resulting in distinct biological traits, such as a low endotoxicity, an increased resistance to macrophage degradation, and a low immune response induction [61–64]. *It differs in its whole structure from the canonical E. coli LPS and harbours a different O-chain, central core, and lipid A [65]. The particular O-chain of Brucella LPS allows bacteria (except B. ovis and B. canis that produce a rough LPS without O-chain) to enter hosts cells through interactions with lipid rafts on their surface and to inhibit lysosome fusion in murine macrophages [66]. Brucella O-chain also impairs complement deposition and the activation of the lectin pathway of complement [67]*."

8. In Fig 3a, given that both Ba-wt and Ba-wadC LPS administration generated an early accumulation of Ly6G+ neutrophils in the omentum of mice, is deletion Ba-wadC able to establish chronic infection in omentum?

As shown in Fig. S1f, indeed at Day 15 p.i. (early chronic phase of infection), *Brucella* is detected both in the spleen and omentum of mice infected with the *wadC* strain. CFU are lower in these two organs when compared to those obtained after infection with the wt strain of *B. abortus*., indicating that 1- this strain is attenuated and 2- the Ba-*wadC* strain is also able to establish a chronic infection in the omentum. Noteworthily, while CFU/organ between the wt and the *wadC* strains vary in the spleen by a 1.17 Log fold, this variation is of 2 in the omentum. This marked effect in the omentum supports a paramount role of omentum in brucellosis. Text, line 212, has been modified as such: "Noteworthily, while CFU/organ between the wt and the wadC strains varied in the spleen by a 1.17 Log fold, this variation was of 2 in the omentum, supporting a paramount role of this organ in brucellosis.".

9. In Fig 6b, how long does IL-1RA exist after Brucella infection? Does it help brucella establish chronic infection?

To address Reviewer #3's question, we have measured IL-1RA by ELISA at the acute (D8 p.i.) and chronic phase (D30 p.i.) of infection. These results now included in Fig. 6g show that like after Ba LPS administration, IL-1RA is present in the omentum during the acute phase until the chronic phase of infection. Technically, we could not keep infected animals much longer in the animal house of the BSL3. This means that we cannot directly answer the question of Reviewer #3 about how long IL1-RA exists after *Brucella* infection. Concerning the other highly pertinent question related to the importance of IL-1-RA in establishing a chronic infection, please refer to our answer to Reviewer #2 (point 6). Basically, this question is at the present time impossible to address for technical reasons and difficulty to work in a BSL3 with very strict restricted access and regulations. In Fig. 7 legend, we are now more cautious and say "IL1-RA..." penultimate line, "is produced until the chronic phase of infection in the omentum, most likely favoring bacterial persistence.".

10. In Fig.7, the influx of macrophage from peritoneal fluid to omentum was not demonstrated.

Reviewer #3 is right indeed. No arrow was shown suggesting it in the scheme of Fig. 7 nor in the Fig. legend. The text clearly states, second sentence after fig. title, "*B. abortus* mobilizes macrophages, monocytes and neutrophils to the peritoneal cavity and the omentum, nearby milky spots." . No more.

11. Vascular and lymphatic connections are important part of omental, however, lymphatic vessel structure is not depicted in Fig. 7.

Reviewer #3 is right again and scheme has been modified accordingly with lymphatic vessels now depicted in Fig. 7.

12. Is IL-1RA secreted only by PD-L1+Sca-1+ omental neutrophils?

To simplify the study, already complex, and to focus on its novelty, i.e. the first immunosuppressive role for omental neutrophils, we did not include in our original submitted manuscript other cell types. This information is now included in the revised version of our manuscript in Fig. 6h, which shows that indeed, PD-L1^{+}Sca-1^{+} monocytes and F4/80^{int}MHCII^{hi} SPM macrophages are also producing IL-1RA during infection, with probably distinct functions (see Fig. 6j). Please see accompanying text line 362: "Most importantly, during infection in the mouse, IL-1RA" line 361 "produced by omental neutrophils, as well as monocytes and SPM at day 8 p.i. (Fig.6h). The IL-1RA^{+}Ly6G^{+} neutrophils were mostly PD-L1+Sca-1+ (Fig. 6i). Analysis of the correlation between each of the IL-1RA+ myeloid cell type with the total, PD-1^{+}, LAG-3^{+} or LAG-3^{+}PD-1^{+} CD4^{+} or CD8^{+} or Treg T cells during *B. abortus* infection revealed that IL1-RA+Ly6G+ neutrophils positively associated with CD4^{+} and CD8^{+} expressing both PD-1 and LAG-3 suppressive markers (Fig.6j, S4h) …. IL-1RA^{+} macrophages.". Please see also added text in discussion line 456: "During *B. abortus* infection, the positive association of IL-1RA-producing neutrophils with CD4^{+} and CD8^{+} T cells expressing both PD-1 and LAG-3 suggests that this T cell immunosuppressive mechanism is definitely acting. Yet, IL1-RA is additionally produced by monocytes; the privileged link of IL1-RA-producing monocytes with PD-1^{+}LAG-3^{+} CD4^{+} T cells and Treg during *B. abortus* infection opens up a complementary and specific role for these cells in immunosuppression, which needs future investigations.

13. There are no evidence that LPS core moiety directly contributed to neutrophils up-regulation of IL-1RA expression.

We agree with Reviewer #3 on this point. We now show that Ba-*wadC* LPS-challenged omental neutrophils decrease T cell responsiveness similarly to Ba-wt LPS-challenged omental or peritoneal neutrophils (Fig 5c). Moreover, *B. abortus* infection elicits IL-RA production by mostly PD-L1^{+}Sca-1^{+} neutrophils (as well as monocytes and SPM) (Fig. 6h, i). These findings demonstrate that the *wadC*-

encoded determinant of the *Brucella* LPS core does not control the immunosuppressive ability of Ba-LPS challenged neutrophils, but mediates the persistence of the PD-L1+Sca-1+ myeloid cells in the omentum during infection (Fig. 3b, S2c-e).

14. What is special about the omentum in terms of its structure, biochemistry and immunity that makes it a long-term storage site for Brucellae?

Please refer to our review (reference #1), which details all the advantages of the omentum as a reservoir for intracellular bacteria and *Brucella* in particular. To cite a few: its high vascularization by both blood and lymphatic vessels; the presence of immune cells and their induced influx in order for the bacteria to take over the immune response at its own advantage (immunosuppression); a rich nutrient source, since it is full of adipocytes.

15. Why did Milky spots become portals for Mycosis to invade the omentum?

We do not understand the question of Reviewer #3. What was he thinking of? For brucellosis, as mentioned in our review (reference #1) the omentum offers a number of assets, detailed in our answer to point 14. They make this organ a privileged reservoir for these intracellular bacteria, which most likely reach the omentum during natural infection through the blood and lymphatic vessels.

16. LPS should be extracted and the difference between LPS of wad C knockout bacteria and wild type bacteria should be compared by silver staining, which could be a challenge.

We have been using highly purified LPS which was extracted from the wt- or *wadC* strains of *Brucella abortus*. An experiment comparing these two LPS as suggested by Reviewer #3 has been already done and published (see our reference `#21 Conde-Alvarez et al., 2012 PloS Pathogens 2012).

17. Is the omentum, as a storage organ, specific to B. abortus or universal to Brucella species? More evidence are needed.

To answer Reviewer #3's question, we have infected mice with *B. melitensis* and determined CFU in the omentum, spleen and peritoneal fluid fluid at D8 and D30 p.I.. It appears that *B.melitensis* gives a similar pattern to that observed after *B. abortus* infection at day 8 p.i.., with a significant colonization of the omentum and much fewer CFU in the peritoneal fluid. However, only CFU in the spleen persisted at Day 30 p.i. with *B. melitensis*. This denotes a specificity of *B. melitensis*, that is out of the scope of this manuscript, which describes a novel immunosuppressive mechanism and organ reservoir for *B. abortus*. This is why we chose not to include these data in the present version of our manuscript. Therefore, we have dampened our conclusion. Please now note that for accuracy *Brucella* has been changed to *Brucella abortus* in the title, and to *B. abortus* in the abstract line 54, introduction lines 86, 87 and 89, results lines 202 and 249, discussion lines 390, 395, 411, 413 and 473, Figure 7 legend; lines 1051 and 1057.

18. Will the proliferation titer of Brucella in the omentum be enhanced by direct injection of IL-1RA into mice? Will chronic infection be established after injection?

Please read our reply to Reviewer #2's comment #6, which describes this experiment that we did twice, and explains why we did not see any difference between saline- and ANAKINRA-injected animals. We'd like to mention that technically it is impossible to 1- dispense higher doses subcutaneously without two or three injections daily, which we cannot do, 2- inject intraperitoneally daily the drug, as it would perturb the myeloid cell equilibrium in the peritoneal cavity, 3- inject through a peristaltic pump the drug, as we do not have the authorization for such procedure in the BSL3 of our collaborators, which would take more than a year to get.

19. The omentum is rich in adipocytes. Do adipocytes in omentum play a role in Brucella infection of the omentum?

Experiments with preadipocytes in culture have shown that *B. abortus* (added at a high MOI 100 or 1000) is able to infect these cells as well as differentiated ones but have a negative impact on MMP production in the undifferentiated cells only (Reference #4). The fact that the authors show that *B. abortus*-infected macrophages inhibit adipogenesis in a TNFalpha-dependent manner in vitro reveals the existence of a crosstalk between preadipocytes-adipocytes and macrophages. In our Ds-Red *B. abortus* infection experiments (in total n=6), the only cells that we detected positive for Ds-Red were macrophages (see Fig.1c-d and our comment to Reviewer #1's comment 1).

20. What is the accurate function of Brucella LPS core? Does it regulate the recruitment of macrophages, monocytes and neutrophils to the omentum, or the secretion of IL-1RA by neutrophils?

By showing that 1- the *wadC*-encoded determinant of *B. abortus* LPS core is involved in the persistence of PD-L1^{+}Sca-1^{+} neutrophils, monocytes and macrophages in the omentum (Fig. 3), 2- Ba-*wadC* LPS-challenged omental neutrophils are immunosuppressive in a manner comparable to that of Ba-wt LPS-challenged omental and peritoneal neutrophils (Fig. 5b, c and Fig. S4c-f), we now formally demonstrate what we had suggested in the original version of our manuscript, e. g. that the accurate function of the *wadC*-encoded core determinant is to maintain these immunosuppressive cells in omentum during infection. We have experimental data showing that omental Ba-*wadC* LPS-challenged neutrophils, like the Ba-wt-LPS challenged ones, similarly express IL-1RA. However, we chose not to include them, because results in Fig.5C and S4f present a further step, i. e. a demonstration of their suppressive ability, and also because text is already long. Main text has been unchanged (see our conclusion line 418 'We now show… Ba-wt-LPS dependent fashion". However, in the legend of Fig. 7, please now read: "These cells harbor an immunosuppressive phenotype, characterized by the co-expression of PD-L1 and Sca-1 that remains from the acute to the chronic phase of infection and is driven by *Brucella* LPS. Maintenance of these immunosuppressive cells in the omentum depends on the wadC-encoded determinant of *Brucella* LPS. ".

Point-by-point rebuttal to Reviewers' comments

We thank the Editor and the Reviewers for accepting our manuscript entitled "*Brucella abortus* impairs T lymphocyte responsiveness by mobilizing IL-1RA-secreting omental neutrophils" for publication in Nature Communications. Reviewers' comments undoubtfully helped us to improve our initial manuscript by performing additional experiments and clarifying a number of points in the text, including our first revised version. We are grateful to them for that. Given the extent of experiments done as well as technology involved (spectral cytometry) during the first revision of our manuscript, more authors were added and the order of the first co-authors had been changed to be fair (You will find the signature of all authors acknowledging these changes in the dedicated file). Please find below a point-by-point rebuttal to the few comments of the Reviewers on our first revised version (NCOMMS-23-35030A).

Answers to Reviewer #1's comments:

I appreciate the clarifications and additional data that the authors have provided. All of my concerns have been adequately addressed by either adding additional information or performing additional experiments.

We sincerely thank Reviewer #1 for his comments acknowledging the efforts done to end up with a manuscript fulfilling acceptance requirements.

Answer to Reviewer #2's comments:

The authors were responsive to all of my prior concerns. The new data clearly strengthen the role for omentum as a possible brucellae reservoir following infection.

We show appreciation to Reviewer #2 for recognizing the complete answer to his prior concerns. We also agree with him on the fact that the additional experiments performed bolster the role of omentum as a novel possible reservoir for *B. abortus* as well as the role of IL-1RA produced by neutrophils as a suppressive cytokine in brucellosis.

However, several minor concerns need to be addressed:

1) Figure legend 1 needs to be modified showing that B. abortus strain 2308 is being used. Outside of the Materials & Methods, the authors failed to define which B. abortus strain is being used.

To comply with Reviewer #2's request, please now read in the legend of Fig. 1 line 944: "**a** C57BL/6 mice were injected intraperitoneally (i.p.) with $1x10^6$ *B. abortus* strain 2308 CFU. At the indicated time…").

2) Figure legend 1 needs to be modified to include the doses used for IP and oral gavage infections. Again, these are not mentioned in the Results nor figure legend.

To follow Reviewer #2's request, please now read in the legend of Fig. 1 line 944: "**a** C57BL/6 mice were injected intraperitoneally (i.p.) with $1x10^6$ *B. abortus* strain 2308 CFU. At the indicated time …"), and line 948: "**b** C57BL/6 mice were infected by gavage with $1x10^9$ *B. abortus* strain 2308 CFU. At 30 days …".

3) Line 373: No where in the manuscript was Tregs defined. No mention of intracellular staining for Foxp3 was described nor antibody used for detection listed in Table S1. Figure S4h failed to define how Tregs were defined. Hence, the text and Figure S4 must be amended describing how Tregs were defined. The absence of intracellular Foxp3 staining would negate any conclusions regarding the presence of Tregs. Staining for CD25 alone is insufficient since CD25 is also increased on activated CD4+ T cells.

We thank Reviewer #2 for pointing out this oversight and his sharp reviewing. Although gating strategy for Treg was shown in Fig. 1f, we forgot to add the link to it in the text. We had not defined in the text Treg as these T cells are as well-known as macrophages or neutrophils. Text, line 357, is now modified as

such: “Analysis of the correlation between each of the IL-1RA$^+$ myeloid cell type with the total, PD-1$^+$, LAG-3$^+$ or LAG-3$^+$PD-1$^+$ CD4$^+$ or CD8$^+$ or CD4$^+$ CD62L$^-$CD44$^+$CD25$^+$GITR$^+$ T regulatory (Treg)(ref#46 Ronchetti et al., 2015 J Immunol Res 2015:171520 added) T cells during *B. abortus* infection… “. Please note that all references following #46 have been modified accordingly.

We agree with Reviewer #2 that staining for CD25 alone is insufficient to define T regulatory cells because CD25 is also expressed on CD4$^+$ activated T cells, as shown in our gating strategy for Treg presented in Fig. S1f (and not lacking, as mentioned by Reviewer who considered Fig. S4 only). From the whole activated CD4$^+$ population (CD62L$^-$CD44$^+$), Foxp3 is indeed required to identify Treg. GITR, because it is upregulated by Foxp3 (see McHugh et al., 2002 Immunity Vol. 16, 311-323, February; Tone et al., 2014 J. Immunol 192 (8): 3915-3924) has emerged a decade ago as another marker for functionally active Treg (Ronchetti et al., 2015 J Immunol Res 2015:171520. doi: 10.1155/2015/171520 now ref#46). Therefore, in our analysis, Treg were defined as CD4$^+$CD62L$^-$CD44$^+$CD25$^+$GITR$^+$ cells as shown in Fig. S1f. To comply with Reviewer #2’s request, you can now read in the text line 358 “or CD4$^+$CD62L$^-$CD44$^+$CD25$^+$GITR$^+$ T regulatory (Treg) (Ref# 46) T cells during *B. abortus* infection “, and in the legend of Fig. S1f “Spectral cytometry gating strategy for the analysis of F4/80^{hi} TIM4$^+$ (LPM) and F4/80^{int}MHCII^{hi} (SPM) macrophages, Ly6C^{hi} monocytes, Ly6G$^+$ neutrophils, CD4$^+$ and CD8$^+$ Tcells, and CD4$^+$CD62L$^-$CD44$^+$CD25$^+$GITR$^+$ Treg T cells with…“. and Fig.S4h “Spectral flow analysis of *B. abortus*-infected omenta showing normalized absolute numbers of total CD4$^+$, CD8$^+$ and CD4$^+$CD62$^-$LCD44$^+$CD25$^+$GITR$^+$ Treg T cells, or …“.

Answer to Reviewer #3’s comments:

The authors carried out all necessary experiments suggested by this reviewer,and addressed all my concerns for the primary manuscripts. The current paper is acceptable for publication in Nature Communications.

We thank Reviewer #3 for highlighting that all required experiments to address his previous concerns were conducted, leading to the acceptance of our manuscript for publication in Nature Communications.