Author’s response to reviews

Title: Mass cytometry analysis reveals a distinct immune environment in peritoneal fluid in endometriosis: a characterisation study

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Version: 2 Date: 11 Nov 2019

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Editorial comments:

1.) Please ensure Additional file 2 is referenced in the main text.
• We have referenced Additional file 2 in the main text (line 12, page 12).

2.) Authors Catherine Shang and Christian M Becker still need to confirm authorship for this manuscript. We will resend theamil requesting this, but would appreciate it if you could encourage them to complete this step.

• Author Christian M Becker stated that he has confirmed authorship. Author Catherine Shang has moved to a new work place. We have updated her current address through the online submission system and notified her to confirm authorship.

3.) Regarding your 'Change of authorship' form confirming the change in corresponding authors, please note that ALL authors need to confirm this change - not just the corresponding authors. Please submit a new form which includes signatures from all authors.

• We have included confirmation emails as a series of pdf from all co-authors showing their approval for the changing of corresponding authorship, this is in addition to 'Change of authorship' form signed by corresponding authors and the senior author.

Reviewer report:

Reviewer #1: Wendy Fantl

Guo et al. have submitted substantial revisions to their manuscript that have markedly improved it. In the interests of further clarity given the complexity of the subject matter and descriptive nature of the analysis, the following issues need to be addressed before publication.

• We appreciate the reviewer’s previous comments regarding our manuscript and we hope that the manuscript has substantially improved

Figure 1: the presentation of the MST in Figure 1B would be much clearer for the audience if it took on the format of Figure 3D and E where the specific immune cell subsets are named. This would be more meaningful to an audience than cell proportions out of a parent population. Additionally, this is the first time the audience will be introduced to MSTs.

• We have changed Figure 1B to the format of Figure 3D and referenced the MST plot in the text (line 11, page 12).

Although Figure 1D focuses on a macrophage branch of 1B, as mentioned in the text, nowhere are macrophages indicated on the figure making things confusing for the reader. In fact, monocytes are called out. For Figure 1D, please include a marker with negative expression such as CD3.
We thank the reviewer for pointing it out. We have corrected Figure 1B so that those cells are labelled as macrophages. Figure 1D shows the expression of activation markers on macrophage groups. However, expression levels of other markers on these groups are shown in Figure 1C. We have added a marker with negative expression (CD20) in Figure 1D on your request.

Page 16, line 12 to 14 states that PCA did not detect differences between the PF CD69+ cells from endometrial versus control PF samples. The case is made that the expression levels of activation markers on CD69+ cells in PF was lower than in blood immune cells. How then would CD69+ cells be used to define endometriosis?

We agree with the reviewer that our results do not claim CD69 defining endometriosis. We showed that CD69 cells define a distinct phenotype in T cell lineages from PF compared to blood counterparts and these CD69+ cells are increased in endometriosis samples compared to control samples. We have modified our statements in the manuscript to avoid confusing readers (line 17, page 2; line 15, page 14).

In the absence of any functional data, "CD69-associated cell phenotypes" should be omitted from the title. The study is a characterization of the endometrial immune environment and a resource for researchers and clinicians alike.

Based on the reviewer's request, we have deleted “CD69-associated T cell phenotypes” from the title.

Line 14 - use of the word "massive" is subjective. Please quote the percentages. There are other places in the text with this usage.

We agree with the reviewer that “massive” is subjective, we have removed this in the manuscript (line 14, page 16; line 19, page 17)

Figure 5B: No indication as to whether the changes in memory and naïve T cells are CD4 or CD8. Please clarify on Figure 5B.

We have changed Figure 5B to show both CD4 and CD8 memory and naïve T cells.

Again, I must return to the gating in the supplementary Figures S1 and S2. In their response, the authors correctly cite the use of DNA, event length and rhodium to gate viable single cell populations. Figure S1 shows that the initial gate was "cells" (presumably DNA) by "dead" (rhodium). However, gating first by DNA1 and DNA2 with a tight gate starts you off with single cells eliminating doublets or other cell aggregates at the beginning of the gating. This can then be followed with DNA by rhodium and the inclusion of other gates with event length. For Figure S2, unless the labeling is incorrect, gating a population especially a minor one (e.g DCs with CD123) straight out of CD45+ cells is not a typical practice and a hierarchical gating strategy is implemented to get the purest populations of cells that are used for downstream analysis and the
plots presented. A hierarchical gating strategy needs to be performed for the plots presented in 5C and elsewhere in the manuscript.

- We thank the reviewer for the diligent focus on our gating strategies. We indeed do use hierarchical gating throughout our work, as shown in Figure S2. Considering that our arrows in Figure S2 may be perceived as confusing, we have modified Figure S2 to clarify that downstream cell populations are derived from parent populations by connecting them using arrows.

- As discussed during our last revision, we compared our pre-gating (Dead/DNA1 first and then DNA1/DNA2) and the reviewer’s suggested gating strategy (DNA1/DNA2 first), results from these comparisons and downstream analyses show high resemblance between the two strategies. In addition, we also cited three publications to show that it is effective to use “dead”, “DNA1”, “DNA2” and “event length” in slightly different orders as pre-gating strategies. Therefore, we suggest to keep our initial pre-gating strategy.