Reviewer’s report

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

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Reviewer: Wendy Fantl

Reviewer's report:

Guo et al. present a revised and improved version of their manuscript entitled: "Mass cytometry analysis reveals a distinct Immune environment and CD69-associated T cell phenotype in peritoneal fluid in endometriosis". Specifically, the inclusion of all the specifications that ensure the samples are of high quality has greatly strengthened this manuscript. However, significant flaws need to be addressed as described below.

1. As acknowledged by the investigators this paper is a resource describing the immune cell landscape in endometriosis. Although the CD69 result is intriguing, no mechanism is provided as to its potential role in endometriosis. Greater emphasis should be placed on the resource aspect. In fact this should be stated in both the title and in the body of the manuscript.

2. In the abstract the writing needs editing. Sentence 1 does not imply the existence of an inflammatory response as written in sentence 2. "Our results demonstrate the presence of more than 40 different distinct immune cell types within the peritoneal cavity. This suggests that there is a complex and highly heterogeneous inflammatory response underpinning the pathology of endometriosis".

3. Page 9, line 16 - presumably the authors are talking about total CD3 T cells.

4. Page 11, line 10 - please clarify what is meant by "controls free of endometriosis…"

5. What was the age range of the patients and controls?

6. Regarding the CD45-negative cell populations no percentages are provided. Indeed for gating, proportions of cells is an important metric. This information is missing from all the gating in Figures S2 and S3. It needs to be compared patient by patient and compared with controls, donor-by-donor. Since the range of immune cell population frequencies is known for healthy PBMCs this is a critical control as a reference for the patient PFCs and PBMCs.

7. Regarding the gating strategy, while in their letter the authors claim that they adapted it from three citations, I find that there is minimal resemblance between those papers and what is presented in this manuscript. Furthermore, for the additional figure in the letter, no percentages of cell populations are provided making it impossible to say whether there...
were any changes in populations used for downstream analysis. I also note that in the added figure one of the biaxial plots has an axis labeled "dead" which is missing in figure S2 and S3. Is this the rhodium viability "dye"? Critical to include this in all gating. The gating strategy in Figure S2 is acceptable but Figure S3 is not. The populations shown need to be determined by hierarchical gating as I pointed out in my prior review. The NK cell gating is rather odd, especially taking our CD56 from the CD45 population. Also it seems that the authors are focusing on the expression of NK inhibitory receptors (NKG2A/CD94 and KIR2DL2-3) although no explanation is given for this choice versus other NK receptors such as TIGIT, DNAM-1, CD96. What was the strategy for choosing NK cell receptors?

8. Figure 1B - for this MST and all others what markers were used to perform the clustering? How many cells were sampled from each FSC file? For the MST in figure 1B it is unclear what parameter the colors are representing. Also, much clearer for the reader to encircle and point out the cell types, rather than cram numbers on circles which we can see clearly are large or small. The cell type identification is nicely shown in Figure 3D and E, but should be shown when the first MST is presented in Figure 1B.

9. For Figures 1A and 1B, they presumably represent composite plots. For readability, please mention this. It is very informative to look at viSNE and MST plots on a patient-by-patient basis. This would be in keeping with the goal of this work to provide a reference for the scientific and medical community (see Bendall et al Science 2011 and Gonzalez et al. Cell Reports 2018).

10. For the heat-map, Figure 1C, indicate that rows are individual clusters.

11. Page 11 line 14 - use of the word "cluster" here is confusing as viSNE doesn't cluster cells.. Suggest "groupings of cells" or "dense regions"?

12. The data in Figure 2A clearly show different immune cell frequencies between PFCs and PBMCs from patients. This figure would be enhanced by including the immune cell type distribution from healthy donors. Please also specify composite versus patient-by-patient in figure legend. Figure 2G could be presented in a clearer way as it is really difficult to get the overall picture of what is going on.

13. For figure 3D and E, it seems that the X-shift clustering and subsequent MST generation was performed separately for PBMCs and PFCs. This should be repeated by combining FCS files from both and clustering which may enhance the differences in the immune landscape between each sample type. Ealthy control samples should also be included. Again, markers used for clustering should be noted.

14. For the data in Figure 5, the interpretation is provided that the CD69+ T cells have reduced activation and cytotoxicity. However, this is only based on marker expression and in order to make this statement functional assays need to be performed.

15. Typo "Ealthy control samples..".
Are the methods appropriate and well described?
If not, please specify what is required in your comments to the authors.

Yes

Does the work include the necessary controls?
If not, please specify which controls are required in your comments to the authors.

Yes

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