Responses to reviewers’ comments

We first would like to thank the reviewers for their time and efforts to review our manuscript. We have considered each of their insightful comments and edited the manuscript accordingly. In response to Comment 4 from Reviewer 3, we have re-run the entire analysis using only primary chemonaïve tumors before they received chemotherapy (pre-NACT). This was a valuable suggestion that strengthened the findings of our paper. Notably, separating the analysis based on chemotherapy exposure uncovered a prognostic relationship of CD68+ macrophages and CD4+ T cells. The manuscript has been updated to reflect these results.

Below we describe the specific changes we have made. Corresponding changes to the manuscript are colored in blue in the revised manuscript for ease of identification.

# Comments from Reviewer 2

*Steinhart et al. provide an interesting study on immune infiltrate of HGSOC and demonstrate increased survival with B cell infiltration. Importantly, the authors demonstrate that interaction of B cells with macrophages increases survival. This study is of importance since as the reviewers mention, there is discrepancy in the information on B cells and HGSOC survival data and the authors raise the point that interaction with other immune cells warrant further study to better understand the TME in HGSOC. There are a few minor revisions that may strengthen the paper.*

Thank you for a favorable assessment of our work and its potential significance. Below we carefully address your recommendations.

1. *The paper highlights the importance of understanding the effects of immune cells on treatment response. While the authors assess overall survival, there is not an assessment of disease-free survival. It would be helpful to also see disease-free survival from standard of care treatment and their association with B cell infiltrates (and B cell and macrophage interactions).*

Thank you for your comment. We agree that disease-free survival is an important additional outcome to consider along with our original outcome, overall survival. To address your concerns, we have added conducted an analysis of disease-free survival and its association with B cell infiltrates and B cell and macrophage interactions. The results of this analysis are presented in the supplemental material as Kaplan Meier curves (Supplemental Figure 1) and a table of median survival days (Supplemental Table 1). Supplemental Table 1 is also reproduced below. Neither B cell percentage (p = 0.83) nor B cell to macrophage interaction (p = 0.43) have a statistically significant effect on disease-free survival (Supplemental Figure 1). However, we see improvement in median survival time for the high vs. low B cell presence groups and for the high vs. low B cell – macrophage interaction groups for both survival outcomes. This indicates that the direction of the effect for disease-free survival is the same as the direction of the effect for overall survival.

We believe these effects are not statistically significant for the disease-free survival outcome because the small number of subjects without a disease-free event for this outcome (6-7 subjects) lead to insufficient power to detect a significant effect. However, these results are promising and should be explored in a larger study. We have also added the following line to the Results section of the revised manuscript on page 4:

“Kaplan-Meier curves were also constructed to assess the relationship between disease-free survival and B cell infiltrates (Supplemental Figure 1). Subjects with high B cell infiltration and high B cell – macrophage interaction expressed higher median disease-free survival, though results were not statistically significant (Supplemental Table 1).”

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Outcome** | **Variable** | **N** | | **Median Survival in Days (95% CI)** |
| **No event** | **event** |
| **Overall Survival** | B cell Presence High | 24 | 28 | 2608 (1795-3075) |
| B cell Presence Low | 11 | 39 | 1559 (1157-1698) |
| **Disease-free Survival** | B cell Presence High | 6 | 46 | 686 (604-884) |
| B cell Presence Low | 7 | 43 | 664 (569-864) |
| **Overall Survival** | B cell – macrophage Interaction High | 24 | 27 | 2301 (1884-2738) |
| B cell – macrophage Interaction Low | 11 | 40 | 1559 (1075-1698) |
| **Disease-free Survival** | B cell – macrophage Interaction High | 7 | 44 | 692 (604-943) |
| B cell – macrophage Interaction Low | 6 | 45 | 661 (510-853) |

Supplemental Table 1: Median survival time (in days) for overall survival and disease-free survival outcomes, split by high and low B cell presence and high and low B cell – macrophage interaction.

1. *The visual overview is quite useful however without a legend, it is unclear what is being displayed in B and C. It would also be beneficial to see the cell coordinates and phenotypes image for a low interaction and a high interaction example.*

We are glad you found our visual overview useful and believe our edits to the overview in response to your comments have clarified its interpretation for future readers. The updated visual overview is attached to our revised manuscript. Specifically, we have added text to images B and C of the upper left panel of the visual overview to indicate that B is a cartoon representation of a multi-spectral immunohistochemistry image, and C shows cell and nuclei segmentation within that image. We have also added a picture of the cell coordinates to the phenotypes image for each (low and high) interaction example.

1. *Why was 25 microns chosen as the cutoff for interaction?*

This is a great question. Macrophages are typically 20-21 microns in diameter and B cells are typically 5-7 microns in diameter. Our cell locations are measured from the center of the cell, so a 25 micron maximum interaction distance indicates that the outer edges of the interacting B cell and macrophage are within very close proximity (12-14 microns apart at most). However, to ensure that our findings are robust to other interaction distance cutoffs we performed a sensitivity analysis at 20 and 30 micron distance cutoffs. Specifically, we reran the adjusted Cox Proportional Hazards model described in Table 1B of our original manuscript at interaction cutoffs of 20 and 30 microns. The results of this sensitivity analysis are provided in Supplemental Table 2, and recapitulate our main conclusion from the original manuscript that higher B cell-macrophage interaction is associated with increased overall survival (p = .002 and p = .004 at 20 micron and 30 micron cutoffs, respectively). We believe this sensitivity analysis bolsters the original findings in our paper, and have added the following sentence to the Methods section of our revised manuscript on page 3:

*“To verify the robustness of our interaction variable we performed a sensitivity analysis with interaction cutoffs and 20 and 30 microns. Results of this sensitivity analysis reflect those at a 25 micron interaction cutoff and are provided in the supplementary material.”*

# Comments from Reviewer 3.

*The manuscript describes spatial interactions between immune cell populations within ovarian tumors and try to establish correlations with clinical outcomes by using a TMA with clinical correlates and VECTRA staining for several immune markers. Description of neighboring cells within the TME of ovarian cancer is interesting, but lack of validation or mechanistic confirmation weakens the strength of the findings.*

Thank you for your positive evaluation of the importance of our work. We appreciate your suggestions for improvement, and we provide responses to each item below.

1. *Multiple prior reports have established a positive correlation between presences of CD8+ cells and clinical outcome in ovarian cancer. This is not confirmed in the current dataset and should be explained.*

We thank the reviewer for this comment. As noted by the reviewer, several reports have defined a positive prognostic value for the presence of CD8+ T cells within the tumor microenvironment ([1], [2] , [3]). While we did not observe CD8+ T cells were significantly associated with improved survival, there was a trend towards improved survival (Supplementary Figure 3). The discrepancy can be explained as a variation between “high” and “low” infiltration. Analysis of the multispectral IHC (mIHC) provides improves both spatial and composition resolution of immune cell infiltration into both the tumor and stromal compartments. Further, with mIHC the percentage cell type infiltrated is normalized to total cell numbers based on the number of nuclei, thus even tumor sections with only a few cells of certain type can be analyzed. In contrast, other studies ([1], [2], [3]) have used traditional DAB IHC staining and cut-offs of certain cell number to assign “positive” or “negative.” Given mIHC provides a continuous variable for T cell infiltration, the sample size is also limiting in the presented study. When evaluated as a correlation versus a KM curve, increased CD3+CD8+ infiltration correlates to improved overall survival in the primary tumor samples (excluding recurrent and NACT) (Response Fig. 2, Spearman r=0.2118, p=0.0517). These limitations and data have been described and included within the Discussion.



Response Figure 2. CD3+CD8+ cell infiltration correlates to improved overall survival.

1. *The main finding that presence of B cells and the proximity between B cells and macrophages are predictive of outcome is interesting and novel, however the percentages of these cells are very small. Have statistical analyses been corrected for multiple variable testing?*

We are thrilled that you find our main results novel and interesting! We agree that the rarity of the immune cells we are interested in makes them challenging to study, and have highlighted this point in the Discussion section of our revised manuscript.

Statistical analyses have not been corrected for multiple comparisons, and we apologize that this was not stated more clearly in the original manuscript. There are several reasons we chose not to correct for multiple comparisons in this analysis. The first is the highly exploratory nature of this analysis. Our analyses of this data were conducted without strong a-priori hypotheses because no previous work existed on the spatial interactions of immune subsets in the TME for ovarian cancer. In exploratory analyses without an a priori hypothesis and with a small number of primary tests it is recommended not to correct for multiple comparisons because this correction can increase type 1 error rate i.e. increase the change of missing out on true novel scientific findings ([4], [5] , [6]). However, a subsequent study with preplanned hypotheses should be conducted to confirm the observed associations in our manuscript. To be transparent to future readers that we have not corrected for multiple comparison in this analysis, we have made the following addition to the Methods section of the revised manuscript:

“Due to the exploratory nature of this analysis, p-values have not been corrected for multiple comparisons.”

1. *It is not clear why information about use of PARPi is relevant to the current analysis*

Deficiencies in homologous repair mechanisms, which can be exploited by PARP inhibitor treatment, are very common in epithelial ovarian cancer and so PARPi status was recorded during data collection. Though information regarding PARP inhibitor use is included with the TMA, the authors agree with the reviewer and that PARP inhibitor use is not relevant for the current study. Information about PARP inhibitor treatments has been removed from the adjusted Cox Proportional Hazards models in the revised manuscript. Our Table 1b from the revised manuscript reflects this change. Information about PARP inhibitor treatments has also been removed from the descriptive characteristics of the data provided in Table 1a, but can be made available upon request.

1. *The dataset is also not uniform. In particular inclusion of tumors collected post NACT(~18% of tumors) could skew results and change immune cell subsets compared to tumors collected prior to chemotherapy. Tumors post NACT should be analyzed separately or excluded from main analysis.*

Thank you for this insightful comment. We agree with the reviewer that neoadjuvant chemotherapy (NACT) induces nontrivial changes to the tumor microenvironment. In response to the reviewer’s comment, we have rerun the entire analysis from our original manuscript submission using only primary chemonaïve tumors (i.e., without NACT treatment). Notably, stratifying based on prior chemotherapy exposure revealed a significant relationship between CD4+ T cells and CD68+ macrophages. Moreover, while not significant, the presence of CD8+ T cells in the primary chemonaïve tumors did convey an improved survival, which is consistent with previous reports. The tables and figures in our revised manuscript now present results from analyzing only images from these primary chemonaïve tumors.

**References**

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