

Peer Review File

Single-cell transcriptome analysis unveils fatty acid metabolism-mediated metastasis and immunosuppression in male breast cancer



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Editorial Note:

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Reviewers' comments:**Reviewer #1 (Remarks to the Author):**

The manuscript "Single-cell transcriptome analysis reveals fatty acid metabolism mediated metastasis and immunosuppression in male breast cancer" is an interesting effort to characterize the differences between breast cancer in men and women, which is an understudied topic. The Authors performed single-cell analysis to identify notable differences in the two groups, such as ESR1 and AR activity and fatty acid metabolism mainly mediated by FASN expression. Moreover, the Authors describe higher levels of tumor purity, cell cycle genes and pathways related to tumor invasiveness, as well as lower immune infiltration in male breast cancer compared to samples from women, findings in line with bulk tumor samples in the TCGA. The Authors also describe the presence of immune-epithelial cells, and the potential relationship between fatty acid metabolism and metastasis-related programs. A prognostic role of these features is also suggested, using bulk data from the TCGA, as well as a potential therapeutic role for FASN inhibition as demonstrated by previous works. The relationship between fatty acid synthesis, immunosuppression and tumor progression is intriguing, but may require some clarification.

The following may need to be addressed:

- As single-cell experiments were performed in 3 male and 2 post-menopausal female breast cancer samples, the explorative nature of the findings should be addressed throughout the manuscript.
- Clinicopathological characteristics such as tumor stage may be relevant when comparing the male and female samples, to ensure that differences observed were not related to differences in the staging (e.g. larger and more advanced tumors may be associated with immune exhaustion). As per supplementary table 1, tumor stage was not available for

female breast cancer samples. The Authors should specify this in the Methods section (lines 406-411). Whether samples are from primary, untreated breast cancers should also be specified.

- Supplementary Table 1 and lines 406-411: Were ER/PR/HER2 positivity defined as per ASCO/CAP guidelines? How was AR positivity defined? What do the Authors mean by “Molecular classification”? Is it based on PAM50 subtyping or defined by IHC? In the latter case, “IHC classification” may be more appropriate. Furthermore, while it is true that all samples were ER-positive, Authors should also mention the presence of HER2-positive samples (1/3 in the male cohort and 2/2 in the female cohort), as biology of HER2-negative and positive tumors is substantially different. Was HER2 status (or ERBB2 expression levels) considered by the Authors in the comparative analysis?

- The Authors may want to specify in the Methods section how correlations were computed (Pearson? Spearman?). Moreover, whether P values are adjusted for multiple testing or not should also be specified.

- Line 176: “up-regulated” may be replaced with “upregulation”.

- Lines 219-226: The Authors describe a positive correlation between FASN expression and tumor purity, and a negative correlation not only with immune cells but also with CAFs and endothelial cells. The Authors then suggest that elevated expression of FASN may promote immune escape. Since higher tumor purity is also necessarily associated with lower stroma content (including immune cells), the cause-effect relationship suggested by the Authors may not be necessarily proved by these findings. This section may need to be adjusted accordingly.

- Similarly, the positive correlation with metastasis-related pathways (e.g. suggested in line 374-376) may not necessarily mean “causation”, as many other factors could play a role.

- Line 270 – Paragraph “MBC-specific T cells that co-expressed epithelial and immune markers were in the apoptosis stage”: here, the Authors describe the presence of T cells

showing both T and epithelial cell markers, suggesting the existence of “epithelial-T cells”. The Authors tried to exclude that this finding were to be related to technical artifacts in the single-cell analysis, and showed the coexistence of CD3E and KRT8 markers with immunofluorescence experiments.

Although intriguing, further validation in other available single cell datasets (e.g. doi: 10.1038/s41588-021-00911-1) is in my opinion warranted, as it would give more robustness to this finding.

Moreover, the section included in lines 278-284 may need to be explained in a clearer way. Indeed, as per Supplementary Figure 6, panel A (Differentially expressed genes and functional analysis of T cells between male and female patients), and as suggested by the Authors (lines 274-278), it seems that KRT genes are expressed only in T cells from sample M3 (and maybe to a lesser extent M1). Confirming these findings in other single-cell datasets would be useful to exclude that the presence of epithelial-T cells are patient-specific, as one may argue that they may not be specific of male breast cancer.

Moreover, the presence of several genes related to dissociation or cellular stress (e.g. mitochondrial gene, FOSB, JUNB, heat-shock protein genes) and ribosomal genes, may raise a concern regarding contamination at the droplet level by dying cells (that would go in the same direction of what is stated in lines 308-313, when the Authors mention the high expression of apoptosis-related genes in this cell type). In this regard, can the Authors exclude that the finding of epithelial-T cells is not related to a potential issue of contamination? Indeed, in case of contamination, filtering by the number of genes may not be enough to identify technical artifacts.

With regards to immunofluorescence (Figure 5 B), can the Authors quantify the number of cells with co-expression of the KRT8 and CD3E marker?

- Lines 504-509: Which statistical method was used to compare KEGG metabolic pathways in male and female clusters?

- Line 558: To ensure reproducibility, which criteria were used for the selection of ER-positive samples in the TCGA? Since some samples in the single-cell cohorts were HER2-positive according to Supplementary Table 1, why was this group excluded from the TCGA analysis?

- Lines 567-571: To further validate the reliability of gene sets derived from the single-cell dataset, these findings may be compared to those derived from available immune-deconvolution tools (e.g. MCP-counter, EPIC, TIMER, xCell...) including the cell types of interest (e.g. in terms of correlations).
- Line 229: From Supplementary Figure 4, it seems that FASN high is significantly ($P = 0.04$) associated with OS in female breast cancer, and not in males ($P = 0.27$), although only 12 male breast cancer samples were present in the TCGA. However, in the text, it is stated that “high expression of FASN could predict poor OS of male patients with BRCA”. This part may need to be rephrased. Related to the comment on lines 588-595, other survival end-points, especially in the breast cancer, may be more informative than OS for evaluating the prognostic value of FASN expression.
- Lines 588-595, Survival analysis: Since overall survival data has to be interpreted carefully in the TCGA, especially for luminal breast cancer (doi:10.1016/j.cell.2018.02.052), did the authors tested also other survival end-points (PFI, DFI)?
- Line 247: For the SingleR tool analysis, which reference dataset was used? From Supplementary Fig. 5 it seems that some cell types are relatively “mixed” together and not well defined in the t-SNE. Did the Authors double checked manually if the automatic annotations were reliable?
- Line 433 and 437: The Cell Ranger versions mentioned are discordant (v2.1.0 and 3.0.2). Is this correct?
- Line 449: Please clarify if cells with more or less than 2000 expressed genes were retained.
- Lines 454-455: Please rephrase specifying in a clearer way if UMI count and MT genes were used as regression terms in the ScaleData function(also, Authors may replace “ScaleDate” with “ScaleData” in the text).
- Line 465: Please specify if the default parameters were used in the IntegrateData function.

Was default integration from Seurat applied from the beginning on all cells, or just for specific cell types?

- Lines 475-480: Please explain in a clearer way this section. What did the “normal cell cluster” used in inferCNV included (e.g. normal epithelial breast cells, stromal cells, immune cells...)? Was it formed by “any other cell” that was not tagged as malignant?

- Line 492: Are P values adjusted for multiple testing or not? This should be stated in the method sections for the other analyses as well.

- Line 509: Which statistical test was used to perform this comparison of metabolic pathways between male and female clusters?

- Figure 3, panel G: Can the Authors add a value for the correlations showed?

- Figure 3, panel J: The difference between the groups of comparisons (cell types and FASN high/low cells) is not clear and may be specified in the Figure legend. Are the 4 main columns representing interactions with opposite directions?

Reviewer #2 (Remarks to the Author):

The is a well written and comprehensive manuscript describing the immune and metabolic landscape of male breast cancer.

The premise of this paper that male and female breast cancers are immunological and metabolically different is very compelling and may potentially provide new insights into therapeutic strategies. The investigators have carefully evaluated a broad range of proliferation, angiogenesis, and metabolic pathways as well as detailed immune characterization. The study includes a limited number (3 and 2) reference cases. The study is expanded by data from the TCGA.

Strength of the study include the clearly distinctive patterns that the evaluated male and female breast cancers. The single cell sequencing is elegantly done, and the figures are beautifully outlined and clearly delineated.

A major concern of the study is that the female breast cancers neither have ER expression (ESR1) nor ER activity. Male breast cancer is mostly ER+, whereas female breast cancer has a broad diversity ranging from triple negative disease to ER+ and HER2 positive disease. The immune landscape, EMT, angiogenesis is vastly different in these subtypes. Particularly, TNBC stand out in their immune profile. The data would be very much strengthened if the authors provided data on ER+ female breast cancer, to show how this is similar or different from an ER+ male breast cancer.

Furthermore, a more in-depth explanation on the significance of the findings. The error bars appear very wide in a large number of examples. How are the p-values adjusted for significance in this multi-parameter assessment?

TCGA data while compelling is not novel and may not provide sufficient annotations to clinical

Reviewer #3 (Remarks to the Author):

Male breast cancer (MBC) is associated with worse prognosis compared to female breast cancer and the cellular and molecular differences between the two remain unclear. The researchers used single-cell RNA (scRNA) sequencing and T cell receptor (scTCR) sequencing characterize the tumor microenvironment of MBC. They sequenced three MBC and two post-menopausal ER+ female breast cancers (FBC) and show evidence that MBC have lower immune infiltration, activated ER and AR regulons, higher fatty acid synthase (FASN) expression, and exhausted CD8 T cells. The authors identify a subset of T-cells that express epithelial cytokeratins. However, the manuscript is lacking good quality evidence for the existence of these epithelial-T cells. The authors should consider removing that entire section or provide additional experiments to validate their findings. Androgens have long been known to drive fatty acid synthase PMID: 9067276, and the authors show good evidence of AR regulon activation in MBC, perhaps more focus on the androgen receptor

would tie this story together. Overall, the study is of interest, but more experiments and analysis are needed for this study.

Specific comments

1. While two of the three MBC samples have low immune infiltrate, one actually has similar levels to the two other FBC samples (Figure 1e). Therefore, one cannot conclude that there are less immune cells in MBC, as this may just be a sampling artefact.

2. Please supply raw p-value and statistical test used in Fig.1g. There are only 12 male samples compared to 1085 female samples in the TCGA, therefore one likely cannot assume the MBC will represent a normal distribution unless proven.

3. Statistical test for Figure 1i needed in figure legend.

4. Representative IHC for foxp3 positive staining appears to be nonspecifically stain tumor cells (Figure 1h). The investigators perhaps should perform dual IF to demonstrate the FOXP3 staining is confined to Treg cells (CD4+). The details of the cohort in Figure 1 needs to be in the figure legend or text.

5. What does IHC look like for FASN and AR in this cohort from Figure 1h?

6. A hallmark of prostate cancer progression is dysregulation of lipid metabolism via overexpression of fatty acid synthase (FASN), a key enzyme in de novo fatty acid synthesis. Why was prostate cancer (PRAD) left out of the survival analysis stratified by FASN levels? Please include citation and discussion of targeting FASN in prostate cancer (PMID: 30578319).

7. Supplementary Fig. 2 legend description inadequate. What fold change and significance and testing performed?

8. Supplementary Fig. 4 legend needs more detail. How were FASN high and low cutoffs determined?

9. The fact that FASN and the ER- and AR-response genesets were significantly enriched by the up-regulated genes of “epithelial-T” co-expression cells, suggests that there may be mixing of epithelial and T cell RNA in these dual positive cells. Therefore, additional experiments are needed for the existence of “epithelial-T cells”. The authors provide dual immunofluorescence (IF), however the staining in Figure 5B is unconvincing. The legend states the scale bar is 50uM, but there is no scale bar and thus hard to interpret. It is not clear whether the staining is from a single mitotic cell or many cells at a distance. The DAPI does not even show uniform nuclear localization. The staining appears to be an artifact. The researchers need to show additional validation of the for IF using positive and negative control tissues. In addition, the investigators need to quantify the CD3 only and epithelial T cells for the IF. The authors should also provide another independent method to support their findings such as flow cytometry (KRT and CD3) of dissociated T cells from fresh tumor tissue if possible.

10. Supplemental Fig. S6a is described as differentially expressed gene across five samples. What are the individual values? Aggregated expression of all the single cells for each tumor? Perhaps showing the expression of KRT8/18/19 and CD3 across all cells annotated by cell type for each tumor would be more convincing for the existence of an epithelial T-cell. This will show the relative KRT levels in true epithelial cells relative to the T cells.

11. Supplemental Fig. 6b shows the percentage of T cells that express KRT (epithelial-T cells) is around 40%, and similar in Fig 5C, however in Fig 5A there it appears that nearly all cells co-expressed CD3 and KRTs. What are the proportions of epithelial T cells in the other MBCs and FBCs or is this just an occurrence in the M3 tumor?

12. The authors should consider evaluating several other scRNA breast cancer datasets for evidence of epithelial T cells.

13. Data availability section is weak, and data are not publicly deposited (this can be blinded

until publication but available for reviewers).

14. The authors should consider evaluating the role of AR in MBC in more detail. Such as performing IHC on specimens, evaluating the RNA-seq for existence to alternative splicing in the androgen receptor.

15. The authors need more detail in most figure legends. It is sometimes hard to interpret the data. For example, Figure 2g and h show expression and activation of transcription factors, but what cell types were evaluated (just epithelial)? There appears to be a bimodal distribution in these blots suggesting there the cells are either in an on or off state. It would be interesting to see what cells are on vs. off.

1 Reviewers' comments:

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3 **Reviewer #1**

4 The manuscript “Single-cell transcriptome analysis reveals fatty acid metabolism
5 mediated metastasis and immunosuppression in male breast cancer” is an interesting
6 effort to characterize the differences between breast cancer in men and women, which
7 is an understudied topic. The Authors performed single-cell analysis to identify notable
8 differences in the two groups, such as ESR1 and AR activity and fatty acid metabolism
9 mainly mediated by FASN expression. Moreover, the Authors describe higher levels of
10 tumor purity, cell cycle genes and pathways related to tumor invasiveness, as well as
11 lower immune infiltration in male breast cancer compared to samples from women,
12 findings in line with bulk tumor samples in the TCGA. The Authors also describe the
13 presence of immune-epithelial cells, and the potential relationship between fatty acid
14 metabolism and metastasis-related programs. A prognostic role of these features is also
15 suggested, using bulk data from the TCGA, as well as a potential therapeutic role for
16 FASN inhibition as demonstrated by previous works. The relationship between fatty
17 acid synthesis, immunosuppression and tumor progression is intriguing, but may
18 require some clarification.

19 The following may need to be addressed:

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21 1. As single-cell experiments were performed in 3 male and 2 post-menopausal female
22 breast cancer samples, the explorative nature of the findings should be addressed
23 throughout the manuscript.

24 **Response:** Thank you for your comments. In order to further support and validate the
25 conclusion in this study, we expand the sample size of both male and female breast
26 cancer. In this revised version, six MBC and thirteen FBC samples were included, in
27 which eleven FBC samples were from a previous study by Wu et al. (Nature genetics,
28 2021, 53(9): 1334-1347. doi: 10.1038/s41588-021-00911-1) and other samples were in-
29 house. All of the collected samples were ER⁺. The transcriptome of 58,578 and 52,460
30 single-cells was sequenced in MBC and FBC, respectively (**Response Figure 1**). By
31 performing the same analysis procedure using this updated dataset, we found that the
32 main results were consistent with the previous version, and demonstrated the followings:
33 (1) scRNA-seq, bulk transcriptome, and immunohistochemistry consistently
34 demonstrated that MBC had a significantly lower degree of T cell infiltration than FBC;
35 (2) metastasis-related programs such as cell migration, epithelial-mesenchymal
36 transition (EMT), and angiogenesis were more active in cancer cells from MBC than
37 FBC; (3) the activated fatty acid metabolism involved by FASN was related to the
38 cancer cell metastasis and low immune infiltration of MBC; (4) different characteristics

39 of T cell subpopulations between MBC and FBC were identified. T cells in MBC
40 showed activation of p38 MAPK and lipid oxidation pathways, indicating the
41 dysfunctional state. In contrast, T cells in FBC exhibited a higher expression level of
42 cytotoxic markers such as GZMK and KLRB1, and activated pathways mediated by
43 immune-modulatory cytokines; (5) the inhibitory interactions between cancer cells and
44 T cells in the MBC microenvironment were identified, such as cell-cell
45 communications mediated by TGF- β , TIGIT, and VSIR. (6) KRT⁺ T cells with high
46 level of fatty acid metabolism were enriched in the MBC microenvironment. These
47 observations were further validated in bulk-RNAseq data and molecular experiments.

48 Despite the rarity of MBC occurrence and the stringent sample requirements of
49 single-cell experiments, we had collected and sequenced six MBC samples as possible
50 as we can. As far as we know, this study is the first to characterize the differences
51 between MBC and FBC at the single-cell resolution. Benefiting from the enlarged
52 sample size (6 MBC vs. 13 FBC), we could statistically evaluate the significance of the
53 observed differences between MBC and FBC samples. On the other hand, we also
54 discussed the explorative nature of this preliminary study in the revised manuscript as
55 follows (**Lines 529-533**): “Due to the rarity of MBC occurrence and the stringent
56 sample requirements of single-cell experiments, only limited MBC samples were
57 included in this study. However, this explorative study identified notable differences
58 between MBC and FBC, especially the distinct metabolic and immunological
59 characteristics of MBC patients. These observations need to be further validated with
60 larger sample sizes in the future.”

61 [FIGURE REDACTED]

62 **Response Figure 1 (Related to Figure 1a in revised manuscript). Schematic workflow for data**
63 **collection and single-cell analysis in this study.**

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65 2. Clinicopathological characteristics such as tumor stage may be relevant when
66 comparing the male and female samples, to ensure that differences observed were not
67 related to differences in the staging (e.g. larger and more advanced tumors may be
68 associated with immune exhaustion). As per supplementary table 1, tumor stage was
69 not available for female breast cancer samples. The Authors should specify this in the
70 Methods section (lines 406-411). Whether samples are from primary, untreated breast
71 cancers should also be specified.

72 **Response:** Thank you for your professional suggestions. We added the tumor size and
73 TNM staging of each sample in the revised supplementary table 1. Besides, we
74 compared the clinical characteristics of the collected MBC and FBC samples. Results

75 showed that there were no significant differences in age, HER2 status, KI67 level, and
 76 extent of the tumor (T) between the FBC and MBC groups (**Response table 1**),
 77 avoiding the influence of these factors on the comparison. Due to the absence of tumor
 78 size and metastasis information of samples from Wu et al.’s study, only the categories
 79 of the tumor extent (T1 ~ T4) were compared between the two groups. Continuous
 80 variables, including age and Ki67 level, were compared using 2-sided Mann-Whitney
 81 U test. Categorical variables, including HER2 status and tumor extent, were compared
 82 using Fisher’s exact test. We added these comparison results in the revised manuscript
 83 as follows (**Lines 126-132**): “Considering some clinicopathological characteristics such
 84 as tumor stage may be associated with the immune microenvironment and metabolism
 85 of patients, we compared the clinical characteristics of the collected MBC and FBC
 86 samples. Results showed that there were no significant differences in age, HER2 status,
 87 KI67 level, and extent of the tumor (T1 ~ T4) between the FBC and MBC groups
 88 (**Supplementary Table 2**), avoiding the influence of these factors on the comparison.”

89 **Response table 1 (Related to Supplementary table 2 in the revised manuscript).**
 90 **Comparison of the clinical characteristics of the collected MBC and FBC samples**

	male(n=6)	female(n=13)	p-value
Age, <i>Median(IQR)*</i>	63.5(54.8-73)	55(52-67)	0.4293
HER2			
	2+ (n=1)	2+ (n=5)	
	+ (n=0)	+ (n=4)	0.1625
	- (n=5)	- (n=4)	
Ki67 (%) , <i>median(IQR)*</i>	25(12.5-30)	15(10-50)	0.9293
T Stage			
	T1 (n=2)	T1 (n=2)	
	T2 (n=3)	T2 (n=5)	0.7007
	T3 (n=1)	T3 (n=5)	
	T4 (n=0)	T4 (n=1)	

91 **IQR: interquartile range*

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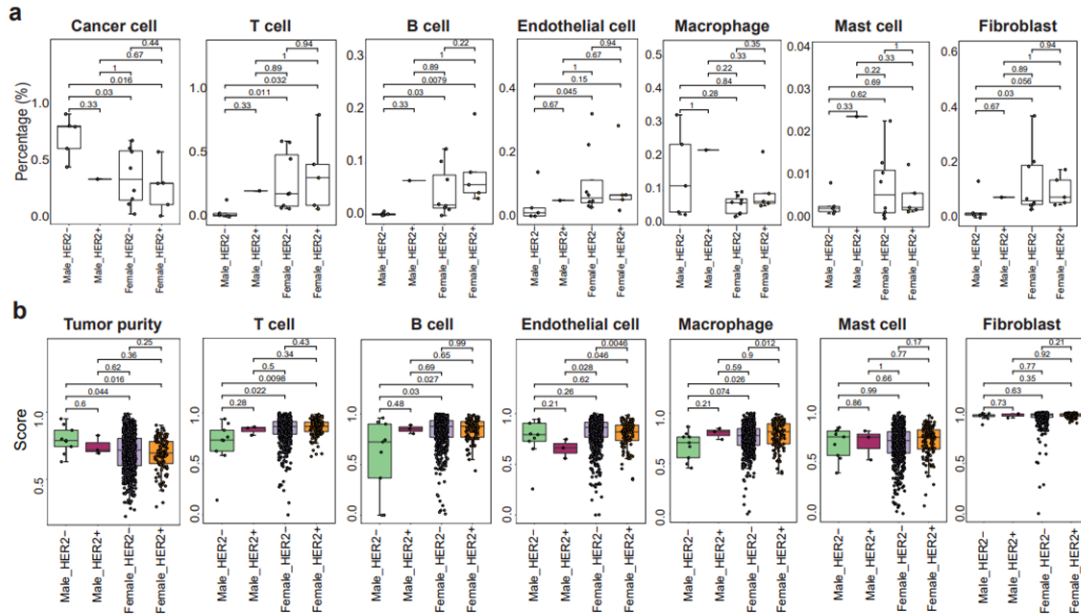
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94 Moreover, 18/19 samples were from primary untreated ER+ breast cancers, and
 95 FBC8 was from an ER+ female patient treated with neoadjuvant chemotherapy
 96 (**supplementary table 1**). We added the corresponding description in the revised
 97 Method section as follows (**Lines 550-552**): “Besides, 18/19 samples were from
 98 primary untreated ER+ breast cancers, and FBC8 was from an ER+ female patient
 99 treated with neoadjuvant therapy”.

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101 3. Supplementary Table 1 and lines 406-411: Were ER/PR/HER2 positivity defined as
102 per ASCO/CAP guidelines? How was AR positivity defined? What do the Authors
103 mean by “Molecular classification”? Is it based on PAM50 subtyping or defined by IHC?
104 In the latter case, “IHC classification” may be more appropriate. Furthermore, while it
105 is true that all samples were ER-positive, Authors should also mention the presence of
106 HER2-positive samples (1/3 in the male cohort and 2/2 in the female cohort), as biology
107 of HER2-negative and positive tumors is substantially different. Was HER2 status (or
108 ERBB2 expression levels) considered by the Authors in the comparative analysis?

109 **Response:** The ER/PR/HER2 positivity was defined according to the ASCO guidelines.
110 We defined the ER, PR, HER2, and KI67 status using IHC, and further evaluated the
111 amplification of HER2 based on FISH. The updated information was shown in the
112 revised supplementary table 1. All the collected samples (including MBC and FBC)
113 were negative for HER2 amplification evaluated by FISH (supplementary table 1). In
114 order to figure out whether the HER2 status evaluated by IHC was related to the
115 observation in this study, we further compared the immune infiltration, FASN
116 expression, and metastasis signature scores among groups of ER⁺HER2⁺ MBC,
117 ER⁺HER2⁻ MBC, ER⁺HER2⁺ FBC, and ER⁺HER2⁻ FBC samples. The following
118 results were found: (1) Both the scRNA-seq data and TCGA-BRCA data consistently
119 showed that the ER⁺HER2⁻ MBC samples had the highest level of cancer cell
120 enrichment and significantly lower level of T cell and B cell percentage (**Response**
121 **Figure 2**). Besides, it seemed that the T and B cell percentages were higher in
122 ER⁺HER2⁺ MBC than in ER⁺HER2⁻ MBC samples, although further evaluation was
123 needed in a larger cohort. (2) Cancer cells from MBC samples showed higher
124 expression of FASN than FBC samples, independent of the HER2 status (**Response**
125 **Figure 3**). (3) Cancer cells of MBC samples, including ER⁺HER2⁺ and ER⁺HER2⁻
126 samples, showed higher scores of metastasis-related signatures than FBC samples,
127 especially angiogenesis and cell migration (**Response Figure 4**). These results
128 indicated that MBC samples had a lower level of immune infiltration, especially
129 ER⁺HER2⁻ MBC samples. Both ER⁺HER2⁺ and ER⁺HER2⁻ MBC samples had more
130 active FASN expression and metastasis-related signatures than FBC samples.



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Response Figure 2 (Related to Supplementary Figure 4b-c in revised manuscript).

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Comparison of cellular components in ER⁺HER2⁻ MBC, ER⁺HER2⁺ MBC, ER⁺HER2⁻ FBC, and ER⁺HER2⁺ FBC samples in the scRNA-seq and TCGA dataset.

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(a) Boxplot showing the percentage of cancer cells, T cells, B cells, endothelial cells, macrophages, mast cells and fibroblasts

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in ER⁺HER2⁻ MBC, ER⁺HER2⁺ MBC, ER⁺HER2⁻ FBC, and ER⁺HER2⁺ FBC samples for ScRNA-seq data. HER2 status is defined by IHC experiments.

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(b) Boxplot showing the tumor purity and signature scores of T cells, B cells, endothelial cells, macrophages, mast cells and fibroblasts in

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ER⁺HER2⁻ MBC, ER⁺HER2⁺ MBC, ER⁺HER2⁻ FBC, and ER⁺HER2⁺ FBC in TCGA ER⁺ BRCA cohort. HER2 status is based on the IHC results in the clinical information of the TCGA-BRCA

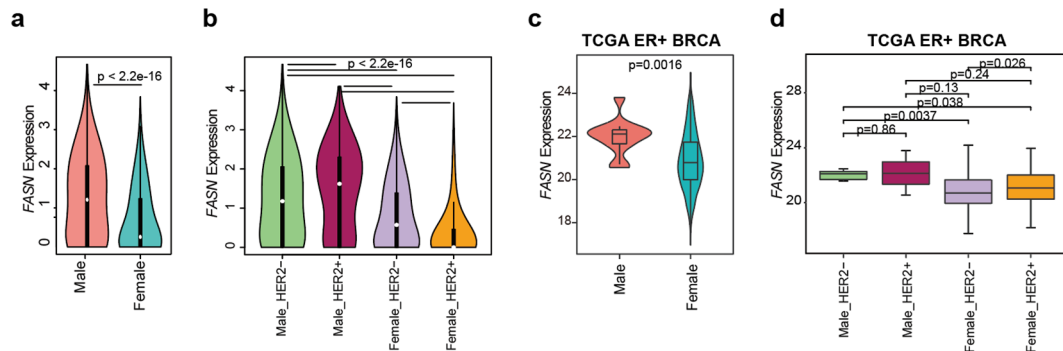
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dataset. P-value was calculated by two-sided Wilcoxon rank-sum test.

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Response Figure 3 (Related to Figure 4c-d and Supplementary Figure 8b, d in revised

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manuscript). The comparison of expression levels of FASN between MBC and FBC samples.

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(a) Violin plot of FASN expression in cancer cells from male and female samples. P-value was

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calculated by two-sided Wilcoxon rank-sum test. (b) Violin plot showing FASN expression in

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cancer cells from ER⁺HER2⁻ MBC, ER⁺HER2⁺ MBC, ER⁺HER2⁻ FBC, and ER⁺HER2⁺ FBC

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samples in our scRNA-seq dataset. P-value was calculated by two-sided Wilcoxon rank-sum test.

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(c) Violin-boxplots showing the FASN expression among male and female samples in TCGA ER⁺

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BRCA cohort. P-value was calculated by two-sided Wilcoxon rank-sum test. (d) Boxplot showing

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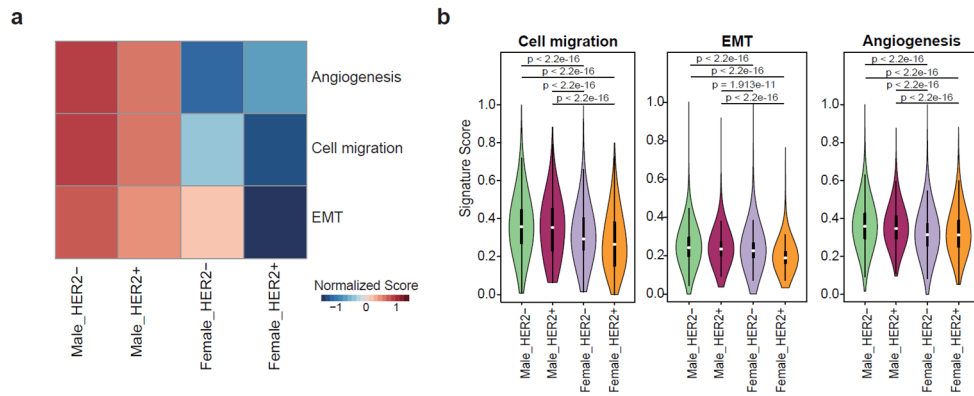
the FASN expression among ER⁺HER2⁻ MBC, ER⁺HER2⁺ MBC, ER⁺HER2⁻ FBC, and ER⁺HER2⁺

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FBC samples in TCGA BRCA cohort. P-value was calculated by two-sided Wilcoxon rank-sum

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test.



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Response Figure 4 (Related to Supplementary Figure 6a-b in revised manuscript). Comparison of metastasis signature scores of cancer cells in ER⁺HER2⁻ MBC, ER⁺HER2⁺ MBC, ER⁺HER2⁻ FBC, and ER⁺HER2⁺ FBC samples. (a) Heatmap showing the average ssGSEA scores of cell migration, EMT and angiogenesis in cancer cells from ER⁺HER2⁻ MBC, ER⁺HER2⁺ MBC, ER⁺HER2⁻ FBC, and ER⁺HER2⁺ FBC samples. (b) Violin plots comparing the scores of cell migration, EMT and angiogenesis of cancer cells from ER⁺HER2⁻ MBC, ER⁺HER2⁺ MBC, ER⁺HER2⁻ FBC, and ER⁺HER2⁺ FBC samples. P-value was calculated by two-sided Wilcoxon rank-sum test.

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We added the corresponding description in the revised Method section as follows (Lines 544-550): “Single-cell transcriptomic data from six MBC and thirteen FBC samples were analyzed, in which eleven FBC samples were collected from a previous study by Wu et al.⁵⁷, and other samples were in-house. All of the collected samples were ER⁺. We defined the ER, PR, HER2, and KI67 status using IHC, and further evaluated the amplification of HER2 based on FISH. The clinicopathological characteristics were shown in **supplementary table 1**. All the collected samples (including MBC and FBC) were negative for HER2 amplification evaluated by FISH.”

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Also, the corresponding results of immune infiltration were updated in the revised Results section as follows (Lines 166-175): “In order to figure out whether the HER2 status has an influence on the comparison of cellular components between MBC and FBC, we further compared the immune infiltration among groups of ER⁺HER2⁺ MBC, ER⁺HER2⁻ MBC, ER⁺HER2⁺ FBC, and ER⁺HER2⁻ FBC samples. Both the scRNA-seq data and TCGA-BRCA data consistently showed that the ER⁺HER2⁻ MBC samples had the highest level of cancer cell enrichment and significantly lower level of T cell and B cell percentages (Supplementary Figure 4b, c). Besides, it seemed that the T and B cell percentages were higher in ER⁺HER2⁺ MBC than in ER⁺HER2⁻ MBC samples, although further evaluation was needed in a larger cohort.”

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The comparison results of FASN expression among groups of ER⁺HER2⁺ MBC, ER⁺HER2⁻ MBC, ER⁺HER2⁺ FBC, and ER⁺HER2⁻ FBC samples were added in the revised Results section as follows (Lines 224-226): “Single cancer cells from both ER⁺HER2⁺ and ER⁺HER2⁻ MBC samples showed higher expression of FASN than FBC samples, independent of HER2 status (Supplementary Figure 8b).”

187 The comparison results of metastasis signature scores of cancer cells from
188 ER⁺HER2⁺ MBC, ER⁺HER2⁻ MBC, ER⁺HER2⁺ FBC, and ER⁺HER2⁻ FBC samples
189 were added in the revised Results section as follows (**Lines 195-197**): “Besides, cancer
190 cells from both ER⁺HER2⁺ and ER⁺HER2⁻ MBC showed higher scores of metastasis-
191 related signatures than FBC, especially angiogenesis and cell migration
192 (**Supplementary Figure 6**).”

193
194 4. The Authors may want to specify in the Methods section how correlations were
195 computed (Pearson? Spearman?). Moreover, whether P values are adjusted for multiple
196 testing or not should also be specified.

197 **Response:** Thank you for pointing this out. We apologize for not making this clear. The
198 correlations were calculated by the Pearson correlation coefficient (PCC). The p-values
199 here were not adjusted because each test was performed separately. We added the
200 corresponding description in the revised Method section as follows (**Lines 735-738**):
201 “Based on these signatures (Supplementary Table 4), we used ssGSEA to assess the
202 scores of tumor metastasis. The Pearson correlation coefficient between fatty acid
203 metabolism score and metastasis-related signature scores was calculated by the “cor.test”
204 function for TCGA pan-cancer samples.” The description in the Results section was
205 also revised as follows (**Lines 253-256**) “As our above results showed that cancer cells
206 from MBC patients had higher metastasis-related signature scores, we further explored
207 the correlations between fatty acid metabolism and metastasis in ER⁺ breast cancers of
208 the TCGA dataset by calculating the Pearson correlation coefficient (PCC)”. Besides,
209 the method of correlation analysis were added in the revised figure legends of Figure
210 4h-k, supplementary figure 3a, and 8g.

211
212 5. Line 176: “up-regulated” may be replaced with “upregulation”.

213 **Response:** Thank you. We replaced the “up-regulated” with “upregulation” in the
214 revised manuscript (**Line 203**).

215
216 6. Lines 219-226: The Authors describe a positive correlation between FASN
217 expression and tumor purity, and a negative correlation not only with immune cells but
218 also with CAFs and endothelial cells. The Authors then suggest that elevated expression
219 of FASN may promote immune escape. Since higher tumor purity is also necessarily
220 associated with lower stroma content (including immune cells), the cause-effect
221 relationship suggested by the Authors may not be necessarily proved by these findings.
222 This section may need to be adjusted accordingly.

223 **Response:** Thank you for your comments. We agree with the reviewer’s concern.
224 Accordingly, we revised the corresponding part as follows (**Lines 273-279**): “Thus, we
225 performed a pan-cancer analysis to evaluate the association between FASN expression

226 and immune infiltration in TCGA datasets. Results showed that FASN expression and
227 tumor purity were positively correlated in most cancers, while the infiltration scores of
228 T cells and B cells were negatively associated with FASN expression (**Supplementary**
229 **Figure 8h**). These results implied that the elevated expression of FASN may be
230 associated with the immune exclusion.”

231

232 7. Similarly, the positive correlation with metastasis-related pathways (e.g. suggested
233 in line 374-376) may not necessarily mean “causation”, as many other factors could
234 play a role.

235 **Response:** Thank you for your comments. We apologize for the inappropriate statement.
236 We revised the corresponding part as follows (**Lines 502-505**): “Notably, the fatty acid
237 metabolism showed a positive correlation with metastasis, and a negative correlation
238 with immune infiltration, implying the activated fatty acid metabolism might involve
239 in the immunological suppression and metastasis of MBC.”

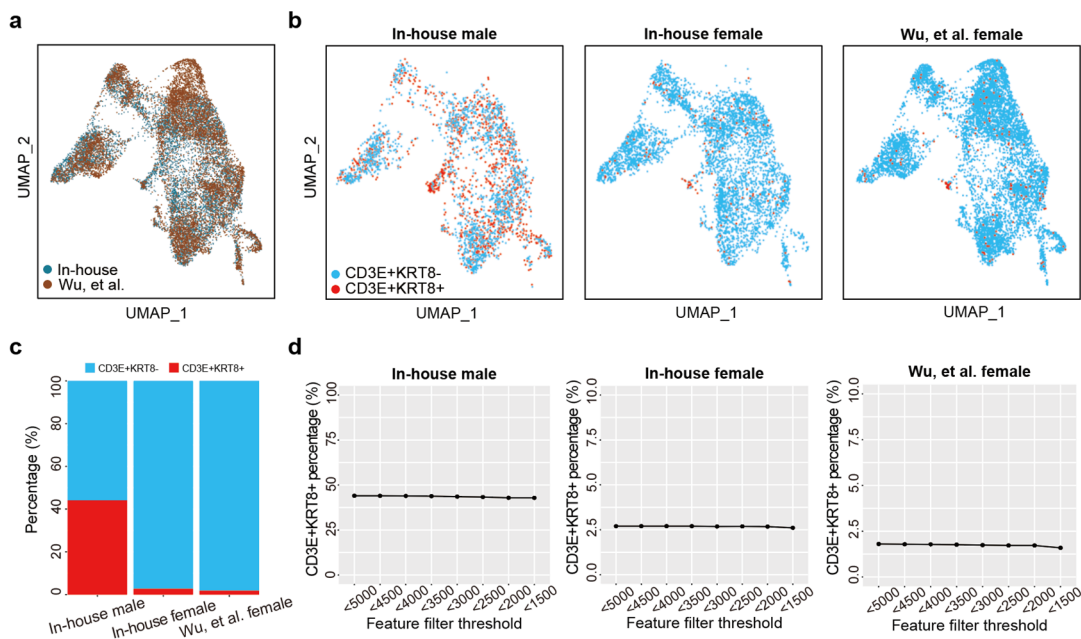
240

241 8. Line 270 – Paragraph “MBC-specific T cells that co-expressed epithelial and immune
242 markers were in the apoptosis stage”: here, the Authors describe the presence of T cells
243 showing both T and epithelial cell markers, suggesting the existence of “epithelial-T
244 cells”. The Authors tried to exclude that this finding were to be related to technical
245 artifacts in the single-cell analysis, and showed the coexistence of CD3E and KRT8
246 markers with immunofluorescence experiments.

247 Although intriguing, further validation in other available single cell datasets (e.g. doi:
248 10.1038/s41588-021-00911-1) is in my opinion warranted, as it would give more
249 robustness to this finding.

250 **Response:** Thank you for your valuable suggestion. With the development of single-
251 cell techniques, we could investigate the cellular characteristics at high resolution and
252 identify the previously unappreciated cells. Intriguingly, a study from Hu et al. reported
253 a non-traditional CD45⁺EpCAM⁺ cell population in the fallopian tube epithelial layer
254 of ovarian cancer patients (Hu et al., Cancer Cell, 2020, 37(2), 226-242). This
255 population was also positive for CD3, CD44, CD69, and CD103, suggesting that these
256 cells are possibly tissue-resident memory T lymphocytes (TRMs). They identified these
257 cells by scRNA-seq (Smart-Seq2) and validated them using immunofluorescence
258 experiments. However, the biological and clinical implications of this population are
259 unclear yet. To further validate the existence of “epithelial-T cells” in breast cancer, we
260 downloaded and performed an integrated analysis for the scRNA-seq data of ER⁺
261 BRCA from the previous study (Wu et al., Nature genetics, 2021, 53(9): 1334-1347)
262 suggested by the reviewer, in which all the samples were from female patients. By
263 integrating the transcriptomic data of T cells from in-house and Wu et al. (**Response**

264 **Figure 5a**), we calculated the percentage of CD3E⁺KRT8⁺ T cells of in-house MBC,
 265 in-house FBC, and Wu et al.'s FBC samples, respectively. Results showed that MBC
 266 samples had a significantly higher percentage of CD3E⁺KRT8⁺ T cells than the FBC
 267 samples from the two datasets (**Response Figure 5b, 5c**). Besides, the percentages of
 268 CD3E⁺KRT8⁺ T cells were similar in in-house and Wu et al.'s FBC samples (**Response**
 269 **Figure 5c**), suggesting the existence of CD3E⁺KRT8⁺ T cells and the enrichment of
 270 these cells in male samples. We also excluded the influence of doublets or multipl
 271 by evaluating the CD3E⁺KRT8⁺ T cell percentage under different cell-filtering criteria
 272 for in-house MBC, in-house FBC, and Wu et al.'s FBC datasets. Considering there may
 273 be more expressed genes that could be detected in doublets or multipl
 274 the number of expressed genes within each single cell using different cutoffs, rang
 275 from 1500 to 5000. Results showed that, in all of the three datasets, the percentag
 276 CD3E⁺KRT8⁺ T cells did not decline with the screening criteria became strict and
 277 remained at a robust level in all tests (**Response Figure 5d**), partially avoiding the
 278 technical artifacts caused by doublets or multipl
 279 that CD3E⁺KRT8⁺ T cells existed in both in-house and Wu et al.'s data, especially in
 280 MBC samples. We added these results in the revised manuscript (**Lines 355-361**) as
 281 follows: "To further validate the existence of these cells, we calculated the percentag
 282 of CD3E⁺KRT8⁺ T cells of in-house MBC, in-house FBC, and Wu et al.'s FBC sampl
 283 respectively (**Supplementary Figure 13a, b**). Results showed that the percentages of
 284 CD3E⁺KRT8⁺ T cells were similar in in-house and Wu et al.'s FBC samples
 285 (**Supplementary Figure 13c**). MBC samples had a significantly higher percentage of
 286 CD3E⁺KRT8⁺ T cells than the FBC samples from the two datasets (**Supplementary**
 287 **Figure 13c**)."



288
 289

Response Figure 5 (Related to Supplementary Figure 13a-b and Supplementary Figure 14a in

290 **revised manuscript). Evaluation of the existence of CD3E⁺KRT8⁺ T cells in the scRNA-seq**
291 **dataset. (a)** T-SNE plot of T cells colored by data sources. **(b)** T-SNE plots showing the distribution
292 of CD3E⁺KRT8⁻ and CD3E⁺KRT8⁺ T cells in in-house MBC samples (left), in-house FBC samples
293 (middle), and FBC samples from Wu et al. (right). **(c)** Barplot showing the percentage of
294 CD3⁺KRT8⁺ T cells in different datasets. **(d)** The line chart showing the percentage of
295 CD3E⁺KRT8⁺ T cells under different feature filter thresholds in in-house MBC samples (left), in-
296 house FBC samples (middle), and FBC samples from Wu et al. (right).

297

298 9. Moreover, the section included in lines 278-284 may need to be explained in a clearer
299 way.

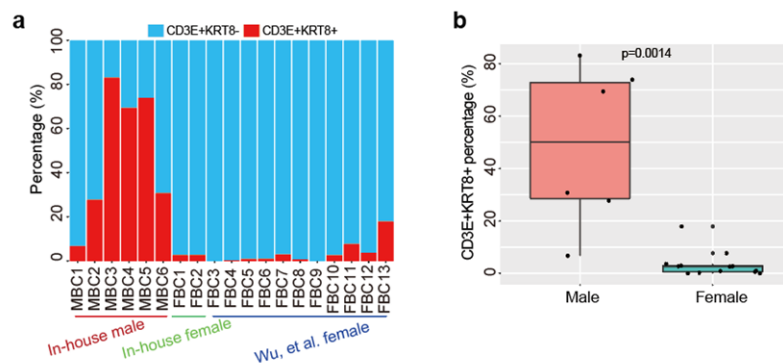
300 **Response:** Thanks for mention it. We revised the corresponding explanation as follows
301 **(Lines 378-390):** “By evaluating the CD3E⁺KRT8⁺ T cell percentage under different
302 cell-filtering criteria, we excluded the influence of low-quality cells that would be
303 possibly included during the tissue dissociation, including the doublets or multiplets
304 and broken/dying cells. Considering there may be more expressed genes that could be
305 detected in doublets or multiplets, we limited the number of expressed genes within
306 each single cell using different cutoffs, ranging from 1500 to 5000. Also, dying or
307 broken cells often exhibit extensive mitochondrial contamination. Thus, we calculated
308 the percentage of reads that mapped to the mitochondrial genome in each single cell.
309 Gradient cell-filtering criteria were performed to limit the number of expressed genes
310 and mitochondrial reads percentage. Results showed that the percentage of
311 CD3E⁺KRT8⁺ T cells did not decline with the screening criteria becoming strict and
312 remained at a robust level in all tests **(Supplementary Figure 14a, b)**, partially
313 avoiding the technical artifacts caused by low-quality cells.”

314

315 10. Indeed, as per Supplementary Figure 6, panel A (Differentially expressed genes and
316 functional analysis of T cells between male and female patients), and as suggested by
317 the Authors (lines 274-278), it seems that KRT genes are expressed only in T cells from
318 sample M3 (and maybe to a lesser extent M1). Confirming these findings in other
319 single-cell datasets would be useful to exclude that the presence of epithelial-T cells are
320 patient-specific, as one may argue that they may not be specific of male breast cancer.

321 **Response:** In order to figure out whether the observed CD3E⁺KRT8⁺ T cells were
322 patient-specific or generally existed, we evaluated the percentage of CD3E⁺KRT8⁺ T
323 cells across 19 samples, including 6 in-house MBC samples, 2 in-house FBC samples,
324 and 11 FBC samples from Wu et al.. It turned out that 17/19 breast cancer samples had
325 CD3E⁺KRT8⁺ T cells with different degrees, ranging from 0.2% to 83.1% **(Response**
326 **Figure 6a)**. Especially, MBC samples showed higher percentage of CD3E⁺KRT8⁺ T
327 cell component (6.7% ~ 83.1%), and FBC samples had relatively lower percentage (0.2%
328 ~ 17.9%). The Wilcoxon rank-sum test showed a significant difference of
329 CD3E⁺KRT8⁺ T cell enrichment between MBC and FBC groups **(Response Figure 6b;**

330 p-value: 0.0014). We added these results in the revised manuscript (Lines 361-377) as
 331 follows: “In order to figure out whether the observed CD3E⁺KRT8⁺ T cells were
 332 patient-specific or generally existed, we evaluated the percentage of CD3E⁺KRT8⁺ T
 333 cells across 19 samples, including 6 in-house MBC samples, 2 in-house FBC samples,
 334 and 11 FBC samples from Wu et al.. It turned out that 17/19 breast cancer samples had
 335 CD3E⁺KRT8⁺ T cells with different degrees, ranging from 0.2% to 83.1%
 336 (Supplementary Figure 13d). Especially, MBC samples showed higher percentage of
 337 CD3E⁺KRT8⁺ T cell component (6.7% ~ 83.1%), and FBC samples had relatively lower
 338 percentage (0.2% ~ 17.9%). We re-clustered the cells from each sample and then
 339 visualized all cell types and marker expressions at the single-cell level. MBC and FBC
 340 samples with the highest percentage of CD3⁺KRT⁺ cells were shown in Supplementary
 341 Figure 13e, f. To further evaluate the expression of KRT8/18/19 in T cells, we also
 342 showed the aggregated expression of these markers of epithelial and T cells in each
 343 sample using the dot-plot (Supplementary Figure 13g). The T cells from MBC2, MBC3,
 344 MBC4, MBC5, MBC6, and FBC13 had KRT8/18/19 expression, but were lower than
 345 these levels in epithelial cells. The Wilcoxon rank-sum test showed a significant
 346 difference of CD3E⁺KRT8⁺ T cell enrichment between MBC and FBC groups
 347 (Supplementary Figure 13h; p-value: 0.0014).”

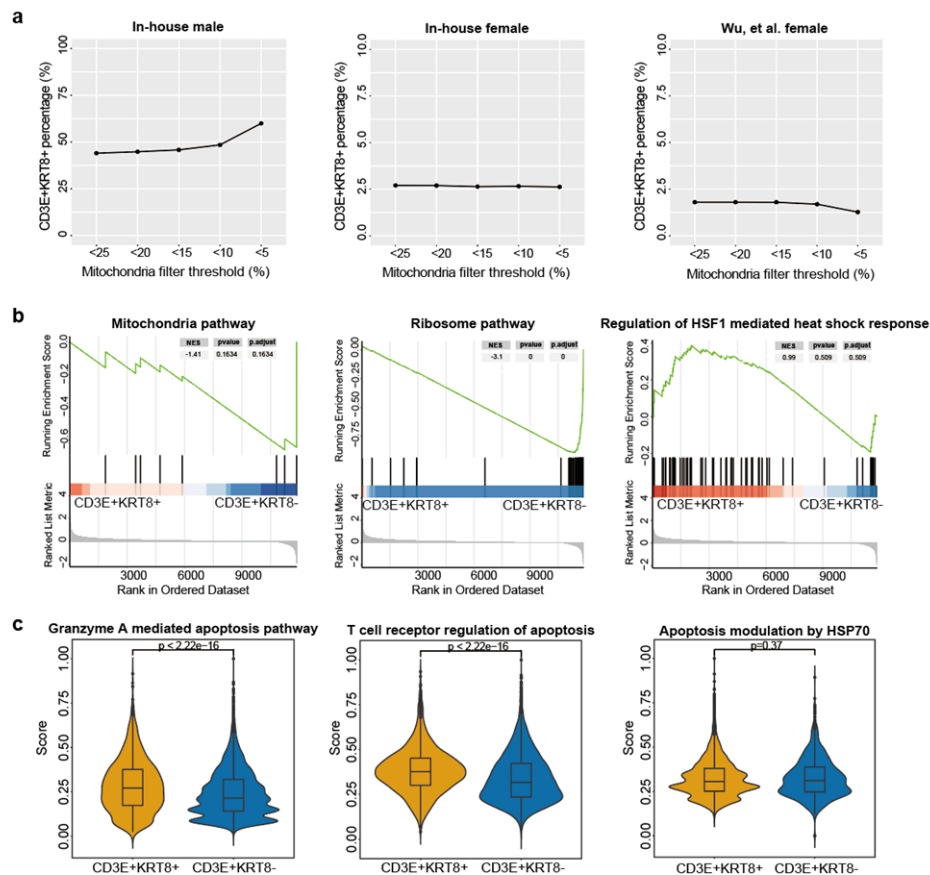


348 **Response Figure 6 (Related to Supplementary Figure 13d, h in revised manuscript).**
 349 **Evaluation of the existence of CD3E⁺KRT8⁺ T cells in the scRNA-seq dataset.** (a) Barplot
 350 showing the percentage of CD3⁺KRT8⁺ T cells in each MBC and FBC sample. (b) Boxplot
 351 comparing the percentage of CD3E⁺KRT8⁺ T cells between MBC and FBC samples. P-value was
 352 calculated by two-sided Wilcoxon rank-sum test.
 353

354
 355 11. Moreover, the presence of several genes related to dissociation or cellular stress (e.g.
 356 mitochondrial gene, FOSB, JUNB, heat-shock protein genes) and ribosomal genes, may
 357 raise a concern regarding contamination at the droplet level by dying cells (that would
 358 go in the same direction of what is stated in lines 308-313, when the Authors mention
 359 the high expression of apoptosis-related genes in this cell type). In this regard, can the
 360 Authors exclude that the finding of epithelial-T cells is not related to a potential issue
 361 of contamination? Indeed, in case of contamination, filtering by the number of genes
 362 may not be enough to identify technical artifacts.

363 Response: Thank you for your insightful comments. We agree with the reviewer that
364 some low-quality cells would be possibly included during the tissue dissociation,
365 including the stressed, broken, or dying cells, and doublets or multiplets. Firstly, by
366 performing the standard cell-filtering procedures that are commonly used in many
367 scRNA-seq studies, we had tried to limit the dissociation-related artifacts of multiplets
368 and broken/dying cells. Specifically, cells with expressed genes less than 200 or greater
369 than 6000 were excluded to remove the empty droplets and multiplets. Considering that
370 dying cells often exhibit extensive mitochondrial contamination, we calculated the
371 percentage of reads that mapped to the mitochondrial genome and filtered cells that
372 had >25% mitochondrial reads. Secondly, gradient cell-filtering criteria were
373 performed to limit the number of expressed genes and mitochondrial reads percentage.
374 Results showed that the percentage of CD3E⁺KRT8⁺ T cells did not decline with the
375 mitochondria filtering threshold (**Response Figure 7a**), indicating that the observation
376 of these cells may be not caused by technical artifacts. Thirdly, to further address the
377 concern of cellular stress and dying cell contamination, we performed GSEA analyses
378 using the signature of mitochondria, ribosome, and heat-shock protein for the gene
379 expression profile of T cells. Results showed that the up-regulated genes of
380 CD3E⁺KRT8⁺ T cells were not enriched by these signatures (**Response Figure 7b**).
381 Furthermore, we found that the CD3E⁺KRT8⁺ T cells had significantly higher
382 expression levels of genes related to ‘Granzyme A mediated apoptosis pathway’ and ‘T
383 cell receptor regulation of apoptosis’, but not enriched in ‘Apoptosis modulation by
384 HSP70’ (**Response Figure 7c**), indicating that the apoptosis of these cells was induced
385 by immune response rather than cellular stress. We added the description of the above
386 results in the revised manuscript as follows (**Lines 378-394**): “By evaluating the
387 CD3E⁺KRT8⁺ T cell percentage under different cell-filtering criteria, we excluded the
388 influence of low-quality cells that would be possibly included during the tissue
389 dissociation, including the doublets or multiplets and broken/dying cells. Considering
390 there may be more expressed genes that could be detected in doublets or multiplets, we
391 limited the number of expressed genes within each single cell using different cutoffs,
392 ranging from 1500 to 5000. Also, dying or broken cells often exhibit extensive
393 mitochondrial contamination. Thus, we calculated the percentage of reads that mapped
394 to the mitochondrial genome in each single cell. Gradient cell-filtering criteria were
395 performed to limit the number of expressed genes and mitochondrial reads percentage.
396 Results showed that the percentage of CD3E⁺KRT8⁺ T cells did not decline with the
397 screening criteria becoming strict and remained at a robust level in all tests
398 (Supplementary Figure 14a, b), partially avoiding the technical artifacts caused by low-
399 quality cells. To further address the concern of cellular stress and dying cell
400 contamination, we performed GSEA analyses using the signature of mitochondria,

401 ribosome, and heat-shock protein for the gene expression profile of T cells. Results
 402 showed that the up-regulated genes of CD3E⁺KRT8⁺ T cells were not enriched in these
 403 signatures (Supplementary Figure 14c).” and (Lines 422-425): “We found that the
 404 CD3E⁺KRT8⁺ T cells had significantly higher expression levels of genes related to
 405 apoptosis induced by the immune response, such as granzyme-A and T cell receptor
 406 mediated apoptosis pathway, but not enriched in the apoptosis related to cellular stress
 407 (Supplementary Figure 14d).”

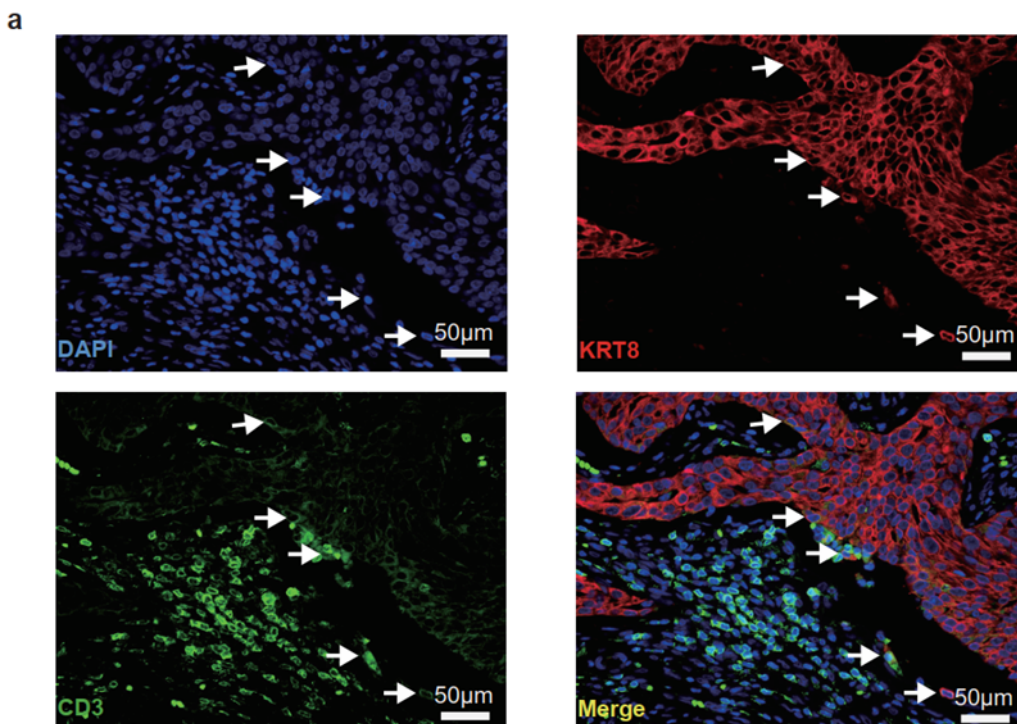


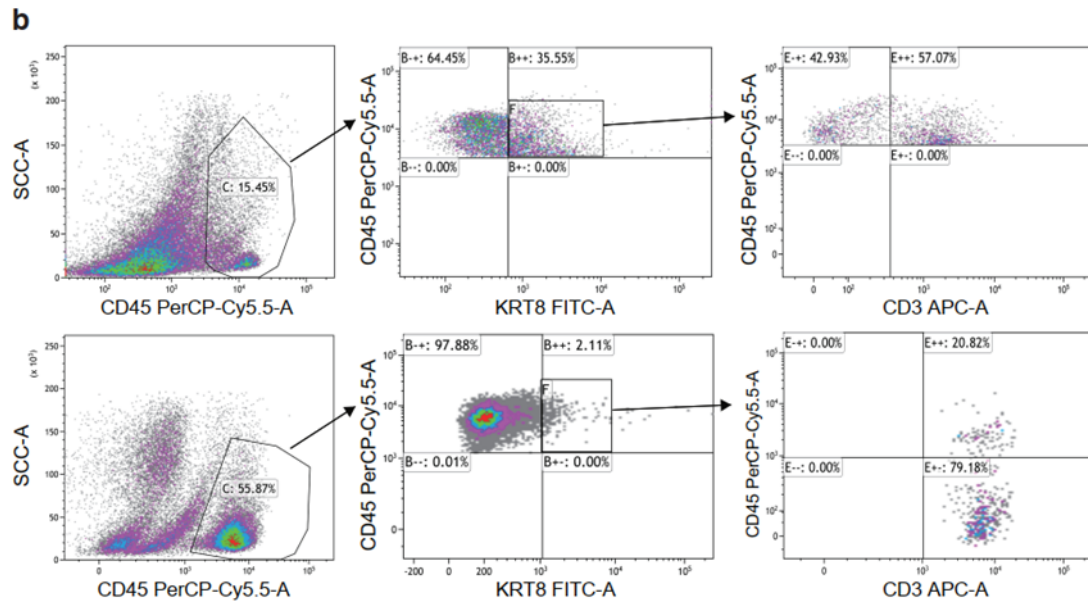
408
 409 **Response Figure 7 (Related to Supplementary Figure 14b-d in revised manuscript). Validation**
 410 **and functional analysis of CD3E⁺KRT8⁺ T cells.** (a) The line chart showing the percentage of
 411 CD3E⁺KRT8⁺ T cells under different mitochondria filter thresholds in in-house MBC samples (left),
 412 in-house FBC samples (middle), and FBC samples from Wu et al. (right). (b) GSEA analysis of
 413 mitochondria (left), ribosome (middle) and regulation of HSF-1 mediated heat shock response (right)
 414 pathway between CD3E⁺KRT8⁺ and CD3E⁺KRT8⁻ T cells. (c) Violin plots showing the scores of
 415 apoptosis-related pathways in CD3E⁺KRT8⁺ and CD3E⁺KRT8⁻ T cells.

416
 417 12. With regards to immunofluorescence (Figure 5 B), can the Authors quantify the
 418 number of cells with co-expression of the KRT8 and CD3E marker?

419 **Response:** Thank you for your professional suggestion. We are sorry for the unclear
 420 immunofluorescence results in the previous version. According to the advice from
 421 reviewer #3, we performed the immunofluorescence experiments again and showed the
 422 cells with different phenotypes, including CD3E⁺KRT8⁻, CD3E⁻KRT8⁺, and

423 CD3E⁺KRT8⁺ cells. According to the immunofluorescence, CD3E⁺KRT8⁺ cells were
424 located at the interface between KRT8⁺ epithelial cells and CD3⁺ T cells (**Response**
425 **Figure 8a**). Furthermore, flow cytometry of KRT8 and CD3 was performed using fresh
426 tumor tissues from two MBC patients to validate and quantify the number of
427 CD3E⁺KRT8⁺ cells (**Response Figure 8b**). We gated the CD45⁺ immune cells and
428 evaluated the expression of KRT8 of these cells. Results showed that there were 35.55%
429 and 2.11% CD45⁺KRT8⁺ cells in two samples, respectively. Notably, 57.07% and 20.82%
430 of these KRT8⁺ immune cells were CD3⁺ T cells in two samples. Thus, the
431 immunofluorescence and flow cytometry experiments indicated that the CD3⁺KRT8⁺
432 cells existed with various percentage in MBC samples. We added these corresponding
433 evidence in the revised manuscript as follows (**Lines 395-405**): “Further validation
434 using immunofluorescence experiments for the MBC sample confirmed the above
435 observation and showed that the CD3⁺KRT8⁺ cells were located at the interface
436 between KRT8⁺ epithelial cells and CD3⁺ T cells (Figure 6c). Furthermore, flow
437 cytometry of KRT8 and CD3 was performed using fresh tumor tissue from two MBC
438 patients to validate and quantify the number of CD3⁺KRT8⁺ cells (Figure 6d). We gated
439 the CD45⁺ immune cells and evaluated the expression of KRT8 in these cells. Results
440 showed that there were 35.55% and 2.11% CD45⁺KRT8⁺ cells in two samples,
441 respectively. Notably, 57.07% and 20.82% of these KRT8⁺ immune cells were CD3⁺ T
442 cells in two samples. Therefore, these results indicated the biological existence of
443 CD3⁺KRT8⁺ T cells and the enrichment of these cells with various percentages in MBC
444 samples.”





446

447 **Response Figure 8 (Related to Figure 6c-d in revised manuscript). Validation of the existence**
 448 **of CD3⁺KRT8⁺ T cells by the immunofluorescence and flow cytometry experiments. (a) The**
 449 **immunofluorescence staining of KRT8 and CD3 in an MBC sample. White arrows indicate the**
 450 **CD3⁺KRT8⁺ T cells. Scale bar, 50 μ m. (b) Flow cytometry showing the percentage of CD3⁺KRT8⁺**
 451 **cells in two MBC samples.**

452

453 12. Lines 504-509: Which statistical method was used to compare KEGG metabolic
 454 pathways in male and female clusters?

455 **Response:** Sorry for our unclear description. The differentially activated metabolic
 456 pathways between male and female cancer cell clusters were identified by the Wilcoxon
 457 rank-sum test. We revised the corresponding description as follows (**Lines 644-651**):
 458 **“The analysis of the metabolic pathways was performed as described previously by**
 459 **Xiao et al.⁶². Single-sample GSEA (ssGSEA) scores were calculated for 85 Kyoto**
 460 **Encyclopedia of Genes and Genomes (KEGG) metabolic pathways based on gene**
 461 **expression levels⁶³. The activity difference of KEGG metabolic pathways between male**
 462 **and female cancer cell clusters was measured by two-sided Wilcoxon rank-sum test. P-**
 463 **values were adjusted for multiple testing using the Benjamini-Hochberg method.**
 464 **Pathways with adjusted p-value less than 0.05 were identified as differentially activated**
 465 **pathways between male and female cancer cell clusters.”**

466

467 13. Line 558: To ensure reproducibility, which criteria were used for the selection of
 468 ER-positive samples in the TCGA? Since some samples in the single-cell cohorts were
 469 HER2-positive according to Supplementary Table 1, why was this group excluded from
 470 the TCGA analysis?

471 **Response:** We selected the ER⁺ TCGA-BRCA samples based on the clinical

472 information in the XenaBrowser website (<https://xenabrowser.net/datapages/>).
473 Specifically, 835 primary tumor samples with positive
474 breast_carcinoma_estrogen_receptor_status were selected, including both HER2⁺ and
475 HER2⁻ samples. Samples without RNA-seq data were further removed. Finally, we
476 obtained the transcriptomic and clinical data of 722 ER⁺ TCGA-BRCA samples,
477 including 598 ER⁺HER2⁻ FBC, 112 ER⁺HER2⁺ FBC, 9 ER⁺HER2⁻ MBC, and 3
478 ER⁺HER2⁺ MBC samples. To figure out the influence of HER2 status on the
479 observation in this study, we further compared the immune infiltration, FASN
480 expression, and metastasis signatures scores among these four groups (**Response**
481 **Figure 2-4**). We added the description for sample selection of TCGA data in the revised
482 Methods section as follows (**Lines 711-720**): “Bulk transcriptomic data and clinical
483 information from The Cancer Genome Atlas (TCGA) database were downloaded and
484 extracted from the XenaBrowser website <https://xenabrowser.net/datapages/>. We
485 selected the ER⁺ TCGA-BRCA samples based on the clinical information. Specifically,
486 835 primary tumor samples with positive breast_carcinoma_estrogen_receptor_status
487 were selected, including both HER2⁺ and HER2⁻ samples. Samples without RNA-seq
488 data were further removed. Finally, we obtained the transcriptomic and clinical data of
489 722 ER⁺ TCGA-BRCA samples, including 598 ER⁺HER2⁻ FBC, 112 ER⁺HER2⁺ FBC,
490 9 ER⁺HER2⁻ MBC, and 3 ER⁺HER2⁺ MBC samples. HER2 status is based on the IHC
491 results in the clinical information of the TCGA-BRCA dataset.”

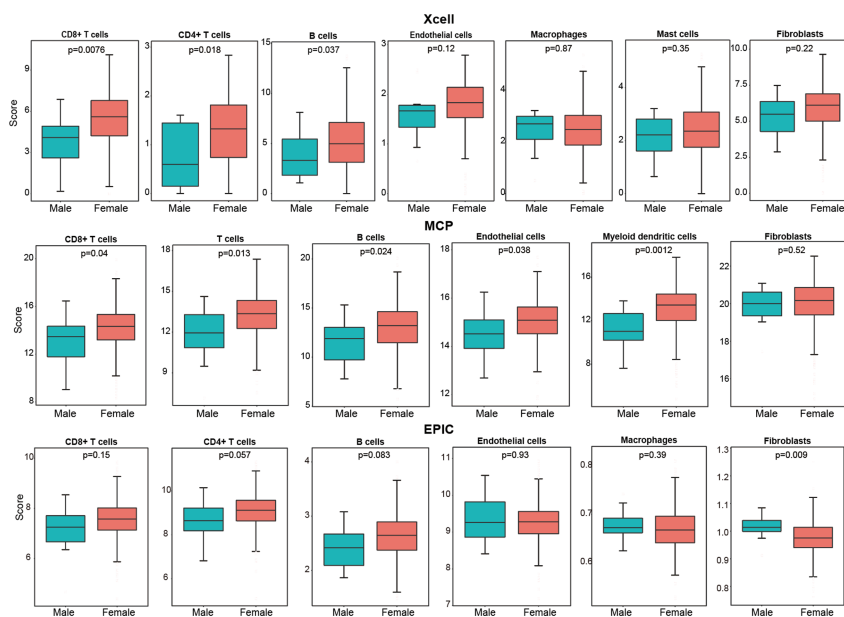
492

493 14. Lines 567-571: To further validate the reliability of gene sets derived from the
494 single-cell dataset, these findings may be compared to those derived from available
495 immune-deconvolution tools (e.g. MCP-counter, EPIC, TIMER, xCell...) including the
496 cell types of interest (e.g. in terms of correlations).

497 **Response:** Thank you for your professional suggestion. We performed the immune-
498 deconvolution analysis for the 722 ER⁺ TCGA-BRCA samples using MCP-counter
499 (Becht E, et al. Genome biology, 2016), EPIC (Racle J, Gfeller D. Bioinformatics for
500 Cancer Immunotherapy, 2020), and xCell (Aran D, et al. Genome biology, 2017). By
501 comparing the scores of immune or stromal cell types calculated by these tools between
502 MBC and FBC samples, we found that the results of immune-deconvolution tools were
503 largely consistent with our previous observation based on signatures derived from the
504 single-cell dataset (**Response Figure 9**). Notably, results from both single-cell
505 signature and immune-deconvolution tools showed that the levels of T cells and B cells
506 were significantly higher in MBC samples than in FBC samples. Besides, we evaluated
507 the correlation of putative cell type levels derived from single-cell signatures and
508 immune-deconvolution tools and found a significantly positive correlation between
509 these methods (**Response Figure 10**), indicating the reliability of gene signatures

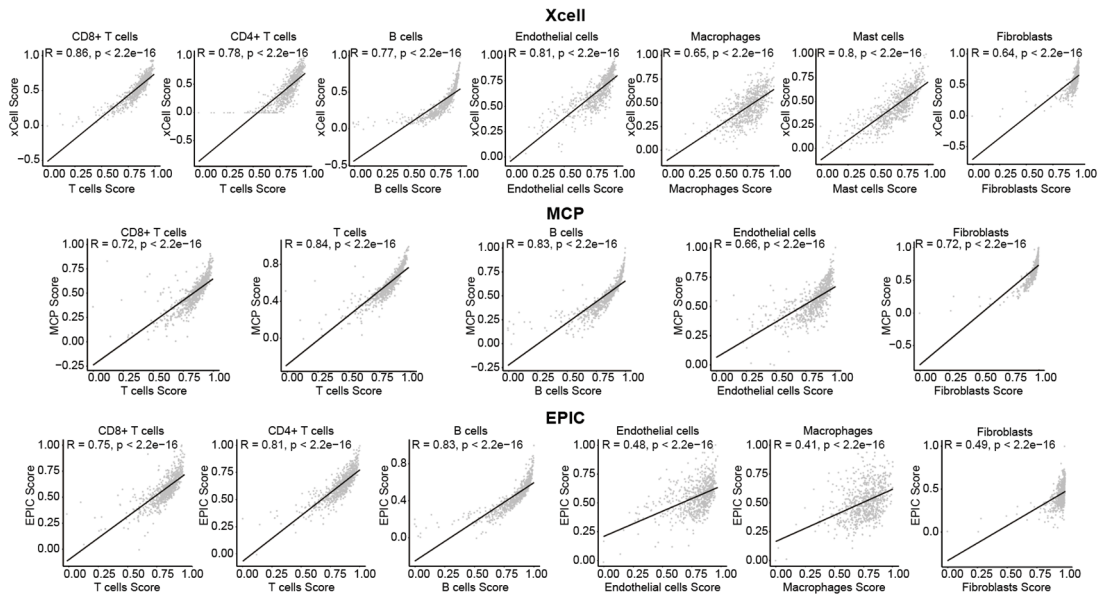
510 derived from our single-cell dataset, as well as the immunological difference between
 511 MBC and FBC. We added the above validation in the revised Results section as follows
 512 (Lines 146-159): “To further validate this result, we calculated the scores of various
 513 cell types for 722 ER⁺ TCGA-BRCA samples based on the gene signatures derived
 514 from our single-cell data (see Methods; Figure 2f). These scores between
 515 premenopausal and postmenopausal FBC patients were also compared (Figure 2g).
 516 Results verified that MBC had a relatively higher tumor purity and lower proportions
 517 of T cells and B cells, consistent with the observation at the single-cell level. The
 518 immunological components of TCGA samples were also verified using three immune-
 519 deconvolution tools, including MCP-counter¹⁷, EPIC¹⁸, and xCell¹⁹. We evaluated the
 520 correlation of putative cell type levels derived from single-cell signatures and immune-
 521 deconvolution tools and found a significantly positive correlation between these
 522 methods (Supplementary Figure 3a). Consistently, results from immune-
 523 deconvolution tools indicated that the levels of T cells and B cells were significantly
 524 lower in MBC samples than in FBC samples of the TCGA dataset (Supplementary
 525 Figure 3b)”.

526 We also added the description of the corresponding validation procedures in the
 527 revised Methods section as follows (Lines 723-730): “We identified the top ten genes
 528 with the highest fold-changes of each cell type in our single-cell data and then
 529 calculated the ssGSEA scores of these gene signatures for bulk samples. The scores of
 530 immune or stromal cells were compared between MBC and FBC samples using two-
 531 sided Wilcoxon rank-sum test, which was a non-parametric test that did not assume
 532 known distributions⁶⁵. To further validate the reliability of gene signatures derived
 533 from the single-cell dataset, we measured the enrichment of TME cells by using
 534 immune-deconvolution tools MCP-counter¹⁷, EPIC¹⁸, and xCell¹⁹.”



535

536 **Response Figure 9 (Related to Supplementary Figure 3b in revised manuscript). Cellular**
 537 **components in TCGA MBC and FBC ER+ samples inferred by immune-deconvolution tools.**
 538 **Boxplot showing the scores of immune and stromal cells in TCGA MBC and FBC ER+ samples**
 539 **inferred by xCell, MCP, and EPIC. P-value was calculated by two-sided Wilcoxon rank-sum test.**



540
 541 **Response Figure 10 (Related to Supplementary Figure 3a in revised manuscript). The Pearson**
 542 **correlation analysis of putative cell type levels derived from single-cell signatures and**
 543 **immune-deconvolution tools.**

544 15. Line 229: From Supplementary Figure 4, it seems that FASN high is significantly
 545 ($P = 0.04$) associated with OS in female breast cancer, and not in males ($P = 0.27$),
 546 although only 12 male breast cancer samples were present in the TCGA. However, in
 547 the text, it is stated that “high expression of FASN could predict poor OS of male
 548 patients with BRCA”. This part may need to be rephrased.

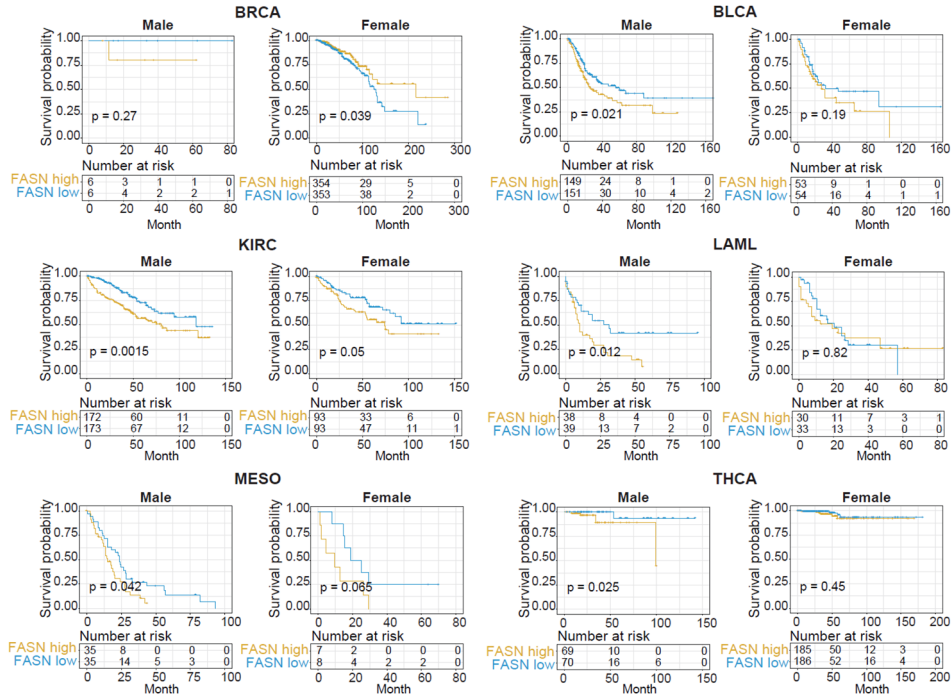
549 Related to the comment on lines 588-595, other survival end-points, especially in
 550 the breast cancer, may be more informative than OS for evaluating the prognostic value
 551 of FASN expression. Lines 588-595, Survival analysis: Since overall survival data has
 552 to be interpreted carefully in the TCGA, especially for luminal breast cancer
 553 (doi:10.1016/j.cell.2018.02.052), did the authors tested also other survival end-points
 554 (PFI, DFI)?

555 **Response:** Thanks for your valuable suggestion. We apologize for the inaccurate
 556 statement. The significant results of overall survival analyses were shown in the revised
 557 supplementary figure (**Response Figure 11**). We agree with the reviewer that
 558 progression-free interval (PFI), disease-free interval (DFI), or disease-specific survival
 559 (DSS) are important for evaluating the prognostic value of FASN expression, especially
 560 for luminal breast cancer. Accordingly, we also performed survival analyses for PFI and
 561 DSS of TCGA pan-cancer datasets by categorizing the patients into FASN-high and
 562 FASN-low groups for each dataset according to the median of FASN expression. The
 563 analysis of DFI was not included due to the missing data of MBC samples. Results

564 showed that FASN expression was prognostic for the OS, PFI, and DSS of many types
565 of cancers, especially for male cancer patients (**Response Figure 11-13**). Male BRCA
566 patients with higher expression of FASN had a relatively poor prognosis but were not
567 statistically significant possibly due to that only 12 MBC samples were present in the
568 TCGA. Besides, high expression of FASN could predict poor OS and PFI of male
569 patients with bladder urothelial carcinoma (BLCA) and kidney renal clear cell
570 carcinoma (KIRC). The PFI of FASN-high male patients with kidney renal papillary
571 cell carcinoma (KIRP) and uveal melanoma (UVM) was also significantly poor. The
572 DSS of lung squamous cell carcinoma (LUSC) male patients with high FASN
573 expression was significantly poorer than those with low FASN expression. However,
574 the prognosis of female patients with these cancers was not associated with the FASN
575 expression.

576 We revised the corresponding description and added the undated results as follows
577 (**Lines 280-298**): “We performed analyses for overall survival (OS), progression-free
578 interval (PFI), and disease-specific survival (DSS) of TCGA pan-cancer datasets ²⁸ by
579 categorizing the patients into FASN-high and FASN-low groups for each dataset
580 according to the median of FASN expression. Results showed that FASN expression
581 was prognostic for the OS, DSS, and PFI of many types of cancers, especially for male
582 cancer patients (**Supplementary Figure 9-11**). Male BRCA patients with higher
583 expression of FASN had a relatively poor prognosis but were not statistically significant
584 possibly due to that only 12 MBC samples were present in the TCGA. Besides, high
585 expression of FASN could predict poor OS and PFI in male patients with bladder
586 urothelial carcinoma (BLCA) and kidney renal clear cell carcinoma (KIRC). The PFI
587 of FASN-high male patients with kidney renal papillary cell carcinoma (KIRP) and
588 uveal melanoma (UVM) was also significantly poor. The DSS of lung squamous cell
589 carcinoma (LUSC) male patients with high FASN expression was significantly poorer
590 than those with low FASN expression. However, the prognosis of female patients with
591 these cancers was not associated with the FASN expression. Notably, higher FASN
592 expression was prognostic for the poor DSS of PRAD patients, consistent with a
593 previous study that demonstrated that targeting FASN could inhibit the aggressive and
594 resistant PRAD²⁴. This result suggested that FASN may be a potential therapeutic target
595 for male patients with these cancers.”

overall survival



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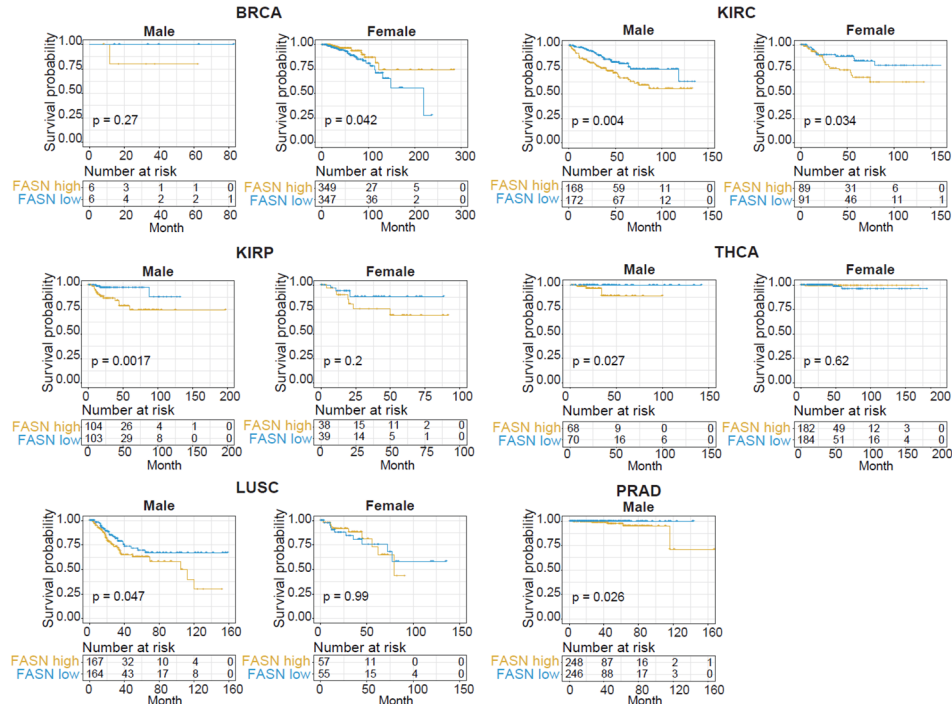
600

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Response Figure 11 (Related to Supplementary Figure 9 in revised manuscript). Overall survival analysis of male and female patients in various cancer types based on the FASN expression. Patients are categorized into FASN-high and FASN-low groups for each dataset according to the median of FASN expression. BRCA: Breast invasive carcinoma; BLCA: Bladder Urothelial Carcinoma; KIRC: Kidney renal clear cell carcinoma; LAML: Acute Myeloid Leukemia; MESO: Mesothelioma; THCA: Thyroid carcinoma.

disease-specific survival

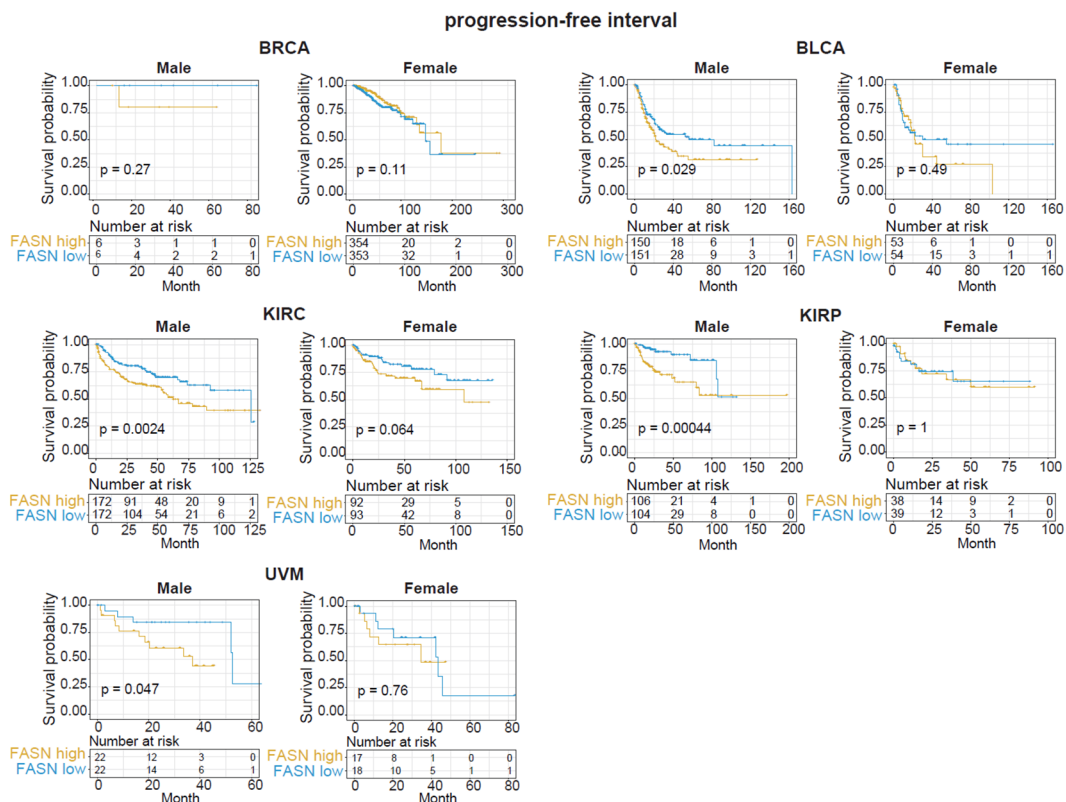


603

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Response Figure 12 (Related to Supplementary Figure 10 in revised manuscript). Disease-specific survival analysis of male and female patients in various cancer types based on the FASN expression. Patients are categorized into FASN-high and FASN-low groups for each dataset according to the median of FASN expression. BRCA: Breast invasive carcinoma; KIRC: Kidney renal clear cell carcinoma; KIRP: Kidney renal papillary cell carcinoma; THCA: Thyroid carcinoma; LUSC: Lung squamous cell carcinoma; PRAD: Prostate adenocarcinoma.

605 **specific survival analysis of male and female patients in various cancer types based on the**
 606 **FASN expression.** Patients are categorized into FASN-high and FASN-low groups for each dataset
 607 according to the median of FASN expression. BRCA: Breast invasive carcinoma; KIRC: Kidney
 608 renal clear cell carcinoma; KIRP: Kidney renal papillary cell carcinoma; THCA: Thyroid carcinoma;
 609 LUSC: Lung squamous cell carcinoma; PRAD: Prostate adenocarcinoma.
 610

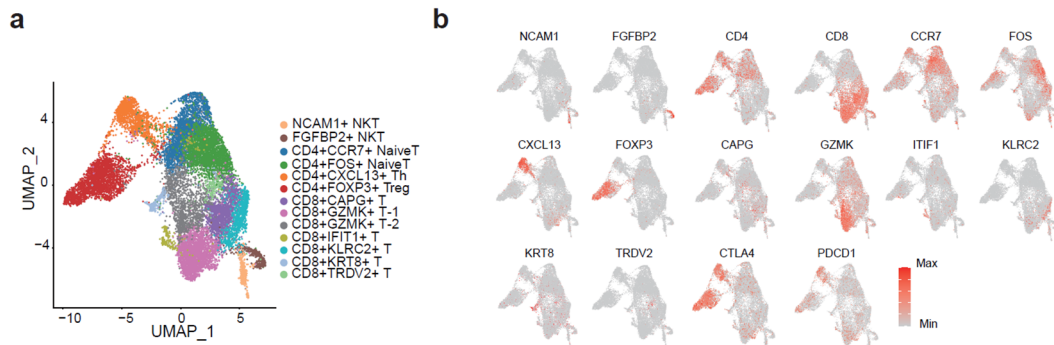


611 **Response Figure 13 (Related to Supplementary Figure 11 in revised manuscript). Progression-**
 612 **free interval analysis of male and female patients in various cancer types based on the FASN**
 613 **expression.** Patients are categorized into FASN-high and FASN-low groups for each dataset
 614 according to the median of FASN expression. BRCA: Breast invasive carcinoma; BLCA: Bladder
 615 Urothelial Carcinoma; KIRC: Kidney renal clear cell carcinoma; KIRP: Kidney renal papillary cell
 616 carcinoma; UVM: Uveal Melanoma.
 617

618
 619 16. Line 247: For the SingleR tool analysis, which reference dataset was used? From
 620 Supplementary Fig. 5 it seems that some cell types are relatively “mixed” together and
 621 not well defined in the t-SNE. Did the Authors double checked manually if the
 622 automatic annotations were reliable?

623 **Response:** Thank you for your comment. We agree with the reviewer that the
 624 annotations of T cell subtypes using reference “MonacoImmuneData” in the SingleR
 625 package were confusing. When analyzing the updated transcriptomic dataset of 15,690
 626 single T cells from 19 BRCA samples, we had tried to annotate the subpopulations using
 627 multiple references from SingleR package but got some “mixed” results possibly due
 628 to the complicated phenotypes of T cells in different tissues and conditions. Therefore,
 629 we manually defined the T cell subpopulations based on the specifically-expressed

630 genes of each cell cluster in the revised manuscript, as shown in **Response Figure 14**.
 631 Besides, the top 30 genes that were specifically expressed in each subpopulation were
 632 listed in the revised supplementary table 6.



633
 634 **Response Figure 14 (Related to Figure 5a and Supplementary Figure 12a in revised**
 635 **manuscript). Identification of T cell subpopulations. (a)** T-SNE plot showing the subpopulations
 636 **of T cells. (b)** Expression levels of representative genes in each subpopulation.

637
 638 17. Line 433 and 437: The Cell Ranger versions mentioned are discordant (v2.1.0 and
 639 3.0.2). Is this correct?

640 **Response:** Thank you for your kind comment. Sorry for our mistake. The Cell Ranger
 641 version used for our data analysis pipeline is 3.0.2. We have further clarified this in the
 642 revised manuscript as follows (**Lines 585-586**): “The Cell Ranger v3.0.2 pipeline was
 643 performed to analyze the raw data and generate gene count data using the default and
 644 recommended parameters”.

645
 646 18. Line 449: Please clarify if cells with more or less than 2000 expressed genes were
 647 retained.

648 **Response:** We apologize for the unclear description. In order to remove the empty
 649 droplets and multiplets, cells with expressed genes less than 200 or greater than 6000
 650 were excluded. We modified the description for quality control in the revised Methods
 651 section as follows (**Lines 593-595**): “To eliminate the influence of low-quality cells
 652 such as empty droplets and multiplets, cells with expressed genes less than 200 or
 653 greater than 6000 were excluded.”

654
 655 19. Lines 454-455: Please rephrase specifying in a clearer way if UMI count and MT
 656 genes were used as regression terms in the ScaleData function(also, Authors may
 657 replace “ScaleDate” with “ScaleData” in the text).

658 **Response:** We apologize for not making this point clear. By using the default parameters,
 659 UMI count and MT genes were not regressed out in the ScaleData function. We revised
 660 the corresponding description as follows (**Lines 600-604**): “We identified the top 2000

661 variable features using the “vst” method for each dataset. Datasets were then anchored
662 and integrated using the integration procedure from the Seurat package to eliminate the
663 batch effects among the samples. ScaleData function was used to perform a linear
664 scaling transformation on the identified variable features using default parameters.”

665

666 20. Line 465: Please specify if the default parameters were used in the IntegrateData
667 function. Was default integration from Seurat applied from the beginning on all cells,
668 or just for specific cell types?

669 Response: Sorry for our ambiguous description. The integration procedure from the
670 Seurat package was performed at the beginning on all cells, not just for specific cell
671 types. In the revised manuscript, we rephrased this description according to the order
672 of data processing as follows (Lines 600-612): “We identified the top 2000 variable
673 features using the “vst” method for each dataset. Datasets were then anchored and
674 integrated using the integration procedure from the Seurat package to eliminate the
675 batch effects among the samples. ScaleData function was used to perform a linear
676 scaling transformation on the identified variable features using default parameters.
677 Principal component analysis (PCA) was performed on the scaled data to reduce the
678 dimensionality. The statistical significance of the PCA scores was determined using the
679 JackStraw function. The first 25 principal components were used for identifying the
680 neighbors and clustering the cells with a resolution of 1.5. The cell clusters were
681 visualized using 2D uniform manifold approximation projection (UMAP) or t-
682 distributed stochastic neighbor embedding (tSNE) plots. The FindAllMarkers function
683 was used to identify the genes specifically expressed in each cell cluster. We identified
684 the cell types based on the expression of well-established gene markers.”

685

686 21. Lines 475-480: Please explain in a clearer way this section. What did the “normal
687 cell cluster” used in inferCNV included (e.g. normal epithelial breast cells, stromal cells,
688 immune cells...)? Was it formed by “any other cell” that was not tagged as malignant?

689 Response: We apologize for the unclear description. The “normal cell clusters” included
690 immune cells (T cells, B cells, macrophages, mast cells) and stromal cells (fibroblasts
691 and endothelial cells). We revised the corresponding description in the manuscript
692 (Lines 616-622) as follows: “First, we identified malignant epithelial cells using the
693 marker genes EPCAM, KRT18, KRT14, and KRT19. To verify the identified cancer
694 cells more accurately, we also used the inferCNV R package⁶⁰ to evaluate copy number
695 variants (CNVs) levels, using immune cells (T cells, B cells, macrophages, and mast
696 cells) and stromal cells (fibroblasts and endothelial cells) as the control group and
697 epithelial cells as the test group.”

698

699 22. Line 492: Are P values adjusted for multiple testing or not? This should be stated in
700 the method sections for the other analyses as well.

701 Response: Thank you for pointing this out. P values were adjusted for multiple testing
702 when identifying differentially expressed genes or pathways throughout the whole
703 study. We updated the description of p-value adjustment in the revised manuscript as
704 follows: (1) **Lines 626-629**: “we identified genes with log2 fold change greater than
705 0.25 and adjusted p-value less than 0.01 for each cluster. Based on the order of log2
706 fold change, the top 100 genes were further identified as markers of each cluster.”. (2)
707 **Lines 647-651**: “The activity difference of KEGG metabolic pathways between male
708 and female cancer cell clusters were measured by two-sided Wilcoxon rank-sum test.
709 P-values were adjusted for multiple testing using the Benjamini-Hochberg method.
710 Pathways with adjusted p-value less than 0.05 were identified as differentially activated
711 pathways between male and female cancer cell clusters”. (3) **Lines 740-741**: “The gene
712 lists were submitted to Enrichr (<https://maayanlab.cloud/Enrichr/>) online tool, and the
713 top ten terms were retained according to the adjusted p-value”.

714

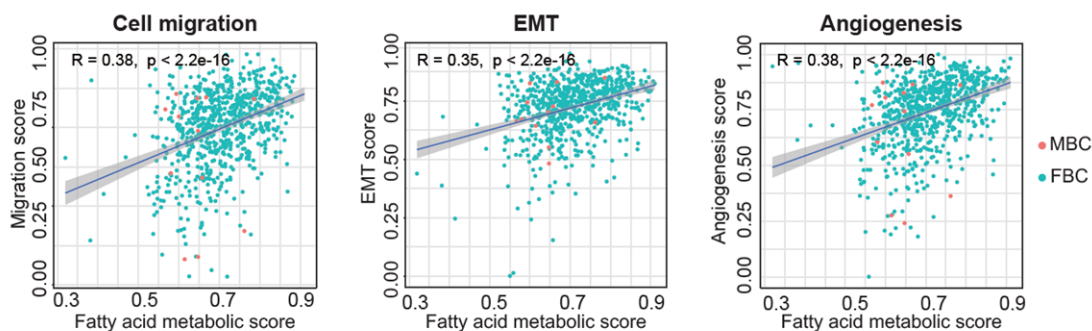
715 23. Line 509: Which statistical test was used to perform this comparison of metabolic
716 pathways between male and female clusters?

717 Response: Sorry for our unclear description. The differentially activated metabolic
718 pathways between male and female cancer cell clusters were identified by the two-side
719 Wilcoxon rank-sum test. We revised the corresponding description as follows (**Lines**
720 **647-651**): “The activity difference of KEGG metabolic pathways between male and
721 female cancer cell clusters were measured by two-sided Wilcoxon rank-sum test. P-
722 values were adjusted for multiple testing using the Benjamini-Hochberg method.
723 Pathways with adjusted p-value less than 0.05 were identified as differentially activated
724 pathways between male and female cancer cell clusters.”

725

726 24. Figure 3, panel G: Can the Authors add a value for the correlations showed?

727 Response: We apologize for forgetting to show the p-values and correlation coefficients.
728 Both p-values and correlation coefficients were added in the corresponding figure
729 (**Response Figure 15, related to Figure 4j in the revised version**).

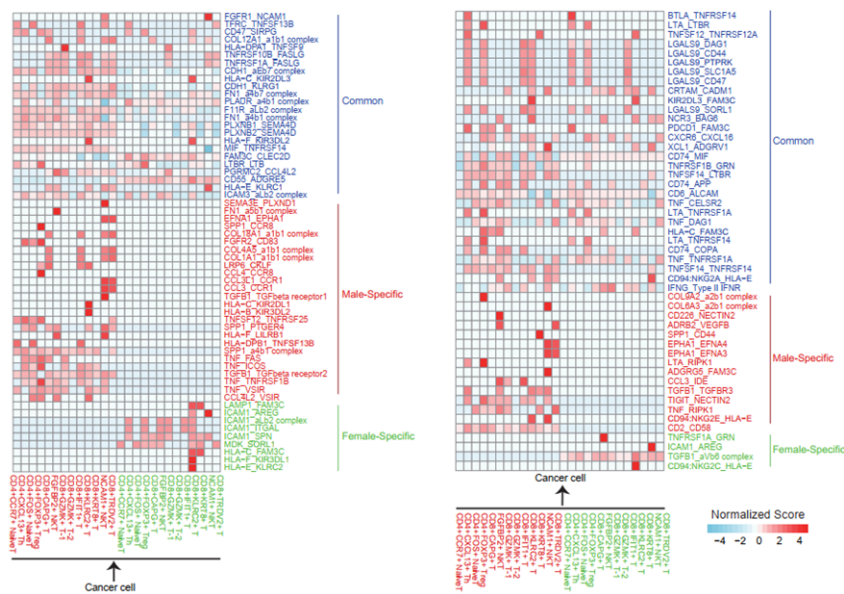


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731 **Response Figure 15 (Related to Figure 4j in revised manuscript). The Pearson correlation**
 732 **analysis between the scores of metastasis-related signatures and fatty acid metabolic pathway**
 733 **in TCGA ER+ BRCA cohort.**

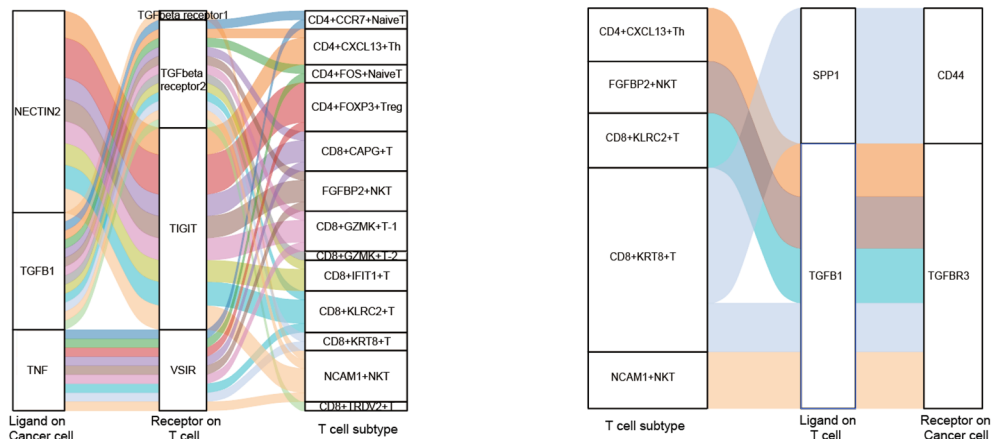
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 735 25. Figure 3, panel J: The difference between the groups of comparisons (cell types and
 736 FASN high/low cells) is not clear and may be specified in the Figure legend. Are the 4
 737 main columns representing interactions with opposite directions?

738 **Response:** We apologize for the confusing visualization and unclear description in the
 739 previous version. To comprehensively illustrate the cell-cell communications in MBC
 740 and FBC samples, we re-analyzed the inter-cellular interactions using the updated
 741 single-cell datasets. The ligand-receptor interactions were visualized using heatmaps
 742 and Sankey plots, as shown in **Response Figures 16 and 17**. To visualize more clearly,
 743 we split the interactions with opposite directions into two plots, and marked the
 744 ‘common’, ‘male-specific’, and ‘female-specific’ interactions using different font
 745 colors (**Response Figure 16**). We also clarified the corresponding descriptions in the
 746 revised figure legends.



747
 748 **Response Figure 16 (Related to Figure 7c-d in revised manuscript). Heatmap showing the**
 749 **common, male-specific and female-specific ligand-receptor pairs in MBC and FBC samples.**

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Response Figure 17 (Related to Figure 7e-f in revised manuscript). Sankey plot showing the representative examples of male-specific ligand-receptor pairs.

Response References:

1. Wu S Z, Al-Eryani G, Roden D L, et al. A single-cell and spatially resolved atlas of human breast cancers[J]. Nature genetics, 2021, 53(9): 1334-1347.
2. Hu Z, Artibani M, Alsaadi A, et al. The repertoire of serous ovarian cancer non-genetic heterogeneity revealed by single-cell sequencing of normal fallopian tube epithelial cells[J]. Cancer Cell, 2020, 37(2): 226-242.
3. Becht E, Giraldo N A, Lacroix L, et al. Estimating the population abundance of tissue-infiltrating immune and stromal cell populations using gene expression[J]. Genome biology, 2016, 17(1): 1-20.
4. Racle J, Gfeller D. EPIC: a tool to estimate the proportions of different cell types from bulk gene expression data[M]. Bioinformatics for Cancer Immunotherapy. Humana, New York, NY, 2020: 233-248.
5. Aran D, Hu Z, Butte A J. xCell: digitally portraying the tissue cellular heterogeneity landscape[J]. Genome biology, 2017, 18(1): 1-14.

771 **Reviewer #2**

772

773 This is a well written and comprehensive manuscript describing the immune and
774 metabolic landscape of male breast cancer.

775

776 1. The premise of this paper that male and female breast cancers are immunological and
777 metabolically different is very compelling and may potentially provide new insights
778 into therapeutic strategies. The investigators have carefully evaluated a broad range of
779 proliferation, angiogenesis, and metabolic pathways as well as detailed immune
780 characterization. The study includes a limited number (3 and 2) reference cases. The
781 study is expanded by data from the TCGA.

782 **Response:** We are grateful for your comments. In order to further support and validate
783 the conclusion in this study, we expand the sample size of both male and female breast
784 cancer. In this revised version, six MBC and thirteen FBC samples were included, in
785 which eleven FBC samples were from a previous study by Wu et al. (Nature genetics,
786 2021, 53(9): 1334-1347. doi: 10.1038/s41588-021-00911-1) and other samples were in-
787 house. All of the collected samples were ER⁺. The transcriptome of 58,578 and 52,460
788 single-cells was sequenced in MBC and FBC, respectively. By performing the same
789 analysis procedure using this updated dataset, we found that the main results were
790 consistent with the previous version, and demonstrated the followings: (1) scRNA-seq,
791 bulk transcriptome, and immunohistochemistry consistently demonstrated that MBC
792 had a significantly lower degree of T cell infiltration than FBC; (2) metastasis-related
793 programs such as cell migration, epithelial-mesenchymal transition (EMT), and
794 angiogenesis were more active in cancer cells from MBC than FBC; (3) the activated
795 fatty acid metabolism involved by FASN was related to the cancer cell metastasis and
796 low immune infiltration of MBC; (4) different characteristics of T cell subpopulations
797 between MBC and FBC were identified. T cells in MBC showed activation of p38
798 MAPK and lipid oxidation pathways, indicating the dysfunctional state. In contrast, T
799 cells in FBC exhibited a higher expression level of cytotoxic markers such as GZMK
800 and KLRB1, and activated pathways mediated by immune-modulatory cytokines; (5)
801 the inhibitory interactions between cancer cells and T cells in the MBC
802 microenvironment were identified, such as cell-cell communications mediated by TGF-
803 β , TIGIT, and VSIR. (6) KRT8⁺ T cells with high level of fatty acid metabolism were
804 enriched in the MBC microenvironment. These observations were further validated in
805 bulk-RNAseq data and molecular experiments.

806 Despite the rarity of MBC occurrence and the stringent sample requirements of
807 single-cell experiments, we had collected and sequenced six MBC samples as possible
808 as we can. As far as we know, this study is the first to characterize the differences
809 between MBC and FBC at the single-cell resolution. Benefiting from the enlarged

810 sample size (6 MBC vs. 13 FBC), we could statistically evaluate the significance of the
811 observed differences between MBC and FBC samples. On the other hand, we also
812 discussed the explorative nature of this preliminary study in the revised manuscript as
813 follows (Lines 529-533): “Due to the rarity of MBC occurrence and the stringent
814 sample requirements of single-cell experiments, only limited MBC samples were
815 included in this study. However, this explorative study identified notable differences
816 between MBC and FBC, especially the distinct metabolic and immunological
817 characteristics of MBC patients. These observations need to be further validated with
818 larger sample sizes in the future.”

819 [FIGURE REDACTED]

820
821 **Response Figure 18 (Related to Figure 1a in revised manuscript). Schematic workflow for**
822 **data collection and single-cell analysis in this study.**

823
824 2. Strength of the study include the clearly distinctive patterns that the evaluated male
825 and female breast cancers. The single cell sequencing is elegantly done, and the figures
826 are beautifully outlined and clearly delineated.

827 **Response: Thank you for the positive evaluation of our work.**

828
829 3. A major concern of the study is that the female breast cancers neither have ER
830 expression (ESR1) nor ER activity. Male breast cancer is mostly ER+, whereas female
831 breast cancer has a broad diversity ranging from triple negative disease to ER+ and
832 HER2 positive disease. The immune landscape, EMT, angiogenesis is vastly different
833 in these subtypes. Particularly, TNBC stand out in their immune profile. The data would
834 be very much strengthened if the authors provided data on ER+ female breast cancer,
835 to show how this is similar or different from an ER+ male breast cancer.

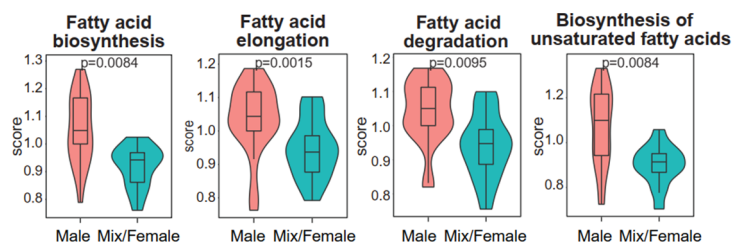
836 **Response: All of the collected male and female samples were from ER+ breast cancer**
837 **patients without HER2 amplification. The clinicopathological characteristics of the**
838 **collected samples were listed in the revised supplementary table 1, including age, ER**
839 **status, PR status, IHC results for HER2, FISH results for HER2, KI67 level, tissue size,**
840 **and TNM stage. Accordingly, we clarified the description of clinicopathological**
841 **characteristics of the collected samples in the revised Methods section as follows (Lines**
842 **544-550): “Single-cell transcriptomic data from six MBC and thirteen FBC samples**
843 **were analyzed, in which eleven FBC samples were collected from a previous study by**
844 **Wu et al.⁵⁷, and other samples were in-house. All of the collected samples were ER+.**
845 **We defined the ER, PR, HER2, and KI67 status using IHC, and further evaluated the**
846 **amplification of HER2 based on FISH. The clinicopathological characteristics were**
847 **shown in supplementary table 1. All the collected samples (including MBC and FBC)**

848 were negative for HER2 amplification evaluated by FISH.”

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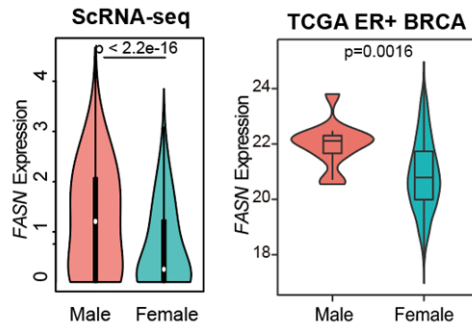
850 4. Furthermore, a more in-depth explanation on the significance of the findings. The
851 error bars appear very wide in a large number of examples. How are the p-values
852 adjusted for significance in this multi-parameter assessment?

853 Response: Thank you for pointing this out. In order to address this concern, we used
854 the violin-boxplots to better visualize the distribution of data in the revised Figure 4
855 and 5 (Response Figure 19-21). Specifically, the shape of violins represents the data’s
856 density: the thicker part means the values in that section of the violin have higher
857 frequency, and the thinner part implies lower frequency. Boxplots were also added
858 inside the violins to show the medians, ranges and variabilities of the data. P values
859 were adjusted for multiple testing when identifying differentially expressed genes or
860 pathways throughout the whole study. We updated the description of p-value adjustment
861 in the revised manuscript as follows: (1) Lines 626-629: “we identified genes with log2
862 fold change greater than 0.25 and adjusted p-value less than 0.01 for each cluster. Based
863 on the order of log2 fold change, the top 100 genes were further identified as markers
864 of each cluster.” (2) Lines 647-651: “The activity difference of KEGG metabolic
865 pathways between male and female cancer cell clusters was measured by two-sided
866 Wilcoxon rank-sum test. P-values were adjusted for multiple testing using the
867 Benjamini-Hochberg method. Pathways with adjusted p-value less than 0.05 were
868 identified as differentially activated pathways between male and female cancer cell
869 clusters”. (3) Lines 740-741: “The gene lists were submitted to Enrichr
870 (<https://maayanlab.cloud/Enrichr/>) online tool, and the top ten terms were retained
871 according to the adjusted p-value”.



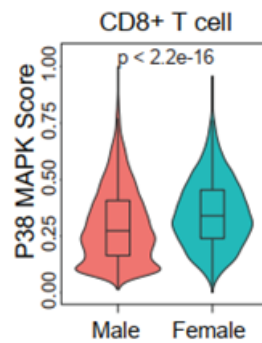
872

873 Response Figure 19 (Related to Figure 4a in revised manuscript). Violin-boxplots showing the
874 signature scores of fatty acid metabolic pathways in cancer cells of male and mixed/female
875 clusters. P-value was calculated by two-sided Wilcoxon rank-sum test and adjusted for multiple
876 testing using the Benjamini-Hochberg method.



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Response Figure 20 (Related to Figure 4c-d in revised manuscript). The expression levels of FASN between MBC and FBC samples in ScRNA-seq data and TCGA ER+ BRCA cohort.



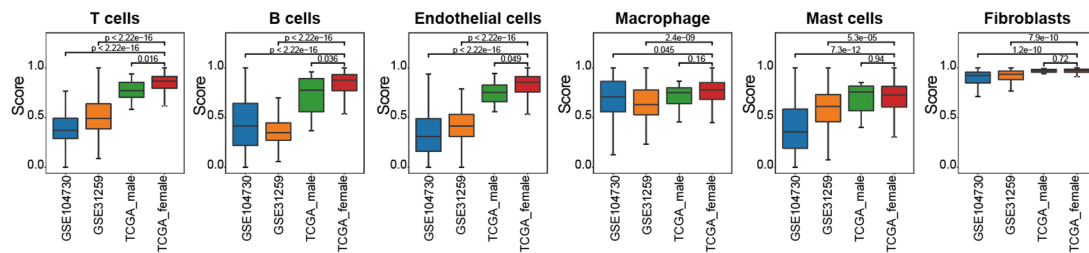
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Response Figure 21 (Related to Figure 5e in revised manuscript). Violin plot of p38 MAPK activity in CD8+ T cells from MBC and FBC samples. P-value was calculated by two-sided Wilcoxon rank-sum test.

885 5. TCGA data while compelling is not novel and may not provide sufficient annotations
886 to clinical

887 Response: Thank you for your professional advice. We agree with the reviewer that it
888 would be more convincing to validate the findings of this study using multiple
889 independent datasets of male breast cancer (MBC). Besides, the limited number of
890 MBC samples in the TCGA dataset may not sufficient for the comparison between FBC
891 and MBC samples. Thus, we collected two gene expression profiles of MBC samples
892 from previous studies, GSE104730 (RNA-seq, 46 samples, Severson T M, et al. Nature
893 communications, 2018) and GSE31259 (microarray data, 74 samples, Johansson I, et
894 al. Breast Cancer Research, 2012). Using the analysis procedure based on the ssGSEA
895 algorithm, we calculated the scores of immune or stromal cells for MBC samples from
896 TCGA, GSE104730, GSE31259, as well as for FBC samples from the TCGA dataset.
897 These scores were compared between MBC and FBC samples using two-sided
898 Wilcoxon rank-sum test. Results showed that the scores of T cells and B cells were
899 significantly lower in MBC samples from three independent datasets than in FBC
900 samples, confirming the results of low immune infiltration in MBC samples observed
901 in the single-cell dataset (**Response Figure 22**). We added this validation result in the
902 revised manuscript as follows (**Lines 159-166**): “**To further verify this result with larger**
903 **MBC sample size, we also collected two gene expression profiles of MBC samples**

904 from previous studies, including RNA-seq data of 46 MBC samples (GSE104730)⁶ and
 905 microarray data of 74 MBC samples (GSE31259)²⁰. We calculated and compared the
 906 scores of immune or stromal cells for MBC samples from three datasets, and for FBC
 907 samples from the TCGA dataset. Results showed that the scores of T cells and B cells
 908 were significantly lower in MBC samples from three independent datasets than in FBC
 909 samples (Supplementary Figure 4a), further confirming the results of low immune
 910 infiltration in MBC samples.” However, we failed to collect the survival data of MBC
 911 patients except for the TCGA dataset, possibly due to the rarity of MBC occurrence.



912
 913 **Response Figure 22 (Related to Supplementary Figure 4a in revised manuscript). Comparison**
 914 **of cellular components between MBC and FBC in independent datasets.** Boxplots showing the
 915 signature scores of T cells, B cells, endothelial cells, macrophages, mast cells and fibroblasts in ER⁺
 916 MBC samples from GSE104730, GSE31259, and TCGA datasets, as well as ER⁺ FBC samples
 917 from TCGA dataset. P-value was calculated by two-sided Wilcoxon rank-sum test.

918
 919 **Response References**

- 920 1. Wu S Z, Al-Eryani G, Roden D L, et al. A single-cell and spatially resolved atlas of human
 921 breast cancers[J]. Nature genetics, 2021, 53(9): 1334-1347.
- 922 2. Severson T M, Kim Y, Joosten S E P, et al. Characterizing steroid hormone receptor
 923 chromatin binding landscapes in male and female breast cancer[J]. Nature communications,
 924 2018, 9(1): 1-12.
- 925 3. Johansson I, Nilsson C, Berglund P, et al. Gene expression profiling of primary male breast
 926 cancers reveals two unique subgroups and identifies N-acetyltransferase-1 (NAT1) as a
 927 novel prognostic biomarker[J]. Breast Cancer Research, 2012, 14(1): 1-15.

928

929 **Reviewer #3**

930

931 Male breast cancer (MBC) is associated with worse prognosis compared to female
932 breast cancer and the cellular and molecular differences between the two remain unclear.
933 The researchers used single-cell RNA (scRNA) sequencing and T cell receptor (scTCR)
934 sequencing characterize the tumor microenvironment of MBC. They sequenced three
935 MBC and two post-menopausal ER⁺ female breast cancers (FBC) and show evidence
936 that MBC have lower immune infiltration, activated ER and AR regulons, higher fatty
937 acid synthase (FASN) expression, and exhausted CD8 T cells. The authors identify a
938 subset of T-cells that express epithelial cytokeratins. However, the manuscript is lacking
939 good quality evidence for the existence of these epithelial-T cells. The authors should
940 consider removing that entire section or provide additional experiments to validate their
941 findings. Androgens have long been known to drive fatty acid synthase PMID: 9067276,
942 and the authors show good evidence of AR regulon activation in MBC, perhaps more
943 focus on the androgen receptor would tie this story together. Overall, the study is of
944 interest, but more experiments and analysis are needed for this study.

945

946 Specific comments:

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948 1. While two of the three MBC samples have low immune infiltrate, one actually has
949 similar levels to the two other FBC samples (Figure 1e). Therefore, one cannot conclude
950 that there are less immune cells in MBC, as this may just be a sampling artefact.

951 **Response:** We are grateful for your comments. We agree with the reviewer that the
952 conclusions are not convincing due to the limited sample size for the scRNA analysis.
953 In order to further support and validate the conclusion in this study, we expand the
954 sample size of both male and female breast cancer. In this revised version, 6 MBC and
955 13 FBC samples were included, in which eleven FBC samples were from a previous
956 study by Wu et al. (Nature genetics, 2021, 53(9): 1334-1347. doi: 10.1038/s41588-021-
957 00911-1) and other samples were in-house. All of the collected samples were ER⁺. The
958 transcriptome of 58,578 and 52,460 single-cells was sequenced in MBC and FBC,
959 respectively (**Response Figure 23**). By performing the same analysis procedure using
960 this updated dataset, we found that the main results were consistent with the previous
961 version. Benefiting from the enlarged sample size of scRNA-seq data, we could
962 statistically evaluate the significance of the cellular component difference between
963 MBC and FBC samples. Results showed that compared with FBC, MBC showed a
964 significantly higher proportion of cancer cells and a lower proportion of immune cells,
965 such as T cells and B cells, indicating a lower level of immune infiltration (**Response**
966 **Figure 24a-d**). These immune cell proportions had no obvious differences between

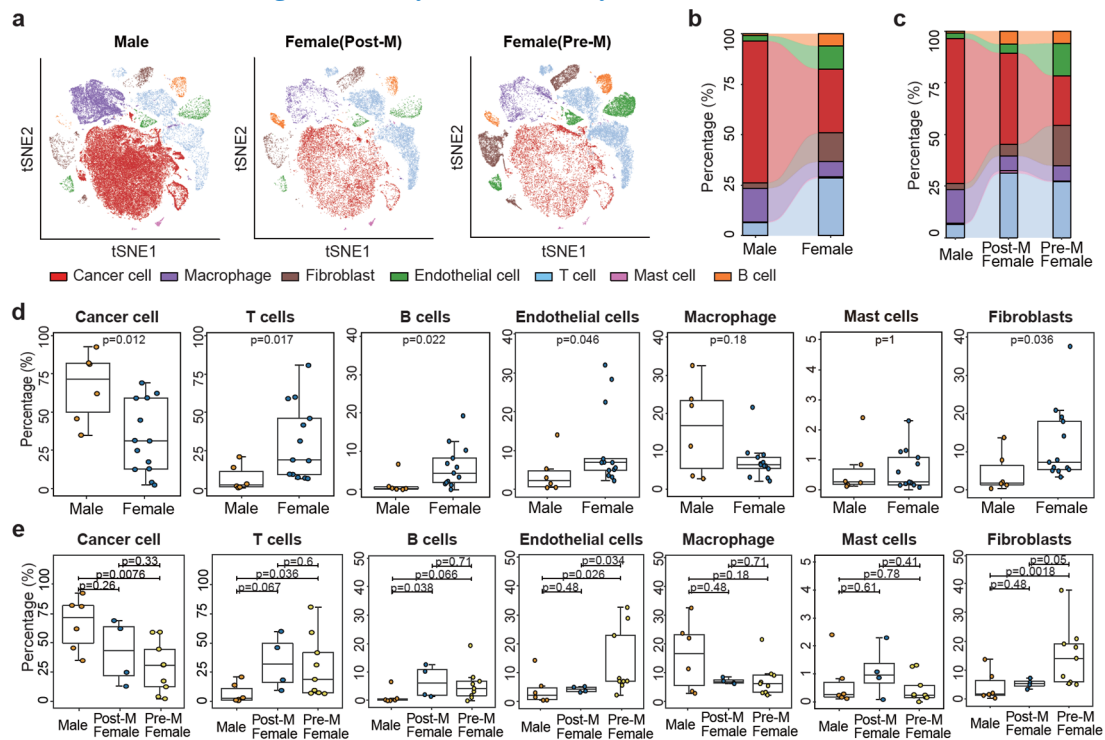
967 premenopausal and postmenopausal FBC patients (**Response Figure 24e**). To further
968 validate this result, we calculated the scores of various cell types for 722 ER⁺ TCGA-
969 BRCA samples based on the gene signatures derived from single-cell data (**Response**
970 **Figure 24f**). These scores between premenopausal and postmenopausal FBC patients
971 were also compared (**Response Figure 24g**). Results verified that MBC had a relatively
972 higher tumor purity and lower proportions of T cells and B cells, consistent with the
973 observation at the single-cell level. These observations of immunological components
974 of TCGA samples were also verified using three immune-deconvolution tools,
975 including MCP-counter, EPIC, and xCell. Consistently, results from these immune-
976 deconvolution tools indicated that the levels of T cells and B cells were significantly
977 lower in MBC samples than in FBC samples of the TCGA dataset (**Response Figure**
978 **25**). To further verify this result with larger MBC sample size, we also collected two
979 gene expression profiles of MBC samples from previous studies, including RNA-seq
980 data of 46 MBC samples (GSE104730) and microarray data of 74 MBC samples
981 (GSE31259). Using the analysis procedure based on the ssGSEA algorithm, we
982 calculated and compared the scores of immune or stromal cells for MBC samples from
983 three datasets, and for FBC samples from TCGA dataset. Results showed that the scores
984 of T cells and B cells were significantly lower in MBC samples from three independent
985 datasets than in FBC samples (**Response Figure 26**). Furthermore, we performed
986 immunohistochemistry (IHC) analysis for T cell markers CD4 and CD8 in 30 ER⁺ MBC
987 and 30 ER⁺ FBC samples. Results suggested that T cell markers had a lower expression
988 proportion in MBC than in FBC (**Response Figure 27**). Therefore, the analysis of
989 scRNA-seq, bulk transcriptome and IHC consistently demonstrated that MBC had a
990 significantly lower degree of immune cell infiltration than FBC.

991 We added the above results in the revised manuscript as follows (**Lines 142-166**):
992 “Results showed that compared with FBC, MBC showed a significantly higher
993 proportion of cancer cells and a lower proportion of immune cells, such as T cells and
994 B cells, indicating a lower level of immune infiltration (**Figure 2a-d**). These immune
995 cell proportions had no obvious differences between premenopausal and
996 postmenopausal FBC patients (**Figure 2e**). To further validate this result, we calculated
997 the scores of various cell types for 722 ER⁺ TCGA-BRCA samples based on the gene
998 signatures derived from our single-cell data (**see Methods; Figure 2f**). These scores
999 between premenopausal and postmenopausal FBC patients were also compared
1000 (**Figure 2g**). Results verified that MBC had a relatively higher tumor purity and lower
1001 proportions of T cells and B cells, consistent with the observation at the single-cell level.
1002 The immunological components of TCGA samples were also verified using three
1003 immune-deconvolution tools, including MCP-counter¹⁷, EPIC¹⁸, and xCell¹⁹. We
1004 evaluated the correlation of putative cell type levels derived from single-cell signatures

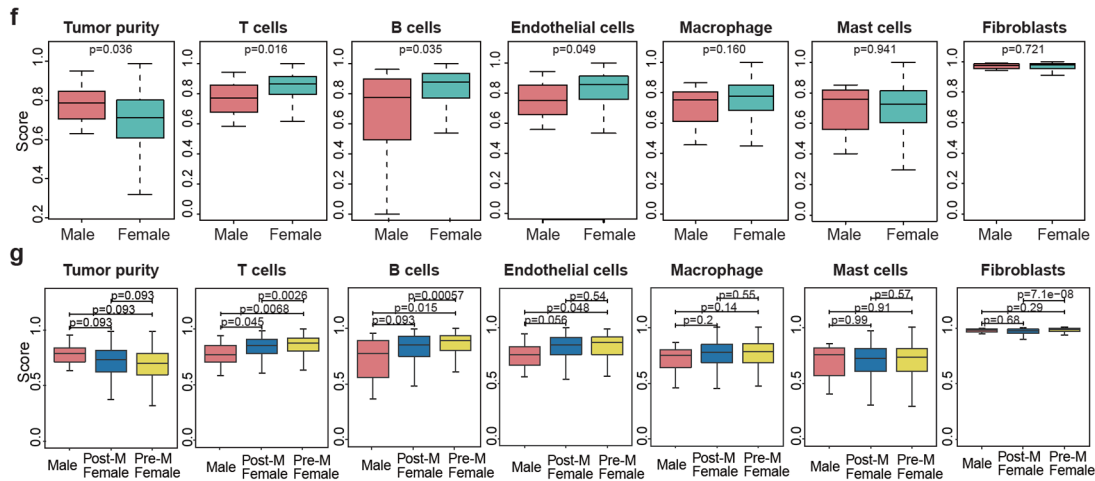
1005 and immune-deconvolution tools and found a significantly positive correlation between
 1006 these methods (**Supplementary Figure 3a**). Consistently, results from immune-
 1007 deconvolution tools indicated that the levels of T cells and B cells were significantly
 1008 lower in MBC samples than in FBC samples of the TCGA dataset (**Supplementary**
 1009 **Figure 3b**). To further verify this result with larger MBC sample size, we also collected
 1010 two gene expression profiles of MBC samples from previous studies, including RNA-
 1011 seq data of 46 MBC samples (GSE104730)⁶ and microarray data of 74 MBC samples
 1012 (GSE31259)²⁰. We calculated and compared the scores of immune or stromal cells for
 1013 MBC samples from three datasets, and for FBC samples from the TCGA dataset.
 1014 Results showed that the scores of T cells and B cells were significantly lower in MBC
 1015 samples from three independent datasets than in FBC samples (**Supplementary Figure**
 1016 **4a**), further confirming the results of low immune infiltration in MBC samples.”

1017 [FIGURE REDACTED]

1018 **Response Figure 23 (Related to Figure 1a in revised manuscript). Schematic workflow for**
 1019 **data collection and single-cell analysis in this study.**



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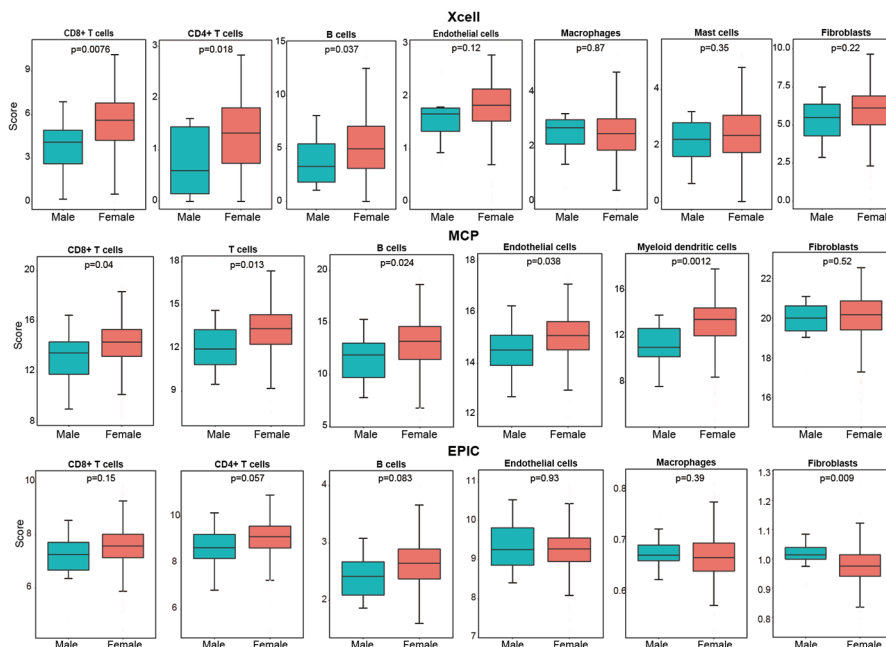
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Response Figure 24 (Related to Figure 2a-g in revised manuscript). Comparison of cellular components between MBC and FBC samples. (a) The t-SNE plot of MBC, postmenopausal and premenopausal FBC samples. Colors represent cell types. **(b)** Sankey diagram showing the fraction of each cell type between male and female samples. **(c)** Sankey diagram showing the fraction of each cell type between MBC, postmenopausal and premenopausal FBC samples. **(d)** Boxplot showing the percentage of cancer cells, T cells, B cells, endothelial cells, macrophages, mast cells and fibroblasts in MBC and FBC samples. P-value was calculated by two-sided Wilcoxon rank-sum test. **(e)** Boxplot showing the percentage of cancer cells, T cells, B cells, endothelial cells, macrophages, mast cells and fibroblasts in MBC, postmenopausal and premenopausal FBC samples. P-value was calculated by two-sided Wilcoxon rank-sum test. **(f)** Boxplot showing the tumor purity and signature scores of various cell types between MBC and FBC in TCGA ER⁺ BRCA cohort. P-value was calculated by two-sided Wilcoxon rank-sum test. **(g)** Boxplot showing the tumor purity and signature scores of various cell types between MBC, postmenopausal and premenopausal FBC samples in TCGA ER⁺ BRCA cohort. P-value was calculated by two-sided Wilcoxon rank-sum test.



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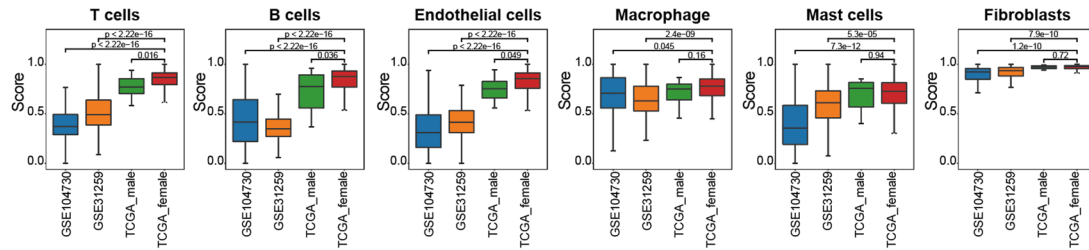
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Response Figure 25 (Related to Supplementary Figure 3b in revised manuscript). Cellular components in TCGA MBC and FBC ER⁺ samples inferred by immune-deconvolution tools. Boxplot showing the scores of immune and stromal cells in TCGA MBC and FBC ER⁺ samples inferred by xCell, MCP, and EPIC. P-value was calculated by two-sided Wilcoxon rank-sum test.



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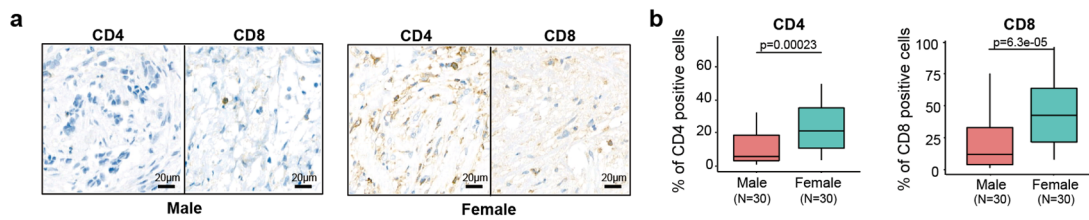
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Response Figure 26 (Related to Supplementary Figure 4a in revised manuscript). Comparison of cellular components between MBC and FBC in independent datasets. Boxplots showing the signature scores of T cells, B cells, endothelial cells, macrophages, mast cells and fibroblasts in ER⁺ MBC samples from GSE104730, GSE31259, and TCGA datasets, as well as ER⁺ FBC samples from TCGA dataset. P-value was calculated by two-sided Wilcoxon rank-sum test.



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Response Figure 27 (Related to Figure 2h-i in revised manuscript). Statistical quantification of CD4 and CD8 staining in MBC and FBC. (a) IHC images representing MBC and FBC samples stained for T cell markers CD4 and CD8. Scale bar, 20 μm. (b) Boxplot indicating the IHC scores of CD4 and CD8 in 30 ER⁺ male and 30 ER⁺ female patients (identified by the percentage of positive cells). P-value was calculated by two-sided Wilcoxon rank-sum test.

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Moreover, we also discussed the explorative nature of this preliminary study in the revised manuscript as follows (Lines 529-533): “Due to the rarity of MBC occurrence and the stringent sample requirements of single-cell experiments, only limited MBC samples were included in this study. However, this explorative study identified notable differences between MBC and FBC, especially the distinct metabolic and immunological characteristics of MBC patients. These observations need to be further validated in more samples in the future.”

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2. Please supply raw p-value and statistical test used in Fig.1g. There are only 12 male samples compared to 1085 female samples in the TCGA, therefore one likely cannot assume the MBC will represent a normal distribution unless proven.

Response: Thanks for your professional suggestions. We showed raw p-values in all figures of the revised version, including Figure 2, 3, 4, 5, 6, and Supplementary Figure 3-11. We selected the ER⁺ TCGA-BRCA samples based on the clinical information in the XenaBrowser website (<https://xenabrowser.net/datapages/>). Specifically, 835 primary tumor samples with positive breast_carcinoma_estrogen_receptor_status were selected. Samples without RNA-seq data were further removed. Finally, we obtained the transcriptomic and clinical data of 722 ER⁺ TCGA-BRCA samples. The tumor

1072 purity and signature scores of immune cells between 12 MBC and 710 FBC were
1073 compared using two-sided Wilcoxon rank-sum test, which was a non-parametric test
1074 that did not assume known distributions (Hogg, R.V. and Tanis, E.A., Probability and
1075 Statistical Inference, 7th Ed, Prentice Hall, 2006). We added description of the
1076 statistical test in the revised Methods as follows (**Lines 725-728**): “The scores of
1077 immune or stromal cells were compared between MBC and FBC samples using two-
1078 sided Wilcoxon rank-sum test, which was a non-parametric test that did not assume
1079 known distributions ⁶⁵.” Besides, the legend of this figure was revised to specify the
1080 statistical test: “Boxplot showing the tumor purity and signature scores of various cell
1081 types between MBC and FBC in TCGA ER⁺ BRCA cohort. P-value was calculated by
1082 two-sided Wilcoxon rank-sum test.”

1083

1084 3. Statistical test for Figure 1i needed in figure legend.

1085 Response: We apologize for the unclear legend. The two-sided Wilcoxon rank-sum test
1086 was used to measure the differences between two groups. The legend was revised to
1087 “Boxplot indicating the IHC scores of CD4 and CD8 in 30 ER⁺ male and 30 ER⁺ female
1088 patients (identified by the percentage of positive cells). P-value was calculated by two-
1089 sided Wilcoxon rank-sum test.”

1090

1091 4. Representative IHC for foxp3 positive staining appears to be nonspecifically stain
1092 tumor cells (Figure 1h). The investigators perhaps should perform dual IF to
1093 demonstrate the FOXP3 staining is confined to Treg cells (CD4+). The details of the
1094 cohort in Figure 1 needs to be in the figure legend or text.

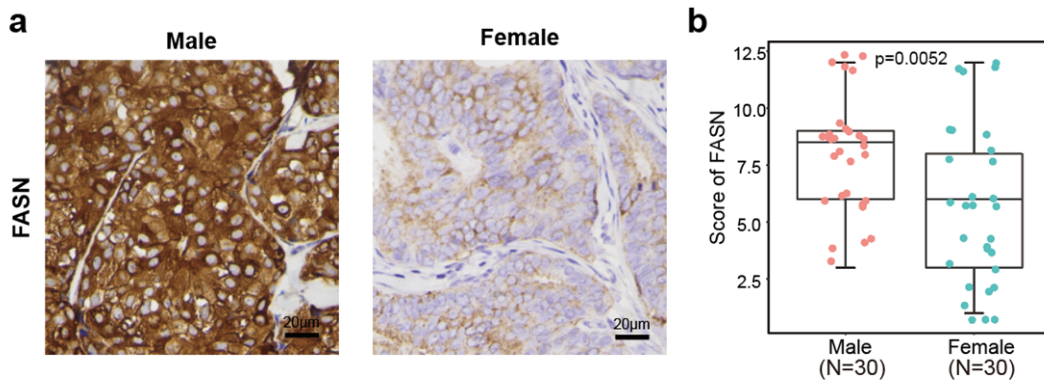
1095 Response: Thank you for pointing this out. We agree with the reviewer that the IHC
1096 staining of FOXP3 was not specific. It would be better to perform dual staining for both
1097 CD4 and FOXP3 to identify the Tregs. However, these IHC staining results were used
1098 to validate the significantly higher enrichment of T cells in FBC samples than in MBC
1099 samples, which was observed in the single-cell data and bulk RNA-seq data.
1100 Considering the Treg infiltration is not the concern of this context, we only retained the
1101 IHC staining of CD4 and CD8 in the revised manuscript, as shown in **Response Figure**
1102 **27** (Figure 2h in the revised manuscript). This validation cohort includes 30 ER⁺ MBC
1103 and 30 ER⁺ FBC samples. The details of this cohort were described in the revised
1104 manuscript as follows (**Lines 175-178**): “Furthermore, we performed
1105 immunohistochemistry (IHC) analysis for T cell markers CD4 and CD8 in 30 ER⁺ MBC
1106 and 30 ER⁺ FBC samples. Results suggested that these T cell markers had a lower
1107 expression proportion in MBC than in FBC samples (**Figure 2h, i**)”. We also added the
1108 corresponding description in the figure legend as “Boxplot indicating the IHC scores
1109 of CD4 and CD8 in 30 ER⁺ male and 30 ER⁺ female patients (identified by the

1110 percentage of positive cells). P-value was calculated by two-sided Wilcoxon rank-sum
1111 test”.

1112

1113 5. What does IHC look like for FASN and AR in this cohort from Figure 1h?

1114 Response: Thank you for your valuable suggestion. Accordingly, we performed the IHC
1115 staining for FASN in the same cohort, including 30 ER⁺ MBC and 30 ER⁺ FBC samples.
1116 Results showed that the protein levels of FASN were remarkably higher in MBC than
1117 in FBC samples (Wilcoxon rank-sum test, p-value: 0.0052; **Response Figure 28**). We
1118 added this result in the revised manuscript as follows (**Lines 229-234**): “Moreover, the
1119 IHC staining for FASN in 30 ER⁺ MBC samples and 30 ER⁺ FBC samples were
1120 compared. Results showed that the protein levels of FASN were remarkably higher in
1121 MBC than in FBC samples (Wilcoxon rank-sum test, p-value: 0.0052; Figure 4e-f).
1122 This observation indicated that fatty acids played an important role in tumor cell energy
1123 metabolism in MBC patients.”

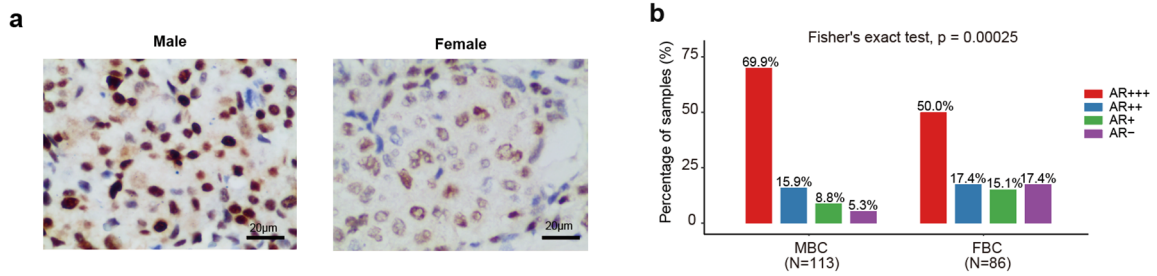


1124

1125 **Response Figure 28 (Related to Figure 4e-f in revised manuscript). Statistical quantification**
1126 **of FASN staining in MBC and FBC.** (a) IHC images of MBC and FBC samples stained for FASN;
1127 Scale bar, 20 μm. (b) Boxplot indicating the IHC score of FASN. P-value was calculated by two-
1128 sided Wilcoxon rank-sum test.

1129 Due to the absence of available qualified tissue samples, we are sorry that it is
1130 unable to perform the IHC experiments for AR in this cohort. But alternatively, based
1131 on the clinical diagnosis information, we retrospectively investigated the AR levels
1132 evaluated by IHC in a large sample cohort, including 113 ER⁺ MBC and 86 ER⁺ FBC
1133 samples (**Response Figure 29**). Results showed that the percentage of AR⁻ patients was
1134 significantly lower in MBC than in FBC samples (5.3% vs. 17.4% in MBC and FBC
1135 samples, respectively), whereas the percentage of AR⁺⁺⁺ patients was higher in MBC
1136 than in FBC samples (69.9% vs. 50.0% in MBC and FBC samples, respectively). This
1137 result further validated the activated AR regulon in MBC patients observed at the
1138 single-cell level. We added this result in the revised manuscript as follows (**Lines 206-**
1139 **213**): “To further evaluate the observation of AR, we retrospectively investigated the
1140 AR levels evaluated by IHC in a large sample cohort, including 113 ER⁺ MBC and 86

1141 ER⁺ FBC samples (**Figure 3i-j**). Results showed that the percentage of AR-negative
 1142 patients was significantly lower in MBC than in FBC samples (5.3% vs. 17.4% in MBC
 1143 and FBC samples, respectively), whereas the percentage of AR+++ patients was higher
 1144 in MBC than in FBC samples (69.9% vs. 50.0% in MBC and FBC samples,
 1145 respectively). This result further validated the activated AR regulon in MBC patients
 1146 observed at the single-cell level.”

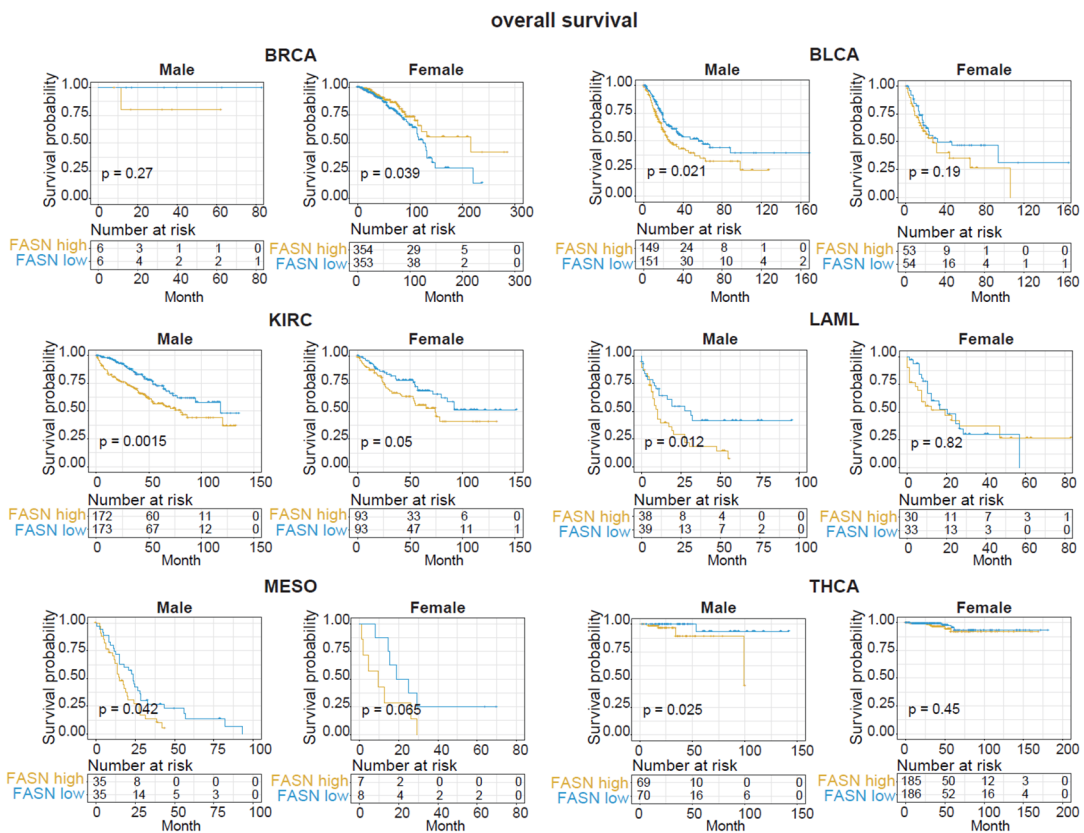


1147 **Response Figure 29 (Related to Figure 3i-j in revised manuscript). The expression levels of AR**
 1148 **in MBC and FBC samples. (a)** IHC images representing MBC and FBC samples stained for AR.
 1149 Scale bar, 20 μm. **(b)** Barplot showing the percentage of AR-negative, AR+, AR++, and AR+++
 1150 samples from MBC and FBC ER⁺ patients. P-value was calculated by fisher's exact test.
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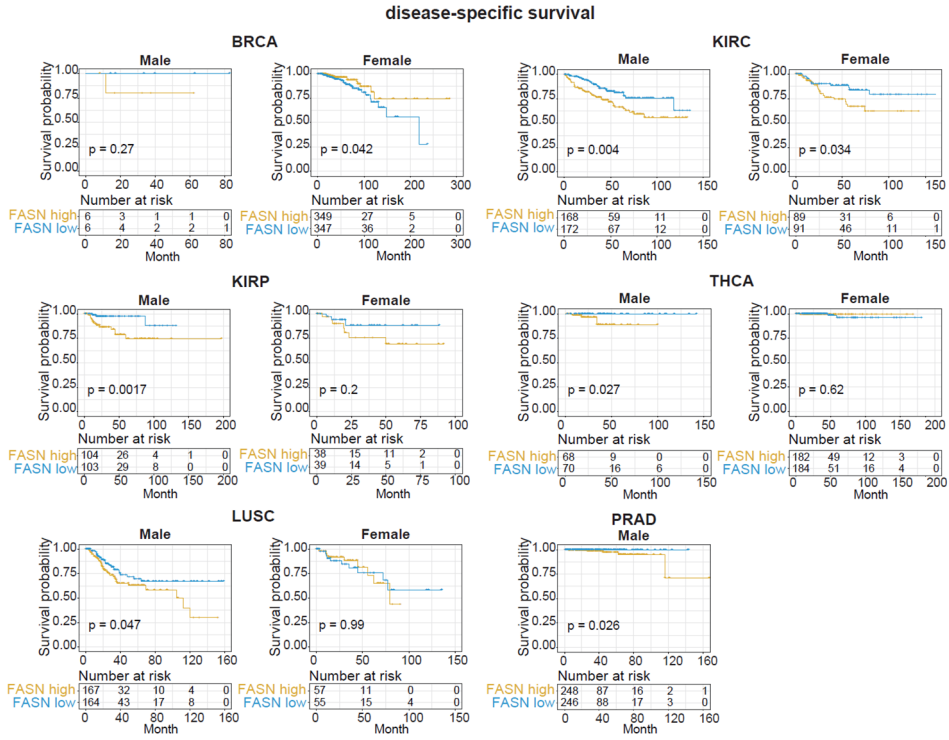
1153 6. A hallmark of prostate cancer progression is dysregulation of lipid metabolism via
 1154 overexpression of fatty acid synthase (FASN), a key enzyme in de novo fatty acid
 1155 synthesis. Why was prostate cancer (PRAD) left out of the survival analysis stratified
 1156 by FASN levels? Please include citation and discussion of targeting FASN in prostate
 1157 cancer (PMID: 30578319).

1158 Response: Thank you for your professional advice. In the previous version, we
 1159 performed the analysis for overall survival (OS) of male and female patients with
 1160 different cancer types and showed that FASN expression was prognostic for male
 1161 patients but not for female patients in some cancers, such as bladder urothelial
 1162 carcinoma (BLCA) and kidney renal clear cell carcinoma (KIRC). Prostate cancer
 1163 (PRAD) was previously not included in the analysis due to the absence of female
 1164 patients. In order to address the reviewer’s concern, we performed survival analyses for
 1165 OS, disease-specific survival (DSS), and progression-free interval (PFI) of TCGA pan-
 1166 cancer datasets by categorizing the patients into FASN-high and FASN-low groups for
 1167 each dataset according to the median of FASN expression (**Response Figure 30-32**).
 1168 Results showed that higher FASN expression was prognostic for the poor DSS of PRAD
 1169 patients, suggesting the involvement of FASN in PRAD progression (**Response Figure**
 1170 **31**). We have carefully read Zadra et al.’s study you recommended and are very inspired
 1171 to know the association between FASN and AR signaling, as well as with the
 1172 aggressiveness and resistance of PRAD (Zadra G, et al. *Proceedings of the National*
 1173 *Academy of Sciences*, 2019). We added the results of survival analysis for PRAD in the
 1174 revised manuscript as follows (**Lines 294-296**): “Notably, higher FASN expression was

1175 prognostic for the poor DSS of PRAD patients, consistent with a previous study that
 1176 demonstrated that targeting FASN could inhibit the aggressive and resistant PRAD²⁴.”
 1177 In addition, we added discussion about the association between FASN and disease
 1178 progression of patients with hormone-receptor-positive cancers as follows (Lines 497-
 1179 502): “Notably, a previous study demonstrated that lipid metabolism dysregulation
 1180 driven by FASN upregulation was important in the PRAD progression and castration
 1181 resistance mediated by AR signaling²⁴. Our analysis also indicated the association
 1182 between FASN expression and poor prognosis in PRAD. These results consistently
 1183 suggested that FASN-mediated lipid metabolism dysregulation was a potential
 1184 therapeutic target for hormone-receptor-positive cancers.”

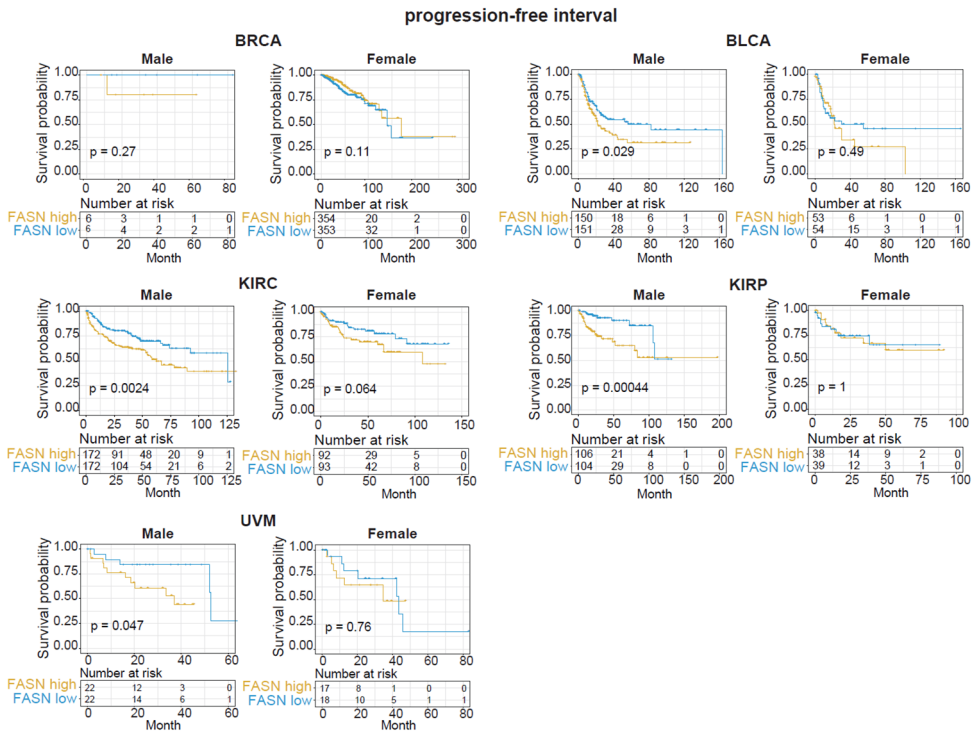


1185
 1186 **Response Figure 30 (Related to Supplementary Figure 9 in revised manuscript). Overall**
 1187 **survival analysis of male and female patients in various cancer types based on the FASN**
 1188 **expression.** Patients are categorized into FASN-high and FASN-low groups for each dataset
 1189 according to the median of FASN expression. BRCA: Breast invasive carcinoma; BLCA: Bladder
 1190 Urothelial Carcinoma; KIRC: Kidney renal clear cell carcinoma; LAML: Acute Myeloid Leukemia;
 1191 MESO: Mesothelioma; THCA: Thyroid carcinoma.
 1192



1193

1194 **Response Figure 31 (Related to Supplementary Figure 10 in revised manuscript). Disease-specific survival analysis of male and female patients in various cancer types based on the**
 1195 **FASN expression. Patients are categorized into FASN-high and FASN-low groups for each dataset**
 1196 **according to the median of FASN expression. BRCA: Breast invasive carcinoma; KIRC: Kidney**
 1197 **renal clear cell carcinoma; KIRP: Kidney renal papillary cell carcinoma; THCA: Thyroid carcinoma;**
 1198 **LUSC: Lung squamous cell carcinoma; PRAD: Prostate adenocarcinoma.**
 1199



1200

1201 **Response Figure 32 (Related to Supplementary Figure 11 in revised manuscript). Progression-**
1202 **free interval analysis of male and female patients in various cancer types based on the FASN**
1203 **expression.** Patients are categorized into FASN-high and FASN-low groups for each dataset
1204 according to the median of FASN expression. BRCA: Breast invasive carcinoma; BLCA: Bladder
1205 Urothelial Carcinoma; KIRC: Kidney renal clear cell carcinoma; KIRP: Kidney renal papillary cell
1206 carcinoma; UVM: Uveal Melanoma

1207

1208 7. Supplementary Fig. 2 legend description inadequate. What fold change and
1209 significance and testing performed?

1210 Response: We apologize for the unclear description. To compare the characteristics of
1211 cancer cells from MBC and FBC samples, we integrated cancer cells from 19 samples
1212 and identified 36 clusters by unsupervised clustering. Using the MAST method with
1213 default parameters in the Seurat package, we identified genes with log₂ fold change
1214 greater than 0.25 and adjusted p-value less than 0.01 for each cluster. Based on the order
1215 of log₂ fold change, the top 100 genes were further identified as markers of each cluster.
1216 By calculating the proportion of cancer cells from MBC samples in each cluster, we
1217 defined male, female, and mixed clusters. Specifically, clusters with a proportion of
1218 male cancer cells higher than 70% were defined as male clusters, those with a
1219 proportion lower than 50% were defined as female clusters, and the others were defined
1220 as mixed clusters. To identify the genes specifically expressed in male clusters, gene
1221 markers that presented in at least three male clusters were selected, and markers of
1222 female or mixed clusters were further removed from this list. We re-phrased the
1223 description of this procedure in the revised Method section, and revised the figure
1224 legend as follows: “The expression levels of specifically expressed genes of male
1225 cancer cell clusters. Genes with log₂ fold change greater than 0.25 and adjusted p-value
1226 less than 0.01 for each cluster were identified using the MAST method with default
1227 parameters. Gene markers that presented in at least three male clusters were selected,
1228 and markers of female or mixed clusters were further removed from this list.”

1229

1230 8. Supplementary Fig. 4 legend needs more detail. How were FASN high and low
1231 cutoffs determined?

1232 Response: Thank your pointing this out. We categorized the patients into FASN-high
1233 and FASN-low groups for each dataset according to the median of FASN expression.
1234 The corresponding legend was revised as: “Overall survival analysis of male and female
1235 patients in various cancer types based on the FASN expression. Patients are categorized
1236 into FASN-high and FASN-low groups for each dataset according to the median of
1237 FASN expression. BRCA: Breast invasive carcinoma; BLCA: Bladder Urothelial
1238 Carcinoma; KIRC: Kidney renal clear cell carcinoma; LAML: Acute Myeloid
1239 Leukemia; MESO: Mesothelioma; THCA: Thyroid carcinoma.”

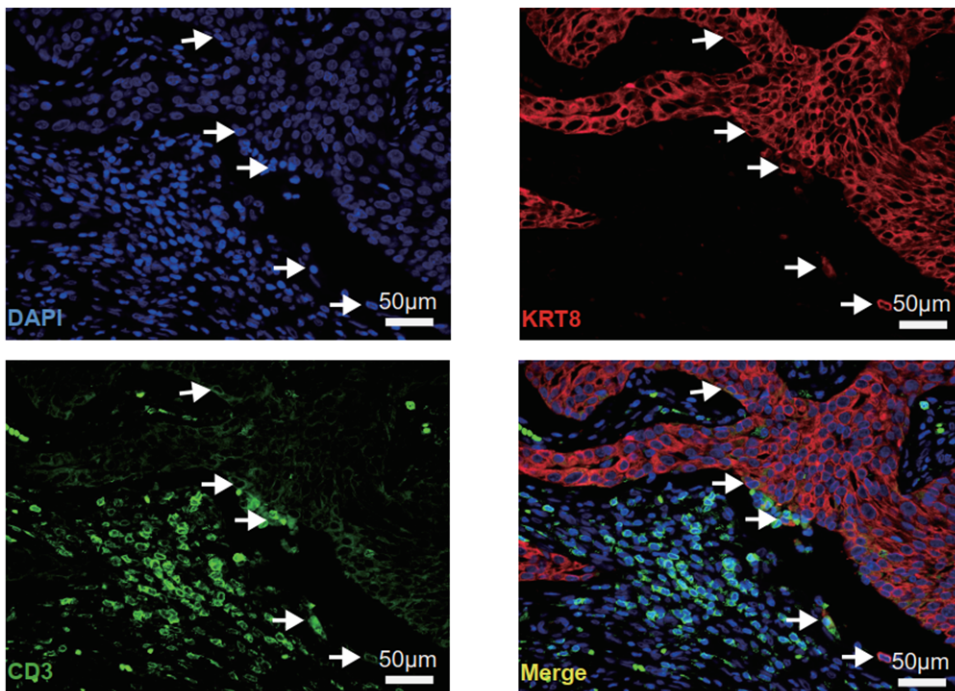
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1241 9. The fact that FASN and the ER- and AR-response genesets were significantly
1242 enriched by the up-regulated genes of “epithelial-T” co-expression cells, suggests that
1243 there may be mixing of epithelial and T cell RNA in these dual positive cells. Therefore,
1244 additional experiments are needed for the existence of “epithelial-T cells”. The authors
1245 provide dual immunofluorescence (IF), however the staining in Figure 5B is
1246 unconvincing. The legend states the scale bar is 50uM, but there is no scale bar and thus
1247 hard to interpret. It is not clear whether the staining is from a single mitotic cell or many
1248 cells at a distance. The DAPI does not even show uniform nuclear localization. The
1249 staining appears to be an artifact. The researchers need to show additional validation of
1250 the for IF using positive and negative control tissues. In addition, the investigators need
1251 to quantify the CD3 only and epithelial T cells for the IF. The authors should also
1252 provide another independent method to support their findings such as flow cytometry
1253 (KRT and CD3) of dissociated T cells from fresh tumor tissue if possible.

1254 **Response:** We appreciate the reviewer for highlighting this concerns. With the
1255 development of single-cell techniques, we could investigate the cellular characteristics
1256 at high resolution and identify the previously unappreciated cells. Intriguingly, a study
1257 from Hu et al. also reported a non-traditional CD45⁺EpCAM⁺ cell population in the
1258 fallopian tube epithelial layer of ovarian cancer patients (Hu et al., Cancer Cell,
1259 2020, 37(2), 226-242). This population was also positive for CD3, CD44, CD69, and
1260 CD103, suggesting that these cells are possibly tissue-resident memory T lymphocytes
1261 (TRMs). They identified these cells by scRNA-seq (Smart-Seq2) and validated using
1262 immunofluorescence experiments. However, the biological and clinical implications of
1263 this populations are unclear yet. We are sorry for the unclear immunofluorescence
1264 results in the previous version. We performed the immunofluorescence experiments
1265 again and showed the cells with different phenotypes, including CD3⁺KRT8⁻, CD3⁻
1266 KRT8⁺, and CD3⁺KRT8⁺ cells. According to the immunofluorescence, CD3⁺KRT8⁺
1267 cells were located at the interface between KRT8⁺ epithelial cells and CD3⁺ T cells
1268 (**Response Figure 33a**). Furthermore, flow cytometry for antibody of KRT8 and CD3
1269 was performed using fresh tumor tissue from two MBC patients to validate and quantify
1270 the number of CD3⁺KRT8⁺ cells (**Response Figure 33b**). We gated the CD45⁺ immune
1271 cells and evaluated the expression of KRT8 of these cells. Results showed that there
1272 were 35.55% and 2.11% CD45⁺KRT8⁺ cells in two samples, respectively. Notably,
1273 57.07% and 20.82% of these KRT8⁺ immune cells were CD3⁺ T cells in two samples.
1274 Thus, the immunofluorescence and flow cytometry experiments indicated that the
1275 CD3⁺KRT8⁺ cells existed with various percentage in MBC samples. We added these
1276 corresponding evidence in the revised manuscript as follows (**Lines 395-405**): “Further
1277 validation using immunofluorescence experiments for the MBC sample confirmed the

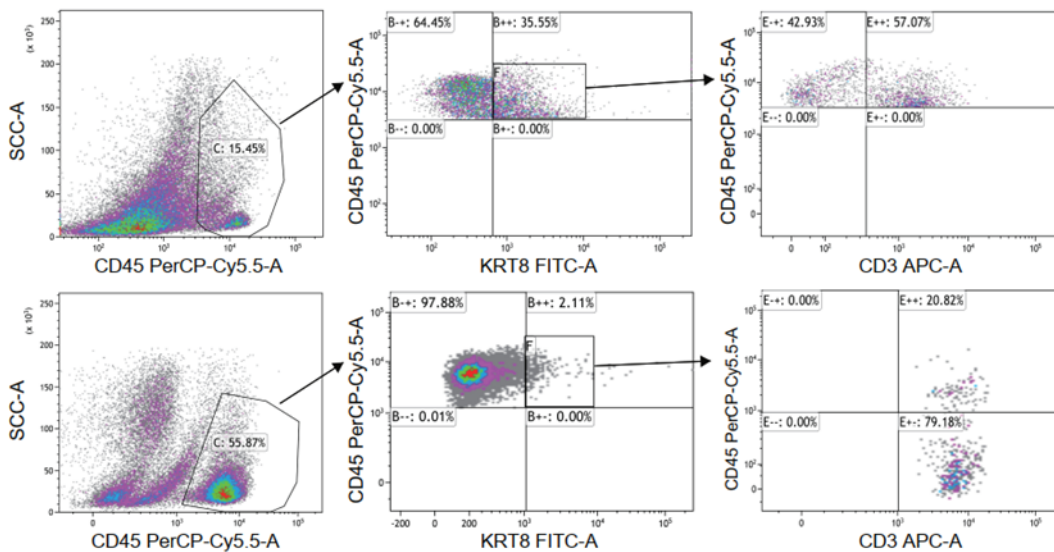
1278 above observation and showed that the CD3⁺KRT8⁺ cells were located at the interface
 1279 between KRT8⁺ epithelial cells and CD3⁺ T cells (**Figure 6c**). Furthermore, flow
 1280 cytometry for antibodies of KRT8 and CD3 was performed using fresh tumor tissue
 1281 from two MBC patients to validate and quantify the number of CD3⁺KRT8⁺ cells
 1282 (**Figure 6d**). We gated the CD45⁺ immune cells and evaluated the expression of KRT8
 1283 in these cells. Results showed that there were 35.55% and 2.11% CD45⁺KRT8⁺ cells in
 1284 two samples, respectively. Notably, 57.07% and 20.82% of these KRT8⁺ immune cells
 1285 were CD3⁺ T cells in two samples. Therefore, these results indicated the biological
 1286 existence of CD3⁺KRT8⁺ T cells and the enrichment of these cells with various
 1287 percentages in MBC samples.”

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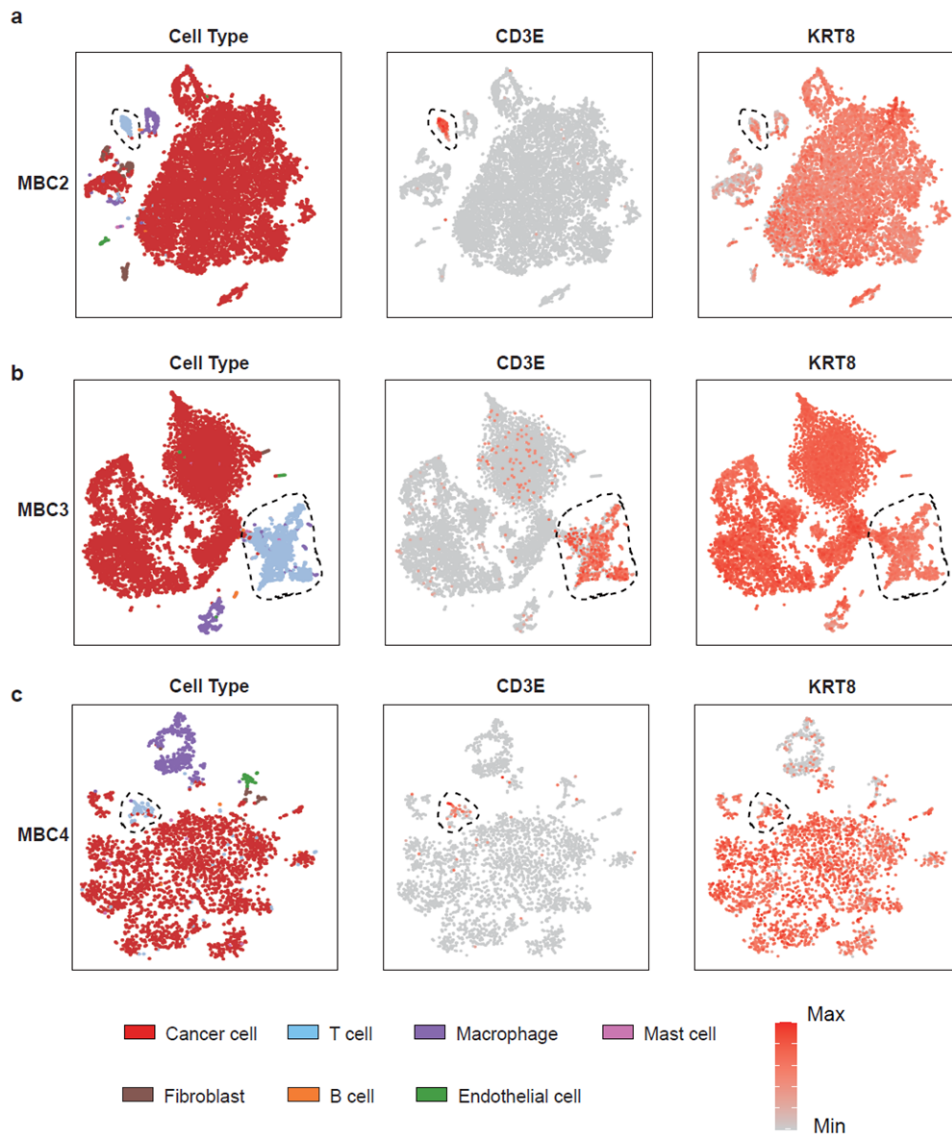
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1290 **Response Figure 33 (Related to Figure 6c-d in revised manuscript). Validation of the existence**
1291 **of CD3⁺KRT8⁺ T cells by the immunofluorescence and flow cytometry experiments. (a)** The
1292 immunofluorescence staining of KRT8 and CD3 in an MBC sample. White arrows indicate the
1293 CD3⁺KRT8⁺ T cells. Scale bar, 50 μ m. **(b)** Flow cytometry showing the percentage of CD3⁺KRT8⁺
1294 cells in two MBC samples.

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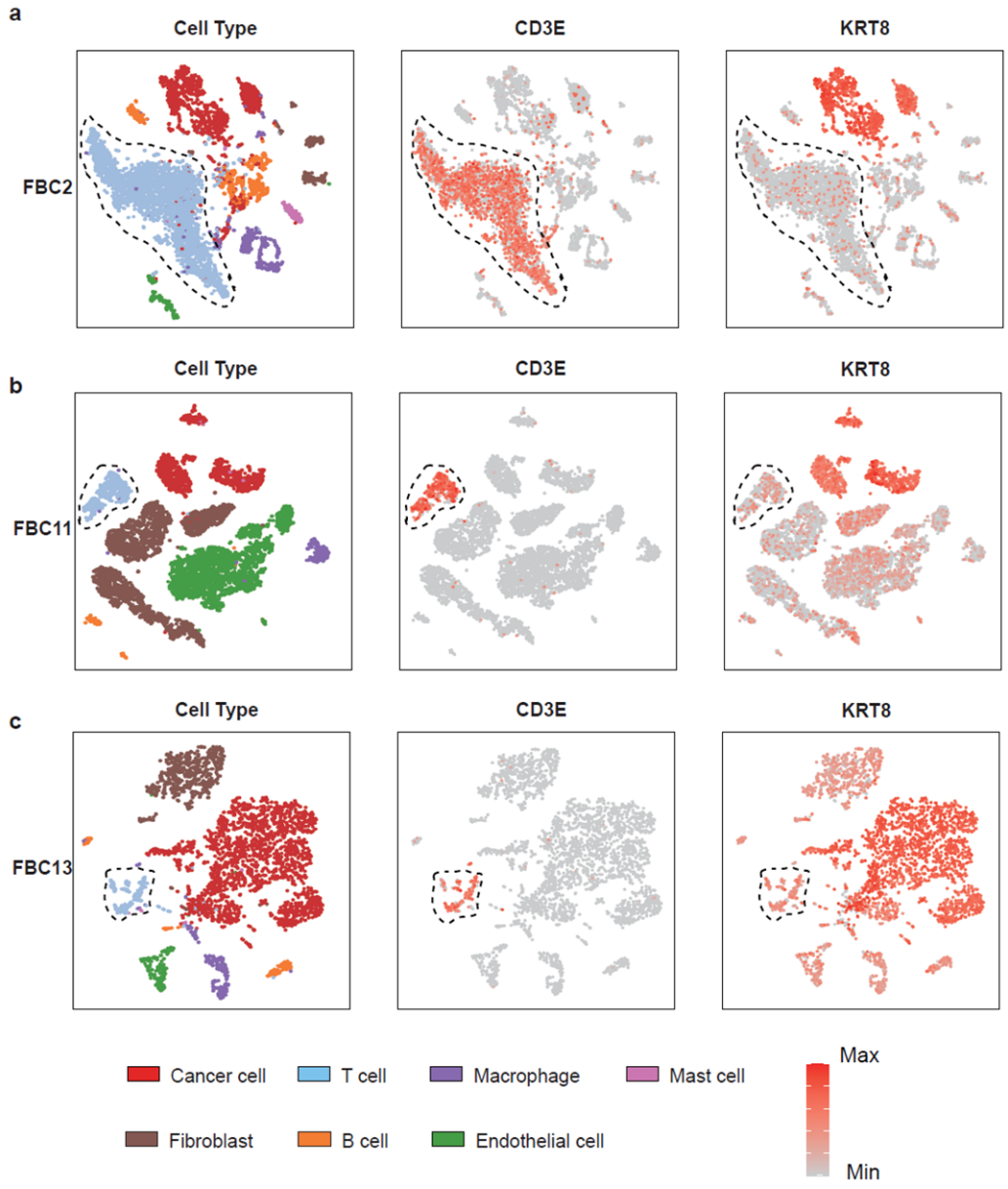
1296 10. Supplemental Fig. S6a is described as differentially expressed gene across five
1297 samples. What are the individual values? Aggregated expression of all the single cells
1298 for each tumor? Perhaps showing the expression of KRT8/18/19 and CD3 across all
1299 cells annotated by cell type for each tumor would be more convincing for the existence
1300 of an epithelial T-cell. This will show the relative KRT levels in true epithelial cells
1301 relative to the T cells.

1302 **Response:** We agree with the reviewer about this concern. It is important to show the
1303 expression of epithelial markers and T cell markers across all cell types. Accordingly,
1304 we re-clustered the cells from each sample and then visualized all cell types and marker
1305 expressions at the single-cell level. MBC and FBC samples with high percentage of
1306 CD3⁺KRT⁺ cells were shown in **Response Figure 34 and 35**. To further evaluate the
1307 expression of KRT8/18/19 in T cells, we also showed the aggregated expression of these
1308 markers of epithelial and T cells in each sample using the dot-plot (**Response Figure**
1309 **36**). The T cells from MBC2, MBC3, MBC4, MBC5, MBC6, and FBC13 had
1310 KRT8/18/19 expression, but were lower than these levels in epithelial cells. We added
1311 these corresponding results in the revised manuscript as follows (**Lines 368-375**): “**We**
1312 **re-clustered the cells from each sample and then visualized all cell types and marker**
1313 **expressions at the single-cell level. MBC and FBC samples with the highest percentage**
1314 **of CD3⁺KRT⁺ cells were shown in Supplementary Figure 13e, f. To further evaluate**
1315 **the expression of KRT8/18/19 in T cells, we also showed the aggregated expression of**
1316 **these markers of epithelial and T cells in each sample using the dot-plot**
1317 **(Supplementary Figure 13g). The T cells from MBC2, MBC3, MBC4, MBC5, MBC6,**
1318 **and FBC13 had KRT8/18/19 expression, but were lower than these levels in epithelial**
1319 **cells.”**



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Response Figure 34 (Related to Supplementary Figure 13e in revised manuscript). The expression of CD3E and KRT8 in various cell types from representative MBC samples. CD3E⁺KRT⁺ T cells were circled with dashed lines.



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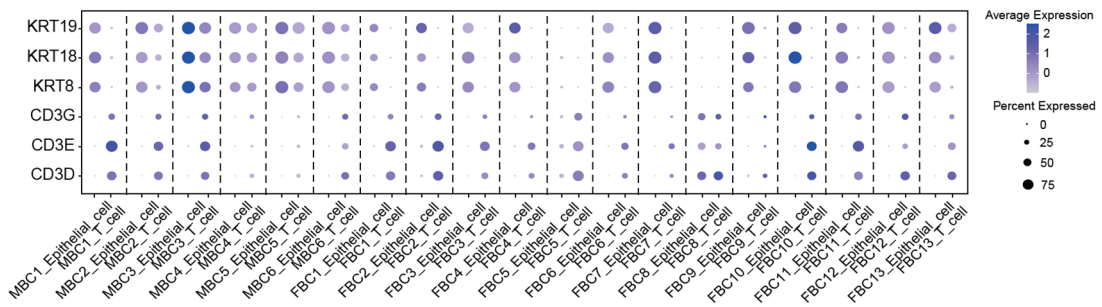
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Response Figure 35 (Related to Supplementary Figure 13f in revised manuscript). The expression of CD3E and KRT8 in various cell types from representative FBC samples. CD3E⁺KRT⁺ T cells were circled with dashed lines.



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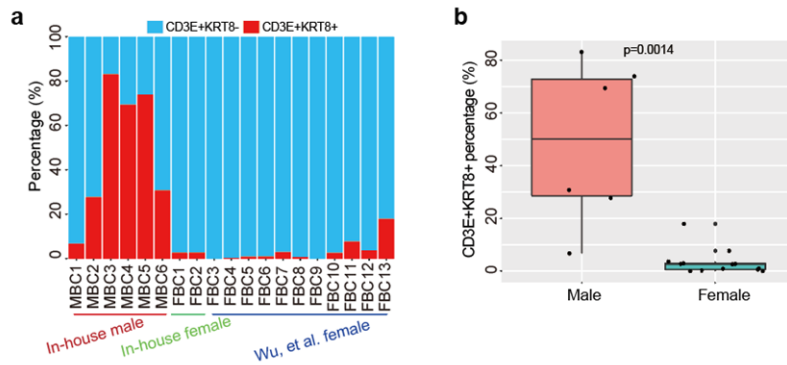
Response Figure 36 (Related to Supplementary Figure 13g in revised manuscript). Dotplot depicting aggregated expression of KRT8/KRT18/KRT19 and CD3D/CD3E/CD3G in epithelial and T cells from MBC and FBC samples.

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1334 11. Supplemental Fig. 6b shows the percentage of T cells that express KRT (epithelial-
1335 T cells) is around 40%, and similar in Fig 5C, however in Fig 5A there it appears that
1336 nearly all cells co-expressed CD3 and KRTs. What are the proportions of epithelial T
1337 cells in the other MBCs and FBCs or is this just an occurrence in the M3 tumor?

1338 **Response:** We thank the reviewer for highlighting this issue. The previous Figure 5A
1339 showed the expression of CD3 and KRT8 by taking MBC3 as an example, which had
1340 the highest percentage (83.1%) of CD3-KRT8 co-expressed cells comparing with other
1341 samples. Thus, due to the high enrichment of CD3-KRT8 co-expressed cells in MBC3,
1342 it visually seems that nearly all cells co-expressed CD3 and KRTs in that figure. The
1343 Supplementary Figure 6b in the previous version showed the percentage of co-
1344 expressed cells in all MBC samples, around 40%. We apologize for the unclear
1345 description and visualization in the previous version. In order to figure out whether the
1346 observed CD3E⁺KRT8⁺ T cells were patient-specific or generally existed, we evaluated
1347 the percentage of CD3E⁺KRT8⁺ T cells using the updated datasets, including 6 in-house
1348 MBC samples, 2 in-house FBC samples, and 11 FBC samples from Wu et al.. It turned
1349 out that 17/19 breast cancer samples had CD3E⁺KRT8⁺ T cells with different degree,
1350 ranging from 0.2% to 83.1% (**Response Figure 37a**). Especially, MBC samples showed
1351 higher percentage of CD3E⁺KRT8⁺ T cell component (6.7% ~ 83.1%), and FBC
1352 samples had relatively lower percentage (0.2% ~ 17.9%). The wilcoxon rank sum test
1353 showed a significant difference of CD3E⁺KRT8⁺ T cell enrichment between MBC and
1354 FBC groups (**Response Figure 37b**; p-value: 0.0014). We added this results in the
1355 revised manuscript (**Lines 361-377**) as follows: “In order to figure out whether the
1356 observed CD3E⁺KRT8⁺ T cells were patient-specific or generally existed, we evaluated
1357 the percentage of CD3E⁺KRT8⁺ T cells across 19 samples, including 6 in-house MBC
1358 samples, 2 in-house FBC samples, and 11 FBC samples from Wu et al.. It turned out
1359 that 17/19 breast cancer samples had CD3E⁺KRT8⁺ T cells with different degrees,
1360 ranging from 0.2% to 83.1% (Supplementary Figure 13d). Especially, MBC samples
1361 showed higher percentage of CD3E⁺KRT8⁺ T cell component (6.7% ~ 83.1%), and
1362 FBC samples had relatively lower percentage (0.2% ~ 17.9%). We re-clustered the cells
1363 from each sample and then visualized all cell types and marker expressions at the
1364 single-cell level. MBC and FBC samples with the highest percentage of CD3⁺KRT⁺
1365 cells were shown in Supplementary Figure 13e, f. To further evaluate the expression of
1366 KRT8/18/19 in T cells, we also showed the aggregated expression of these markers of
1367 epithelial and T cells in each sample using the dot-plot (Supplementary Figure 13g).
1368 The T cells from MBC2, MBC3, MBC4, MBC5, MBC6, and FBC13 had KRT8/18/19
1369 expression, but were lower than these levels in epithelial cells. The Wilcoxon rank-sum
1370 test showed a significant difference of CD3E⁺KRT8⁺ T cell enrichment between MBC

1371 and FBC groups (Supplementary Figure 13h; p-value: 0.0014).”



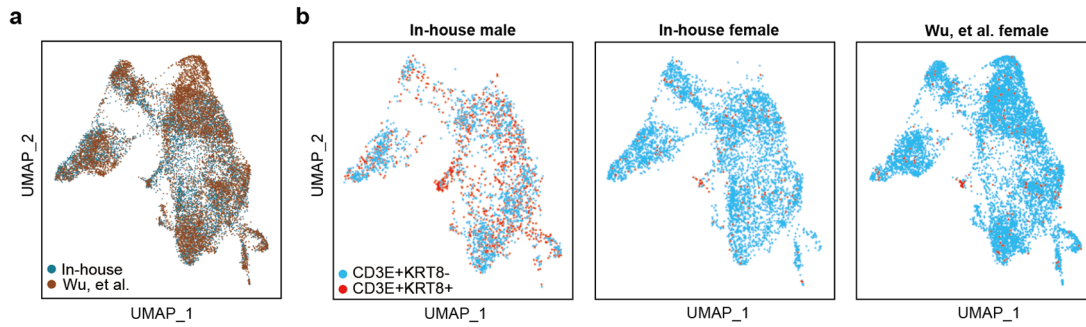
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1373 **Response Figure 37 (Related to Supplementary Figure 13d, h in revised manuscript).**
1374 **Evaluation of the existence of CD3E+KRT8+ T cells in the scRNA-seq dataset. (a)** Barplot
1375 showing the percentage of CD3E+KRT8+ T cells in each MBC and FBC sample. **(b)** Boxplot
1376 comparing the percentage of CD3E+KRT8+ T cells between MBC and FBC samples. P-value was
1377 calculated by two-sided Wilcoxon rank-sum test.

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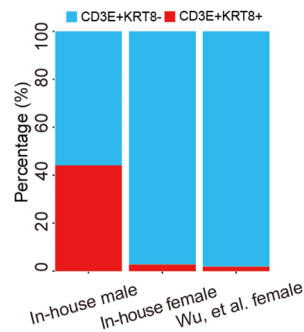
1379 12. The authors should consider evaluating several other scRNA breast cancer datasets
1380 for evidence of epithelial T cells.

1381 **Response:** We agree with the reviewer’s concern regarding the evaluation of
1382 CD3E+KRT8+ cells in other scRNA dataset. To further validate the existence of these
1383 cells, we downloaded and performed an integrated analysis for the scRNA-seq data of
1384 ER+ BRCA from the previous study (Wu et al., *Nature Genetics*, 2021), in which all the
1385 samples were from female patients. By integrating the transcriptomic data of T cells
1386 from in-house and Wu et al. (**Response Figure 38a-b**), we calculated the percentage of
1387 CD3E+KRT8+ T cells of in-house MBC, in-house FBC, and Wu’s FBC samples,
1388 respectively. Results showed that MBC samples had a significantly higher percentage
1389 of CD3E+KRT8+ T cells than the FBC samples from two datasets. Besides, the
1390 percentages of CD3E+KRT8+ T cells were similar in in-house and Wu et al.’s FBC
1391 samples(**Response Figure 39**), suggesting the existence of CD3E+KRT8+ T cells and
1392 the enrichment of these cells in male samples. We added this results in the revised
1393 manuscript (**Lines 355-361**) as follows: “To further validate the existence of these cells,
1394 we calculated the percentage of CD3E+KRT8+ T cells of in-house MBC, in-house FBC,
1395 and Wu et al.’s FBC samples, respectively (**Supplementary Figure 13a, b**). Results
1396 showed that the percentages of CD3E+KRT8+ T cells were similar in in-house and Wu
1397 et al.’s FBC samples (**Supplementary Figure 13c**). MBC samples had a significantly
1398 higher percentage of CD3E+KRT8+ T cells than the FBC samples from the two datasets
1399 (**Supplementary Figure 13c**).”



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Response Figure 38 (Related to Supplementary Figure 13a-b in revised manuscript). Evaluation of the existence of CD3E⁺KRT8⁺ T cells in the scRNA-seq dataset. (a) T-SNE plot of T cells colored by data sources. (b) T-SNE plots showing the distribution of CD3E⁺KRT8⁻ and CD3E⁺KRT8⁺ T cells in in-house MBC samples (left), in-house FBC samples (middle), and FBC samples from Wu et al. (right).



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Response Figure 39 (Related to Supplementary Figure 13c in revised manuscript). Barplot showing the percentage of CD3⁺KRT8⁺ T cells in different datasets.

1411 13. Data availability section is weak, and data are not publicly deposited (this can be
1412 blinded until publication but available for reviewers).

1413 **Response:** We appreciate your comment and apologize for forgetting to include the
1414 access link. The single-cell RNA-seq data of this study have been deposited in Genome
1415 sequence Archive database with accession number HRA001341. Reviewers can access
1416 these sequence files via the link: <https://ngdc.cncb.ac.cn/gsa-human/s/Mv4xF4IP>. The
1417 data will be publicly accessed after the publication of this study.

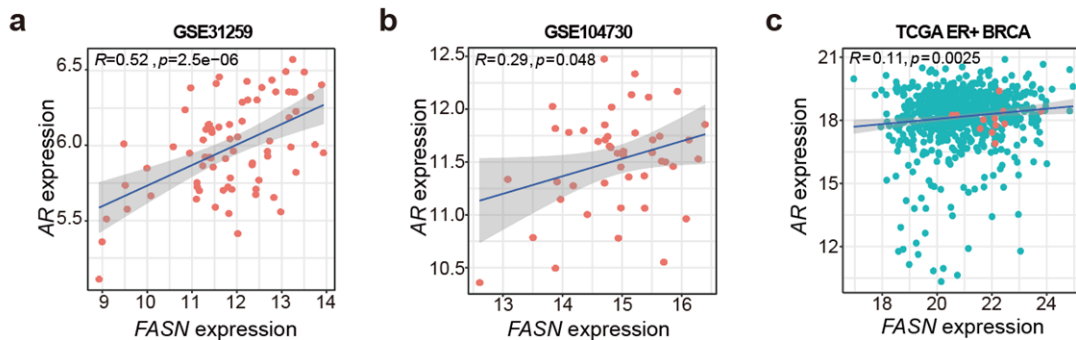
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1419 14. The authors should consider evaluating the role of AR in MBC in more detail. Such
1420 as performing IHC on specimens, evaluating the RNA-seq for existence to alternative
1421 splicing in the androgen receptor.

1422 **Response:** We thank the reviewer for this helpful suggestion. Accordingly, we
1423 retrospectively investigated the AR levels evaluated by IHC in a large sample cohort,
1424 including 113 ER⁺ MBC and 86 ER⁺ FBC samples (**Response Figure 29**). Results
1425 showed a significantly higher percentage of ER⁺ samples in MBC than in FBC (Fisher's
1426 exact test, p-value: 0.00025). We added this result in the revised manuscript as follows
1427 (**Lines 206-213**): "To further evaluate the observation of AR, we retrospectively

1428 investigated the AR levels evaluated by IHC in a large sample cohort, including 113
1429 ER⁺ MBC and 86 ER⁺ FBC samples (**Figure 3i-j**). Results showed that the percentage
1430 of AR-negative patients was significantly lower in MBC than in FBC samples (5.3%
1431 vs. 17.4% in MBC and FBC samples, respectively), whereas the percentage of AR+++
1432 patients was higher in MBC than in FBC samples (69.9% vs. 50.0% in MBC and FBC
1433 samples, respectively). This result further validated the activated AR regulon in MBC
1434 patients observed at the single-cell level.”

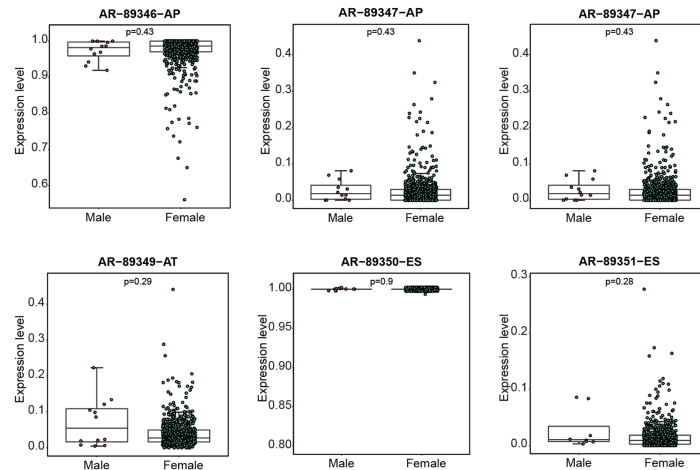
1435 In addition, inspired by the previous studies that demonstrated the fatty acid
1436 metabolism driven by AR in PRAD (Zadra G, et al. *Proceedings of the National*
1437 *Academy of Sciences*, 2019; Swinnen J V, et al. *Cancer research*, 1997), we investigated
1438 the association between AR and FASN expression in breast cancer samples (**Response**
1439 **Figure 40**). Results showed that the expression of AR and FASN had significantly
1440 positive correlations in MBC samples from GSE31259 and GSE104730, but had no
1441 obvious correlations in the TCGA dataset possibly due to the limited number of MBC
1442 samples in TCGA. We added this result in the revised manuscript as follows (**Lines**
1443 **247-252**): “In addition, inspired by the previous studies that demonstrated the fatty acid
1444 metabolism driven by AR in PRAD^{24,25}, we investigated the association between AR
1445 and FASN expression in MBC and FBC samples (**Figure 4i**). Results showed that the
1446 expressions of AR and FASN were positively correlated in MBC samples from
1447 GSE104730⁶ and GSE31259²⁰, but had no obvious correlations in the FBC samples of
1448 the TCGA dataset.”



1449 **Response Figure 40 (Related to Figure 4i in revised manuscript). The Pearson correlation**
1450 **analysis between the expression of FASN and AR in independent breast cancer datasets.**
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1453 Although alternative splicing in AR has been reported to play an important role in
1454 the progression and resistance of PRAD (Antonarakis E S, et al. *New England Journal*
1455 *of Medicine*, 2014; Zadra G, et al. *Proceedings of the National Academy of Sciences*,
1456 2019), the existence of AR alternative splicing in MBC remains unexplored. However,
1457 using the 10X Genomics Chromium (10X) approach, our scRNA-seq data capture
1458 transcripts through poly(A) tails and have 3'-bias in coverage, limiting the capability of
1459 performing the alternative splicing analysis on the single-cell level (Wang X, et al.

1460 *Genomics, proteomics & bioinformatics*, 2021). We analyzed the alternative splicing
1461 events of ER⁺ TCGA-BRCA samples based on the data from TCGASpliceSeq database
1462 (Ryan M, et al. *Nucleic acids research*, 2016). Result showed that there was no
1463 significant differences in the expression of AR isoforms between ER⁺ MBC and FBC
1464 samples in the TCGA dataset (**Response Figure 41**).



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Response Figure 41. The expression of AR splicing isoforms between MBC and FBC samples in TCGA ER+ dataset. AP: Alternate Promoter; ES: Exon Skip.

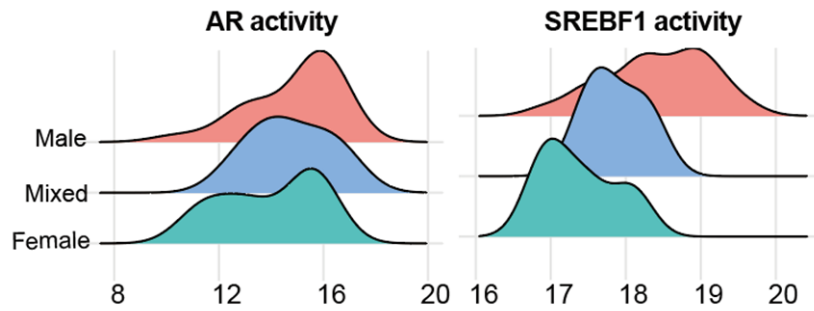
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1469 15. The authors need more detail in most figure legends. It is sometimes hard to
1470 interpret the data. For example, Figure 2g and h show expression and activation of
1471 transcription factors, but what cell types were evaluated (just epithelial)? There appears
1472 to be a bimodal distribution in these blots suggesting there the cells are either in an on
1473 or off state. It would be interesting to see what cells are on vs. off.

1474 **Response:** We thank reviewer for highlighting these concerns. We apologize for the
1475 unclear figure legends. The previous Figure 2 (Figure 3 in the revised manuscript) had
1476 shown the comparison analysis of transcriptome of cancer cells from MBC and FBC
1477 samples. Thus, all the plots in this Figure showed the results of cancer (epithelial) cells.

1478 We revised the legend as follows: “Heatmap showing the activity scores of transcription
1479 factors (TFs) in cancer cells from male, female, and mixed clusters” and “Ridgeline
1480 plot showing the activity levels of MBC-specific TFs in cancer cells from male, female,
1481 and mixed clusters”. Using the updated dataset, we performed the transcriptional
1482 regulon analysis for cancer cells from six MBC samples and thirteen FBC samples.
1483 Results showed that the distribution of AR and SREBF1 activity was not obviously
1484 bimodal (**Response Figure 42**).



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Response Figure 42 (Related to Figure 3g in revised manuscript). Ridgeline plot showing the activity levels of MBC-specific TFs in cancer cells from male, female, and mixed clusters.

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Response References

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1. Wu S Z, Al-Eryani G, Roden D L, et al. A single-cell and spatially resolved atlas of human breast cancers[J]. *Nature genetics*, 2021, 53(9): 1334-1347.

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2. Hogg, R.V. and Tanis, E.A., *Probability and Statistical Inference*, 7th Ed, Prentice Hall, 2006

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3. Zadra G, Ribeiro C F, Chetta P, et al. Inhibition of de novo lipogenesis targets androgen receptor signaling in castration-resistant prostate cancer[J]. *Proceedings of the National Academy of Sciences*, 2019, 116(2): 631-640.

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5. Swinnen J V, Esquenet M, Goossens K, et al. Androgens stimulate fatty acid synthase in the human prostate cancer cell line LNCaP[J]. *Cancer research*, 1997, 57(6): 1086-1090.

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6. Wang X, He Y, Zhang Q, et al. Direct comparative analyses of 10X genomics chromium and smart-seq2[J]. *Genomics, proteomics & bioinformatics*, 2021, 19(2): 253-266.

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7. Ryan M, Wong W C, Brown R, et al. TCGASpliceSeq a compendium of alternative mRNA splicing in cancer[J]. *Nucleic acids research*, 2016, 44(D1): D1018-D1022.

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8. Antonarakis E S, Lu C, Wang H, et al. AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer[J]. *New England Journal of Medicine*, 2014, 371(11): 1028-1038.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors addressed adequately my comments and improved considerably the manuscript.

I still have a few minor questions and remarks:

1. Regarding the existence of CD3D+/KRT8+ cells, did the authors try to use a software such as CellBender to decontaminate the raw UMI matrices and further investigate if this specific population of cells still remain in the resulting clustering ?
2. It seems that the authors are using CD79A to identify B-cells. However, CD79A is expressed by both B and plasma cells, and two different clusters are visible within the umap. Did the authors try to use MS4A1 and JCHAIN to identify B-cells and plasma cells respectively ?
3. A similar remark can be done for endothelial cells. Did the authors tried to discriminate between vascular and lymphatic endothelial cells using specific markers ?
4. The authors used Dorothea to analyze regulon activity (<https://bioconductor.org/packages/release/data/experiment/vignettes/dorothea/inst/doc/dorothea.html>). However, it seems from the documentation that Dorothea is a database of regulons used as input for other statistical methods such as decoupleR or SCENIC. Did the authors used such packages to analyze regulons activity ?

Reviewer #2 (Remarks to the Author):

The responses are extensive and extremely helpful. No further comments.

Reviewer #3 (Remarks to the Author):

The authors have made substantial improvements to the manuscript and the additional experimentation and analysis have cleared up all my concerns but one. The evidence for KRT8+ CD3 T cells is still insufficient for conclusively identifying this novel cell type. The authors have added single cell analysis (Supplemental figs 13e and f), however, but “KRT8/18/19 expression, but were lower than these levels in epithelial cells.” Since there are no scale bars, and min/max should be different for each gene analyzed, it is hard to determine if these CD3 cells are truly KRT8 positive or that the scales are set so low that all cells have some KRT8 positivity.

The new IF images (Figure 6C) show that the only KRT18 positive T cells are at the interface with the tumor cells. In fact, the cell in the top arrow is clearly KRT8 positive, but does not appear to be CD3 positive and is likely an exposure artefact. The additional cells identified appear to be sectioning artefacts in which multiple layers of cells are stained. I would anticipate that some CD3 cells away from the tumor would have to be KRT8 positive to truly identify this novel cell type.

The flow cytometry in Response Figure 8 is also unconvincing because it lacks a positive control for KRT8 (epithelial cells). Additionally, the gate in upper panel of response 8b appears to be loosely gated for CD45 and potentially including CD45 negative cells. Clearly all the T cells (CD3D) in figure 1B are negative for KRT8 and I suspect KRT18 as well. In Figure 5 F, all the male T cells are either KRT18 or KRT8 positive, which is different than figure 1B.

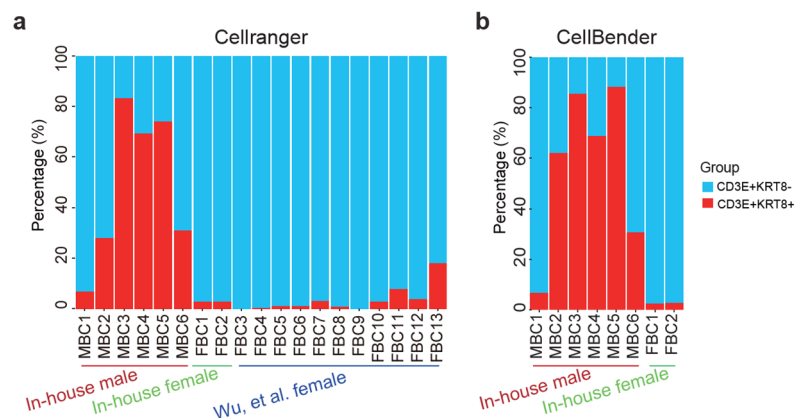
These discrepancies need to be addressed to conclusively state that KRT8 positive T cell exist.

Reviewer #1 (Remarks to the Author):

The authors addressed adequately my comments and improved considerably the manuscript. I still have a few minor questions and remarks:

1. Regarding the existence of CD3D+/KRT8+ cells, did the authors try to use a software such as CellBender to decontaminate the raw UMI matrices and further investigate if this specific population of cells still remain in the resulting clustering?

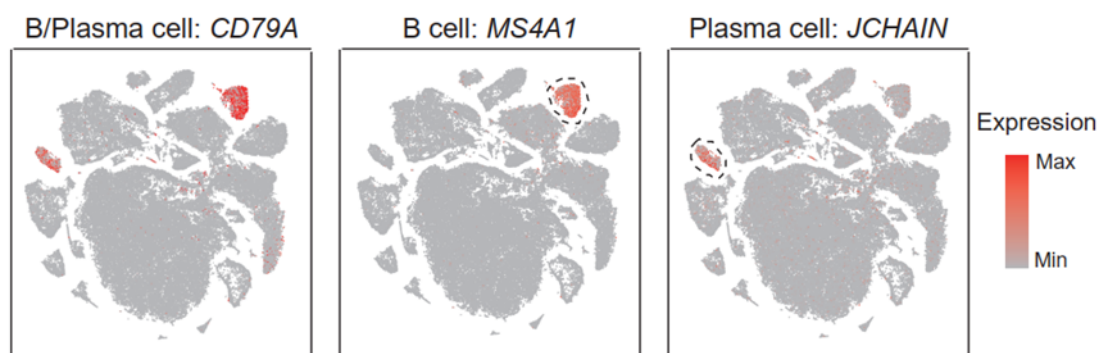
Response: Thank you for your valuable suggestion. Accordingly, we used CellBender[1] to decontaminate the in-house scRNA-seq data, of which the raw UMI matrices were available. After removing the empty droplets and retrieving background-free gene expression profiles by CellBender, we found that CD3E⁺KRT8⁺ cells still existed in all samples (**Response Figure 1**), keeping consistent with the previous results based on Cell Ranger. This result double-confirmed the existence of CD3E⁺KRT8⁺ cells and avoided the potential influence of technical contamination. We added the corresponding result in the revised manuscript as follows (**Lines 402-408**): “Moreover, we used CellBender³⁶ to decontaminate the in-house scRNA-seq data, of which the raw UMI matrices were available. After removing the empty droplets and retrieving background-free gene expression profiles, we found that CD3E⁺KRT8⁺ cells still existed in all samples (**Supplementary Figure 15c**), keeping consistent with the results based on Cell Ranger (**Supplementary Figure 14a**). This result double-confirmed the existence of CD3E⁺KRT8⁺ cells and avoided the potential influence of technical contamination.”



Response Figure 1 (Related to Supplementary Figure 14a and 15c in the revised manuscript). Evaluation of the existence of CD3E⁺KRT8⁺ T cells in the scRNA-seq dataset. (a) Barplot showing the percentage of CD3E⁺KRT8⁺ T cells in each MBC and FBC sample. (b) Barplot showing the percentage of CD3E⁺KRT8⁺ T cells in in-house samples after decontamination analysis by CellBender.

2. It seems that the authors are using CD79A to identify B-cells. However, CD79A is expressed by both B and plasma cells, and two different clusters are visible within the umap. Did the authors try to use MS4A1 and JCHAIN to identify B-cells and plasma cells respectively?

Response: We didn't notice this problem before. Many thanks for the reviewer's suggestion. Accordingly, we evaluated the expression levels of *MS4A1* and *JCHAIN* in each single-cell cluster. It turned out that the smaller B/plasma cluster (1356 cells) had a specific *JCHAIN* expression, and was annotated as plasma cluster; the larger B/plasma cluster (2292 cells) had specific *MS4A1* expression and was annotated as B cluster (**Response Figure 2**). The corresponding figures and text were modified in the revised manuscript (*please also see the response to the relevant Comment #3*).

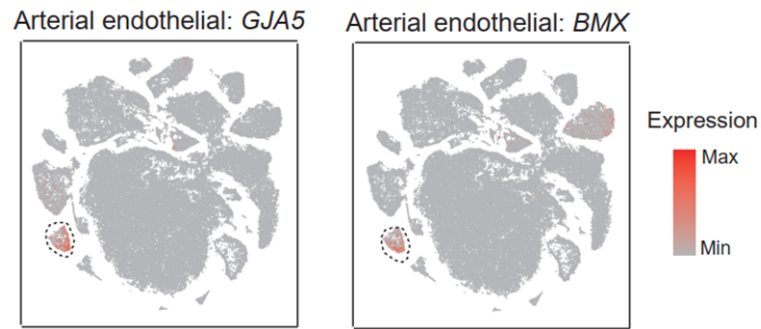


Response Figure 2 (Related to Figure 1b in the revised manuscript). Identification of B cells and Plasma cells.

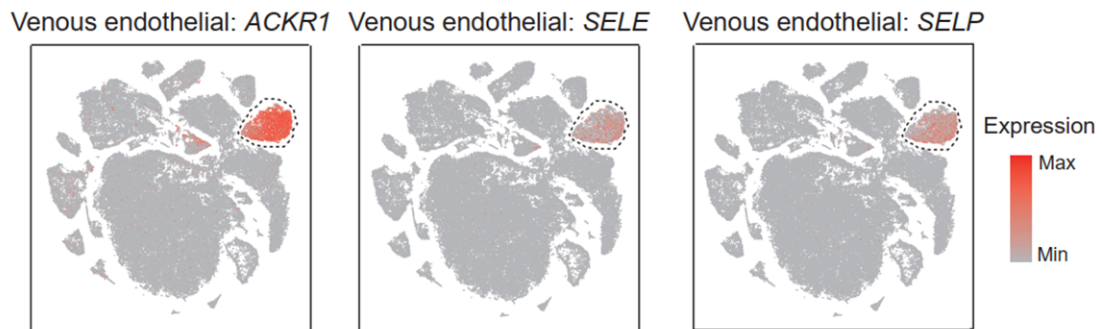
3. A similar remark can be done for endothelial cells. Did the authors tried to discriminate between vascular and lymphatic endothelial cells using specific markers?

Response: According to the reviewer's suggestion, we annotated the endothelial cell clusters in detail by using the following marker genes: arterial endothelial cells (*GJA5* and *BMX*), venous endothelial cells (*SELE*, *ACKR1*, and *SELP*), capillary endothelial cells (*PLVAP* and *RAMP3*), and lymphatic endothelial cells (*PDPN* and *PROX1*). Results showed that the endothelial cell clusters could be further categorized into arterial endothelial cells (**Response Figure 3**), venous endothelial cells (**Response Figure 4**), and capillary endothelial cells (**Response Figure 5**). But we did not identify the lymphatic endothelial cells in our data (**Response Figure 6**). We updated the feature-plots and cell type annotations in Figure 1 of the revised manuscript (**Response Figure 7**). Accordingly, the text was updated in the revised manuscript as follows (**Lines 133-137**): “By analyzing the expression of marker genes, we annotated the

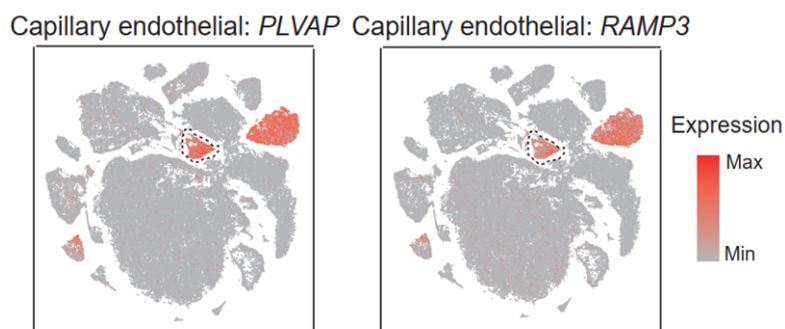
various cell types in the BRCA ecosystem, including epithelial cells, T cells, B cells, plasma cells, macrophages, mast cells, myofibroblasts, cancer-associated fibroblasts (CAFs), arterial endothelial cells, venous endothelial cells, and capillary endothelial cells (**Figure 1b, c and Supplementary Table 3**).”



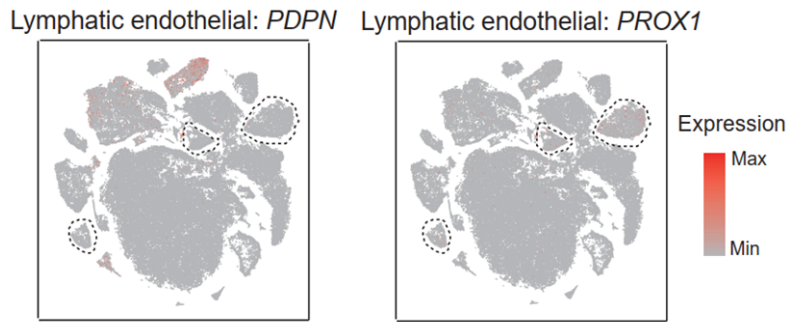
Response Figure 3 (Related to Figure 1b in the revised manuscript). Identification of arterial endothelial cells.



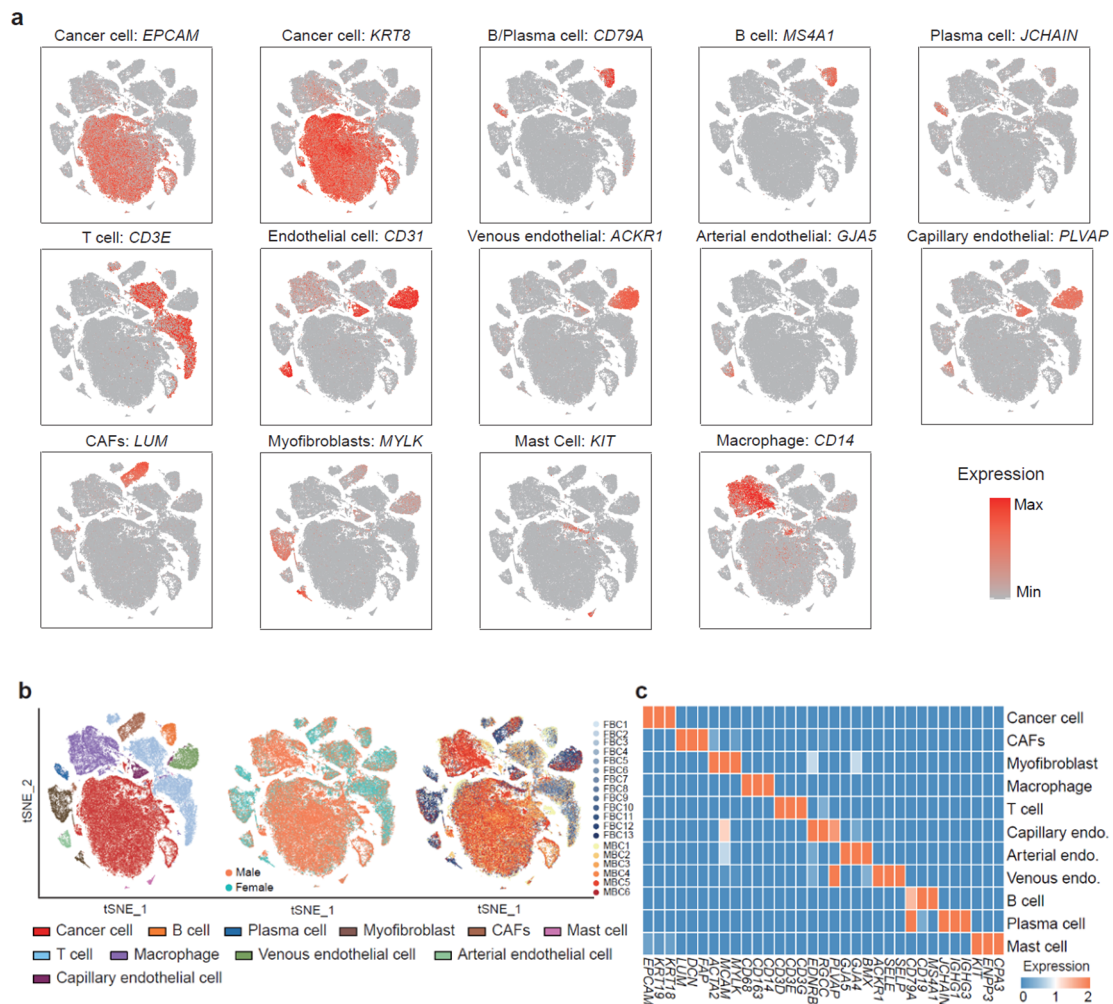
Response Figure 4 (Related to Figure 1b in the revised manuscript). Identification of venous endothelial cells.



Response Figure 5 (Related to Figure 1b in the revised manuscript). Identification of capillary endothelial cells.



Response Figure 6. Identification of lymphatic endothelial cells.



Response Figure 7 (Related to Figure 1 in the revised manuscript). Cell type annotations of scRNA-seq data of breast cancer patients. (a) The expression of marker genes of each cell type. (b) The t-distributed stochastic neighbor embedding (t-SNE) plots of cells types and resources profiled in this study. (c) Heatmap showing genes (columns) that were differentially expressed across various cell types (rows).

4. The authors used Dorothea to analyze regulon activity (<https://bioconductor.org/packages/release/data/experiment/vignettes/dorothea/inst/doc/dorothea.html>). However, it seems from the documentation that Dorothea is a database of regulons used as input for other statistical methods such as decoupleR or SCENIC. Did the authors use such packages to analyze regulon activity?

Response: Thank you for pointing this out. We apologize for not providing a detailed description of the version of the R package we used. We analyzed the regulon activity by using the R package Dorothea (version 1.72), which combined the database of regulons and TF activity inference methods together in one single package. A detailed description of this package can be found in the corresponding publication “Luz Garcia-Alonso, et al., Benchmark and integration of resources for the estimation of human transcription factor activities, *Genome Research*, 2019, 29(8): 1363-1375. doi: 10.1101/gr.240663.118” [2]. In April 2022, the developer of Dorothea uncoupled this R package into two parts, one is for the regulon database (version 1.8 of Dorothea), and the other is for TF activity inference (decoupleR[3]). Based on the TF regulons curated in the Dorothea database, we calculated the regulon activity using VIPER[4], a statistical method included in Dorothea (version 1.72). Besides, only regulons with confidence levels A, B, and C were selected to better estimate TF activities. We updated the description of this analysis process in the revised Method section as follows (**Lines 659-663**): “We analyzed the regulon activity by using the R package Dorothea (version 1.72)⁶², which combined the database of regulons and TF activity inference methods together. Only regulons with confidence levels A, B, and C were selected to better estimate TF activities. Regulon score was calculated for each single cell using VIPER⁶³, a statistical test based on the average ranks of the targets.”

Reviewer #2 (Remarks to the Author):

The responses are extensive and extremely helpful. No further comments.

Reviewer #3 (Remarks to the Author):

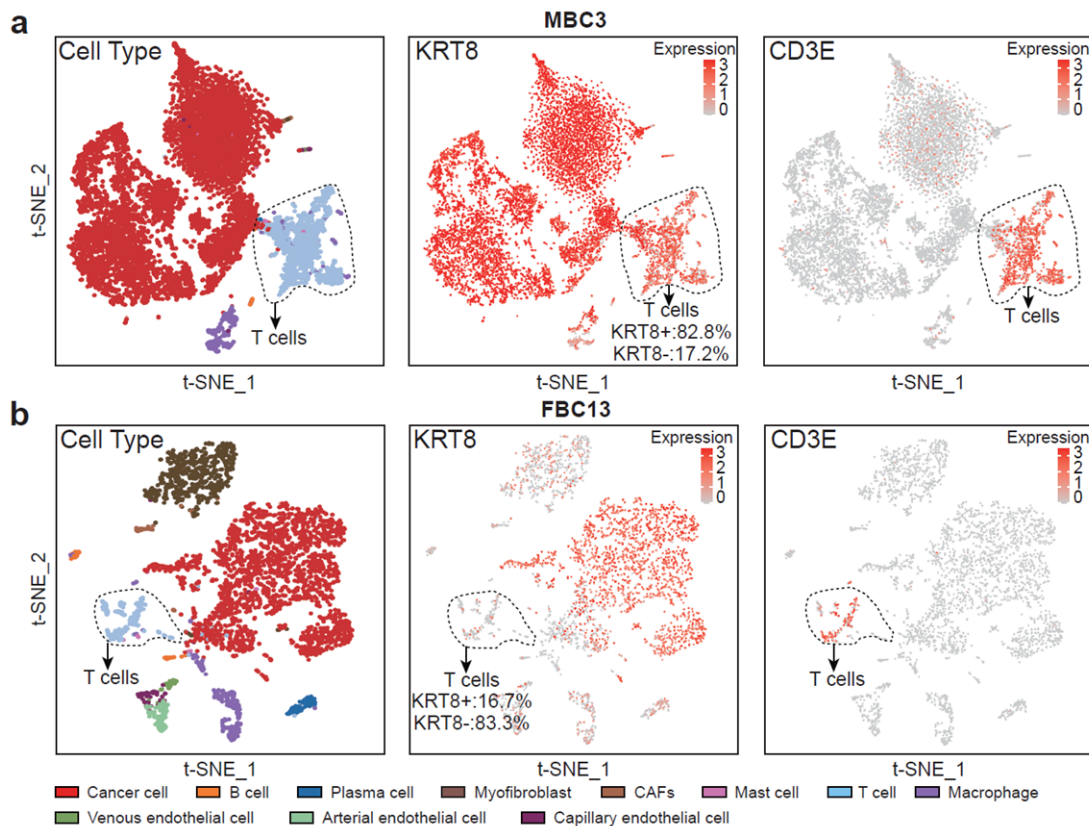
1. The authors have made substantial improvements to the manuscript and the additional experimentation and analysis have cleared up all my concerns but one. The evidence for KRT8⁺ CD3 T cells is still insufficient for conclusively identifying this novel cell type. The authors have added single cell analysis (Supplemental figs 13e and f), however, but “KRT8/18/19 expression, but were lower than these levels in epithelial cells.” Since there are no scale bars, and min/max should be different for each gene analyzed, it is hard to determine if these CD3 cells are truly KRT8 positive or that the scales are set so low that all cells have some KRT8 positivity.

Response: Thank you for the professional comment. We apologize for missing the detailed scale bars in the previous Supplementary Figure 13e, f. The CD3 and KRT8 expression feature-plots in MBC3 and FBC13 were updated with identical scale bars in the revised figures (**Response Figure 8**). The expression level of KRT8 in T cells intuitively seemed to be lower than in epithelial cells, due to hundreds of cells overlapping in the feature-plot. The visualization of feature-plots could be affected by the proportion of cells with KRT8 expression. Actually, almost all epithelial cells are KRT8⁺ (red in feature-plot; 99.4% in MBC3 and 86.2% in FBC13), but only a part of T cells are KRT8⁺ (82.8% in MBC3 and 16.7% in FBC13; **Response Figure 9**), resulting in the overlapping of KRT8⁺ (red) and KRT8⁻ (grey) cells in the area of T cell cluster. In order to illustrate the expression of KRT8 more clearly, we split the feature-plot into two separate parts based on whether KRT8 was positive in T cells (**Response Figure 10**). Violin plots were used to further statistically compare the KRT8 expression among epithelial cells, KRT8⁺ T cells, and KRT8⁻ T cells. Results showed that KRT8⁺ T cells had a similar or lower level of KRT8 expression as epithelial cells (**Response Figure 11**).

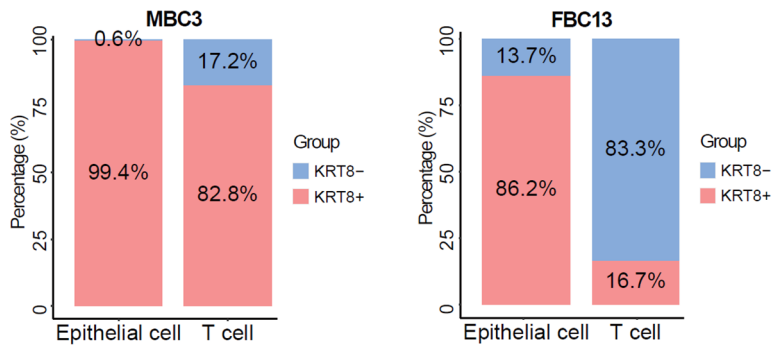
More important, to verify the existence of KRT8⁺ T cells, we evaluated the expression of CD3 and KRT8 in MBC samples by performing immunofluorescence

staining and flow cytometry according to the reviewer's comment #2 and #3 (*Please refer to the response to comment #2 and #3 as well*).

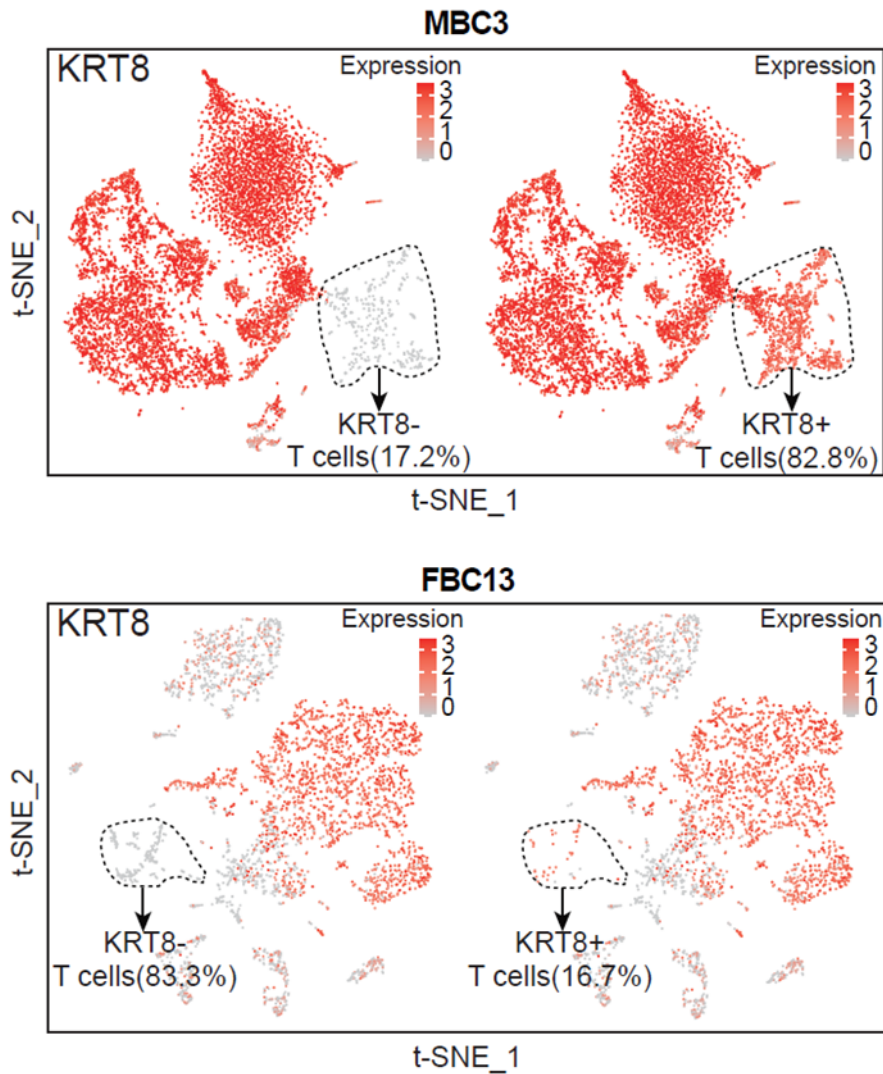
We added these results in the revised Supplementary Figure 14, and the corresponding description in the revised manuscript as follows (**Lines 375-383**): “MBC and FBC samples with the highest percentage of CD3E⁺KRT8⁺ cells were shown in Supplementary Figure 14b, c. Because only a part of T cells were KRT8⁺ (Supplementary Figure 14d, e), we split the feature-plot into two separate parts based on whether KRT8 was positive in T cells to clearly illustrate the expression of KRT8. We found that some T cells did express KRT8 but others had no expression (Supplementary Figure 14f, g). Violin plots were used to further statistically compare the KRT8 expression levels among epithelial cells, KRT8⁺ T cells, and KRT8⁻ T cells, suggesting that KRT8⁺ T cells had a similar or lower level of KRT8 expression compared with epithelial cells (Supplementary Figure 14h, i)”.



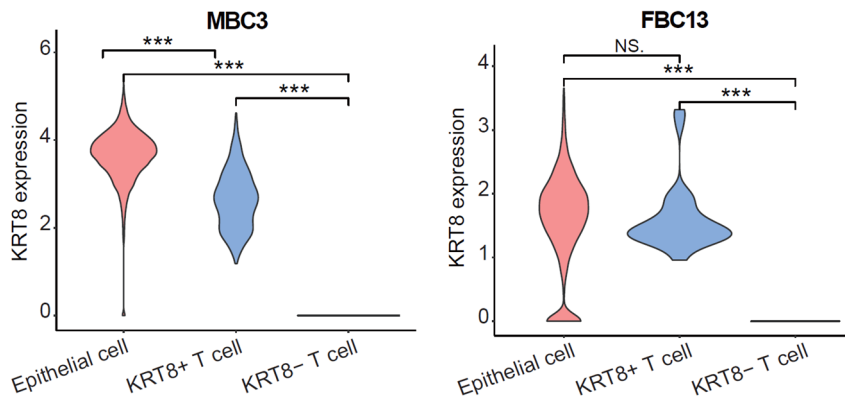
Response Figure 8 (Related to Supplementary Figure 14b, c in the revised manuscript). T-SNE plots showing the cell types, and expression of KRT8 and CD3E in MBC3 (a) and FBC13 (b). T cells were circled with dashed lines.



Response Figure 9 (Related to Supplementary Figure 14d, e in the revised manuscript). Barplots showing the percentage of KRT8⁺ epithelial cells and T cells in MBC3 (left) and FBC13 (right).



Response Figure 10 (Related to Supplementary Figure 14f, g in the revised manuscript). T-SNE plots showing the KRT8 expression of epithelial cells, KRT8⁺ T cells, and KRT8⁻ T cells in MBC3 (upper panel) and FBC13 (bottom panel). T cells were circled with dashed lines. The feature-plots were split into two separate parts based on whether KRT8 was positive in T cells.



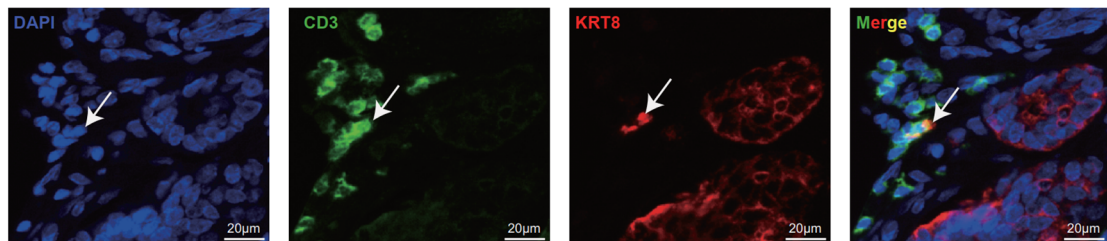
Response Figure 11 (Related to Supplementary Figure 14h, i in the revised manuscript). Violin plots showing the expression of KRT8 among epithelial cells, KRT8⁺ T cells and KRT8⁻ T cells in MBC3 (left) and FBC13 (right).

2. The new IF images (Figure 6C) show that the only KRT18 positive T cells are at the interface with the tumor cells. In fact, the cell in the top arrow is clearly KRT8 positive, but does not appear to be CD3 positive and is likely an exposure artefact. The additional cells identified appear to be sectioning artefacts in which multiple layers of cells are stained. I would anticipate that some CD3 cells away from the tumor would have to be KRT8 positive to truly identify this novel cell type.

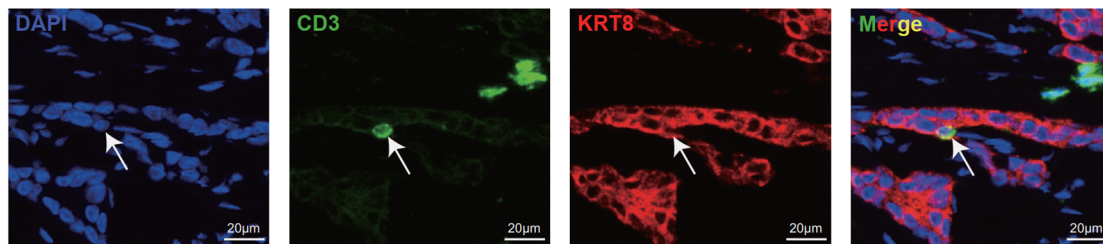
Response: We agree with the reviewer that providing solid evidence for the existence of CD3⁺KRT8⁺ cells is essential. According to your suggestion, we found some instances of CD3⁺KRT8⁺ cells that were away from tumor or T cells to avoid the exposure artifact, although finding these cells is challenging due to the relatively low proportion (*please also see the response to the relevant Comment #3*). As shown in **Response Figure 12 and 13**, the CD3⁺KRT8⁺ cells were not necessarily to be located at the interface between tumor cells and T cells. In order to further avoid the artifacts from multiple layers of cells, we obtained a series of Z-stack confocal images of one single CD3⁺KRT8⁺ cell with a confocal microscope (CarlZeiss LSM880 with NLO & Airyscan; **Response Figure 14**). We hope that these images could prove the existence of CD3⁺KRT8⁺ cells in MBC samples. These results were added in the Figure 6c and Supplementary Figure 16 in the revised manuscript. The corresponding description was revised as follows (**Lines 413-417**): “Further validation using immunofluorescence experiments for the MBC sample confirmed the above observation and showed the

existence of CD3⁺KRT8⁺ cells (Figure 6c and Supplementary Figure 16a). In order to avoid the artifacts from multiple layers of cells, we further obtained a series of Z-stack confocal images of one single CD3⁺KRT8⁺ cell with a confocal microscope (Supplementary Figure 16b)’’.

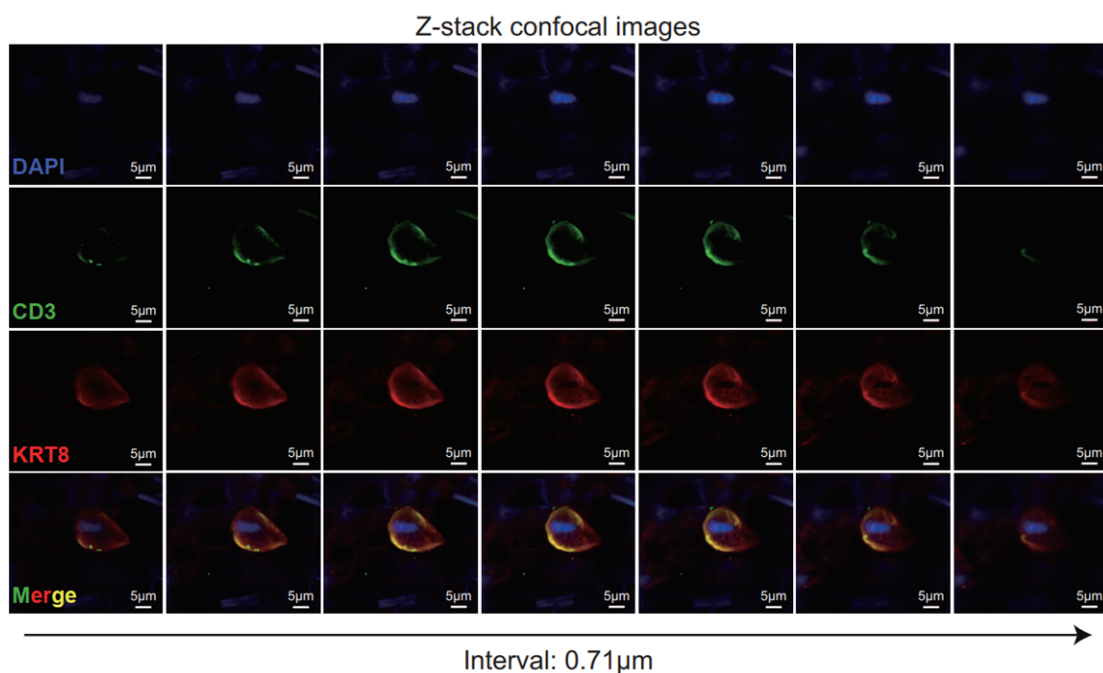
Notably, recent literature has reported cumulative evidence for the existence of cells co-expressing T-cell and epithelial-cells markers in various tissues [5-9]. For example, Hu et al.[8] reported a non-traditional CD45⁺EpCAM⁺ cell population in the fallopian tube epithelial layer of ovarian cancer patients (Hu et al., Cancer cell, 2020, 37(2), 226-242). This population was also positive for CD3, CD44, CD69, and CD103, suggesting that these cells are possibly tissue-resident memory T lymphocytes (TRMs). They identified these cells by scRNA-seq (Smart-Seq2) and validated them using immunofluorescence experiments. Besides, using scRNA-seq, flow cytometry, cell co-culture experiments, RNA-FISH, and immunofluorescent staining, another study from Chen et al.[9] reported that infiltrated CD8⁺ effector T cells expressed tumor markers in prostate cancer (Chen et al., Nature cell biology, 2021, 23(1): 87-98). They also demonstrated that these cells were induced by the extracellular vesicle (EV) derived from prostate tumor cells, and associated with the micrometastases. Moreover, other studies also found cells expressing both epithelial and immune cell markers in ovarian carcinoma and colon adenocarcinoma, etc.[5-7]. Our observation of CD3⁺KRT8⁺ cells in MBC will be an important complement to these previous findings that characterized this non-traditional cell type.



Response Figure 12 (Related to Figure 6c in the revised manuscript). The immunofluorescence staining of KRT8 and CD3 in an MBC sample. White arrow indicates the CD3⁺KRT8⁺ T cell. Scale bar, 20 μm.



Response Figure 13 (Related to Supplementary Figure 16a in the revised manuscript). The immunofluorescence staining of KRT8 and CD3 in an MBC sample. White arrow indicates the CD3⁺KRT8⁺ T cell. Scale bar, 20 μm.

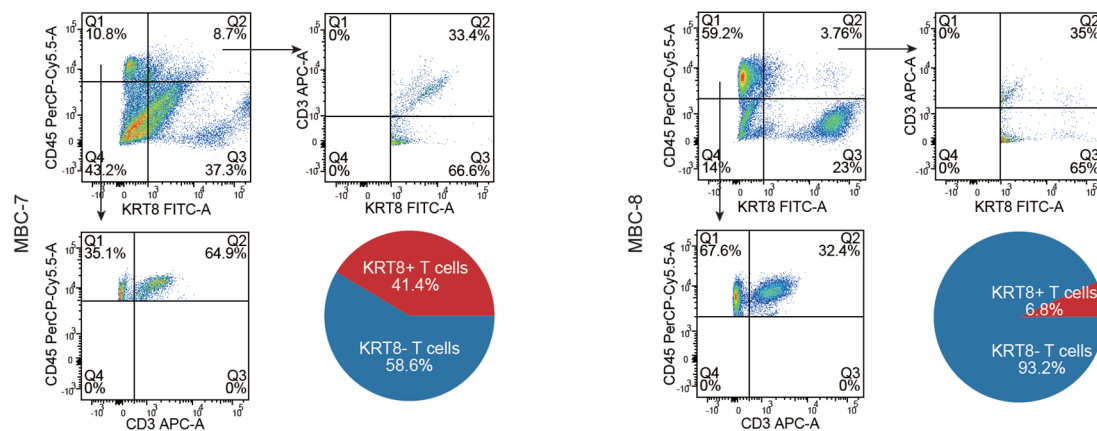


Response Figure 14 (Related to Supplementary Figure 16b in the revised manuscript). Z-stack confocal images of one CD3⁺KRT8⁺ T cell from an MBC sample. Scale bar, 5 μm. The interval for Z-stack was 0.71 μm.

3. The flow cytometry in Response Figure 8 is also unconvincing because it lacks a positive control for KRT8 (epithelial cells). Additionally, the gate in upper panel of response 8b appears to be loosely gated for CD45 and potentially including CD45 negative cells.

Response: Thank you for pointing this out. Accordingly, we re-analyzed the flow cytometry data to show the epithelial cells, T cells and KRT8⁺CD45⁺CD3⁺ cells in two MBC samples (**Response Figure 15**). Firstly, KRT8 and CD45 were used to distinguish the epithelial cells (KRT8⁺CD45⁻), immune cells (KRT8⁻CD45⁺), and KRT8⁺CD45⁺ cells. There were 8.7% and 3.76% KRT8⁺CD45⁺ cells, in which 33.4% and 35% were

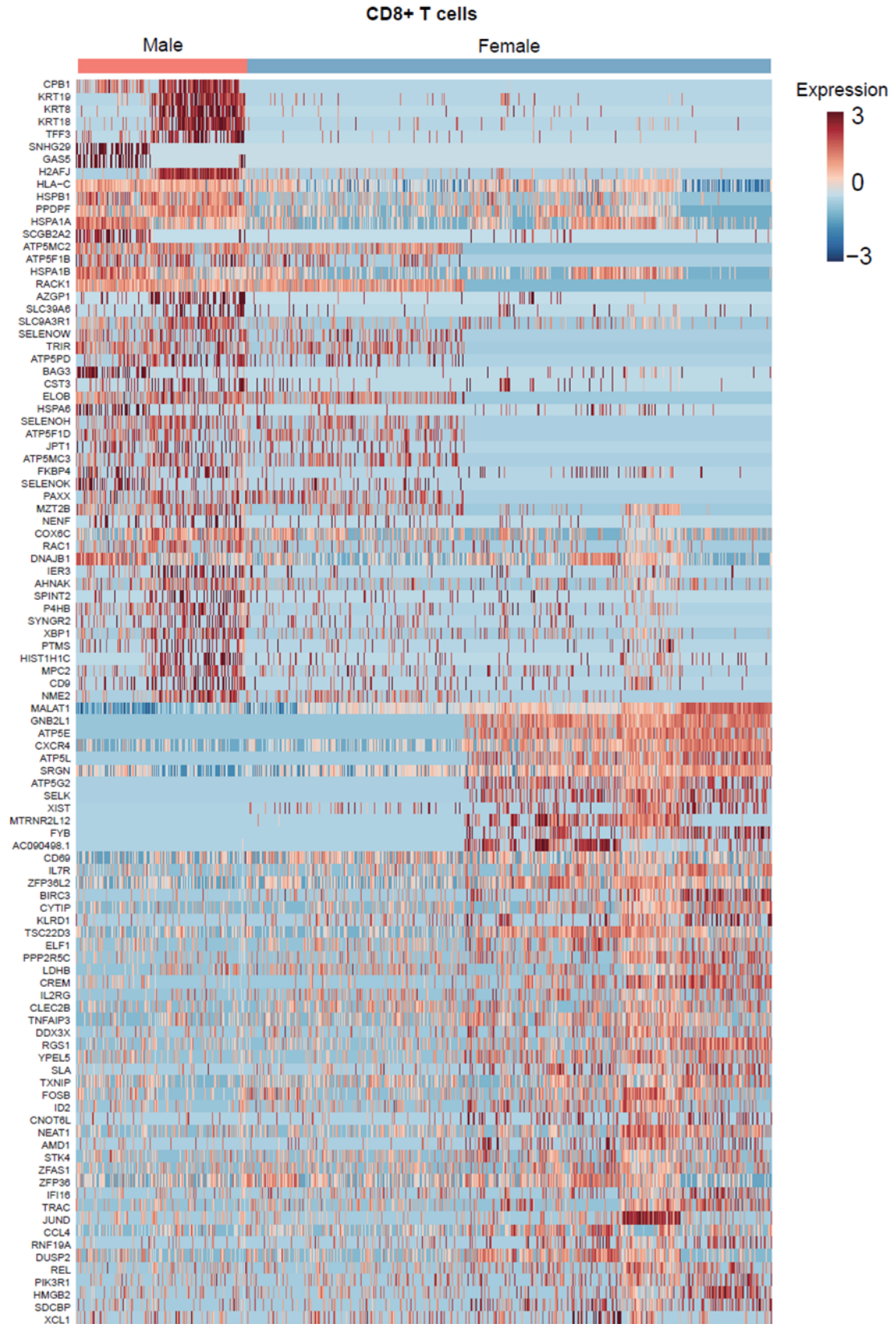
CD3⁺ T cells in MBC-7 and MBC-8, respectively. Among all T cells, KRT8⁺ T cells accounted for 41.4% and 6.8% in MBC-7 and MBC-8, respectively. Therefore, these results indicated the biological existence of KRT8⁺CD45⁺CD3⁺ T cells and the enrichment of these cells with various percentages in different MBC samples. This result was updated in the revised Figure 6d. The corresponding description was revised as follows (Lines 418-426): “Besides, we performed flow cytometry experiments for fresh tumor tissues from two MBC patients to validate and quantify the number of CD3⁺KRT8⁺ cells (Figure 6d). Firstly, KRT8 and CD45 were used to distinguish the epithelial cells (KRT8⁺CD45⁻), immune cells (KRT8⁻CD45⁺), and KRT8⁺CD45⁺ cells. There were 8.7% and 3.76% KRT8⁺CD45⁺ cells, in which 33.4% and 35% were CD3⁺ T cells in MBC-7 and MBC-8, respectively. Among all T cells, KRT8⁺ T cells accounted for 41.4% and 6.8% in MBC-7 and MBC-8, respectively. Therefore, these results indicated the biological existence of KRT8⁺CD45⁺CD3⁺ T cells and the enrichment of these cells with various percentages in different MBC samples”.



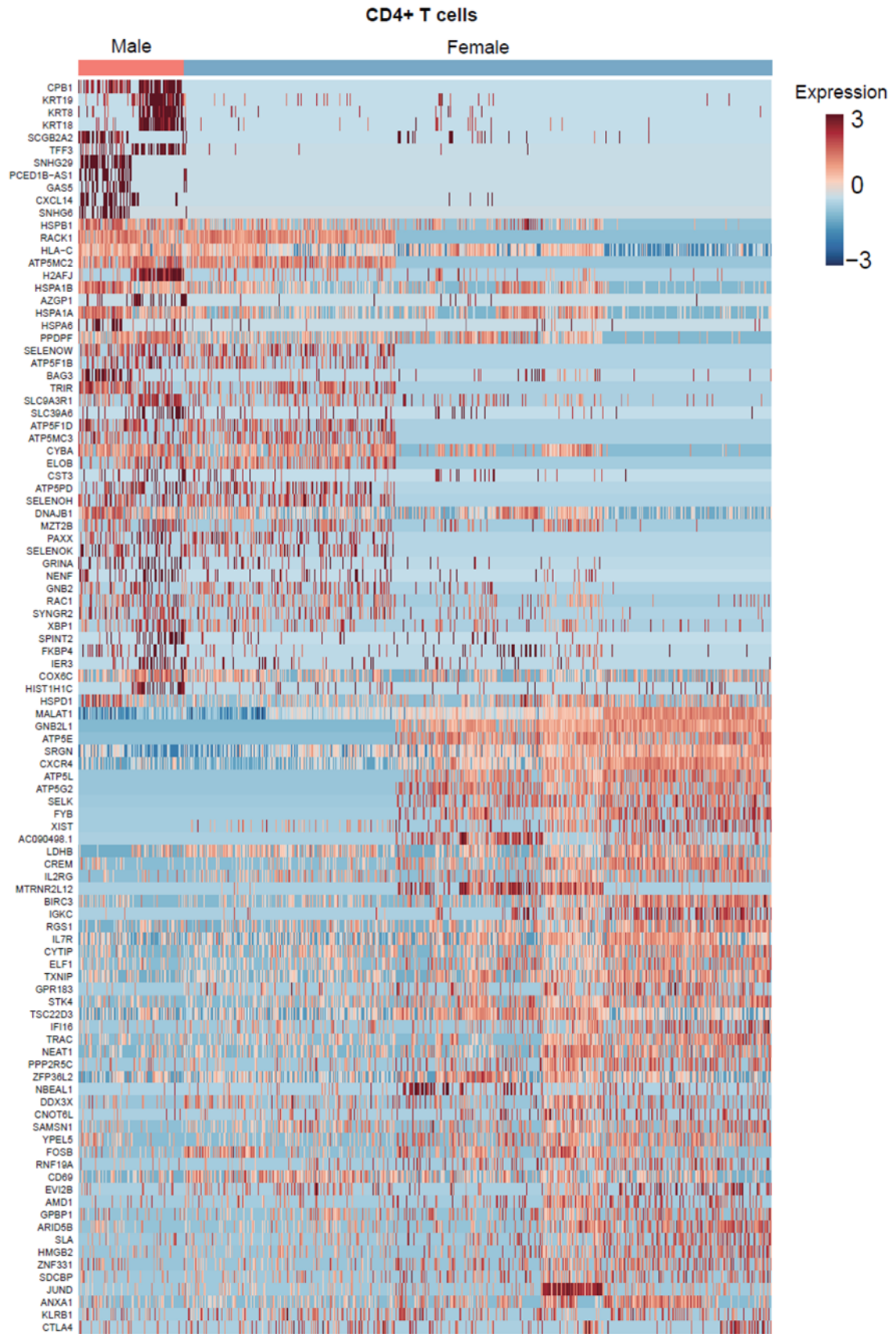
Response Figure 15 (Related to Figure 6d in the revised manuscript). Flow cytometry analysis showing the identification of epithelial cells (KRT8⁺CD45⁻), T cells (KRT8⁻CD45⁺CD3⁺) and KRT8⁺CD45⁺CD3⁺ cells in two MBC samples.

4. Clearly all the T cells (CD3D) in figure 1B are negative for KRT8 and I suspect KRT18 as well. In Figure 5 F, all the male T cells are either KRT18 or KRT8 positive, which is different than figure 1B. These discrepancies need to be addressed to conclusively state that KRT8 positive T cell exist.

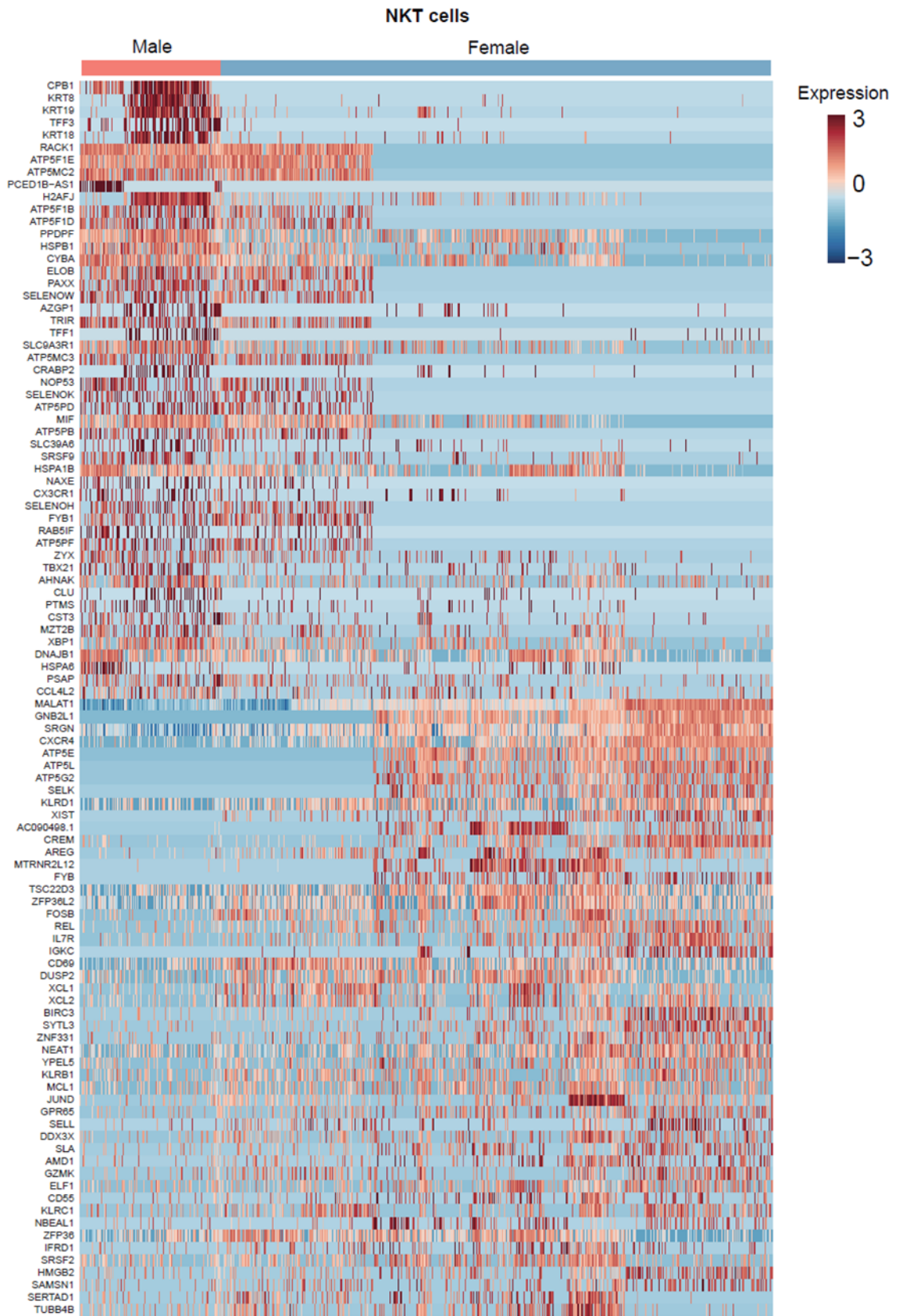
Response: We apologize for the confusing visualization of Figure 5F in the previous submission and appreciate the reviewer's comment. Because as many as 100 differentially expressed genes (including 50 up-regulated genes and 50 down-regulated genes) were included in the heatmap, it is impossible to show the names of all genes in Figure 5F due to the limited space. Thus, only some representative gene names were shown beside the heatmap. Maybe the inexact pointing of KRT8/KRT18/KRT19 in the previous submission caused the misunderstanding of the proportion of MBC T cells with positive expression. We showed the original heatmaps for CD8⁺, CD4⁺, and NKT cells, with all gene names being displayed in **Response Figures 16, 17, and 18**. These figures showed that nearly half of MBC T cells were positive for KRT8/KRT18/KRT19 expression. Accordingly, we corrected the pointing of gene names and tried our best to make sure that the representative genes were exactly pointed beside the corresponding rows in the revised Figure 5F (**Response Figure 19**). Besides, the whole list of differentially expressed genes between T cells from MBC and FBC samples were shown in **Supplementary Tables 7, 8, and 9**.



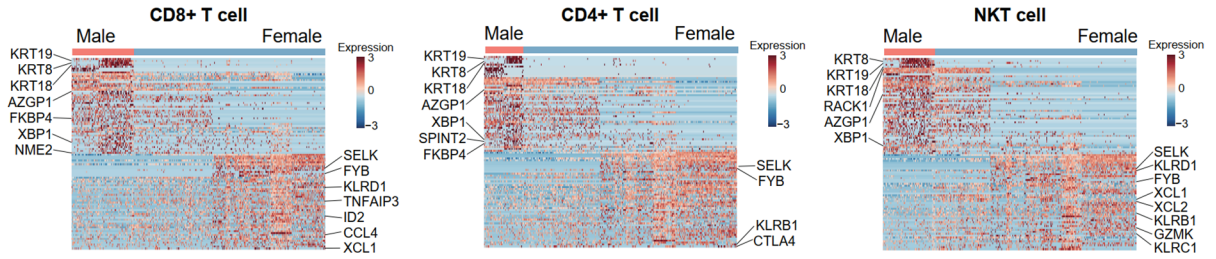
Response Figure 16 (Related to Supplementary table 7 in the revised manuscript). Heatmap showing the differentially expressed genes between MBC and FBC CD8⁺ T cells.



Response Figure 17 (Related to Supplementary table 8 in the revised manuscript). Heatmap showing the differentially expressed genes between MBC and FBC CD4⁺ T cells.



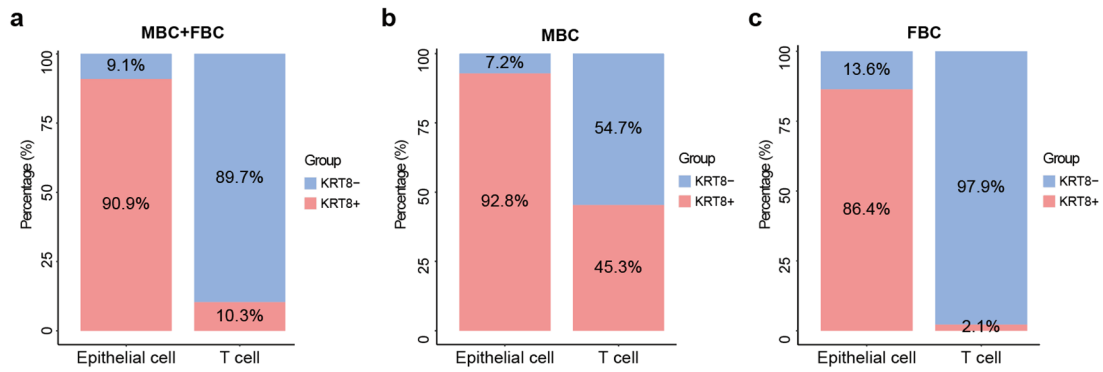
Response Figure 18 (Related to Supplementary table 9 in the revised manuscript). Heatmap showing the differentially expressed genes between MBC and FBC NKT cells.



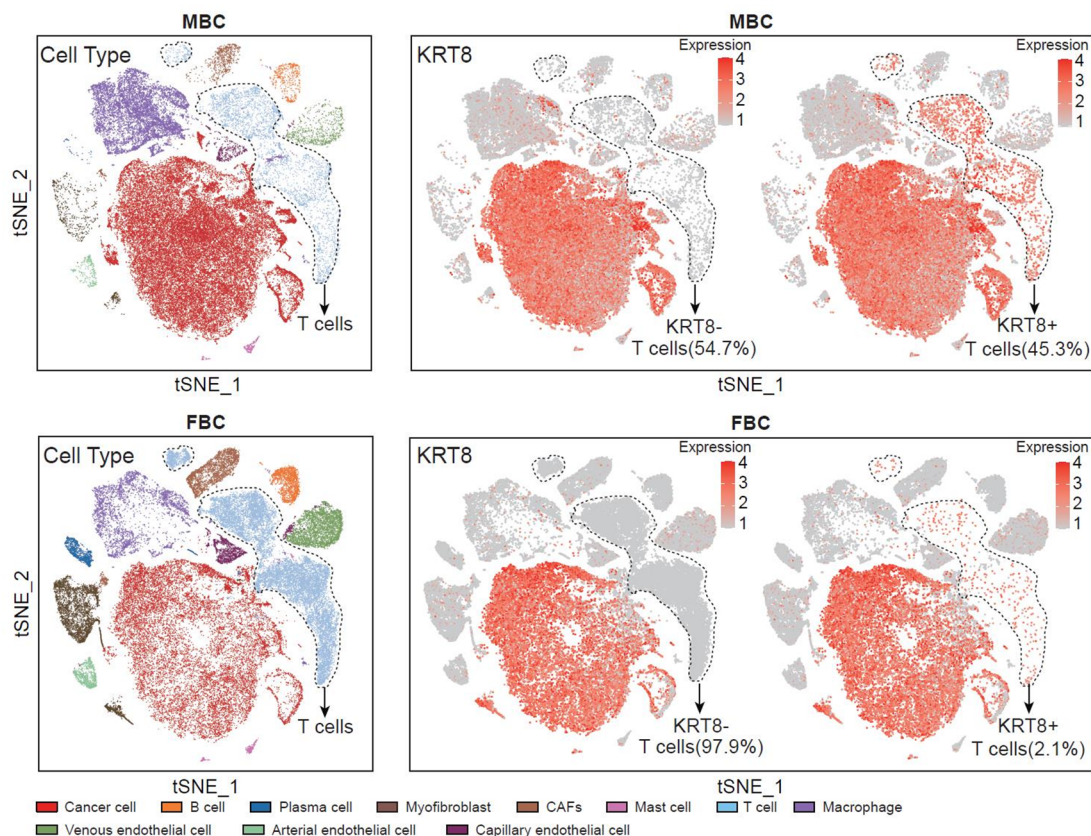
Response Figure 19 (Related to Figure 5f in the revised manuscript). Heatmap showing the differentially expressed genes between MBC and FBC T cells, including CD4⁺, CD8⁺, and NKT cells.

The reason for why T cell clusters seem to be KRT8-negative in Figure 1B is similar to the issue of feature-plot visualization mentioned in *comment #1*. Specifically, the integrated t-SNE plot in Figure 1B included all cells from both MBC and FBC samples. Almost all epithelial cells (90.9%) were KRT8⁺, while only 10.3% of T cells were KRT8⁺ (**Response Figure 20a**). The low proportion of KRT8⁺ T cells resulted in the overlapping of red and grey, showing a dominant grey color in the area of T cell cluster in figure 1B. The percentage of KRT8⁺ T cells was significantly different between MBC and FBC (**Response Figure 20b, c**). KRT8⁺ T cells accounted for 45.3% in T cells from MBC (**Response Figure 20b**), consistent with the observation in Figure 5F (**Response Figure 16-19**). In contrast, only 2.1% of T cells were KRT8⁺ in FBC (**Response Figure 20c**). Thus, we showed the KRT8 expression intensity on the t-SNE plot based on sex and whether T cells were KRT8⁺ (**Response Figure 21**). The results clearly showed that KRT8 was expressed on some T cells, especially the T cells from MBC samples (**Response Figure 21**). These results were added in the revised Supplementary Figure 13. The corresponding description was revised as follows (**Lines 362-366**): “About 50% of T cells from MBC were KRT8⁺, while only 2.1% of T cells from FBC were KRT8⁺ (Supplementary Figure 13d, e). We showed the KRT8

expression intensity on the t-SNE plot based on sex and whether T cells were KRT8⁺, and found that KRT8 was expressed on some T cells, especially the T cells from MBC samples (Supplementary Figure 13f, g)



Response Figure 20 (Related to Figure 13d, e in the revised manuscript). Barplot showing the percentage of KRT8⁺ epithelial cells and T cells in all samples (a), MBC samples (b) and FBC samples (c).



Response Figure 21 (Related to Supplementary Figure 13f, g in the revised manuscript). T-SNE plots showing the cell types and KRT8 expression in MBC (upper panel) and FBC (bottom panel) samples. T cells were circled with dashed lines. The feature-plots were split based on sex and whether T cells were KRT8⁺.

Response References

1. Fleming, S.J., J.C. Marioni, and M. Babadi, *CellBender remove-background: a deep generative model for unsupervised removal of background noise from scRNA-seq datasets*. BioRxiv, 2019: p. 791699.
2. Garcia-Alonso, L., et al., *Benchmark and integration of resources for the estimation of human transcription factor activities*. Genome Res, 2019. **29**(8): p. 1363-1375.
3. Badia-i-Mompel, P., et al., *decoupleR: ensemble of computational methods to infer biological activities from omics data*. Bioinformatics Advances, 2022. **2**(1).
4. Alvarez, M.J., et al., *Functional characterization of somatic mutations in cancer using network-based inference of protein activity*. Nat Genet, 2016. **48**(8): p. 838-47.
5. Sutton, T.L., B.S. Walker, and M.H. Wong, *Digesting the Importance of Cell Fusion in the Intestine*. Cell Mol Gastroenterol Hepatol, 2021. **11**(1): p. 299-302.
6. Ramakrishnan, M., S.R. Mathur, and A. Mukhopadhyay, *Fusion-derived epithelial cancer cells express hematopoietic markers and contribute to stem cell and migratory phenotype in ovarian carcinoma*. Cancer Res, 2013. **73**(17): p. 5360-70.
7. Gast, C.E., et al., *Cell fusion potentiates tumor heterogeneity and reveals circulating hybrid cells that correlate with stage and survival*. Sci Adv, 2018. **4**(9): p. eaat7828.
8. Hu, Z., et al., *The Repertoire of Serous Ovarian Cancer Non-genetic Heterogeneity Revealed by Single-Cell Sequencing of Normal Fallopian Tube Epithelial Cells*. Cancer Cell, 2020. **37**(2): p. 226-242 e7.
9. Chen, S., et al., *Single-cell analysis reveals transcriptomic remodellings in distinct cell types that contribute to human prostate cancer progression*. Nat Cell Biol, 2021. **23**(1): p. 87-98.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

No additional comments.

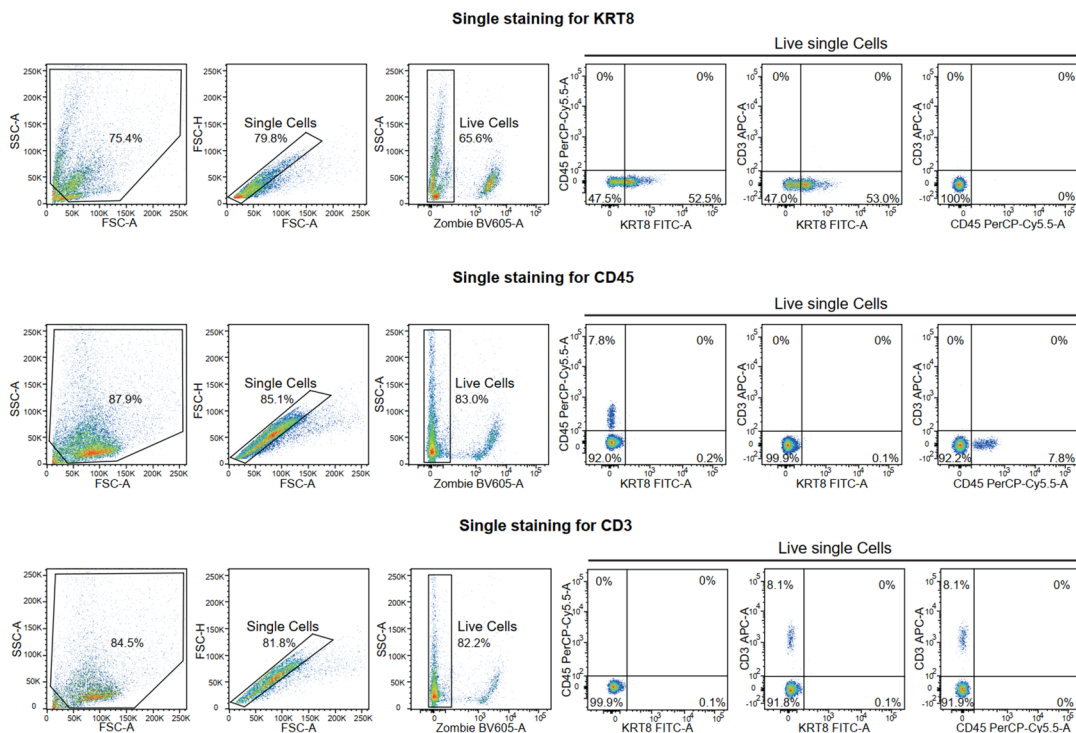
Reviewer #3 (Remarks to the Author):

While the additional flow cytometry data is unconvincing, the additional scRNA analyses and immunofluorescence (especially the confocal z stack) provide sufficient evidence for the existence of KRT8 positive CD3+ cells. The authors have addressed all my concerns in this revised manuscript.

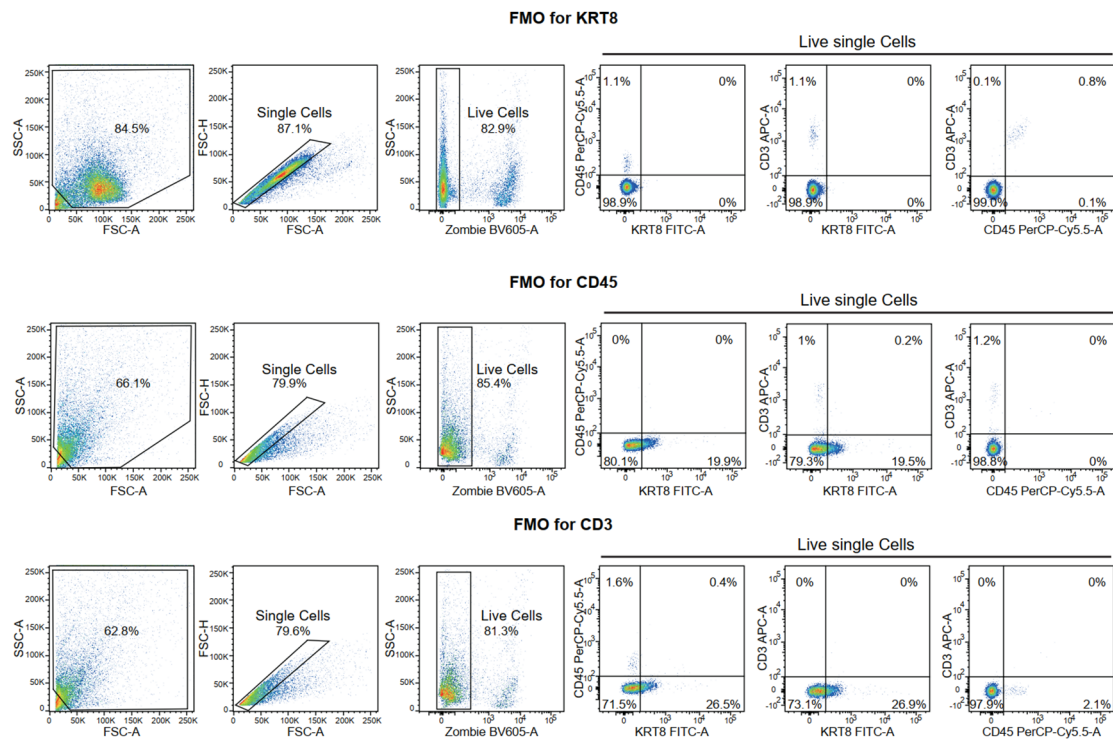
Response to reviewer #3:

While the additional flow cytometry data is unconvincing, the additional scRNA analyses and immunofluorescence (especially the confocal z stack) provide sufficient evidence for the existence of KRT8 positive CD3+ cells. The authors have addressed all my concerns in this revised manuscript.

Response: Thank you for your comments. We are pleased to receive your positive evaluation regarding the confocal z-stack images for proving the existence of KRT8⁺CD3⁺ cells, and terribly sorry for the unsatisfying flow cytometry experiments in the previous submission. In order to address the concern of flow cytometry evidence, we made a thorough revision and re-performed the experiments to further exclude the potentially confounding factors and provide convincing results. We used single antibody-labeled compensation samples and fluorescence minus one (FMO) controls to determine where the gates should be appropriately set (**Response Figure 1, 2**). Doublets were excluded according to the FSC-A/FSC-H profile (**Response Figure 1, 2, 3**). Zombie Yellow (Cat: 423104, Biolegend) was used to stain the live/dead cells to exclude the effect of dead cells (**Response Figure 1, 2, 3**).

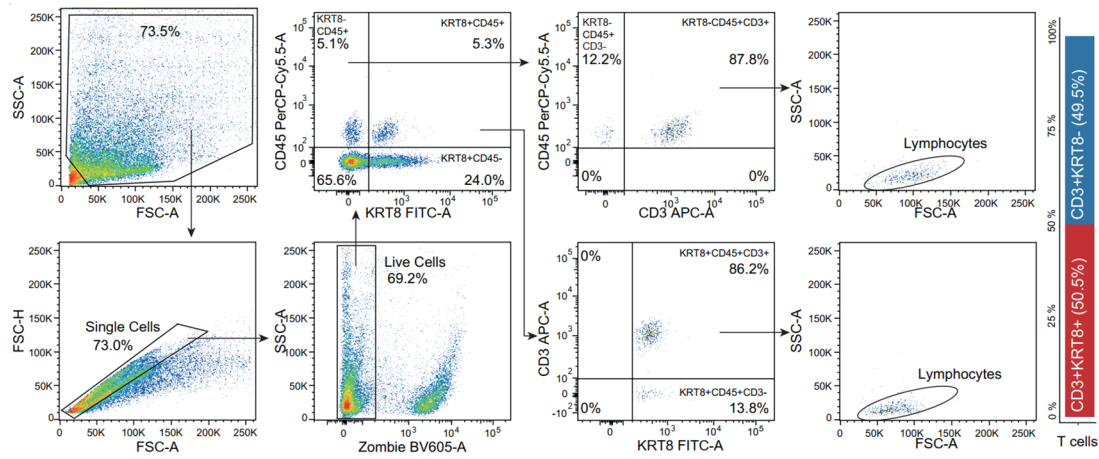


Response Figure 1 (Related to Supplementary Figure 17 in the revised manuscript). Single antibody-labeled compensation controls of flow cytometry analysis for KRT8, CD45, and CD3.



Response Figure 2 (Related to Supplementary Figure 18 in the revised manuscript). Fluorescence minus one (FMO) controls flow cytometry analysis for KRT8, CD45, and CD3.

Due to the scarcity of male breast cancer (MBC, only accounting for 1% of all breast cancers) samples, the experiments of single staining controls and FMO controls were performed using female breast cancer (FBC) samples with the same subtypes (ER⁺). The full staining experiments were performed using fresh tumor tissues from an MBC patient. **Response Figure 3** showed the full gating strategy of the MBC sample. Firstly, debris was excluded by forward and side scatters gating, and single cells were gated using the FSC-A/FSC-H profile. Dead cells were further excluded using live/dead staining by Zombie. Secondly, KRT8 and CD45 were used to distinguish the epithelial cells (KRT8⁺CD45⁻, 24.0%), immune cells (KRT8⁻CD45⁺, 5.1%), and KRT8⁺CD45⁺ double-positive cells (5.3%). Among the KRT8⁺CD45⁺ double-positive cells, 86.2% were KRT8⁺CD45⁺CD3⁺ T cells. Similarly, 87.8% of KRT8⁻CD45⁺ immune cells were CD3⁺ T cells. To better determine the T cell subpopulations, the KRT8⁺CD45⁺CD3⁺ and KRT8⁻CD45⁺CD3⁺ T cells were backgated and overlaid onto the FSC-A/SSC-A plots. Results showed that both KRT8⁺CD45⁺CD3⁺ and KRT8⁻CD45⁺CD3⁺ T cells were located in the lymphocyte gate. Among all T cells (CD45⁺CD3⁺), KRT8⁺ and KRT8⁻ cells accounted for 50.5% and 49.5% in this MBC sample, respectively.



Response Figure 3 (Related to Figure 6d in the revised manuscript). Full gating strategy of flow cytometry analysis for the identification of KRT8⁺ and KRT8⁻ T cells in an MBC sample.

The raw FCS files (including the single staining controls, FMO controls, and full-staining experiments) of the above flow cytometry data have been submitted to Mendeley Data (<https://data.mendeley.com/datasets/wwm9xv56ry/1>).

Correspondingly, the results of flow cytometry experiments were revised in this submission (*Lines 427-443*): “Besides, we performed flow cytometry experiments for fresh tumor tissues from an MBC patient to validate and quantify CD3⁺KRT8⁺ double-positive T cells (Figure 6d). Single antibody-labeled compensation samples and fluorescence minus one (FMO) controls were used to determine where the gates should be set (Supplementary Figure 17, 18). Firstly, debris was excluded by forward and side scatters gating, and single cells were gated using the FSC-A/FSC-H profile. Dead cells were further excluded using live/dead staining by Zombie. Secondly, KRT8 and CD45 were used to distinguish the epithelial cells (KRT8⁺CD45⁻, 24.0%), immune cells (KRT8⁻CD45⁺, 5.1%), and KRT8⁺CD45⁺ double-positive cells (5.3%). Among the KRT8⁺CD45⁺ double-positive cells, 86.2% were KRT8⁺CD45⁺CD3⁺ T cells. Similarly, 87.8% of KRT8⁻CD45⁺ immune cells were CD3⁺ T cells. To better determine the T cell subpopulations, the KRT8⁺CD45⁺CD3⁺ and KRT8⁻CD45⁺CD3⁺ T cells were backgated and overlaid onto the FSC-A/SSC-A plots. Results showed that both KRT8⁺CD45⁺CD3⁺ and KRT8⁻CD45⁺CD3⁺ T cells were located in the lymphocyte gate. Among all T cells (CD45⁺CD3⁺), KRT8⁺ and KRT8⁻ cells accounted for 50.5% and

49.5% in this MBC sample, respectively. Therefore, these results indicated the biological existence of KRT8⁺CD45⁺CD3⁺ T cells”. In addition, we added the corresponding methodological description in the revised manuscript as follows (*Lines 744-754*): “Flow cytometry was performed using a FACSLyric flow cytometer (BD Biosciences). The intrinsic spectral overlap of the different fluorochromes was corrected using compensation matrices. Due to the scarcity of MBC samples, the experiments of single antibody-labeled compensation controls and FMO controls were performed using ER⁺ FBC samples. The full staining experiments were performed using fresh MBC tumor tissues. Doublets were excluded according to the FSC-A/FSC-H profile. Zombie Yellow (Cat: 423104, Biolegend) was used to exclude the dead cells. All the flow cytometry data were analyzed using FlowJo software (Version 10.8.1, FlowJo LLC). The raw FCS files are deposited in Mendeley Data (<https://data.mendeley.com/datasets/wwm9xv56ry/1>)”.

Therefore, evidence of scRNA-seq analysis, immunofluorescence (including confocal z-stack images), and flow cytometry collectively demonstrated the existence of KRT8⁺CD3⁺ T cells in MBC samples (see **main Figures 5, 6 and Supplementary Figures 13, 14, 15, 16, 17, 18, 19 in this submission**).

REVIEWERS' COMMENTS

Reviewer #3 (Remarks to the Author):

The added FACS analysis and gating strategy have provided additional evidence for the existence of KRT8+/CD3+ cells and the authors have addressed all my concerns

The fourth revision

Reviewer #3 (Remarks to the Author):

The added FACS analysis and gating strategy have provided additional evidence for the existence of KRT8+/CD3+ cells and the authors have addressed all my concerns

Response: We thank the reviewer for providing many professional suggestions and advice. We are delighted that all concerns have been successfully addressed.