nature portfolio

Peer Review File

Cross-tissue multicellular coordination and its rewiring in cancer

Corresponding Author: Professor Zemin Zhang

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
In this manuscript, Shi et al. characterised cross-tissue co-occurring cellular modules (CMs) based on a pan-tissue single-cell atlas and a novel computational framework called CoVarNet. They show that these CMs are differentially abundant across tissues and their results point that they are important for understanding tissue-level functional coordination. The association of the inflammatory gene program enriched in exposed human tissues with responsiveness to anti-PD-1 therapies was very interesting. They extend these analyses on cancer samples. The method in my opinion will be useful for future studies in both healthy and disease conditions. It is also an impressive collection of datasets shared as a repository that enables users to explore and visualise the data. As such, it presents a valuable resource for the cancer research community. My main criticism is about more stringent validation of the existence of the modules and their functional significance, as well as more novelty with regards to the role of these CMs in cancer.

Major comments:

For the validation with GTEx data, it was not clear to me how well the enrichment of different CMs in distinct tissues in bulk samples matches with results from the single-cell analysis. I don't see too much similarity between S9a and S10b, but it was difficult to see with the many different colours, can the authors provide some other way of visualisation to validate this?

Why were 12 CMs used when S7A shows k=14 is a more stable solution? The authors mention in the Methods that "optimal rank was then identified as the one at which CCC is maximized"?

The CMs both in normal and cancer should be described more in detail in the text. The cancer part was especially brief. It is well known that many immune and stromal cells dramatically change their phenotype in cancer, the authors should emphasise the novelty that inference of CMs bring in this part. They didn't comment much about enrichment of specific CMs in different cancer types.

The authors should perform a quantification of cellular modules using spatial data to show more reliably that the cell subsets in each CM are colocalised across different patients. This can be done for example by identification of cellular niches across several samples with something like CellCharter or SpatialDE2. In line with the above point, the authors note that "Further investigation focused on CM08, whose components exhibited moderate spatial consistency (Fig. 4a)" - CM08 seems to have weak spatial consistency using Spearman's correlation, how do the authors explain this in terms of how functionally important is this specific CM?

In Line 205-210, S12a the authors claim "Fibroblasts express more ligands….These findings can be harmonized by a notion that spatial adjacence enhances the efficiency of intercellular communication". However, in figure 4a shows that the CMs with a higher stromal composition have the worst colocalization score, how would you explain this?

Fig 5d, the authors write "we observed a notable increase in cell-subset co-occurrence within cCM02 in tumor samples compared to adjacent non-tumor tissues, providing discernible evidence of malignant progression" - it was not clear to me whether all subsets are present both in tumor and adjacent tumor samples, or if the increase in cell-subset co-occurrence in tumor is because some subsets are not present in adjacent tumor samples.

The cytokine analysis both in healthy and cancer samples can lead to interesting hypotheses, it would add a lot more relevance of the functional significance of these CMs if there was validation of some of them. For example, for those cytokines found enriched in some CMs and not others, can you show that they are spatially enriched in the niches/microenvironments representing those CMs compared to other niches?

Minor comments:

It would be helpful to include a brief explanation of the method CoVarNet in the main text.

Fig. S20c, a brief explanation of how this plot was derived should be added in the legend.

For the spatial methods section, why n=5 for cell2location, did the authors perform segmentation?

(Remarks on code availability)
The installation and running of the method worked very well for me with the example data provided. The tutorial could be a bit more detailed.

Referee #2

(Remarks to the Author)
In this study the authors assemble a single-cell RNA-seq atlas of cells across 35 healthy human tissues and 26 cancer types, identifying shared patterns of non-epithelial (immune, stromal, and endothelial) cell type abundancy across tissues. The research aims are broad and forward-looking, exploring important research problems relevant to efforts such as the Human Cell Atlas, with an imminent potential to generate biological insights across multiple tissues. As such, the manuscript describes a method to detect cellular communities that form functional organisational units in human tissues from scRNA-seq data at large sample sizes (n=706). Although not an entirely novel idea, it is highly valuable for the research community to understand how this approach compares to alternative methods based on spatial transcriptomics, multiplex imaging, and bulk deconvolution (Luca et al. 2021). However, there is currently more excitement in the field for using spatial transcriptomics to discover multicellular coordination with approaches such as CellCharter (Varrone et al. 2024) – or from modelling contributions such as hierarchical organisation of tissue unites in CODEX imaging data (Hickey et al. 2023).

Altogether, this assessment is reinforced by the scarcity of original findings presented in the manuscript, with one notable exception being the depletion of universal fibroblasts in the reproductive system, which is a noteworthy finding but never expanded on. Although the study identifies age-associated thymic involution as one of the 12 cellular community clusters, it is presently unclear how this adds to our current understanding of the thymus. Similarly, Peyer's patches are identified and validated with spatial transcriptomics, but no novel findings are presented about them. Another main result is also already well established, namely that inflammation is a predictor of the response to anti PD-1 therapy (Damotte et al. 2019). In comparison, more focussed studies of the tumour immune microenvironment have more clearly delineated the effect on immunotherapy along with subtypes of stromal-immune microenvironments across cancer types (Bagaev et al. 2021), which is not mentioned here.

While the scope of the study is exciting, I think the paper currently lacks fundamental and lucid insights into cellular organisation that would be of sufficiently broad interest to the readers of Nature. The paper is logically structured (except perhaps in the lack of comparison between healthy and cancer cell communities), well written, and the atlas curation and methods exploration represents important work. In addition, there is substantial theoretical originality in the overall approach and, in particular, the use of DIALOGUE to model coupled cellular programs and Immune Dictionary perturbation data for cytokine-mediated interactions. These results certainly seem promising in providing fundamental insight into the role of cytokines in organising tissue microenvironments during tumour progression. But, as these themes are currently disjointed from first half of manuscript on healthy tissues, this reviewer wonders if there could be some insights supported by such data relating immunity during tumour progression to specialised fibroblasts, thymic involution, or Peyer's patches, improving the coherence of the work as a whole. Lastly, I have provided some more specific suggestions and comments categorised by minor/major importance.

Major comments

The main part of the CoVarNet method is a one line function call to non-negative matrix factorization (NMF) in addition to computing correlations between cell type abundances. While this is technically a novel approach applied to cell type abundances from scRNA-seq data it does not constitute a substantial advance to computational biology methodology – and one could argue that the authors are overselling this approach by packaging it as its own named method. One (optional) suggestion would be to apply CoVarNet directly to spatial transcriptomics data, which could be timely and expand the package functionality with additional computational tools.

In the analysis corresponding to Fig 2b, what is the justification for excluding epithelial cells when inferring cell modules? I would expect epithelial cells to be key contributors to many of the tissues analysed in the study. Some explanation is provided in methods lines 428-431, which might be worthwhile expanding on and commenting on in the main text. Perhaps it would be clearer to rephrase this part around stromal-immune cellular organisation and the tumour microenvironment

rather than as a broad survey of all cells.

Fig 3f, lines 188-191, In the CODEX data, CD8 expression alone is insufficient to classify gamma-delta T cells.

Fig 4h is missing controls for general inflammation and cytotoxic T cell profiles to ascertain whether the association with treatment response is specific to the CM08-related inflammation signature. Otherwise these results may simply reflect the well-known observation that immune infiltration is a predictor of anti-PD1 response.

How has ambient RNA been taken into account during the study design or analysis? To my knowledge the standard in the field of atlas construction is to run ambient RNA removal such as CellBender. The lack of such analysis here introduces the possibility of technical confounding during annotation and hence in the identified cellular communities.

Lines 205-210: This interpretation of the number of ligands identified with CellphoneDB is highly speculative. It seems more plausible to this reviewer that the number of ligand-receptor pairs identified would indicate potential diversity of intercellular signalling in a variety of tissue contexts – some of which could be occurring in the identified CMs depending on whether the ligands and receptors are translated and posttranslationally regulated by mechanisms such as trafficking. The use of ligand 'numbers' here is misrepresented to indicate ligand abundance – there isn't necessarily "more messaging molecules" according to the data presented and hence no support for the diffusion over distance argument. I would recommend rephrasing these arguments.

Minor comments

Line 131, the description of "mutually exclusive CM types" conflicts with the methodology of decomposition by NMF which theoretically allows for co-occurring CM types.
If by the authors on line 137-138, "all CMTs could be well recovered [in GTEx bulk data]" then wouldn't that indicate that the 12 cell modules could have been discovered using the GTEx data?

Line 377-378: how is "without excessive enrichment for specific cell types" defined?

Line 425-426: On "we also got assistance from CellTypist", which CellTypist models were used?

Fig. 1f seems out of place in the main figure. In addition, Peyer's patches are well characterised wherefore these results are not novel and used merely in the text for illustration purposes. Could consider swapping Fig S5e as validation data in support of your observation of the universal and specialised fibroblasts distinction in reproductive tissues. Or, could perhaps consider moving the CODEX figure to Figure 2.

In addition, the associated text lines 110-111 "These observations prompted us to ask" is a type of statement on personal motivation which is typically excluded from research papers. I would recommend shortening the introductory paragraph in lines 104-113.

Lines 562-563 in methods and Fig 4b has several unclear descriptions. Firstly, there is a lack of clear definition of the CM abundance threshold used per sample, in addition to what samples have been used – I'm assuming that these are the suspension data, but would be good to clarify. Relatedly, are the 'interaction count' shown in Fig 4b all CellphoneDB results or those specific to the cell types comprising each CM?

Fig4c and g, the ImmuneDictionary analysis is highly interesting, but the circular network plots shown here are difficult to comprehend; excessive abbreviations, busy network depiction with unclear overlapping edges, confusing use of colours for both cell types and cytokines, and the origin of edges at the bottom of the barplots makes it difficult to see the origin cell type. I would recommend rethinking the visualisation style and representation of these plots according to guidelines formulated by Edward Tufte.

References

Bagaev, Alexander, Nikita Kotlov, Krystle Nomie, Viktor Svekolkin, Azamat Gafurov, Olga Isaeva, Nikita Osokin, et al. 2021. "Conserved Pan-Cancer Microenvironment Subtypes Predict Response to Immunotherapy." Cancer Cell 39 (6): 845–65.e7.

Damotte, Diane, Sarah Warren, Jennifer Arrondeau, Pascaline Boudou-Rouquette, Audrey Mansuet-Lupo, Jérôme Biton, Hanane Ouakrim, et al. 2019. "The Tumor Inflammation Signature (TIS) Is Associated with Anti-PD-1 Treatment Benefit in the CERTIM Pan-Cancer Cohort." Journal of Translational Medicine 17 (1): 357.

Hickey, John W., Winston R. Becker, Stephanie A. Nevins, Aaron Horning, Almudena Espin Perez, Chenchen Zhu, Bokai Zhu, et al. 2023. "Organization of the Human Intestine at Single-Cell Resolution." Nature 619 (7970): 572–84.

Luca, Bogdan A., Chloé B. Steen, Magdalena Matusiak, Armon Azizi, Sushama Varma, Chunfang Zhu, Joanna Przybyl, et al. 2021. "Atlas of Clinically Distinct Cell States and Ecosystems across Human Solid Tumors." Cell 184 (21): 5482–96.e28.

Varrone, Marco, Daniele Tavernari, Albert Santamaria-Martínez, Logan A. Walsh, and Giovanni Ciriello. 2024. "CellCharter Reveals Spatial Cell Niches Associated with Tissue Remodeling and Cell Plasticity." Nature Genetics 56 (1): 74–84.

Report written by Dr Simon Koplev under supervision of Professor Sarah A. Teichmann.

(Remarks on code availability)
The code is available only for the 'CoVarNet' method rather than comprehensively for analyses presented throughout the manuscript. On review, the available code does look sensible, but is somewhat underwhelming in terms of software scope. Reimplementing this from scratch would take a couple of hours and as such does not represent a substantial contribution to computational biology. The authors have presented more in-depth analysis elsewhere in the manuscript, in particular with regards to the spatial transcriptomics, MCP, and use of Immune Dictionary, which it would be of greater interest and use to the research community.

Referee #3

(Remarks to the Author)
In this manuscript, the authors sought to understand tissue-level coordination between cell types using the novel concept of the cellular modules to identify collections of cell types that potentially function together. By studying the frequencies of different cell types across a large library of single cell studies in healthy human tissue that they curated from previous studies, they generated twelve cross-tissue cellular modules. While the idea is intriguing, new biology does not really result or is claimed from this analysis. Moreover the interesting claims that are made lack additional support.

The authors do not provide supporting evidence for the existence of the CMs. This is particularly important as the strength of the cell type subset pairs in the CMs appears to be weak in some CMs (particularly in CM07 and CM11) with no accompanying statistics. Also the correlation coefficient cutoff was relatively low (>0.2) and the FDR was relatively high (<0.1). Also, the analysis of bulk and scRNA-Seq to find CMTs appears to be heavily processed. It is unclear how many samples were removed from the analysis based on stringent cutoffs.

The authors state that distribution of exhausted T cells in both tumor and adjacent regions suggest a "broad sensing of malignancy" (line 294). This seems to be a strong claim but no supporting evidence or explanation is provided.

Regarding the interesting result that the inflammatory program increases in melanoma patients that respond to PD1 therapy, and that this increase is absent in non-responders: it is unclear what the inflammatory program is–whether it is CM08 or something different. Also can the authors comment on the precise novelty of this result? The inflammatory program is known to be involved in such aspects, and so is the novelty the association with an entire cellular module?

How do the authors address the possibility that there are no study-specific differences in cell type composition, since sample preparation (ex: dissociation protocol) can drastically affect cell types recovered.

In the abstract, the authors refer to in vivo perturbation data, however it is unclear to what result in the paper this corresponds.

The authors make numerous claims where the data does not appear to be supporting their claim, despite the statistically significant p values, or make claims that are not supported by any quantification: (1) the authors make claims regarding cell type proportions being different across different tissues but do not include statistics to support this claim (Figure 1c). (2) In Figure 3a the authors claim that the CMs are mainly determined by tissue type (system preferences) but it does not appear that way by the annotation. The authors should consider annotating by tissue "type" in addition to the system. (3) Figure 3c - The authors make claims involving the expression of CM09 and cell states across age in the thymus. Although the authors state that these correlations are statistically significant, all of these plots appear to be heavily driven by few data points. (4) The authors make the claim that CM02 and CM03 have distinct functional roles because of the relative spatial organization of expression of CD8 and CD38 (Figure 3f), however, no quantification is done to support this claim and the use of the word 'functional' is not supported by spatial organization of marker expression. (5) The authors make the claim that cCM02, cCM04, and cDM08 are in distinct regions of the tissue (Figure 5c), but quantifications were not performed to support this claim.

In Figure 4 it is unclear which dataset the authors are using for the analysis shown in Figure 4a and how the lineage proportions were calculated. It is also unclear if CellPhone DB was performed on the spatial dataset or a single cell dataset. The claims regarding cellular communication between CMs should be validated experimentally. The authors utilize a package called DIALOGUE to determine which CMs are triggered as a response to the local environment, however this method is not thoroughly explained. The authors look at MCP gene expression, however it is unclear if this is CM08 or an MCP generated from DIALOGUE.

The authors claim that cCM02 has an increase in cell subset co-occurrence in tumor tissue compared to that of adjacent normal tissue (Figure 5d). It is unclear if the authors performed this analysis iteratively in each sample containing both tumor and tumor adjacent tissues or if this analysis was performed taking into account all samples. More broadly in this manuscript there is often a lack of explanation of (1) datasets used for each analysis and (2) a clear explanation of the analysis performed in the methods section.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
The authors have addressed all of my comments and the manuscript has been very much improved.

(Remarks on code availability)
The tutorial is now much more detailed and the authors also provide relevant code for generation of the figures.

Referee #2

(Remarks to the Author)
The revised manuscript “Cross-tissue multicellular coordination and its rewiring in cancer” provides substantially improved narrative and presentation. The authors have addressed most of my concerns and the work as a whole appears strengthened as appropriately outlined in the response to referees letter. As also pointed out by the other reviewers, the original manuscript was lacking in clearly novel biological insights. The main advantage of the study was, and still is, pioneering methodological contributions on the analysis of high sample scRNA-seq data. As discussed in the rebuttal, discovery based on single-cell RNA-seq data, rather than spatial transcriptomics, is indeed a potentially valuable approach, which would guide future thought and analysis of single cell atlases with higher sample sizes (n>100). Altogether, the study is therefore of broad interest to the field of single-cell genomics.

For these reasons, improvements made to the CoVarNet package and descriptions in figures and text are noteworthy and commendable. The authors could even consider, if appropriate, adding functionality for the new trajectory inference based on cell abundance frequency by sample. However, given the technical nature of trajectory inference, it would be important to clarify either precedence of this approach or justification for the use of PHATE and Palantir, which have been tested only (and incompletely so) on single-cell representations of gene expression space – rather than as used here by the authors to analyze patterns in cell type frequency.

The primary outstanding question for consideration for publication in Nature, however, is whether the added and reorganized findings are sufficiently insightful, convincing, and cohesive, providing evidence that the developed approach can be used for making new discoveries from single-cell atlases. As argued below, regarding the new findings on intestinal mucosa, menopausal fibroblasts in breast tissue, spleen aging, and ecosystem rewiring in cancer, the original criticism on novelty still applies albeit to a lesser extent. For instance, the revised work shows changes to fibroblast subtypes in menopausal breast tissue concordant with a reduction of one cellular program (CM12). While in Fig. S17g this pattern is not significantly detected for age (>50 years), the trend appears similarly and does not fully exclude potential confounding with age, which is clearly correlated with menopause according to Fig. 4k. Some associations that are currently missing would be whether, as in Fig. 4h and Fig S17c, age is associated with the 3 fibroblast subtypes highlighted. Nevertheless, this reviewer would expect there could be both some genuine overlap between the effects of aging and menopause along with an understandable infeasibility from distangling such effects based on clinical observational data alone.

In addition, there is a lack of presented background and discussion on how these CM12 results tie in with literature on stromal fibroblasts in breast tissue. Are the CM12 results not consistent with aging-associated fibroblast phenotypes (https://doi.org/10.1002/bies.201100104), including cytokine-secreting macrophages which likely corresponds to your identification of Mph_NLRP3 cells (see https://www.cell.com/trends/biochemical-sciences/fulltext/S0968-0004(16)30148-7)? On a separate note, while it is interesting that CM12 is found also in skin, I would recommend that the authors briefly review relevant literature in the field on the known effects of menopause (such as https://doi.org/10.3109/09513590.2011.613970), clarifying in greater detail which findings related to CM12 have been reported before and what new insight are supported by the data. Perhaps links to breast cancer risk would be worthwhile highlighting (https://doi.org/10.1016/j.critrevonc.2007.09.001), potentially linking these results better with the subsequent section on cancer immunotherapy.

The results on aging in the spleen are interesting and more convincing than the thymic involution results alone, but may be explainable by T cell quiescence (https://www.jci.org/articles/view/85329) and/or T cell exhaustion, which the authors note but which would not in itself be a new finding. In addition, please note the important differences between CD4+ and CD8+ T cells in the paper that you cite (https://www.nature.com/articles/s41586-019-0979-8). Here, your cell type associated with age are CD4+ T cells specifically, which should inform the interpretation of these results. Also, the B cell annotations (B05_Bm_NR4A2) of the aging-related results could be controversial since, at least in mice, B cells are known to lack Nr4a2 expression altogether (https://doi.org/10.1111/imr.13072):

“Since Nr4a2 is virtually absent in B cells, we hypothesized that deletion of Nr4a1 and Nr4a3 should largely eliminate all NR4A function in B cells.”

Some explanation for this discrepancy would be required. Given the lack of ambient RNA removal (such as by CellBender), of which I understand and empathise with not being able to do at this scale despite the suggestion in the previous peer review, one possibility would be myeloid cell contamination in the B cell cluster.

More broadly, and as a suggestion for improved presentation, it would be helpful to have clearer annotations of all identified

CMs indicative of the hypothesized functional roles in tissue architecture. I would suggest adding spelled out descriptive labels to each module in Fig. 2d – or provide a table of each module, clarifying the prevailing hypothesis for each module in greater detail, some of which is already described in the main text. Relatedly, the link from the intestinal Peyer's patches shown in Fig. 2a to other stories is presently unclear and disjointed from those presented elsewhere in Fig. 2 and Fig. 3. This is compounded somewhat by the exclusion of epithelial cells, which are key components of Peyer's patches. One suggestion would be to clarify which of the CMs corresponds to barrier tissue stromal-immune architecture, including Peyer's patches and cross-organ counterparts. Alternatively, the Peyer's patches data may fit better with the intestinal mucosa results shown in Fig. 3c-f.

Lastly, on line 155, what does 'notable system preferences' mean? I suppose this means tissue-specificity?

In summary, the manuscript has improved substantially but some issues remain. Some of the points raised are inherently due to an emphasis of broad scope rather than specific details. The manuscript is well written, timely, and the overall presentation has improved substantially.

(Remarks on code availability)
The associated software package has been expanded and the code for figures are provided as notebooks.

Referee #3

(Remarks to the Author)
The authors have done impressive work in revising their manuscript. They have now convinced me that the cellular module concept is important, comprehensive and robust. I appreciate very much the process of identification and characterization that the authors have gone through to identify the cellular modules. Using this approach they show impressive results with the aging spleen and menopause, as well as the ecosystems in cancer. I believe that this is an important paper that is ready for publication.

Version 2:

Reviewer comments:

Referee #2

(Remarks to the Author)
The authors have fully addressed my concerns – and I would like to congratulate them on the near-complete study, which will undoubtedly be an important contribution to the field of single-cell genomics.

The revised manuscript has a clear and convincing narrative with several minor improvements made during this second revision. The recent changes have added more depth to the interpretation of key results. The rebuttal was well argued and has increased my confidence in the validity of presented findings. Any remaining limitations on the originality of findings seem related to the broad scope of the study and are unlikely to improve on further revision.

Referee #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

1st round of review

Response to Referees Letter

We sincerely thank all three reviewers for their constructive and insightful feedback, which has significantly guided the enhancement of our manuscript. We have thoroughly addressed all comments and suggestions, incorporating additional data and analyses to strengthen our study. Major improvements in the revised manuscript include:

(1) **Spatial characterization of cellular modules (CMs):** We have characterized and validated CMs using extensive Visium and Xenium datasets from various tissues. In addition, we performed orthogonal validation by identifying cellular niches with CellCharter based on spatially resolved transcriptomics data.

(2) **Validation of cytokine-based interactions:** Spatial analyses confirmed that cytokines derived from single-cell analysis are spatially enriched in tissue microenvironments associated with CMs, further supporting their functional relevance.

(3) **CM associations with individual phenotypes:** By linking CMs with phenotypic data, we revealed several fundamental insights, including multicellular dynamics associated with human aging and menopause in women. These findings demonstrate the value of our CM framework in understanding tissue-level coordination.

(4) **Coordinated multicellular rewiring in cancer:** We conducted comparative analyses using paired healthy, tumor, and adjacent non-tumor samples across multiple cancer types. These analyses highlight the simultaneous rewiring of two types of multicellular ecosystems during tumor progression: the loss of tissue-specific organizations and the emergence of a convergent cancerous ecosystem. These results have also helped refine the logical structure of our manuscript.

(5) **Upgraded CoVarNet:** We optimized CoVarNet by implementing more stringent statistical methods and expanded its functionality to include spatial transcriptomics data. Additionally, we have provided more detailed tutorials to facilitate its use and included a discussion comparing our CM framework with alternative methods.

(6) **Enhanced presentation and explanation of results:** We have included more detailed explanations of the datasets and methods used for each analysis, improving the quantification and clarity of our results. These revisions further substantiate our claims with enhanced supporting data.

(7) **Public availability of datasets and code:** All curated datasets and key analysis code are now publicly accessible, ensuring transparency and reproducibility of our work.

We believe these revisions have substantially strengthened our study, and we are confident that the manuscript will make a significant contribution to the research community. Below, please find a point-by-point response to each reviewer comment.

Referee #1 (Remarks to the Author):

In this manuscript, Shi et al. characterised cross-tissue co-occurring cellular modules (CMs) based on a pan-tissue single-cell atlas and a novel computational framework called CoVarNet. They show that these CMs are differentially abundant across tissues and their results point that they are important for understanding tissue-level functional coordination. The association of the inflammatory gene program enriched in exposed human tissues with responsiveness to anti-PD-1 therapies was very interesting. They extend these analyses on cancer samples. The method in my opinion will be useful for future studies in both healthy and disease conditions. It is also an impressive collection of datasets shared as a repository that enables users to explore and visualise the data. As such, it presents a valuable resource for the cancer research community. My main criticism is about more stringent validation of the existence of the modules and their functional significance, as well as more novelty with regards to the role of these CMs in cancer.

We thank the reviewer for the positive comments regarding the concept of cross-tissue CMs, the single-cell atlas, and their promising potential. We also appreciate the reviewer's constructive critique concerning the need for more rigorous validation of CMs and their functional significance. We agree that additional insights into the role of these CMs in cancer are important for strengthening the novelty of our findings. These points have been addressed in detail in our subsequent responses.

Major comments:

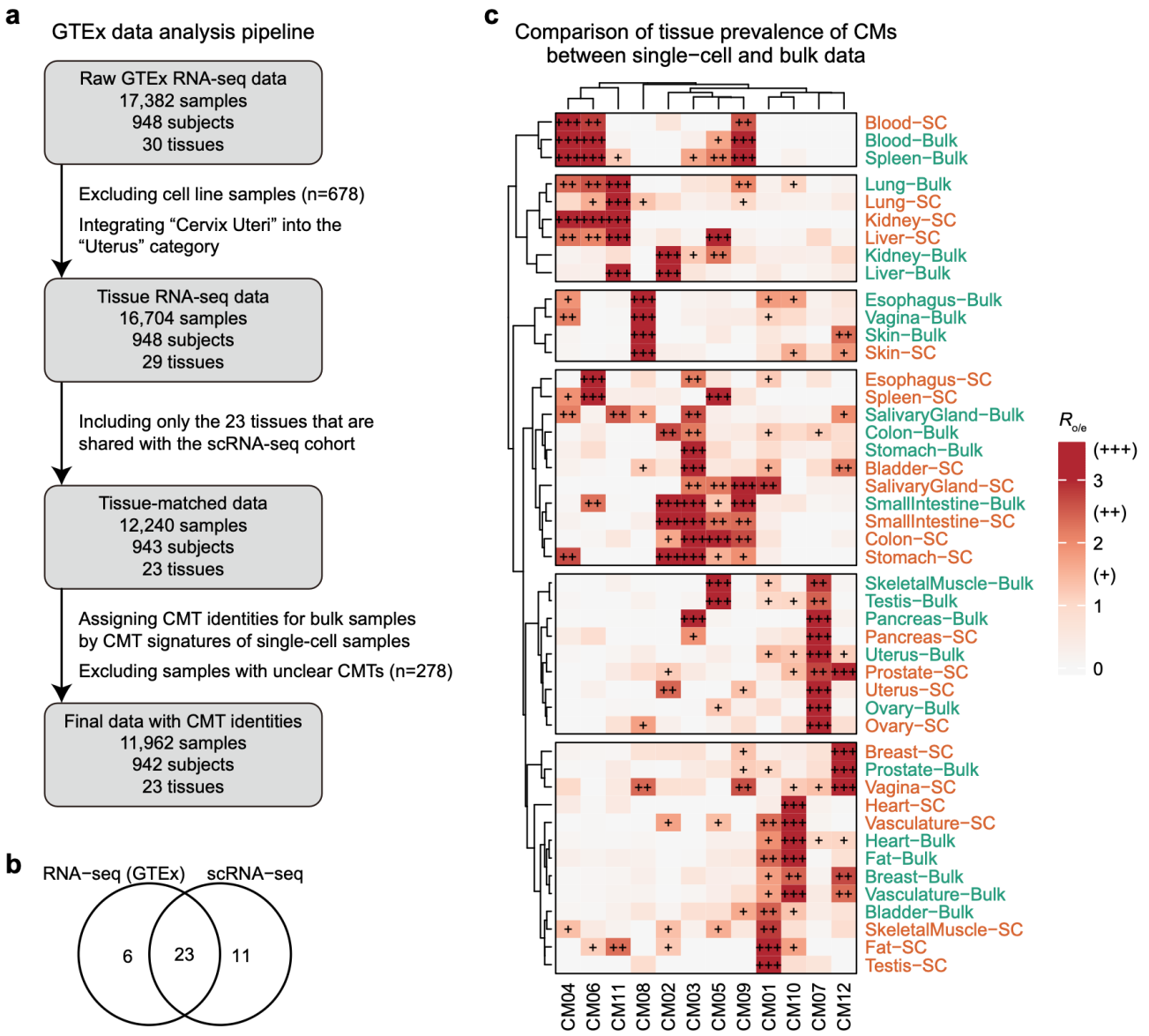
Major Comment 1

For the validation with GTEx data, it was not clear to me how well the enrichment of different CMs in distinct tissues in bulk samples matches with results from the single-cell analysis. I don't see too much similarity between S9a and S10b, but it was difficult to see with the many different colours, can the authors provide some other way of visualisation to validate this?

We thank the reviewer for the insightful comment, which has prompted us to refine the comparison of CM enrichment between bulk and single-cell analyses. The discrepancy observed between the original Fig. S9a and S10b can be attributed to the fact that the single-cell analysis encompassed 34 tissues, whereas the bulk data covered only 23 of these tissues (**Response Fig. 1a-b**).

To address this, we performed a focused comparison using the 23 overlapping tissues. By integrating and clustering these data based on CM profiles, we observed a highly consistent CM enrichment pattern between the two data types (**Response Fig. 1c**). Given the cross-tissue nature of CMs and the potential biases from sequencing library, this concordance provides robust validation for the existence of the CMs, strengthening the reliability of our findings.

We have incorporated these updated results into the revised manuscript (**Lines 155-156 and Fig. S9**).



Response Fig. 1. Validating CMs with GTEx bulk RNA-seq data. (also Fig. S9)

(a) GTEx data analysis pipeline.

(b) Venn diagram of intersecting tissues in GTEx RNA-seq and curated scRNA-seq cohorts.

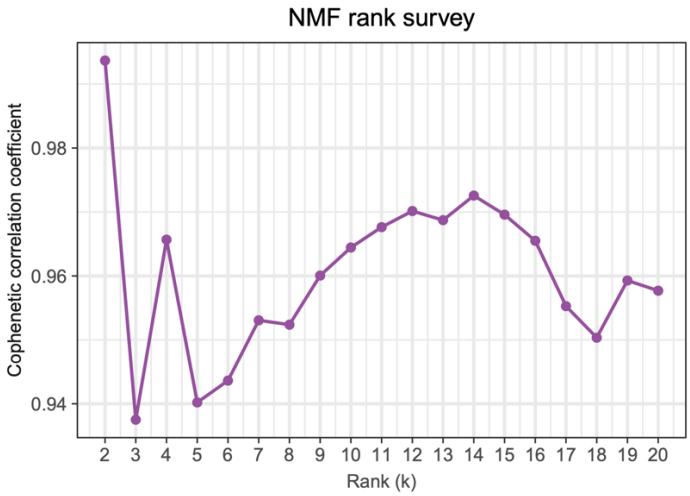
(c) Heatmap illustrating the tissue prevalence of CMs measured by $R_{o/e}$ (the ratio of observed to expected CM abundance) for tissues profiled using both single-cell (SC) and bulk RNA-seq.

Major Comment 2

Why were 12 CMs used when S7A shows k=14 is a more stable solution? The authors mention in the Methods that "optimal rank was then identified as the one at which CCC is maximized"?

We thank the reviewer for raising this important technical point. Although k=14 showed a higher CCC, it was not consistent, as indicated by the non-consecutive changes in CCC values ($CCC_{12} > CCC_{13} < CCC_{14}$) (**Response Fig. 2**). We selected the optimal rank by considering both the CCC and the consistency of the results. Therefore, we chose k=12, which exhibited consistent stability ($CCC_{10} < CCC_{11} < CCC_{12}$) while maintaining one of the highest CCC values.

We have revised the Methods section to provide further clarification on this rationale (**Lines 581-584 and Fig. S6**).



Response Fig. 2. NMF rank selection. (also Fig. S6)
Survey of NMF ranks based on cophenetic correlation coefficients (CCCs). The optimal rank K was selected by considering both the CCC and consistency, indicated by consecutive increases in CCC values leading up to K. The final rank was determined to be 12.

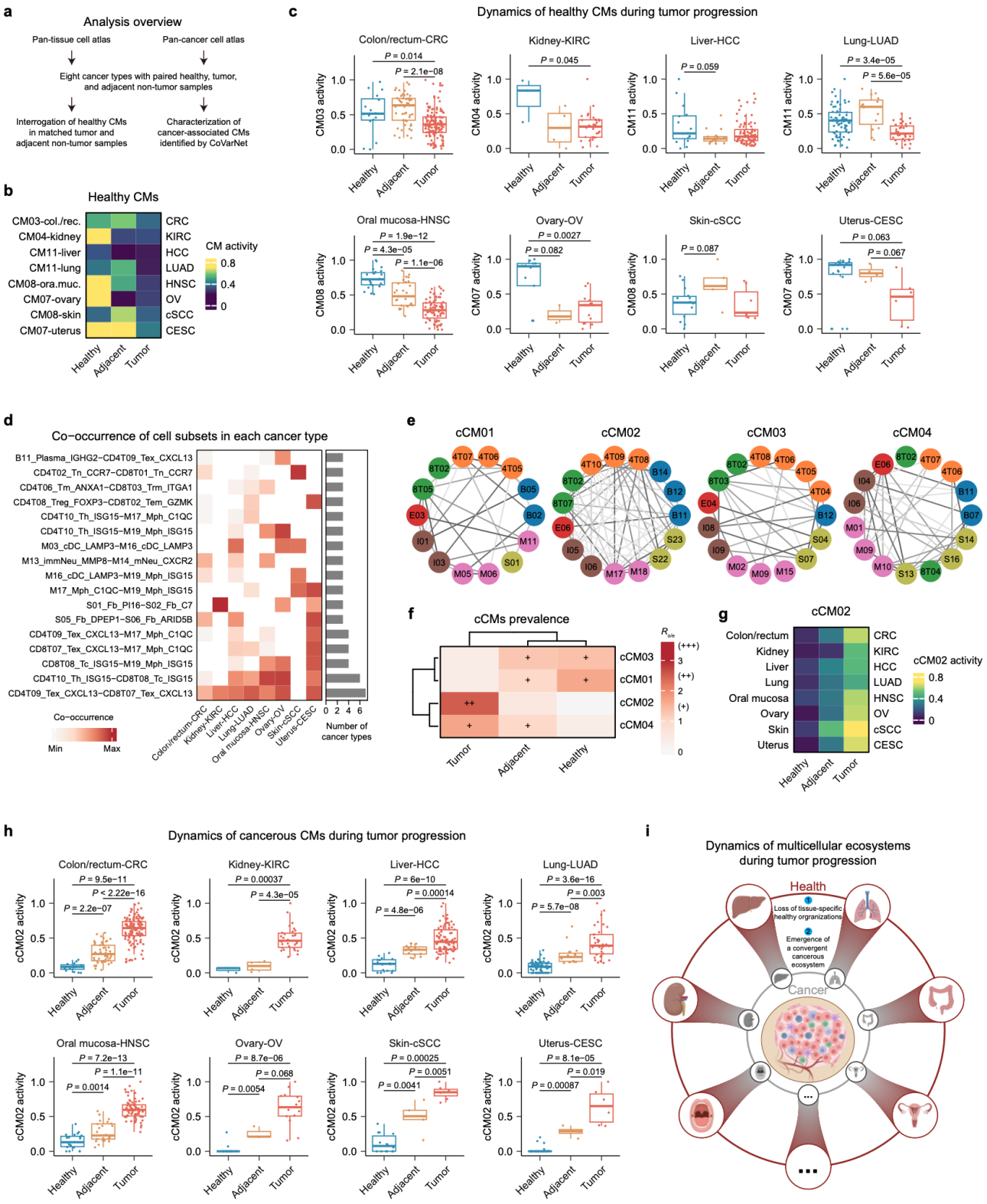
Major Comment 3

The CMs both in normal and cancer should be described more in detail in the text. The cancer part was especially brief. It is well known that many immune and stromal cells dramatically change their phenotype in cancer, the authors should emphasise the novelty that inference of CMs bring in this part. They didn't comment much about enrichment of specific CMs in different cancer types.

We thank the reviewer for this valuable suggestion. We agree that a more detailed description of the CMs linking normal and cancer tissues is warranted. Our CM framework offers a unique opportunity to characterize cellular changes in cancer in a coordinated and comprehensive manner.

Leveraging the comprehensive data across healthy, tumor, and adjacent non-tumor tissues, we investigated the multicellular dynamics during tumor progression. To address this, we conducted two types of multicellular analyses (**Response Fig. 3a**). First, we examined healthy CMs in tumor and adjacent non-tumor tissues, revealing significant disruption of these CMs in cancer (**Response Fig. 3b-c**). Second, we identified a convergent multicellular ecosystem emerging across cancer types (**Response Fig. 3d-h**). These findings highlight the simultaneous rewiring of two types of multicellular ecosystems during tumor progression: the loss of tissue-specific organizations and the emergence of a convergent cancerous ecosystem (**Response Fig. 3i**).

We appreciate the reviewer's insightful feedback, which helps underscore the significance of our conceptual framework in understanding cancer. Detailed results can be found in the revised manuscript (**Lines 387-412, Fig. 5, and Fig. S22-23**).



Response Fig. 3. Rewiring of multicellular ecosystems in cancer (also Fig. 5 and Fig. S22-S23).

(**a**) Overview of multicellular analysis in cancer.

(**b**) Sample-averaged activities of dominant healthy CMs in healthy, adjacent non-tumor, and tumor tissues across cancer types (healthy tissues).

(c) Healthy CM activities in healthy, adjacent non-tumor, and tumor tissues. Dots represent individual samples. Two-tailed unpaired Wilcoxon test.

(d) Heatmap showing the co-occurrence of cell-subset pairs in individual cancer types (healthy tissues). Bar plots on the right show the number of cancer types where individual pairs are detected. Only pairs detected in at least three cancer types are shown.
(e) Network plots illustrating the four cancer-associated CMs. Nodes represent cell subsets labeled by short names and colored by cell types. The edge color represents the specificity of correlation between paired subsets.
(f) Tissue prevalence of cCMs measured by Ro/e (the ratio of observed to expected cCM activities).
(g) Sample-averaged cCM02 activities in healthy, adjacent non-tumor, and tumor tissues across cancer types (healthy tissues).
(h) cCM02 activities in healthy, adjacent non-tumor, and tumor tissues across cancer types. Dots represent individual samples.
(i) Schematic representation of rewiring of multicellular ecosystems during tumor progression.

Major Comment 4

The authors should perform a quantification of cellular modules using spatial data to show more reliably that the cell subsets in each CM are colocalised across different patients. This can be done for example by identification of cellular niches across several samples with something like CellCharter or SpatialDE2. In line with the above point, the authors note that "Further investigation focused on CM08, whose components exhibited moderate spatial consistency (Fig. 4a)" - CM08 seems to have weak spatial consistency using Spearman's correlation, how do the authors explain this in terms of how functionally important is this specific CM?

We thank the reviewer for the insightful suggestions regarding spatial quantification and validation of CMs. We fully acknowledge the importance of spatial data in understanding the colocalization and functional relevance of CMs. To address this, we performed several spatial analyses using multiple Visium datasets from various tissues (**Table S3**):

(1) We assessed spatial relationships among cell subsets within the same CMs by calculating colocalization and aggregation scores (**Response Fig. 4**).

(2) For tissues enriched with specific CMs, such as skin for CM08, we quantified spatial distribution using six skin sections from different donors (**Response Fig. 5**).

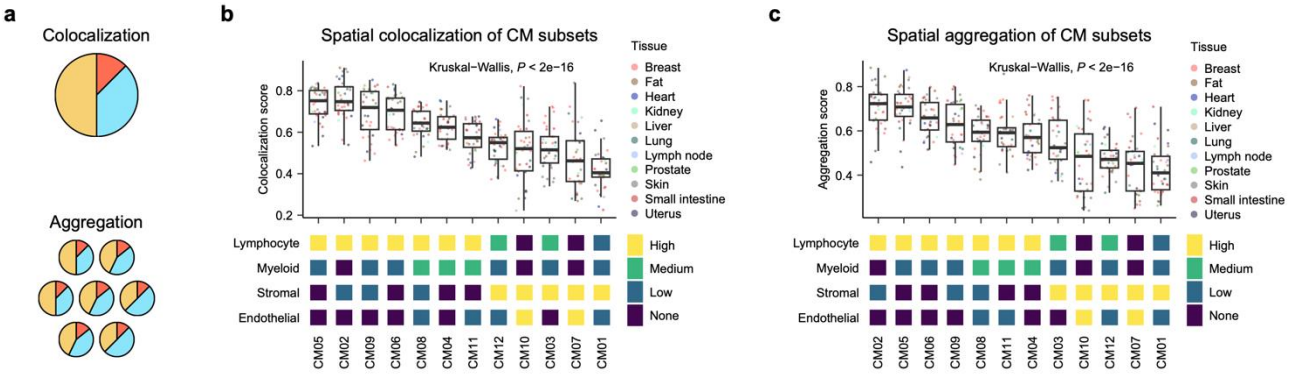
(3) We performed orthogonal validation by identifying cellular niches using CellCharter based on spatial transcriptomics data (**Response Fig. 6**).

Overall measurement of spatial distribution of CMs

We evaluated the spatial colocalization of cell subsets within the same CM (**Response Fig. 4a, top**). For each CM, we calculated correlation coefficients between subset pairs where at least one subset is within this CM, resulting in a set of correlation coefficients denoted as S. The median correlation coefficient within the CM is termed r. The colocalization score for each CM is defined as the proportion of correlations in S that are less than or equal to r, providing a measure of colocalization relative to global contexts. Additionally, we introduced an aggregation score using global Moran's I to assess spatial adjacency of cell subsets within the CM (**Response Fig. 4a, bottom**).

Both colocalization and aggregation scores revealed consistent patterns, reinforcing the robustness of our findings (**Response Fig. 4b-c**). We observed a notable association between cell-subset colocalization and lineage proportions within CMs, with the highest spatial concordance in CMs containing a higher proportion of lymphocytes, followed by myeloid cells, stromal cells, and endothelial cells. (**Response Fig. 4b-c**). These insights emphasize the relationship between spatial organization and the cellular composition of CMs.

We have updated these results in the revised manuscript (**Lines 257-261, Fig. 4a, and Fig. S15**).



Response Fig. 4. Overall measurement of spatial distribution of CMs. (also Fig. 4a and Fig. S15)
(a) Schematic representation of two spatial distribution metrics for CMs using 10x Visium data. The colocalization score evaluates the extent to which two cell subsets colocalize within the same spots, whereas the aggregation score measures the spatial adjacency of two cell subsets, encompassing both colocalization and broader spatial relationships. **(b-c)** Spatial colocalization (b) and aggregation (c) of CMs. Top: Box plots showing cell-subset colocalization or aggregation scores within individual CMs, as determined by spatial transcriptomics across various tissues. Dots represent individual samples colored by tissues. The middle line represents the median; box edges denote the 25th and 75th percentiles; whiskers indicate the most extreme points within ± IQR × 1.5. CMs are ordered by the median colocalization scores across samples. The Kruskal−Wallis *P* value is indicated. Bottom: Heatmap displaying lineage proportion categories (color code) for CMs (columns) across different cellular lineages (rows). Categories: None = 0 < Low < 0.2 ≤ Medium < 0.4 ≤ High. IQR, interquartile range.

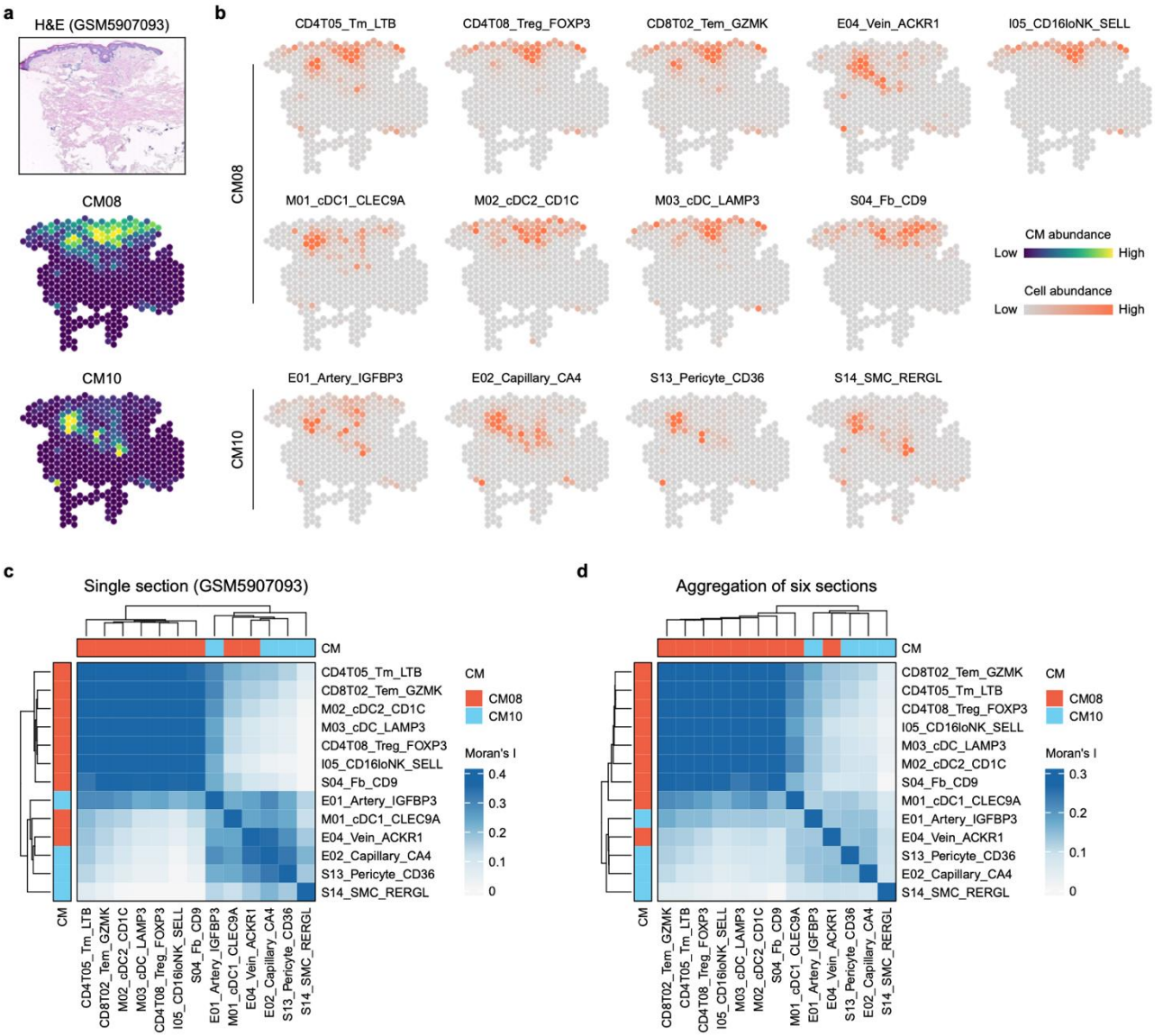
Spatial characteristics of CMs in specific context

We also examined the spatial associations of CMs in a context-specific manner, focusing on skin-enriched CM08 and CM10. Our analysis revealed prominent spatial colocalization of cell subsets within these CMs (**Response Fig. 5a-c**). CM08, which includes various immune cells such as dendritic cells (DCs) and T cells, localized within the epidermis and adjacent dermis layers, playing a vital role in immune defense. CM10, composed of endothelial cells, pericytes, and smooth muscle cells, corresponds to blood vessel components primarily in the dermis layer. While CM08 subsets show high colocalization within the CM, some cells, like vein endothelial cells (E04), also overlapped with CM10 subsets, highlighting the interconnection of different CMs within the tissue microenvironment. These findings were consistently observed across samples from six different donors (**Response Fig. 5d**), underscoring the robust existence of CMs.

Despite CM08 demonstrating moderate spatial consistency among all CMs (**Response Fig. 4b-c**), our context-specific analysis underscores its functional significance in skin immune surveillance and

defense. Therefore, the colocalization and aggregation scores, even when not exceedingly high, reflect meaningful spatial relationships within tissue microenvironments.

We have incorporated these results in the revised manuscript (**Lines 174-183 and Fig. S10**).



Response Fig. 5. Spatial colocalization of CMs in the skin. (also Fig. S10)

(a) Hematoxylin and eosin (H&E) staining and spatial mapping of CM08 and CM10 performed on a section of normal skin using spatial transcriptomics.

(b) Spatial mapping of the constituent cell subsets within CM08 and CM10, as shown in (a).

(c) Heatmap quantifying spatial aggregation (based on Moran's I) among the constituent cell subsets of CM08 and CM10, as shown in (b).

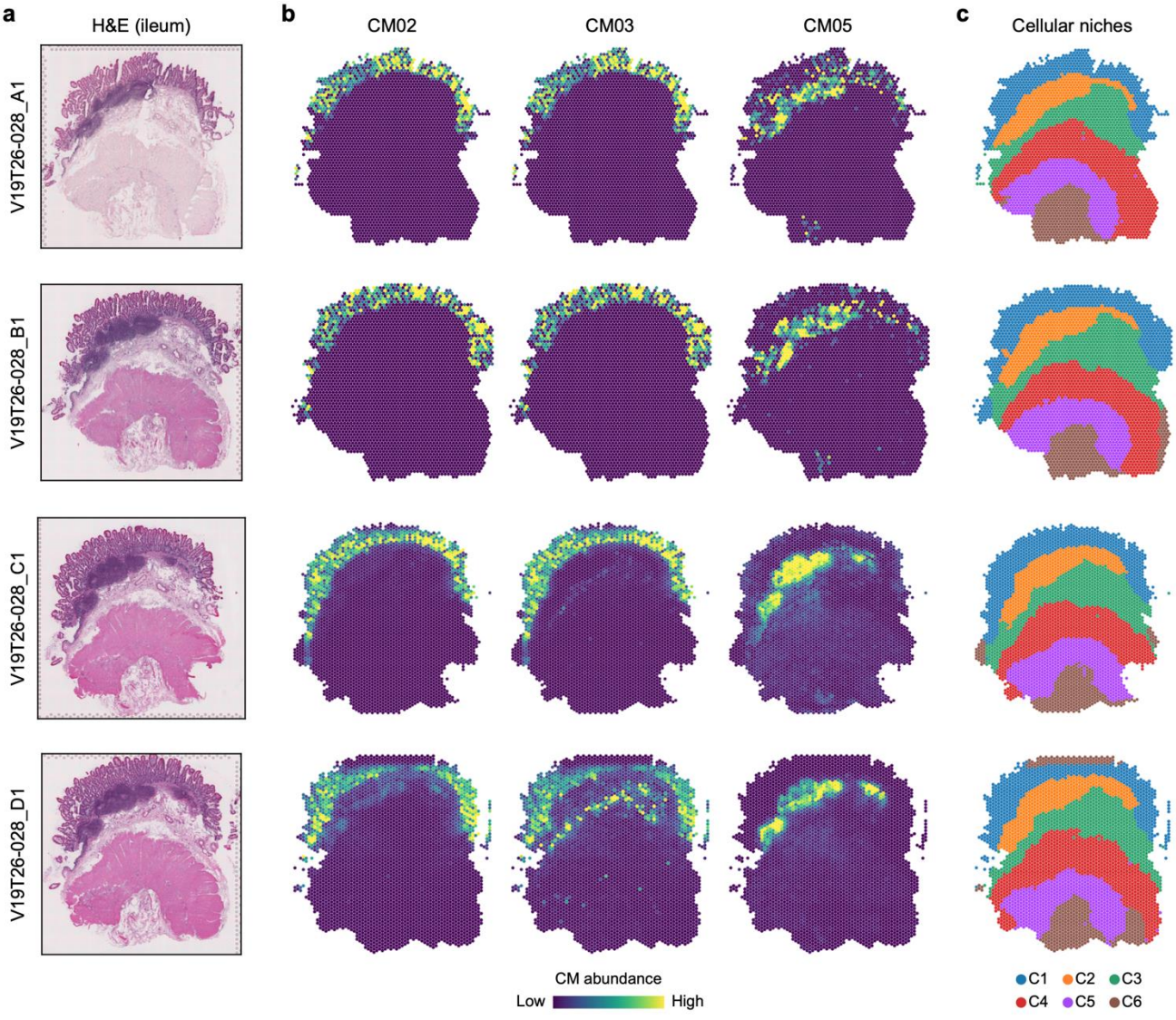
(d) Corresponding heatmap to (c), generated from the aggregated data of six spatial transcriptomics sections from six different donors.

Validation of CMs by cellular niches identified by CellCharter

Following the reviewer's suggestion, we performed spatial clustering directly based on the spatial transcriptomics data using CellCharter (Varrone et al. 2024). We illustrate this analysis using four adjacent ileum sections from the same donor to minimize individual variability.

We mapped small intestine-enriched CMs (CM02, CM03, and CM05) onto these spatial sections, revealing highly consistent spatial patterns across the sections (**Response Fig. 6a-b**). Specifically, CM05 corresponded with the Peyer's patch region, rich in naïve B cells and T cells, which serves as a critical site for antigen recognition and immune activation. In contrast, CM02 and CM03 were more prominent in the intestinal mucosa, aligning with their composition (e.g., tissue-resident T cells and IgA-producing plasma cells). Furthermore, the cellular niches identified by CellCharter were consistent with the spatial locations of these CMs: CM05 colocalized with the C2 niche, while CM02 and CM03 aligned with the C1 niche (**Response Fig. 6c**). This analysis confirmed the spatial patterns of CMs and provided a more reliable demonstration of cell-subset colocalization.

We have incorporated these results in the revised manuscript (**Lines 184-196, Fig. 3c-e, and Fig. S11**).



Response Fig. 6. Validation of CMs and cytokine responses by cellular niches in the small intestine. (also Fig. 3c-e and Fig. S11)

(a) Hematoxylin and eosin (H&E) staining of four adjacent sections of the small intestine from one donor.

(b) Spatial mapping of CM02, CM03, and CM05 on the four spatial transcriptomics sections.

(c) Spatial mapping of cellular niches identified by CellCharter (Varrone et al. 2024).

References:

Varrone, M., Tavernari, D., Santamaria-Martínez, A., Walsh, L. A. & Ciriello, G. CellCharter reveals spatial cell niches associated with tissue remodeling and cell plasticity. *Nat. Genet.* **56**, 74-84 (2024).

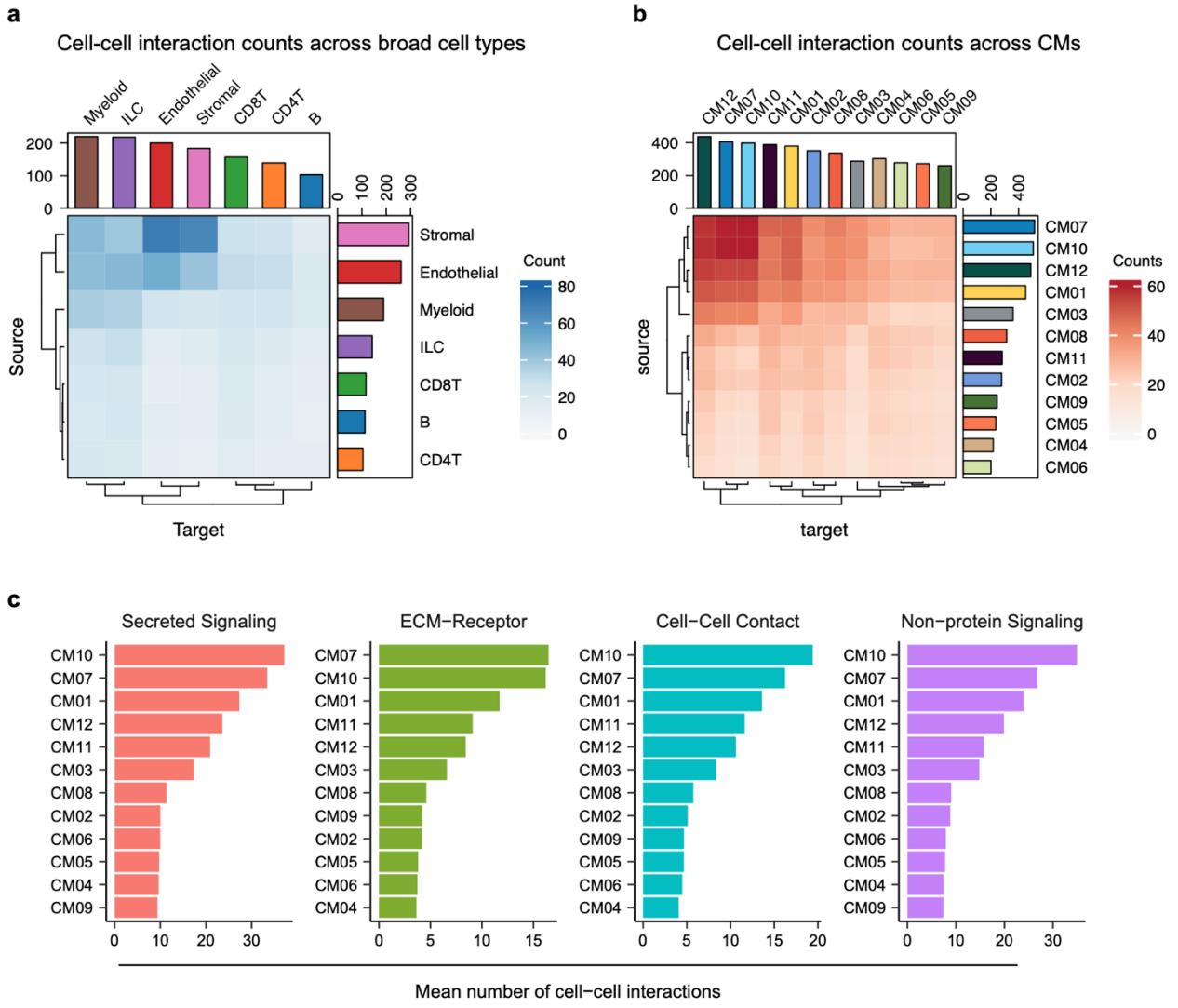
Major Comment 5

In Line 205-210, S12a the authors claim "Fibroblasts express more ligands….These findings can be harmonized by a notion that spatial adjacence enhances the efficiency of intercellular communication". However, in figure 4a shows that the CMs with a higher stromal composition have the worst colocalization score, how would you explain this?

We apologize for any confusion caused by our initial statement. The claim that "spatial adjacency enhances the efficiency of intercellular communication" was intended to convey the idea that closer proximity between cells generally facilitates more efficient interactions, potentially reducing the need for numerous messaging molecules. This concept implies that CMs with higher stromal composition, while exhibiting lower colocalization scores, may produce the larger number of messaging molecules to support communication over longer distances.

However, as pointed out in Major Comment 6 from Referee #2, interaction counts from CellPhoneDB should be interpreted as reflecting interaction diversity, rather than the quantity of signaling molecules. We also validated our findings using another tool, CellChat (**Response Fig. 7**). In the revised manuscript, we have updated our explanation to clarify these insights as follows (**Lines 257-270**):

"To explore this, we first investigated the spatial organization of CM components using spatially resolved transcriptomics (Visium) across various tissues (**Table S3**). Our analysis revealed a strong association between CM spatial organization and composition ($P < 2.2 \times 10^{-16}$, Kruskal-Wallis test). The highest spatial concordance was observed in CMs with a greater proportion of lymphocytes, followed by myeloid cells, stromal cells, and endothelial cells (**Fig. 4a and Fig. S15a-b**). To further understand the implications of these spatial patterns, we analyzed ligand-receptor-mediated cell-cell communication using single-cell data with CellPhoneDB. Intriguingly, endothelial cells and stromal cells produced a broader variety of ligands compared to lymphocytes (B cells, T cells, and innate lymphoid cells) (**Fig. 4b**). This aligns with the notion that spatial proximity may enhance the specificity of intercellular interactions, suggesting that lymphocyte-enriched CMs foster particular interactions within local niches. Conversely, CMs with a higher proportion of stromal and endothelial cells—often situated farther apart—generated a more diverse array of signaling molecules, facilitating broader signaling interactions over greater distances (**Fig. S15c-d**). These insights highlight the relationship among spatial organization, cellular composition, and intercellular communication."



Response Fig. 7. Cell-cell interaction analysis. (also Fig. 4b and Fig. S15)

(a-b) Average CellPhonDB cell-cell interaction counts between subsets within and across broad cell types (a) or CMs (b). Bar plots on the top and right represent sums of columns and rows, respectively.

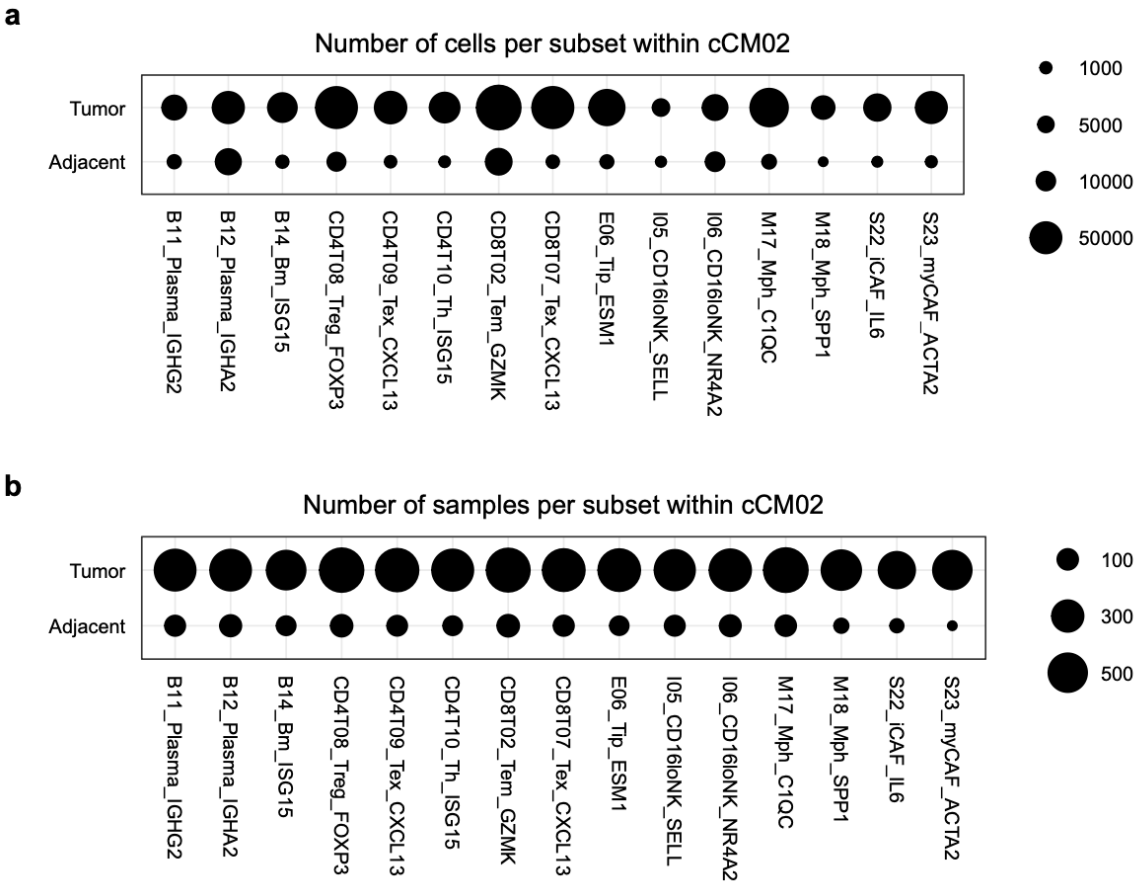
(c) Mean numbers of CellChat cell-cell interactions within individual CMs. All ligand-receptor pairs are categorized into different types, including "Secreted Signaling", "ECM-Receptor", "Cell-Cell Contact", and "Non-protein Signaling".

Major Comment 6

Fig 5d, the authors write "we observed a notable increase in cell-subset co-occurrence within cCM02 in tumor samples compared to adjacent non-tumor tissues, providing discernible evidence of malignant progression" - it was not clear to me whether all subsets are present both in tumor and adjacent tumor samples, or if the increase in cell-subset co-occurrence in tumor is because some subsets are not present in adjacent tumor samples.

We thank the reviewer for raising this important point. We confirm that all subsets of cCM02 are

present in both tumor and adjacent non-tumor samples (**Response Fig. 8**). The observed increase in cell-subset co-occurrence within cCM02 in tumor samples reflects enhanced interactions and coordinated activity among these subsets within the tumor microenvironment, rather than the appearance of previously absent subsets.



Response Fig. 8. The number of cells per subset of cCM02 in tumor and adjacent non-tumor samples. The dot size indicates number of cells.

Major Comment 7

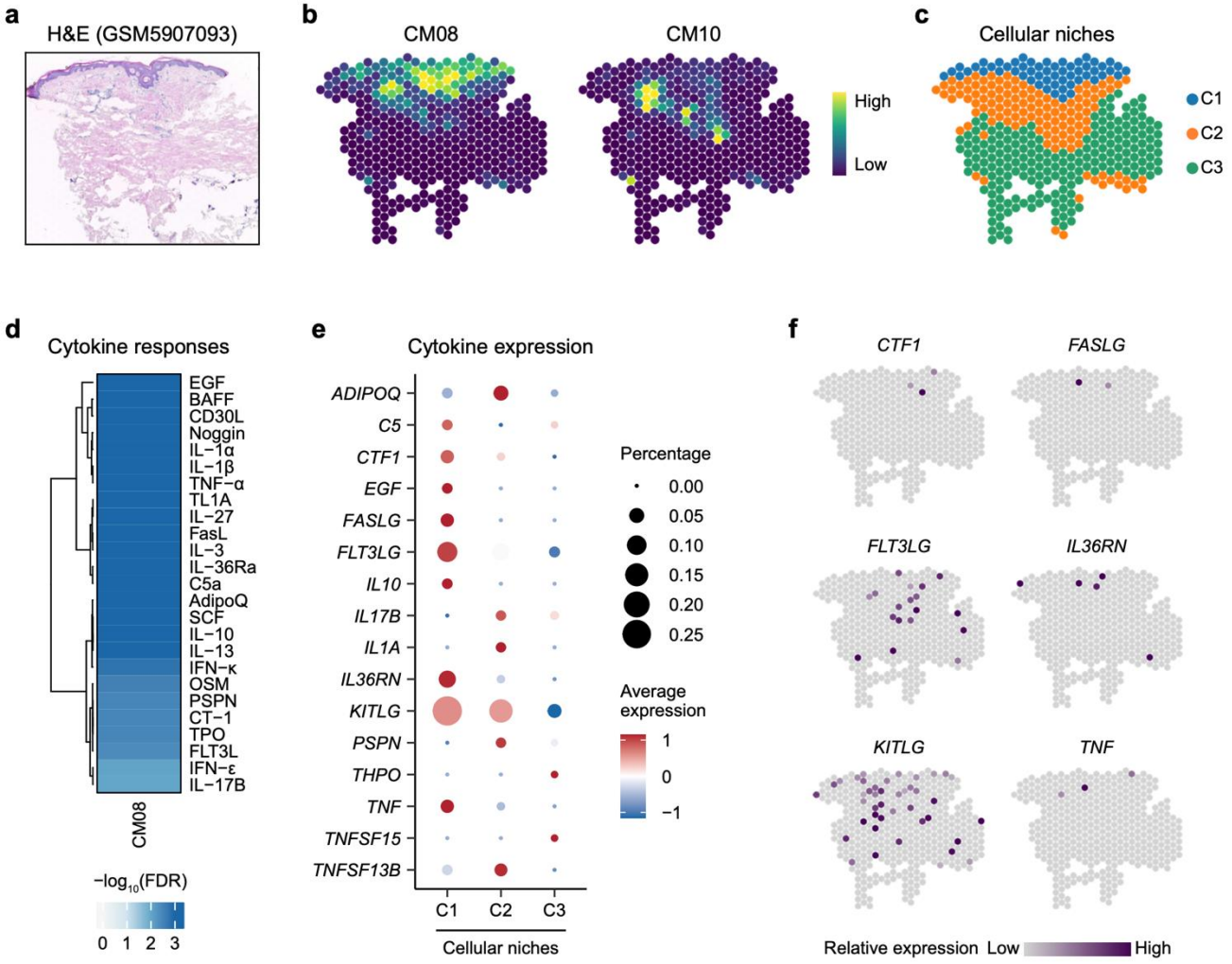
The cytokine analysis both in healthy and cancer samples can lead to interesting hypotheses, it would add a lot more relevance of the functional significance of these CMs if there was validation of some of them. For example, for those cytokines found enriched in some CMs and not others, can you show that they are spatially enriched in the niches/microenvironments representing those CMs compared to other niches?

We greatly appreciate the positive feedback on our cytokine analysis and the insightful suggestions for additional spatial validation. Recognizing the importance of validating cytokine expression within the spatial context of CMs, we have conducted a detailed spatial analysis.

We utilized spatial Visium data from skin (**Response Fig. 9**) and small intestine (**Response Fig. 10**) to investigate the spatial distribution of cytokines within specific CMs. Despite the typically transient and low-level expression of many cytokines, we successfully identified distinct spatial enrichment

patterns consistent with our single-cell cytokine analysis. For example, CM08, which corresponds to the C1 niche in the skin, exhibited notable cytokine responses (**Response Fig. 9a-d**), with specific enrichment observed in the C1 niche and its adjacent regions (**Response Fig. 9e-f**). In the small intestine, our prior analysis aligned CM02/CM03 and CM05 with the C1 and C2 niches, respectively (**Response Fig. 6 and Response Fig. 10a-c**). Further analysis confirmed high expression levels of cytokines within these CMs in their corresponding niches (**Response Fig. 10d-f**).

These findings validate the spatial organization of cytokines within specific niches and highlight the functional roles of different CMs. We thank the reviewer for this insightful suggestion, which has significantly strengthened the depth and impact of our study. These results have been incorporated into the revised manuscript (**Lines 285-289, Lines 348-350, Fig. 4e, Fig. S16, and Fig. S18**).



Response Fig. 9. *In situ* validation of cytokine responses in the skin. (also Fig. S10 and S18)

(a) Hematoxylin and eosin (H&E) staining of a skin section.

(b) Spatial mapping of CM08 and CM05 on the spatial transcriptomics sections.

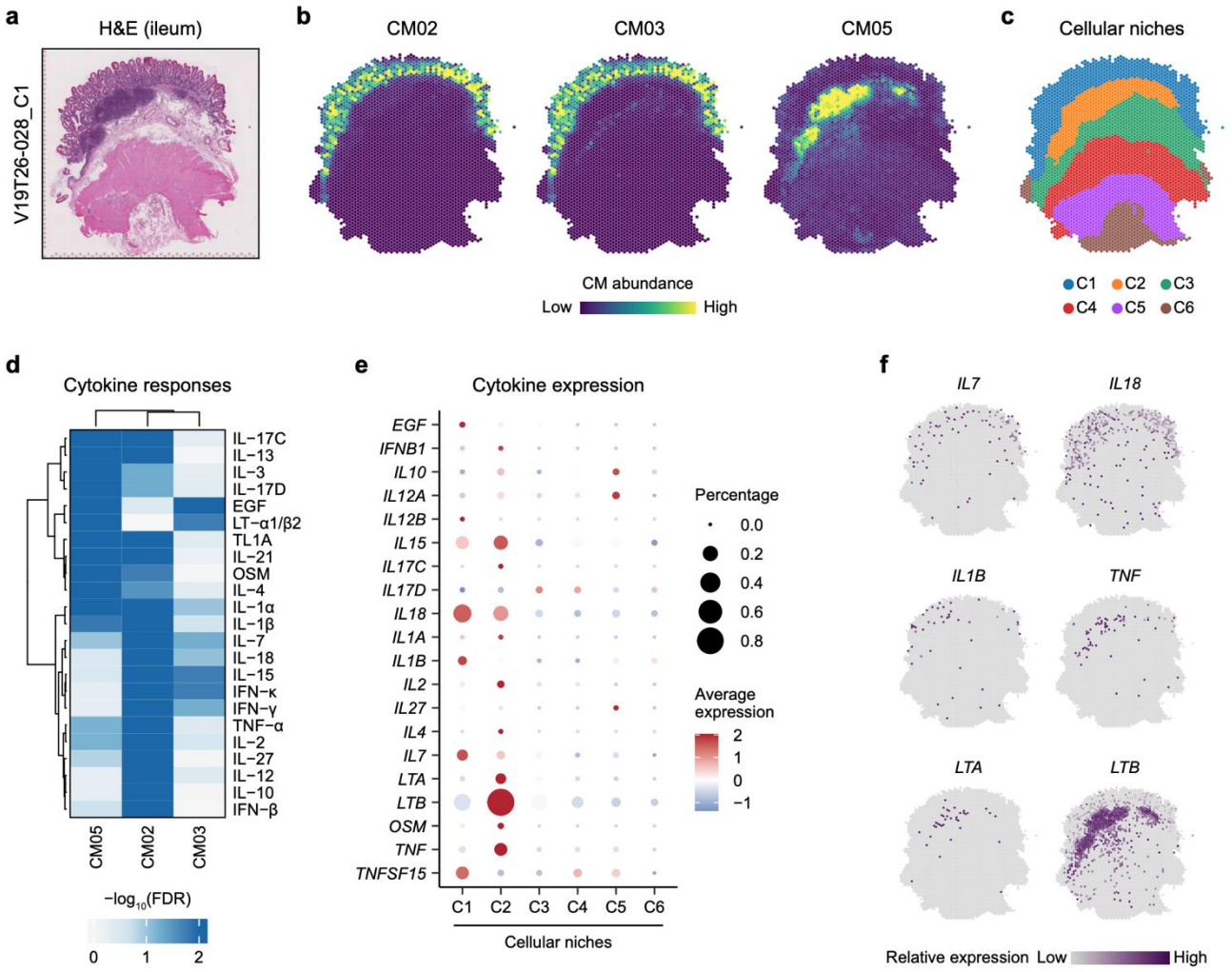
(c) Spatial mapping of cellular niches identified by CellCharter (Varrone et al. 2024).

(d) Heatmap showing cytokine responsiveness in CM08. The CM FDR denotes the minimum FDR value among CM subsets.

(e) Cytokine gene expression profiles across the cellular niches illustrated in (c). Only cytokines detected by spatial

Visium data are shown.

(f) Spatial distribution of select cytokines in the spatial transcriptomics sections.



Response Fig. 10. *In situ* validation of cytokine responses in the small intestine. (also Fig. 4d-e and Fig. S11 and S16)

(a) Hematoxylin and eosin (H&E) staining of a section of the small intestine.

(b) Spatial mapping of CM02, CM03, and CM05 on the spatial transcriptomics section.

(c) Spatial mapping of cellular niches identified by CellCharter (Varrone et al. 2024).

(d) Heatmap showing cytokine responsiveness among CM02, CM03, and CM05. The CM FDR denotes the minimum FDR value among CM subsets. Only cytokines enriched in at least one CM are shown.

(e) Cytokine gene expression profiles across the cellular niches illustrated in (c). Only cytokines detected by spatial Visium data are shown.

(f) Spatial distribution of select cytokines within the small intestine.

References:

Varrone, M., Tavernari, D., Santamaria-Martínez, A., Walsh, L. A. & Ciriello, G. CellCharter reveals spatial cell niches associated with tissue remodeling and cell plasticity. *Nat. Genet.* **56**, 74-84 (2024).

Minor comments:

Minor Comment 1

It would be helpful to include a brief explanation of the method CoVarNet in the main text.

Great suggestion. We have added a brief introduction to the CoVarNet method in the main text as follows (**Lines 121-127**):

"CoVarNet consists of two parallel modules that jointly establish CM networks. The first module employs non-negative matrix factorization (NMF) to identify a set of factors, prioritizing cell subsets based on their weights. The top subsets from each factor serve as co-occurring nodes in the CM network. The second module determines specifically correlated subset pairs, which act as potential edges in the network. Multiple CM networks are then constructed by connecting these co-occurring nodes through the potential edges, followed by topological and statistical evaluations."

Minor Comment 2

Fig. S20c, a brief explanation of how this plot was derived should be added in the legend.

As part of the manuscript refinement for cancer, we have removed this figure panel in the revised version. A more detailed explanation of the original Fig. S20c is provided below:

"c, Compositional rewiring between healthy and cancerous CMs. Each line represents an individual cell subset, linking the healthy CM it belongs to (left) and the cancerous CM it belongs to (right). Subsets involved in multiple CMs are shown separately in each relevant CM."

Minor Comment 3

For the spatial methods section, why n=5 for cell2location, did the authors perform segmentation?

We did not perform segmentation for our analysis. The choice of n=5 for cell2location was based on the recommended guidelines for Visium data provided by the authors of cell2location, as detailed in their document:

(https://github.com/BayraktarLab/cell2location/blob/master/docs/images/Note_on_selecting_hyperparameters.pdf). This clarification has been added to the revised Methods section (**Lines 651-652**).

Referee #1 (Remarks on code availability):

The installation and running of the method worked very well for me with the example data provided. The tutorial could be a bit more detailed.

We thank the reviewer for the positive feedback regarding the usability of our CoVarNet software. We have enhanced the CoVarNet repository with additional functionalities and a more detailed tutorial to improve the user experience (https://github.com/QiangShiPKU/CoVarNet).

Referee #2 (Remarks to the Author):

In this study the authors assemble a single-cell RNA-seq atlas of cells across 35 healthy human tissues and 26 cancer types, identifying shared patterns of non-epithelial (immune, stromal, and endothelial) cell type abundancy across tissues. The research aims are broad and forward-looking, exploring important research problems relevant to efforts such as the Human Cell Atlas, with an imminent potential to generate biological insights across multiple tissues. As such, the manuscript describes a method to detect cellular communities that form functional organisational units in human tissues from scRNA-seq data at large sample sizes (n=706). Although not an entirely novel idea, it is highly valuable for the research community to understand how this approach compares to alternative methods based on spatial transcriptomics, multiplex imaging, and bulk deconvolution (Luca et al. 2021). However, there is currently more excitement in the field for using spatial transcriptomics to discover multicellular coordination with approaches such as CellCharter (Varrone et al. 2024) – or from modelling contributions such as hierarchical organisation of tissue unites in CODEX imaging data (Hickey et al. 2023).

We thank the reviewer for the thorough review and constructive feedback on our manuscript. We appreciate the recognition of our study's broad and forward-looking research aims and its potential contributions to large-scale initiatives like the Human Cell Atlas. Below, we provide a discussion comparing our framework with alternative methods.

Harnessing the power of diverse data

Spatial data offer spatially resolved molecular profiles but are often limited by either the number of genes profiled (imaging-based methods) or the lack of single-cell resolution (sequencing-based methods), hindering comprehensive delineation of multicellular ecosystems. For example, while CODEX provides single-cell resolution, its ability to identify subtle cell subtypes is constrained by limited protein coverage (Hickey et al. 2023). In contrast, our approach leverages single-cell data to identify fine-grained cellular modules (CMs), offering both single-cell resolution and whole-transcriptome coverage. This facilitates further CM characterization by mapping them onto spatial data, effectively harnessing the strengths of diverse datasets. Notably, by integrating single-cell-derived CMs with spatial data (Visium and Xenium), we revealed distinct spatial distributions for CM02 and CM03 that were not evident from Visium data alone (**Fig. 3c-g**).

Additionally, bulk RNA-seq lacks both single-cell resolution and spatial context. Bulk deconvolution methods often fail to capture fine-grained cell subtypes, with uncertainty and noise in cell state identification leading to ambiguous results. For instance, a bulk deconvolution study reported that nearly one-third of deconvoluted cell states were previously uncharacterized (Luca et al. 2021), complicating the biological interpretability of these states and subsequent multicellular analyses.

Our approach stands out by combining the precision of single-cell data with additional characterization and validation through spatial (**Response Fig. 4-6**) and bulk data. We utilized bulk RNA-seq to confirm the existence and prevalence of CMs across tissues, underscoring the complementary strengths of various data types (**Fig. 2d-e and Response Fig. 1**). This integrated methodology ensures robust and biologically meaningful CM characterization, providing an open framework for further multimodal analysis across diverse contexts.

The multidimensional view of multicellular coordination

Alternative methods typically rely on spatial proximity to model intercellular relationships, thereby limiting the scope of multicellular coordination. Such methods may not reveal the cellular dynamics that operate beyond immediate spatial relationships. Instead, our single-cell analysis, grounded in comprehensive cell frequency profiling, can identify co-occurring CMs involved in a wide array of interactions, ranging from spatially proximate to distally connected communications. This inclusive approach may be particularly effective in uncovering complex intercellular networks, such as systemic immunity, cross-tissue modulation, and other forms of long-range coordination.

Broad scope of application

Our approach leverages single-cell data, offering a cost-effective and accessible foundation supported by an expanding array of datasets across diverse biological systems. Advanced single-cell data integration technologies enable scalable analysis of multicellular coordination in both health and disease. This broad applicability facilitates comparative studies across tissues, conditions, and disease states.

We thank the reviewer for raising this excellent point. Comparing our approach to alternative methods further highlights the broad applicability and unique strengths of our CM framework. These discussions have been incorporated into the revised manuscript (**Lines 456-472**).

References:

Hickey, J. W. *et al*. Organization of the human intestine at single-cell resolution. *Nature* **619**, 572–584 (2023).

Luca, B. A. *et al*. Atlas of clinically distinct cell states and ecosystems across human solid tumors. *Cell* **184**, 5482–5496 e5428 (2021).

Altogether, this assessment is reinforced by the scarcity of original findings presented in the manuscript, with one notable exception being the depletion of universal fibroblasts in the reproductive system, which is a noteworthy finding but never expanded on. Although the study identifies age-associated thymic involution as one of the 12 cellular community clusters, it is presently unclear how this adds to our current understanding of the thymus. Similarly, Peyer's patches are identified and validated with spatial transcriptomics, but no novel findings are presented about them. Another main result is also already well established, namely that inflammation is a predictor of the response to anti PD-1 therapy (Damotte et al. 2019). In comparison, more focussed studies of the tumour immune microenvironment have more clearly delineated the effect on immunotherapy along with subtypes of stromal-immune microenvironments across cancer types (Bagaev et al. 2021), which is not mentioned here.

While the scope of the study is exciting, I think the paper currently lacks fundamental and lucid insights into cellular organisation that would be of sufficiently broad interest to the readers of Nature. The paper is logically structured (except perhaps in the lack of comparison between healthy and cancer cell communities), well written, and the atlas curation and methods exploration represents important work. In addition, there is substantial theoretical originality in the overall approach and, in particular, the use of DIALOGUE to model coupled cellular programs and Immune Dictionary perturbation data for cytokine-mediated interactions. These results certainly seem promising in providing fundamental insight into the role of cytokines in organising tissue microenvironments during tumour progression. But, as these themes are currently disjointed from first half of manuscript on healthy tissues, this reviewer wonders if there could be some insights supported by such data relating immunity during tumour progression to specialised fibroblasts, thymic involution, or Peyer's patches, improving the coherence of the work as a whole. Lastly, I have provided some more specific suggestions and comments categorised by minor/major importance.

We thank the reviewer for these constructive comments. We understand the need to emphasize the new biological insights derived from our framework rather than merely validating existing knowledge. Our initial focus was to ensure that our framework was seen as valid and reliable by emphasizing its consistency with known biological data. In retrospect, this approach may have overshadowed the novel biological discoveries our framework can reveal. To address this, we have performed extensive multicellular analyses within specific contexts.

First, we examined the associations between CM activity and various phenotypes, including sex, age, and other tissue-specific factors. A noteworthy age-related association was observed in the spleen, where CM05 increased with age and CM06 decreased. Specifically, the expansion of four CM05 subsets (B03, B05, CD4T03, and I06) was more pronounced with aging compared to the previously reported age-associated B cells (ABCs). These results in the spleen, the largest peripheral immune organ in adults, highlight coordinated behavior at the molecular, cellular, and multicellular levels. Additionally, we found other CM dynamics linked to various phenotypes, such as alcohol consumption in the lymph node, childhood tuberculosis in the lung, and periodic menstrual stages in the uterus. These findings are detailed in the revised manuscript (**Lines 207-251, Fig. 3h-j, and Fig. S12-14**).

Second, our analyses of spatial organization, intercellular communication, and cytokine response show that CMs, as fundamental tissue configurations, recapitulate the complexity of multicellular ecosystems. These refined analyses provide insights into multicellular organization across diverse contexts. For instance, by modeling the frequencies of CM12-related cellular subsets, we reconstructed a pseudo-time trajectory that aligned with the progression of menopause, which was linked to dynamics of certain specialized fibroblasts. Detailed results are included in the revised manuscript (**Lines 297-334, Fig. 4f-l, and Fig. S17**).

Third, we focused on the rewiring of multicellular ecosystems during tumor progression. First, we examined healthy CMs in tumor and adjacent non-tumor tissues, revealing significant disruption of these CMs in cancer. Second, we identified a convergent multicellular ecosystem emerging across cancer types. These findings illustrate the simultaneous rewiring of two types of multicellular ecosystems during tumor progression: the loss of tissue-specific organizations and the emergence of a convergent cancerous ecosystem. Detailed results are provided in the revised manuscript (**Lines 387-412, Fig. 5, and Fig. S22-23**). These insights not only deepen our understanding of multicellular

rewiring in cancer but also strengthen the logical structure and coherence of the manuscript.

Together, these new findings provide valuable insights into multicellular ecosystems in both health and disease and further underscore the significance of our conceptual framework. We thank the reviewer for the positive and encouraging comments regarding the logical structure, quality of writing, and the significance of our data and methods. It's particularly gratifying to hear that the reviewer recognizes substantial theoretical originality in our approach. The specific comments and suggestions the reviewer raised have been addressed in detail in our subsequent responses.

Major comments

Major Comment 1

The main part of the CoVarNet method is a one line function call to non-negative matrix factorization (NMF) in addition to computing correlations between cell type abundances. While this is technically a novel approach applied to cell type abundances from scRNA-seq data it does not constitute a substantial advance to computational biology methodology – and one could argue that the authors are overselling this approach by packaging it as its own named method. One (optional) suggestion would be to apply CoVarNet directly to spatial transcriptomics data, which could be timely and expand the package functionality with additional computational tools.

We appreciate the reviewer's feedback on the CoVarNet method. While the technical components, such as NMF, represent a straightforward approach, the innovation of CoVarNet lies in its conceptual framework. It provides a systematic method to uncover cross-tissue multicellular coordination using large-scale single-cell data, addressing fundamental biological questions. In our view, the evolution of computational methods often starts with a strong conceptual foundation, followed by refinements as the field advances. In response to this feedback, we have optimized CoVarNet by implementing more stringent statistical and topological methods (**Lines 578-607 and Fig. 2c**).

We also thank the reviewer for suggesting the application of CoVarNet to spatial transcriptomics data, an enhancement that significantly expands its capabilities. In the revised manuscript, we have incorporated several spatial analyses using Visium datasets from diverse tissues, revealing the spatial characteristics of CMs, such as the colocalization of cell subsets within CMs (**Response Fig. 4-6**). These spatial analyses have been integrated into CoVarNet, along with new features enabling the recovery of CMs identified from single-cell data in external spatial transcriptomics data. To support these updates, we have provided more detailed tutorials to enhance the user experience: https://github.com/QiangShiPKU/CoVarNet

Major Comment 2

In the analysis corresponding to Fig 2b, what is the justification for excluding epithelial cells when inferring cell modules? I would expect epithelial cells to be key contributors to many of the tissues analysed in the study. Some explanation is provided in methods lines 428-431, which might be worthwhile expanding on and commenting on in the main text. Perhaps it would be clearer to rephrase this part around stromal-immune cellular organisation and the tumour microenvironment rather than

as a broad survey of all cells.

We thank the reviewer for raising the question and providing a suggestion regarding the exclusion of epithelial cells in our analysis. Our decision to exclude epithelial cells was grounded in our primary goal to identify cross-tissue CMs with coordinated patterns. Epithelial cells, due to their high tissue specificity, could potentially obscure the identification of these cross-tissue patterns. By concentrating on stromal and immune cells, which are more consistent across tissues, we were able to discern broadly applicable patterns of multicellular organization. Following the reviewer's suggestion, we have added this rationale to the main text of the revised manuscript, clarifying our emphasis on stromal-immune cellular organization (**Lines 129-131**) and the tumor microenvironment (**Lines 379-381**).

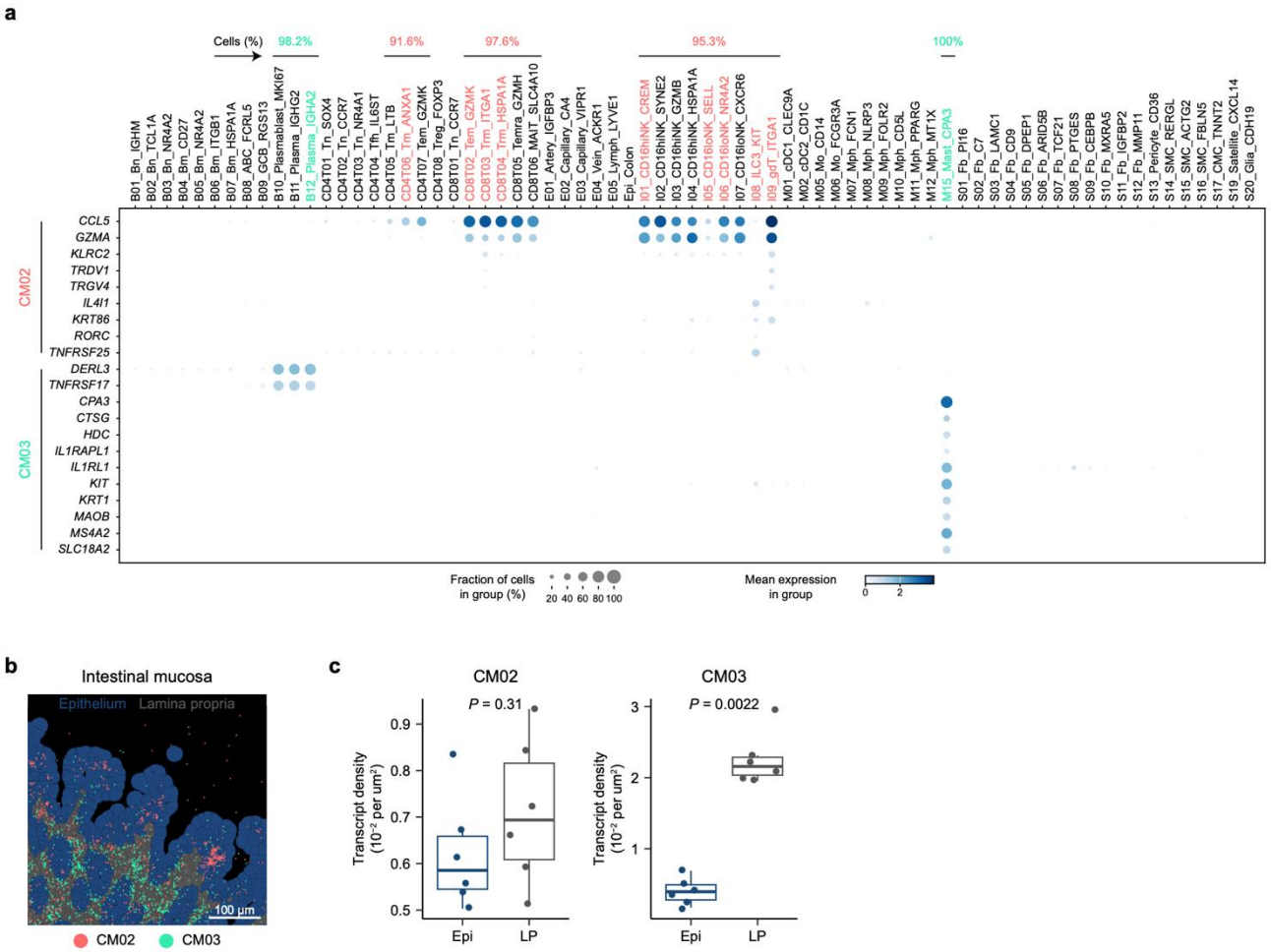
Major Comment 3

Fig 3f, lines 188-191, In the CODEX data, CD8 expression alone is insufficient to classify gamma-delta T cells.

We thank the reviewer for this insightful comment. We agree that CD8 expression alone is insufficient for the accurate identification of γδ T cells. In the context of intestine, our goal is to demonstrate that CM02 and CM03 occupy distinct spatial locations based on high-resolution CODEX data, despite both appearing enriched in the intestinal mucosa in spot-resolution Visium data. However, the limited marker scope of CODEX restricts the ability to more precisely identify cell subsets within these CMs.

To address this limitation, we turned to an alternative spatial data, Xenium, capable of *in situ* characterization of hundreds to thousands of genes in cells and tissues with ultra-precise single-cell spatial imaging. Using Xenium intestinal data from 10x Genomics (**Table S3**), we designed a gene panel to distinguish multiple cell subsets within CM02 and CM03 (**Response Fig. 11a**). We then considered the gene transcript density to represent the intensity of the respective CMs. Our analysis revealed a notable enrichment of CM03 in the lamina propria compared to the epithelium, whereas CM02 displayed a consistent distribution across the tissue (**Response Fig. 11b-c**).

These results have been incorporated into the revised manuscript (**Lines 197-205, Fig. 3f-g, and Fig. S11**).



Response Fig. 11. Distinguishing spatial locations of CM02 and CM03. (also Fig. 3f-g and Fig. S11)

(a) Dot plot illustrating the gene panel expression that differentiates CM02 and CM03 among various cell subsets in the colon. The percentages of colored subsets among similar subsets are indicated at the top. For example, 98.2% of the plasma cells (B10, B11, and B12) are B12.

(b) Representative Xenium imaging of an intestinal mucosal section.

(c) Quantification from six sections demonstrating the distinct anatomical distribution of CM02 and CM03.

Major Comment 4

Fig 4h is missing controls for general inflammation and cytotoxic T cell profiles to ascertain whether the association with treatment response is specific to the CM08-related inflammation signature. Otherwise these results may simply reflect the well-known observation that immune infiltration is a predictor of anti-PD1 response.

We sincerely thank the reviewer for this insightful suggestion. We appreciate the opportunity to provide additional clarity and detail on our findings. Specifically, we have re-designated the CM08-associated multicellular program to the "CM08 program", avoiding the previous "inflammatory program" terminology, to accurately reflect its detailed characteristics as outlined below.

Characteristics of the CM08 program

The CM08 program, identified through DIALOGUE analysis in healthy tissues (Jerby-Arnon et al. 2022), reflects the intrinsic characteristics of barrier tissues that are constantly interfacing with the external environment, such as skin, oral mucosa, and vagina (**Fig. 3a and Fig. S18a-c**). Analysis of bulk RNA-seq samples from the GTEx database confirmed the robust expression of the CM08 program in barrier tissues like skin, vagina, salivary gland, and esophagus (**Response Fig. 12a**). Additionally, a comparative analysis of the CM08 program against established tumor inflammation (Ayers et al. 2017) and cytotoxic signatures (Bagaev et al. 2021) revealed limited overlap (**Response Fig. 12b**), This underscores that the CM08 program is specific to the intrinsic features of barrier tissues under healthy condition, rather than being directly tied to the tumor microenvironment.

Therapeutic response indicator in melanoma

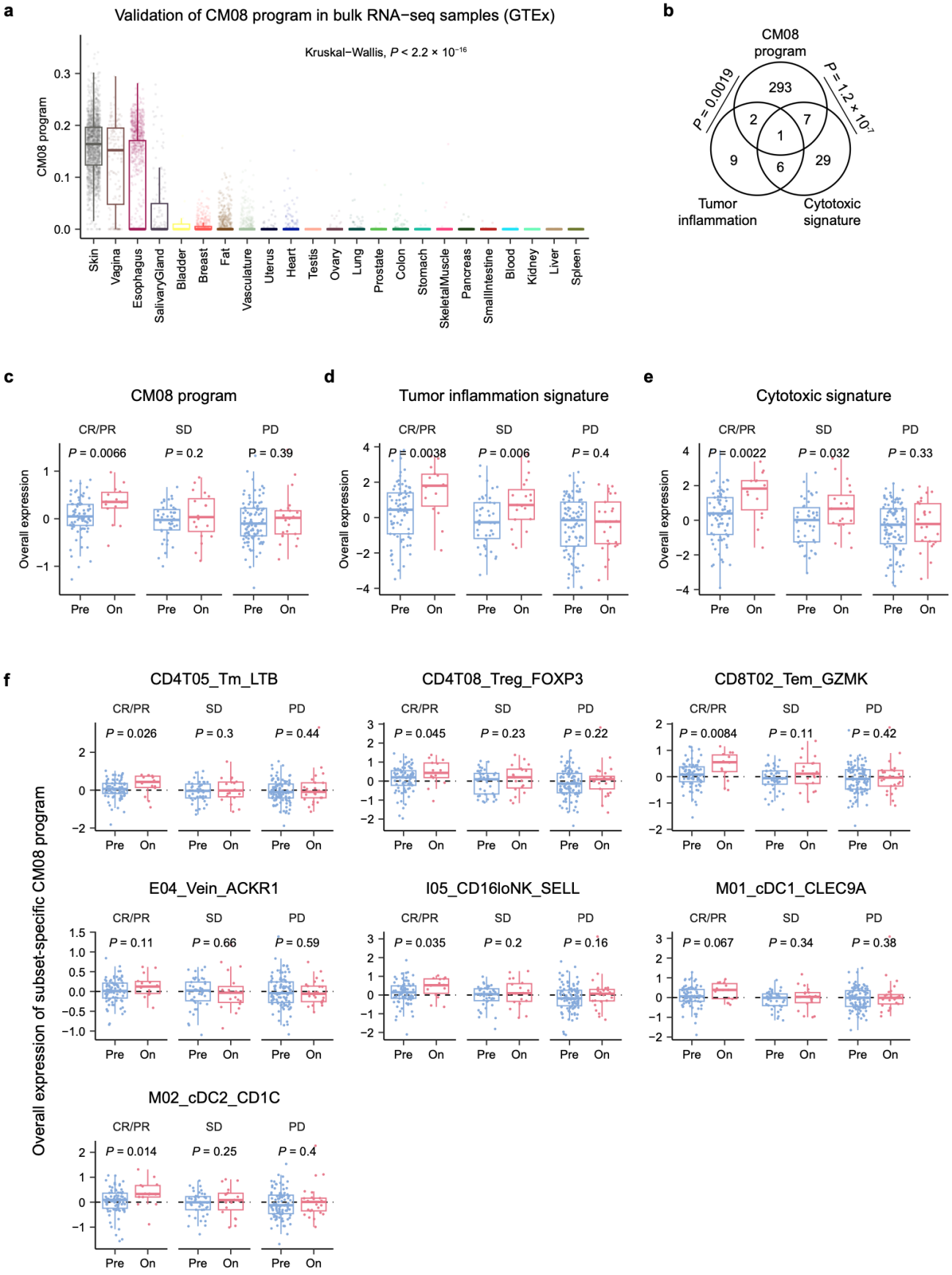
We analyzed the expression of the CM08 program alongside tumor inflammation and cytotoxic signatures in melanoma patients undergoing anti-PD-1 therapy (**Response Fig. 12c-e**). In patients with complete (CR) or partial (PR) responses, a notable increase in the expression of all three signatures was observed post-treatment compared to pre-treatment levels. In contrast, patients with stable disease (SD) showed significant upregulation of the tumor inflammation and cytotoxic signatures post-treatment, while the CM08 program exhibited no such increase. These findings suggest that the CM08 program may serve as a more precise indicator of treatment efficacy in response to anti-PD-1 therapy.

Importantly, this coordinated response was also observed within individual cell subsets of the CM08 (**Response Fig. 12f**). The gene expression patterns of each subset align with the broader CM08 program, reinforcing the concept of functional coordination among these cell subsets within the CM08.

Additional implications

In our multicellular analysis in cancer, we found that CM08 maintained consistent activity across healthy, adjacent non-tumor, and tumor tissues, suggesting that the multicellular ecosystem in healthy tissues was relatively well-preserved in cutaneous squamous cell carcinoma (cSCC) (**Fig. S22a**). This finding aligns with the superior response to immunotherapy observed in skin cancers, such as melanoma and cSCC, compared to other cancer types. These findings suggest that understanding the multicellular properties of healthy tissue could offer valuable insights into disease progression and treatment efficacy. Further investigation is needed to explore the mechanisms connecting these properties in both homeostatic and pathological states.

We have incorporated these additional results and discussions into the revised manuscript (**Lines 361-374, Lines 393-398, and Figs. S18-S19**). We thank the reviewer once again for these valuable comments.



Response Fig. 12. CM08-associated multicellular program. (also Fig. S18-S19)

(a) Box plots showing CM08 program expression across tissues. Dots represent individual samples profiled by bulk

RNA-seq in GTEx. Tissues are ordered by the mean of CM08 program expression across samples.
(b) Venn diagram illustrating the overlap of signature genes of the CM08 program, tumor inflammation signature, and cytotoxic signature. The *P* values are calculated with hypergeometric tests.
(c-e) Overall expression of the CM08 program (c), tumor inflammation signature (d), and cytotoxic signature (e) in bulk RNA-seq from 226 patients with advanced melanoma before (pre) and on PD-1 blockade therapies, stratified to responders (CR/PR) and non-responders (SD and PD).
(f) The same plot as in **c** but showing the gene expression of individual cell subsets.

References:

Jerby-Arnon, L. & Regev, A. DIALOGUE maps multicellular programs in tissue from single-cell or spatial transcriptomics data. *Nat. Biotechnol*. **40**, 1467–1477 (2022).

Ayers, M. *et al*. IFN-γ-related mRNA profile predicts clinical response to PD-1 blockade. *J. Clin. Invest.* **127**, 2930-2940 (2017).

Bagaev, A. *et al*. Conserved pan-cancer microenvironment subtypes predict response to immunotherapy. *Cancer Cell* **39**, 845–865 e847 (2021).

Major Comment 5

How has ambient RNA been taken into account during the study design or analysis? To my knowledge the standard in the field of atlas construction is to run ambient RNA removal such as CellBender. The lack of such analysis here introduces the possibility of technical confounding during annotation and hence in the identified cellular communities.

We thank the reviewer for raising this important question. We understand that ambient RNA removal is becoming increasingly standard in scRNA-seq data analysis. After a comprehensive review of studies regarding single-cell atlas construction and ambient RNA removal, we opted not to apply ambient RNA removal techniques for the following considerations:

First, our study included only scRNA-seq datasets from fresh samples generated using 10x single-cell assays. These protocols typically result in fewer ruptured or degraded cells and less residual cytoplasmic debris compared to those using frozen samples or single-nucleus RNA-seq (snRNA-seq) assays. The CellBender paper (Fleming et al. 2023) notes that snRNA-seq is particularly susceptible to cytoplasmic RNA contamination owing to the nuclear extraction process, thereby intensifying ambient RNA concerns. In contrast, scRNA-seq is less affected by this problem.

Second, applying CellBender is not feasible for our curated public data. CellBender requires the raw, unfiltered gene expression matrix, which includes empty droplets, to model and remove background noise. However, our dataset predominantly comprises filtered, high-quality gene expression matrices that have minimal empty droplets. Specifically, the integrated Human Lung Cell Atlas (HLCA), which combined 14 human lung datasets from 107 individuals into a unified atlas (Sikkema et al. 2023), aimed to annotate previously undescribed cell types and serve as a reference for further lung research. Despite this broad aim, only 3 out of the 14 datasets in HLCA were preprocessed for ambient RNA correction, indicating the practical challenges in integrating public datasets with such preprocessing.

Third, ambient RNA primarily affects the identification of differentially expressed genes. Our study focuses on clustering common cell types and subtypes rather than discovering new cell types or markers. We received assistance with cell annotation from CellTypist, a robust tool known for accurate cell type classification (Dominguez Conde et al. 2022). Furthermore, we cross-checked our results against annotations provided in original publications and databases. These efforts were crucial for ensuring the accuracy of our cell annotations and mitigating any potential influence of ambient RNA.

In summary, while recognizing the importance of ambient RNA removal in enhancing data quality, we believe that this factor has a limited impact on our study based on the points above. In future studies, we will integrate such tools if applicable. We thank the reviewer for highlighting this important aspect.

References:

Fleming, S. J. *et al*. Unsupervised removal of systematic background noise from droplet-based single-cell experiments using CellBender. *Nat. Methods* **20**, 1323–1335 (2023).

Sikkema, L. *et al.* An integrated cell atlas of the lung in health and disease. *Nat. Med.* **29**, 1563–1577 (2023).

Dominguez Conde, C. *et al*. Cross-tissue immune cell analysis reveals tissue-specific features in humans. *Science* **376**, eabl5197 (2022).

Major Comment 6

Lines 205-210: This interpretation of the number of ligands identified with CellphoneDB is highly speculative. It seems more plausible to this reviewer that the number of ligand-receptor pairs identified would indicate potential diversity of intercellular signalling in a variety of tissue contexts – some of which could be occurring in the identified CMs depending on whether the ligands and receptors are translated and posttranslationally regulated by mechanisms such as trafficking. The use of ligand 'numbers' here is misrepresented to indicate ligand abundance – there isn't necessarily "more messaging molecules" according to the data presented and hence no support for the diffusion over distance argument. I would recommend rephrasing these arguments.

We greatly appreciate this insightful feedback on our interpretation of the CellphoneDB findings. We concur that the number of ligand-receptor pairs detected likely reflects the diversity of intercellular signaling across different tissue contexts, rather than indicating the abundance of messaging molecules. In light of this feedback, we have revised our interpretation to more accurately reflect these findings (**Lines 257-270**) as follows:

"To explore this, we first investigated the spatial organization of CM components using spatially resolved transcriptomics (Visium) across various tissues (**Table S3**). Our analysis revealed a strong association between CM spatial organization and composition ($P < 2.2 \times 10^{-16}$, Kruskal-Wallis test). The highest spatial concordance was observed in CMs with a greater proportion of lymphocytes, followed by myeloid cells, stromal cells, and endothelial cells (**Fig. 4a and Fig. S15a-b**). To further understand the implications of these spatial patterns, we analyzed ligand-receptor-mediated cell-cell communication using single-cell data with CellPhoneDB. Intriguingly, endothelial cells and stromal

cells produced a broader variety of ligands compared to lymphocytes (B cells, T cells, and innate lymphoid cells) (**Fig. 4b**). This aligns with the notion that spatial proximity may enhance the specificity of intercellular interactions, suggesting that lymphocyte-enriched CMs foster particular interactions within local niches. Conversely, CMs with a higher proportion of stromal and endothelial cells—often situated farther apart—generated a more diverse array of signaling molecules, facilitating broader signaling interactions over greater distances (**Fig. S15c-d**). These insights highlight the relationship among spatial organization, cellular composition, and intercellular communication."

Minor comments

Minor Comment 1

Line 131, the description of "mutually exclusive CM types" conflicts with the methodology of decomposition by NMF which theoretically allows for co-occurring CM types.

We thank the reviewer for this detailed concern. We have revised the text to avoid this conflict and ensure alignment with the NMF methodology (**Lines 141-142**).

If by the authors on line 137-138, "all CMTs could be well recovered [in GTEx bulk data]" then wouldn't that indicate that the 12 cell modules could have been discovered using the GTEx data?

We thank the reviewer for this valuable inquiry. In response, we would like to provide further clarifications regarding the description of CMTs and their application in our study.

(1) *Definition of CM types (CMTs).* Using the coefficient matrix from the NMF, each sample was assigned a CMT label based on its most abundant CM. For instance, if a sample exhibited the highest activity of CM01 among all CMs, it was labeled as "CMT01". All healthy single-cell samples used across tissues were stratified into 12 CMT groups (**Fig. S8a**).

(2) *Recovery of CMTs in GTEx bulk data.* The statement that "all CMTs could be well recovered [in GTEx bulk data]" means that GTEx samples are assigned CMT labels based on the similarity of their expression profiles to those observed in single-cell data. This assignment, however, does not imply the direct identification of individual CMs within the GTEx samples. Instead, GTEx samples are labeled with a CMT identifier to facilitate comparison and integration with single-cell data.

These clarifications have been incorporated into the revised manuscript (**Lines 608-612 and Lines 623-629**).

Minor Comment 2

Line 377-378: how is "without excessive enrichment for specific cell types" defined?

We thank the reviewer for this important question. We categorized the collected samples into three distinct groups based on cell-type enrichment: 1) no cell-type enrichment; 2) a mixture of immune, epithelial, endothelial, and stromal compartments; 3) enrichment for either immune or non-immune cell populations. In the context of identifying CMs, we focused on the first two categories to ensure a

balanced representation and avoid bias toward any specific cell type (**Lines 493-495 and Lines 563-565**).

Minor Comment 3

Line 425-426: On "we also got assistance from CellTypist", which CellTypist models were used?

We used two CellTypist models for cell annotation: "Immune_All_High" and "Immune_All_Low". These models were selected for their capacity to accurately classify a broad range of immune cells, ensuring robust annotation across diverse tissue contexts. We have clarified this in the revised manuscript (**Lines 541-542**).

Minor Comment 4

Fig. 1f seems out of place in the main figure. In addition, Peyer's patches are well characterised wherefore these results are not novel and used merely in the text for illustration purposes. Could consider swapping Fig S5e as validation data in support of your observation of the universal and specialised fibroblasts distinction in reproductive tissues. Or, could perhaps consider moving the CODEX figure to Figure 2.

We appreciate the reviewer's perceptive suggestion regarding the figure arrangement in our manuscript. We have replaced the CODEX data (**original Fig. 1f**) with spatial data (**original Fig. S5e**) to better illustrate the distinction between universal and specialized fibroblasts in reproductive tissues. Additionally, the CODEX data (**original Fig. 1f**) has been moved to **Fig. 2a** to improve the flow and coherence of the figures.

Minor Comment 5

In addition, the associated text lines 110-111 "These observations prompted us to ask" is a type of statement on personal motivation which is typically excluded from research papers. I would recommend shortening the introductory paragraph in lines 104-113.

We appreciate the reviewer's guidance on enhancing the clarity and focus of our introduction. In line with this recommendation, we have condensed and refined the introductory section (**Lines 110-117**) to align with the conventions of scientific communication.

Minor Comment 6

Lines 562-563 in methods and Fig 4b has several unclear descriptions. Firstly, there is a lack of clear definition of the CM abundance threshold used per sample, in addition to what samples have been used – I'm assuming that these are the suspension data, but would be good to clarify. Relatedly, are the 'interaction count' shown in Fig 4b all CellphoneDB results or those specific to the cell types comprising each CM?

We appreciate the reviewer's feedback regarding the clarity of the descriptions in the methods and data used. Below are the clarifications:

(1) The samples used for the analysis in original Fig 4b (**revised Fig. 4c**) were derived from suspension data (scRNA-seq).

(2) Regarding the CM abundance threshold, we did not apply a hard cutoff. Consistent with our approach to defining CMTs as outlined in our response to the Minor Comment 1 of this reviewer, samples designated as CMT01 were categorized under the "High" group, whereas samples not falling under CMT01 were placed in the "Low" group.

(3) The interaction counts depicted in **original Fig. 4b** (**revised Fig. 4c**) are specific to the cell types that constitute each CM.

These clarifications have been incorporated into the revised manuscript for enhanced clarity (**Line 272, Lines 608-612, Lines 726-733, and Fig. 4c and its legend**).

Minor Comment 7

Fig4c and g, the ImmuneDictionary analysis is highly interesting, but the circular network plots shown here are difficult to comprehend; excessive abbreviations, busy network depiction with unclear overlapping edges, confusing use of colours for both cell types and cytokines, and the origin of edges at the bottom of the barplots makes it difficult to see the origin cell type. I would recommend rethinking the visualisation style and representation of these plots according to guidelines formulated by Edward Tufte.

We appreciate the reviewer's detailed feedback on the visualization of our Immune Dictionary analysis and acknowledge the importance of clear and effective figure presentation. In response to these suggestions, we have redesigned the visualizations to display the circular network of individual cytokines, along with annotated cell subsets, to improve clarity (**Fig. S18e**). These revisions aim to make the intricate interactions identified in the Immune Dictionary analysis more accessible and comprehensible for readers, in line with the visualization guidelines proposed by Edward Tufte.

References

Bagaev, Alexander, Nikita Kotlov, Krystle Nomie, Viktor Svekolkin, Azamat Gafurov, Olga Isaeva, Nikita Osokin, et al. 2021. "Conserved Pan-Cancer Microenvironment Subtypes Predict Response to Immunotherapy." Cancer Cell 39 (6): 845–65.e7.

Damotte, Diane, Sarah Warren, Jennifer Arrondeau, Pascaline Boudou-Rouquette, Audrey Mansuet-Lupo, Jérôme Biton, Hanane Ouakrim, et al. 2019. "The Tumor Inflammation Signature (TIS) Is Associated with Anti-PD-1 Treatment Benefit in the CERTIM Pan-Cancer Cohort." Journal of Translational Medicine 17 (1): 357.

Hickey, John W., Winston R. Becker, Stephanie A. Nevins, Aaron Horning, Almudena Espin Perez, Chenchen Zhu, Bokai Zhu, et al. 2023. "Organization of the Human Intestine at Single-Cell Resolution." Nature 619 (7970): 572–84.

Luca, Bogdan A., Chloé B. Steen, Magdalena Matusiak, Armon Azizi, Sushama Varma, Chunfang Zhu, Joanna Przybyl, et al. 2021. "Atlas of Clinically Distinct Cell States and Ecosystems across Human Solid Tumors." Cell 184 (21): 5482–96.e28.

Varrone, Marco, Daniele Tavernari, Albert Santamaria-Martínez, Logan A. Walsh, and Giovanni Ciriello. 2024. "CellCharter Reveals Spatial Cell Niches Associated with Tissue Remodeling and Cell Plasticity." Nature Genetics 56 (1): 74–84.

Report written by Dr Simon Koplev under supervision of Professor Sarah A. Teichmann.

Dear Prof. Teichmann and Dr. Koplev,

We would like to extend our heartfelt thanks for your thorough and insightful review of our manuscript. Your constructive feedback has been invaluable in shaping the revisions and improving the quality of our work. We deeply appreciate the time and effort you dedicated to our study.

Zemin

Referee #2 (Remarks on code availability):

The code is available only for the 'CoVarNet' method rather than comprehensively for analyses presented throughout the manuscript. On review, the available code does look sensible, but is somewhat underwhelming in terms of software scope. Reimplementing this from scratch would take a couple of hours and as such does not represent a substantial contribution to computational biology. The authors have presented more in-depth analysis elsewhere in the manuscript, in particular with regards to the spatial transcriptomics, MCP, and use of Immune Dictionary, which it would be of greater interest and use to the research community.

We appreciate the feedback on the code availability for the "CoVarNet" method and the insightful suggestion to extend its application to spatial transcriptomics data. In response, we have substantially upgraded CoVarNet, incorporating advanced analyses and expanding its scope to include spatial transcriptomics. We have also provided comprehensive tutorials and documentation to facilitate user engagement and ensure the broad utility of our software: https://github.com/QiangShiPKU/CoVarNet.

Furthermore, we have now released the code for in-depth analyses related to spatial transcriptomics, MCP, and the use of the Immune Dictionary: https://zenodo.org/records/14175262. We believe these resources will be of significant interest and use to the research community, enhancing the reproducibility and impact of our work.

Zenodo token for reviewers:

https://zenodo.org/records/14175262?token=eyJhbGciOiJIUzUxMiJ9.eyJpZCI6ImQ1MGE2ZjY4LTZmNzQtNDgyMy05YThhLWIzODMxYzY5MTkyZSIsImRhdGEiOnt9LCJyYW5kb20iOiI2NzU3MTFiZGZkZWQxYWJkNmY0MTkxYjBkMWFlMjA0YyJ9.RZl2fW_aWDWXD8pXCGLzj58mE_ijKJMQkG3ojml17zqGZinhFB_oiPk_b93PrG9v4vfbT8-vuPzYM7RHp9mf6Q

Referee #3 (Remarks to the Author):

In this manuscript, the authors sought to understand tissue-level coordination between cell types using the novel concept of the cellular modules to identify collections of cell types that potentially function together. By studying the frequencies of different cell types across a large library of single cell studies in healthy human tissue that they curated from previous studies, they generated twelve cross-tissue cellular modules. While the idea is intriguing, new biology does not really result or is claimed from this analysis. Moreover the interesting claims that are made lack additional support.

We are grateful for the recognition of the novel concept we proposed to understand tissue-level cellular coordination. We agree the need to emphasize the new biological insights derived from our framework rather than merely validating existing knowledge. Our initial focus was to ensure that our framework was seen as valid and reliable by emphasizing its consistency with known biological data. In retrospect, this approach may have overshadowed the novel biological discoveries our framework can reveal. To address this, we have performed extensive multicellular analyses within specific contexts.

First, we examined the associations between CM activity and various phenotypes, including sex, age, and other tissue-specific factors. A noteworthy age-related association was observed in the spleen, where CM05 increased with age and CM06 decreased. Specifically, the expansion of four CM05 subsets (B03, B05, CD4T03, and I06) was more pronounced with aging compared to the previously reported age-associated B cells (ABCs). These results in the spleen, the largest peripheral immune organ in adults, highlight coordinated behavior at the molecular, cellular, and multicellular levels. Additionally, we found other CM dynamics linked to various phenotypes, such as alcohol consumption in the lymph node, childhood tuberculosis in the lung, and periodic menstrual stages in the uterus. These findings are detailed in the revised manuscript (**Lines 207-251, Fig. 3h-j, and Fig. S12-14**).

Second, our analyses of spatial organization, intercellular communication, and cytokine response show that CMs, as fundamental tissue configurations, recapitulate the complexity of multicellular ecosystems. These refined analyses provide insights into multicellular organization across diverse contexts. For instance, by modeling the frequencies of CM12-related cellular subsets, we reconstructed a pseudo-time trajectory that aligned with the progression of menopause, which was linked to dynamics of certain specialized fibroblasts. Detailed results are included in the revised manuscript (**Lines 297-334, Fig. 4f-l, and Fig. S17**).

Third, we focused on the rewiring of multicellular ecosystems during tumor progression. First, we examined healthy CMs in tumor and adjacent non-tumor tissues, revealing significant disruption of these CMs in cancer. Second, we identified a convergent multicellular ecosystem emerging across cancer types. These findings illustrate the simultaneous rewiring of two types of multicellular ecosystems during tumor progression: the loss of tissue-specific organizations and the emergence of a convergent cancerous ecosystem. Detailed results are provided in the revised manuscript (**Lines 387-412, Fig. 5, and Fig. S22-23**). These insights not only deepen our understanding of multicellular rewiring in cancer but also strengthen the logical structure and coherence of the manuscript.

Together, these new findings provide valuable insights into multicellular ecosystems in both health and disease and further underscore the significance of our conceptual framework. The specific comments and suggestions of the reviewer have been thoroughly addressed in our detailed responses below.

Comment 1

The authors do not provide supporting evidence for the existence of the CMs. This is particularly important as the strength of the cell type subset pairs in the CMs appears to be weak in some CMs (particularly in CM07 and CM11) with no accompanying statistics. Also the correlation coefficient cutoff was relatively low (>0.2) and the FDR was relatively high (<0.1). Also, the analysis of bulk and scRNA-Seq to find CMTs appears to be heavily processed. It is unclear how many samples were removed from the analysis based on stringent cutoffs.

We thank the reviewer for pointing out insightful comments on the identification and validation of the CMs. We appreciate the opportunity to provide further clarification and supporting evidence. In response to these concerns, we conducted several analyses on multiple spatial datasets, GTEx RNA-seq, as well as curated scRNA-seq.

(1) We refined the CoVarNet workflow and cutoff selection strategy, leading to a more robust delineation of co-occurring cell subsets and CMs (**Response Fig. 13**).

(2) We conducted additional spatial analyses to reinforce the validation of CMs (**Response Fig. 5, 6, and 14**).

(3) We examined our previous integrative analysis based on GTEx bulk RNA-seq datasets and provided further clarification (**Response Fig. 1**).

Optimizing CoVarNet workflow for robust CM identification

We refined the computational workflow to robustly identify co-occurring cell subsets. Specifically, we utilized both the correlation (coefficient and FDR) and specificity score to determine specifically correlated subset pairs (**Response Fig. 13a**). Regarding the concern about the cutoff, we examined the distribution of correlation coefficients and found that the cutoff used (0.2) represented the 92nd percentile for the context of healthy datasets, although its absolute value was not excessively high (**Response Fig. 13b**). This cutoff is intended to account for scenarios in which a single cell subset co-occurs with multiple others, potentially participating in various CMs. Additionally, we incorporated the correlation coefficient cutoff as a functional parameter in the CoVarNet package to accommodate diverse datasets. The correlation coefficient cutoff can be specified as either an absolute value or a quantile.

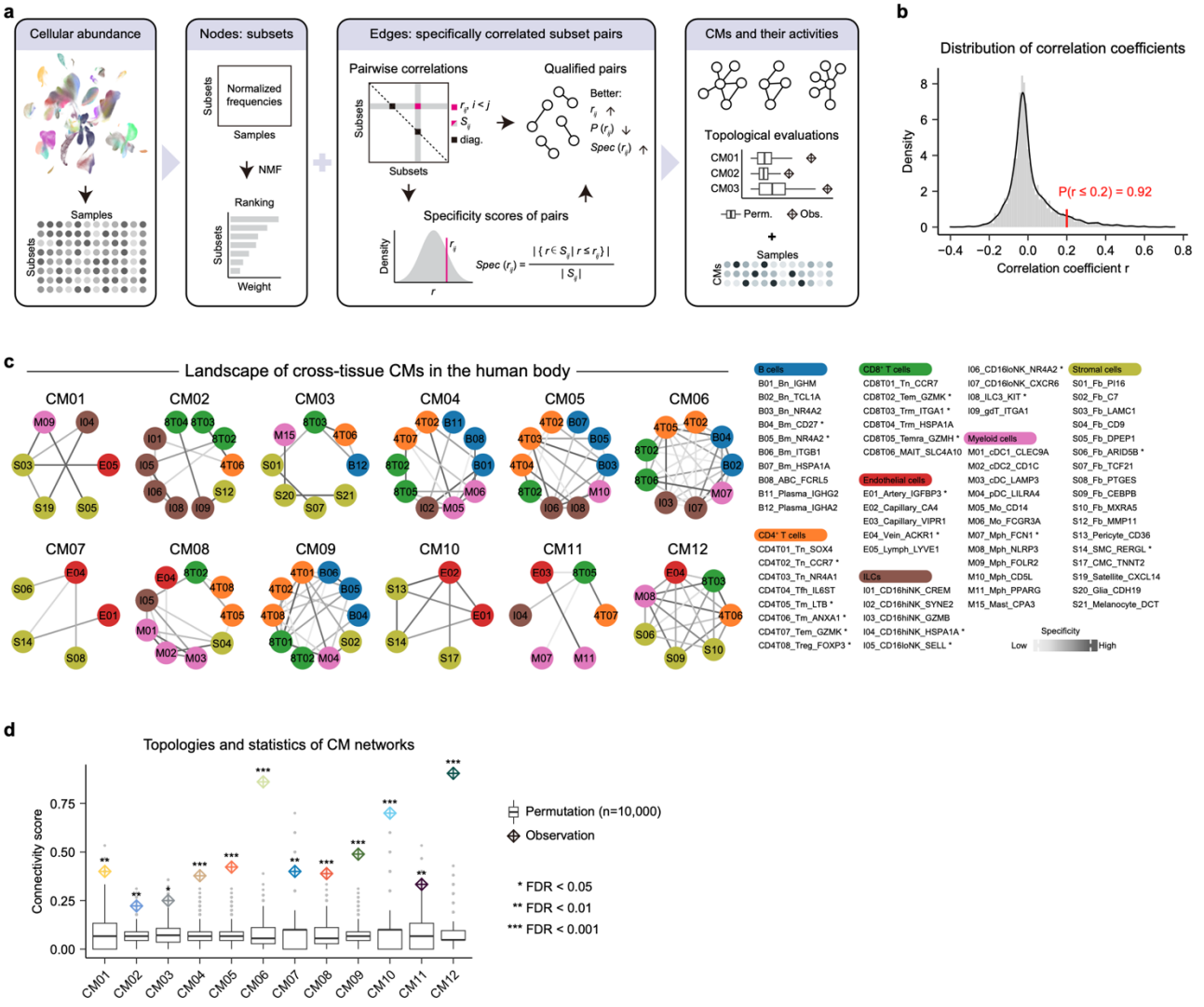
Furthermore, we introduced an automatic method to determine the specificity cutoff, further streamlining and expediting the CM identification process (**Response Fig. 13a**). Specifically, if n and N represent the assumed number of subsets in each CM and the total number of subsets, then the specificity cutoff $spec(n,N)$ will be determined as:

$$spec(n, N) = 1 - \frac{(n-1) * 2 - 1}{(N-1) * 2 - 1}$$

This approach allows for a balanced assessment of the number of subsets within CMs and the extent of their co-occurrence. These enhancements have substantially improved the identification of CMs.

Notably, despite adding one cell subset to CM04 and CM11, and two subsets to CM08, the stability of the 12 previously identified CMs has been largely maintained (**Response Fig. 13c**). This stability confirms the robustness of our approach, demonstrating its capacity to adapt to modifications without affecting the coherence of the CMs. Moreover, we established a connectivity score for each CM, which is defined as the ratio of observed edges to the total possible edges among all nodes within the CM network. To evaluate the statistical significance of this score, we conducted a permutation test on the node labels. Our results demonstrated that all CMs exhibited robust topological structures (**Response Fig. 13d**).

These revisions have been incorporated into the revised manuscript accordingly (**Lines 121-127, Lines 585-607, Fig. 2b-c, and Fig. S7**).



Response Fig. 13. Optimization of CoVarNet workflow and cutoff selection. (also Fig. 2b-c and Fig. S7)

(a) Schematic diagram illustrating the correlation analysis in CoVarNet.

(b) Distribution of correlation coefficients from the pan-tissue single-cell atlas, with the 0.2 cutoff indicating the 92nd percentile.

(c) Network plots illustrating the 12 CMs identified from the pan-tissue analysis. Nodes represent cell subsets with

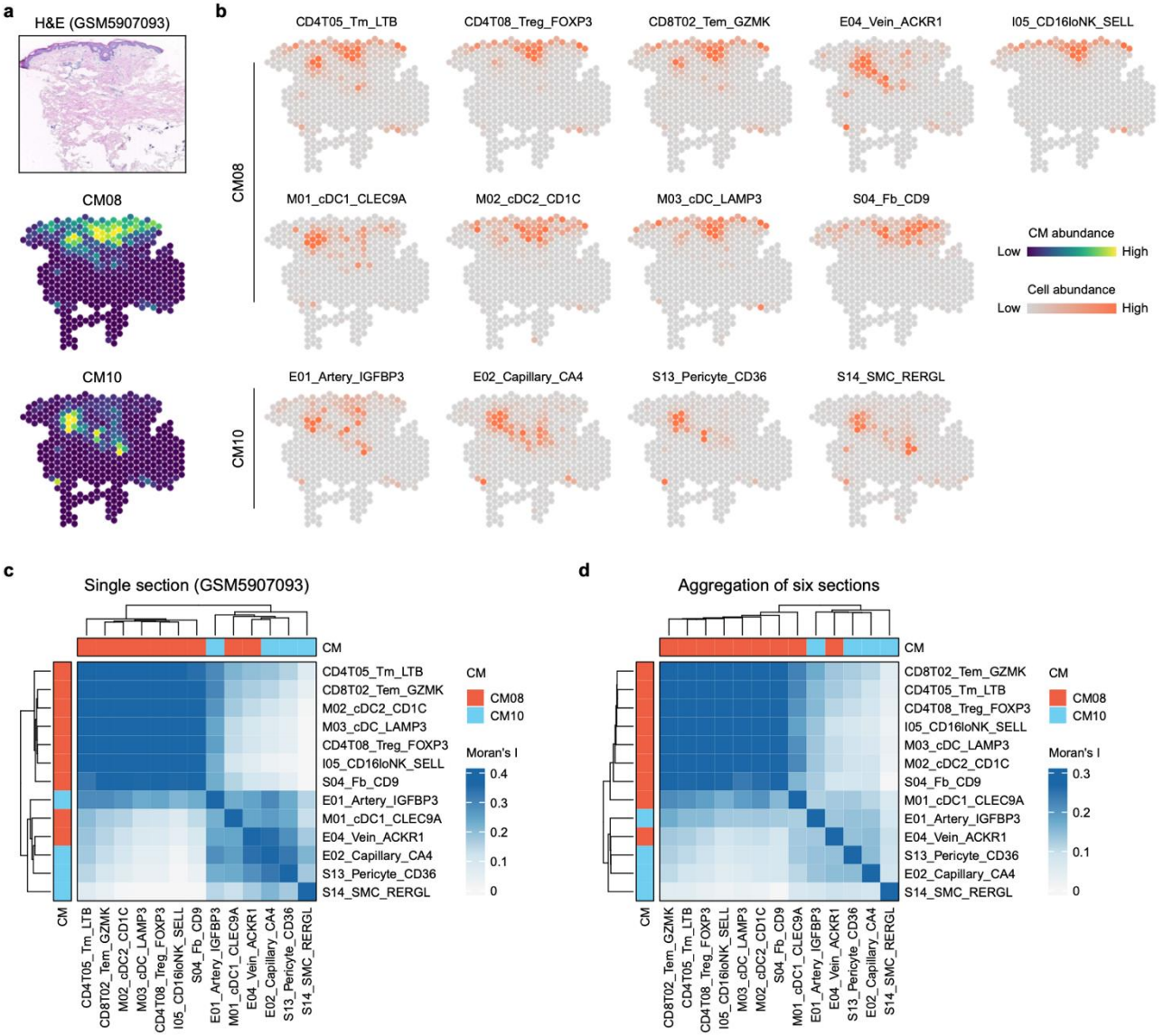
short names. Node colors correspond to broad cell types, while the edge color gradient represents the specificity of correlation between paired subsets. Cell subsets involved in multiple CMs are noted with asterisks.
(d) Connectivity scores and statistical significance of CM networks. The connectivity score is defined as the ratio of observed edges to the total possible edges among all nodes within the CM network. The statistical significance of connectivity score was assessed by permutation tests.

Spatial validation of CMs in diverse tissues

Spatial transcriptomics data, by resolving gene expression spatially, enable the visualization of CMs within their tissue context. In the revied manuscript, we conducted several spatial analyses on multiple Visium datasets from various tissues (**Table S3**). We systematically assessed the spatial relationships among cell subsets both within the same CM and across different CMs in various spatial samples. For example, we examined the spatial associations of skin-enriched CM08 and CM10 and demonstrated prominent spatial colocalization of cell subsets within them (**Response Fig. 5a-c**). While subsets within CM08 exhibit high colocalization, certain cell types, such as venous endothelial cells (E04), also show overlap with CM10, underscoring the interconnectedness of CMs within the tissue microenvironment (**Response Fig. 5a-c**). Notably, these findings were consistently observed across sections from six different donors (**Response Fig. 5d**), reinforcing the reliability of our findings. We have incorporated these results in the revised manuscript (**Lines 174-183 and Fig. S10**).

Additionally, we mapped small intestine-enriched CMs (CM02, CM03, and CM05) onto four adjacent sections from the same donor, revealing remarkably consistent spatial patterns (**Fig. 3c-d and Response Fig. 6a-b**). Notably, CM05 was found to correspond with the Peyer's patch region, known for its abundance of naïve B cells and T cells. In contrast, CM02 and CM03 exhibited greater prominence in the intestinal mucosa, consistent with the cellular composition of tissue-resident T cells and IgA-producing plasma cells. As an orthogonal validation, we leveraged CellCharter (Varrone et al. 2024) to delineate spatial niches using spatial transcriptomics data. The spatial niches identified by CellCharter corroborated the locations of these CMs, with CM05 colocalizing to the C2 niche and CM02 and CM03 aligning with the C1 niche (**Response Fig. 6c**). We have incorporated these results in the revised manuscript (**Lines 184-196, Fig. 3c-e, and Fig. S11**).

Our spatial analyses have delineated variable colocalization patterns among endothelial and fibroblast cell subsets, indicating their unique contributions to tissue coordination (**Response Fig. 4**). Despite the variability in colocalization presenting challenges for multicellular modeling, our methodology successfully identified related CMs, such as CM07 and CM10. We further characterized these CMs using spatial transcriptomics data to elucidate their tissue-specific behaviors. Notably, CM07 displayed a pronounced preference for reproductive tissues, specifically the uterus, ovary, and prostate. Upon mapping CM07 and its components onto two spatial sections of proliferative endometrium, we observed a consistent distribution of these subsets (**Response Fig. 14a-b**). Furthermore, a heightened spatial aggregation of these subsets was observed in the secretory phase endometrium (**Response Fig. 14c-d**). This finding illuminates the dynamic interplay of multicellular coordination across the menstrual cycle, offering a novel perspective on tissue-level regulation. Additionally, mapping CM11 components onto lung spatial sections revealed their aggregation within non-stromal regions (**Response Fig. 14e-f**). We have incorporated these results in the revised manuscript (**Fig. S14**).



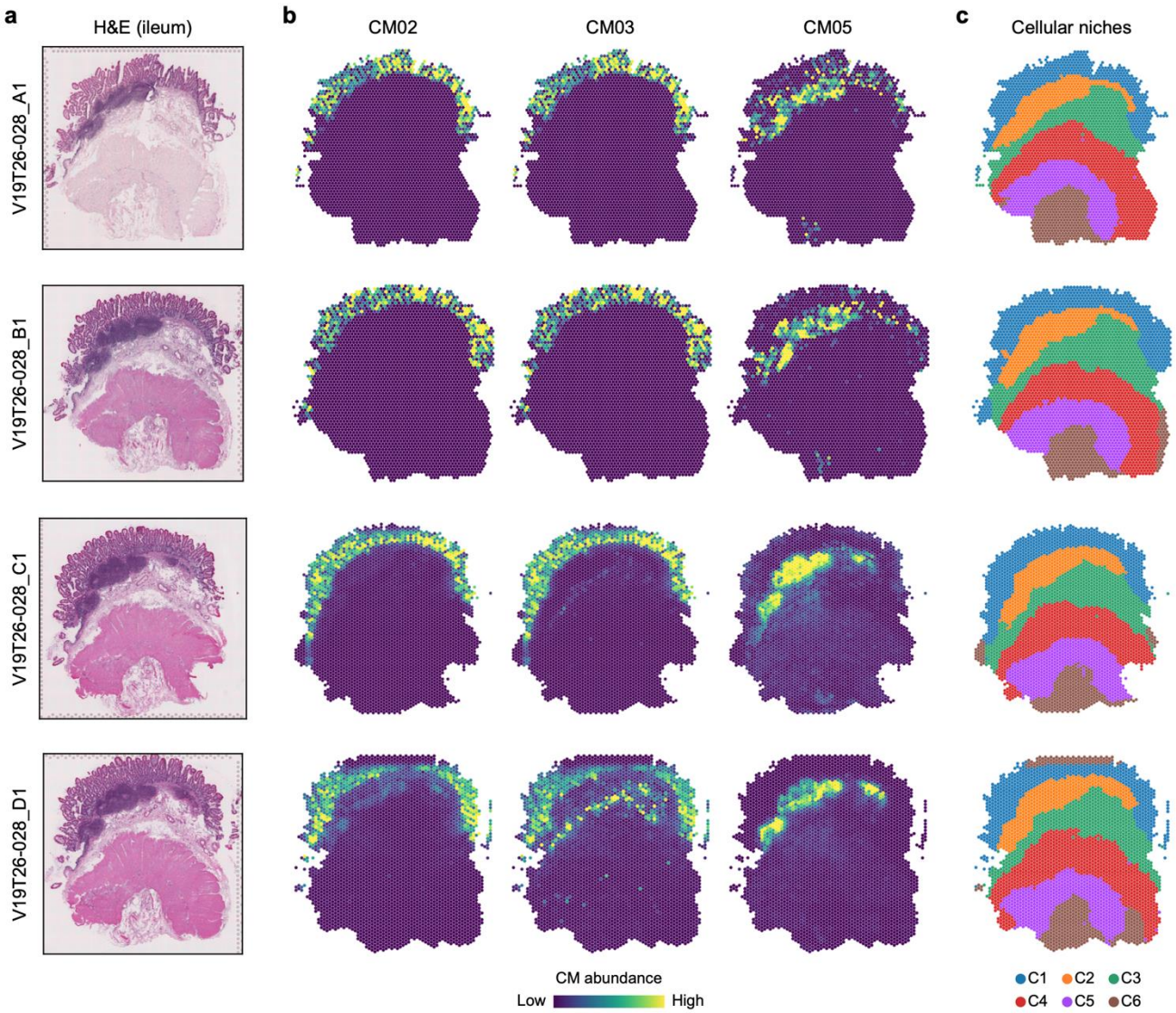
Response Fig. 5. Spatial colocalization of CMs in the skin. (also Fig. S10)

(a) Hematoxylin and eosin (H&E) staining and spatial mapping of CM08 and CM10 performed on a section of normal skin using spatial transcriptomics.

(b) Spatial mapping of the constituent cell subsets within CM08 and CM10, as shown in (a).

(c) Heatmap quantifying spatial aggregation (based on Moran's I) among the constituent cell subsets of CM08 and CM10, as shown in (b).

(d) Corresponding heatmap to (c), generated from the aggregated data of six spatial transcriptomics sections from six different donors.

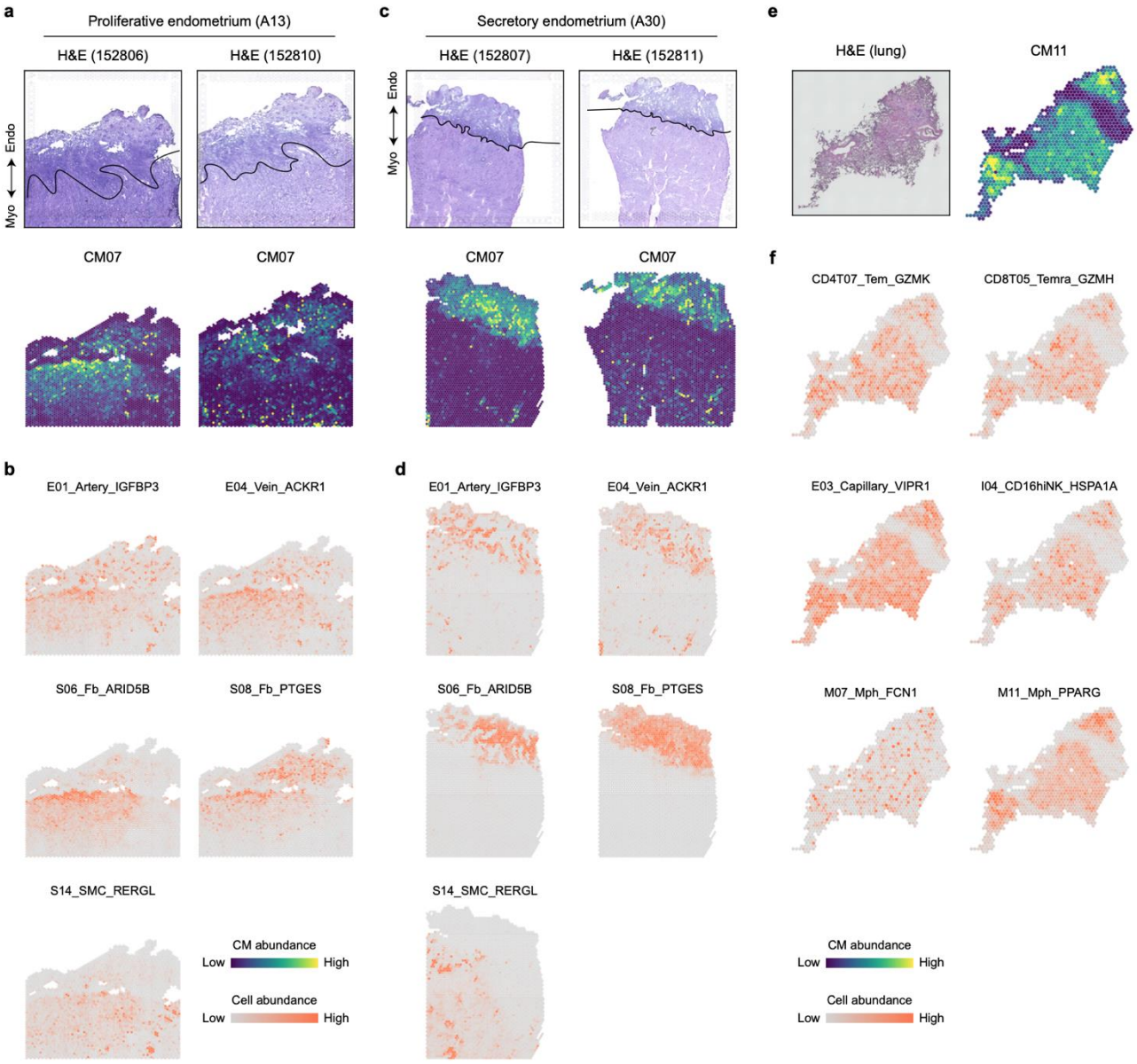


Response Fig. 6. Validation of CMs and cytokine responses by cellular niches in the small intestine. (also Fig. 3c-e and Fig. S11)

(a) Hematoxylin and eosin (H&E) staining of four adjacent sections of the small intestine from one donor.

(b) Spatial mapping of CM02, CM03, and CM05 on the four spatial transcriptomics sections.

(c) Spatial mapping of cellular niches identified by CellCharter (Varrone et al. 2024).



Response Fig. 14. Spatial validation of CMs in context. (also Fig. S14)

(a, c) Hematoxylin and eosin (H&E) staining and spatial mapping of CM07 performed on two sections of proliferative (a) or secretory (c) endometrium using spatial transcriptomics. Endo, endometrium; Myo, myometrium.

(b, d) Spatial mapping of the constituent cell subsets within CM07 on the proliferative (b, 152806) or secretory (d, 152807) endometrium sections.

(e) H&E staining and spatial mapping of CM11 performed on a lung section (GSM5388415) using spatial transcriptomics.

(f) Spatial mapping of the constituent cell subsets within CM11 on the lung section.

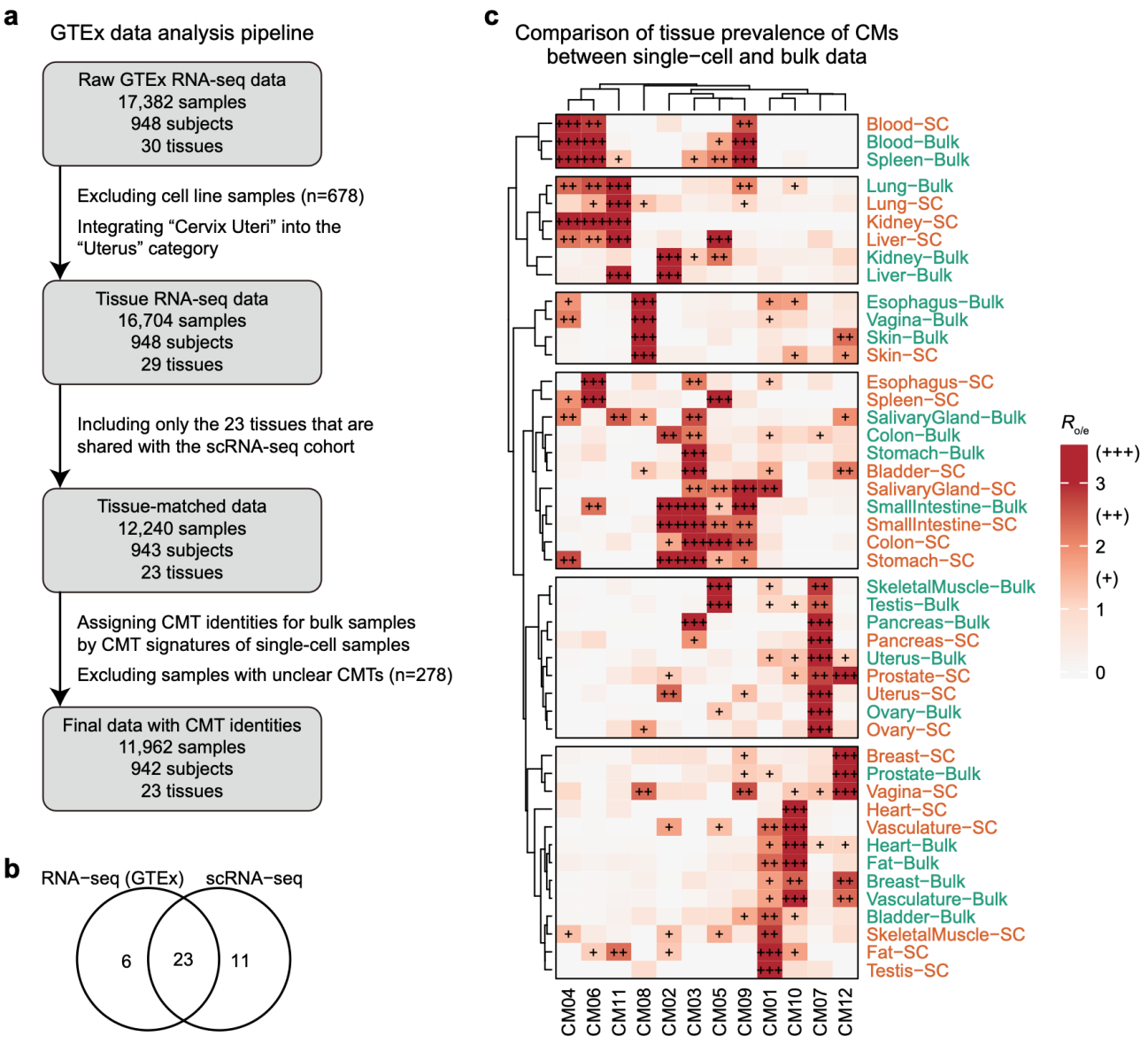
Validating CMs with GTEx bulk RNA-seq data

In the original manuscript, we conducted integrative analysis using bulk and single-cell RNA-Seq data to validate the CMs. To ensure dataset comparability, we restricted our analysis to tissue types present in both bulk and single-cell datasets (**Response Fig. 1a-b**). We did not apply any additional pre-

screening or sample exclusion criteria beyond ensuring tissue type compatibility. During the determination of CMT identities in bulk samples, we excluded 2.3% (278 of 12,240) of samples lacking clear CM enrichment. This validation confirms that CMs represent genuine biological phenomena, highlighting their robustness across diverse data types beyond single-cell analyses.

We have incorporated these details into the revised manuscript (**Lines 614-629 and Fig. S9**).

Collectively, these enhancements substantially bolster the robustness and substantiation of our CMs. We thank the reviewer for these valuable comments, which have greatly guided the enhancement of our manuscript.



Response Fig. 1. Validating CMs with GTEx bulk RNA-seq data. (also Fig. S9)

(a) GTEx cohort analysis pipeline.

(b) Venn diagram of intersecting tissues in GTEx RNA-seq and curated scRNA-seq cohorts.

(c) Heatmap illustrating the tissue prevalence of CMs measured by $R_{o/e}$ (the ratio of observed to expected CM abundance) for tissues profiled using both single-cell (SC) or bulk RNA-seq.

References:

Varrone, M., Tavernari, D., Santamaria-Martínez, A., Walsh, L. A. & Ciriello, G. CellCharter reveals spatial cell niches associated with tissue remodeling and cell plasticity. *Nat. Genet.* **56**, 74-84 (2024).

Comment 2

The authors state that distribution of exhausted T cells in both tumor and adjacent regions suggest a "broad sensing of malignancy" (line 294). This seems to be a strong claim but no supporting evidence or explanation is provided.

We apologize for any confusion caused by our original phrasing. The phrase "broad sensing of malignancy" was intended to suggest that the distribution of cCM02 across both tumor and adjacent non-tumor regions may reflect a response to tumor-associated signals in the surrounding tissue. However, upon reflection, we agree that this statement was speculative and lacked sufficient supporting evidence. Therefore, we have removed this sentence in the revised manuscript to avoid potential misinterpretation. We thank the reviewer for this important point.

Comment 3

Regarding the interesting result that the inflammatory program increases in melanoma patients that respond to PD1 therapy, and that this increase is absent in non-responders: it is unclear what the inflammatory program is–whether it is CM08 or something different. Also can the authors comment on the precise novelty of this result? The inflammatory program is known to be involved in such aspects, and so is the novelty the association with an entire cellular module?

We thank the reviewer for this insightful comment regarding the CM08-associated multicellular program. We appreciate the opportunity to provide additional clarity and detail on our findings. Specifically, we have re-designated the CM08-associated multicellular program to the "CM08 program", avoiding the previous "inflammatory program" terminology, to accurately reflect its detailed characteristics as outlined below.

Characteristics of the CM08 program

The CM08 program, identified through DIALOGUE analysis in healthy tissues (Jerby-Arnon et al. 2022), reflects the intrinsic characteristics of barrier tissues that are constantly interfacing with the external environment, such as skin, oral mucosa, and vagina (**Fig. 3a and Fig. S18a-c**). Analysis of bulk RNA-seq samples from the GTEx database confirmed the robust expression of the CM08 program in barrier tissues like skin, vagina, salivary gland, and esophagus (**Response Fig. 12a**). Additionally, a comparative analysis of the CM08 program against established tumor inflammation (Ayers et al.

2017) and cytotoxic signatures (Bagaev et al. 2021) revealed limited overlap (**Response Fig. 12b**), This underscores that the CM08 program is specific to the intrinsic features of barrier tissues under healthy condition, rather than being directly tied to the tumor microenvironment.

Therapeutic response indicator in melanoma

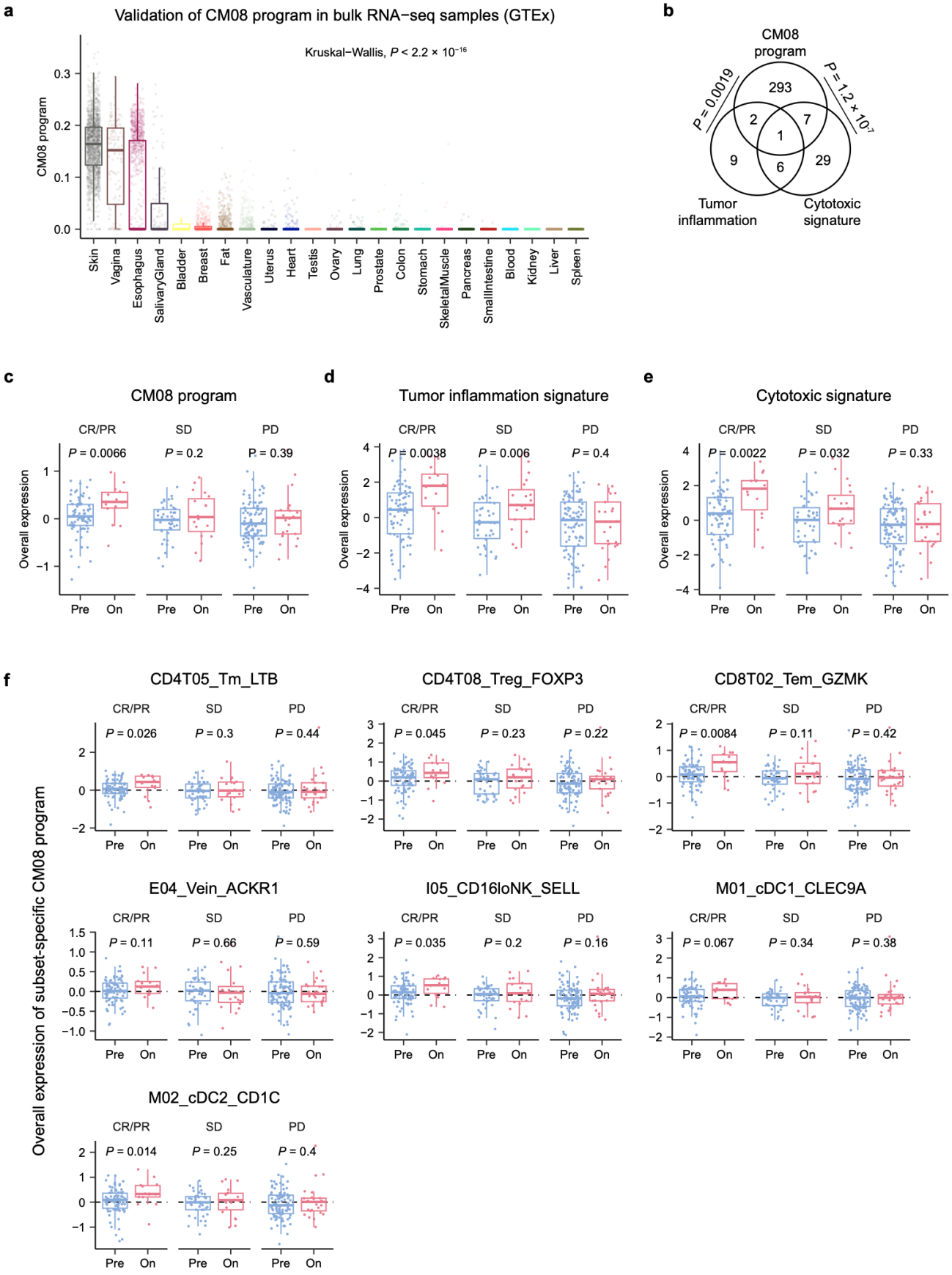
We analyzed the expression of the CM08 program alongside tumor inflammation and cytotoxic signatures in melanoma patients undergoing anti-PD-1 therapy (**Response Fig. 12c-e**). In patients with complete (CR) or partial (PR) responses, a notable increase in the expression of all three signatures was observed post-treatment compared to pre-treatment levels. In contrast, patients with stable disease (SD) showed significant upregulation of the tumor inflammation and cytotoxic signatures post-treatment, while the CM08 program exhibited no such increase. These findings suggest that the CM08 program may serve as a more precise indicator of treatment efficacy in response to anti-PD-1 therapy.

Importantly, this coordinated response was also observed within individual cell subsets of the CM08 (**Response Fig. 12f**). The gene expression patterns of each subset align with the broader CM08 program, reinforcing the concept of functional coordination among these cell subsets within the CM08.

Additional implications

In our multicellular analysis in cancer, we found that CM08 maintained consistent activity across healthy, adjacent non-tumor, and tumor tissues, suggesting that the multicellular ecosystem in healthy tissues was relatively well-preserved in cutaneous squamous cell carcinoma (cSCC) (**Fig. S22a**). This finding aligns with the superior response to immunotherapy observed in skin cancers, such as melanoma and cSCC, compared to other cancer types. These findings suggest that understanding the multicellular properties of healthy tissue could offer valuable insights into disease progression and treatment efficacy. Further investigation is needed to explore the mechanisms connecting these properties in both homeostatic and pathological states.

We have incorporated these additional results and discussions into the revised manuscript (**Lines 361-374, Lines 393-398, and Figs. S18-S19**). We thank the reviewer once again for these insightful comments.



Response Fig. 12. CM08-associated multicellular program. (also Fig. S18-S19)

(a) Box plots showing CM08 program expression across tissues. Dots represent individual samples profiled by bulk

RNA-seq in GTEx. Tissues are ordered by the mean of CM08 program expression across samples.
(b) Venn diagram illustrating the overlap of signature genes of the CM08 program, tumor inflammation signature (Damotte et al. 2019), and cytotoxic signature (Bagaev et al. 2021). The *P* values are calculated with hypergeometric tests.
(**c-e)** Overall expression of the CM08 program (c), tumor inflammation signature (d), and cytotoxic signature (e) in bulk RNA-seq from 226 patients with advanced melanoma before (pre) and on PD-1 blockade therapies, stratified to responders (CR/PR) and non-responders (SD and PD).
(f) The same plot as in **c** but showing the gene expression of individual cell subsets.

References:

Jerby-Arnon, L. & Regev, A. DIALOGUE maps multicellular programs in tissue from single-cell or spatial transcriptomics data. *Nat. Biotechnol*. **40**, 1467–1477 (2022).

Ayers, M. *et al*. IFN-γ-related mRNA profile predicts clinical response to PD-1 blockade. *J. Clin. Invest.* **127**, 2930-2940 (2017).

Bagaev, A. *et al*. Conserved pan-cancer microenvironment subtypes predict response to immunotherapy. *Cancer Cell* **39**, 845–865 e847 (2021).

Comment 4

How do the authors address the possibility that there are no study-specific differences in cell type composition, since sample preparation (ex: dissociation protocol) can drastically affect cell types recovered.

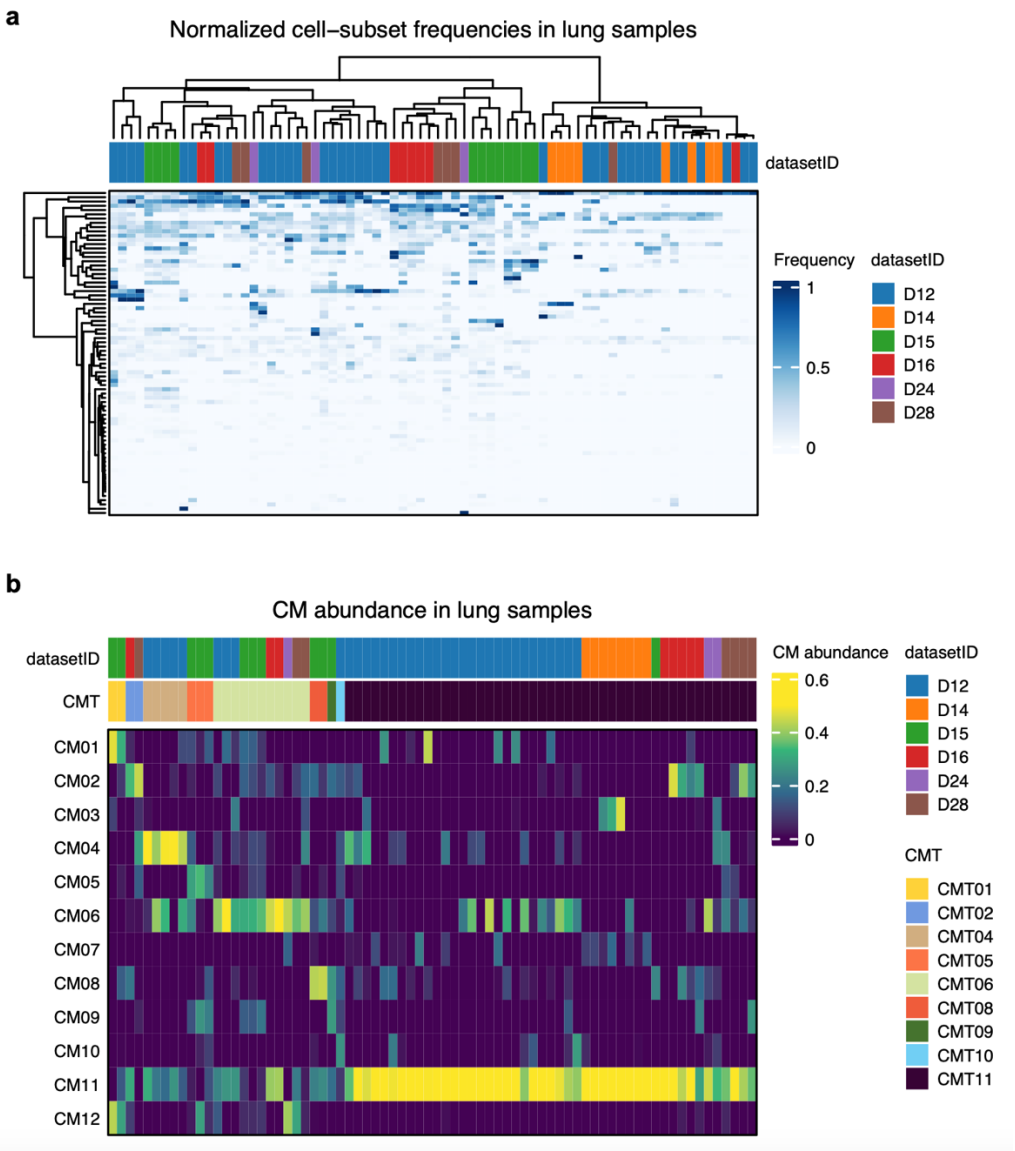
We thank the reviewer for highlighting the potential impact of sample preparation on cell type composition. To address this concern, we implemented several strategies:

(1) *Stringent data inclusion criteria*. We included only single-cell (not single-nucleus) RNA-seq datasets from fresh (not frozen) samples generated using the 10x Genomics platform, which reduces cell degradation and minimizes study-specific biases. Samples with experimental cell-type enrichment were excluded from our frequency analysis to avoid distortion.

(2) *Normalization of cell frequency input*. We calculated cell subset frequencies within broad cell categories and applied min-max scaling to mitigate variations caused by differences in study protocols.

(3) *Assessment of Study-Specific Bias*. We analyzed 74 lung samples from six distinct studies and found no evidence of clustering by study origin, indicating minimal study-specific differences (**Response Fig. 15a**). Furthermore, the consistency of the resulting CM profiles reinforced the robustness of our findings (**Response Fig. 15b**).

These strategies helped minimize the influence of study-specific effects, ensuring the reliability of our results.



Response Fig. 15. Evaluation of study-specific effects.

(a) Heatmap showing normalized cell-subset (rows) frequencies in lung samples (columns). Sample are annotated by their dataset sources (studies).

(b) CM abundance profiles in single-cell lung samples. Sample are annotated by their dataset sources (studies) and CMT identities. All samples are ordered by their CMT identities and dataset sources. CM, cellular module; CMT, CM type.

Comment 5

In the abstract, the authors refer to in vivo perturbation data, however it is unclear to what result in the paper this corresponds.

We thank the reviewer for pointing this out. The *in vivo* perturbation data mentioned in the abstract refers to our cytokine analysis using the Immune Dictionary, which provides a comprehensive overview of cell type-specific responses to a spectrum of cytokines (Cui et al. 2024). This analysis was crucial for understanding cytokine-mediated interactions within the CMs. We have clarified this

connection in the revised manuscript (**Lines 277-279**).

Reference:

Cui, A. *et al.* Dictionary of immune responses to cytokines at single-cell resolution. *Nature* **625**, 377–384 (2024).

Comment 6

The authors make numerous claims where the data does not appear to be supporting their claim, despite the statistically significant p values, or make claims that are not supported by any quantification:

We appreciate the reviewer's insightful comment regarding the need for stronger support for our claims. In response, we have revised the manuscript to include additional statistical analyses and more detailed quantifications to better substantiate our findings.

(1) the authors make claims regarding cell type proportions being different across different tissues but do not include statistics to support this claim (Figure 1c).

This is a valid concern. To support our claims regarding the differences in cell type proportions across different tissues, we performed a chi-square test of independence, revealing a significant association between broad cell types and tissues (Chi-squared test, $P < 2.2 \times 10^{-16}$). This addition enhances our observations as presented in **Fig. 1c**. We have revised the manuscript accordingly (**Lines 83-84**).

(2) In Figure 3a the authors claim that the CMs are mainly determined by tissue type (system preferences) but it does not appear that way by the annotation. The authors should consider annotating by tissue "type" in addition to the system.

We thank the reviewer for raising this important point, and we apologize for any confusion caused. To ensure clarity and consistency with related cross-tissue studies (Dominguez Conde et al. 2022), we have revised the manuscript to replace "tissue types" with "tissues", referring specifically to the 35 anatomical tissues analyzed, such as lung, liver, and kidney.

In **Fig. 3a**, each column exactly represents a specific tissue, as clearly indicated in the labeling. We believe this representation effectively illustrates the relationship between CMs and the specific tissues and systems.

Reference:

Dominguez Conde, C. *et al*. Cross-tissue immune cell analysis reveals tissue-specific features in humans. *Science* **376**, eabl5197 (2022).

(3) Figure 3c - The authors make claims involving the expression of CM09 and cell states across age in the thymus. Although the authors state that these correlations are statistically significant, all of these plots appear to be heavily driven by few data points.

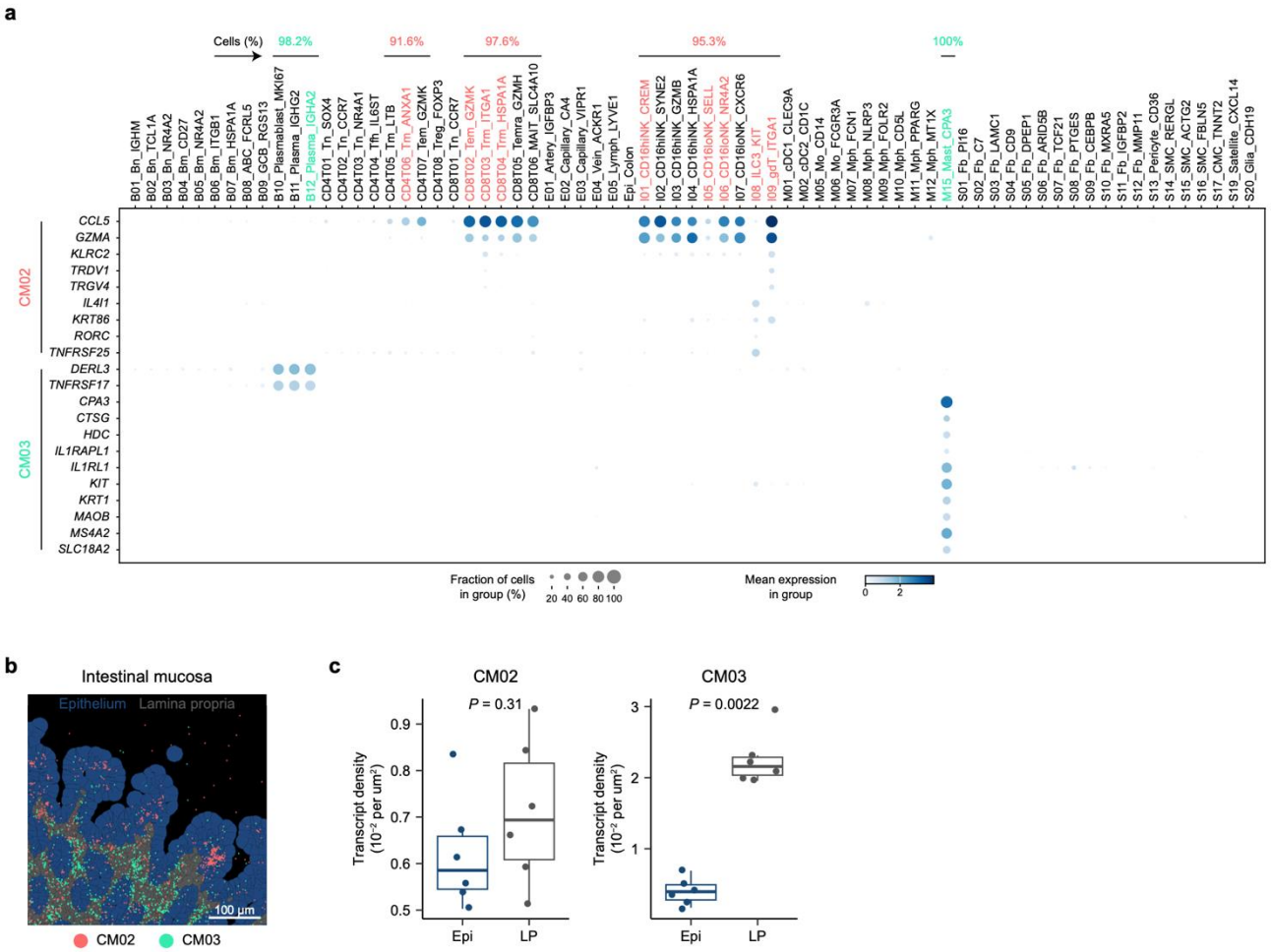
We thank the reviewer for this important feedback. We understand the concern that the correlations involving the expression of CM09 and cell states across age in the thymus may be influenced by a limited number of data points. To address this, we have stratified all samples into two groups based on age (young vs. old) for a more robust comparison. This stratification allows us to present the analysis more accurately, focusing on the differences between these age groups. We have revised the manuscript accordingly (**Fig. S12d**).

(4) The authors make the claim that CM02 and CM03 have distinct functional roles because of the relative spatial organization of expression of CD8 and CD38 (Figure 3f), however, no quantification is done to support this claim and the use of the word 'functional' is not supported by spatial organization of marker expression.

We thank the reviewer for highlighting this issue. We understand the need for quantification to support this claim. We aimed to demonstrate that CM02 and CM03 occupy distinct spatial locations using high-resolution CODEX data, even though both appear enriched in the intestinal mucosa when observed through spot-resolution Visium data. However, the limited marker scope of CODEX hinders the achievement of a more refined identification of cell subsets within the CMs.

To address this limitation, we turned to an alternative spatial data, Xenium, capable of in situ characterization of hundreds to thousands of genes in cells and tissues with ultra-precise single-cell spatial imaging. Using Xenium intestinal data from 10x Genomics (**Table S3**), we designed a gene panel to distinguish multiple cell subsets within CM02 and CM03 (**Response Fig. 11a**). We then considered the gene transcript density to represent the intensity of the respective CMs. Our observations and quantifications revealed a notable enrichment of CM03 in the lamina propria compared to the epithelium, whereas CM02 displayed a consistent distribution across the tissue (**Response Fig. 11b-c**).

Additionally, we have avoided the use of the term "functional" based solely on spatial marker expression patterns without additional evidence. These results have been incorporated into the revised manuscript (**Lines 197-205, Fig. 3f-g, and Fig. S11**).



Response Fig. 11. Distinguishing spatial locations of CM02 and CM03. (also Fig. 3f-g and Fig. S11)

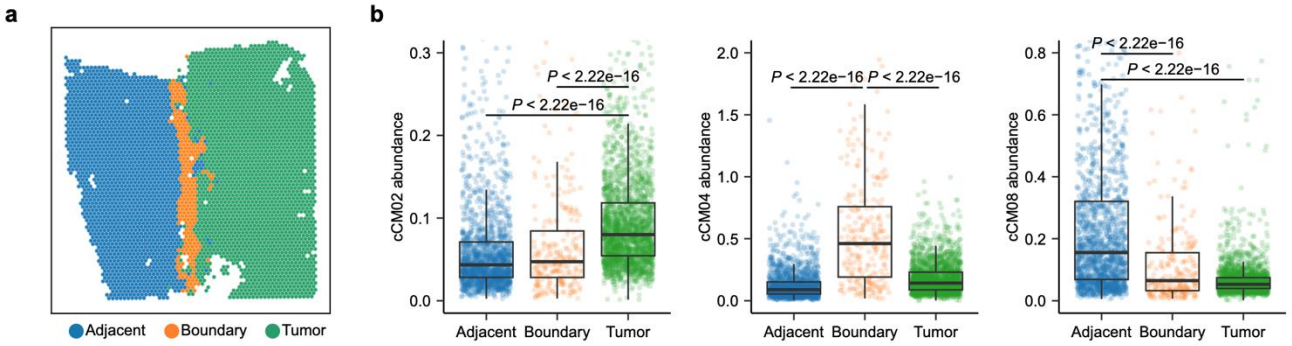
(a) Dot plot illustrating the gene panel expression that differentiates CM02 and CM03 among various cell subsets in the colon. The percentages of colored subsets among similar subsets are indicated at the top. For example, 98.2% of the plasma cells (B10, B11, and B12) are B12.

(b) Representative Xenium imaging of an intestinal mucosal section.

(c) Quantification from six sections demonstrating the distinct anatomical distribution of CM02 and CM03.

(5) The authors make the claim that cCM02, cCM04, and cDM08 are in distinct regions of the tissue (Figure 5c), but quantifications were not performed to support this claim.

We appreciate this helpful comment on the need for quantification to support our claim regarding the distinct tissue localization of cCM02, cCM04, and cCM08. In response, we have conducted a detailed classification of all spots on the leading-edge Visium section of liver cancer into three distinct regions—adjacent, tumor, and boundary—employing unsupervised clustering with minor manual adjustments (**Response Fig. 16a**). The subsequent quantification analysis revealed that cCM08 is highly enriched in adjacent non-tumor regions, cCM02 is present in both tumor and adjacent non-tumor regions, and cCM04 constitutes the stromal barrier around the tumor regions (**Response Fig. 16b**). These findings provide substantial evidence for the distinct tissue localization of the different CMs, reinforcing our original claims.



Response Fig. 16. Quantifications of cCMs.

(a) A leading-edge section of HCC, showing H&E staining (left) and three regions determined by H&E staining and transcriptome (right).

(b) Box plots showing the abundance of cCM02, cCM04, and cCM08 in a leading-edge region of HCC.

Comment 7

(1) In Figure 4 it is unclear which dataset the authors are using for the analysis shown in Figure 4a and how the lineage proportions were calculated.

We apologize for any confusion. The box plots presented in **Figure 4a** utilizes multiple spatial transcriptomics datasets (Visium) across tissues (**detailed in Table S3**). We evaluated the spatial associations of cell subsets within the same CM across spatial sections using the colocalization score.

The lineage proportions depicted in the figure were calculated based on the relative abundance of each cell subtype within the identified CMs. For example, CM06 comprises 8 lymphocyte subsets (including T cells, B cells, and NK cells) and 1 myeloid subset (**Fig. 2c**). Thus, the proportion of lymphocytes is calculated as 88.9% (8 out of 9 total subsets).

We thank the reviewer for bringing this concern, and we have revised the text accordingly (**Lines 257-261**).

(2) It is also unclear if CellPhone DB was performed on the spatial dataset or a single cell dataset.

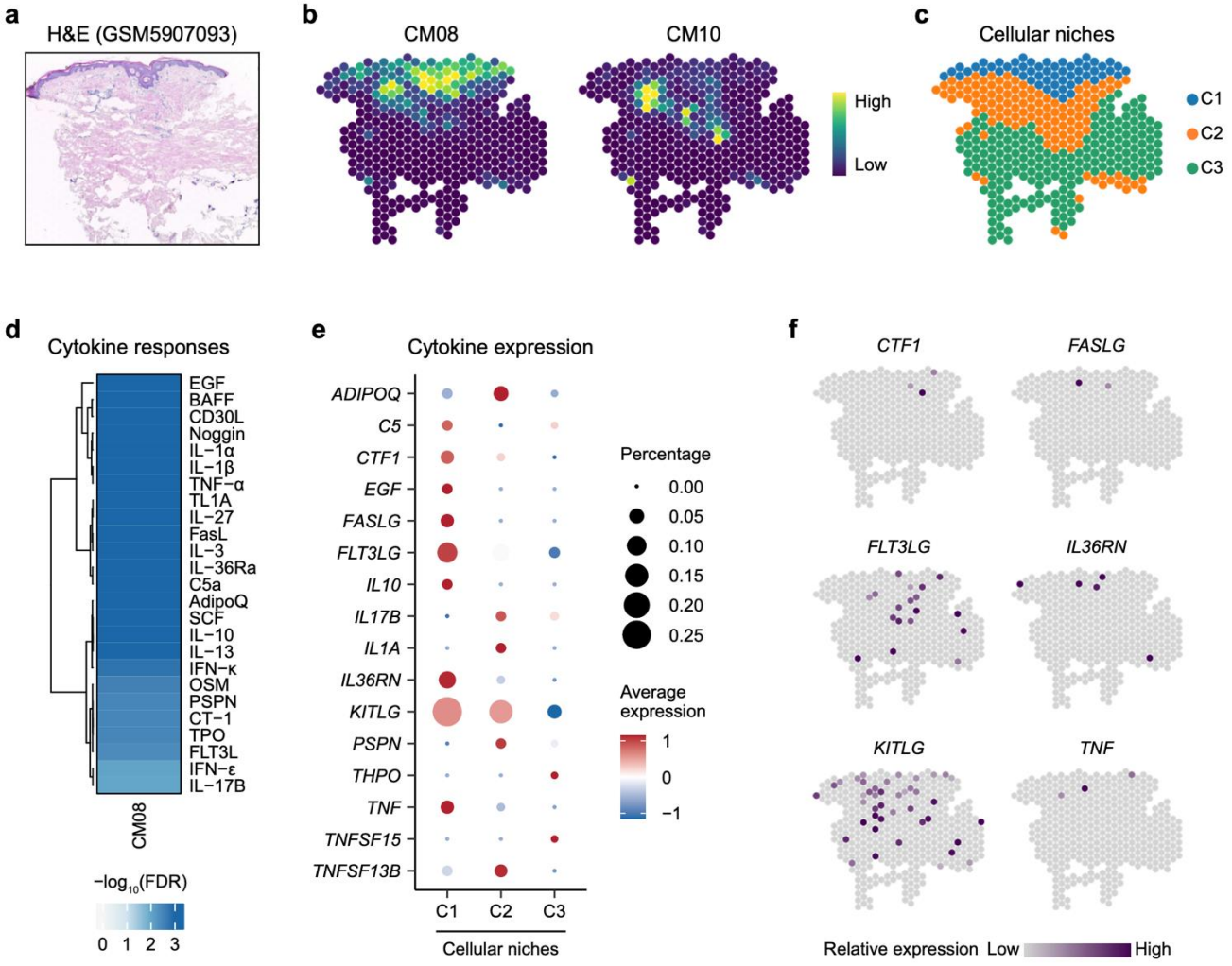
The CellPhoneDB analysis presented in Figure 4 was conducted using single-cell data rather than spatial transcriptomics data. This clarification has been added to the manuscript accordingly (**Lines 261-263, Lines 271-277, and Fig. 4b-c and their legends**).

(3) The claims regarding cellular communication between CMs should be validated experimentally.

We appreciate the comment on the need for additional validation of cellular communication within CMs. Our assertions regarding cellular communication were primarily based on cytokine analysis, as previously discussed in response to the Major Comment 7 of Referee 1.

To address the request for additional validation, we conducted a comprehensive spatial analysis to demonstrate the enrichment of cytokines within specific cellular niches corresponding to their respective CMs. For instance, within the skin section, CM08 aligns with the C1 niche, and our experiments confirmed the presence of various CM08-enriched cytokines within this niche (**Response Fig. 9**). Similarly, for the small intestine, our results validated the high expression levels of cytokines enriched in CM02/CM03 (C1 niche) and CM05 (C2 niche), as observed in our spatial analysis (**Response Fig. 10**).

These orthogonal validations reinforce the spatial organization of cytokines within specific niches and underscore the functional roles of different CMs in mediating cellular communication. We have incorporated these results in the revised manuscript (**Lines 286-289, Lines 348-350, Fig. 4e, Fig. S16, and Fig. S18**).



Response Fig. 9. *In situ* validation of cytokine responses in the skin. (also Fig. S10 and S18)

(a) Hematoxylin and eosin (H&E) staining of a skin section.

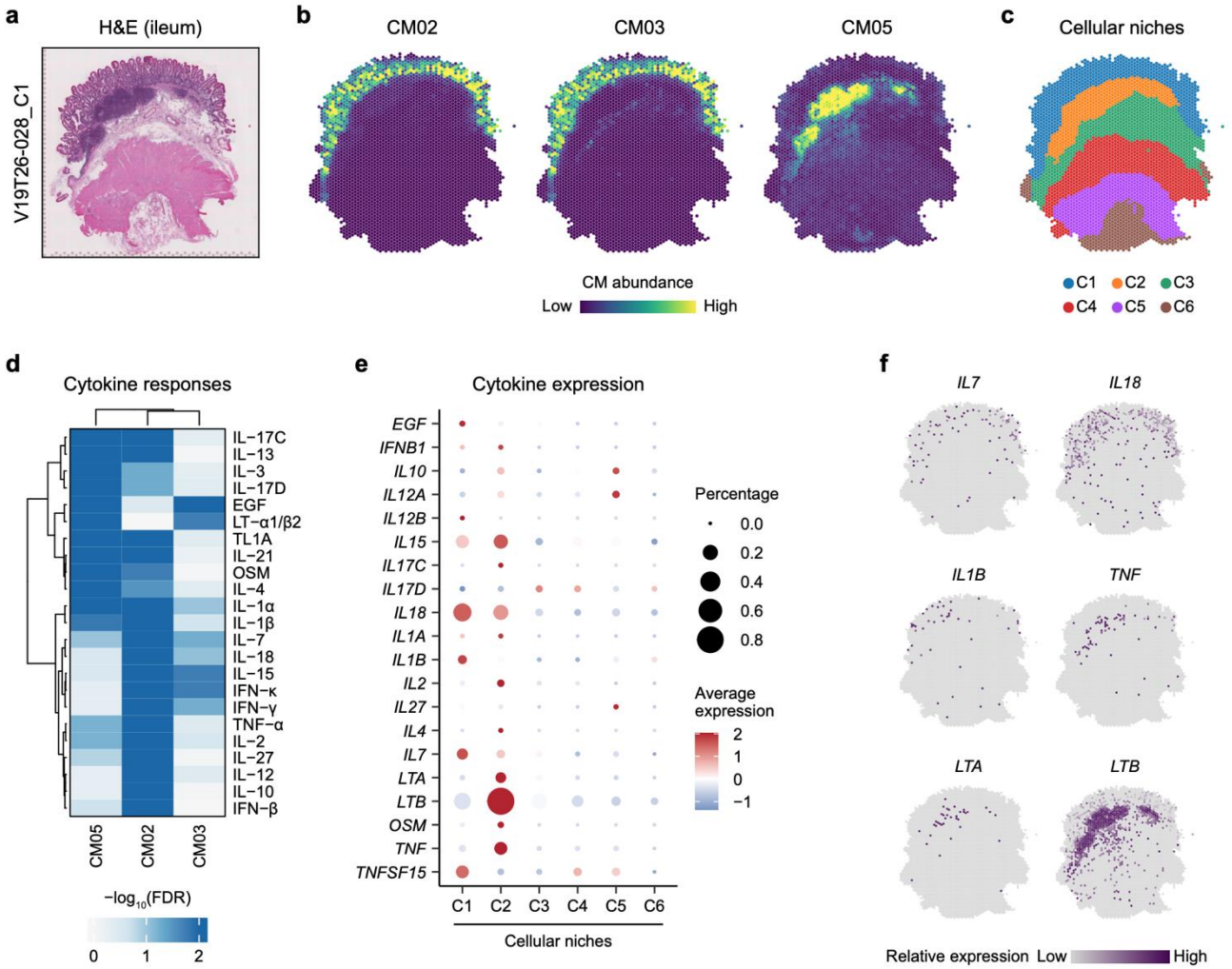
(b) Spatial mapping of CM08 and CM05 on the spatial transcriptomics sections.

(c) Spatial mapping of cellular niches identified by CellCharter (Varrone et al. 2024).

(d) Heatmap showing cytokine responsiveness in CM08. The CM FDR denotes the minimum FDR value among CM subsets.

(e) Cytokine gene expression profiles across the cellular niches illustrated in (c). Only cytokines detected by spatial transcriptomics data are shown.

(f) Spatial distribution of select cytokines in the spatial transcriptomics sections.



Response Fig. 10. ***In situ*** **validation of cytokine responses in the small intestine. (also Fig. 4d-e and Fig. S11 and S16)**

(a) Hematoxylin and eosin (H&E) staining of a section of the small intestine.

(b) Spatial mapping of CM02, CM03, and CM05 on the spatial transcriptomics section.

(c) Spatial mapping of cellular niches identified by CellCharter (Varrone et al. 2024).

(d) Heatmap showing cytokine responsiveness among CM02, CM03, and CM05. The CM FDR denotes the minimum FDR value among CM subsets. Only cytokines enriched in at least one CM are shown.

(e) Cytokine gene expression profiles across the cellular niches illustrated in (c). Only cytokines detected by spatial transcriptomics data are shown.

(f) Spatial distribution of select cytokines within the small intestine.

(4) The authors utilize a package called DIALOGUE to determine which CMs are triggered as a response to the local environment, however this method is not thoroughly explained. The authors look

at MCP gene expression, however it is unclear if this is CM08 or an MCP generated from DIALOGUE.

We appreciate the concern about the application of the DIALOGUE method and the generation of the MCP in our study. Here are further clarifications:

(1) *DIALOGUE*. Cells within the same CM encounter analogous stimuli within a shared tissue microenvironment, potentially triggering coordinated responses across various cell types. DIALOGUE (Jerby-Arnon et al. 2022) was utilized to identify a multicellular program (MCP), which represents genes modulated in a coordinated manner across various cell types within the same CM, reflecting a harmonized response to shared tissue microenvironmental stimuli.

(2) *Explanations for original Figure 4d-f.* In the context of CM08, DIALOGUE was applied to derive a coordinated gene set, termed the "CM08 MCP" or "CM08 program". It is important to note that while the MCP was generated by DIALOGUE, the identification of CM08 and other CMs were determined by our CoVarNet workflow (**Fig. 2b-c**).

In the revised manuscript, we also performed DIALOGUE analysis for CM12 in the breast context. These results and clarifications have been incorporated into the revised manuscript (**Lines 300-302, Lines 341-343, and Lines 762-767, Fig. 4f-g, and Fig. S18**).

References:

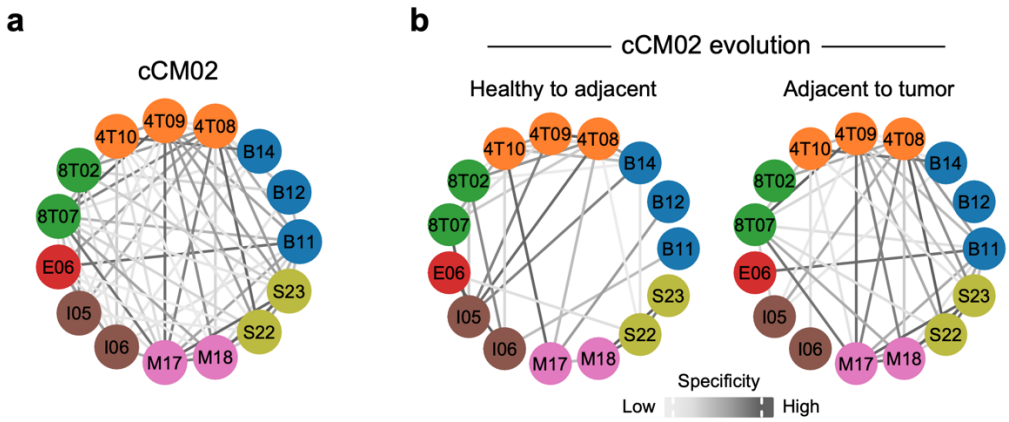
Varrone, M., Tavernari, D., Santamaria-Martínez, A., Walsh, L. A. & Ciriello, G. CellCharter reveals spatial cell niches associated with tissue remodeling and cell plasticity. *Nat. Genet.* **56**, 74-84 (2024).

Jerby-Arnon, L. & Regev, A. DIALOGUE maps multicellular programs in tissue from single-cell or spatial transcriptomics data. *Nat. Biotechnol*. **40**, 1467–1477 (2022).

Comment 8

The authors claim that cCM02 has an increase in cell subset co-occurrence in tumor tissue compared to that of adjacent normal tissue (Figure 5d). It is unclear if the authors performed this analysis iteratively in each sample containing both tumor and tumor adjacent tissues or if this analysis was performed taking into account all samples.

We apologize for the lack of clarity. Due to limited number of samples containing both tumor and adjacent non-tumor tissues, we performed this analysis taking into all samples. Specifically, for established cCM02 nodes (**Response Fig. 17a**), we re-measured cell subset co-occurrence across all healthy and adjacent samples (**Response Fig. 17b, left**) or all tumor and adjacent samples (**Response Fig. 17b, right**). We have incorporated this clarification into the revised manuscript (**Lines 849-853**).



Response Fig. 17. cCM02 evolution. (also Fig. 5i and Fig. S22)

(a) cCM02 identified by CoVarNet using all samples from healthy, tumor adjacent non-tumor tissues.

(b) cCM02 evolution. Nodes are the same as **(a)**, but edges derived from all healthy and adjacent non-tumor samples (left) or all tumor and adjacent non-tumor samples (right).

More broadly in this manuscript there is often a lack of explanation of (1) datasets used for each analysis and (2) a clear explanation of the analysis performed in the methods section.

We thank the reviewer for this helpful feedback regarding clarity in dataset descriptions and analysis methods in our manuscript. To address these concerns comprehensively, we have provided detailed descriptions of the datasets utilized for each analysis. In the Methods section, we also provided concise and explicit descriptions of each analysis, detailing the procedures, parameters, and tools used to ensure transparency and reproducibility. These improvements are aimed at enhancing the accessibility and understanding of our study's methodologies and datasets.

2nd round of review

Response to Referees Letter

Referee #1 (Remarks to the Author):

The authors have addressed all of my comments and the manuscript has been very much improved.

Referee #1 (Remarks on code availability):

The tutorial is now much more detailed and the authors also provide relevant code for generation of the figures.

We sincerely thank the reviewer for the positive evaluation of our revisions. We greatly appreciate the constructive comments and valuable suggestions, which have significantly contributed to improving the quality of our manuscript.

Referee #2 (Remarks to the Author):

The revised manuscript "Cross-tissue multicellular coordination and its rewiring in cancer" provides substantially improved narrative and presentation. The authors have addressed most of my concerns and the work as a whole appears strengthened as appropriately outlined in the response to referees letter. As also pointed out by the other reviewers, the original manuscript was lacking in clearly novel biological insights. The main advantage of the study was, and still is, pioneering methodological contributions on the analysis of high sample scRNA-seq data. As discussed in the rebuttal, discovery based on single-cell RNA-seq data, rather than spatial transcriptomics, is indeed a potentially valuable approach, which would guide future thought and analysis of single cell atlases with higher sample sizes (n>100). Altogether, the study is therefore of broad interest to the field of single-cell genomics.

We sincerely thank the reviewer for the positive evaluation of the revised manuscript. We are pleased to hear that the study is now considered to be of broad interest to the single-cell genomics community. We greatly appreciate the reviewer's acknowledgment of our methodological contributions and the potential value of our approach for future research in the field.

For these reasons, improvements made to the CoVarNet package and descriptions in figures and text are noteworthy and commendable. The authors could even consider, if appropriate, adding functionality for the new trajectory inference based on cell abundance frequency by sample. However, given the technical nature of trajectory inference, it would be important to clarify either precedence of this approach or justification for the use of PHATE and Palantir, which have been tested only (and incompletely so) on single-cell representations of gene expression space – rather than as used here by the authors to analyze patterns in cell type frequency.

We sincerely thank the reviewer for the valuable suggestions regarding the technical improvements.

While PHATE[1] and Palantir[2] were initially designed for single-cell gene expression analysis, their underlying mathematical principles—such as manifold learning and diffusion maps—are broadly applicable to high-dimensional data, including cell type frequency space. In this context, the relationships between samples can be represented as a high-dimensional matrix, where each dimension corresponds to the frequency of a specific cell type. PHATE and Palantir are well-suited for uncovering latent structures and trajectories in such data, as they excel at capturing nonlinear relationships and transitions between states. This approach is particularly useful for studying tissue-level dynamics, where changes in cell type composition reflect biological processes like aging, disease progression, or developmental transitions.

Notably, a recent study has demonstrated the successful application of PHATE and Palantir to analyze dynamic cellular proportions in brain samples[3], further supporting their broader utility. In response to the reviewer's suggestion, we have incorporated this reference into the Methods section (**Lines 789-790**) and added trajectory inference functionality to the CoVarNet package. We thank the reviewer once again for these valuable comments.

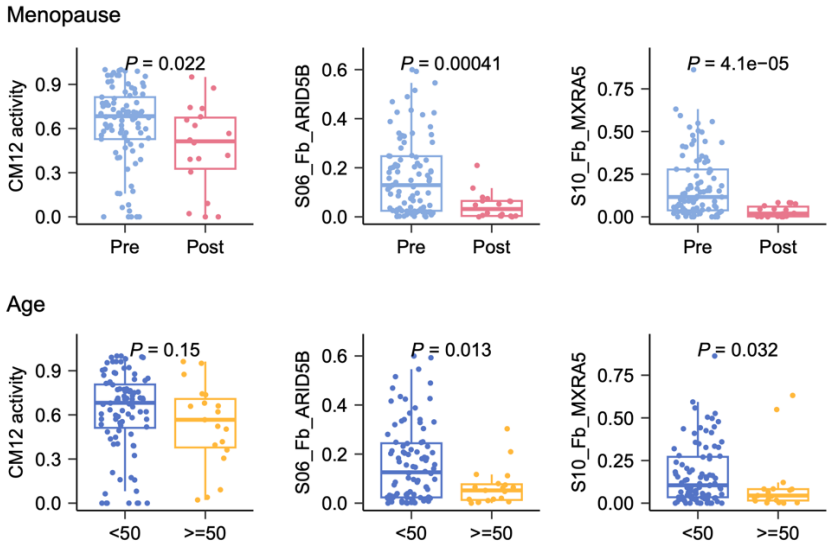
The primary outstanding question for consideration for publication in Nature, however, is whether the added and reorganized findings are sufficiently insightful, convincing, and cohesive, providing evidence that the developed approach can be used for making new discoveries from single-cell atlases. As argued below, regarding the new findings on intestinal mucosa, menopausal fibroblasts in breast tissue, spleen aging, and ecosystem rewiring in cancer, the original criticism on novelty still applies albeit to a lesser extent. For instance, the revised work shows changes to fibroblast subtypes in menopausal breast tissue concordant with a reduction of one cellular program (CM12). While in Fig. S17g this pattern is not significantly detected for age (>50 years), the trend appears similarly and does not fully exclude potential confounding with age, which is clearly correlated with menopause according to Fig. 4k. Some associations that are currently missing would be whether, as in Fig. 4h and Fig S17c, age is associated with the 3 fibroblast subtypes highlighted. Nevertheless, this reviewer would expect there could be both some genuine overlap between the effects of aging and menopause along with an understandable infeasibility from distangling such effects based on clinical observational data alone.

We sincerely thank the reviewer for their thoughtful feedback. We agree that menopause is strongly correlated with age, but it represents a distinct set of biological changes influenced by both intrinsic and extrinsic factors. While the patterns observed in fibroblast subtypes are similar for both age and menopause, we note that CM12 activity and fibroblast frequencies (S06 and S10) show a more significant reduction in post-menopausal samples compared to those over 50 (**Response Fig. 1**). This suggests that CM12 captures menopause-associated biological changes rather than merely reflecting age-related effects.

We appreciate the reviewer's recognition of the difficulty in disentangling these factors. Tailored studies will be essential to better understand the specific contributions of aging and menopause, as well as their potential interactions. These points have been further discussed in the revised manuscript (**Lines 317-319, Lines 439-441, and Fig. S17g**).

More broadly, while we have presented several new findings in this manuscript, our approach has

significant promise for advancing the understanding of multicellular coordination. As integrated atlases from initiatives like the Human Cell Atlas continue to develop, our method will provide valuable insights into specific biological and pathological contexts. Additionally, we note the Editor's comment that no further biological insights are requested at this stage. We believe that, with the methodological advancements and insights presented, this manuscript represents a meaningful contribution to the research community.



Response Fig. 1. Comparison of menopause and age-related changes.

CM12 activity and frequencies of S06 and S10 fibroblasts in samples grouped by menopause (top) and age (bottom). Two-tailed unpaired Wilcoxon tests.

In addition, there is a lack of presented background and discussion on how these CM12 results tie in with literature on stromal fibroblasts in breast tissue. Are the CM12 results not consistent with aging-associated fibroblast phenotypes (https://doi.org/10.1002/bies.201100104), including cytokine-secreting macrophages which likely corresponds to your identification of Mph_NLRP3 cells (see https://www.cell.com/trends/biochemical-sciences/fulltext/S0968-0004(16)30148-7)? On a separate note, while it is interesting that CM12 is found also in skin, I would recommend that the authors briefly review relevant literature in the field on the known effects of menopause (such as https://doi.org/10.3109/09513590.2011.613970), clarifying in greater detail which findings related to CM12 have been reported before and what new insight are supported by the data. Perhaps links to breast cancer risk would be worthwhile highlighting (https://doi.org/10.1016/j.critrevonc.2007.09.001), potentially linking these results better with the subsequent section on cancer immunotherapy.

We sincerely thank the reviewer for their insightful suggestions regarding the contextualization of our CM12 results. In response, we have conducted a thorough review of the relevant literature and revised the manuscript to better highlight the significance of our findings in relation to existing studies on cellular phenotypes in breast tissue.

Aging-associated cellular phenotype

We acknowledge potential differences in cellular phenotypes, including fibroblasts and macrophages, between our findings and previous reports. Upon re-examining our analysis, we confirmed a reduction in inflammation in post-menopausal breast samples, particularly a decrease in *NLRP3*-high macrophages (M08)[4] (**Response Fig. 2a**). This observation aligns with the original breast study from which our curated single-cell breast data are derived. That study found that pre-menopausal women exhibit a decrease in anti-inflammatory macrophages (macro-m2-CXCL cells)[5] (**Response Fig. 2b**). In single-cell studies of mouse models, aged ductal macrophages (M08 in our atlas, see Fig. 4i and Fig. S17f) upregulate immunosuppressive ligands such as Cd274/Pd-l1 and Lilrb4/Ilt3[6], further supporting our findings. Additionally, our data revealed that ECM-related (S10) and lobular (S06) fibroblasts show notable decreases in post-menopausal samples, whereas ductal fibroblasts (S09) show little change.

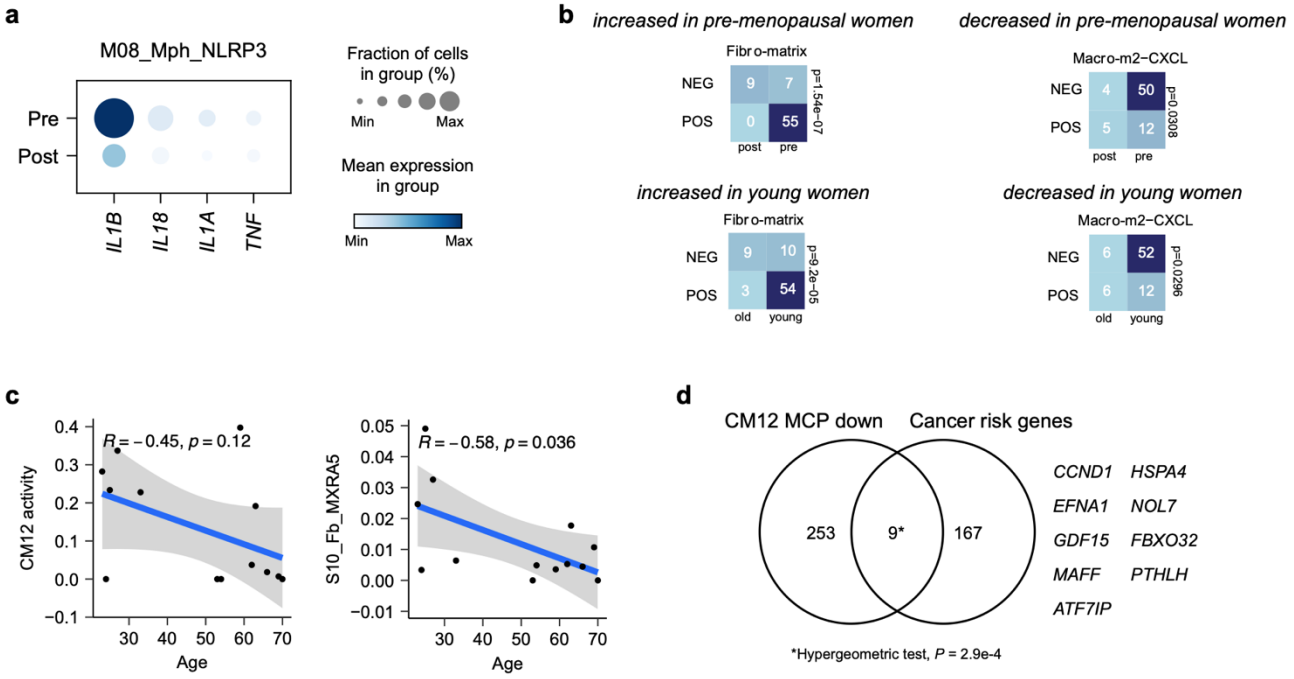
Additional studies provide further complexity regarding the relationship between aging and inflammation. For instance, age-adjusted analyses have shown that both pro-inflammatory markers (e.g., IL-6, TNF-α) and anti-inflammatory markers (e.g., IL-10) are inversely associated with complete lobular involution in the breast[7]. Furthermore, fibroblasts from centenarian exhibit an anti-inflammatory molecular profile characterized by low levels of IL-6, IFN-β, and pro-inflammatory microRNAs[8].

While our data may seem at odds with literature suggesting a systemic increase in inflammation with aging[9], we hypothesize that breast tissue, as a reproductive organ, is more strongly influenced by estrogen levels, leading to localized reductions in inflammation. Estrogen has been shown to have dual effects on the NLRP3 inflammasome[10], suggesting that the relationship between estrogen and inflammation in the breast may be more complex than previously understood. Furthermore, much of the existing research on aging-related inflammation focuses on disease contexts, which differ substantially from healthy tissue. Many studies of fibroblast aging also rely on *in vitro* senescence models, which may not fully capture the complexities of *in vivo* aging[9].

Broader discussion on menopause, aging, and CM12

In skin, CM12 activity and ECM-related fibroblasts (S10) exhibited a trend of chronological decreases (**Response Fig. 2c**), consistent with studies reporting collagen atrophy in post-menopausal skin[11]. Moreover, a study mapping genomic regions associated with breast cancer risk identified 191 likely target genes enriched in known breast cancer driver genes and transcription factors[12]. We found that many of the down-regulated genes in CM12 MCP (i.e. up-regulated genes in post-menopausal breast samples) are included in this breast cancer risk gene set (**Response Fig. 2d**). For instance, the oncogenic role of *MAFF* has been identified as an activator of the IL11/STAT3 pathway in breast cancer[13]. These observations link the non-epithelial multicellular characteristics of CM12 with age-related breast cancer risk[14], highlighting the broader potential of our approach.

We have incorporated these points into the revised manuscript (**Lines 441-444**) to provide a more comprehensive context for our findings and further align them with existing literature. We thank the reviewer once again for these valuable suggestions.



Response Fig. 2. Additional characteristics of CM12.

(a) Expression of pro-inflammatory factors in M08 macrophages from pre- and post-menopausal breast samples.

(b) Significant associations of breast cell states with menopause status (top) or age groups (bottom) using Fisher's exact test. This figure was extracted from Extended Data Fig. 12 of the published paper[5].

(c) Scatter plots showing the correlation between age and CM12 activity or the frequencies of ECM-related fibroblasts (S10) in skin samples. Smoothed conditional means and their 95% confidence intervals from fitted linear models are shown. Pearson correlation coefficients and significance are noted.

(d) Venn diagram showing the overlap of down-regulated genes in CM12 MCP (i.e. up-regulated genes in post-menopausal breast samples) and previously predicted target genes associated with breast cancer risk[12]. The nine shared genes are highlighted.

The results on aging in the spleen are interesting and more convincing than the thymic involution results alone, but may be explainable by T cell quiescence (https://www.jci.org/articles/view/85329) and/or T cell exhaustion, which the authors note but which would not in itself be a new finding. In addition, please note the important differences between CD4+ and CD8+ T cells in the paper that you cite (https://www.nature.com/articles/s41586-019-0979-8). Here, your cell type associated with age are CD4+ T cells specifically, which should inform the interpretation of these results. Also, the B cell annotations (B05_Bm_NR4A2) of the aging-related results could be controversial since, at least in mice, B cells are known to lack Nr4a2 expression altogether (https://doi.org/10.1111/imr.13072):

"Since Nr4a2 is virtually absent in B cells, we hypothesized that deletion of Nr4a1 and Nr4a3 should largely eliminate all NR4A function in B cells."

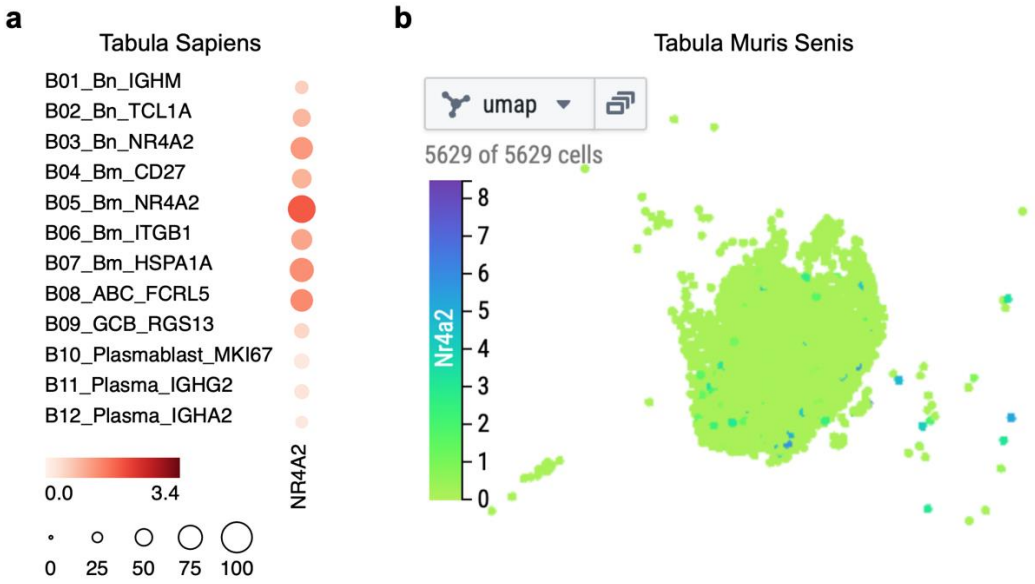
Some explanation for this discrepancy would be required. Given the lack of ambient RNA removal (such as by CellBender), of which I understand and empathise with not being able to do at this scale despite the suggestion in the previous peer review, one possibility would be myeloid cell contamination

in the B cell cluster.

We sincerely thank the reviewer for their insightful comments on aging-related immune dynamics in the spleen. These comments have prompted us to refine the explanation and discussion of these findings. Below, we address the reviewer's concerns regarding *NR4A2* expression in B cells, T cell quiescence and exhaustion, and the broader implications of our findings.

NR4A2 expression in B cells

To address the concern regarding *NR4A2* expression in B cells, we first investigated the potential contribution of ambient RNA. Using the Tabula Sapiens[15] dataset (which applies DecontX[16] for ambient RNA removal), we confirmed that human B cells express *NR4A2* even after ambient RNA removal (**Response Fig. 3a**). In contrast, the Tabula Muris[17] dataset confirmed that murine B cells virtually lack *NR4A2* expression[18] (**Response Fig. 3b**). This species-specific difference is important to highlight, and we thank the reviewer for raising this issue.

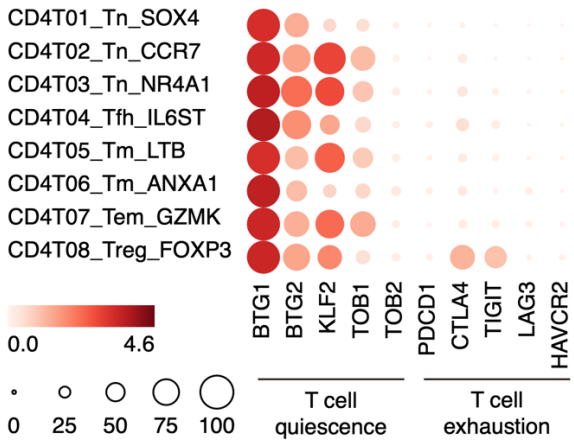


Response Fig. 3. *NR4A2* expression in B cells.
(a) *NR4A2* expression in B cells from the Tabula Sapiens dataset. The data is sourced from our curated pan-tissue single-cell atlas, available at: http://cm.cancer-pku.cn/.
(b) *Nr4a2* expression in B cells from the Tabula Muris Senis dataset. The data is available from a cellxgene session for Smart-seq2 data: https://cellxgene.cziscience.com/e/98e5ea9f-16d6-47ec-a529-686e76515e39.cxg/.

T cell quiescence and exhaustion

To investigate whether *NR4A1*-high CD4+ T cells (CD4T03) exhibit quiescent or exhausted states, we examined the expression of canonical markers associated with these conditions in CD4+ T cells using the Tabula Sapiens[15] dataset. We found no significant enrichment of quiescence or exhaustion markers in CD4T03 cells compared to other CD4+ T cells (**Response Fig. 4**). While *NR4A1* has been linked to T cell quiescence[19] and exhaustion[20], our findings suggest that *NR4A1* may play a role in multiple contexts.

T cell exhaustion is typically associated with chronic infections or cancer, where T cells are terminally differentiated and express multiple inhibitory receptors[21]. Given the naïve phenotype of CD4T03 cells in homeostatic conditions, it is unlikely these cells represent exhausted T cells.



Response Fig. 4. Gene expression in CD4+ T cells.
Expression of canonical markers associated with T cell quiescence[22-25] and T cell exhaustion[21] in CD4+ T cells from the Tabula Sapiens dataset. These markers are based on previously published literature.

Additional discussion on aging and immune tolerance

The accumulation of *NR4A1*-high CD4+ T cells in the aging spleen can be attributed to both immune tolerance and thymic involution. Chronic exposure to pathogens and environmental antigens drives T cells toward a tolerant phenotype, characterized by high *NR4A1* expression[20]. In mouse models, NR4A family members have been reported to regulate T cell tolerance and immune homeostasis, thereby helping to suppress autoimmunity[26,27]. We propose that the accumulation of *NR4A1*-high CD4+ T cells in the aging spleen represents an adaptive mechanism that preserves immune balance and prevents autoimmunity, which is particularly important in the aging immune system. Concurrently, the decline in thymic output reduces the influx of naïve T cells, leading to homeostatic changes in the T cell pool, including an increase in tolerant T cells.

We also hypothesize that the functional roles of CD4+ and CD8+ T cells may contribute to the observed differences. CD4+ T cells primarily regulate immune responses by secreting cytokines that activate other immune cells, such as B cells and CD8+ T cells. In contrast, CD8+ T cells are cytotoxic, directly killing infected or malignant cells. The accumulation of *NR4A1*-high CD4+ T cells with aging may therefore reflect an increased role in immune regulation under aging conditions.

We have revised the manuscript to incorporate these points in the discussion (**Lines 217-219**). We thank the reviewer once again for these constructive comments.

More broadly, and as a suggestion for improved presentation, it would be helpful to have clearer annotations of all identified CMs indicative of the hypothesized functional roles in tissue architecture. I would suggest adding spelled out descriptive labels to each module in Fig. 2d – or provide a table of each module, clarifying the prevailing hypothesis for each module in greater detail, some of which is

already described in the main text. Relatedly, the link from the intestinal Peyer's patches shown in Fig. 2a to other stories is presently unclear and disjointed from those presented elsewhere in Fig. 2 and Fig. 3. This is compounded somewhat by the exclusion of epithelial cells, which are key components of Peyer's patches. One suggestion would be to clarify which of the CMs corresponds to barrier tissue stromal-immune architecture, including Peyer's patches and cross-organ counterparts. Alternatively, the Peyer's patches data may fit better with the intestinal mucosa results shown in Fig. 3c-f.

We sincerely thank the reviewer for the valuable suggestions to improve the presentation of our findings. We agree that clearer annotations of the identified CMs, along with their functional roles in tissue architecture, would enhance the manuscript. In response, we have reorganized Fig. 2 and Fig. 3 and added a table summarizing the prevailing hypothesis for each CM based on our current understanding (**Fig. 2f**). We have also revised the narrative in the main text accordingly. While we have made our best efforts to characterize cross-tissue multicellular communities, further tailored studies are necessary to fully elucidate their functional significance.

Regarding the Peyer's patches data, we acknowledge that their inclusion in Fig. 2a may have caused some confusion. These data were initially included to illustrate the CM concept. Following the referee's suggestion, we have relocated the CODEX data of Peyer's patches to the supplementary figures (**Fig. S11d**) to better support the intestinal mucosa results presented in Fig. 3a-d. We believe these revisions provide a more cohesive presentation of the data and enhance the manuscript's overall clarity. We thank the reviewer again for these constructive suggestions.

Lastly, on line 155, what does 'notable system preferences' mean? I suppose this means tissue-specificity?

We thank the reviewer for pointing this out. To ensure greater precision and clarity, we have revised the phrasing to "notable preferences for specific human body systems" (**Lines 148**).

In summary, the manuscript has improved substantially but some issues remain. Some of the points raised are inherently due to an emphasis of broad scope rather than specific details. The manuscript is well written, timely, and the overall presentation has improved substantially.

Referee #2 (Remarks on code availability):

The associated software package has been expanded and the code for figures are provided as notebooks.

We sincerely thank the reviewer for their positive comments and thoughtful suggestions. We have carefully addressed these concerns and have made the necessary revisions to ensure that the manuscript clearly conveys its broad scope of findings while providing the appropriate level of detail. We believe that these changes have significantly improved the quality and clarity of our manuscript.

Referee #3 (Remarks to the Author):

The authors have done impressive work in revising their manuscript. They have now convinced me that the cellular module concept is important, comprehensive and robust. I appreciate very much the

process of identification and characterization that the authors have gone through to identify the cellular modules. Using this approach they show impressive results with the aging spleen and menopause, as well as the ecosystems in cancer. I believe that this is an important paper that is ready for publication.

We sincerely thank the reviewer for the positive evaluation of our revised manuscript and for the insightful comments, which have significantly enhanced the quality of our work. We are pleased that the manuscript is now considered ready for publication.

References

1 Moon, K. R. *et al.* Visualizing structure and transitions in high-dimensional biological data. *Nat. Biotechnol.* **37**, 1482–1492 (2019).

2 Setty, M. *et al.* Characterization of cell fate probabilities in single-cell data with Palantir. *Nat. Biotechnol.* **37**, 451–460 (2019).

3 Green, G. S. *et al.* Cellular communities reveal trajectories of brain ageing and Alzheimer's disease. *Nature* **633**, 634–645 (2024).

4 He, Y., Hara, H. & Nunez, G. Mechanism and Regulation of NLRP3 Inflammasome Activation. *Trends Biochem. Sci.* **41**, 1012–1021 (2016).

5 Kumar, T. *et al.* A spatially resolved single-cell genomic atlas of the adult human breast. *Nature* **620**, 181–191 (2023).

6 Li, C. M. *et al.* Aging-Associated Alterations in Mammary Epithelia and Stroma Revealed by Single-Cell RNA Sequencing. *Cell Rep* **33**, 108566 (2020).

7 Hanna, M. *et al.* Association between local inflammation and breast tissue age-related lobular involution among premenopausal and postmenopausal breast cancer patients. *PLoS One* **12**, e0183579 (2017).

8 Storci, G. *et al.* Genomic stability, anti-inflammatory phenotype, and up-regulation of the RNAseH2 in cells from centenarians. *Cell Death Differ.* **26**, 1845–1858 (2019).

9 Bonafe, M., Storci, G. & Franceschi, C. Inflamm-aging of the stem cell niche: breast cancer as a paradigmatic example: breakdown of the multi-shell cytokine network fuels cancer in aged people. *Bioessays* **34**, 40–49 (2012).

10 Dong, W. *et al.* Estrogen plays an important role by influencing the NLRP3 inflammasome. *Biomed Pharmacother* **167**, 115554 (2023).

11 Calleja-Agius, J. & Brincat, M. The effect of menopause on the skin and other connective tissues. *Gynecol Endocrinol* **28**, 273–277 (2012).

12 Fachal, L. *et al.* Fine-mapping of 150 breast cancer risk regions identifies 191 likely target genes. *Nat. Genet.* **52**, 56–73 (2020).

13 Moon, E. J. *et al.* The HIF target MAFF promotes tumor invasion and metastasis through IL11 and STAT3 signaling. *Nat Commun* **12**, 4308 (2021).

14 Benz, C. C. Impact of aging on the biology of breast cancer. *Crit Rev Oncol Hematol* **66**, 65–74 (2008).

15 Tabula Sapiens, C. *et al.* The Tabula Sapiens: A multiple-organ, single-cell transcriptomic atlas of humans. *Science* **376**, eabl4896 (2022).

16 Yang, S. *et al.* Decontamination of ambient RNA in single-cell RNA-seq with DecontX. *Genome Biol* **21**, 57 (2020).

17 Tabula Muris, C. *et al.* Single-cell transcriptomics of 20 mouse organs creates a Tabula Muris. *Nature* **562**, 367–372 (2018).
18 Hiwa, R., Brooks, J. F., Mueller, J. L., Nielsen, H. V. & Zikherman, J. NR4A nuclear receptors in T and B lymphocytes: Gatekeepers of immune tolerance. *Immunol. Rev.* **307**, 116–133 (2022).
19 Boddupalli, C. S. *et al.* ABC transporters and NR4A1 identify a quiescent subset of tissue-resident memory T cells. *J. Clin. Invest.* **126**, 3905–3916 (2016).
20 Liu, X. *et al.* Genome-wide analysis identifies NR4A1 as a key mediator of T cell dysfunction. *Nature* **567**, 525–529 (2019).
21 Wherry, E. J. & Kurachi, M. Molecular and cellular insights into T cell exhaustion. *Nat. Rev. Immunol.* **15**, 486–499 (2015).
22 Buckley, A. F., Kuo, C. T. & Leiden, J. M. Transcription factor LKLF is sufficient to program T cell quiescence via a c-Myc-dependent pathway. *Nat. Immunol.* **2**, 698–704 (2001).
23 Tzachanis, D. *et al.* Tob is a negative regulator of activation that is expressed in anergic and quiescent T cells. *Nat. Immunol.* **2**, 1174–1182 (2001).
24 Hwang, S. S. *et al.* mRNA destabilization by BTG1 and BTG2 maintains T cell quiescence. *Science* **367**, 1255–1260 (2020).
25 Kuo, C. T., Veselits, M. L. & Leiden, J. M. LKLF: A transcriptional regulator of single-positive T cell quiescence and survival. *Science* **277**, 1986–1990 (1997).
26 Hiwa, R., Nielsen, H. V., Mueller, J. L., Mandla, R. & Zikherman, J. NR4A family members regulate T cell tolerance to preserve immune homeostasis and suppress autoimmunity. *JCI Insight* **6** (2021).
27 Nielsen, H. V. *et al.* Nr4a1 and Nr4a3 redundantly control clonal deletion and contribute to an anergy-like transcriptome in auto-reactive thymocytes to impose tolerance in mice. *Nat Commun* **16**, 784 (2025).

3rd round of review

Response to Referees Letter

Referee #2 (Remarks to the Author):

The authors have fully addressed my concerns – and I would like to congratulate them on the near-complete study, which will undoubtedly be an important contribution to the field of single-cell genomics.

The revised manuscript has a clear and convincing narrative with several minor improvements made during this second revision. The recent changes have added more depth to the interpretation of key results. The rebuttal was well argued and has increased my confidence in the validity of presented findings. Any remaining limitations on the originality of findings seem related to the broad scope of the study and are unlikely to improve on further revision.

We sincerely appreciate the reviewer's thoughtful feedback and kind words. We are truly grateful for the time and effort invested in evaluating our study throughout the review process. The insightful comments have greatly contributed to improving the clarity, depth, and presentation of our work. We are especially encouraged by the reviewer's recognition of our study's contribution to the field of single-cell genomics.

Once again, we extend our heartfelt thanks for the constructive suggestions and the positive assessment of our revised manuscript.

Referee #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

We sincerely appreciate the time and effort that the reviewer dedicated to reviewing our manuscript. The contributions, along with those of the co-reviewer, have been invaluable in strengthening our study. We are grateful for the thoughtful assessment and support.