nature portfolio

Peer Review File

Mast cells boost anti-tumor potency of MAIT cells via inflammasome-dependent secretion of IL-18

Corresponding Author: Professor Haiquan Chen

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, Fan et al provide an extensive characterization of mast cells infiltrating lung adenocarcinomas, using both bulk and single cell sequencing, flow cytometry, and cell cultures. They could identify a tumor-infiltrating subset of mast cells expressing activation markers and secreting IL-18 through NLRP3 activation and pyroptosis. Tumor-infiltrating MAIT cells were identified as potential targets of the mast cell derived IL-18, and both mast cell and MAIT cell infiltration into tumors corelated to a better patient outcome. The manuscript provides interesting information on mast cell presence and function in lung tumors, and also adds to the knowledge of tumor-infiltrating MAIT cells. There are however several aspects of the manuscript that can be improved.

The English language could be considerably improved, and it is recommended to consult a native speaking colleague to better this aspect.

The manuscript gives a somewhat unkempt impression. Some sentences are hard to interpret, (e.g. line 189-190, 372, 411), and sometimes, the call out for figures is not correct (e.g. line 155, 318 and 324) or missing (line 565)

Furthermore, several of the figures are hard to interpret, as both labeling in the figures themselves and the figure legends are sparse with information.
Fig. 1C: Mast cell percentage is shown, but percentage of what?
Fig. 1G: What is the definition of a favorable and adverse outcome? Are the hazard ratios significant? If so, at what level?
Fig. 2C: The labelling of some of the lanes appears wrong, some + should probably be -.
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Fig. 5I: Which subset is shown in grey and which in yellow?
Fig. S1E: Mast cell percentage is shown, but percentage of what?
Fig. S2I: Which comparison yields $p<0.0001$? And what does the histograms and score show?
Fig. S6D: Which panel shows which condition?
Fig. S6E: Which panel shows which cohort?

The introduction is very short and could be expanded, for example on the topic of mast cell function in the tumor microenvironment and on mast cell pyroptosis in general, outside of tumors.

Please indicate the purity of the different sorted cell populations used in the experiments.

Please also define the Opal pairing with the different monoclonals used for immunofluorescence.

The single cell transcriptomic analysis yielded 9 mast cells clusters defined by their expression of certain molecules (line 157-158). It would be nice to see the expression of these respective markers in the t-SNE visualization of the clusters.

Several of the clusters in Fig. 2A are provide by cells from a single data set, such as the FcER1A-MC, RPL-MC, and CD74-MC. I find this worrying, as they may not represent actual clusters present in most tumors, but the handling and/or analysis parameters of a certain study.

In line 189-190, what is meant by IL-18- mast cells (as I assume the sentence is supposed to mean)? All other clusters but IL18+-MC, or on the single cell level comparing actual IL-18+ and IL-18- mast cells?

Fig. 2E shows NLRP1 and NLRP3 expression in LUVA cells, contrary to the text (line 236-237) that states that there is a selective expression of NLRP3.

In Fig. 3A, the percentage of FLICA+ cells is very similar in healthy lung and tumor tissue, still, the text states that it is higher in the tumors (lines 253-256).

In the discussion, it is stated that “no research has ever focused on the heterogeneity and function of MCs in the tumor microenvironment”: This is simply not true, see for example these recent reviews PMID: 38462035, 38323271, 37852570.

Likewise, the statement that “the characteristics of MAIT cells in lung adenocarcinoma remain unveiled” is also not correct. See PMID 36657812 and 36435063. In contrast, the reference given on line 311, ref 51, does not contain information on lung adenocarcinoma, as the authors claim.
CD69 on MAIT cells from tumor tissue is regarded as an activation marker but should rather be seen as a marker of tissue residency.

In the discussion, the authors claim that mast cells induce MAIT cell activation in a TCR-independent way (line 415-416). When this may be likely, it has not been tested by i.e. blocking MR1 interactions with the TCR, and thus the statement would have to be tempered.

The authors show that mast cell-derived IL-18 activates MAIT cells, but the discussion should also take into account other sources of IL-18 in the tumor microenvironment (for example macrophages and monocytes), which may be more numerous than mast cells.

Reviewer #2

(Remarks to the Author)
The paper entitled "Mast cells boost anti-tumor potency of MAIT cells via inflammasome activation" is informative, but there are some concerns that need to be addressed as follows.

Major Concerns

The author showed that mast cells (MCs)-derived IL-18 enhances the innate-like anti-tumor effects of mucosal-associated invariant T (MAIT) cells through the cytokine activation pathway, however, is there any possibility that MAIT cells are triggered by a tumor-derived antigen presented via IL-18+MCs?

Contrary to mucosal cancers like lung adenocarcinoma (LUAD), gastric cancer GC) and colorectal cancer (CRC), the frequency of tumor-infiltrating MAIT cells in hepatocellular carcinomas (HCC) is known to decrease.
In this case, what makes the difference in the levels of MCs-derived IL-18 between mucosal and nonmucosal cancers?

A recent report has shown that MAIT cells enhance the anti-tumor function of NK cells in an IFN-γ-dependent manner.
We therefore suggest that the author investigates whether MCs-derived IL-18 augmented MATT cells regulate NK cell-mediated tumor immunity.

Reviewer #3

(Remarks to the Author)
The manuscript by Haiquan Chen et al. presents an analysis of mast cells in non-small cell lung cancer and their implications for the activation of MAIT cells in the context of tumor immunology. While the authors state to present a comprehensive mast cell "atlas" in lung cancer with 9 MC subsets, they focus almost exclusively on IL-18+ mast cells. While this is an interesting subset, their claim to present an atlas would deserve in my opinion a more detailed appreciation of the other 8 subsets, and their associations with clinico-pathological data although I understand that the data set may not be sufficiently clinically annotated and large enough to performa survival analyses for all subsets. The authors do show that mast cell counts are negatively correlated with increasing tumor aggressiveness as defined by a shift from precursor lesions, in-situ carcinoma and fully invasive lung cancer.
The authors also provide data on several aspects of IL-18 associated mechanisms in mast cells such as NLRP3 inflammasome activation and GSDMD expression. The authors then establish a connection of IL-18+ MCs to the activation of MAIT cells which belong to the innate immune system and have been shown to exert anti-tumor immunity. Their in vitro data on the IFNg stimulation and MAIT cell activation through IL-18 and MCs appears convincing. However, while the MC-IL18-MAIT axis can in my opinion be derived from the presented data, I see a major limitation of the study in the translation of the results in terms of their clinical interpretation. While the authors start showing a negative correlation of MC density and tumor aggressiveness in their MC lung cancer atlas, they finish by stating that MAIT cell positively correlated with overall and recurrence free survival. If the axis they propose is right, larger MC numbers should lead to MAIT cell activation. However, MC numbers are increased in less aggressive tumors (precursors, in situ lesions) according to their results which leads to the trivial conclusion that increasing MC-MAIT-cell activity is associated with improved survival. The authors should

perform a normalization to demonstrate that the effects on survival are independent of tumor subtype/stage to make the paper a strong candidate for Nature Communications.

Specific comments:

p. 3. LUAD can be distinguished indolence from invasiveness[27] ?

p. 3.
What do the authors mean by "preference of MCs", to me the data look as if there is no association between MCs and any of the shown parameters apart from radiology and pathology.
Integrating MCs infiltration profiling with genomic,
127 radiologic, pathologic, and clinical features revealed a remarkable variety and preference of MCs
128 distribution (Figure 1B)
Percentage? Decrease of MCs with increasing solidity of tumors.

What do the authors mean by:
we generated 24 in-house single-cell RNA sequencing (scRNA-seq) "data"
Does this refer to the numbers of tumors?

p. 7: complicated sentence, not clear:
However, unique aspects of IL-18
304 biology in potent IFN- inducer and preferential activation of cytotoxic lymphocytes prompted us
305 to explore the function mechanism of IL-18-producing MCs, as MCs were the main cellular source
306 of IL-18 in LUAD.

p. 8. unclear, what does "one tissue" mean in this context:
Clusters largely contained cells from one tissue, suggesting the
335 imprint of local tumor microenvironment.

p. 9
differentiation trajectory of CXCR4+IFNG+ MAIT cells 353 was from blood MAIT cells (Figure 5F),
354 suggesting the microenvironment imprinting partly owing to exposure to IL-18.

p. 10
"LAUD" please correct.

In my opinion this is too speculative:
427 thus reasonable to speculate MCs may play a key role in maintaining LUAD indolent nature for a
428 long time away from invasiveness.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript has been considerably improved, and I only have two minor remarks.

Fig. 1G: What is the definition of a favorable and adverse outcome. It may be in the M&M, but I can't find it.

Fig. S2K: Which comparison yield a p-value of <0.0001? Total differences across different immune subtypes?

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

In response to the reviewer's comments, the authors performed additional analyses and experiments. The revised manuscript has been greatly improved.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
The authors have addressed my concern, in principle. The authors concede that due to the nature of the data set (limited sizes and clinico-pathological annotations), questions remain open that would be addressable by a more comprehensive data set. Nevertheless, this would not preclude in my opinion the publication in Nature Commun.
A minor remaining point is that the while the multivariate analysis confirms that MC density is an independent prognostic factor, MAIT cells are not but mentioned as relevant in the proposed model. The authors should at least state clearly this limitation of the relevance of MAIT cells and discuss possible reasons.

(Remarks on code availability)

Response to the Reviewer's Comments:
Reviewer #1 (Remarks to the Author)

In this manuscript, Fan et al provide an extensive characterization of mast cells infiltrating lung adenocarcinomas, using both bulk and single cell sequencing, flow cytometry, and cell cultures. They could identify a tumor-infiltrating subset of mast cells expressing activation markers and secreting IL-18 through NLRP3 activation and pyroptosis. Tumor-infiltrating MAIT cells were identified as potential targets of the mast cell derived IL-18, and both mast cell and MAIT cell infiltration into tumors corelated to a better patient outcome. The manuscript provides interesting information on mast cell presence and function in lung tumors, and also adds to the knowledge of tumor-infiltrating MAIT cells. There are however several aspects of the manuscript that can be improved.

Reviewer 1#' Comment 1:
The English language could be considerably improved, and it is recommended to consult a native speaking colleague to better this aspect.
Response to Reviewer 1#' Comment 1:
Thank you for your thoughtful and constructive feedback on our manuscript. We greatly appreciate your positive comments regarding our work and your valuable suggestions have been instrumental in refining the manuscript.
We fully acknowledge your comment on the English language quality. We recognize the importance of clear and precise language in effectively communicating our findings. We have enlisted the assistance of a native English-speaking colleague to perform a comprehensive revision of the manuscript. All modifications have been highlighted in yellow in the manuscript highlight version. We are confident that these changes have enhanced both the readability and academic rigor of the text.

Reviewer 1#' Comment 2:
"The manuscript gives a somewhat unkempt impression. Some sentences are hard to interpret, (e.g. line 189-190, 372, 411), and sometimes, the call out for figures is not correct (e.g. line 155, 318 and 324) or missing (line 565)
Furthermore, several of the figures are hard to interpret, as both labeling in the figures themselves and the figure legends are sparse with information.
Fig. 1C: Mast cell percentage is shown, but percentage of what?
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Fig. S2I: Which comparison yields $p<0.0001$? And what does the histograms and score show?
Fig. S6D: Which panel shows which condition?
Fig. S6E: Which panel shows which cohort?"
Response to Reviewer 1#' Comment 2:

Thank you for your meticulous and constructive review of our manuscript. We greatly appreciate the time and effort you have invested in providing such detailed suggestions. We have addressed each of your comments and made the necessary revisions to improve both the clarity and presentation of the manuscript.

Line 189-190: *"Furthermore, a direct comparison of IL-18+ versus IL-18+ MCs demonstrated that IL-18+ MCs displayed significantly enhanced proinflammatory signatures, as indicated by GSEA (Figure 1K)."*
Changes in the manuscript: Page 5, Line 204:
*"A direct comparison between IL-18$^+$ MCs and other MC subsets revealed significantly enhanced proinflammatory signatures in IL-18$^+$ MCs, as evidenced by Gene Set Enrichment Analysis (**Figure 1K**)."*

Line 372: *Moreover, CDR3β amino acid length and sequence were higher variable than CDR3α and no difference was not observed among MAIT cell subsets (Figure S5G and Figure S5H).*
Changes in the manuscript: Page 10, Line 403:
*"Furthermore, the variability in CDR3β amino acid length and sequence was greater than that observed for CDR3α. No significant differences were found between the MAIT cell subsets in this regard (**Figure 5J** and **S5G-S5H**)."*

Line 411: *"The transcriptional heterogeneity of MCs remained elusive among tumor-infiltrating innate immune cells, leaving their controversial roles across human cancers [9]."*
Changes in the manuscript: Page 11, Line 458:
"The transcriptional heterogeneity of MCs within the tumor-infiltrating innate immune compartment remains poorly understood, leaving their roles across various human cancers ambiguous [2]."

Line 155: *"A high-quality MCs single-cell transcriptional atlas was compiled after removing batch effects through Harmony, containing 14,590 MCs deriving from 115 samples covering 51 nLung, 11 lung benign lesions, and 53 lung cancer, 50.5% of which were in-house data and the average infiltration percentage of MCs was 3.07% (Figure 2A and Figure S2C)."*
Changes in the manuscript: Page 4, Line 165:
*"The resulting MC atlas comprised 14,590 MCs derived from 115 samples, including 51 nLung, 11 benign lung inflammation, and 53 tumors, with 50.5% of the data originating from in-house datasets (**Figure 1D** and **S2C**). The average infiltration percentage of MCs across all samples was 3.07% (**Figure 1D**)."*

Line 318: *"MAIT cells signatures from three studies [52-54] (Table S8) strongly correlated with IL-18 MCs signature, indicating the relevance of their infiltrating levels, while plasma cells and Treg were negatively associated with MCs (Figure 4D, Figure S4D, and Table S9), which was also validated in FUSCC_150 cohort (Figure S4E)."*
Changes in the manuscript: Page 8, Line 340:
*"The MAIT cell signatures (**Table S8**), derived from three independent studies [51-53], which demonstrated strong consistency in the assessment of MAIT cell infiltration (**Figure S4C**). MAIT*

*cell signatures showed a strong positive correlation with the IL-18$^+$ MC signature, suggesting that the infiltrating levels of these cells are closely linked (**Figure 4D** and **S4D, Table S9**). This finding was also validated in the FUSCC_150 cohort (**Figure S4C-S4D**). Conversely, plasma cells and Tregs exhibited a negative correlation with IL-18$^+$ MCs (**Figure 4D**, **Table S9**)."*

Line 324: *"In agreement with previous findings, MAIT cells ranked highest in the expression of both IL18R1 and IL18RAP (Figure 4F), which was validated by flow cytometry (Figure 4H and Figure S4F)."*

Changes in the manuscript: Page 8, Line 349:

*"As corroborated by previous studies, MAIT cells exhibited the highest expression levels of both IL18R1 and IL18RAP (**Figure 4F**), a finding further confirmed by flow cytometry (**Figure 4G, S4E-S4F**)."*

Line 565: *"The specific markers for these cell types could be found in Table."*

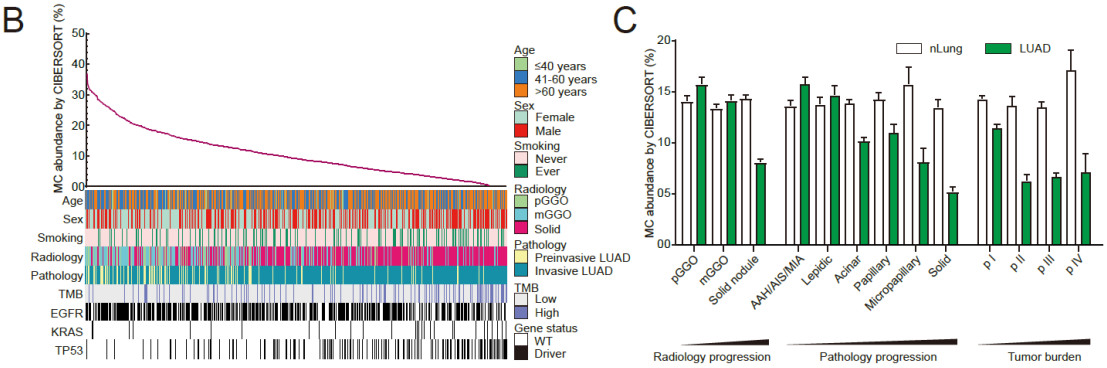
Changes in the manuscript: Page 15, Line 626:

*"A comprehensive list of the specific markers used for each cell type is provided in **Table S12**."*

Figure 1C: *"Fig. 1C: Mast cell percentage is shown, but percentage of what?"*

Response to Reviewer 2#' Comment 2:

In Figure 1C, we calculated infiltration abundance of MCs among tumor-infiltrating immune cells by the CIBERSORT algorithms.



Changes in the manuscript: Figure 1B and 1C:

Changes in the manuscript: Page 24, Line 1044:

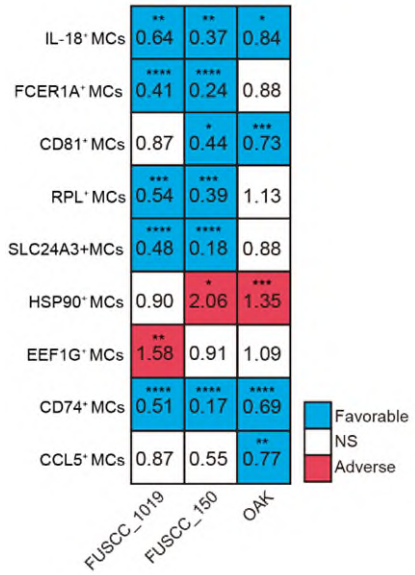
Figure 1B legend: *"The infiltration abundance of MCs among LUAD-infiltrating immune cells deconvoluted by CIBERSORT. The clinical features and mutation status for selected genes are shown at the bottom."*

Figure 1C legend: *"(C) The dynamic infiltration pattern of MCs during LUAD progression (white, nLung; green, LUAD). The infiltration abundance of MCs was deconvoluted by CIBERSORT. Data were presented as mean + SEM."*

Fig. 1G: *"Fig. 1G: What is the definition of a favorable and adverse outcome? Are the hazard ratios significant? If so, at what level?"*

Response to Reviewer 1#' Comment 2:

Thank you for your insightful comments regarding Fig. 1G. We have conducted statistical analyses to evaluate the significance of the hazard ratios. The hazard ratios colored by blue (favorable, HR<1) and red (adverse, HR>1) in Figure 1G, are statistically significant, with a p value <0.05, while the hazard ratios colored by white (NS) are statistically non-significant. Following your suggestion, each p value has been added in the Figure 1G.



Changes in the manuscript: Page 24, Line 1052, Figure 1G legend:

*"(G) The prognostic value of each MCs subset signature in the FUSCC_1019 cohort, the FUSCC-150 cohort, and OAK cohort, with numbers in each square showing the hazard ratio value. NS, not significant. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001."*

Fig. 2C: *"Fig. 2C: The labelling of some of the lanes appears wrong, some + should probably be -."*

Changes in the manuscript: Figure 2C:

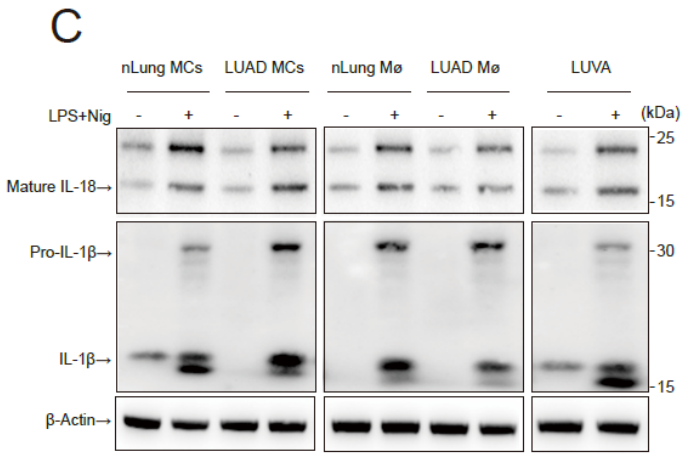


Fig. 4D: *"Fig. 4D: Is the labeling of the intensity scale correct?"*

Response to Reviewer 1#' Comment 2:

Thank you for your meticulous work, we have updated the incorrect labeling of the intensity scale.

Changes in the manuscript: Figure 4D:

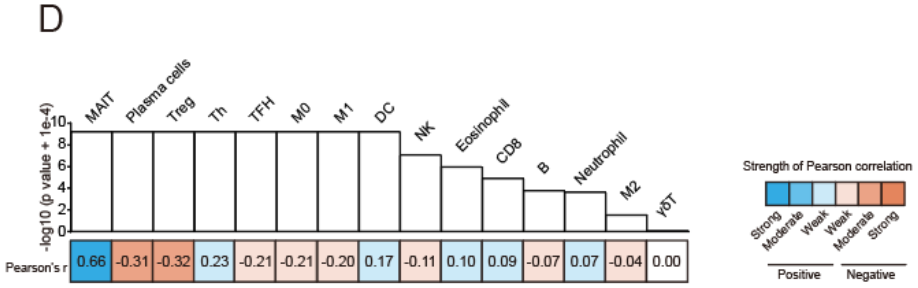
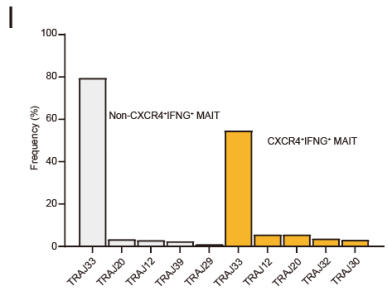


Fig. 5I: *"Fig. 5I: Which subset is shown in grey and which in yellow?"*

Changes in the manuscript: Figure 5I:



Changes in the manuscript: Page 27, Line 1154, Figure 5I legend:

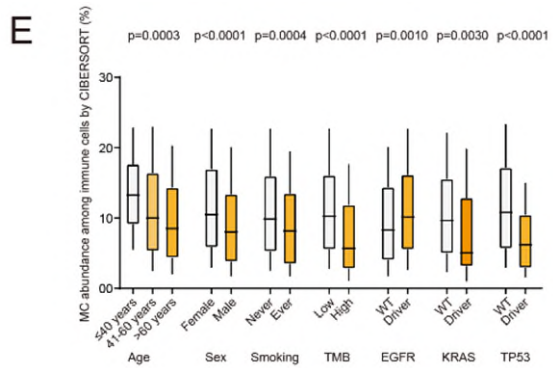
Figure 5I legend: *"(I) Percentage of top 5 TRAJ gene usage generated from CXCR4$^+$IFNG$^+$ MAIT cells (yellow) and non-CXCR4$^+$IFNG$^+$ MAIT cells (grey)."*

Fig. S1E: Fig. S1E: Mast cell percentage is shown, but percentage of what?

Response to Reviewer 1#' Comment 2 regrading Fig. S1E:

In Figure S1E, we calculated infiltration abundance of MCs among tumor-infiltrating immune cells by the CIBERSORT algorithms.

Changes in the manuscript: Figure S1E:



Changes in the supplementary materials: Figure S1E legend:

"(E) Quantification of MCs infiltration level according to clinic features by CIBERSORT in the FUSCC_1019 cohort. p values were determined by ANOVA test and two-tailed Student's t-test."

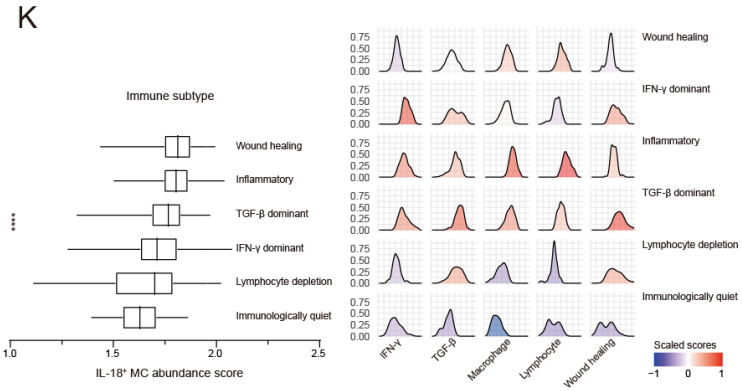
Fig. S2I: *"Fig. S2I: Which comparison yields p<0.0001? And what does the histograms and score show?"*

Response to Reviewer 1#' Comment 2 regrading Fig. S2I:

We performed the ANOVA test to compare the infiltration score of IL-18$^+$ MC subset across

different immune subtypes. The score showed the infiltration score of IL-18$^+$ MC subset in each LUAD sample calculated by single-sample Gene Set Enrichment Analysis (ssGSEA). The histogram showed the infiltration score of IL-18$^+$ MC subset in each LUAD sample across different immune subtype. The center of the boxes indicated the median value and the upper and lower bounds of the boxes indicated the 25th and 75th percentile of data.

Changes in the manuscript: Figure S2I (updated as Figure S2K):



Changes in the supplementary materials: Figure S2K legend:

*"(K) IL-18$^+$ MC infiltration abundance score of each LUAD sample in the FUSCC_1019 cohort across different immune subtypes. The scores were calculated by single-sample Gene Set Enrichment Analysis (ssGSEA) and the ANOVA test was performed. The center of the boxes indicated the median value and the upper and lower bounds of the boxes indicated the 25th and 75th percentile of data. ****: p<0.0001."*

Fig. S6D: *"Fig. S6D: Which panel shows which condition?"*

Changes in the manuscript: Figure S6D:

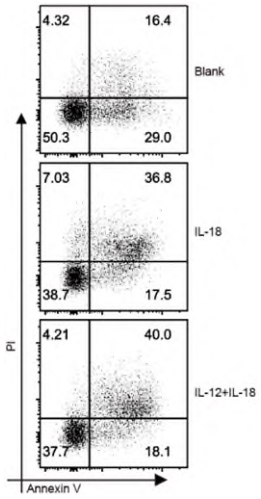
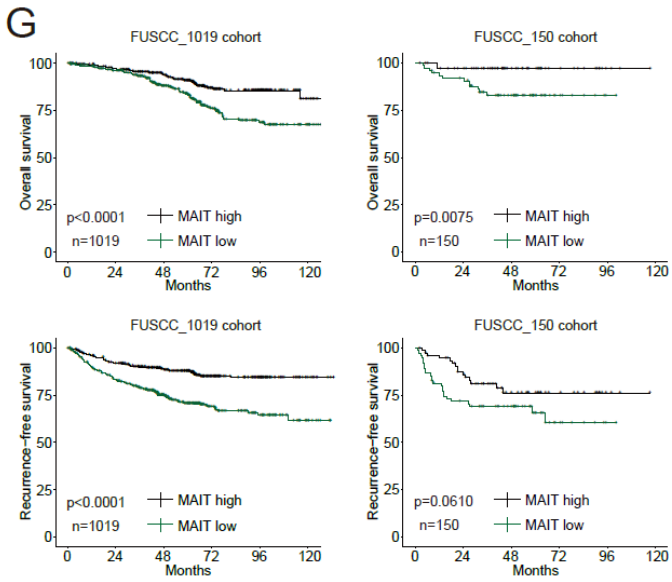


Fig. S6E: Fig. S6E: Which panel shows which cohort?

Changes in the manuscript: Figure S6E (updated as Figure S6G):



Reviewer 1#' Comment 3:

The introduction is very short and could be expanded, for example on the topic of mast cell function in the tumor microenvironment and on mast cell pyroptosis in general, outside of tumors.

Response to Reviewer 1#' Comment 3:

We thank the reviewer for the constructive feedback. In response, we have expanded the introduction to offer a more comprehensive overview of MC function in the tumor microenvironment. This includes a discussion of inflammatory mediators, cytokines, and chemokines released by MCs, as well as intricate crosstalk between MCs with other immune cells, tumor cells, and stromal cells. Additionally, we have highlighted the emerging role of MCs as potential therapeutic targets in cancer immunotherapy. Furthermore, we have broadened the scope of the discussion on mast cell pyroptosis outside of tumors, including anaphylaxis, Cryopyrin-associated periodic syndromes (CAPS), experimental autoimmune encephalomyelitis (EAE). These revisions strengthen the introduction and provide a clearer context for our study.

Changes in the manuscript: Page 3, Line 90:

"Mast cells (MCs), as evolutionarily conserved and ancient innate immune cells, are well-known for their culprit roles in IgE-mediated allergic disorders [1]. The specialized sentinel identity and potent immunomodulatory properties have sparked an increasing recognition of the importance of MCs in the context of tumor, extending beyond their traditional roles in allergies and parasitic infection [2-5]. The observation of robust infiltration of MCs across cancers has highlighted their involvement in the tumor microenvironment, such as tumor angiogenesis and microenvironment remodeling [6-8]. The exceptional advantage of MCs resides in high content of cytoplasmic electron-dense secretory granules containing substantial preformed inflammatory mediators, peptides, proteases, cytokines, and chemokines, which are closely associated with the effects that MCs have on the tumor microenvironment in many instances [9-11]. Moreover, the intricate crosstalk between MCs and other immune cells, tumor cells, and stromal cells within the tumor microenvironment has deepened our understanding of the complex function of MCs [12-15]. Given their versatile repertoires, unique differentiation and maturation trajectory occurring locally, and location proximity to blood vessels and periphery nerves, MCs present a compelling target for

therapeutic interventions in the tumor immunotherapy [16].

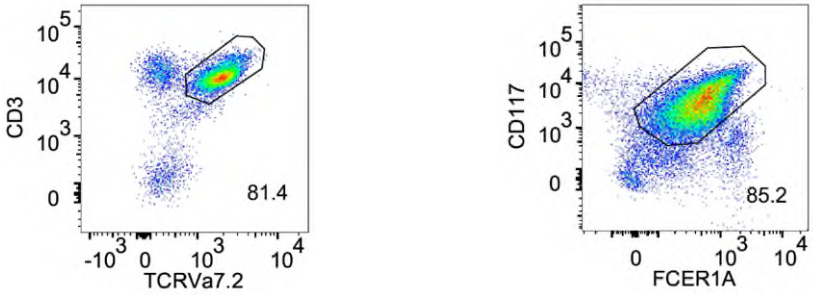
Inflammasomes are critical sentinels of the innate immune system, forming supramolecular complexes composed of a cytosolic sensor protein, an adaptor protein, apoptosis-associated speck-like protein containing a CARD (ASC), and a downstream effector protein, pro-caspase-1[17, 18]. The assembly of inflammasomes triggers the activation of caspase-1, leading to the maturation and secretion of proinflammatory IL-1β and IL-18, which in turn drive inflammation and pyroptosis, a form of inflammatory cell death [19, 20]. Most studies on inflammasomes have primarily focused on macrophages, dendritic cells, and certain subsets of T cells [21-24]. Previous researches have demonstrated that MCs can release appreciable amounts of IL-1β through NLRP3 inflammasome activation in various non-neoplastic conditions, including anaphylaxis, Cryopyrin-associated periodic syndromes (CAPS), experimental autoimmune encephalomyelitis (EAE) [25-29]. However, despite the recognized role of inflammasomes in tumor immunity [30, 31], no studies have yet investigated the involvement of inflammasome activation in MCs in the tumor microenvironment."

Reviewer 1#' Comment 4:

"Please indicate the purity of the different sorted cell populations used in the experiments."

Response to Reviewer 1#' Comment 4:

We thank the reviewer for the valuable suggestion. We have included detailed information regarding the purity of the sorted cell populations including MCs and MAIT cells. The purity of each sorted population was assessed by flow cytometry (left Figure, MAIT cells; right Figure, mast cells), and we have appended the corresponding purity percentages to the revised manuscript.



Changes in the manuscript: Page 17, Line 726:

"The purity of the sorted cell populations, including MAIT cells and MCs, exceeded 80%."

Reviewer 1#' Comment 5:

Please also define the Opal pairing with the different monoclonals used for immunofluorescence.

Response to Reviewer 1#' Comment 5:

Thanks for your kind suggestion! The details of the Opal fluorophores pairing with the different antibodies used for multiplex immunofluorescence have been provided in the Materials and Methods section.

Changes in the manuscript: Page 18, Line 786:

"*The following antibodies were used: TCRVa7.2 (BioLegend, Cat#351702, paired with Opal 690), Tryptase (BioLegend, Cat#B208014, paired with Opal 570), PanCK (Novus, Cat#IHC-M067, paired with Opal 650), NLRP3 (Proteintech, Cat#68102-1-Ig, paired with Opal 690), ASC (Santa Cruz, Cat#sc-514414, paired with Opal 620), Asp275 (cleaved Gasdermin D, CST, Cat#36425, paired with Opal 690), and IL-18 (Proteintech, Cat#60070-1-Ig, paired with Opal 520), along with*

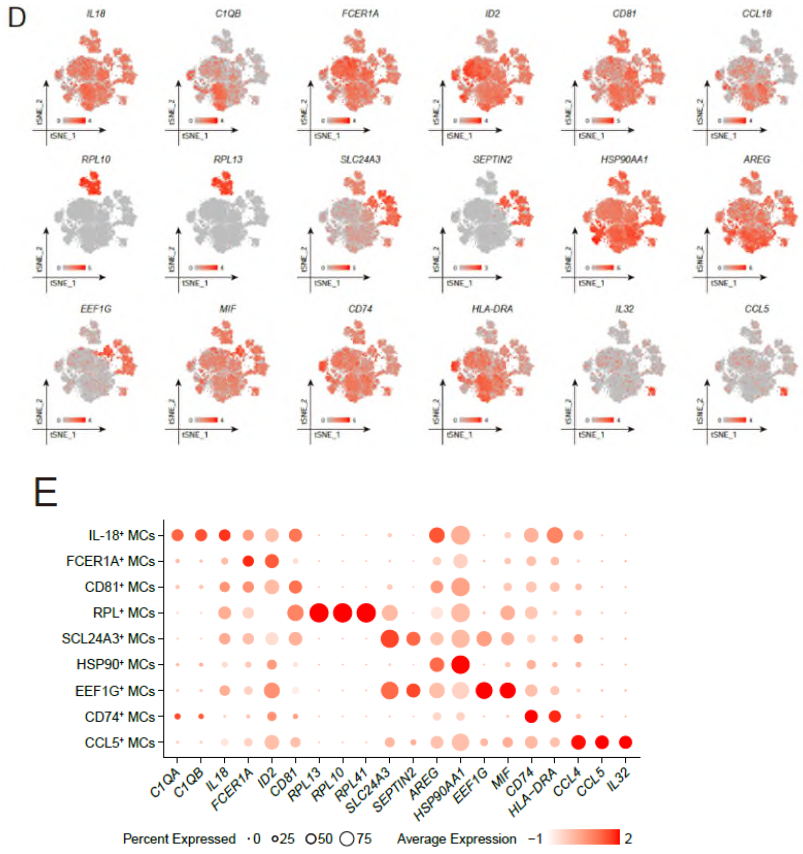
DAPI (BioLegend, Cat#422801). The signal for antibody complexes was visualized using corresponding Opal Fluorophore Reagents."

Reviewer 1#' Comment 6:

The single cell transcriptomic analysis yielded 9 mast cells clusters defined by their expression of certain molecules (line 157-158). It would be nice to see the expression of these respective markers in the t-SNE visualization of the clusters.

Response to Reviewer 2#' Comment 6:

We greatly appreciate the kind remind. In response to your suggestion, we have included the t-SNE visualization and bubble plot showing the expression of the respective markers in the Figure S2D-S2E.



Changes in the manuscript: Page 4, Line 170:

*"The unbiased clustering analysis revealed nine 9 distinct MCs subsets with different maturation and functional states: IL-18$^+$, FCERIA$^+$, CD81$^+$, RPL$^+$, SLC24A3$^+$, HSP90$^+$, EEF1G$^+$, CD74$^+$, and CCL5$^+$ MCs, according to the expression of canonical gene markers (**Figure 1D** and **S2D-S2E**, **Table S2**)."*

Changes in the supplementary materials: Figure S2D-S2E legends:

"(D) t-SNE plot showing the expression of the representative marker genes in each mast cells subset. (E) Dot plot for the expression of marker genes in each mast cells subset. Colors represent average expression of marker genes, and the size represents the percentage of cells expressing these genes."

Reviewer 1#' Comment 7:

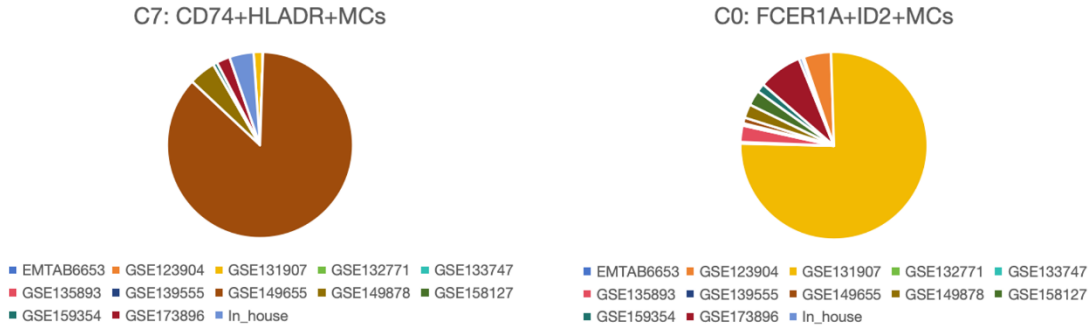
Several of the clusters in Fig. 2A are provide by cells from a single data set, such as the FcER1A-

MC, RPL-MC, and CD74-MC. I find this worrying, as they may not represent actual clusters present in most tumors, but the handling and/or analysis parameters of a certain study.

Response to Reviewer 1#' Comment 7:

Thanks for pointing this key point out. We also place great importance on this phenomenon. All scRNA-seq data from both public and in-house datasets underwent rigorous quality control and preprocessing before any downstream analysis. This process involved filtering out low-quality samples, such as those with a low proportion of mast cells, as they may compromise the stability of the analysis. Additional quality control measures included removing cells with abnormal gene expression levels (<= 200 or >= 5000 expressed genes) or excessive mitochondrial gene percentage (>=15%), ensuring that only high-quality cells were retained. The data were then normalized and scaled to harmonize gene expression levels. During the preprocessing of the data, we applied Harmony[1], a widely used algorithm for batch effect removal, with the default parameters.

Regarding the subpopulations you raised concerns about, we quantified the distribution of different MC subgroups across various states and observed distinct state preferences among the subgroups. The FCER1A^{+} MC and CD74^{+} MC subsets originate from multiple datasets. The RPL^{+} MC subset primarily comes from our in-house data. We believe that this phenomenon is reasonable based on the following considerations. (1) Unlike other types of immune cells that develop and mature in the bone marrow (BM) before migrating to the periphery, MCs leave the BM as progenitors rather that as circulating end-stage mature cells. MCs completed their development and maturation in the periphery tissues. Thus, the function and transcriptional signature of MCs are largely shaped by the local microenvironment. (2) In contract to public datasets, our own in-house single-cell datasets came from early-stage lung cancer, especially indolent GGO-featured LUAD. (3) Indeed, due to the inherent challenge of fully distinguishing biological variation from batch effects[2], it is difficult to entirely eliminate batch effects in scRNA-seq analyses. After applying Harmony for batch effect correction and integrating the existing statistical results, we believe that batch effects have been effectively controlled.



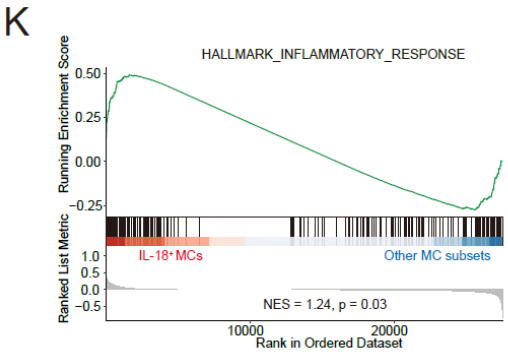
Reviewer 1#' Comment 8:

In line 189-190, what is meant by IL-18- mast cells (as I assume the sentence is supposed to mean)? All other clusters but IL18+-MC, or on the single cell level comparing actual IL-18+ and IL-18- mast cells?

Response to Reviewer 1#' Comment 8:

Thank you for your thoughtful question. We apologize for the lack of clarity in this regard. We conducted the comparison between IL-18^{+} MC subset and other MC subsets through Gene Set Enrichment Analysis. We have revised both the text and Figure 1K to enhance clarity and accuracy, in response to your feedback. We appreciate your keen observation, which has helped us improve

the precision of our manuscript.



Changes in the manuscript: Page 5, Line 204:

*"A direct comparison between IL18$^+$ MCs and other MC subsets revealed significantly enhanced proinflammatory signatures in IL18$^+$ MCs, as evidenced by Gene Set Enrichment Analysis (**Figure 1K**)."*

Reviewer 1#' Comment 9:

Fig. 2E shows NLRP1 and NLRP3 expression in LUVA cells, contrary to the text (line 236-237) that states that there is a selective expression of NLRP3.

Response to Reviewer 1#' Comment 9:

Thank you for your careful review and insightful feedback. We confirm that both *NLRP1* and *NLRP3* are expressed in LUVA cells, as shown in the **Figure 2E**. We have revised the manuscript to accurately reflect this observation and have corrected the relevant text accordingly. Once again, we appreciate your attention to this matter.

Changes in the manuscript: Page 6, Line 253:

*"Transcriptomic analysis of LUVA cells further confirmed high expression of NLRP3 and NLRP1, with no other members of the inflammasome sensor family detected (**Figure 2E**)."*

Reviewer 1#' Comment 10:

In Fig. 3A, the percentage of FLICA+ cells is very similar in healthy lung and tumor tissue, still, the text states that it is higher in the tumors (lines 253-256).

Response to Reviewer 1#' Comment 10:

Thank you for your careful review of our manuscript. We appreciate your observation regarding the discrepancy between the data presented in Fig. 3A and the statement in the text. We acknowledge the error in describing the proportion of FLICA$^+$ MCs in the representative graph, instead of the mean value of FLICA$^+$ MCs ratio. We have corrected the statement regarding the proportion of FLICA$^+$ MCs from nLung and LUAD samples.

Changes in the manuscript: Page 7, Line 269:

*"In our study, we identified approximately 12.2% and 12.3% activated FLICA$^+$ (fluorochrome-labeled inhibitor of caspases assay) MCs from nLung and LUAD (**Figure 3A** and **S3E**)."*

Reviewer 1#' Comment 11:

In the discussion, it is stated that "no research has ever focused on the heterogeneity and function of MCs in the tumor microenvironment": This is simply not true, see for example these recent reviews PMID: 38462035, 38323271, 37852570.

Response to Reviewer 1#' Comment 11:

Thank you for your insightful comments. We appreciate you bringing the recent reviews (PMID: 38462035, 38323271, 37852570) to our attention. We also elucidated the heterogeneity and function of MCs in GGO-featured lung adenocarcinoma[3]. We have revised the relevant statement in the manuscript accordingly. We are grateful for your valuable comments, which has significantly improved the accuracy and depth of our manuscript.

Changes in the manuscript: Page 11, Line 480:

"*Despite significant advances in the understanding of tumor-infiltrating innate immune compartments, including macrophages and neutrophils [59], the heterogeneity and functions of MCs within the tumor microenvironment have received limited attention*."

Reviewer 1#' Comment 12:

Likewise, the statement that "the characteristics of MAIT cells in lung adenocarcinoma remain unveiled" is also not correct. See PMID 36657812 and 36435063. In contrast, the reference given on line 311, ref 51, does not contain information on lung adenocarcinoma, as the authors claim.
CD69 on MAIT cells from tumor tissue is regarded as an activation marker but should rather be seen as a marker of tissue residency.

Response to Reviewer 1#' Comment 12:

(1) Thanks for your insightful comments and for pointing out the references related to the characteristics of MAIT cells in lung adenocarcinoma (PMID 36657812 and 36435063). We apologize for the inaccurate statement of regarding MAIT cells characteristics in lung adenocarcinoma. We have now revised the statement in the manuscript, incorporating the references you provided.

Changes in the manuscript: Page 12, Line 501:

"Alterations in MAIT cell frequency, activation status, and cytotoxicity within tumor microenvironment have been well-documented, including lung cancers [52, 57, 62-64]. However, the characteristics cytokine-activated MAIT cells in LUAD, as explored through multi-omics approaches, remain insufficiently defined."

(2) Regarding the reference on line 311, ref 51, we appreciate your clarification. Upon further review, we recognize that this reference does not specifically address lung adenocarcinoma, as we initially claimed. In fact, what we want to claim in this context is that, in contrast to murine models, MAIT cells represent the predominant population of innate-like T cells in humans. We have corrected this statement in the manuscript and removed the reference.

Changes in the manuscript: Page 8, Line 336:

*"~~In agreement with previous findings [44],~~ Flow cytometric analysis revealed that MAIT cells were the predominant innate-like T cell subset in LUAD, while γδT cells and NKT cells were relatively scarce (**Figure 4C and S4B**)."*

(3) We agree with your point that CD69 on MAIT cells is more accurately described as a marker of tissue residency. Although CD69 can also be considered an early-activation marker of T cells, we acknowledge that, in the context of tumor tissue, CD69 is more appropriately considered a marker of tissue residency rather than a definitive activation marker. We have updated this statement in the manuscript. We are grateful for your helpful suggestions, which have allowed

us to improve the clarity and accuracy of our manuscript.

Changes in the manuscript: Page 9, Line 372:

*"A comprehensive analysis of the transcriptional signatures and differential gene expression revealed that the CXCR4^{+}IFNG^{+} MAIT cells, the predominant subcluster in LUAD, were distinguished by heightened expression of activation markers, including ICOS ~~and CD69,~~ and IFN-γ (**Figure S5C**)."*

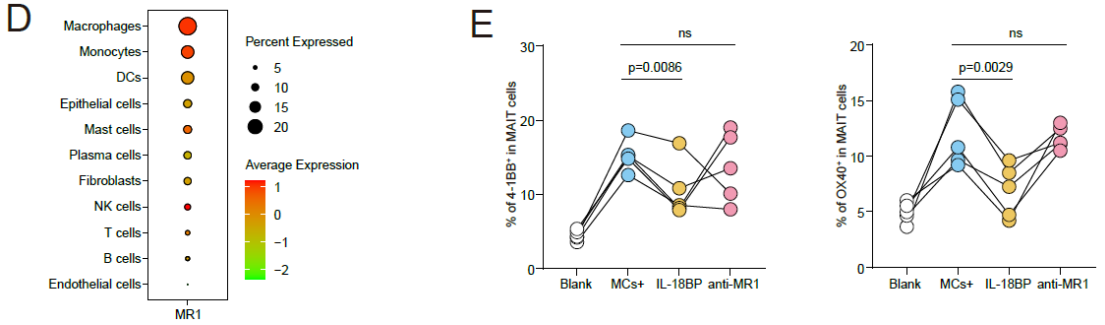
Reviewer 1#' Comment 13:

In the discussion, the authors claim that mast cells induce MAIT cell activation in a TCR-independent way (line 415-416). When this may be likely, it has not been tested by i.e. blocking MR1 interactions with the TCR, and thus the statement would have to be tempered.

Response to Reviewer 1#' Comment 13:

We thank the reviewer for this insightful comment. Regarding the mechanism of MCs-mediated activation of MAIT cells, we perform additional analyses and experiments to further investigate it. We first assessed the expression of MR1 on various types of cells in lung adenocarcinoma. We found that macrophages expressed the highest level of MR1, while the expression level of MR1 on MCs was similar to plasma cells, fibroblasts and NK cells (Figure S6D). Then, we tested the activation mechanism of MAIT cells through in vitro experiments by adding MR1 (anti-MR1 antibody, Biolegend, 361110) or IL-18 inhibitors (IL-18BP, MCE, HY-P7211). IL-18BP effectively inhibits MCs-mediated activation of MAIT cells, as evidenced by the reduced expression of 4-1BB and OX40, while blocking MR1 does not notably impact MCs-mediated activation of MAIT cells (Figure S6E). Collectively, these results demonstrate that the activation of MAIT cells mediated by MCs is largely dependent on cytokine signaling. Moreover, the potential of MCs to function as antigen-presenting cells (APCs), particularly in presenting MR1-specific antigens to MAIT cells, requires further investigation.

Changes in the manuscript: Figure S6D and S6E:



Changes in the manuscript: Page 10, Line 427:

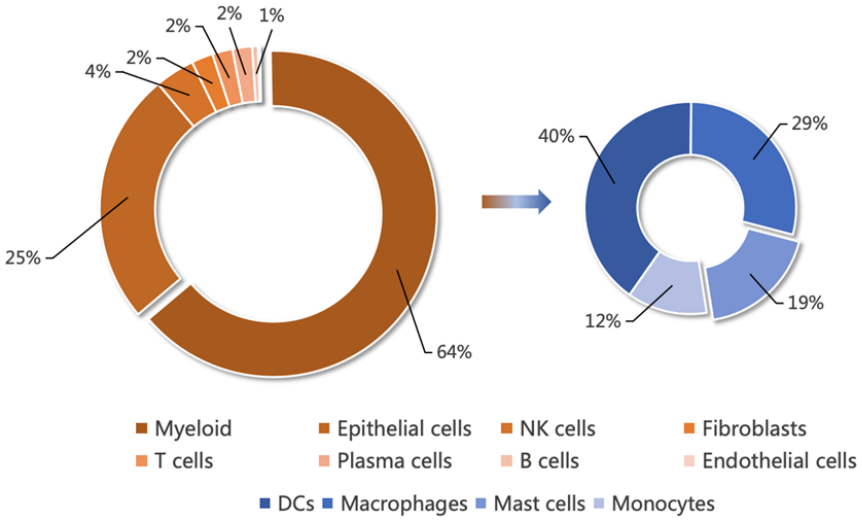
*"To determine whether MCs-mediated activation of MAIT cells is IL-18-dependent or MR1-dependent, we first assessed the expression of MR1 across various types of cells in LUAD. We found that macrophages exhibited the highest levels of MR1 expression, whereas the expression of MR1 on MCs was low, which was comparable to that on plasma cells, fibroblasts, and NK cells (**Figure S6D**). Then, we conducted in vitro blocking experiments using MR1 or IL-18 inhibitors (IL-18BP). IL-18BP effectively inhibited MCs-mediated activation of MAIT cells, as demonstrated by the reduced expression of 4-1BB and OX40. In contrast, blocking MR1 did not significantly affect MCs-mediated activation of MAIT cells (**Figure S6E**)."*

Reviewer 1#' Comment 14:

The authors show that mast cell-derived IL-18 activates MAIT cells, but the discussion should also take into account other sources of IL-18 in the tumor microenvironment (for example macrophages and monocytes), which may be more numerous than mast cells.

Response to Reviewer 1#' Comment 14:

Thank for your insightful suggestions! We have assessed the expression of IL-18 in each cell type within the tumor microenvironment in **Figure 2A**. Following your kind advise, we calculated the contribution of each cell type within the microenvironment to IL-18 secretion by multiplying the cell subpopulation counts by the average expression level of the *IL18* gene. Our analysis revealed that, aside from MCs, macrophages also expressed significant amounts of *IL18*. We will include this information in the discussion section. Once again, we sincerely appreciate your suggestion, which has greatly enhanced the completeness of our study.



Changes in the manuscript: Page 12, Line 490:

"Besides, it is important to note that macrophages may serve as a significant source of IL-18 through inflammasome activation, potentially outnumbering MCs within the tumor microenvironment. Our study emphasizes the identification of inflammasome activation in MCs, which can nonspecifically activate MAIT cells via IL-18 secretion, thereby providing a valuable supplement in this field."

Reviewer #2 (Remarks to the Author)

The paper entitled "Mast cells boost anti-tumor potency of MAIT cells via inflammasome activation" is informative, but there are some concerns that need to be addressed as follows.

Major Concerns

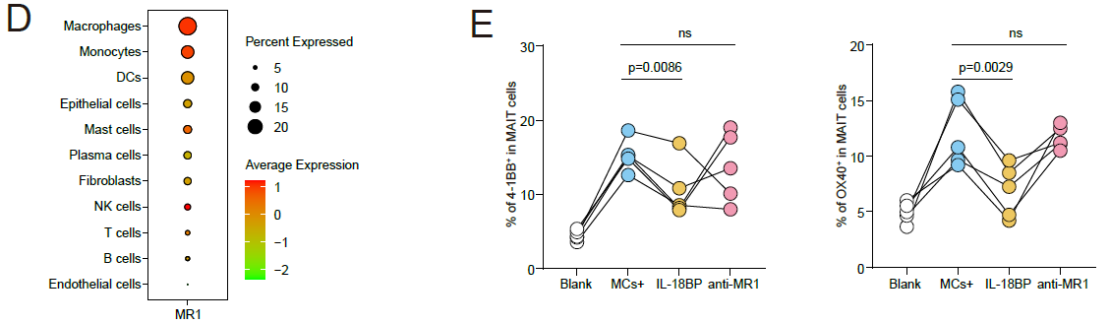
The author showed that mast cells (MCs)-derived IL-18 enhances the innate-like anti-tumor effects of mucosal-associated invariant T (MAIT) cells through the cytokine activation pathway, however, is there any possibility that MAIT cells are triggered by a tumor-derived antigen presented via IL-18+MCs?

Response to Reviewer 2#' Comment 1:

We sincerely appreciate your recognition of our work and the insightful feedback you have provided.

The questions you have raised are thought-provoking and associated with fundamental principles in immunology. Regarding the mechanism of MCs-mediated activation of MAIT cells, we perform additional analyses and experiments to further investigate it. We first assessed the expression of MR1 on various types of cells in lung adenocarcinoma. We found that macrophages expressed the highest level of MR1, while the expression level of MR1 on MCs was similar to plasma cells, fibroblasts and NK cells (Figure S6D). Then, we tested the activation mechanism of MAIT cells through in vitro experiments by adding MR1 (anti-MR1 antibody, Biolegend, 361110) or IL-18 inhibitors (IL-18BP, MCE, HY-P7211). IL-18BP effectively inhibits MCs-mediated activation of MAIT cells, as evidenced by the reduced expression of 4-1BB and OX40, while blocking MR1 does not notably impact MCs-mediated activation of MAIT cells (Figure S6E). Collectively, these results demonstrate that the activation of MAIT cells mediated by MCs is largely dependent on cytokine signaling. Moreover, the potential of MCs to function as antigen-presenting cells (APCs), particularly in presenting MR1-specific antigens to MAIT cells, requires further investigation.

Changes in the manuscript: Figure S6D and S6E:



Changes in the manuscript: Page 10, Line 427:

*"To determine whether MCs-mediated activation of MAIT cells is IL-18-dependent or MR1-dependent, we first assessed the expression of MR1 across various types of cells in LUAD. We found that macrophages exhibited the highest levels of MR1 expression, whereas the expression of MR1 on mast cells was low, which was comparable to that on plasma cells, fibroblasts, and NK cells (**Figure S6D**). Then, we conducted in vitro blocking experiments using MR1 or IL-18 inhibitors (IL-18BP). IL-18BP effectively inhibited MCs-mediated activation of MAIT cells, as demonstrated by the reduced expression of 4-1BB and OX40. In contrast, blocking MR1 did not significantly affect MCs-mediated activation of MAIT cells (**Figure S6E**)."*

Reviewer 2#' Comment 2:

Contrary to mucosal cancers like lung adenocarcinoma (LUAD), gastric cancer GC) and colorectal cancer (CRC), the frequency of tumor-infiltrating MAIT cells in hepatocellular carcinomas (HCC) is known to decrease.

In this case, what makes the difference in the levels of MCs-derived IL-18 between mucosal and nonmucosal cancers?

Response to Reviewer 2#' Comment 2:

We appreciate the reviewer's thoughtful question. It is very interesting to observe the differential frequency of tumor-infiltrating MAIT cells between mucosal and non-mucosal cancers. Our laboratory reported the decreased infiltration and impaired function of MAIT cell in hepatocellular carcinomas (HCC)[4]. Similarly, the infiltration of MCs was mainly observed in mucosal cancers, like lung cancer, gastric cancer, and colorectal cancer, while the infiltration of MCs in non-mucosal

cancers, like HCC, was scarce[5].

Regarding the difference in MC-derived IL-18 levels between mucosal and non-mucosal cancers, we propose that the following factors may contribute to this phenomenon. (1) Distinct tissue-specific microenvironment and cytokine profiles between mucosal and non-mucosal tissues. For example, MCs-favor cytokines, SCF, IL-3, IL-4, IL-13 may be enriched in the mucosal tissues. (2) MCs are predominantly localized in mucosal tissues, including airways, the gastrointestinal and genitourinary tracts, not non-mucosal tissues. (3) Mucosal tissues, in particular respiratory tracts, are localized at the host-environment interface, which results in high local inflammatory burden and enhanced of recruitment and activation of MCs as well as other immune cells, due to exposure to various respiratory pathogens and external stimuli. Inflammatory microenvironment could further induce MC inflammasome activation and IL-18 secretion. (4) The heterogeneity of MC subsets between mucosal and non-mucosal tissues. MCs can be categorized into mucosal MCs (MC_T, expressing tryptase alone, located in the mucosal tissues) and connective tissue MCs (MC_{TC} subset, expressing both tryptase and chymase, located in the connective tissues). We speculate that distinct composition of MC subsets between mucosal and non-mucosal cancers may also account for the difference in the levels of MCs-derived IL-18.

Reviewer 2#' Comment 3:

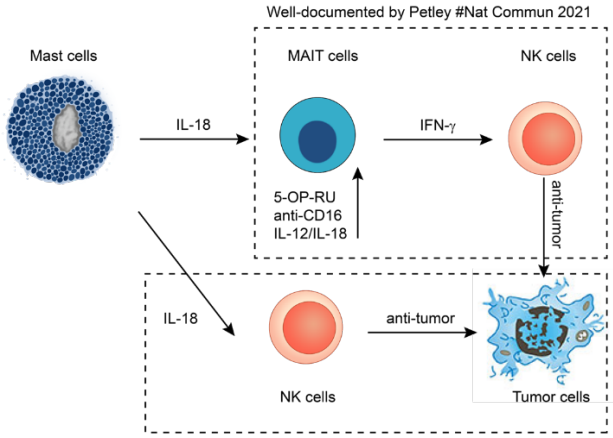
A recent report has shown that MAIT cells enhance the anti-tumor function of NK cells in an IFN-γ-dependent manner.

We therefore suggest that the author investigates whether MCs-derived IL-18 augmented MATT cells regulate NK cell-mediated tumor immunity.

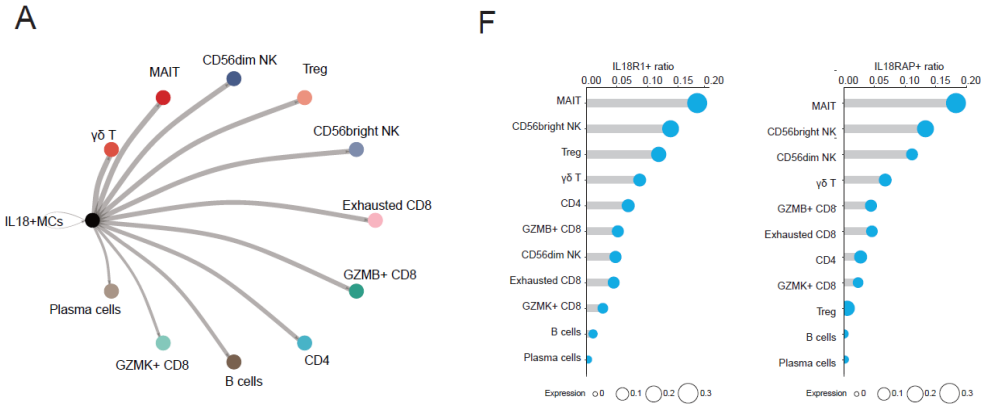
Response to Reviewer 2#' Comment 3:

Thank you for your insightful suggestion. We also noticed this interesting study on the enhance of NK cell-mediated anti-tumor immunity by MAIT cells via an IFN-γ-dependent mechanism. This study detailed the MAIT-IFNγ-NK axis in the context of tumor immunology. The well-documented MAIT-IFNγ-NK axis by Petley was validated both in vivo and in vitro. Moreover, these results were robustly observed through different MAIT cell activation ways, including 5-OP-RU, anti-CD16, and IL-12/IL-18. In our study, we confirmed that MCs-derived IL-18 augmented the secretion of IFN-γ in MAIT cells. It is reasonable that MAIT cells could enhance the anti-tumor function of NK cells via IFN-γ secretion. We believed this research served as a valuable addition to MC-IL-18-MAIT axis and diversified the repertoire of MAIT cells in tumor immunology. Thus, it is unnecessary to repeated the founding that MAIT cells could enhance the anti-tumor role of NK cells via IFN-γ. We appreciate your suggestion and have addressed this detailly in the discussion section. Moreover, it is hard to perform in vitro experiments to mimic the MCs-IL-18-MAIT-IFNγ-NK-tumor axis based on the following considerations. This axis involves four types cells and several cytokines or mediators. Both mast cells, MAIT cells, and NK cells are not abundant in periphery

blood and tumor samples.



Moreover, IL-18, which was also known as IFN-γ-inducing factor, augments an array of effector lymphocytes, such as NK and $CD8^+$ T cells[6]. We believed mast cell-derived IL-18 could directly enhance the IFN-γ expression and anti-tumor function of NK cells (above illustration). In our study, we found that $IL\text{-}18^+$ MCs also interacted closely with NK cells based on the ligand-receptor crosstalk analysis (Figure 4A). Moreover, NK cells also express high levels of IL-18 receptors (Figure 4F).



Besides, the infiltration of NK cells is scarce, accounting for only 1-2% of the immune cells on average from our previous study. We believed that MAIT-derived IFN-γ may be likely to enhance the cytotoxicity of other cells, such as CTLs.

Changes in the manuscript: Page 12, Line 511:

"Activated MAIT cells upregulated IFN-γ, which was reported to enhance the anti-tumor function of NK cells [70]. Further endeavors are warranted to examine the interplay between MAIT cells and other immune cell types, in particular NK cells and MCs, as these insights may pave the way for innovative MAIT-centered immunotherapies [70-72]. For instance, in vitro stimulation of MAIT cells using IL-18 or MCs prior to CAR-MAIT delivery may represent a promising therapeutic strategy [73]. Moreover, in our study, we found that $IL\text{-}18^+$ MCs also interacted closely with NK cells and NK cells also express high levels of IL-18 receptors (IL18R1 and IL18RAP). We speculated that MCs-derived IL-18 could also augment the activation and cytotoxicity of NK cells, in addition to MAIT cells."

Reviewer #3 (Remarks to the Author):

Reviewer 3#' Comment 1:

The manuscript by Haiquan Chen et al. presents an analysis of mast cells in non-small cell lung cancer and their implications for the activation of MAIT cells in the context of tumor immunology. While the authors state to present a comprehensive mast cell "atlas" in lung cancer with 9 MC subsets, they focus almost exclusively on IL-18+ mast cells. While this is an interesting subset, their claim to present an atlas would deserve in my opinion a more detailed appreciation of the other 8 subsets, and their associations with clinico-pathological data although I understand that the data set may not be sufficiently clinically annotated and large enough to performa survival analyses for all subsets. The authors do show that mast cell counts are negatively correlated with increasing tumor aggressiveness as defined by a shift from precursor lesions, in-situ carcinoma and fully invasive lung cancer.

The authors also provide data on several aspects of IL-18 associated mechanisms in mast cells such as NLRP3 inflammasome activation and GSDMD expression. The authors then establish a connection of IL-18+ MCs to the activation of MAIT cells which belong to the innate immune system and have been shown to exert anti-tumor immunity. Their in vitro data on the IFNg stimulation and MAIT cell activation through IL-18 and MCs appears convincing. However, while the MC-IL18-MAIT axis can in my opinion be derived from the presented data, I see a major limitation of the study in the translation of the results in terms of their clinical interpretation. While the authors start showing a negative correlation of MC density and tumor aggressiveness in their MC lung cancer atlas, they finish by stating that MAIT cell positively correlated with overall and recurrence free survival. If the axis they propose is right, larger MC numbers should lead to MAIT cell activation. However, MC numbers are increased in less aggressive tumors (precursors, in situ lesions) according to their results which leads to the trivial conclusion that increasing MC-MAIT-cell activity is associated with improved survival. The authors should perform a normalization to demonstrate that the effects on survival are independent of tumor subtype/stage to make the paper a strong candidate for Nature Communications.

Response to Reviewer 3#' Comment 1:

Thank you for your thorough and constructive review of our manuscript. We greatly appreciate your insights, which will undoubtedly help improve the clarity and rigor of our study.

(1) We agree that a more detailed discussion of the other MC subsets and their associations with clinic-pathological data would strengthen the manuscript. Due to the limitations in the clinical annotations (external data) and the size of our dataset, we were unable to conduct comprehensive survival analyses and fully explore the clinic-pathological associations of all MC subsets. However, we have tried our best to explore the characteristics of other 8 MC subsets, including visualization of marker genes, function and pathway enrichment analysis, cytokine and chemokine profiling, and metabolic activity analysis (Figure 1E, 1F, 1J, S2E, S2J). Among other 8 MC subsets, the chemotactic $CCL5^+$ MC subset has been extensively studied in our previous work[3], while the antigen-presenting $CD74^+$ MC subset has been introduced by other researches[7]. Larger and more comprehensively annotated datasets are required for the exploration and validation of the remaining MC subsets.

(2) As you rightly pointed out, our data suggest that MC density is higher in precursor lesions and

in situ carcinomas, while it decreases in more invasive tumors. This creates a paradox when correlating MC density with survival, as less aggressive tumors show higher MC counts. To address this concern, we included a more detailed analysis to account for tumor stage and other clinical and pathological features in the survival analyses. Specifically, we performed multivariate analyses incorporating MC-MAIT, sex, age, smoking history, pathological TNM stage by Cox regression model. This analysis demonstrated that the observed associations between MCs and survival are independent of tumor stage, as suggested. This will clarify that the positive correlation between MCs and survival is not confounded by tumor stage, and will strengthen the clinical interpretation of the MC-MAIT axis (Figure S6H).

Multivariate analysis for overall survival of LUAD (Cox Regression model)

Feature	HR	95% CI	p Value
MC	0.019	0.001-0.397	0.011
MAIT cells	0.502	0.166-1.519	0.223
Sex			
Male	1.162	0.792-1.707	0.443
Female	1.000		
Age[a]	1.031	1.013-1.050	0.001
Smoking			
Smoker	1.000		
Nonsmoker	0.772	0.511-1.165	0.218
Stage			
AAH/AIS.MIA	1.000		
Stage I	4.515	1.086-18.779	0.038
Stage II	8.078	1.749-37.316	0.007
Stage III	21.203	5.042-89.153	<0.001
Stage IV	27.800	5.519-149.789	<0.001

[a]Calculated as continuous valuable.
HR, hazard ratio; CI, confidence interval;

We hope that these revisions will address your concerns and improve the clinical relevance of the manuscript. We are committed to ensuring that the results are presented in the most robust and interpretable manner.

Changes in the manuscript: Page 11, Line 444:

*"Furthermore, multivariate analyses by Cox regression model revealed that MCs infiltration was a significant prognostic factor for overall survival of patients with LUAD, which was independent of tumor stage (**Figure S6H**)."*

Reviewer 3#' Comment 2:

Specific comments:

p. 3. LUAD can be distinguished indolence from invasiveness[27] ?

Response to Reviewer 3#' Comment 2:

We sincerely appreciate that you pointed this out. LUAD can be characterized by varying degrees of aggressiveness, with some cases exhibiting indolent behavior while others showing invasive and metastatic potential. In clinical practice, ground-glass opacity (GGO) featured LUAD in radiology or atypical adenomatous hyperplasia (AAH), adenocarcinoma in situ (AIS), minimally invasive adenocarcinoma (MIA), lepidic growth in pathology are indolent. Studies from our center or others showed that these indolent LUAD can remain stable for a long time without progression. Radiologic pure-GGO featured LUAD and pathological AIS/MIA have no recurrence during 10-year follow-up after resection[8-10]. These indolent LUAD can be cured by surgery. To clarify, we have revised the sentence to better reflect the current understanding in the field. We hope this revision resolves the ambiguity and provides a clearer understanding.

Changes in the manuscript: Page 4, Line 143:

"LUAD can be characterized by varying degrees of aggressiveness, with some cases exhibiting indolent behavior while others show invasive and metastatic potential [34]. Indolent LUAD typically remains stable for extended periods without progression, often manifesting as ground-glass opacity (GGO) in radiological evaluations, which may be associated with pathological conditions such as atypical adenomatous hyperplasia (AAH), adenocarcinoma in situ (AIS), minimally invasive adenocarcinoma (MIA), and lepidic adenocarcinoma [35, 36]."

Reviewer 3#' Comment 3:

p. 3. What do the authors mean by "preference of MCs", to me the data look as if there is no association between MCs and any of the shown parameters apart from radiology and pathology.

Integrating MCs infiltration profiling with genomic, 127 radiologic, pathologic, and clinical features revealed a remarkable variety and preference of MCs 128 distribution (Figure 1B)

Percentage? Decrease of MCs with increasing solidity of tumors.

Response to Reviewer 3#' Comment 3:

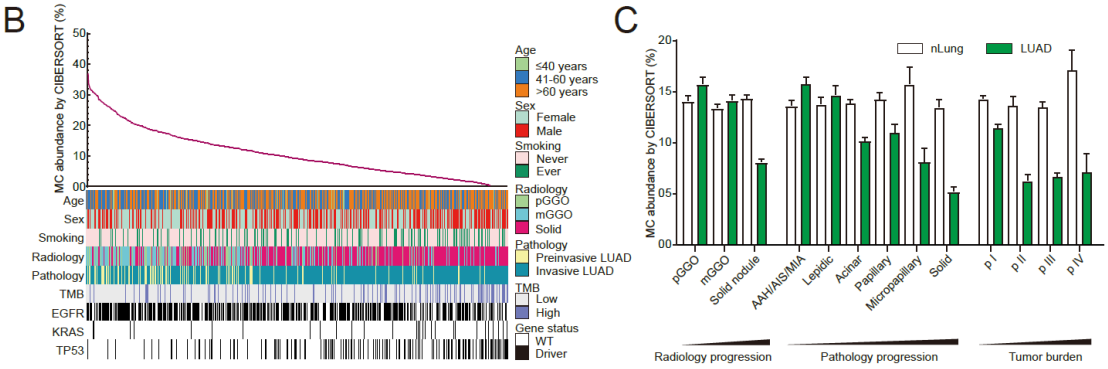
(1) Thank you for your thoughtful comment and for carefully reviewing our data. When we refer to the "preference of MCs," we are describing the observed variation in MCs distribution across different tumor characteristics, as revealed by our integrated analysis. Specifically, the term "preference" is used to emphasize the distinct patterns of MC infiltration, particularly the enriched presence of MCs in indolent LUAD subtypes.

To improve precision, we have revised the relevant section to specify that we are referring to the distribution patterns of MCs in relation to different tumor characteristics, including radiological and pathological features.

Changes in the manuscript: Page 4, Line 136:

"Integrating MCs infiltration profiling with genomic, radiologic, pathologic, and clinical features revealed significant variability in the distribution of MCs, which showed distinct patterns in relation to radiological and pathological characteristics (Figure 1B)."

(2) Regarding your point about the percentage of MCs decreasing with increasing tumor solidity, we deconvoluted the infiltration abundance of MCs among tumor-infiltrating immune cells by the CIBERSORT algorithms. We have revised it in the Figure and Figure Legend.



Changes in the manuscript: Page 24, Line 1044; Figure 1B-1C legend:

"(B) The infiltration abundance of MCs among LUAD-infiltrating immune cells deconvoluted by CIBERSORT. The clinical features and mutation status for selected genes are shown at the bottom. (C) The dynamic infiltration pattern of MCs during LUAD progression (white, nLung; green, LUAD). The infiltration abundance of MCs was deconvoluted by CIBERSORT. Data were presented

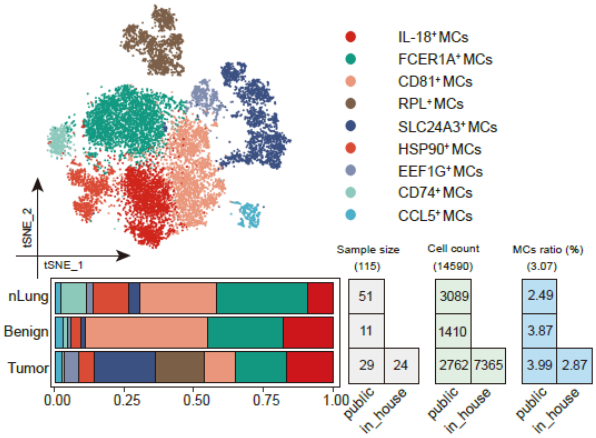
as mean + SEM."

Reviewer 3#' Comment 4:

What do the authors mean by: we generated 24 in-house single-cell RNA sequencing (scRNA-seq) "data". Does this refer to the numbers of tumors?

Response to Reviewer 3#' Comment 4:

Thank you for your insightful comment. To clarify, "*we generated 24 in-house single-cell RNA sequencing (scRNA-seq) data*", is referring to 24 individual tumor samples that were subjected to scRNA-seq analysis (Figure 1D). We have revised the manuscript to make this statement clearer for the reader.



Changes in the manuscript: Page 4, Line 161:

*"To construct a comprehensive single-cell transcriptome atlas for infiltrating MCs, we generated in-house single-cell RNA sequencing (scRNA-seq) dataset (n=24) and integrated them with published scRNA-seq data (**Figure 1D** and **S2A**)."*

Reviewer 3#' Comment 5:

p. 7: complicated sentence, not clear: However, unique aspects of IL-18 304 biology in potent IFN-g inducer and preferential activation of cytotoxic lymphocytes prompted us 305 to explore the function mechanism of IL-18-producing MCs, as MCs were the main cellular source 306 of IL-18 in LUAD.

Response to Reviewer 3#' Comment 5:

Thank you for your valuable feedback. We agree that the original sentence may have been somewhat complex and unclear. To enhance clarity and precision, we have revised the sentence as follows: "Unique feature of IL-18 in IL-1 family cytokine is the potent ability to induce IFN-γ production by cytotoxic lymphocytes. We then sought to investigate the functional mechanisms associated with IL-18-producing MCs, which were a major source of IL-18 in LUAD."

Changes in the manuscript: Page 8, Line 328:

"Unique feature of IL-18 in IL-1 family cytokine is the potent ability to induce IFN-γ production by cytotoxic lymphocytes. We then sought to investigate the functional mechanisms associated with IL-18-producing MCs, which were a major source of IL-18 in LUAD."

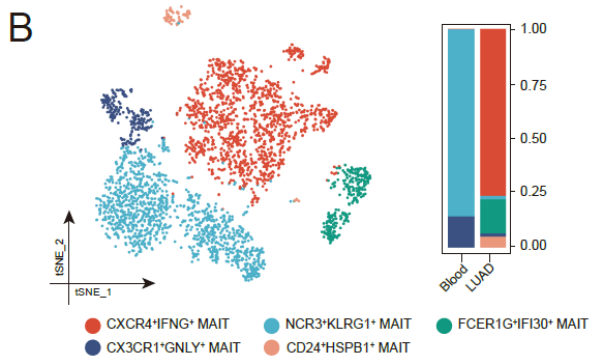
Reviewer 3#' Comment 6:

p. 8. unclear, what does "one tissue" mean in this context: Clusters largely contained cells from one

tissue, suggesting the 335 imprint of local tumor microenvironment.

Response to Reviewer 3#' Comment 6:

Thank for your comment. We agree that the phrase "one tissue" should be clarified. In this context, "one tissue" refers to MAIT cell clusters derived from a single tissue type (blood or tumor). We hypothesize that this may be due to the influence of the tumor microenvironment on the transcriptional profile of MAIT cells. We have revised this statement in the manuscript to clarify this point. The updated sentence: *"Most MAIT cell clusters were primarily composed of cells from a single tissue type, which may be due to the influence of the local tumor microenvironment on MAIT cell transcriptional signature."*



Changes in the manuscript: Page 9, Line 363:

"Most MAIT cell clusters were primarily composed of cells from a single tissue type, which may be due to the influence of the local tumor microenvironment on MAIT cell transcriptional signature."

Reviewer 3#' Comment 7:

p. 9 differentiation trajectory of CXCR4+IFNG+ MAIT cells 353 was from blood MAIT cells (Figure 5F), 354 suggesting the microenvironment imprinting partly owing to exposure to IL-18.

Response to Reviewer 3#' Comment 7:

Thanks for your insightful comment regarding this point. This statement is too speculative based only on the pseudotime analysis. According to the pseudotime analysis, we can only observe that the differentiation trajectory of MAIT cell clusters was from blood to tumor. Thank you for your constructive feedback again. We have revised this speculative statement in the manuscript.

Changes in the manuscript: Page 9, Line 384:

"Additionally, we observed continuous differentiation along MAIT cell clusters, with the most terminal pseudotime value observed for MAIT cell clusters from LUAD (Figure 5F)."

Reviewer 3#' Comment 8:

p. 10 "LAUD" please correct.

Response to Reviewer 3#' Comment 8:

Thanks for your valuable feedback. We apologize for the oversight. The term "LAUD" has been corrected as per your suggestion.

Changes in the manuscript: Page 10, Line 441:

"To evaluate the prognostic significance of the MCs-MAIT cells axis in patients with LUAD, we performed a Kaplan-Meier survival analysis on two ***LUAD*** *cohorts (the FUSCC_1019 cohort and the FUSCC_150 cohort)."*

Reviewer 3#' Comment 9:

In my opinion this is too speculative: 427 thus reasonable to speculate MCs may play a key role in maintaining LUAD indolent nature for a 428 long time away from invasiveness.

Response to Reviewer 3#' Comment 9:

Thank you for your constructive feedback. We understand your concern regarding the speculative nature of the statement. This is a hypothesis based on current observations, rather than an established conclusion. Thank you for your suggestion again, we have removed the relevant statement.

Changes in the manuscript: Page 10, Line 427:

~~*"It is thus reasonable to speculate MCs may play a key role in maintaining LUAD indolent nature for a long time away from invasiveness."*~~

Reference

1. Korsunsky, I., et al., *Fast, sensitive and accurate integration of single-cell data with Harmony.* Nat Methods, 2019. **16**(12): p. 1289-1296.
2. Wu, Y. and K. Zhang, *Tools for the analysis of high-dimensional single-cell RNA sequencing data.* Nat Rev Nephrol, 2020. **16**(7): p. 408-421.
3. Fan, F., et al., *Elevated Mast Cell Abundance Is Associated with Enrichment of CCR2+ Cytotoxic T Cells and Favorable Prognosis in Lung Adenocarcinoma.* Cancer Res, 2023. **83**(16): p. 2690-2703.
4. Duan, M., et al., *Activated and Exhausted MAIT Cells Foster Disease Progression and Indicate Poor Outcome in Hepatocellular Carcinoma.* Clin Cancer Res, 2019. **25**(11): p. 3304-3316.
5. Cheng, S., et al., *A pan-cancer single-cell transcriptional atlas of tumor infiltrating myeloid cells.* Cell, 2021. **184**(3): p. 792-809 e23.
6. Landy, E., et al., *Biological and clinical roles of IL-18 in inflammatory diseases.* Nature Reviews Rheumatology, 2023. **20**(1): p. 33-47.
7. Stelekati, E., et al., *Mast cell-mediated antigen presentation regulates CD8+ T cell effector functions.* Immunity, 2009. **31**(4): p. 665-76.
8. Li, D., et al., *Ten-Year Follow-up Results of Pure Ground-Glass Opacity-Featured Lung Adenocarcinomas After Surgery.* Ann Thorac Surg, 2023. **116**(2): p. 230-237.
9. Li, D., et al., *Ten-year follow-up of lung cancer patients with resected adenocarcinoma in situ or minimally invasive adenocarcinoma: Wedge resection is curative.* J Thorac Cardiovasc Surg, 2022. **164**(6): p. 1614-1622 e1.
10. Ye, T., et al., *Lung Adenocarcinomas Manifesting as Radiological Part-Solid Nodules Define a Special Clinical Subtype.* J Thorac Oncol, 2019. **14**(4): p. 617-627.

Response to the Reviewer's Comments:

Reviewer #1 (Remarks to the Author):

Reviewer 1#' Comment 1:

The manuscript has been considerably improved, and I only have two minor remarks.

Fig. 1G: What is the definition of a favorable and adverse outcome. It may be in the M&M, but I can't find it.

Response to Reviewer 1#' Comment 1:

We sincerely appreciate your recognition of our work and the insightful feedback you have provided. Your valuable comments have greatly strengthened the quality of our manuscript. In Fig. 1G, we the prognostic value of each MCs subset signature in the FUSCC_1019 cohort, the FUSCC-150 cohort, and OAK cohort. We performed statistical analyses to evaluate the significance of the hazard ratios (HR). When the p-value was <0.05 and the hazard ratio (HR) was <1, the variable was considered a favorable factor; conversely, when the p-value was <0.05 and the HR was >1, it was considered an adverse factor. We have added this description in the Methods and Materials part.

Changes in the manuscript: Page 13, Line 561:

"To evaluate the prognostic value of each MCs subset signature (Supplementary Data 4), we performed ssGSEA analysis (Supplementary Data 5) and Cox univariate analysis in the FUSCC_1019 cohort, FUSCC_150 cohort, and OAK cohort. A p-value greater than 0.05 is considered indicative of no statistically significant difference. When the p-value was <0.05 and the hazard ratio (HR) was <1, the variable was considered a favorable factor; conversely, when the p-value was <0.05 and the HR was >1, it was considered an adverse factor."

Reviewer 1#' Comment 2:

Fig. S2K: Which comparison yield a p-value of <0.0001? Total differences across different immune subtypes?

Response to Reviewer 1#' Comment 2:

In Fig. S2K, we performed the ANOVA test to compare the infiltration score of IL-18$^+$ MC subset across different immune subtypes. The infiltration score of IL-18$^+$ MC subset in each LUAD sample calculated by single-sample Gene Set Enrichment Analysis (ssGSEA). The p value indicated total differences across different immune subtypes.

Changes in the manuscript: Figure S2K (updated as Supplementary Figure 2K):

*"(K) IL-18$^+$ MC infiltration abundance score of each LUAD sample in the FUSCC_1019 cohort across different immune subtypes. The scores were calculated by single-sample Gene Set Enrichment Analysis (ssGSEA) and the ANOVA test was performed. The p value indicated total differences across different immune subtypes. The center of the boxes indicated the median value and the upper and lower bounds of the boxes indicated the 25th and 75th percentile of data. ****: p<0.0001."*

Reviewer 2#' Comment:

Reviewer #2 (Remarks to the Author):

In response to the reviewer's comments, the authors performed additional analyses and experiments. The revised manuscript has been greatly improved.

Response to Reviewer 2#' Comment:

Thank you for your constructive review of our manuscript. We sincerely appreciate your valuable insights, which will undoubtedly enhance the clarity and rigor of our study. We are deeply grateful once again for the time and effort you have dedicated to reviewing our manuscript.

Reviewer 3#' Comment:

Reviewer #3 (Remarks to the Author):

The authors have addressed my concern, in principle. The authors concede that due to the nature of the data set (limited sizes and clinico-pathological annotations), questions remain open that would be addressable by a more comprehensive data set. Nevertheless, this would not preclude in my opinion the publication in Nature Commun.

A minor remaining point is that the while the multivariate analysis confirms that MC density is an independent prognostic factor, MAIT cells are not but mentioned as relevant in the proposed model. The authors should at least state clearly this limitation of the relevance of MAIT cells and discuss possible reasons.

Response to Reviewer 3#' Comment:

Thank you for your insightful suggestion. We acknowledge that, although MAIT cell density showed prognostic relevance in univariate analysis, it did not retain independent significance in the Cox multivariate model. We believe this is partly attributable to the limitation of the MAIT cell infiltration deconvolution using RNA-seq data. The infiltration level of MAIT cells is very low, which affects the accuracy of transcriptome-based estimation of MAIT cell infiltration levels. Moreover, the transcriptome characteristics of MAIT cells overlap with those of other T cells.

Changes in the manuscript: Page 11, Line 476:

"In our study, although MAIT cell density demonstrated prognostic relevance in the univariate analysis, it did not remain an independent prognostic factor in the Cox multivariate model. We believe this may be partly attributable to limitations in the deconvolution of MAIT cell infiltration using RNA-seq data. Specifically, the low infiltration levels of MAIT cells likely reduce the accuracy of transcriptome-based estimation. Additionally, the transcriptomic signatures of MAIT cells overlap substantially with those of other T cell subsets, further complicating their distinct quantification."