nature portfolio

Peer Review File

Comprehensive profiling of smoke-induced T cells reveals clonal γδT17 cells as a hallmark of COPD

Corresponding Author: Professor Pixin Ran

Parts of this Peer Review File have been redacted as indicated to remove third-party material.

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)
The authors exposed mice to cigarette smoke for 2 or 6 months and characterized the T cell populations in the lungs by labeling for CD4+ and CD8+ cells and by using single cell RNA sequencing. The findings suggest that cigarette smoke increased the number of T cells, specifically of gδ T cells in the airways, but reduced the percentages in the vasculature. The profile of T cells sorted from the spleen and the lung were clustered, and annotations using 41 marker genes suggested that the number of gδ T cells was reduced in the lungs exposed to tobacco smoke. The gδ T cells were driven to express higher levels of IL-17a as exposures progressed over 2 and 6 months. Analyses of the scRNAseq data using scVelo suggest that the gδ T cells irreversibly differentiate into IL-17a expressing cells. Finally, adoptive transfer of gδ T17 cells into Tcrd-/- mice indicated that gδ T17 cells were protective from emphysema development and lung function decline in mice exposed to smoke for 6 months.

General comments
1. The description of the cigarette smoke exposure system suggests that the particulate matter concentration in the exposure chamber was not assessed. Without that quantification, it is difficult to standardize the exposure conditions. The exact components of the commercial tobacco used, the size of the exposure chamber, and the rate of airflow in the chamber during exposures are not defined. The number of cigarettes used per time over a 4-hour exposure period daily does not allow for a reliable reproduction of the exposure conditions. Even under well-defined exposure conditions, the effect of tobacco smoke on mice is highly variable.
2. It is assumed that the observed T cell diversity observed in mice exposed to tobacco smoke indicates an antigen encounter following smoke exposure. The length and diversity of the CDR3 region is increased; however, it is not clear what antigen these mice may be exposed to.
3. Increased expression of IL-17a in the gδ T cell clones was detected in mice exposed to smoke not only in the lung but also in the spleen. The gδ T cells in the spleen showed distinct characteristics compared to those in the lung tissue, and this is assumed to be due to inconsistent changes across different “lung regions.” This should likely be “changes across different organs.”
4. The adoptive transfer experiment of the gδ t17 cells into Tcrd-/- is not well characterized. Did the cells engraft? Are these cells detected in the Tcrd-/- cells before and after smoke exposure? If so, is IL-17a alone sufficient to protect from smoke-induced lung destruction?
5. Studies conducted in this field suggest that IL-17a does not protect but enhances chronic lung diseases (PMID #s 27913421, 26024895, 36267325) and inhibitors of IL17A protect from inflammation. While it is not novel that gδ T cells produce IL-17, it is not clear how the findings from this report are contradicting what has been observed in previous studies. If, as mentioned in the Discussion, IL-17a that is produced in response to an antigen has a different effect from what has been reported, it is important to show why the same cytokine can cause a protective or detrimental effect in the lung.

Reviewer #2

(Remarks to the Author)
The study characterizes the T cell immune response to smoke exposure in a model of Chronic Obstructive Pulmonary

Disease (COPD). A significant finding is the identification of γδT17 cells (γ6δ4 T cells) as a hallmark of COPD, which has potential therapeutic implications. The authors utilized single-cell RNA sequencing (scRNA-seq) and T-cell receptor (TCR) sequencing to investigate T cell subsets and clonal expansion. However, the study critically lacks functional validation of the role of this cell subset in COPD inflammation, especially on human samples and have several other limitations.

• The study indicates an increase in PD-1 expression on γδT17 cells following smoke exposure; however, it does not investigate whether PD-1-positive cells exhibit decreased functionality. The exhaustion status of these cells needs to be examined in terms of cell activation, cytokine production, and survival. This could be further validated using patient samples from individuals with COPD. This is very critical to better understand the functionality of these cell subsets.
• This study lacks human validation, as the data relies solely on a murine model of COPD. The authors could enhance their findings by incorporating publicly available human single-cell RNA-seq datasets to verify the presence of γδT17 cells in COPD patients. Further validation using flow cytometry could also be conducted.
• What are the clinical implications and potential therapeutic targets suggested by this study, given the conflicting protective and pro-inflammatory roles previously attributed to γδT17 cells?
• The sample size for each experiment is not always clearly stated. Please specify the number of mice used per group in the figures and methods.

Reviewer #3

(Remarks to the Author)
Overall, the authors have provided an interesting manuscript focusing on t-cell subsets in a model of COPD. However, there are some concerns about some of the experimental setup that need to be addressed.
Major
1. There are no air-treated mice in the single cell experiment. As the smoke exposure was for 6 months ageing would have a large influence on the immune cell populations. Can the authors provide evidence that ageing did not influence the results? I understand this was corrected in the KO model where air exposure mice were used.
2. Some human replication is required to prove these results are relevant to COPD, especially the t-cell sub populations
3. Were multiple test corrections applied to figures where multiple t-test were conducted?
4. How were the cell types of the clusters in the single cell determined? Was an atlas used to make sure naming convention was maintained?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all the concerns for this Reviewer. However, the additional studies that were done are documented in the response letter but are not included in the manuscript or the supplemental information.
The question about what antigen the T cells are reacting to is well addressed in the responses. This response should be included in the Discussion, as it is helpful for others to consider addressing this issue in the future.

Reviewer #2

(Remarks to the Author)
The authors have addressed my comments. No further changes required.

Reviewer #1 (Remarks to the Author)

The authors exposed mice to cigarette smoke for 2 or 6 months and characterized the T cell populations in the lungs by labeling for CD4+ and CD8+ cells and by using single cell RNA sequencing. The findings suggest that cigarette smoke increased the number of T cells, specifically of gδ T cells in the airways, but reduced the percentages in the vasculature. The profile of T cells sorted from the spleen and the lung were clustered, and annotations using 41 marker genes suggested that the number of gδ T cells was reduced in the lungs exposed to tobacco smoke. The gδ T cells were driven to express higher levels of IL-17a as exposures progressed over 2 and 6 months. Analyses of the scRNAseq data using scVelo suggest that the gδ T cells irreversibly differentiate into IL-17a expressing cells. Finally, adoptive transfer of gδ T17 cells into Tcrd-/- mice indicated that gδ T17 cells were protective from emphysema development and lung function decline in mice exposed to smoke for 6 months.

General comments

1. The description of the cigarette smoke exposure system suggests that the particulate matter concentration in the exposure chamber was not assessed. Without that quantification, it is difficult to standardize the exposure conditions. The exact components of the commercial tobacco used, the size of the exposure chamber, and the rate of airflow in the chamber during exposures are not defined. The number of cigarettes used per time over a 4-hour exposure period daily does not allow for a reliable reproduction of the exposure conditions. Even under well-defined exposure conditions, the effect of tobacco smoke on mice is highly variable.

Response: Thank the Reviewer for the insightful comments regarding standardization of the smoke exposure system. We have revised the Methods section to include the requested details. Specifically, commercial cigarettes (Hongmei brand; tar: 13 mg/cigarette, nicotine: 0.8 mg/cigarette) were combusted using a whole-body passive smoking system (Liuwei Hardware & Electromechanical Co., Ltd., Guangzhou, China). Each exposure session involved 12 cigarettes burned within a 60 × 57 × 100 cm chamber over 10 minutes, reaching a total particulate matter (TPM) concentration of 500 mg/m^3. Mice were exposed four times daily for 1 hour per session, with 15-minute fresh-air intervals between sessions to prevent CO_2 accumulation. This corresponds to a daily exposure of 48 cigarettes over 4 hours. Exposures were conducted 6 days per week for 2 or 6 months. Concurrently, Age-matched controls were housed in a clean-air chamber under identical conditions.

2. It is assumed that the observed T cell diversity observed in mice exposed to tobacco smoke indicates an antigen encounter following smoke exposure. The length and diversity of the CDR3 region is increased; however, it is not clear what antigen these mice may be exposed to.

Response: Indeed, this is an excellent question what antigen these mice may be exposed to, which is a recognized challenge in the field, and we don't really know. Our study was designed to test the hypothesis that tobacco smoke introduces exogenous stimuli—such as physicochemical agents or microbial products—that activate innate and

structural airway cells, resulting in the release of inflammatory cytokines and the creation of a pro-inflammatory microenvironment. Simultaneously, smoke-induced epithelial damage may lead to regulated cell death (e.g., apoptosis or necrosis), exposing self-antigens that further contribute to immune activation and airway remodeling. Thus, both exogenous and self-antigens may contribute to the recruitment and clonal expansion of antigen-specific T cells. The observed increase in TCR diversity, including longer and more diverse CDR3 regions, is consistent with antigen-driven T cell activation, though it does not pinpoint specific antigenic triggers. We honestly acknowledge the limitations of our current data. Due to the extensive diversity of TCRs and the complexity of antigen-epitope-TCR interactions (where a single antigen can contain multiple epitopes, each potentially recognized by a variety of TCRs), our current results cannot directly clarify the nature of antigen specificity in $CD4^+$ and $CD8^+$ T cells within COPD airways. We believe that leveraging TCR diversity as a proxy for antigen encounter is a valid and widely accepted approach. We sincerely appreciate the reviewer for raising this critical point, which has identified an important direction for future research. Future work may focus on elucidating the nature of these antigens using emerging technologies such as peptide–MHC tetramer screens or antigen discovery platforms.

3. Increased expression of IL-17a in the gδ T cell clones was detected in mice exposed to smoke not only in the lung but also in the spleen. The gδ T cells in the spleen showed distinct characteristics compared to those in the lung tissue, and this is assumed to be due to inconsistent changes across different “lung regions.” This should likely be “changes across different organs.”

Response: We thank the Reviewer to point out this error, which we have now corrected in the revised manuscript.

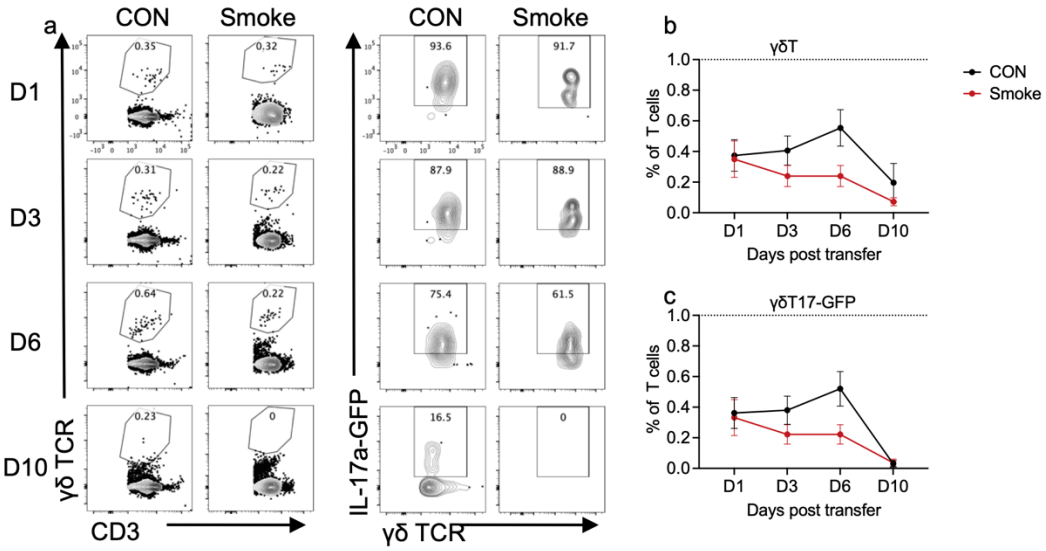
4. The adoptive transfer experiment of the gδ t17 cells into Tcrd-/- is not well characterized. Did the cells engraft? Are these cells detected in the Tcrd-/- cells before and after smoke exposure? If so, is IL-17a alone sufficient to protect from smoke-induced lung destruction?

Response: Thank the Reviewer for these insightful comments. We have carefully addressed the characterization of γδT17 cell adoptive transfer. Our results confirmed successful engraftment of transferred γδT17 cells in the *Tcrd*$^{-/-}$ mice. IL-17A$^+$ γδT cells were detectable in peripheral blood at Days 1, 3, and 6 post-transfer in both air control (CON) and smoke exposure groups (Supplementary Fig. 8). However, by Day 10, these cells were no longer observed in circulation, likely due to their migration into lung tissues.

To evaluate the long-term persistence of transferred cells, we performed scRNA-seq and scTCR-seq on $CD3^+$ T cells sorted from the lungs of *Tcrd*$^{-/-}$ mice reconstituted with γδT17 cells (*Tcrd*$^{-/-}$-T17) after 6 months of smoke exposure (pooled from 3 biological replicates, each comprising cells from 3 mice). Among 4,917 paired TCR sequences, we identified 13 cells bearing Vδ TCR chains (0.26%), demonstrating long-term persistence in the lung. While the recovery rate was low, this finding suggests successful

tissue engraftment and warrants further investigation. This part has not been published yet.

Regarding the functional role of IL-17A, existing literature indicates that IL-17A is not sufficient to protect against cigarette smoke-induced lung destruction. Rather, IL-17A functions as a pro-inflammatory mediator, contributing to neutrophilic infiltration and emphysema progression. Studies show that IL-17A or IL-17R deficiency mitigates CS-induced pulmonary inflammation and alveolar destruction (PMIDs: 21647421, 24097560, 21456954). However, certain models report that IL-17A deficiency may predispose mice to spontaneous alveolar damage (PMID: 26024895), suggesting a context-dependent role. Although the reasons remain unclear, possibly due to differences in experimental models, exposure to cigarette smoke still led to lung tissue damage in IL-17-deficient mice, despite a partial reduction in inflammatory mediator expression compared to wild-type controls (PMID: 26024895). Furthermore, a Phase II clinical trial targeting IL-17A (CNTO 6785) did not demonstrate clinical benefit in COPD patients (PMID: 28753067). Taken together, while γδT17 cells persist and produce IL-17A in vivo, IL-17A is unlikely to mediate protection against CS-induced lung damage.



Supplementary Fig. 8. *Tcrd*$^{-/-}$ mice in air control group (CON) and smoke exposure group (Smoke) were tansfered with γδT17 cells.

a Representative flow cytometry of γδ T cells and γδ T17 cells from blood of *Tcrd*$^{-/-}$ mice. **b-c** The percentage of γδ T cells (b) and γδ T17 cells (c) from blood were monitored at the indicated time points post transfer. n = 5 mice/group/time point.

5. Studies conducted in this field suggest that IL-17a does not protect but enhances chronic lung diseases (PMID #s 27913421, 26024895, 36267325) and inhibitors of IL17A protect from inflammation. While it is not novel that gδ T cells produce IL-17, it is not clear how the findings from this report are contradicting what has been observed in previous studies. If, as mentioned in the Discussion, IL-17a that is produced in

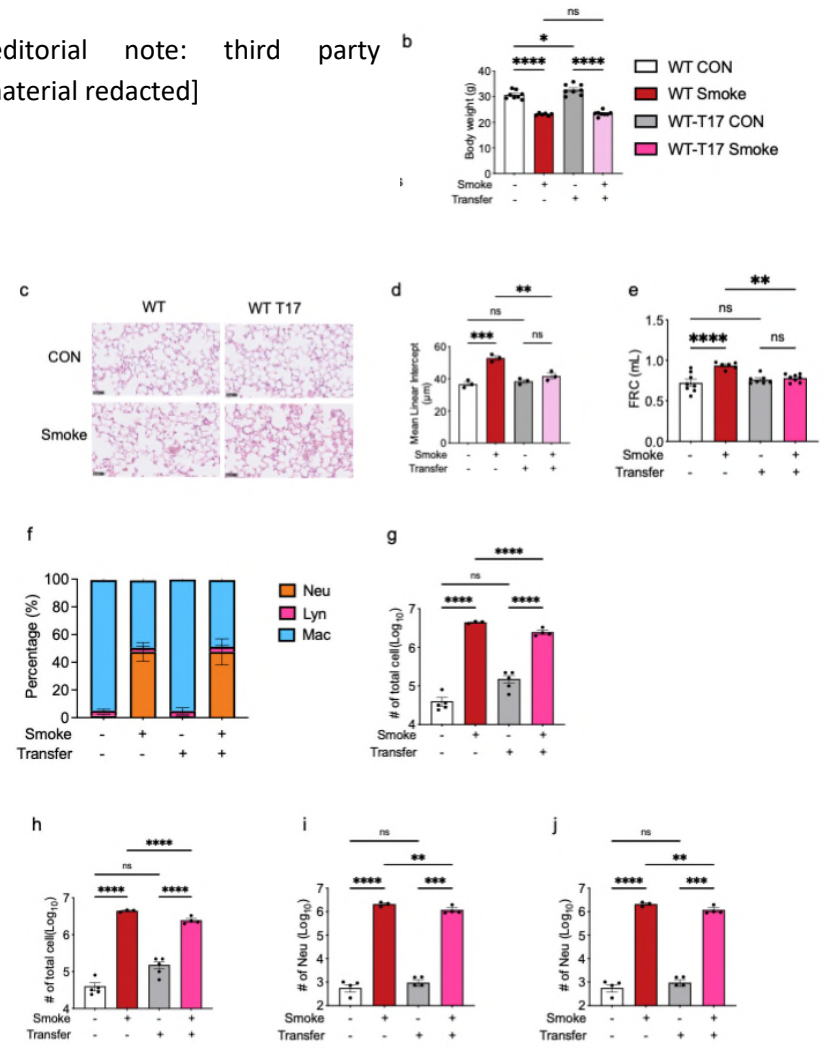
response to an antigen has a different effect from what has been reported, it is important to show why the same cytokine can cause a protective or detrimental effect in the lung.

Response: Thank the Reviewer for raising this important point—this is indeed a critical issue that warrants further attention. We fully agree that IL-17A, rather than providing protection, generally promotes chronic lung disease by driving neutrophilic inflammation, tissue remodeling, and emphysema progression (PMID: 27913421, 26024895, 36267325). However, our experimental observations reveal a distinct, context-dependent role when IL-17A is derived from γδ T cells.

We first noted a reduced percentage of γδ T cells within the airways in COPD models (Fig. 2), alongside clonal expansion of γ6δ4 T cells— a subset of γδ T cells with high *Il17a* expression (Fig. 4). Functional studies further demonstrated that depletion of these γδ T cells exacerbated disease susceptibility, whereas reconstitution of γδT17 cells (IL-17A-producing γδ T cells) restored their protective capacity, rendering the system more resistant to COPD progression (Fig. 6).

In our recent experiments (unpublished), adoptive transfer of γδT17 cells into wild-type recipients alleviated an emphysema-like phenotype and airway inflammation following six months of tobacco smoke exposure (Response letter Fig. 1). This suggests that γδ T cells, particularly γδT17 subsets, may play a dual role—participating in both immune activation and tissue repair. This is consistent with previous reports that γδ T cell deficiency worsens disease, suggesting that these cells may also contribute to tissue protection or repair, potentially via IL-17A–independent pathways (PMID: 22261033). Importantly, we hypothesize that γδ T cells contribute to immune homeostasis in the lung, especially under stress conditions such as tobacco smoke exposure. While smoke induces clonal expansion and IL-17A production by γδ T cells, their overall functional role may extend beyond IL-17A secretion. Therefore, we propose that the IL-17A response may reflect an early, smoke-induced immune activation, whereas the broader protective effect of γδ T cells might involve IL-17A–independent mechanisms, or a temporally restricted IL-17A function. Further mechanistic studies are required to dissect how γδT17 cells balance inflammatory and reparative functions during COPD progression.

[editorial note: third party material redacted]



Response letter Fig. 1. γδ T17 cells ameliorate emphysema-like phenotype and airway inflammations.

a, b C57BL/6J wild-type (WT) mice were randomly divided into two groups: the air control group (CON) and the tobacco smoke exposure group (Smoke). Mice were placed in a fume box and exposed to tobacco smoke for 6 months, respectively. Twenty-four hours prior to tobacco smoke exposure, mice were randomly divided into two groups: the transferred γδT17 cell (T17) group and the PBS control group, with body weights recorded at 6 months (6M), after exposure (n = 6-8 mice/group). **c** Representative images of hematoxylin and eosin-stained paraffin sections (40×, scale bar = 50 μm) of the WT, and WT-T17 groups show that after 6 months of tobacco smoke exposure the Smoke group exhibited marked airway inflammation and fused alveolar damage compared to the CON group. **d** Mean linear intercept (MLI) values are presented (n = 3 mice/group). **e** Functional residual capacity (FRC) measurements are shown (n = 6–8 mice/group). **f–j** The proportion (**f**) and absolute counts (**g–j**) of inflammatory cells in bronchoalveolar lavage fluid (BALF) are shown (n = 3–6 mice/group). Statistical significance is indicated as *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Data are presented as mean ± SEM. One-way ANOVA was used for comparisons among multiple groups when data were normally distributed, while the non-parametric Kruskal–Walli's test was applied for non-normally distributed data.

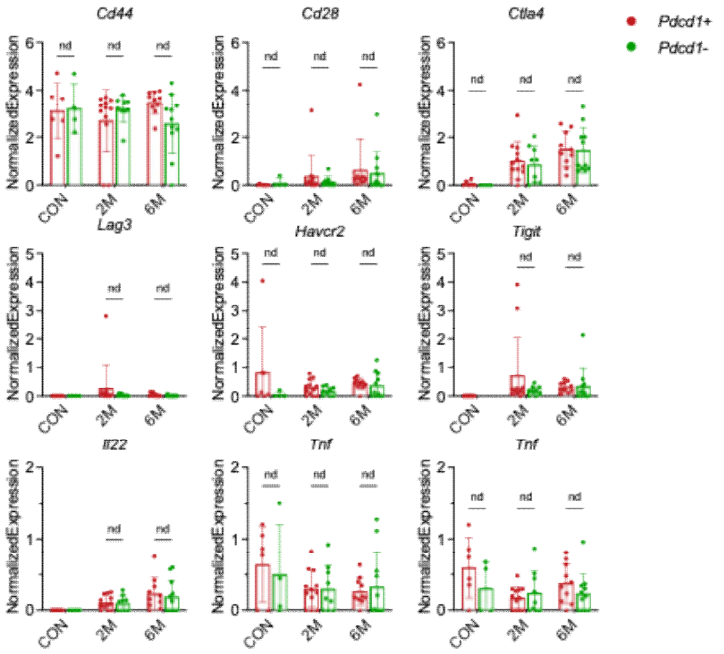
Reviewer #2 (Remarks to the Author)

The study characterizes the T cell immune response to smoke exposure in a model of Chronic Obstructive Pulmonary Disease (COPD). A significant finding is the identification of γδT17 cells (γ6δ4 T cells) as a hallmark of COPD, which has potential therapeutic implications. The authors utilized single-cell RNA sequencing (scRNA-seq) and T-cell receptor (TCR) sequencing to investigate T cell subsets and clonal expansion. However, the study critically lacks functional validation of the role of this cell subset in COPD inflammation, especially on human samples and have several other limitations.

Response: We thank the Reviewer for recognizing the significance of our identification of γδT17 cells as a hallmark of COPD, which may offer new avenues for targeted therapeutic implications. In response to the Reviewer's comment regarding functional validation, we have now incorporated additional data to assess the role of γδT17 cells in human COPD samples. Furthermore, we have addressed each of the Reviewer's concerns in detail below. We hope that these substantial revisions have improved the quality and clarity of our manuscript and that it is now suitable for publication.

1. The study indicates an increase in PD-1 expression on γδT17 cells following smoke exposure; however, it does not investigate whether PD-1-positive cells exhibit decreased functionality. The exhaustion status of these cells needs to be examined in terms of cell activation, cytokine production, and survival. This could be further validated using patient samples from individuals with COPD. This is very critical to better understand the functionality of these cell subsets.

Response: We sincerely thank the Reviewer for this insightful comment and fully agree with the importance of investigating the functional status of PD-1^{+} γδT17 cells in the context of smoke exposure. To address this point, we re-analyzed our single-cell RNA-seq data from the TTA γδ T cell dataset. We specifically extracted *Il17a*-expressing cells to define the γδT17 subset and further stratified them into *Pdcd1*$^{+}$ and *Pdcd1*$^{-}$ populations (Response letter Fig. 2). Comparative transcriptomic analysis revealed no significant differences in the expression of activation markers (*Cd44, Cd28*), exhaustion markers (*Ctla4, Lag3, Havcr2, Tigit*), or effector cytokines (*Il22, Tnf, Csf2*) between these two groups. These data suggest that PD-1 expression on γδT17 cells does not necessarily indicate functional exhaustion following smoke exposure.



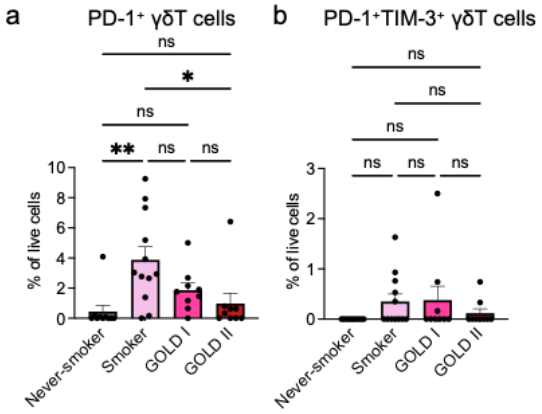
Response letter Fig.2. Gene expression in *Pdcd1*$^+$ (red) and *Pdcd1*- γδT (green) cells.
CON: air control group, n = 3; 2M: 2-month tobacco smoke exposure group, n = 3; 6M: 6-month tobacco smoke exposure group, n = 3. CON: air control group, pooled from 3 biological replicates, each comprising cells from 20 mice (total n = 60 mice). 2M: 2-month tobacco smoke exposure group, pooled from 3 biological replicates, each comprising cells from 4 mice (total n = 12 mice). 6M: 6-month tobacco smoke exposure group, pooled from 3 biological replicates, each comprising cells from 4 mice (total n = 12 mice)

To further validate this observation in human samples, we performed flow cytometric analysis on induced sputum mononuclear cells (ISMCs) from non-smokers, smokers, and COPD patients smoked (GOLD I and II stages, Supplementary Table 1). All human studies were approved by The First Affiliated Hospital of Guangzhou Medical University or Guangzhou National Laboratory (No. 2018-53) or Guangzhou National Laboratory (No. 2023-0009). We assessed PD-1 and TIM-3 expression as markers of activation and exhaustion. Smokers showed a significantly higher frequency of PD-1$^+$ γδ T cells compared to non-smokers (Response Letter Fig. 3a). Interestingly, GOLD II patients exhibited a substantial reduction in PD-1$^+$ γδ T cells relative to smokers, while the frequency of TIM-3$^+$PD-1$^+$ γδ T cells remained only modestly affected (Response Letter Fig. 3b). Together, these findings suggest that airway γδ T cells in COPD are predominantly in an activated state rather than undergoing functional exhaustion.

Response letter Table 1: Demographic and Clinical Characteristics of Subjects

Characteristic	Never-smoker (n = 10)	Smoker (n = 12)	GOLD I (n = 9)	GOLD II (n=9)
Age, mean ± SD, yr	51 ± 11	58 ± 8	69 ± 7	66 ± 9
BMI, mean ± SD, kg/m^2	25 ± 3	23 ± 2	21 ± 3	21 ± 4

Gender				
Female	8	1	0	0
Male	2	11	9	9
Post-FEV1/FVC, mean ± SD	85 ± 4	81 ± 5	66 ± 5	56 ± 9
Post-FEV1 % predicted, mean ± SD	107 ±11	97 ±10	96 ± 11	70 ± 7

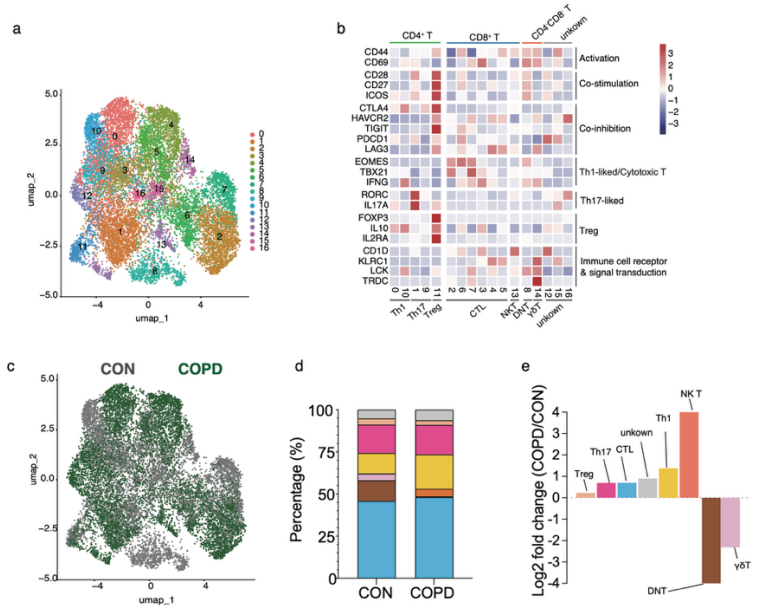


Response letter Fig.3. Flow cytometry analysis of γδT cells in humen induced sputum mononuclear cells (ISMCs)

a-b Flow cytometry analysis of percentage of $PD1^+$ γδT cells, and $PD1^+TIM3^+$ γδT cells in live cells.

2. This study lacks human validation, as the data relies solely on a murine model of COPD. The authors could enhance their findings by incorporating publicly available human single-cell RNA-seq datasets to verify the presence of γδT17 cells in COPD patients. Further validation using flow cytometry could also be conducted.

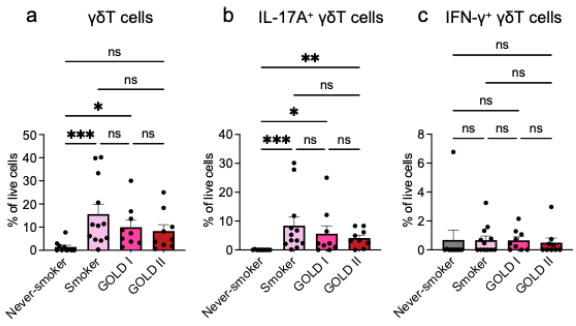
Response: We appreciate the Reviewer's insightful comment and fully agree that human validation is critical for strengthening our findings. To address this, we analyzed the publicly available single-cell RNA-seq dataset GSE136831. We identified T cells by selecting CD3E-expressing cells and further confirmed their identity based on author-provided annotations, which contains lung tissue samples from 17 COPD patients and 26 healthy controls. Among 15,591 T cells, we identified 17 transcriptionally distinct clusters (Response letter Fig. 4a) and annotated them into 7 T cell subsets, including cytotoxic T cells (CTL), Th1 cells, Th17 cells, double-negative T cells (DNT), γδ T cells, regulatory T cells (Treg), and natural killer T cells (NKT), using 22 marker genes (Response letter Fig. 4b). Notably, γδ T cells were detected and found to be significantly reduced in COPD patients compared to controls (Response letter Fig. 4d–e), which is consistent with our findings in the COPD murine model.



Response letter Fig.4. Single-Cell Atlas of humen T cells Immune System

a Single-cell transcriptomes of T cells displayed by UMAP. Seurat-based clustering of 15591 cells colored based on cluster ID. **b** The mean expression of each gene in each cell cluster is presented in different colors as shown in the legend color bar. The values were row scaled. The marker genes for different cell types were listed on the left. **c-e** Cell distribution (**c**), percentage(**d**), and fold changes (**e**) in cluster proportions associated with COPD.

However, we did not detect γδ T17 cells in this dataset, likely due to limitations in sequencing depth, dropout of low-abundance cytokine transcripts, or tissue processing bias. To overcome this, we performed flow cytometry on human induced sputum mononuclear cells (ISMCs) from non-smokers, smokers and COPD patients smoked (GOLD I and II stages). The proportion of γδ T cells among live cells increased significantly in smokers compared to non-smokers (Response letter Fig. 5a), indicating smoking-associated expansion of γδ T cells in the airway. We observed a significant increase in IL-17A$^+$ γδ T cells in smokers and COPD patients relative to non-smokers, and a trend toward a decrease in COPD patients compared with smokers, although the difference was not statistically significant (Response letter Fig. 5b). In contrast, the proportion of IFN-γ$^+$ γδ T cells remained unchanged across all groups (Response letter Fig. 5c).



Response letter Fig.5. Flow cytometry analysis of γδT cells in humen induced sputum mononuclear cells (ISMCs)

a-c Flow cytometry analysis of percentage of (**a**) γδT cells, (**b**) IL-17A$^+$ γδT cells and (**c**) IFN-γ$^+$ γδT cells in live cells.

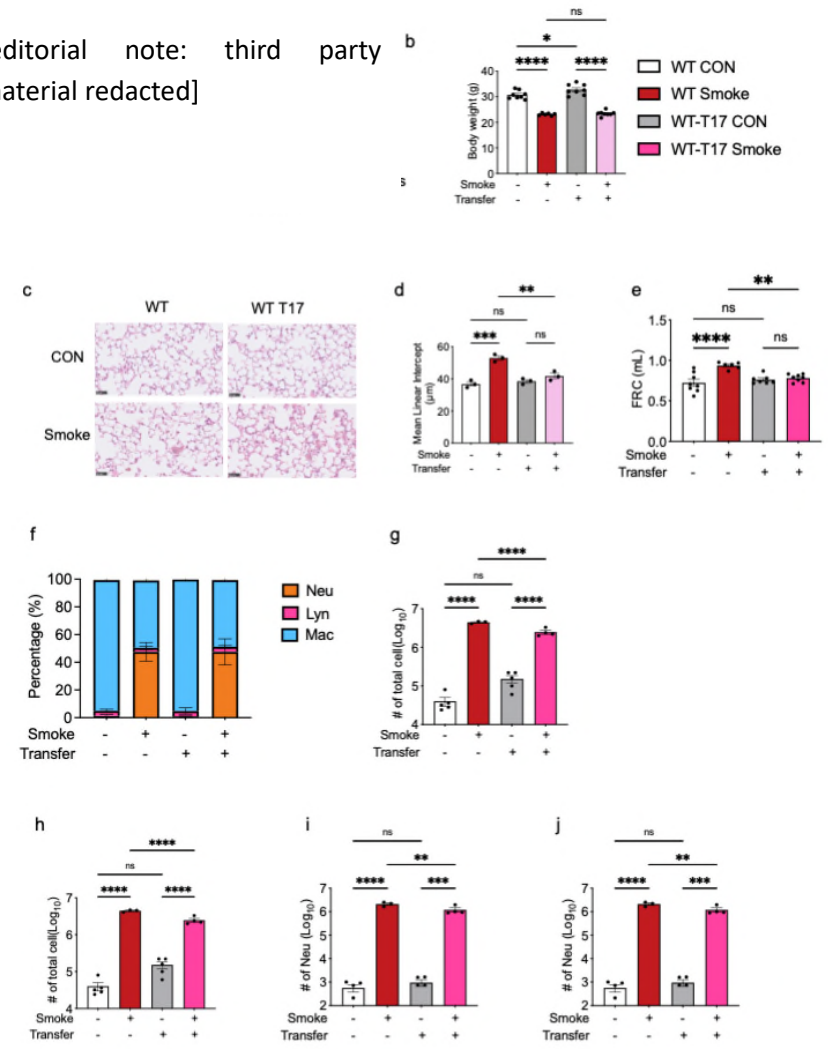
3. What are the clinical implications and potential therapeutic targets suggested by this study, given the conflicting protective and pro-inflammatory roles previously attributed to γδT17 cells?

Response: We appreciate the Reviewer's thoughtful question regarding the seemingly conflicting roles of γδT17 cells. This is indeed a critical issue that warrants further attention. Our study does not aim to contradict the established pro-inflammatory role of IL-17A, but instead to highlight the complex and context-dependent functions of γδ T cells in the lung microenvironment.

While IL-17A itself is primarily pathogenic, our findings suggest that γδT17 cells may have a broader role beyond IL-17A secretion. In our recent experiments (unpublished), adoptive transfer of γδT17 cells into wild-type recipients alleviated an emphysema-like phenotype and airway inflammation following six months of tobacco smoke exposure (Response letter Fig. 1). This suggests that γδ T cells, particularly γδT17 subsets, may play a dual role—participating in both immune activation and tissue repair. This is consistent with previous reports that γδ T cell deficiency worsens disease (PMID: 22261033), suggesting that these cells may also contribute to tissue protection or repair, potentially via IL-17A–independent mechanisms.

From a clinical perspective, our data raise the possibility that selectively modulating γδT17 cell function—rather than globally inhibiting IL-17A—could represent a novel therapeutic approach. For instance, strategies that preserve the tissue-repairing or immunoregulatory functions of γδT cells while minimizing their pathogenic cytokine output might offer a more balanced and safer intervention in COPD. Further mechanistic studies are needed to elucidate the molecular pathways by which γδT17 cells mediate protection, and whether these pathways can be therapeutically targeted. Overall, our findings support the view that γδT17 cells are immunologically adaptable and may represent both a biomarker of disease progression and a therapeutic target to restore immune homeostasis in chronic lung diseases.

[editorial note: third party material redacted]



Response letter Fig. 1. γδ T17 cells ameliorate emphysema-like phenotype and airway inflammations.

a, b C57BL/6J wild-type (WT) mice were randomly divided into two groups: the air control group (CON) and the tobacco smoke exposure group (Smoke). Mice were placed in a fume box and exposed to tobacco smoke for 6 months, respectively. Twenty-four hours prior to tobacco smoke exposure, mice were randomly divided into two groups: the transferred γδT17 cell (T17) group and the PBS control group, with body weights recorded at 6 months (6M), after exposure (n = 6-8 mice/group). **c** Representative images of hematoxylin and eosin-stained paraffin sections (40×, scale bar = 50 μm) of the WT, and WT-T17 groups show that after 6 months of tobacco smoke exposure the Smoke group exhibited marked airway inflammation and fused alveolar damage compared to the CON group. **d** Mean linear intercept (MLI) values are presented (n = 3 mice/group). **e** Functional residual capacity (FRC) measurements are shown (n = 6–8 mice/group). **f–j** The proportion (**f**) and absolute counts (**g–j**) of inflammatory cells in bronchoalveolar lavage fluid (BALF) are shown (n = 3–6 mice/group). Statistical significance is indicated as *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Data are presented as mean ± SEM. One-way ANOVA was used for comparisons among multiple groups when data were normally distributed, while the non-parametric Kruskal–Walli's test was applied for non-normally distributed data.

4. The sample size for each experiment is not always clearly stated. Please specify the number of mice used per group in the figures and methods.

Response: We thank the Reviewer for pointing this out. We apologize for any ambiguity regarding the number of mice used per group in the figures and methods section, and we have made changes.

Reviewer #3 (Remarks to the Author):

Overall, the authors have provided an interesting manuscript focusing on t-cell subsets in a model of COPD. However, there are some concerns about some of the experimental setup that need to be addressed.

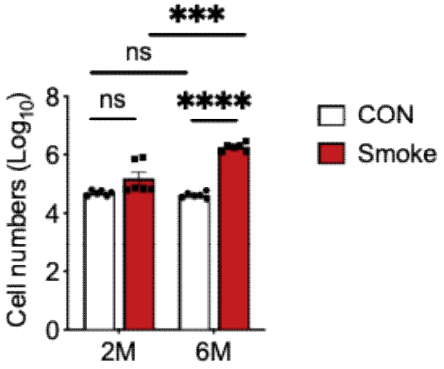
Response: We thank the Reviewer for recognizing the value of our study, which investigates T cell subsets in a COPD model. In response to the concerns raised regarding certain aspects of the experimental design, we have now provided detailed clarifications and revisions as outlined below. We believe these improvements have strengthened both the rigor and clarity of the manuscript, and we hope it is now suitable for publication.

Major

1.There are no air-treated mice in the single cell experiment. As the smoke exposure was for 6 months ageing would have a large influence on the immune cell populations. Can the authors provide evidence that ageing did not influence the results? I understand this was corrected in the KO model where air exposure mice were used.

Response: We thank the Reviewer for raising this important point, which we have carefully considered. Indeed, previous single-cell studies have documented age-associated alterations in immune cell populations across various mouse tissues, including the lung. However, these studies typically define aged mice as those older than 16 months (e.g., PMID: 32669714, 36747595, 37852965, 30814501, 39443746). In our study, although mice were exposed to cigarette smoke for 6 months, their age remained within the mature adult stage and did not meet the criteria for age-related immune remodeling.

To directly assess whether aging may have influenced our results within this timeframe, we collected bronchoalveolar lavage fluid (BALF) from air-exposed control and smoke-exposed mice at both 2 and 6 months. Total immune cell numbers in BALF were comparable between 2- and 6-months air-exposed control, ranging from approximately 40,000 to 60,000 cells (Response letter Fig. 6), suggesting limited age-related effects on immune cell number over this period. Furthermore, to reduce inter-individual variability and enhance dataset robustness, we pooled BALF cells from five mice for flow cytometry and from twenty adult mice for single-cell RNA sequencing. Taken together, these data support the conclusion that the observed T cell alterations are primarily attributable to smoke exposure rather than aging.



Response letter Fig. 6. Cell number of bronchoalveolar lavage fluid (BALF) in mice.

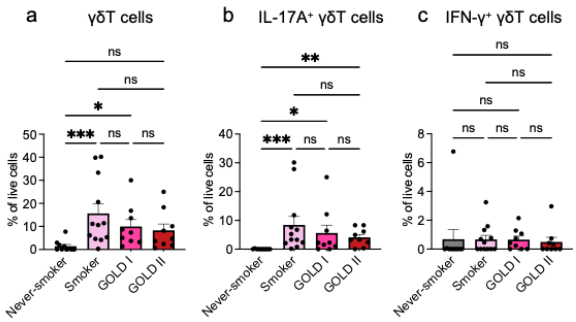
C57BL/6J wild-type (WT) mice were randomly divided into two groups: the air control group (CON) and the tobacco smoke exposure group (Smoke). Mice were placed in a fume box and exposed to tobacco smoke for 2 or 6 months, respectively (n = 6 mice/group/time point). The numbers of cells in BALF are shown. Statistical significance is indicated as *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. Error bars in and represent standard error of the mean (SEM).

2.Some human replication is required to prove these results are relevant to COPD, especially the t-cell sub populations

Response: Thank the Reviewer for pointing this out. Given the emergence of *Il7a* as a key gene in murine smoking models, we analyzed γδ T cells in induced sputum mononuclear cells (ISMCs) from non-smokers, smokers, and COPD patients smoked (GOLD I and II stages, Response letter Table 1). All human studies were approved by The First Affiliated Hospital of Guangzhou Medical University or Guangzhou National Laboratory (No. 2018-53) or Guangzhou National Laboratory (No. 2023-0009). The proportion of γδ T cells among live cells increased significantly in smokers compared to non-smokers (Response letter Fig. 5a), indicating smoking-associated expansion of γδ T cells in the airway. We observed a significant increase in IL-17A$^+$ γδ T cells in smokers and COPD patients relative to non-smokers, and a trend toward a decrease in COPD patients compared with smokers, although the difference was not statistically significant (Response letter Fig. 5b). In contrast, the proportion of IFN-γ$^+$ γδ T cells remained unchanged across all groups (Response letter Fig. 5c).

Response letter Table 1: Demographic and Clinical Characteristics of Subjects

Characteristic	Never-smoker (n = 10)	Smoker (n = 12)	GOLD I (n = 9)	GOLD II (n=9)
Age, mean ± SD, yr	51 ± 11	58 ± 8	69 ± 7	66 ± 9
BMI, mean ± SD, kg/m^2	25 ± 3	23 ± 2	21 ± 3	21 ± 4
Gender				
Female	8	1	0	0
Male	2	11	9	9
Post-FEV1/FVC, mean ± SD	85 ± 4	81 ± 5	66 ± 5	56 ± 9
Post-FEV1 % predicted, mean ± SD	107 ±11	97 ±10	96 ± 11	70 ± 7



Response letter Fig.5. Flow cytometry analysis of γδT cells in humen induced sputum

mononuclear cells (ISMCs)

a-c Flow cytometry analysis of percentage of (**a**) γδT cells, (**b**) IL-17A$^+$ γδT cells and (**c**) IFN-γ$^+$ γδT cells in live cells.

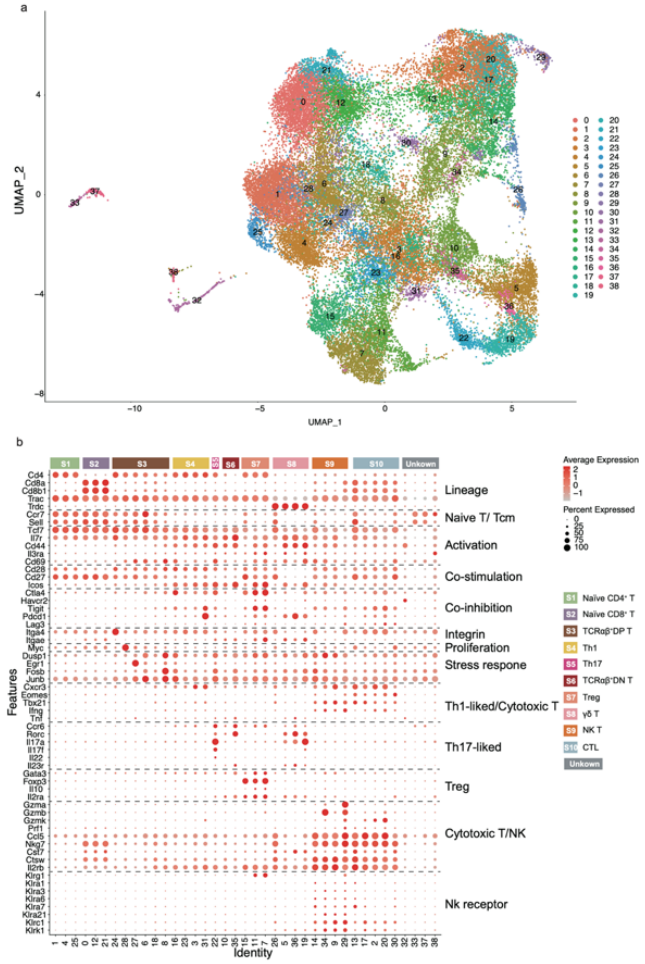
3. Were multiple test corrections applied to figures where multiple t-test were conducted?

Response: We thank the Reviewer for raising this important statistical consideration. We have carefully reviewed the data and confirm that appropriate multiple testing corrections were applied to figures involving multiple t-tests. These corrections were implemented during data analysis using GraphPad Prism 9.0, ensuring that statistical significance was not overestimated. We appreciate the opportunity to clarify this and have revised the figure legends and Methods section.

4.How were the cell types of the clusters in the single cell determined? Was an atlas used to make sure naming convention was maintained?

Response: Thank the Reviewer for the insightful comment. We apologize for not clearly describing our annotation strategy in the original manuscript. In our single-cell analysis, we identified 39 T cell clusters based on transcriptional profiles (Supplementary Fig. 2a). Annotation of these clusters was performed manually using well-established canonical marker genes, based on comprehensive literature references (PMID: 33271118, 34914499, 29942094). To ensure consistency and biological accuracy, we applied a uniform marker gene set across all samples and conditions. While we did not use a reference atlas (such as CellTypist or ImmGen) for automated annotation in this study, our manual curation was grounded in consensus marker definitions and aligns with cell identity frameworks from existing murine single-cell atlases.

We have revised the manuscript accordingly. Specifically, we identified 39 T cell clusters based on gene expression profiles (Supplementary Fig. 2a). Using 41 marker genes, these clusters were annotated into specific T cell subsets, including naïve CD4$^+$ and CD8$^+$ T cells (high *Cd4/Cd8, Ccr7, Sell, Tcf7, Il17r; low Cd44, Cd69, Pdcd1*), double-positive (DP) T cells (*Trac, Cd4, Cd8a, Cd8b, Cd69*), Th1 cells (high *Cd4, Cxcr3*; low *Cxcr6, Rorc, Il17a, Il17f*), Th17 cells (*high Cd4, Cxcr6, Rorc, Il17a, Il17f*; low *Cxcr3*), double-negative (DN) T cells (*Trac, Cd44*; low *Cd4, Cd8a, Cd8b*), γδ T cells (high *Trdc*; low *Trac*), Tregs (*Cd4, Gata3, Foxp3, Il10, Il2ra*), NKT cells (*Klra1, Klrc1, Klrk1*), and cytotoxic T cells (*Cd8a, Cd8b, Cxcr3, Eomes, Tbx21, Ifng*; low *Cxcr6, Rorc, Il17a, Il17f*). (Supplementary Fig. 2b)



Supplementary Fig. 2. T cells from mice in the air control group (CON) and 2mouth or 6 mouth after the tobacco smoke exposure.

a Single-cell transcriptomes of T cells displayed by UMAP. Seurat-based clustering of 40849 cells colored based on cluster ID. **b** Average expression (color scale) and the percentage of expressing cells (size scale) of selected genes in indicated clusters. The values were row scaled. The marker genes for different cell types were listed on the left.

Version 2: Response to the Reviewers

Reviewer #1 (Remarks to the Author):

1. The authors have addressed all the concerns for this Reviewer. However, the additional studies that were done are documented in the response letter but are not included in the manuscript or the supplemental information.

Response: We thank the Reviewer for this final positive comment. As suggested, we have now incorporated all the additional study data and corresponding analyses from our point-by-point response into the main manuscript and the supplementary information. The new figures have been added as Supplementary Fig. 9, with the associated descriptions added to the Results section (Lines 236-239). These additions have significantly improved the completeness of the manuscript.

2. The question about what antigen the T cells are reacting to is well addressed in the responses. This response should be included in the Discussion, as it is helpful for others to consider addressing this issue in the future.

Response: We thank the Reviewer for recognizing our explanation regarding the potential antigens that T cells may be responding to. As suggested, we have incorporated this discussion into the revised manuscript (Discussion section, lines 268–282) to provide a more comprehensive perspective for readers.

Reviewer #2 (Remarks to the Author):

The authors have addressed my comments. No further changes required.

Response: We thank the reviewer for their valuable comments and suggestions, which have helped us improve the quality and completeness of our manuscript.

There are no comments from reviewer #3 for this round.