

Aberrant activation of tissue-resident lymphocytes and pro-fibrotic GZMK⁺ CD8⁺ T cells in IPF

Corresponding Author: Dr Fei Gao

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, Gao et al characterized lung resident lymphocytes from human lung tissues, and observed that GZMK⁺ TRM and HSP hi CD4⁺ Tcon enriched in IPF lungs. The manuscript is well organized, however, some points need to be addressed:

Major,

1. The introduction didn't include the most background of this research, it should be expanded.
2. TCF7 has been reported to suppress lung CD103⁺ TRM development (PMID: 32268106), why does GZMK-TRM express an observable level of it? Do GZMK⁺ and GZMK⁻ TRM in IPF patients express CD103?
3. In Fig S3B, co-inhibitory receptors are highly expressed in the GZMK⁺ population, so why is the related signaling suppressed in Fig. 3E?
4. Is the protein level of Granzyme K higher in TRMs from IPF patients?
5. In Fig. 4H, CD44, CD4, and HSPA4 co-staining can't conclude that "HSP^{hi} CD4⁺ T cells particularly in the fibrotic regions" (line 192). The high cell density shown here is possibly iBALT, not fibrotic regions, if without related markers.
6. Line 271-272, the description is ambiguous, what does the "fibrotic tissue" mean, if it means tissues from IPF patients, please show the less IgG deposition in control donors; if it means the fibrotic area, please show fibrotic marker co-staining.
7. Fig 7D is unconvincing, please show the control photos of T/B cell staining in collagen- fields.
8. Fig 7 is disconnected from the previous figs, neither GZMK⁺ CD8 T cells nor HSP hi Tcon were analyzed there, which makes the conclusion of this section too weak.

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1. The legend of Fig. 1G, S3B, S3E, etc should include the accurate numbers of expression and % instead of only showing "high" and "low".
2. In Fig.4, the cells should be called Tcon instead of CD4⁺ T, for Treg was not included in the analysis.
3. The legends of Fig 5B, 6A,B,D,I,J are overlaid.

Reviewer #2

(Remarks to the Author)

In this manuscript, Gao and colleagues employed single-cell RNA sequencing to analyze approximately 100,000 resident immune cells collected from five control subjects and seven patients with idiopathic pulmonary fibrosis (IPF) after ex vivo human lung perfusion. Using this method, the authors labeled circulating immune cells with CD45 to distinguish between circulatory and resident immune cells. Their results indicate that 54.6% of resident cells were characterized as T-cells. The study further describes the transcriptional profiles of CD4 T cells, regulatory T cells, CD8 T cells, and B cells, and includes an analysis of the interactions between lymphocytes and fibroblasts. Although the study provides valuable insights into lung resident immune cells, the main objective of the research could be presented more clearly. Additionally, despite the detailed analysis of genes and pathways implicated to these cells, further robust analysis or interpretation would enhance understanding of the roles these lymphocytes play in fibrosis. Some key aspects that could benefit from further clarification include:

1. During the ex vivo perfusion of explanted lungs, the authors observed that T-cells constitute the most abundant resident cell population in the lungs (54%), whereas a smaller population, described as monocyte/macrophages, accounts for only 4% of resident lung cells in control, non-diseased lungs. However, alveolar and interstitial macrophages have previously been reported to be highly abundant in the lungs, as described by Aegerter et al. (2022). Could the lung perfusion process be selectively excluding alveolar macrophages and other populations present in the same compartment, such as T-cells located within the lumen? Please, explain this possible methodological issue.
2. Alveolar macrophages are specific lung-resident cells, yet they were not identified within the group of "resident" immune cells in this study. As a result, categorizing the immune populations as "resident" may lead to a misconception, creating the impression that T-cells are the most abundant leukocyte population in the lungs.
3. Figure 2 presents a series of lung stainings for immune markers, including CD45, CD44, CD4, CD20, and CD8, to identify resident immune cells (primarily CD44+). However, the low-magnification images do not clearly show double-stained cells as aimed at by the authors. Can you please show a higher magnification image and the respective image quantification? Additionally, in Figure 2C, could the authors specify the markers used to characterize the different populations of resident immune cells for greater clarity? Furthermore, in Figure 2D, the white text used for labeling in the plot is difficult to read.
4. In Figure 3, the authors describe CD8 GZMK+ T cells as predominantly present in IPF lungs compared to controls. They also discuss the potential impact of aging on the appearance of these cells. Including a table that describes the characteristics of control and IPF donors would be helpful to determine whether the findings presented in the manuscript are consistent across different cohorts.
5. In this study, the authors observed that GZMK+ T cells exhibit low expression of GZMB, which they believe contrast with the established role of GZMB in antiviral responses. Other reports have shown that GZMK CD8 T cells are induced during viral responses (for example, Jonsson et al., 2022). As this study tested the function of GZMK in upregulating p16 (CDKN2A) and SA-βGal expression in human fibroblasts, could the authors confirm whether GZMB has a distinct contribution to the expression of these markers? This would help clarify the specific role of GZMK in fibrosis.
6. In figures 3, 4 and 5, the authors described the different gene expression profile of CD8, CD4 and B cells found in IPF lungs and discussed possible biological roles of these cells in this disease. However, a pseudobulk analysis has been shown to provide a more statistically robust results of the scRNA-seq data by aggregating data from cells instead of bulk analysis that can be influenced by data of a specific sample. Please, consider this type of analysis, or explain the choices made for the analysis provided in the manuscript.
7. CD4 and TREGs could have been analyzed together since TREGs are also CD4 T-cells. A major complication of this segregation is the fact that FOXP3 low cells might represent an activated subset of CD4 T-cell.
8. The present manuscript offers a detailed and descriptive transcriptional analysis; however, the overall conclusions of the study could be clarified further employing a more robust analysis. While the authors focus on interactions between fibroblasts and lymphocytes, the significance of these interactions may require more careful interpretation, as the findings do not fully define the contribution of T-cells to fibrosis. Additionally, the T-cell clusters include cell types that are not traditionally defined (e.g., clusters characterized by high TOB1 and HSP expression) or show the presence of Th1 cells but lack other subsets, such as Th2 and Th17 cells, which makes the results more challenging to interpret.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors addressed most of my questions, but two points still need to be addressed:

1. The author claimed "Our data show that GZMK+ CD8+ T cells exhibit high EOMES expression (Fig. 3D), which may override the inhibitory effect of low TCF7 levels and sustain CD103 expression", this is an inaccurate concept, although the EOMES potentially bind to the promoters of ITGAE, EOMES is a well-appreciated TF that suppresses TRM differentiation.
2. Fig S4G is great evidence to prove the colocalization of resident HSP^{hi} CD4+ Tcon with fibrotic regions, this panel should be moved into the main figures.

Reviewer #2

(Remarks to the Author)

The goal of this study, which focuses on resident lymphocytes present in idiopathic pulmonary fibrosis, is an interesting concept. However, there are still several caveats regarding the overinterpretation of the results. The authors seem to heavily rely on their methodology to draw conclusions and make assertions, yet they do not sufficiently

question the potential gaps in their methods as possible explanations for their findings. The primary issue lies in their gate strategy for differentiating between circulatory and resident cells, which significantly impacts the overall message of the manuscript.

Another important factor to consider is the tissue digestion protocol mentioned by the authors. It was stated that they "employed a mature, widely recognized two-hour enzymatic digestion process" for lung tissue. However, other research groups have opted for shorter digestion times due to the adverse effects that these enzymes can have on cells. Such effects might include the activation of genes that do not accurately represent the disease. Without a more thorough analysis of T-cell activation, resistance to apoptosis, and lower suppression response, the conclusions should be presented with more caution. Twisting the language might facilitate understanding and interpretation for the reader. For example, the authors use the explanation that "CD4+ Tcon cells may exist in plastic or transitional states rather than conforming to the classic Th1/Th2/Th17 paradigm", without considering that technical limitations could have decisive impact on this conclusion. Additionally, some immunofluorescence images or graphics are still unfocused and do not contain appropriate quantifications (figs 3P and 4H, 7C, 7D), which limit interpretation. It might be important to note that the term "tissue-resident lymphocytes" used throughout the text refers to B-cells and T-cell populations present in the lungs in this context of this study and not to a specific population of CD8+CD103+ (ITGAE) CD69+ T-cell. Finally, the discrepancy in gene expression and pathways observed in GZMB+CD8 T cells is puzzling, and although the authors justify this issue in their response to the reviewer, this point is not addressed in the text, nor is the meaning of these findings to the detrimental role of these cells.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors addressed all my questions.

Reviewer #2

(Remarks to the Author)

The authors addressed all comments and made appropriate changes.

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Reviewers' comments:

Reviewer #1 (Remarks to the Author):

In this manuscript, Gao et al characterized lung resident lymphocytes from human lung tissues, and observed that GZMK⁺ TRM and HSP hi CD4⁺ Tcon enriched in IPF lungs. The manuscript is well organized, however, some points need to be addressed:

Major,

1. The introduction didn't include the most background of this research, it should be expanded.

Response: We sincerely thank the reviewer's suggestion. Accordingly, we have expanded the introduction to provide a more comprehensive background. This includes a clearer rationale for focusing on tissue-resident immune cells in IPF, a more detailed introduction of current knowledge and gap in studying human lung-resident immune subsets, and a stronger emphasis on the novelty of combining ex vivo lung perfusion with single-cell RNA-sequencing to address the challenge. Line 27-49

2. TCF7 has been reported to suppress lung CD103⁺ TRM development (PMID: 32268106), why does GZMK-TRM express an observable level of it? Do GZMK⁺ and GZMK⁻ TRM in IPF patients express CD103?

Response: We greatly appreciate the reviewer's insightful comment regarding TCF7 and CD103 expression in tissue-resident T cells, which prompts us to clarify the molecular differences between our human IPF study and previous animal model findings, as well as the potential regulatory mechanisms underlying CD103 expression in human lung tissue-resident T cells.

First, we fully acknowledge the key finding from the study cited by the reviewer (PMID: 32268106): in mouse models, TCF7 knockout significantly increases the number of CD103⁺ tissue-resident T cells. The elevated CD103 expression on TCF7-deficient CD8⁺ T cells is proposed to enhance tissue residency by strengthening interactions with epithelial cells, while also compensating for reduced tissue-resident T cell homeostatic turnover caused by TCF7 loss. This mechanism clearly establishes TCF7 as a negative regulator of CD103⁺ tissue-resident T development in murine systems.

However, our scRNA-seq data reveal a distinct pattern in human IPF lungs: both GZMK⁺ and GZMK⁻ CD8⁺ T lymphocyte subsets express detectable levels of CD103 (encoded by ITGAE) (Fig. S3F, Line 112-113). This observation indicates that the regulatory relationship between TCF7 and CD103 in human IPF tissue-resident T cells is more complex than the straightforward "TCF7 suppression → CD103 upregulation" axis observed in mice. It strongly suggests that additional regulatory pathways or microenvironmental factors modulate CD103 expression in human lung

tissue-resident T cells during IPF pathogenesis, independent of TCF7 alone.

Several potential mechanisms may contribute to this species-specific and disease context-dependent phenomenon:

1. TCF7-independent transcriptional regulation of CD103

In human T cells, CD103 expression is not exclusively controlled by TCF7. Transcription factors such as RUNX3 and EOMES have been shown to directly bind to the ITGAE (CD103) promoter and drive its expression in tissue-resident T cells. Our data show that GZMK⁺ CD8⁺ T cells exhibit high EOMES expression (Fig. 3D), which may override the inhibitory effect of low TCF7 levels and sustain CD103 expression. In contrast, GZMK⁻ subsets retain moderate TCF7 expression but may activate RUNX3 via TGF- β signaling (a key pathway in IPF fibrosis), leading to CD103 upregulation despite incomplete TCF7 downregulation.

2. IPF-specific microenvironmental cues

The fibrotic lung microenvironment in IPF is enriched with pro-fibrotic cytokines (TGF- β , IL-1 β), extracellular matrix (ECM) proteins (collagen, fibronectin), and hypoxia—factors that can induce CD103 expression independently of TCF7. TGF- β , in particular, is highly abundant in IPF lungs and has been demonstrated to upregulate CD103 on human CD8⁺ T cells through SMAD3-mediated signaling, even in the presence of TCF7. Additionally, direct interactions between T cells and activated fibroblasts or ECM proteins (e.g., via integrin $\alpha 5 \beta 1$ -fibronectin binding) can trigger intracellular signaling cascades that enhance CD103 expression, providing a TCF7-independent regulatory loop in the pathological niche.

3. Species-specific differences in tissue-resident T cell biology

Murine and human tissue-resident T cells exhibit inherent differences in transcriptional regulation and functional programming. For example, human tissue-resident T cells rely more heavily on TGF- β and ECM-derived signals for tissue retention, whereas murine tissue-resident T cells are more dependent on TCF7-mediated homeostatic turnover. This evolutionary divergence may explain why the TCF7-CD103 regulatory axis observed in mice is not recapitulated in human IPF tissue-resident T cells.

In summary, our finding that both GZMK⁺ and GZMK⁻ CD8⁺ T cells express CD103 in human IPF lungs reflects the complexity of tissue-resident T cell regulation in a pathological human context. It highlights that CD103 expression in human IPF tissue-resident T cells is governed by a combinatorial network of TCF7-independent transcription factors and microenvironmental signals, which may be critical for sustaining tissue residency of pro-fibrotic and pro-inflammatory T cells in the fibrotic lung niche. These data underscore the importance of studying human tissue-resident immune cells in their native disease environment, as findings from mouse models may not directly translate to human IPF pathogenesis.

3. In Fig S3B, co-inhibitory receptors are highly expressed in the GZMK⁺ population, so why is the related signaling suppressed in Fig. 3E?

Response: The high expression of inhibitory receptors like PDCD1 and CTLA4

indicates an "exhausted" or "dysfunctional" state, which is a hallmark of chronic antigen exposure. However, the IPA pathway analysis in Fig. 3E reflects the net signaling activity inferred from the entire transcriptomic profile. The prediction of "inhibition of PD-1/PDL1 and CTLA4 pathways" suggests that, despite the high receptor expression, the downstream inhibitory signaling within these T cells may be functionally impaired or overridden by stronger co-stimulatory signals (e.g., from CD28). This disconnect between receptor expression and functional pathway suppression is a recognized phenomenon in chronically activated T cells (Andrew Baessler, et al.2024. *Annu Rev Immunol*. PMID: 38166256, Daniel L Barber, et al. *Nature*. PMID: 16382236).

4. Is the protein level of Granzyme K higher in TRMs from IPF patients?

Response: We agree that protein-level validation is important. We have performed additional immunofluorescence (IF) staining for GZMK on lung tissue sections from IPF and control donors. The results confirm a significant increase in GZMK protein within CD8+ T cells in IPF lungs compared to controls. These new data have been added as Fig. 3P. Line 350

5. In Fig. 4H, CD44, CD4, and HSPA4 co-staining can't conclude that "HSP^{hi} CD4+ T cells particularly in the fibrotic regions" (line 192). The high cell density shown here is possibly iBALT, not fibrotic regions, if without related markers.

Response: We thank the reviewer for the insightful comment. To address this, we have performed two complementary experiments: 1) We retained our original immunofluorescence (IF) staining strategy to detect HSP^{hi} CD4+ Tcon cells (co-stained for HSPA6, CD4, and CD44) in lung tissue sections (Fig. 4H); 2) We conducted Masson trichrome staining on adjacent serial sections of the same lung regions to visualize collagen deposition (a hallmark of fibrotic areas) (Fig. S4G). The combined results clearly demonstrate that resident HSP^{hi} CD4+ Tcon cells are preferentially accumulated in regions with intense Masson staining (indicating high collagen deposition), confirming their localization in fibrotic regions rather than solely in iBALT structures. These updated data, including the IF images and corresponding Masson staining of the same regions, have been integrated into the revised Fig. 4H and Fig. S4G. Line 185-188

6. Line 271-272, the description is ambiguous, what does the "fibrotic tissue" mean, if it means tissues from IPF patients, please show the less IgG deposition in control donors; if it means the fibrotic area, please show fibrotic marker co-staining.

Response: We apologize for the ambiguity. We meant that IgG expression is elevated in lung tissues from IPF patients compared with control donors. We have revised the text for clarity and have now included representative IF images for IgG in control lungs in the revised Fig.6L, which demonstrates significantly less IgG deposition compared with IPF lungs. Line 383

7. Fig 7D is unconvincing, please show the control photos of T/B cell staining in collagen- fields.

Response: We have added the images of control lungs in the revised Fig.7D, showing healthy lung tissue co-stained for Collagen, CD3, and CD20. These images demonstrate the absence of dense collagen-positive fibroblastic foci and reveal a distinct spatial distribution of lymphocytes compared with IPF lungs. Line 394

8. Fig 7 is disconnected from the previous figs, neither GZMK+ CD8 T cells nor HSP^{hi} Tcon were analyzed there, which makes the conclusion of this section too weak.

Response: We agree and thank the reviewer's comment. We have now performed a more targeted CellChat analysis to specifically investigate the interactions between fibroblast subsets (Alveolar, HAS1^{hi}, and CTHRC1^{hi}) and the key lymphocyte subsets highlighted in our study (GZMK⁺ CD8⁺ T cells and HSP^{hi} CD4⁺ Tcon cells). The results, added as Fig. S7E and S7F, confirm that CTHRC1^{hi} fibroblasts have the strongest predicted interactions with these aberrant lymphocyte populations. This new analysis strengthens the connection and supports our conclusion that the pathological stromal niche actively engages pro-fibrotic and pro-inflammatory lymphocytes. Line 283-288

Minor,

1. The legend of Fig. 1G, S3B, S3E, etc should include the accurate numbers of expression and % instead of only showing "high" and "low".

Response: We have updated the legends for Fig. 1G, S3B, S3E, etc., to specify that the feature plots show normalized gene expression levels (log-normalized counts).

2. In Fig.4, the cells should be called Tcon instead of CD4⁺ T, for Treg was not included in the analysis.

Response: We have revised the terminology in Fig. 4 and related text to refer to Tcon.

3. The legends of Fig 5B, 6A,B,D,I,J are overlaid.

Response: We have corrected the figure legends for Fig 5B, 6A, B, D, I, J to ensure no text is overlaid.

Reviewer #2 (Remarks to the Author):

In this manuscript, Gao and colleagues employed single-cell RNA sequencing to analyze approximately 100,000 resident immune cells collected from five control subjects and seven patients with idiopathic pulmonary fibrosis (IPF) after ex vivo human lung perfusion. Using this method, the authors labeled circulating immune cells with CD45 to distinguish between circulatory and resident immune cells. Their results indicate that 54.6% of resident cells were characterized as T-cells. The study further describes the transcriptional profiles of CD4 T cells, regulatory T cells, CD8 T cells, and B cells, and includes an analysis of the interactions between lymphocytes and fibroblasts. Although the study provides valuable insights into lung resident immune cells, the main objective of the research could be presented more clearly. Additionally, despite the detailed analysis of genes and pathways implicated to these cells, further robust analysis or interpretation would enhance understanding of the roles these lymphocytes play in fibrosis. Some key aspects that could benefit from further clarification include:

1. During the ex vivo perfusion of explanted lungs, the authors observed that T-cells constitute the most abundant resident cell population in the lungs (54%), whereas a smaller population, described as monocyte/macrophages, accounts for only 4% of resident lung cells in control, non-diseased lungs. However, alveolar and interstitial macrophages have previously been reported to be highly abundant in the lungs, as described by Aegerter et al. (2022). Could the lung perfusion process be selectively excluding alveolar macrophages and other populations present in the same compartment, such as T-cells located within the lumen? Please, explain this possible methodological issue.

2. Alveolar macrophages are specific lung-resident cells, yet they were not identified within the group of "resident" immune cells in this study. As a result, categorizing the immune populations as "resident" may lead to a misconception, creating the impression that T-cells are the most abundant leukocyte population in the lungs.

For question 1&2 response: Thank you for your insightful questions and valuable comments, which have prompted us to conduct a thorough reflection on the methodological details and data interpretation of our study. Alveolar macrophages (AMs) and interstitial macrophages (IMs) are well-recognized as critical lung-resident immune cells, and numerous previous studies—including work by Aegerter et al. (2022), Habermann et al. (2020), and Morse C et al. (2019)—have clearly demonstrated their important roles in IPF pathogenesis. These studies also highlight that the variations in sample digestion and processing methodologies are key factors contributing to differences in cell population profiling across single-cell sequencing studies.

We fully acknowledge your concerns regarding two critical points: first, the relative abundance of T cells (54.6% of resident immune cells) and the apparent

underrepresentation of monocyte/macrophage subsets (only 4%) in control lungs; second, the potential misconception in defining “resident” immune cell populations that might arise from the lack of identified alveolar macrophages. To clarify these issues, we need to emphasize the inherent connection between our experimental design, methodological choices, and the observed cell population characteristics.

First, regarding the ex vivo lung perfusion (EVLP) procedure itself: the perfusion fluid exclusively circulates through the vascular compartment and does not come into contact with the alveolar space or the lumen of the airways. Therefore, EVLP does not selectively exclude alveolar macrophages or luminal T cells. The observed cell population bias originates instead from the subsequent sample processing steps for single-cell suspension preparation. Specifically, our study aimed to focus on tissue-resident lymphocytes, which have been understudied in IPF despite their potential pathogenic roles. To maximize the recovery of these lymphocytes, we employed a mature, widely recognized 2-hour digestion process. In contrast, studies focusing on macrophage biology typically use digestion durations of less than one hour (Adams et al. (2020); Morse C et al. (2019)) to preserve macrophage viability and integrity. This is because alveolar macrophages are highly sensitive to prolonged digestion due to their tight adherence to the alveolar epithelium, making them more prone to loss or damage compared with the relatively robust lymphocytes. This methodological bias inherently leads to higher enrichment of lymphocytes while reducing the recovery of alveolar macrophages and other luminal cell populations (including T cells within the alveolar lumen). Thus, the differences in immune cell subset abundance between our study and reports using shorter digestion times or macrophage-enriched protocols are a direct result of targeted cellular isolation strategies, rather than a flaw in the EVLP technique.

Second, regarding the definition of “resident” immune cells in our study: we strictly defined resident immune cells as CD45⁺/intraarterial (i.a.) anti-CD45⁻ cells based on the EVLP-based labeling strategy, which has been validated to distinguish tissue-resident from circulating immune cells (as shown in Fig. 1C-E). The absence of alveolar macrophages in our “resident” immune cell clusters is not due to an incorrect definition of residency, but rather the aforementioned digestion-related recovery bias. We fully agree that alveolar macrophages are bona fide lung-resident immune cells, and their underrepresentation in our dataset reflects a methodological trade-off made to prioritize lymphocyte recovery—an intentional choice given the understudied status of tissue-resident lymphocytes in IPF.

It is important to note that our findings specifically reflect the transcriptional landscape of lung-resident lymphocytes (including CD4⁺ Tcon cells, regulatory T cells, CD8⁺ T cells, and B cells) and their interactions with fibroblasts in IPF versus control lungs. These results complement, rather than contradict, the well-established roles of macrophages in fibrosis. In future studies, we plan to modify our experimental protocol (e.g., adjusting digestion duration, optimizing enzyme combinations, and incorporating macrophage-specific enrichment steps) to specifically isolate and analyze alveolar and interstitial macrophages. This will enable a comprehensive investigation of crosstalk between macrophages, lymphocytes, and fibroblasts, further

clarifying their coordinated roles in IPF development.

We sincerely appreciate your constructive feedback, which will greatly help us improve the completeness and rigor of our research by highlighting the need for clearer methodological transparency and contextualization of our findings within the broader field of IPF immunology. Line 406-411

3. Figure 2 presents a series of lung stainings for immune markers, including CD45, CD44, CD4, CD20, and CD8, to identify resident immune cells (primarily CD44+). However, the low-magnification images do not clearly show double-stained cells as aimed at by the authors. Can you please show a higher magnification image and the respective image quantification? Additionally, in Figure 2C, could the authors specify the markers used to characterize the different populations of resident immune cells for greater clarity? Furthermore, in Figure 2D, the white text used for labeling in the plot is difficult to read.

Response: We sincerely appreciate your insightful and constructive comments, which have significantly helped us improve the quality of our manuscript. In response to your suggestions:

1. We have revised the images in Fig. 2A, E, G, H, I, and J by incorporating high-magnification insets, which now clearly visualize the double-positive stained cells as intended.
2. The specific markers used to characterize the 13 distinct populations of resident immune cells have been explicitly listed in Supplementary Fig. 2 for greater clarity.
3. To enhance the readability of the plot, we have changed the text color in Fig. 2D from white to black, which ensures optimal contrast against the background. Line 331

4. In Figure 3, the authors describe CD8 GZMK⁺ T cells as predominantly present in IPF lungs compared to controls. They also discuss the potential impact of aging on the appearance of these cells. Including a table that describes the characteristics of control and IPF donors would be helpful to determine whether the findings presented in the manuscript are consistent across different cohorts.

Response: We sincerely appreciate this valuable suggestion, which helps to strengthen the rigor of our cohort analysis. The detailed characteristics of control lung donors and IPF patients are summarized in Supplementary Table 1. To address your concern about the potential confounding effect of aging and further improve the transparency of our cohort information, we have supplemented this table with additional key variables, including smoking history, body mass index (BMI), and tissue acquisition sites. Notably, statistical analysis confirmed that there was no significant difference in age between the control and IPF groups (Fig. S3C), which eliminates age as a confounding factor for the observed enrichment of CD8⁺GZMK⁺ T cells in IPF lungs.

5. In this study, the authors observed that GZMK⁺ T cells exhibit low expression of GZMB, which they believe contrast with the established role of GZMB in antiviral responses. Other reports have shown that GZMK CD8 T cells are induced during viral responses (for example, Jonsson et al.,2022) As this study tested the function of GZMK in upregulating p16 (CDKN2A) and SA-βGal expression in human fibroblasts, could the authors confirm whether GZMB has a distinct contribution to the expression of these markers? This would help clarify the specific role of GZMK in fibrosis.

Response: We thank you for this well-founded and necessary suggestion. To test this, we treated human lung fibroblasts with recombinant GZMB protein, paralleling our GZMK experiments. GZMB treatment led to a significant reduction in the mRNA expression levels of TGFB in human lung fibroblasts, whereas no discernible effect on ACTA2, COL1A1 and CDKN2A expression was observed. This indicates that the pro-fibrotic and pro-senescent effects we observed are specific to GZMK. These new results have been added as Fig.3. Since p16 (CDKN2A) is a well-recognized senescence marker and GZMB treatment had no impact on its expression, we did not perform additional SA-βGal staining assays for the GZMB group. Line 157-164

6. In figures 3, 4 and 5, the authors described the different gene expression profile of CD8, CD4 and B cells found in IPF lungs and discussed possible biological roles of these cells in this disease. However, a pseudobulk analysis has been shown to provide a more statistically robust results of the scRNA-seq data by aggregating data from cells instead of bulk analysis that can be influenced by data of a specific sample. Please, consider this type of analysis, or explain the choices made for the analysis provided in the manuscript.

Response: We thank the reviewer for their careful reading of our manuscript and their insightful suggestion regarding the analytical method. We have carefully studied the literature comparing different differential analysis approaches, specifically the cited work by Courtine et al. (*Nature Communications* 2021), and appreciate the reviewer's point concerning the statistical robustness of pseudobulk methods.

In our study, we performed differential gene expression (DGE) analysis at the single-cell level using the MAST framework. This choice was deliberate and justified for our specific biological question and dataset, based on the following considerations:

1. Alignment with Study Aims and Biological Context:

Our goal was to identify cell-intrinsic transcriptional changes within specific immune cell subsets (CD8⁺ T cells, CD4⁺ T cells, B cells) between IPF and healthy lungs. IPF tissue is notoriously heterogeneous. Analyzing data at the single-cell level preserves this heterogeneity and allows us to detect shifts in gene expression that might be specific to particular cellular states or microenvironments, which could be averaged out during the data aggregation step of a pseudobulk approach.

2. Focus on Lowly Expressed Genes:

As correctly noted by Courtine et al., the differences between pseudobulk and

single-cell methods are minimal for lowly expressed genes, with the most pronounced disparities occurring among highly expressed genes. Our results, as highlighted in Figures 3–5, focus on a curated set of differentially expressed genes (DEGs) that are largely low-to-moderately expressed and show biologically consistent patterns. Therefore, we anticipate that the identification and interpretation of these key genes would be largely consistent regardless of the chosen method.

3. Statistical Rigor of the MAST Model:

The MAST (Model-based Analysis of Single-cell Transcriptomics) method is explicitly designed for scRNA-seq data. It employs a generalized linear mixed model that incorporates cellular detection rate as a covariate to control for technical artifacts (e.g., dropout events) and includes random effects or sample-level covariates to account for inter-sample variation. This modeling strategy directly addresses the concern that results might be driven by a specific sample with a high cell count, thereby providing statistically robust inferences at the single-cell level.

4. Appropriateness for Our Experimental Design:

Our cohort consists of 7 IPF and 5 control samples. While cell numbers per subset vary across donors, the MAST framework appropriately models this variation. Our downstream analysis further strengthened the findings by filtering DEGs based on both statistical thresholds (p-value & avg_log2FC) and biological prior knowledge from the literature. The consistent expression patterns visualized via violin plots provide additional validation for the identified genes.

5. Consistency with Established Practices in the Field:

Single-cell-level DGE analysis using methods like MAST or Wilcoxon rank-sum test is well-established and widely used in key scRNA-seq studies of pulmonary fibrosis (e.g., Habermann et al., *Nature Medicine* 2020; Morse et al., *Cell Reports* 2019). These studies have successfully uncovered disease mechanisms using this approach, demonstrating its validity for questions similar to ours.

We acknowledge the strengths of pseudobulk analysis in certain scenarios, particularly for complex experimental designs or when sample-to-sample cell count differences are extreme. However, for our specific objective—to uncover subtle, cell-type-specific expression changes in a heterogeneous disease, with a focus on genes that are often lowly expressed—the single-cell-level MAST analysis is an appropriate, robust, and field-standard choice. Line 485-493

7. CD4 and TREGs could have been analyzed together since TREGs are also CD4 T-cells. A major complication of this segregation is the fact that FOXP3 low cells might represent an activated subset of CD4 T-cell.

Response: We sincerely appreciate the reviewer's valuable comment regarding the analytical strategy for CD4⁺ T cells and regulatory T cells (Tregs). We fully agree that Tregs are a functionally specialized subset of CD4⁺ T cells, and we have strictly followed a logical analytical process that combines initial integrated profiling with subsequent subset-specific deep analysis. Notably, in the global UMAP projection (Fig. 2C), Tregs form a discrete cluster that does not overlap with conventional CD4⁺

T cell subsets—even when FOXP3^{low} Tregs are considered—confirming their distinct transcriptomic identity at the global profiling stage. The decision to perform separate downstream analyses of conventional CD4⁺T cells and Tregs was driven by their functionally divergent roles in IPF pathogenesis, which require targeted investigation beyond the global profiling stage:

Conventional CD4⁺T cells: Our core objective was to dissect subtype-specific alterations in IPF, such as the pathogenic shift from quiescent (TOB1^{hi}) to pro-inflammatory (HSP^{hi}) subsets (Fig. 4G–J). These subsets are defined by unique transcriptional programs (e.g., HSP-mediated inflammation, T cell activation pathways) that are irrelevant to Treg function and would be masked by combining them with Tregs in downstream analyses.

Tregs: IPF is characterized by a paradox—Treg accumulation (Fig. 2D) yet impaired suppressive function (Fig. 5D). To resolve this, we focused on Treg-intrinsic changes, including FOXP3 downregulation, reduced expression of suppressive molecules (CTLA4, IL2RA), and subset dynamics (FOXP3^{hi} activated vs. FOXP3^{lo} resting Tregs; Fig. 5A–D). These analyses demand a focused lens on Treg-specific transcriptional signatures that are distinct from those of conventional CD4⁺T cells.

8. The present manuscript offers a detailed and descriptive transcriptional analysis; however, the overall conclusions of the study could be clarified further employing a more robust analysis. While the authors focus on interactions between fibroblasts and lymphocytes, the significance of these interactions may require more careful interpretation, as the findings do not fully define the contribution of T-cells to fibrosis. Additionally, the T-cell clusters include cell types that are not traditionally defined (e.g., clusters characterized by high TOB1 and HSP expression) or show the presence of Th1 cells but lack other subsets, such as Th2 and Th17 cells, which makes the results more challenging to interpret.

Response: We sincerely thank the reviewer for this insightful and critical observation. We fully agree that applying the canonical Th1/Th2/Th17 framework does not clearly define the T-cell subsets in our dataset, which indeed poses a challenge for straightforward interpretation. To address this point directly, we have conducted a deeper re-evaluation of our data, and we believe this reveals a significant biological aspect of T-cell biology in the disease state under study.

1. **Acknowledgment of the Expression Patterns:** As the reviewer astutely noted, we have provided expression plots for the key transcription factors TBX21 (Th1), GATA3 (Th2), and RORA (Th17-associated) in our figures (Fig. S4B, D, E). As shown, the expression of these genes is broad and overlapping across the T-cell clusters and does not resolve into mutually exclusive populations. This directly confirms the reviewer's observation regarding the lack of discrete, canonical Th-subset partitioning.

2. **Biological Interpretation: From "Fixed Subsets" to "Continuous States":** This pattern strongly suggests that within the specific fibrotic tissue microenvironment we are investigating, infiltrating T-cells may not exist as fixed, polarized Th1/Th2/Th17 subsets. Instead, they likely exhibit:

(1) High Plasticity or Intermediate States: Many cells co-express genes associated with multiple lineages, indicating they may be in transitional or less-differentiated states.

(2) Microenvironment-Driven, Unique Activation States: Tissue-specific signals (e.g., TGF- β , hypoxia, metabolic stress) may drive T-cells towards disease-specific phenotypes that are not aligned with classic Th dichotomies.

(3) Implication of the "Non-traditional" Clusters (e.g., TOB1+HSP+): The presence of such clusters further supports this notion. These cells likely represent T-cell populations in a specific stress-responsive or regulatory state shaped by the local niche.

We will revise the text in the Discussion section to more clearly acknowledge and interpret this observation. We will state that the expression patterns of canonical transcription factors (TBX21, GATA3, RORA) are broad and overlapping in our dataset, suggesting that T-cells in this fibrotic niche may exist in plastic or transitional states rather than as discrete, polarized subsets. The presence of non-canonical clusters (e.g., TOB1+HSP+) will be discussed as potentially representing microenvironment-specific stress or regulatory states. Line 452-459

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors addressed most of my questions, but two points still need to be addressed:

1. The author claimed "Our data show that GZMK⁺ CD8⁺ T cells exhibit high EOMES expression (Fig. 3D), which may override the inhibitory effect of low TCF7 levels and sustain CD103 expression", this is an inaccurate concept, although the EOMES potentially bind to the promoters of ITGAE, EOMES is a well-appreciated TF that suppresses TRM differentiation.

Response: We sincerely thank the reviewer for this rigorous and astute correction, which points out a critical conceptual error in our previous response and helps us further improve the scientific rigor of our work. We fully acknowledge this mistake and apologize for the erroneous statement in our initial reply that "GZMK⁺CD8⁺ T cells exhibit high EOMES expression (Fig. 3D), which may override the inhibitory effect of low TCF7 levels and sustain CD103 expression". This argument is indeed conceptually flawed, as EOMES is a well-recognized transcription factor that represses TRM cell differentiation, a conclusion well-supported by existing literature in the field.

We wish to clarify that this incorrect interpretation was exclusively present in our initial response to reviewer comments and not included in any section of the revised manuscript. No relevant claims regarding the relationship between EOMES, TCF7 and CD103 expression have been made in the manuscript, and this error has not affected any of our data interpretation, study conclusions or experimental findings.

We have thoroughly reflected on this conceptual mistake, and will strictly follow well-established findings in the field for all subsequent data interpretation and scientific writing. We will also adopt a more rigorous multi-step review process for all study-related analyses and reviewer responses to ensure the accuracy and validity of all our statements. We would like to express our sincere gratitude again for the reviewer's professional and critical feedback, which is invaluable for elevating the quality of our research and avoiding similar inaccuracies in the future.

2. Fig S4G is great evidence to prove the colocalization of resident HSP α CD4⁺ Tcon with fibrotic regions, this panel should be moved into the main figures.

Response: As suggested, we have moved Fig S4G to the main figures (now Fig. 4I) and updated the corresponding figure legend and text accordingly.

Reviewer #2 (Remarks to the Author):

The goal of this study, which focuses on resident lymphocytes present in idiopathic pulmonary fibrosis, is an interesting concept. However, there are still several caveats regarding the overinterpretation of the results.

The authors seem to heavily rely on their methodology to draw conclusions and make

assertions, yet they do not sufficiently question the potential gaps in their methods as possible explanations for their findings. The primary issue lies in their gate strategy for differentiating between circulatory and resident cells, which significantly impacts the overall message of the manuscript.

Response: We appreciate the reviewer's feedback about overinterpretation and agree that a more balanced discussion of methodological limitations is essential. Regarding the gating strategy for distinguishing circulating from resident immune cells, we acknowledge that this is a critical technical aspect that warrants more thorough discussion. Intravenous (i.v.) administration of anti-CD45 antibodies for labeling circulating immune cells has become a widely validated method in both murine and non-human primate models, with minimal impact on cellular properties, including their transcriptional profiles (Anderson et al., Nat Protoc, 2014; Tkachev et al, Sci Transl Med, 2021; Potter et al., Sci Transl Med, 2021). And the EVLP-based intravascular antibody labeling technique we employed has been previously validated for identifying tissue-resident immune cells in human lungs (Snyder et al., Am J Respir Crit Care Med 2021). However, we recognize that this technique has limitations. The 30-minute labeling period may not achieve complete saturation of all circulating cells, and some cells within the vascular compartment might remain unlabeled. Conversely, antibody extravasation into the interstitial space, though minimized by the intact endothelial barrier during EVLP, cannot be entirely excluded. To address these concerns in the revised manuscript, we have added the discussion on study limitations with the following content (highlighted in revisions):

“Our sorting of tissue-resident immune cells relied heavily on the current gating method, which used EVLP-mediated intravascular anti-CD45 antibody labeling. While this technique is widely validated in murine and non-human primate models⁶³⁻⁶⁵ and has been validated for identifying human lung-resident immune cells with minimal impact on cellular properties¹⁶, it is not without limitations. The 30-minute labeling period may not fully saturate all circulating cells, leaving some vascular cells unlabeled and potentially misclassified as resident cells. Additionally, antibody extravasation into the interstitial space, though minimized by the intact endothelial barrier during EVLP, cannot be entirely excluded. ”

Another important factor to consider is the tissue digestion protocol mentioned by the authors. It was stated that they "employed a mature, widely recognized two-hour enzymatic digestion process" for lung tissue. However, other research groups have opted for shorter digestion times due to the adverse effects that these enzymes can have on cells. Such effects might include the activation of genes that do not accurately represent the disease. Without a more thorough analysis of T-cell activation, resistance to apoptosis, and lower suppression response, the conclusions should be presented with more caution. Twisting the language might facilitate understanding and interpretation for the reader. For example, the authors use the explanation that “CD4+ Tcon cells may exist in plastic or transitional states rather than conforming to the classic

Th1/Th2/Th17 paradigm” , without considering that technical limitations could have decisive impact on this conclusion.

Response: We sincerely thank the reviewer for this critical comment on our tissue digestion protocol and potential technical limitations, which has helped us further clarify the methodological rationale and data interpretation in our study.

1. Rationale and literature basis for the 2-hour enzymatic digestion protocol

Our use of a 2-hour enzymatic digestion protocol for lung tissue isolation was not arbitrary but was based on previously established methodologies reported in lung research. Specifically, this protocol was adapted from two prior studies: Kathiriya et al. (Nature Cell Biology, 2022) and Wang et al. (Immunity, 2023), the latter of which focused on lung-resident lymphocytes. This digestion method has been validated in both studies for effective isolation of lung tissue-derived cells while preserving their transcriptional integrity. Consistent with these prior reports, we observed effective recovery of lung-resident lymphocyte populations in our dataset.

2. Addressing potential technical artifacts in T cell activation and phenotypic interpretation

We fully acknowledge the reviewer’s concern that enzymatic digestion might induce artificial gene activation and interfere with the interpretation of T cell phenotypes. To exclude such technical artifacts and ensure the reliability of our conclusions regarding T cell activation, anti-apoptotic features and suppressive function, we have conducted multi-layered validation experiments:

Functional validation:

For the pro-fibrotic phenotype of GZMK⁺CD8⁺T cells, we performed in vitro functional assays by treating human lung fibroblasts with recombinant GZMK protein to validate our transcriptional findings. These assays demonstrated the pro-fibrotic and pro-senescent effects of GZMK, and these functional results are independent of tissue digestion, further confirming the biological relevance of our T cell phenotypic analysis.

Spatial validation:

We combined immunofluorescence staining with Masson trichrome staining of adjacent serial lung sections to confirm the spatial colocalization of pro-inflammatory HSP^{hi}CD4⁺Tcon cells with fibrotic regions in IPF lungs. This spatial evidence is unaffected by enzymatic digestion and validates the in vivo relevance of the T cell phenotypes we identified.

3. Reconfirmation of CD4⁺ Tcon cell plasticity in IPF lungs (excluding technical limitations)

Our conclusion that CD4⁺ Tcon cells in IPF lungs may exist in plastic or transitional states rather than conforming to the classic Th1/Th2/Th17 paradigm is not based solely on single-cell transcriptional profiling. Instead, it is supported by multiple lines of evidence that rule out technical limitations caused by enzymatic digestion:

First, the overlapping expression of canonical Th subset transcription factors

(TBX21 for Th1, GATA3 for Th2, RORA for Th17) was observed in all 7 IPF samples, but not in the 5 control samples processed with the identical digestion protocol. This indicates that this phenotypic feature is disease-specific, rather than a technical artifact.

Second, the identification of non-canonical CD4⁺ Tcon subsets (TOB1^{hi} quiescent cells and HSP^{hi} pro-inflammatory cells) was accompanied by the activation of corresponding signaling pathways (e.g., heat stress response, TNFR2 signaling) and specific spatial localization in fibrotic regions. These features are consistent with the pathological microenvironment of IPF, characterized by high levels of TGF- β , hypoxia and extracellular matrix stress, and cannot be explained by enzymatic digestion alone.

Third, published studies on lung fibrosis T cell biology (e.g., Tomomi Ichikawa et al, Nature Immunology, 2019; Celada LJ et al, Science Translational Medicine, 2018) have also reported the loss of classic Th subset polarization in fibrosis lungs. Their findings are highly consistent with ours, and further support that the plasticity of CD4⁺ Tcon cells is a genuine biological characteristic of IPF, rather than a technical limitation.

We agree with the reviewer's comment that prolonged digestion may, to some extent, affect lymphocytes. Accordingly, we have revised the Discussion section of the manuscript to address this point as follows:

"Finally, our 2-hour enzymatic digestion protocol, referenced from two previous studies^{66,67}, likely contributed to the efficient isolation of more lymphocytes. However, we note that some research groups use digestion times under one hour, as longer digestion may induce enzyme-related stress that affects cells. Optimizing digestion conditions, such as comparing different durations and enzyme formulations, could further refine lung-resident immune cell isolation while reducing in vitro cellular perturbations."

All relevant revisions are highlighted in the revised manuscript.

Additionally, some immunofluorescence images or graphics are still unfocused and do not contain appropriate quantifications (figs 3P and 4H, 7C, 7D), which limit interpretation.

Response: We sincerely thank the reviewer for this valuable comment and apologize for the suboptimal image quality and lack of quantification. We have addressed these concerns as follows:

All unclear images have been replaced with high-resolution versions.

Statistical analysis for Fig. 3P has been added in the new Fig. 3Q, confirming significant enrichment of GZMK⁺CD8⁺ T cells in IPF lungs ($P < 0.01$).

We supplemented quantification for Fig. 4H, verifying that HSPA6⁺CD44⁺CD4⁺ Tcon cells are significantly enriched in IPF fibrotic regions versus non-fibrotic areas ($P < 0.001$). Fig. 7D is visual validation, showing CTHRC1^{hi} fibroblasts (COLLAGEN⁺) closely associated with B/T lymphocytes in IPF fibroblastic foci to illustrate this spatial relationship. In addition, the CellChat heatmap referenced in Fig. 7C is in Fig. 7E, which quantifies interaction strength between fibroblast subsets and lymphocytes.

The revisions are highlighted in the manuscript.

It might be important to note that the term “tissue-resident lymphocytes” used throughout the text refers to B-cells and T-cell populations present in the lungs in this context of this study and not to a specific population of CD8⁺CD103⁺ (ITGAE) CD69⁺ T-cell.

Response: We thank the reviewer for this clarification. We agree that our use of “tissue-resident lymphocytes” encompasses all lymphocyte subsets isolated from the resident fraction after EVLP, including B cells, CD4⁺ T cells, CD8⁺ T cells, and others, rather than being restricted to canonical CD8⁺CD103⁺CD69⁺ tissue-resident memory T cells (TRM). To avoid confusion, we have supplemented the Discussion section with the following content:

“Fifth, we acknowledge limitations in our identification of tissue-resident lymphocytes and the associated gating strategy. Importantly, the “tissue-resident lymphocytes” described in this study refer broadly to B-cell and T-cell populations residing within lung tissue, rather than the canonical CD8⁺CD103⁺CD69⁺ tissue-resident T-cell subset.”

The revisions are highlighted in the manuscript.

Finally, the discrepancy in gene expression and pathways observed in GZMB⁺CD8⁺ T cells is puzzling, and although the authors justify this issue in their response to the reviewer, this point is not addressed in the text, nor is the meaning of these findings to the detrimental role of these cells.

Response: We sincerely thank the reviewer for this insightful comment. To clarify the gene expression differences and pathological roles of GZMB⁺CD8⁺ T cells, we have supplemented the Discussion section with the following content:

“The relative expansion of GZMK⁺ over GZMB⁺ CD8⁺ T cells in IPF lungs has important functional implications. While GZMB mediates classical cytotoxicity via caspase activation, GZMK lacks this activity and instead acts as a pro-inflammatory mediator that induces fibroblast senescence and collagen production. Thus, GZMK⁺ CD8⁺ T cells may contribute to fibrosis not through direct tissue damage via cytotoxicity, but rather through non-cytotoxic, pro-fibrotic remodeling. The predominance of GZMK⁺CD8⁺ T cells in IPF, together with their spatial association with fibroblasts in fibrotic foci, supports a model where GZMK⁺CD8⁺ T cells drive disease progression via stromal cell modulation.”

As noted in our previous response, GZMB and GZMK have distinct roles in pulmonary fibrosis: recombinant GZMB reduced TGFB expression in human lung fibroblasts but had no effect on pro-fibrotic (ACTA2, COL1A1) or senescence (CDKN2A) markers, while GZMK specifically promoted these phenotypes (Fig. 3N-O). These revisions

clarify the biological relevance of GZMB⁺CD8⁺ T cells and provide a clearer mechanistic context for their transcriptional differences in IPF. All revisions are highlighted.