

Pulmonary fibroblast activation during *Aspergillus fumigatus* infection enhances lung defense via immunomodulation and tissue remodeling

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Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this study, the authors investigate the role of pulmonary fibroblasts in response to *Aspergillus fumigatus* infection, demonstrating that fibroblast activation contributes to lung defense via immunomodulatory and extracellular matrix (ECM) remodeling functions. The authors employ novel cell lineage tracing, targeted cell ablation, single-cell RNA sequencing (scRNA-seq), and histological analysis to delineate the response of fibroblasts to fungal infection. Their findings highlight that periostin-expressing (PostnLin) fibroblasts become activated in response to *A. fumigatus* and contribute to infection resolution. The absence of these cells exacerbates disease progression, indicating a protective role of fibroblasts in host defense. The manuscript is very well written, the results are original, clearly communicated, reproducible, and support the conclusions of the authors. However, the significance of the findings is not clear, and the results are generally expected and unsurprising.

Major issues:

- 1) The study is very descriptive, and not mechanistic. The finding that DTA-deletion of Postn+ fibroblasts in aspergillosis increases disease is a significant one. However, a potential mechanism is only discussed (lines 401-403), not investigated. The rest of the study characterizes fibroblast populations in the lung, which is important, but does not increase the potential impact of the study.
- 2) There is no supporting data in human fibroblasts.
- 3) It is unclear why bulk sequencing was done, followed by scRNAseq, when only single cell was necessary. Bulk sequencing might have some issues, especially since there appeared to be multiple variables in the cell populations that were sorted (based on both Pdgfra+/- and infection/non-infection). Also, spatial sequencing would be very appropriate for this project.

Minor issues:

- 1) It is confusing why PostnLin cells are concluded to be distinct from resident PdgfraLin fibroblasts when 60% of PostLin cells are Pdgfra+ (lines 200-202).
- 2) Why was the triaminiclon model of immune suppression used? Why was it necessary to infect 5 hours prior to treatment? More information would be useful for the reader.
- 3) Bulk sequencing results should probably be supplemental, as scRNAseq results are more informative.
- 4) Lines 526-527. "At least three biological replicates were quantified per replicate" is confusing.
- 5) Figure 5. Why is TAM administered one day before fungal inoculation? Is it possible that the damage from DTA-mediated killing is more important than the loss of PostnLin cells? What if TAM was administered several days before infection and mice were allowed recover DTA-mediated damage?

Reviewer #2

(Remarks to the Author)

In this manuscript entitled "Pulmonary fibroblast activation during *Aspergillus fumigatus* infection enhances lung defense via

immunomodulation and tissue remodeling", Guirao-Abad and colleagues investigated the role of fibroblasts during fungal pneumonia. The authors first showed that the lungs became fibrotic after *A. fumigatus* inoculation. The authors used Postn-MerCreMer mice to show Postn+ fibroblasts markedly increased after the infection. The authors showed that those de novo Postn+ cells were mostly fibroblasts in the alveolar regions, and the ablation of Postn+ cells caused increased mortality after *A. fumigatus* inoculation in immunosuppressed mice. By bulk RNA-seq and single cell RNA sequencing, the authors showed that alveolar fibroblasts underwent pro-fibrotic and pro-inflammatory changes after *A. fumigatus* inoculation. Although this manuscript demonstrated the protective function of Postn+ fibroblasts in fungal pneumonia, the authors do not adequately explore the mechanism(s) by which loss of periostin lineage cells increases mortality in immunosuppressed mice inoculated with *A. fumigatus*. Although the authors describe areas of hemorrhage and angio-invasion after deletion of periostin+ cells, the manuscript currently lacks quantitative data regarding the observed phenotypes and detailed information on the method to evaluate the results. There are also no experimental data explaining how the deleted cells might prevent these adverse events.

Since periostin+ cells emerge after most of the organisms were cleared in wild type mice it is not clear how/if these cells would have contributed to immune cell recruitment. At the very least it would be important to perform quantitative immunostaining and/or flow cytometry to determine whether loss of periostin+ cells modulated the recruitment and/or activation of adaptive and innate immune cells. It would also have been useful to see whether differences in mortality were due to effects on vascular permeability.

Additionally, the possibility of Postn-CreER targeting pericytes rather than fibroblasts should be experimentally excluded to prove the authors' claims.

Major concerns

1. The genetic background of the mice used for the experiments should be described in detail. The Postn-MerCreMer mouse distributed in the Jackson Laboratory has been only backcrossed 5 times. How many times did the authors backcross the Postn-MerCreMer mouse? In Figure 5, what mice did the authors use for control? This information is critical since the different genetic backgrounds can show different sensitivity to infection. Additionally, did the control group in Figure 5 also receive tamoxifen treatment? Tamoxifen itself can cause various effects on the lungs. Tamoxifen food can also cause body weight loss sometimes since mice are reluctant to eat it. If the control groups in Figure 5 were not treated by tamoxifen, how do the authors exclude the possibility that the phenotype is caused by tamoxifen?
2. The authors stated that the Postn+ cell ablation resulted in pulmonary hemorrhage and fungal angioinvasion in Figure 5. These phenotypes should be quantified and statistically evaluated.
3. As the authors stated in the discussion, publicly available scRNA-seq data show Postn expression in pericytes at baseline. What portion of Postn-lineage cells are pericytes? Authors should be able to quantify this by Mcam (CD146) staining on flow cytometry. Although authors showed minimal tdTomato+ cells without infection by histology, this should be confirmed by flow cytometry of digested lungs for a more unbiased assessment of whole lung distribution. If there are minimal tdTomato+ cells without infection, can the authors explain why Postn-MerCreMer specifically labels fibroblasts but not pericytes, which highly express Postn at baseline?
4. Since Postn is also highly expressed in pericytes, the authors should experimentally exclude the possibility of pericytes contributing to the phenotypes in Figure 5. The authors should be able to do this by treating the mice with fungus after treating and washing out tamoxifen or using Rosa26-DTR mice.
5. Figure 7E is difficult to understand. Figure 1A shows that there are normal alveoli left on day 1. However, Figure 7E seems to show that all alveolar fibroblasts homogeneously become activated alveolar fibroblasts. Similar results are shown for peribronchiolar fibroblasts. If the clusters are completely driven by treatment and time points, the authors might want to try integration methods such as Harmony or Mutual Nearest Neighbors (MNN), which may allow them to identify newly emerged populations while keeping some normal populations.
6. In Figure 7E, the authors showed only inflammatory genes for the alveolar fibroblast lineage. Differentially expressed genes for each cluster should be shown by heatmap or dot plots for readers to know the specific genes of each cluster.
7. The authors need to include data demonstrating the efficiency of the deletion method used to delete periostin-lineage cells. It would also be good to overlay periostin expression on the uMAP plots and to have included the lineage label in the single cell sequencing experiments so the authors could determine which of the emergent stromal populations they describe were derived from periostin-expressing cells and thus which populations were likely to have been deleted.

Minor comments

8. It would be nice to quantify fibrosis after fungus infection by hydroxyproline assay
9. Figure 7D, colors for activated alv and deactivated alv were swapped.
10. Figure 4 would benefit from addition of staining for specific pericyte markers.

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

This study combines robust in-host assays with newly generated reporter animal lines and scRNAseq to uncover a previously unappreciated role for pulmonary fibroblasts in limiting the severity of *Aspergillus*-induced pulmonary injury. The authors provide strong evidence that collagen is deposited at sites of *Aspergillus*-induced inflammation and that periostin-

positive fibroblasts, localized primarily in the alveoli, are the major contributors. Use of a novel mouse line that can be specifically depleted for this cell type, causes exacerbation of the severity of invasive aspergillosis. In-depth bulk and scRNAseq analyses revealed that infection with *A. fumigatus* triggers pulmonary stromal cells to differentiate into an emergent population of fibroblasts with ECM-producing and immunomodulatory roles. Importantly, the authors also provide data showing that these activated fibroblasts return to their resident state as infection resolves.

Major strengths:

- This manuscript is very well written, and the findings will have broad impact.
- The study uncovers a novel cellular response to *A. fumigatus* infection that promotes appropriate host response to limit tissue damage.
- The study demonstrates that even heat-killed conidia, that are coated in a protective barrier of hydrophobic proteins, can elicit a fibroblast response. This is critical as the hydrophobin layer has always been considered as rendering resting conidia immunologically inert, leaving the initial signals driving host response vague.
- The study confirms that activation of fibroblast subsets also occurs in response to two additional isolates of *A. fumigatus*.

Points to address:

1. Do the authors have any data suggesting that fungal burden is (or is not) impacted by loss of the postn lineage fibroblasts during invasive disease (Figure 5)? Maybe this is just a point for the Discussion, but is it possible that after invasive growth begins, these fibroblasts play a role in limiting fungal growth? Or, is the increased mortality of the DTA mice simply due to heightened levels of tissue damage in response to the same amount of fungal growth?
2. In the same line of thought, the immune competent DTA mice did not experience death after infection (Line 223), but did they have prolonged time to clearance/sterilization? Even negative data may be very informative here and could be included as a Supplemental Figure, if available.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript presents interesting findings that highlight a potentially important role for fibroblasts in shaping immune responses and maintaining lung integrity during fungal infection. The study's use of fibroblast lineage tracing and ablation in a model of invasive aspergillosis is important and novel and contributes to our understanding of fibroblast responses to *A. fumigatus*. However, while the data convincingly show altered immune cell recruitment and gene expression in the absence of periostin-lineage fibroblasts, the mechanistic conclusions advanced in the text—particularly those concerning alveolar hemorrhage, angiogenesis, and vascular compromise—are not directly supported by the experimental data. The authors reference new data additions and state that over 12 figures have been added in the revision, but these appear to primarily represent expanded or reformatted analyses of existing datasets. No new *in vivo* mechanistic experiments were introduced, nor were fibroblast-specific effector functions or endothelial outcomes directly tested. Thus, mechanistic claims remain speculative. Additionally, while the revised manuscript includes clearer figure labeling and additional cytokine profiling, the requested functional studies to demonstrate causality—particularly those needed to link fibroblast ablation to vascular injury or fungal dissemination—are still lacking. Based on these results, TGF-beta may be a candidate for PostLn-specific ablation. The methods are generally sound and reproducible, and the work is likely to be of interest to those studying fungal pathogenesis and pulmonary immunology. However, the authors need to provide additional experimental support for the proposed mechanism.

Reviewer #2

(Remarks to the Author)

The authors have adequately responded to all of the issues raised in my initial review

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

The authors have completed an impressive amount of additional work to directly address all concerns of the previous review. The authors have included 12 new figures and altered existing figures, as well as included major edits to the presentation and discussion of results in the text. Together, these new data and revisions have greatly improved the manuscript and increased the rigor and significance of the work. I have no other critiques for the authors to consider.

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Response to Reviewers for NCOMMS-25-02679

We are grateful to the reviewers for their thoughtful and constructive evaluations of our manuscript. We have addressed all comments, performed additional experiments, and added more than 12 new figures to strengthen the study. To improve the clarity, we have repositioned the section titled ("Postn^{Lin} cells express a unique transcriptional profile of matrix modification compared to resident pulmonary fibroblasts") earlier in the Results.

A central theme across the reviews was the mechanism by which fibroblast ablation increases mortality during *A. fumigatus* infection. To address this, we quantified fungal burden, immune cell recruitment, alveolar hemorrhage, and vascular invasion in both immunosuppressed and immunocompetent mice. Our results in this revised manuscript demonstrate that fungal burden remains unchanged following Postn^{Lin} ablation, whereas myeloid cell infiltration, alveolar hemorrhage, and angioinvasion are significantly increased. These findings establish that Postn^{Lin} fibroblasts protect the host by modulating inflammation and preserving vascular integrity, rather than by directly limiting fungal growth. We believe these new results directly resolve the major mechanistic concerns raised by Reviewers #1, #2, and #4.

Reviewer #1

...However, the significance of the findings is not clear, and the results are generally expected and unsurprising.

We appreciate this perspective and have revised the text (lines 92–97, 559–565) to highlight the novelty of our findings. While fibroblasts are expected to contribute to ECM remodeling, our new data reveal that their response extends beyond matrix deposition to include a profound immunomodulatory role. For the first time, we demonstrate that ablation of activated fibroblasts accelerates mortality in IPA, with new data showing this is accompanied by exacerbated inflammation rather than increased fungal proliferation. We have revised and expanded the data presented in Fig. 6; Fig. 7; Fig. S7; Fig. S8).

1) The study is very descriptive, and not mechanistic. The finding that DTA-deletion of Postn+ fibroblasts in aspergillosis increases disease is a significant one. However, a potential mechanism is only discussed (lines 401-403), not investigated. The rest of the study characterizes fibroblast populations in the lung, which is important, but does not increase the potential impact of the study.

We performed additional experiments to provide mechanistic insights into how fibroblast ablation influences the two major determinants of disease outcome in IPA: fungal burden and the associated inflammatory response. Our results demonstrate that ablation of activated fibroblasts following *A. fumigatus* exposure is associated with an exacerbated inflammatory response, with no detectable alteration in fungal burden. New experiments quantifying fungal burden and immune cell recruitment have been included. Revised results are included in the manuscript at lines 268–307, 470–480; and figures Fig. 6F–L, Fig. S7).

2) There is no supporting data in human fibroblasts.

We thank the reviewer for this comment. To address this point, we have now included new data demonstrating that primary human pulmonary fibroblasts can directly sense and respond to *A. fumigatus* exposure. Specifically, we performed *in vitro*

stimulation experiments using isolated primary fibroblasts and assessed their ability to detect and respond to fungal challenge. The results revealed a strong immunomodulatory response within 24 hours, particularly in response to actively growing hyphae, whereas heat-killed conidia induced only modest activation. These findings provide the first evidence that human pulmonary fibroblasts can directly respond to *A. fumigatus*.

Accordingly, we have added new Figure 7G and Figure S10 (described in lines 422–438) and expanded the Discussion section (lines 532–555) to include recent studies identifying POSTN⁺ fibroblast subsets and inflammatory fibroblast states in human lung disease, including COVID-19 and idiopathic pulmonary fibrosis. While *in vivo* validation through human IPA samples remains a future direction, these new data substantially strengthen the translational relevance of our findings.

3) It is unclear why bulk sequencing was done, followed by scRNAseq, when only single cell was necessary. Bulk sequencing might have some issues, especially since there appeared to be multiple variables in the cell populations that were sorted (based on both *Pdgfra*⁺/⁻ and infection/non-infection).

We apologize for the lack of a clear rationale for doing both bulk RNAseq and scRNA-seq. Our bulk RNA-seq analysis specifically focused on Postn^{Lin} cells, which represent those cells that had activated the Postn promoter during the initial 1-week window, rendering that cell and its progeny permanently tagged with tdTomato. This allows us to characterize their distinct pro-fibrotic profile in response to fungal challenges. In parallel, we performed scRNA-seq at early post-challenge stages (Day 1), revealing an immunomodulatory profile that is distinct from the one observed in Postn^{Lin} cells. It is important to indicate that an isolated Postn⁺ expression fibroblast cluster was not detected by scRNA-seq either at Day 1 or Day 7 and bulk RNA-seq proved to be the most effective approach for analysing this specific population. This can be explained by the fact that fibrotic clusters are predominantly found in conditions that induce acute fibrosis, such as the bleomycin-induced IPF model, whereas the moderate response observed with *A. fumigatus* results in transient Postn expression distributed across all clusters.

To improve the clarity, we have repositioned the section titled (“Postn^{Lin} cells express a unique transcriptional profile of matrix modification compared to resident pulmonary fibroblasts”) earlier in the Results. The text has been revised to clarify these aspects (lines 311-324 and lines 516–531).

4).... spatial sequencing would be very appropriate for this project.

We appreciate this thoughtful suggestion and agree that spatial RNA sequencing would provide valuable insight into the spatial organization and stage-specific responses of fibroblast subsets during *A. fumigatus* infection. We have already initiated plans to incorporate these approaches; however, we encountered a resolution limitation in generating the necessary data. Because of the close proximity of myeloid cells and fibroblasts observed in Fig. 4L, we require single-cell-level spatial sequencing to distinguish these populations.

The 10x Genomics Visium Spatial Gene Expression platform, while capable of unbiased whole-transcriptome profiling, lacks sufficient spatial resolution for this purpose. Therefore, we are pursuing the 10x Genomics Xenium In Situ platform, which allows custom targeted gene panel design and enables single-cell and subcellular spatial mapping of fibroblast-immune-epithelial interactions. This next-generation spatial

strategy will allow us to define how the tissue microenvironment at the host–pathogen interface shapes both host and fungal responses, complementing and extending the findings presented here. These experiments are currently underway and will form the basis of future studies beyond the scope of the present manuscript.

Minor Comments:

1) It is confusing why *Postn^{Lin}* cells are concluded to be distinct from resident *Pdgfra^{Lin}* fibroblasts when 60% of *Post^{Lin}* cells are *Pdgfra⁺* (lines 200-202).

We apologize for this confusion. Resident fibroblasts are *Pdgfra⁺*, but do not express *Postn*. While all quiescent resident fibroblasts are *Pdgfra⁺* (represented in Fig. S4A-D), *Postn^{Lin}* cells represent an activated and differentiated subset that emerges in response to *A. fumigatus*, exhibiting a distinct morphology (Fig. S4E-H) and functional response (Fig. 5). It is important to note that, although *Postn^{Lin}* cells can express *Pdgfra*, their expression of this receptor is reduced compared to resident fibroblasts (Fig. 5D) which is a finding that is supported by our previous study (Kanisicak et al., 2016).

We made the following modifications to clarify that the *Pdgfra⁺* lineage (*Pdgfra^{Lin}*) cells depicted in Fig. S4A-D are quiescent:

-Line 175: "...the morphology of **quiescent** *Pdgfra^{Lin}* resident pulmonary fibroblasts to that of *A. fumigatus*-induced..."

-Line 176: "... **Quiescent** resident *Pdgfra^{Lin}* fibroblasts displayed a thin, elongated morphology..."

-Also added clarity to the figure titles in Fig.4

2) Why was the triamcinolone model of immune suppression used? Why was it necessary to infect 5 hours prior to treatment? More information would be useful for the reader.

All mouse models of IPA require some level of immunosuppression to develop IPA (similar to the situation in humans). This is generally accomplished using a corticosteroid such as triamcinolone, administered as a single dose to create transient immunosuppression, or multiple doses to maintain constant levels of immunosuppression over time. More severe immunosuppression is accomplished by combining the corticosteroid with multiple doses of the chemotherapeutic agent cyclophosphamide. We chose a single dose of triamcinolone to minimize the potential for variability that could arise with the use of multiple doses of two different immunosuppressants. Moreover, our use of a mild and transient immunosuppression protocol allowed us to analyze the immune response to infection by flow cytometry, which would be masked by the use of more severe or chronic immunosuppression protocols. We chose to infect 5 hours prior to immunosuppression to allow the initial host-pathogen interaction to occur in the absence of immunosuppression, which could potentially impact fibroblast activation.

- Expanded rationale for triamcinolone model and infection timing (lines 466–470).

3) Bulk sequencing results should probably be supplemental, as scRNAseq results are more informative.

Since bulk RNA-seq provides key information about a cell type not captured at the time-points represented in the scRNA-seq data, we strongly suggest keeping this information in the main text. To improve clarity, we have repositioned this section titled as stated

above lines 214-252. The text has been revised to clarify the rationale of this complementary and significant aspects. *However, we are happy to move it to the supplementary section if collectively reviewers and editors concur that this is the best way to present the data.*

We have revised rationale in the single cell seq data section as well in lines 311-324 and the discussion section.

4) Lines 526-527. "At least three biological replicates were quantified per replicate" is confusing.

The sentence has been corrected: "At least three biological replicates were quantified per experiment" (Line 640-641)

5) Figure 5. (Revised Figure 6) Why is TAM administered one day before fungal inoculation? Is it possible that the damage from DTA-mediated killing is more important than the loss of Postn^{Lin} cells? What if TAM was administered several days before infection and mice were allowed recover DTA-mediated damage?

We thank the reviewer for raising this important point. Tamoxifen (TAM) was administered one day before fungal inoculation to ensure that Cre recombination occurred concurrently with the induction of Postn expression, which is only activated in fibroblasts upon tissue injury or infection. Administering TAM several days prior to fungal challenge would not result in recombination, as Postn-MerCreMer activity requires de novo promoter activation, and quiescent fibroblasts do not express Postn. Consistent with this, "tamoxifen only" controls exhibited minimal to no tdTomato labeling, even after extended exposure (Fig. 2C, 2F).

Previous kinetic studies have shown that TAM-induced recombinase activation reaches 50% within 12 h and plateaus by 24 h (Feil et al., 1997), and serum levels peak within 6–7 h before declining over 24–48 h (Vanhecke et al., 2024). Thus, TAM administration one day before infection ensures efficient recombination precisely during Postn promoter activation.

In our hands, we determined that the Postn^{Lin} ablation achieves ~30% depletion of Pdgfr α positive fibroblasts (new Fig. 6J). Under immunocompetent conditions even with high dose of fungal challenge, Postn^{Lin} fibroblast loss did not elicit increased lethality, inflammation or vascular damage, demonstrating that the pathological phenotype observed during *A. fumigatus* infection in an immunocompromised setting is driven by the loss of activated Postn⁺ fibroblasts, rather than nonspecific DTA toxicity (new Figure S8 B).

Finally, to further address the reviewer's concern regarding potential off-target effects of fibroblast ablation, we designed a new complementary DTA ablation study in which a modified TAM dose was used to ablate pulmonary fibroblasts utilizing the Pdgfr α CreER;R26-DTA mouse line ("Reviewer's eyes only" figure A-B) to achieve fibroblast depletion (Reviewer Figure C) similar to the depletion reached in Postn-DTA mouse (new Figure 6J), since Pdgfr α is broadly expressed by resident fibroblasts. This design allows us to compare the inflammatory outcomes of partial fibroblast depletion in the absence of fungal infection.

Consistent with prior findings that extensive Pdgfr α ⁺ fibroblast ablation does not cause tissue injury even after prolonged depletion (Kuwabara et al., 2022), our model confirmed that fibroblast loss alone did not elicit an inflammatory response. Instead,

ablation of Pdgfra^+ fibroblasts resulted in decreased macrophage and neutrophil numbers (Reviewer Figure D), opposite to the elevated inflammation observed in the Postn-DTA model during *A. fumigatus* challenge, indicating that the proinflammatory phenotype in the Postn model is specifically driven by the fungal infection.

As this broader fibroblast–immune interaction represents a separate mechanistic focus currently under investigation, these additional data are provided for reviewer reference only and are not included in the revised manuscript to maintain its targeted scope. The text has been updated accordingly (lines 284, 452–472; Fig. 2C, 2F; Fig. 6J) and the aforementioned references were added as follows.

Feil, R., Wagner, J., Metzger, D., & Chambon, P. (1997). Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. *Biochemical and Biophysical Research Communications*, 237(3), 752–757. <https://doi.org/10.1006/bbrc.1997.7124>

Vanhecke, D., Bugada, V., Steiner, R. et al. Refined tamoxifen administration in mice by encouraging voluntary consumption of palatable formulations. *Lab Anim* **53**, 205–214 (2024). <https://doi.org/10.1038/s41684-024-01409-z>

Kuwabara, J. T., Hara, A., Bhutada, S., Gojanovich, G. S., Chen, J., Hokutan, K., Shettigar, V., Lee, A. Y., DeAngelo, L. P., Heckl, J. R., Jahansooz, J. R., Tacdol, D. K., Ziolo, M. T., Apte, S. S., & Tallquist, M. D. (2022). Consequences of $\text{PDGFR}\alpha^+$ fibroblast reduction in adult murine hearts. *eLife*, 11, e69854. <https://doi.org/10.7554/eLife.69854>
[PMC](#)

[REDACTED]

[REDACTED]

Reviewer #2

1) Although this manuscript demonstrated the protective function of Postn⁺ fibroblasts in fungal pneumonia, the authors do not adequately explore the mechanism(s) by which loss of periostin lineage cells increases mortality in immunosuppressed mice inoculated with A. fumigatus.

To address this point, we performed flow cytometric analysis of pulmonary immune cell populations and quantified fungal burden, representing two key determinants of disease outcome in *A. fumigatus* infection. Postn⁺ lineage (Postn^{Lin}) cell ablation resulted in a marked increase in myeloid cell infiltration without a significant change in fungal burden. These results indicate that accelerated mortality in the Postn^{Lin}-ablated mice arises from excessive immune activity, rather than from uncontrolled fungal proliferation. The resulting hyperinflammatory response promotes immune-mediated tissue injury, fungal angioinvasion, and alveolar hemorrhage, contributing to rapid disease progression.

Given that myeloid cells are the principal mediators of antifungal defense, their overrepresentation may also account for the modest reduction in fungal load observed in Postn^{Lin}-ablated mice. Thus, our findings support a model in which Postn⁺ fibroblasts restrain excessive inflammatory activation, and their loss amplifies tissue-destructive immunity.

These results are now incorporated into the revised manuscript and discussed in the indicated sections (lines 85–87, 290–307, 461–480; Fig. 6F–L; Fig. S7).

2) Although the authors describe areas of hemorrhage and angio-invasion after deletion of periostin⁺ cells, the manuscript currently lacks quantitative data regarding the observed phenotypes and detailed information on the method to evaluate the results.

We thank the reviewer for this helpful suggestion. We have now included quantitative analyses of hemorrhagic areas and angioinvasion following Postn⁺ cell ablation, together with expanded methodological details describing how these parameters were assessed. These quantitative results and methodological details have been incorporated into the Results (lines 268–275) and Methods (lines 643–656) sections, and summarized in Figure 6F–G.

3) "...no experimental data explaining how the deleted cells might prevent these adverse events."

As noted above, we have performed additional experiments demonstrating that Postn^{Lin} cells play a key role in mitigating the inflammatory response to *A. fumigatus*. The absence of these cells results in a hyperinflammatory phenotype, consistent with increased immune-mediated injury (Fig. 6F). Importantly, these findings are supported

by single-cell RNA sequencing (scRNA-seq) data obtained at early stages of infection (Day 1), which reveal the emergence of fibroblast populations with immunomodulatory transcriptional profiles characterized by elevated expression of IL6, CXCL1, and CXCL2. These gene programs are associated with myeloid cell recruitment and activation, suggesting that fibroblast-derived inflammatory cues are critical for maintaining balanced immune responses during fungal infection. Together, these data indicate that loss of Postn^{Lin} fibroblasts disrupts this regulatory network, leading to excessive inflammation and tissue injury.

4) “...it would be important to perform quantitative immunostaining and/or flow cytometry to determine whether loss of periostin+ cells modulated the recruitment and/or activation of adaptive and innate immune cells.”

We thank the reviewer for the opportunity to address this important point. As described above, we performed flow cytometric analysis of pulmonary immune cell populations and observed a marked increase in the infiltration of macrophages, neutrophils, dendritic cells, and NK cells following Postn^{Lin} cell ablation. These innate immune populations represent the first line of defense against *A. fumigatus*, and their expansion indicates that loss of Postn^{Lin} fibroblasts trigger a highly specific yet dysregulated inflammatory response.

Importantly, these findings support our proposed mechanism that Postn⁺ fibroblasts normally act to regulate innate immune activation, and that their ablation leads to uncontrolled inflammation and tissue injury during fungal infection.

These new data have been incorporated into the revised manuscript (lines 85–87, 290–307, 461–480; Fig. 6L).

5) It would also have been useful to see whether differences in mortality were due to effects on vascular permeability.

We appreciate this thoughtful comment. Assessing vascular permeability in the immunosuppressed model is technically challenging, as the extensive tissue damage caused by invasive fungal growth in both control and DTA mice obscures accurate quantification of vascular integrity. To overcome this limitation, we performed permeability assays in immunocompetent mice using fluorophore-labeled dextrans of varying molecular sizes to evaluate microvascular leakage following *A. fumigatus* infection. Under these conditions, no significant differences in vascular permeability were detected between *A. fumigatus*-treated control and Postn^{Lin} ablated (Postn^{MCM/+};R26-DTA) mice.

These results indicate that loss of Postn⁺ fibroblasts alone does not compromise microvascular stability, and that the vascular injury observed in the immunosuppressed infection model likely arises secondarily from excessive inflammation and tissue damage.

This new dataset is presented in Figure S8, with corresponding methodological details added (lines 717-728) and text revisions included (lines 276–289, 498-501).

6) Additionally, the possibility of Postn-CreER targeting pericytes rather than fibroblasts should be experimentally excluded to prove the authors' claims.

We thank the reviewer for highlighting this important consideration.

First, to determine whether Postn^{Lin} cells include pericytes, we performed flow cytometric and immunohistochemical analyses using antibodies against Neuron-Glial Antigen 2 (NG2, encoded by Cspg4), a canonical pericyte marker. These analyses revealed no co-labeling between tdTomato⁺ Postn^{Lin} cells and NG2⁺ pericytes, which was further confirmed by confocal microscopy (Fig. 4M–O; Fig. S5H).

Second, while single-cell RNA-seq identified low-level Postn expression in a small subset of pericyte-like cells under baseline conditions, this expression was insufficient to elicit tamoxifen-induced recombination. Consistent with this, mice treated with tamoxifen alone displayed minimal-to-no tdTomato labeling, even after prolonged exposure (Fig. 2C, 2F), demonstrating that recombination occurs only upon robust Postn induction during fibroblast activation.

Finally, if pericytes were significantly targeted or ablated, one would expect loss of vascular stability. However, vascular permeability assays using fluorophore-labeled dextrans of multiple molecular sizes revealed no increase in microvascular leakage following Postn^{Lin} cell ablation (Fig. S8).

Together, these findings confirm that Postn-CreER recombination selectively targets activated fibroblasts, not pericytes, and that pericyte integrity and vascular stability remain intact. This information has been incorporated into the revised manuscript (lines 204–209, 486–501; Fig. 4M–O; Fig. S5H; Fig. S8).

7) How many times did the authors backcross the Postn-MerCreMer mouse? In Figure 5, what mice did the authors use for control? This information is critical since the different genetic backgrounds can show different sensitivity to infection.

The Postn-MerCreMer mouse line used in this study was backcrossed for more than 10 generations onto the C57BL/6J background over the past 9 years and additional mice lines purchased from JAX were already backcrossed and pure C57BL/6J background, ensuring genetic uniformity and consistency with the other transgenic strains employed.

For all experiments, including those shown in Figure 5 (Revised Figure 6), littermate controls were used. Specifically, Postn-MerCreMer; R26-DTA (Postn^{MCM/+};DTA) mice were compared to Cre-negative littermates (R26-DTA) generated from the same breeding pairs. We also utilized lineage tracing mice as controls when appropriate. All experimental and control animals were cohoused, sex- and age-matched, and subjected to identical immunosuppressive and infection protocols to control for environmental and genetic variability.

These details have been clarified in the Methods section of the revised manuscript (lines 595-601).

8) Additionally, ...did the control group in Figure 5 also receive tamoxifen treatment? Tamoxifen itself can cause various effects on the lungs. Tamoxifen food can also cause body weight loss sometimes since mice are reluctant to eat it. If the control groups in Figure 5 were not treated by tamoxifen, how do the authors exclude the possibility that the phenotype is caused by tamoxifen?

All groups in original Figure 5 (Revised Figure 6) received tamoxifen, ensuring that any observed phenotypic differences are not attributable to TAM treatment.

Moreover, no differences in weight loss were detected between immunosuppressed control mice with or without TAM administration (Reviewers only Fig 2). Histological analysis of lung tissues from TAM-only treated mice showed no evidence of inflammation, fibrosis, or structural abnormalities, indicating that the TAM regimen used in our study does not introduce gross phenotypic alterations to tissue architecture.

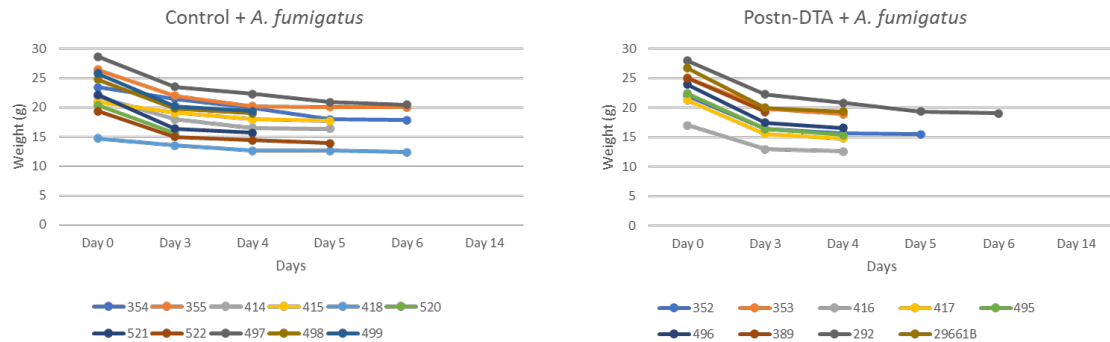


Figure 2. Body weight changes over time in immunocompetent mice challenged with *A. fumigatus*. Mice were treated with TAM via gavage and TAM-infused chow diet for the duration of the experiment. Each color denotes a unique mouse, with weights recorded at the indicated time points until death.

9) The authors stated that the Postn+ cell ablation resulted in pulmonary hemorrhage and fungal angioinvasion in Figure 5. These phenotypes should be quantified and statistically evaluated.

Quantitative data has been added as previously indicated. Lines 268-275, lines 643-656; Fig 6F-G.

10) What portion of Postn-lineage cells are pericytes? Authors should be able to quantify this by Mcam (CD146) staining on flow cytometry. Although authors showed minimal tdTomato+ cells without infection by histology, this should be confirmed by flow cytometry of digested lungs for a more unbiased assessment of whole lung distribution. If there are minimal tdTomato+ cells without infection, can the authors explain why Postn-MerCreMer specifically labels fibroblasts but not pericytes, which highly express Postn at baseline?

We appreciate this comment. As outlined above, we used anti-NG2 (Cspg4) antibodies to identify pericytes, as NG2 is a widely accepted and specific pericyte marker in the lung. We avoided Mcam (CD146) because it is also expressed by smooth muscle and endothelial cells, which could confound flow cytometric analyses.

Flow cytometry and confocal microscopy showed no overlap between tdTomato⁺ Postn^{Lin} cells and NG2⁺ pericytes (Fig. 4M–O; Fig. S5H), and tamoxifen-only controls displayed minimal-to-no tdTomato⁺ labeling by both histology and flow cytometry (Fig. 2C, 2F). Although low baseline Postn expression was detected in pericyte-like clusters by scRNA-seq, this level was insufficient to drive Cre recombination, which occurs only after Postn promoter activation in stimulated fibroblasts.

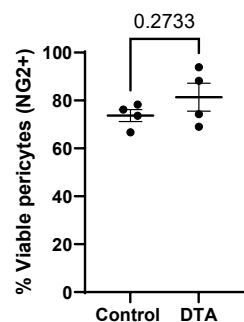
These data confirm that Postn-MerCreMer recombination selectively targets activated fibroblasts, not pericytes.

11) Since *Postn* is also highly expressed in pericytes, the authors should experimentally exclude the possibility of pericytes contributing to the phenotypes in Figure 5. The authors should be able to do this by treating the mice with fungus after treating and washing out tamoxifen or using *Rosa26-DTR* mice.

As described above, our results indicate that *Postn*-CreER recombination selectively targets fibroblasts rather than pericytes. To further confirm that pericytes do not contribute to the phenotypes shown in Figure 5, we quantified NG2⁺ pericytes by flow cytometry in control and *Postn*-DTA mice following infection with *A. fumigatus* (3×10^7 conidia). Mice received tamoxifen as outlined in Fig. S8A, and lungs were harvested 4 days post-inoculation. The results demonstrate that NG2⁺ pericyte numbers were unchanged between DTA-treated and control mice, indicating that *Postn*^{Lin} ablation did not affect pericyte survival.

In addition, tamoxifen-only controls showed minimal-to-no tdTomato labeling by both histology and flow cytometry (Fig. 2C, 2F), confirming that baseline *Postn* expression in pericytes is insufficient to induce Cre-mediated recombination. Consistent with this, vascular permeability assays using fluorophore-labeled dextrans revealed no microvascular leakage or endothelial instability after *Postn*^{Lin} ablation (Fig. S8).

Together, these findings confirm that *Postn*-CreER-mediated recombination and DTA ablation specifically target activated fibroblasts, not pericytes, and that the phenotypes observed in Figure 5 (Revised Figure 6) are not attributable to pericyte loss. A reviewer's-eyes-only figure is provided to illustrate that NG2⁺ pericyte populations remain intact following *Postn*^{Lin} ablation.



Reviewer Figure 3. Flow cytometry quantification of NG2⁺ cells in control and *Postn*-DTA mice after 4-day challenge with 3×10^7 conidia of *A. fumigatus*.

12) Figure 7E is difficult to understand. Figure 1A shows that there are normal alveoli left on day 1. However, Figure 7E seems to show that all alveolar fibroblasts homogeneously become activated alveolar fibroblasts. Similar results are shown for peribronchiolar fibroblasts. If the clusters are completely driven by treatment and time points, the authors might want to try integration methods such as Harmony or Mutual Nearest Neighbors (MNN), which may allow them to identify newly emerged populations while keeping some normal populations.

We appreciate the reviewer's insightful comment and agree that batch effect correction is a critical step in single-cell data analysis. Methods such as Harmony or Mutual Nearest Neighbors (MNN) can mitigate batch-associated variability but may also over-correct, potentially masking genuine biological differences, particularly when samples represent distinct activation states.

In this study, we carefully evaluated batch effects prior to integration. No significant batch-driven segregation was observed between the two biologically similar saline-treated control samples (day 1 and day 7), which were processed in separate batches. Their UMAP embeddings overlapped extensively, confirming the absence of technical bias. Moreover, applying Harmony-based correction yielded a nearly identical cluster distribution, further indicating that batch effects were minimal.

Given these findings, we chose not to apply batch correction for the infection dataset to preserve true biological variation among fibroblast activation states.

13) In Figure 7E, the authors showed only inflammatory genes for the alveolar fibroblast lineage. Differentially expressed genes for each cluster should be shown by heatmap or dot plots for readers to know the specific genes of each cluster.

In the original version, Figure 7E focused on inflammatory and activation-associated genes to highlight the dynamic transcriptional response of fibroblast subsets following *A. fumigatus* infection. To provide a more comprehensive view of fibroblast heterogeneity, we have now included differential gene expression analyses for all stromal clusters.

Specifically, we generated a new supplementary figure showing the top five marker genes (DEGs) defining each stromal subpopulation in dot plot (Fig. S9) and added the complete DEG list as a supplementary data table (Tables S4 and S5). These additions allow readers to evaluate the molecular identity and functional characteristics of each cluster.

Corresponding details have been added to the Results section (lines 331–397).

14) The authors need to include data demonstrating the efficiency of the deletion method used to delete periostin-lineage cells. It would also be good to overlay periostin expression on the uMAP plots and to have included the lineage label in the single cell sequencing experiments so the authors could determine which of the emergent stromal populations they describe were derived from periostin-expressing cells and thus which populations were likely to have been deleted.

We appreciate this important point.

First, we quantified the efficacy of periostin-lineage ablation by flow cytometry as described above via labeling all fibroblast with Pdgfr α . We determined that the Postn^{Lin} ablation achieves ~30% depletion of Pdgfr α positive fibroblasts (new Fig. 6J).

Second, in our droplet scRNA-seq dataset at Day 1, Postn transcripts exhibit pronounced sparsity/dropout and map predominantly to a pericyte-like cluster that shows low baseline Postn expression whereas the fibroblast clusters that become activated after infection display low detectable Postn mRNA at this early time point. This is consistent with (i) the known behavior of matricellular/ECM transcripts (low capture efficiency and high dropout in 3' droplet data), and (ii) our kinetic data indicating that periostin protein peaks later (past 24 hrs), while the Day-1 scRNA profile captures the onset of activation. As a result, a simple Postn-UMAP overlay would be misleading in our dataset. To this end we have expanded our data set and determined that the low expression of Postn in pericytes have not resulted in recombination in pericyte (Fig.4 M-O), nor resulted in ablation (Reviewer figure 3 above). Therefore, our collective data suggests the consequence of Postn^{Lin} ablation is derived from the fibroblasts.

Third (linking to fibroblast activation states). Instead, we clarified the relationship between periostin-lineage biology and the single-cell map by providing a new supplementary figure (Fig. S9) showing the top five marker genes defining each fibroblast subpopulation and a complete DEG table (Table S4 and S5). These analyses align the emergent Day-1 inflammatory fibroblast states with the populations reduced by Postn^{Lin} ablation.

Finally (future lineage-resolved single-cell). Our present scRNA-seq libraries did not include the fluorescent lineage reporter. In ongoing work, we are implementing feature barcoding/CITE-seq for anti-RFP and targeted spatial panels (10x Xenium) to directly map Postn^{Lin} derivatives at single-cell spatial resolution in future studies.

We have added these clarifications to the Results/Methods and included the new DEG visualizations (lines 311–397; Fig. S9; Table S4-5).

Minor comments

1. It would be nice to quantify fibrosis after fungus infection by hydroxyproline assay

Collagen deposition was evaluated by Picrosirius Red staining on lung sections from immunosuppressed control and Postn-DTA mice infected with *A. fumigatus*. Both groups showed comparable levels and distribution of collagen, localized to areas of inflammation, indicating that Postn⁺ cell ablation did not alter total collagen content. These results further imply that pulmonary fibroblast immune modulation to be the central mechanism of action in response to *A. fumigatus* challenge.

These data are shown in Figure 6H and discussed in lines 270–275.

Because this histological and quantitative approach captures both the extent and spatial context of collagen deposition, a hydroxyproline assay was not employed.

2. Figure 7D, colors for activated alv and deactivated alv were swapped.

Thank you! The legend has been corrected.

3. Figure 4 would benefit from addition of staining for specific pericyte markers.

Flow cytometry and confocal immunohistochemistry images using the pericyte marker NG2 have been added, showing a lack of colabeling of TdT⁺ cells with NG2 (Fig. 4 M-O and Fig S5H).

Reviewer #4

1) Do the authors have any data suggesting that fungal burden is (or is not) impacted by loss of the postn lineage fibroblasts during invasive disease (Figure 5)? Maybe this is just a point for the Discussion, but is it possible that after invasive growth begins, these fibroblasts play a role in limiting fungal growth? Or, is the increased mortality of the DTA mice simply due to heightened levels of tissue damage in response to the same amount of fungal growth?

Fungal burden was quantified in immunosuppressed Postn-DTA and control mice during invasive *A. fumigatus* infection. The results show that fungal load remained unchanged following ablation of Postn^{Lin} fibroblasts (Fig. 6K). However, angioinvasion of pulmonary arteries was markedly increased in the DTA group (Fig. 6G), suggesting that

Postn⁺ fibroblasts contribute to maintaining vascular integrity and limiting fungal dissemination.

Collagen quantification by Picrosirius Red staining revealed no difference in total collagen deposition between groups (Fig. 6H), indicating that the observed vascular compromise is not due to reduced ECM deposition. Instead, DTA mice displayed excessive infiltration of macrophages, neutrophils, dendritic cells, eosinophils, and NK cells, along with alveolar hemorrhage (Fig. 6D–F, 6L; Fig. S7).

Together, these findings indicate that fibroblast ablation disrupts immune homeostasis, leading to hyperinflammation and vascular injury, rather than impaired fungal clearance. The increased angiogenesis and hemorrhage, in the context of stable fungal burden, likely account for the accelerated mortality in the DTA model.

This information has been incorporated into the revised manuscript (lines 85–87, lines 267–275, 290–307, 461–480, 643–656, 700–716).

2. In the same line of thought, the immune competent DTA mice did not experience death after infection (Line 223), but did they have prolonged time to clearance/sterilization? Even negative data may be very informative here and could be included as a Supplemental Figure, if available.

To address this point, we quantified fungal burden in immunocompetent Postn- DTA and control mice following *A. fumigatus* infection. At day 4 post-inoculation, which corresponds to the earliest time point at which phenotypic differences emerge in the immunosuppressed model, fungal growth was already minimal in both groups. The number of colony-forming units (CFUs) recovered from lung homogenates was consistently below 20 CFUs per plate, indicating that fungal clearance was essentially complete by this time.

Given this very low baseline burden, no meaningful difference in fungal sterilization kinetics could be determined between groups. These data are summarized in the accompanying supplementary table below for reviewer reference.

Together, these findings indicate that in the immunocompetent setting, the absence of Postn^{Lin} fibroblasts does not impair fungal clearance, consistent with their role in modulating inflammation rather than direct antifungal defense.

Mouse ID	Group	Af inoculum	Dilution	CFUs
1401	Control + Af	3x10 ⁷ conidia	1	13
			1/2	6
1404	Control + Af	3x10 ⁷ conidia	1	20
			1/2	10
1449	Control + Af	3x10 ⁷ conidia	1	9
			1/2	6
1467	Control + Af	3x10 ⁷ conidia	1	15
			1/2	8
1408	DTA + Af	3x10 ⁷ conidia	1	0
			1/2	1
1410	DTA + Af	3x10 ⁷ conidia	1	3
			1/2	2
1414	DTA + Af	3x10 ⁷ conidia	1	1
			1/2	1
1435	DTA + Af	3x10 ⁷ conidia	1	6
			1/2	4

Response to Reviewers for NCOMMS-25-02679A

Reviewer #1:

... However, while the data convincingly show altered immune cell recruitment and gene expression in the absence of periostin-lineage fibroblasts, the mechanistic conclusions advanced in the text—particularly those concerning alveolar hemorrhage, angioinvasion, and vascular compromise—are not directly supported by the experimental data.

We appreciate the reviewer's thoughtful critique and agree that our data do not directly establish mechanistic causality for alveolar hemorrhage, angioinvasion, or vascular compromise. In response, we have revised the manuscript to more carefully constrain our conclusions to associations supported by the experimental data.

Specifically, we have modified the Conclusions section (Lines 303–306) to state that loss of PostnLin cells in the context of IPA is associated with accelerated mortality and altered innate immune responses to *A. fumigatus*, without implying direct mechanistic causation. We have also revised the Discussion (Line 472) to clarify that the observed hyperinflammatory response is consistent with immune-mediated tissue damage contributing to increased mortality, rather than asserting a direct causal mechanism.

These changes ensure that all interpretations regarding vascular injury and fungal invasion remain appropriately bound by the data presented.

List of changes made:

-Line 303-306: "...We conclude that loss of PostnLin cells in the context of IPA is associated with accelerated mortality and a dysregulated innate immune response to *A. fumigatus*, accompanied by pathological features consistent with vascular injury and fungal invasion."

-Line 472: "...This was associated with a hyperinflammatory response, consistent with immune-mediated tissue damage as a potential contributor to the observed increase in mortality (Fig. 6)."

...The authors reference new data additions and state that over 12 figures have been added in the revision, but these appear to primarily represent expanded or reformatted analyses of existing datasets. No new in vivo mechanistic experiments were introduced, nor were fibroblast-specific effector functions or endothelial outcomes directly tested. Thus, mechanistic claims remain speculative. Additionally, while the revised manuscript includes clearer figure labeling and additional cytokine profiling, the requested functional studies to demonstrate causality—particularly those needed to link fibroblast ablation to vascular injury or fungal dissemination—are still lacking. Based on these results, TGF-beta may be a candidate for PostLn-specific ablation

We appreciate the reviewer's assessment and agree that the additional figures primarily extend and refine in vivo analyses rather than introducing endothelial-targeted or single-pathway effector experiments. However, we respectfully note that the revised manuscript does include new in vivo mechanistic data at the level of immune regulation.

Specifically, we incorporated flow cytometric analyses demonstrating heightened activation and expansion of innate myeloid populations following PostnLin fibroblast ablation, providing direct in vivo evidence that these cells restrain inflammatory immune responses during IPA (Fig. 6I–L). Importantly, these changes occur independently of increased fungal burden, supporting a mechanistic role for PostnLin cells in regulating host immune responses rather than pathogen control.

In addition, experiments performed in an immunocompetent background demonstrate that the pathological features observed upon Postn ablation are infection-specific and not attributable to baseline alterations in vascular permeability (Fig. S8). Together with new in vitro data showing that human pulmonary fibroblasts directly sense *A. fumigatus* and acquire immunomodulatory properties (Fig. 7G and Fig. S10), these findings provide mechanistic insight into how fibroblasts shape local immune responses during fungal infection.

While we do not claim identification of a single downstream effector pathway or direct endothelial injury mechanism, we have revised the manuscript to clearly frame our conclusions at the level of immune modulation and tissue homeostasis, which are directly supported by the data presented.