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Peer Review File

Profibrotic monocyte-derived alveolar macrophages are expanded in patients with persistent respiratory symptoms and radiographic abnormalities after COVID-19

Corresponding Author: Dr Alexander Misharin

Version 0:

Decision Letter:

2nd Oct 2023

Dear Dr. Misharin,

Your Article, "Expansion of profibrotic monocyte-derived alveolar macrophages in patients with persistent respiratory symptoms and radiographic abnormalities after COVID-19" has now been seen by 3 referees. While the work is of potential interest, the reviewers have raised substantial concerns that must be addressed. As such, we cannot accept the current manuscript for publication, but would be interested in considering a revised version that addresses these serious concerns, as long as novelty is not compromised in the interim.

Should you find yourself able to thoroughly address the referees' concerns, please let me know. We consider it is important to include recovered post-COVID-19 controls, in addition to the current healthy controls, in order to increase the insight gained into what happens after COVID and increase the impact of the study. Please note that two of the three referees have found the advance incremental or moderate and a revision should be aimed at addressing this point. At resubmission, please include a point-by-point “Response to referees” detailing how you have addressed each referee comment (please specify page and figure number where the new data can be found in the revised manuscript). This response will be sent back to the referees along with the revised manuscript.

In addition, please include a revised version of any required reporting checklist. It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review. A revised checklist is essential for re-review of the paper.

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We hope to receive a suitably revised manuscript within 6 months. If you cannot send it within this time, please let us know.

We will be happy to consider your revision so long as nothing similar has been accepted for publication at Nature Immunology or published elsewhere.

Nature Immunology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

Please do not hesitate to contact me if you have any questions or would like to discuss the required revisions further.

Thank you for the opportunity to review your work.

Sincerely,

Ioana Visan, Ph.D.
Senior Editor
Nature Immunology

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Reviewers' Comments:

Reviewer #2:
Remarks to the Author:
In this prospective, longitudinal, observational cohort study, Bailey et al. investigated patients with post-acute sequelae of COVID-19 (PASC), more specifically PASC with respiratory symptoms and radiographic abnormalities (RPRA) on chest CT. Respiratory symptoms are among the most frequent complaints of patients with PASC, yet the underlying pathobiology remains largely unknown. The authors analyzed immune responses in the upper and lower airways of RPRA patients and controls using flow cytometry and scRNA-Seq, and combined these studies with machine-learning based image analysis of CT scans.
They identified an accumulation of profibrotic monocyte-derived macrophages in the airways of PASC patients, which was positively correlated with fibrotic lesions on CT. They also found elevated levels of CCL2 and increased neutrophils, indicating a continued influx of monocytes and neutrophils, potentially due to insufficient epithelial repair as a result of infection. These results are robust and in line with previous findings associating profibrotic monocytes and macrophages with other fibrotic interstitial lung disease. Their association with PASC may further indicate a general role for this macrophage state in fibrotic lung diseases of different origin and with distinct clinical features and outcomes.
Overall, this detailed, well-conducted, multimodal study provides highly resolved insights into respiratory immune responses and their radiographic correlates in PASC / RPRA. However, the conceptual advance over existing data is moderate. Even though directionality and causality is difficult to establish in human observational studies, the authors may want to consider further investigating the molecular links between the observed macrophage state in PASC and the resulting fibrotic phenotype.

Specific comments
1. The manuscript is very clear and well written.
2. The authors did a great job in assembling this cohort of RPRA patients with longitudinal CT imaging and sampling of BAL and nasal swabs. However, there are some concerns relating to the heterogeneity of the cohorts with regards to demographics, e.g. age (differs substantially between patients and controls), or sex. Also, the time points for CT follow-ups and bronchoscopy vary quite substantially. Recruitment or analysis of additional subjects may be helpful to better streamline the cohorts.
3. The authors observed no apparent correlation between the time to CT follow-up and the fibrosis score. This is a bit unexpected, since it has been reported in numerous studies, that interstitial lung lesions after COVID cleared or partially resolved over time. Could the authors comment on this apparent discrepancy?
4. On page 7, the authors state that "Direct pairwise comparison between healthy volunteers and patients with RPRA demonstrated a significant increase in the relative abundance of neutrophils and monocytes, and a decrease in the relative abundance of CD206high and CD206low alveolar macrophages". Judging from Figure 2b, it seems that CD206lo macrophages are actually increased in RPRA patients, not decreased.
5. Does the heatmap in Figure 2a actually show "percent" (as indicated iin the legend in Fig. 2s) or is this rather a z-score? Please clarify.
6. MoAM-1 cluster of macrophages was labeled as "profibrotic macrophages" based on the expression of selected genes with known or suggested profibrotic function. However, it would be useful if the authors integrated their scRNA-Seq data with

published phenotypes in published data sets or atlases and compared their MoAM phenotype to published proprofibrotic macrophage phenotypes from COVID-19 and other fibrotic conditions. Did the authors systematically assess similarity with previously published profibrotic Macrophages (e.g. from their own work on IPF) ? What are the commonalities and distinctions that could hint at the capacity to reprogramm the macrophages to fibrosis resolution?
7. I feel that Figure 4 is not overly informative. What is the advantage of using SPECTRA as opposed to conventional gene modules or GO-Terms? The differential gene programs identified primarily in TRAMs are only labeled numerically. More meaningful labels could improve the information provide in Fig. 4. Also, the authors should make it more clear what
8. The analysis of nasal cells is of interest, yet the value of comparing nasal sample to BAL samples, particularly the sorted BAL samples, is somewhat limited, since immune cells are strongly underrepresented in the nasal samples and epthelila cells where removed from the BAL samples before analysis.

Reviewer #3:
Remarks to the Author:
The alveolar niche is profoundly affected by infection, inflammation and injury, and the presence of monocyte-derived macrophages expressing a profibrotic signature has been largely documented in the BALF of severe Covid-19 patients: PMID 34914922, PMID 33138195, PMID 32398875, etc. In this report, Bailey, Puritez and colleagues performed an observational cohort study on patients experiencing respiratory symptoms and shortness of breath and exhibiting CT abnormalities more than 90 days after the initial infection event. Concordant with the presence of inflammatory and fibrotic signs on CT images, they find the presence of neutrophils and monocyte-derived macrophages expressing a profibrotic signature. These findings suggest that some conserved immunological changes may drive the persistence of lung symptoms and CT abnormalities in these patients.

Major concerns.

The potential interaction between monocyte-derived macrophages and fibroblasts, and the enriched presence of monocyte-derived macrophages in collagen-rich areas has not been addressed. The proposed function of those monocyte-derived macrophages is only based on transcriptomic data, which is underwhelming.

Patients have been stratified in 4 clusters in Figure 2a based on BALF flow cytometry phenotyping, with cluster 3 containing only RPRA patients and many CD206 low MPs. It would be informative to keep this stratification and assess whether patients from cluster 3 also exhibit more MoAM-1 in the scRNA-seq, and more fibrotic areas on CT scans, even though this piece of information would still remain correlative.

It would be interesting to assess whether blood monocytes are already primed towards a more activated, pro-fibrotic profile in these patients, suggesting systemic changes that would then influence tissue immunity.

Overall, the data represent, in the reviewer's opinion, an incremental advance for a broad immunological audience and would be more appropriate for a specialized journal in the field of respiratory medicine.

Reviewer #4:
Remarks to the Author:
This is a tour-de-force manuscript characterizing clinical and molecular patterns of patients with post-acute sequelae of Covid-19 (PASC) by one of the leading groups in Covid/post-Covid lung research. The authors submit a large body of data analyzing clinical and molecular data from a unique and well-characterized cohort of 35 post-acute Covid patients at Northwestern U and identify cell populations that correlate with extent of fibrosis in these patients (such as monocyte-derived macrophages).

The story of molecular patterns of post-acute (pulmonary) sequelae of Covid (and reversal of lung fibrosis in a subset of these patients) is impressive and will undoubtedly be highly cited. These findings build on previous investigations by the authors on macrophage populations in lung fibrosis and highlight ongoing recruitment of these macrophage (and neutrophil) populations as a common pathophysiological trait of PASC in this single center cohort of PASC patients.

Authors are commended for the extended supplementary data submission, reviewer token, and methods description.

Specific critiques:
1) The patient population is very unique yet rather small when confronted with an array of molecular analysis as presented herein. The authors present a number of molecular analysis from different subgroups of these patients, thereby challenging data integration between technologies (such as immunoprofiling, scRNAseq, and nasal gene expression, e.g.). This is difficult to overcome in clinical reality, but this reviewer wonders if a smaller population with comprehensive analysis of all assays would yield different results. Have the authors performed data integration in just a subgroup of patients (e.g. all patients undergoing bronchoscopy w and w/o steroids)?

2) Why did the authors choose healthy volunteers as a control group instead of acute Covid-19 without detectable PASC? Such a control group would be highly informative and ore appropriate for e.g. scRNAseq analysis.

3) Is there a distinct molecular/immunological profile of the patients which resolved fibrosis over the course of two CT scans compared with those that did not?

4) Minor comment: The results section often reads like a clinical medial journal, highlighting specifics of individual patients, rather than focusing on common immunological observations.

5) Page 7, 2nd paragraph. The authors invalidate all correlations described by stating that "these correlations were not significant". Better to delete the entire passage.

6) The entire passage on microbiological BAL analysis is more appropriate in either supplementary data or even methods section.

7) The last section on comparing transcriptional changes in nasal mucose compared with distal lung is highly interesting (given published data on e.g. lung cancer patients), but entirely underdeveloped. This reviewer would suggest expanding this section due to its findings that are highly relevant.

Author Rebuttal letter:

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R: We are thankful to the reviewers for their challenge to consider our findings within a broader immunologic context and to better highlight their novelty and impact on the field. In response, we have conducted substantial new analyses and have substantially revised our paper to highlight the central immunologic question that our dataset is uniquely poised to answer. The COVID-19 pandemic allowed us to collect alveolar samples from a large group of patients with lung injury and fibrosis induced by the same virus, some of whom went on to resolve their fibrosis and some of whom progressed to require lung transplantation. These data allowed us to compare the transcriptome of alveolar macrophages in resolving or non-resolving patients for evidence of a specific program of tissue repair. Despite our use of several complementary analysis strategies, including Spectra, we were unable to identify such a program in monocyte derived or tissue resident alveolar macrophages.
Our data address a growing problem in alveolar macrophage biology. In many tissues, for example skeletal and cardiac muscle, monocyte-derived macrophages play a central role in tissue repair after injury. However, in all the published data of which we are aware, genetic or pharmacologic strategies targeting monocyte-derived alveolar macrophages ameliorate lung injury and fibrosis, even when they are administered after fibrosis is established. Furthermore, investigators have observed few transcriptomic differences in monocyte-derived alveolar macrophages in response to diverse causes of lung injury in mice and humans. These data suggest a model in which monocyte-derived alveolar macrophages activate a stereotypical program to injury that promotes tissue fibrosis. Animal models suggest this fibrotic program resolves as the monocyte derived alveolar macrophages cease to be recruited and undergo apoptosis or differentiate during injury resolution. Our data lend support for this model in humans with resolving lung fibrosis. This has important implications for therapy—strategies targeting monocyte-derived alveolar macrophages are unlikely to impede repair. With luck, the opportunity to reproduce these data will not occur in our generation, making this an important dataset for the field. We have substantially revised the manuscript and the analyses to focus on this central finding.
We agree that BAL fluid analysis of patients who recovered from COVID-19 without sequelae might be an important additional control. There are pragmatic limitations with this suggestion as patients who recover from COVID-19 infrequently seek care and, in these patients, we would not perform bronchoscopy for clinical indications. The difficulty in obtaining samples like these as part of clinical care or research further highlights the unique features of this dataset.

At resubmission, please include a point-by-point “Response to referees” detailing how you have addressed each referee comment (please specify page and figure number where the new data can be found in the revised manuscript). This response will be sent back to the referees along with the revised manuscript.

Reviewers' Comments:

Reviewer #2:
Remarks to the Author:
C1: In this prospective, longitudinal, observational cohort study, Bailey et al. investigated patients with post-acute sequelae of COVID-19 (PASC), more specifically PASC with respiratory symptoms and radiographic

abnormalities (RPRA) on chest CT. Respiratory symptoms are among the most frequent complaints of patients with PASC, yet the underlying pathobiology remains largely unknown. The authors analyzed immune responses in the upper and lower airways of RPRA patients and controls using flow cytometry and scRNA-seq, and combined these studies with machine-learning based image analysis of CT scans.
They identified an accumulation of profibrotic monocyte-derived macrophages in the airways of PASC patients, which was positively correlated with fibrotic lesions on CT. They also found elevated levels of CCL2 and increased neutrophils, indicating a continued influx of monocytes and neutrophils, potentially due to insufficient epithelial repair as a result of infection. These results are robust and in line with previous findings associating profibrotic monocytes and macrophages with other fibrotic interstitial lung disease. Their association with PASC may further indicate a general role for this macrophage state in fibrotic lung diseases of different origin and with distinct clinical features and outcomes. Overall, this detailed, well-conducted, multimodal study provides highly resolved insights into respiratory immune responses and their radiographic correlates in PASC / RPRA.

R1: Thank you for this excellent summary and emphasizing the fact that the underlying pathobiology of PASC remains largely unknown. In addition to addressing biologic questions related to the pathobiology of PASC, we have substantially revised and clarified the manuscript to focus on the central question of whether monocyte derived alveolar macrophages activate a distinct transcriptional program in resolving compared to progressive fibrosis.

C2: However, the conceptual advance over existing data is moderate. Even though directionality and causality is difficult to establish in human observational studies, the authors may want to consider further investigating the molecular links between the observed macrophage state in PASC and the resulting fibrotic phenotype.

R2: Because all the samples from patients with pulmonary fibrosis analyzed to date (to our knowledge) have included only patients with end-stage disease (e.g. those with progressive fibrotic lung disease or requiring transplant), it is not known whether monocyte-derived alveolar macrophages activate a distinct transcriptional program in resolving fibrosis as they do in other tissues. We show the abundance of profibrotic monocyte-derived alveolar macrophages correlates with fibrosis severity in a population of patients who show improved fibrosis on serial imaging. Furthermore, we did not observe differences in monocyte-derived alveolar macrophages from patients with resolving fibrosis compared to those with progressive disease.

Our results challenge existing paradigms in alveolar macrophage biology and suggest new areas of investigation. First, they challenge the widely held view (which we included in our own review, Watanabe JCI 2019) that alveolar macrophages develop a transcriptional phenotype during injury resolution that actively promotes lung repair. They instead suggest that successful lung repair slows the recruitment of monocyte-derived alveolar macrophages and promotes their maturation toward a homeostatic phenotype that is indistinguishable from tissue resident alveolar macrophages. This view is consistent with data from our group and others suggest deletion of monocyte-derived alveolar macrophages, irrespective of timing, improves fibrosis severity (Misharin et al., JEM 2017; McCubbrey et al., AJRCMB 2018; Aran et al., Nat Immunol 2019; Joshi et.al. ERJ, 2019, and others), and data showing strategies that promote alveolar epithelial repair reduce the recruitment and accelerate the differentiation of monocyte-derived alveolar macrophages (Watanabe et al., PNAS, 2021). Second, our findings open new questions for research, for example, do tissue resident alveolar macrophages express genes that promote repair (i.e., is a repair program inherently part of the differentiation of monocyte-derived alveolar macrophages toward a tissue-resident phenotype) and do tissue resident macrophages present before the injury participate in repair?

We have substantially revised the manuscript both to include these new data and to discuss these conceptual advances.

Specific comments
C3: The manuscript is very clear and well written.

R3: Thank you!

C4: The authors did a great job in assembling this cohort of RPRA patients with longitudinal CT imaging and sampling of BAL and nasal swabs. However, there are some concerns relating to the heterogeneity of the cohorts with regards to demographics, e.g. age (differs substantially between patients and controls), or sex. Also, the time points for CT follow-ups and bronchoscopy vary quite substantially. Recruitment or analysis of additional subjects may be helpful to better streamline the cohorts.
R4: Thank you for this comment. We made extensive efforts to enroll as many patients as possible in this observational study over the course of the pandemic. At this time in the pandemic, due to the success of large-scale vaccination campaigns and virulence of current viral variants, it will not be possible to enroll additional patients. We have made our clinical and molecular data available so that we and others can integrate them with smaller cohorts that might be analyzed in future studies.

C5: The authors observed no apparent correlation between the time to CT follow-up and the fibrosis score. This is a bit unexpected, since it has been reported in numerous studies, that interstitial lung lesions after COVID cleared or partially resolved over time. Could the authors comment on this apparent discrepancy?

R5: Thank you for this question. We were also surprised that the duration of time between CT scans is not correlated with the amount of improvement (Supplemental Figure 1A). At the reviewer's suggestion, we interrogated this further by limiting the analysis to patients in whom the CT improved over time. Even in these subjects, there was little correlation with time, suggesting biological variability in the rates of lung recovery in patients with severe respiratory PASC (new Supplemental figure 1B).

C6: On page 7, the authors state that "Direct pairwise comparison between healthy volunteers and patients with RPRA demonstrated a significant increase in the relative abundance of neutrophils and monocytes, and a decrease in the relative abundance of CD206high and CD206low alveolar macrophages". Judging from Figure 2b, it seems that CD206lo macrophages are actually increased in RPRA patients, not decreased.

R6: The reviewer is correct. We apologize for this mistake and corrected it in the revised manuscript (see page 7).

C7: Does the heatmap in Figure 2a actually show "percent" (as indicated in the legend in Fig. 2s) or is this rather a z-score? Please clarify.

R7: Thank you for noticing this. The heatmap in Figure 2a shows z-scores scaled across the rows, while bar plots in Figure S2a show the percent of CD45 cells. We have corrected the figure legend accordingly.

C8: MoAM-1 cluster of macrophages was labeled as "profibrotic macrophages" based on the expression of selected genes with known or suggested profibrotic function. However, it would be useful if the authors integrated their scRNA-Seq data with published phenotypes in published data sets or atlases and compared their MoAM phenotype to published profibrotic macrophage phenotypes from COVID-19 and other fibrotic conditions. Did the authors systematically assess similarity with previously published profibrotic Macrophages (e.g. from their own work on IPF)? What are the commonalities and distinctions that could hint at the capacity to reprogram the macrophages to fibrosis resolution?

R8: We thank the reviewer for this excellent suggestion. Accordingly, we compared gene expression in patients with resolving versus non-resolving fibrosis in this dataset and extended our analysis to include patients with more severe PASC (who died or required lung transplantation, Bharat et al., Sci Trans Med, 2020) and patients with pulmonary fibrosis requiring pulmonary lung transplantation for other causes, not related to inflammation or pneumonia (Reyfman et al., AJRCCM, 2019) and lungs from "normal" brain dead lung transplant donors (Reyfman et al., AJRCCM, 2019). As the reviewer is likely aware, these older studies used previous version of single cell chemistries. These introduce substantial batch effects that preclude meaningful integration using standard tools. Accordingly, we used a transfer learning approach to identify transcriptionally similar macrophage populations across datasets and combined this with gene programs identified by Spectra to compare the expression of fibrotic and homeostatic genes. While these expression patterns are similar (new Figure 5), we cannot make statistically-supported conclusions about the relative expression of these programs between the datasets. Indeed, the delay in resubmission of this manuscript resulted from multiple attempts to overcome this problem. We suspect this problem will not be easy to solve from a bioinformatic perspective and instead will require better designed studies that prospectively include analysis pipelines to correct for batch effects.
We agree with the reviewer that these results challenge the concept that alveolar macrophages assume a phenotype that actively promotes repair during resolution. Indeed, this suggestion motivated our reworking of the manuscript (thank you). Our findings are consistent with data from our group and others in murine models of lung fibrosis suggest deletion of monocyte-derived alveolar macrophages, irrespective of timing, improves fibrosis severity (Misharin et al., JEM 2017; McCubbrey et al., AJRCMB 2018; Aran et al., Nat Immunol 2019; Joshi et. al. ERJ 2020, and others). Additional data from our group suggest promoting alveolar epithelial repair reduces the recruitment and accelerates the differentiation of monocyte-derived alveolar macrophages (Watanabe et al., PNAS, 2021)..

C9: I feel that Figure 4 is not overly informative. What is the advantage of using SPECTRA as opposed to conventional gene modules or GO-Terms? The differential gene programs identified primarily in TRAMs are only labeled numerically. More meaningful labels could improve the information provide in Fig. 4.

R9: Thank you for this comment. Most authors compare gene expression programs between groups of individuals by clustering single cell RNA sequencing data, aggregating the cells within the clusters by individual (pseudobulk), applying differential gene expression tools designed for bulk RNA-sequencing analysis to each cell cluster, and identifying cellular processes using GSEA, GSVA, or other enrichment tools. This approach has been criticized as the clustering algorithms are designed to minimize differences in gene expression, the "pseudobulking" of the cells loses the advantages of the single cell resolution of the data, and GSVA programs are fixed. Spectra was designed to address these problems by analyzing pre-defined gene programs in each individual cell across the dataset, modifying these gene programs with genes identified in the data (within limits), and examining the expression of the gene programs across cell clusters. Hence the Spectra method is particularly suitable to supporting a null finding—there is no new gene program that emerges in patients with resolving fibrosis in any alveolar macrophage population. Indeed, while we continue to use traditional approaches for differential gene expression, which show no significant differentially expressed genes, we have highlighted Spectra as our primary analysis in the revised manuscript.

C10: The analysis of nasal cells is of interest, yet the value of comparing nasal sample to BAL samples, particularly the sorted BAL samples, is somewhat limited, since immune cells are strongly underrepresented in the nasal samples and epithelial cells where removed from the BAL samples before analysis.

R10: We agree with the reviewer that these data are largely negative, but we would argue they are important. Since SARS-CoV-2 infection invariably starts in the nasopharynx, the lack of persistent inflammation or markers of ongoing airway epithelial injury in the nasopharynx in patients where alveolar inflammation persists, excludes host factors that preclude the resolution of inflammation or epithelial repair is an important finding. Furthermore, these findings have important, if disappointing, implications for research. In a voluntary nasal challenge study (Lindeboom et al., medRxiv, 2023), the Teichmann group used nasal single-cell RNA sequencing analysis to demonstrate immunologic processes that closely mirrored findings we published in an analysis of BAL fluid samples from patients with respiratory failure secondary to SARS-CoV-2 pneumonia (Grant et al., Nature, 2021). Their findings offered the possibility that repeated nasal sampling might report on recovery from SARS-CoV-2 pneumonia noninvasively. Indeed, those data motivated our decision to obtain nasal samples. Our data suggest this strategy is unlikely to be fruitful for PASC or sequelae of other viral pneumonias.

Reviewer #3:
Remarks to the Author:
C11: The alveolar niche is profoundly affected by infection, inflammation and injury, and the presence of monocyte-derived macrophages expressing a profibrotic signature has been largely documented in the BALF of severe Covid-19 patients: PMID 34914922, PMID 33138195, PMID 32398875, etc. In this report, Bailey, Puritez and colleagues performed an observational cohort study on patients experiencing respiratory symptoms and shortness of breath and exhibiting CT abnormalities more than 90 days after the initial infection event. Concordant with the presence of inflammatory and fibrotic signs on CT images, they find the presence of neutrophils and monocyte-derived macrophages expressing a profibrotic signature. These findings suggest that some conserved immunological changes may drive the persistence of lung symptoms and CT abnormalities in these patients.

R11: We are thankful to the reviewer for this comment. In addition to these insights into the pathobiology of PASC, we have conducted substantial new analyses and have substantially revised our paper to highlight the central immunologic question that our dataset is uniquely poised to answer. The COVID-19 pandemic allowed us to collect alveolar samples from a large group of patients with lung injury and fibrosis induced by the same virus, some of whom went on to resolve their fibrosis and some of whom progressed to require lung transplantation. These data allowed us to compare the transcriptome of alveolar macrophages in resolving or non-resolving patients for evidence of a specific program of tissue repair. Despite our use of several complementary analysis strategies, including Spectra, we were unable to identify such a program in monocyte derived or tissue resident alveolar macrophages.

Major concerns.

C12: The potential interaction between monocyte-derived macrophages and fibroblasts, and the enriched presence of monocyte-derived macrophages in collagen-rich areas has not been addressed. The proposed function of those monocyte-derived macrophages is only based on transcriptomic data, which is underwhelming.

R12: We thank the reviewer for this comment. We acknowledge limitations of our observational study in human subjects. However, our conclusions about the role of profibrotic monocyte-derived alveolar macrophages in the pathogenesis of post-COVID-19 pulmonary fibrosis are based on ours and others previous observations from causal mouse models as outlined below. Furthermore, they provide data in humans to address an important question in alveolar macrophage biology—do monocyte derived alveolar macrophages assume a reparative phenotype during fibrosis resolution? Our finding that they do not has therapeutic implications—strategies that target monocyte-derived alveolar macrophages are unlikely to impede repair.

In murine models of lung injury and fibrosis, we and others have shown causal role of monocyte-derived alveolar macrophages in the development of pulmonary fibrosis in mouse models (Misharin et al., JEM 2017; McCubbrey et al., AJRCMB 2018; Aran et al., Nat Immunol 2019; and others). There is a prevalent hypothesis, which we have historically endorsed (e.g., in our review Watanabe et al., JCI, 2019, PMID 31107246), that at some time after injury, monocyte-derived alveolar macrophages take on a reparative phenotype. To test this hypothesis, we leveraged our observation from single-cell RNA-seq data that monocyte-derived alveolar macrophages rely on signaling through CSF1R for their maintenance in the niche, while tissue-resident alveolar macrophages rely on CSF2R. When we administered a CSF1R inhibitor to mice with established asbestos-induced lung fibrosis, we reduced the abundance of monocyte-derived alveolar macrophages. Instead of worsening fibrosis, however, we found fibrosis was improved, suggesting monocyte-derived alveolar macrophages continue to drive even established fibrosis (Joshi, et al., ERJ, 2020). In that study, we used smFISH to localize interactions between these profibrotic monocyte-derived alveolar macrophages with fibroblasts specifically within the fibrotic niche. These findings raised the question of what happens to monocyte-derived alveolar macrophages as fibrosis improves. To address this question, we leveraged our finding that a small molecule inhibitor of the integrated stress response accelerates lung repair by driving the differentiation of alveolar epithelial type 2 cells

independent of alveolar macrophages (Watanabe et al., PNAS 2021, Han et al., Nature 2023). When we administered ISRIB after fibrosis was established, the number of monocyte-derived alveolar macrophages in the lung was reduced and those that remained were transcriptionally more similar to tissue-resident alveolar macrophages.

Collectively, these results suggest a model where injury to the alveolar epithelial/capillary barrier results in the recruitment of monocyte-derived alveolar macrophages spatially restricted to the site of injury. These cells function to promote local fibroblast proliferation and matrix deposition, creating a temporary barrier between the circulation and alveolar space until the epithelial/capillary barrier is restored. Restoration of the barrier precludes further interaction between CSF1-expressing fibroblasts in the interstitium and profibrotic monocyte-derived alveolar macrophages, depriving them of a growth signal necessary for their maintenance in the niche. Simultaneously, the release of CSF2 from the repaired alveolar epithelium promotes the differentiation of monocyte-derived alveolar macrophages toward a mature phenotype. If repair fails, Uri Alon and colleagues have suggested that continued signaling from profibrotic monocyte-derived alveolar macrophages will promote the cell-autonomous expansion of fibroblasts to form a protective scar (PMID 32058955).

C13: Patients have been stratified in 4 clusters in Figure 2a based on BALF flow cytometry phenotyping, with cluster 3 containing only RPRA patients and many CD206 low MPs. It would be informative to keep this stratification and assess whether patients from cluster 3 also exhibit more MoAM-1 in the scRNA-seq, and more fibrotic areas on CT scans, even though this piece of information would still remain correlative.
R13: We thank the reviewer for this helpful suggestion. We now provide additional analysis which shows high agreement between groups of patients identified via flow cytometry and single-cell RNA-seq analysis (see Figure S3e, f).

C14: It would be interesting to assess whether blood monocytes are already primed towards a more activated, pro-fibrotic profile in these patients, suggesting systemic changes that would then influence tissue immunity.

R14: This is an excellent suggestion and interesting question. Unfortunately, in this study PBMCs were not biobanked. While increased number of circulating monocytes was associated with pulmonary fibrosis progression and poor outcomes (PMID 30935881), a recent study found no transcriptomic differences between monocytes from healthy controls, and patients with stable or progressive pulmonary fibrosis (PMID 37163015). Together, these studies suggest that numeric expansion of monocytes, rather than their priming, promotes development of pulmonary fibrosis. Accordingly, we tested whether number of circulating monocytes was associated with fibrosis severity at the time of chest computed tomography assessment. We found no association between number of circulating monocytes and abundance of fibrotic or inflammatory abnormalities in the lung (Figure S1c-e), although our study was underpowered to detect the small differences in previous reports.

C15: Overall, the data represent, in the reviewer's opinion, an incremental advance for a broad immunological audience and would be more appropriate for a specialized journal in the field of respiratory medicine.

R15: We thank the reviewer for the challenge inherent in this concern. We hope the new analyses discussed above will be of interest to both the reviewer and to the broader immunology community.

Reviewer #4:
Remarks to the Author:

C16: This is a tour-de-force manuscript characterizing clinical and molecular patterns of patients with post-acute sequelae of Covid-19 (PASC) by one of the leading groups in Covid/post-Covid lung research. The authors submit a large body of data analyzing clinical and molecular data from a unique and well-characterized cohort of 35 post-acute Covid patients at Northwestern U and identify cell populations that correlate with extent of fibrosis in these patients (such as monocyte-derived macrophages).

The story of molecular patterns of post-acute (pulmonary) sequelae of Covid (and reversal of lung fibrosis in a subset of these patients) is impressive and will undoubtedly be highly cited. These findings build on previous investigations by the authors on macrophage populations in lung fibrosis and highlight ongoing recruitment of these macrophage (and neutrophil) populations as a common pathophysiological trait of PASC in this single center cohort of PASC patients.

Authors are commended for the extended supplementary data submission, reviewer token, and methods description.

R16: We thank the reviewer for these kind comments. We especially appreciate that the reviewer has noticed the connection between the current study in human subjects and our previous mechanistic work in mouse models of pulmonary fibrosis and lung injury.

Specific critiques:
C17: The patient population is very unique yet rather small when confronted with an array of molecular analysis as presented herein. The authors present a number of molecular analysis from different subgroups of these patients, thereby challenging data integration between technologies (such as immunoprofiling, scRNAseq, and

nasal gene expression, e.g.). This is difficult to overcome in clinical reality, but this reviewer wonders if a smaller population with comprehensive analysis of all assays would yield different results. Have the authors performed data integration in just a subgroup of patients (e.g. all patients undergoing bronchoscopy w and w/o steroids)?

R17: Thank you for this question. As noted by the reviewer, the COVID-19 pandemic allowed us to collect alveolar samples from a large group of patients with lung injury and fibrosis induced by the same virus, some of whom went on to resolve their fibrosis and some of whom progressed to require lung transplantation. These data allowed us to compare the transcriptome of alveolar macrophages in resolving or non-resolving patients for evidence of a specific program of tissue repair. Despite our use of several complementary analysis strategies, including Spectra, we were unable to identify such a program in monocyte derived or tissue resident alveolar macrophages. We considered other factors in our study that might have modified the transcriptome, including the use of steroids, but were underpowered to address these questions.

C18: Why did the authors choose healthy volunteers as a control group instead of acute Covid-19 without detectable PASC? Such a control group would be highly informative and or appropriate for e.g. scRNAseq analysis.

R18: We thank the reviewer for this question. We assume that the reviewer means “subjects who have recovered from COVID-19 but do not have detectable PASC”. We agree that such controls would be ideal, and we acknowledge the recruitment bias in our observational study. All patients in our study came to us with persistent respiratory symptoms at a referral center for PASC, so convalescent cases were not included. We are aware of at least one study in the US that recently started recruitment of such subjects, however, this study is still in the recruitment phase and no data are available. As we have done in our previous work, we will share both clinical and molecular phenotypes from our cohort, so they can be reused and integrated with the data from other studies.

C19: Is there a distinct molecular/immunological profile of the patients which resolved fibrosis over the course of two CT scans compared with those that did not?

R1: Thank you for this suggestion. This important question has become the central theme of the revised manuscript and is addressed above.

C20: Minor comment: The results section often reads like a clinical medical journal, highlighting specifics of individual patients, rather than focusing on common immunological observations.

R20: Thank you for this comment. We have now reframed the manuscript around the central question in alveolar macrophage biology our data addresses, namely: Do monocyte derived alveolar macrophages express a distinct transcriptional program during fibrosis resolution.

C21: Page 7, 2nd paragraph. The authors invalidate all correlations described by stating that "these correlations were not significant". Better to delete the entire passage.

R21: Thank you, this has been re-written to state the results of this analysis were null.

C22: The entire passage on microbiological BAL analysis is more appropriate in either supplementary data or even methods section.

R22: Thank you for this recommendation. We would maintain that a description of relevant host and microbiologic findings discussed in the results section adds important context to the epithelial injury and immune response within the alveolar microenvironment in our cohort. We have added a new introductory sentence to this section to better clarify this point.

C23: The last section on comparing transcriptional changes in nasal mucosa compared with distal lung is highly interesting (given published data on e.g. lung cancer patients), but entirely underdeveloped. This reviewer would suggest expanding this section due to its findings that are highly relevant.

R23: Thank you. We agree with the reviewer that these data are largely negative, but we would argue they are important. Since SARS-CoV-2 infection invariably starts in the nasopharynx, the lack of persistent inflammation or markers of ongoing airway epithelial injury in the nasopharynx in patients where alveolar inflammation persists, excludes host factors that preclude the resolution of inflammation or epithelial repair is an important finding. Furthermore, these findings have important, if disappointing, implications for research. In a voluntary nasal challenge study (Lindeboom et al., medRxiv, 2023), the Teichmann group used nasal single-cell RNA sequencing analysis to demonstrate immunologic processes that closely mirrored findings we published in an analysis of BAL fluid samples from patients with respiratory failure secondary to SARS-CoV-2 pneumonia. Their findings offered the possibility that repeated nasal sampling might report on recovery from SARS-CoV-2 pneumonia noninvasively. Indeed, those data motivated our decision to obtain nasal samples. Our data suggest this strategy is unlikely to be fruitful.

Version 1:

Decision Letter:

23rd Jul 2024

Dear Dr Misharin,

Thank you for providing a point-by-point response to the referees' comments on your revised manuscript entitled, "Expansion of profibrotic monocyte-derived alveolar macrophages in patients with persistent respiratory symptoms and radiographic abnormalities after COVID-19", which was seen by 2 of the original referees. We agree with your proposed textual revision as proposed in your response letter. We therefore invite you to revise your manuscript taking into account all reviewer and editor comments. Please highlight all changes in the manuscript text file in Microsoft Word format.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

When revising your manuscript:

* Include a “Response to referees” document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

* If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions at http://www.nature.com/ni/authors/index.html. Refer also to any guidelines provided in this letter.

* Please include a revised version of any required reporting checklist. It will be available to referees to aid in their evaluation of the manuscript goes back for peer review. They are available here:

Reporting summary:
https://www.nature.com/documents/nr-reporting-summary.pdf

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-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

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We hope to receive your revised manuscript within two weeks. If you cannot send it within this time, please let us know. We will be happy to consider your revision so long as nothing similar has been accepted for publication at Nature Immunology or published elsewhere.

Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further.

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We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,

Laurie A. Dempsey, Ph.D. (for Ioana Staicu, Ph.D.)
Senior Editor
Nature Immunology
l.dempsey@us.nature.com
ORCID: 0000-0002-3304-796X

Referee expertise:

Referee #1:

Referee #2:

Referee #3:

Reviewers' Comments:

Reviewer #2:
Remarks to the Author:
Bailey et al. performed additional analyses and carefully revised and rewrote the manuscript text. The manuscript now has a stronger emphasis on the general role of monocyte-derived macrophages in lung fibrosis and repair. Their study contributes valuable insights, demonstrating that the abundance of “profibrotic” monocyte-derived alveolar macrophages corelates with radiographic signs of fibrosis in patients with PASC/RPRA. They propose that acute lung injury generally elicits a “stereotypic” profibrotic macrophage phenotype, as part of a conserved pulmonary damage response program. Their results support previous findings from mouse models, suggesting that abrogation of continued monocyte-derived macrophage recruitment and replacement with homeostatic tissue-resident type cells, rather than a differential transcriptional programming promotes fibrosis resolution and lung repair. In line with this, they demonstrate that transcriptional profiles in resolving and non-resolving COVID-19 associated fibrosis were similar, yet the numbers of profibrotic monocytes-derived macrophages differed, according to clinical outcome. Patients with RPRA showed a continued recruitment of monocyte-derived alveolar macrophages, suggesting a defective resolution of the “stereotypic” lung damage response.

Overall, the manuscript has been much improved. Even though causality and directionality remain difficult to address in this setting, I believe that this study elegantly combines clinical and deep molecular phenotyping and thereby contributes new conceptual insights into macrophage- and pulmonary pathophysiology during acute and post-acute lung injury.

All my comments have been addressed or sufficiently answered by the authors.

Reviewer #3:
Remarks to the Author:
The authors have extensively revised their manuscript and have decided to focus on a novel, central question of whether monocyte
derived alveolar macrophages activate a distinct transcriptional program in resolving compared to progressive fibrosis. The authors found that monocyte-derived and tissue-resident-like alveolar macrophages from patients with "resolving" fibrosis expressed the same set of profibrotic genes as patients with progressive fibrosis, arguing against a distinct reparative phenotype in monocyte-derived alveolar macrophages.

Major comment:

- the reviewer may have missed this information (apologies if this is the case): how were the 5 patients with "non-resolving" fibrosis defined / chosen? Only 2 patients developed severe fibrosis and required lung transplantation, not 5, correct? In addition, how was the "resolving" fibrotic phenotype defined? Is it related to the decrease in the fraction of fibrotic area on the CT scan between scan 1 and 2? In the reviewer's opinion, a decrease in fibrotic areas between 2 time points 4 months apart does not guarantee that the fibrosis will eventually resolve in the future. Are there additional parameters that the authors have taken into consideration to define the resolving phenotype?

- BALF cells may only represent the tip of the iceberg in fibrotic disorders, which mostly occur in the tissue. While the authors point out that monocyte-derived alveolar macrophages have been repeatedly reported to play a pathogenic role in fibrosis / after injury, other lung monocyte-derived macrophages, which are likely not found in the BALF, have been shown to play

beneficial roles after injury in different contexts (PMID: 35624205, 32750316, 28506464). This should be better discussed in the manuscript. One possible scenario is that monocyte-derived alveolar macrophages indeed upregulate a stereotypical pro-fibrotic program in response to injury, regardless of the trigger and outcome, but that the response and functional diversity of tissue monocyte-derived macrophages, rather than the one of monocyte-derived alveolar macrophages, critically influence the development of fibrosis and would eventually dictate disease severity / outcome.

Author Rebuttal letter:

Dear Dr. Dempsey,

Thank you for considering our revised manuscript. Comments from reviewer #3 are great discussion points; however, they do not question the significance of our findings or our conclusions with respect to the association between abundance and gene expression programs in profibrotic monocyte-derived macrophages and radiographic severity of fibrotic abnormalities. Please find the point-by-point response to critiques below. We provide a copy of the manuscript with changes in response to reviewer #3 critiques highlighted in blue and updated Supplementary Data File 1.

***

Reviewer #2: "Overall, the manuscript has been much improved. Even though causality and directionality remain difficult to address in this setting, I believe that this study elegantly combines clinical and deep molecular phenotyping and thereby contributes new conceptual insights into macrophage- and pulmonary pathophysiology during acute and post-acute lung injury. All my comments have been addressed or sufficiently answered by the authors."

Response: We thank Reviewer #2 for their helpful and constructive critique, which helped us to improve our manuscript.

***

Reviewer #3: "The authors have extensively revised their manuscript and have decided to focus on a novel, central question of whether monocyte derived alveolar macrophages activate a distinct transcriptional program in resolving compared to progressive fibrosis. The authors found that monocyte-derived and tissue-resident-like alveolar macrophages from patients with "resolving" fibrosis expressed the same set of profibrotic genes as patients with progressive fibrosis, arguing against a distinct reparative phenotype in monocyte-derived alveolar macrophages."

Response: We thank Reviewer #3, whose helpful critique helped us to improve our manuscript.

Major comment:

C1: The reviewer may have missed this information (apologies if this is the case): how were the 5 patients with "non-resolving" fibrosis defined / chosen? Only 2 patients developed severe fibrosis and required lung transplantation, not 5, correct? In addition, how was the "resolving" fibrotic phenotype defined? Is it related to the decrease in the fraction of fibrotic area on the CT scan between scan 1 and 2? In the reviewer's opinion, a decrease in fibrotic areas between 2 time points 4 months apart does not guarantee that the fibrosis will eventually resolve in the future. Are there additional parameters that the authors have taken into consideration to define the resolving phenotype?

R1: We apologize for not explicitly explaining the definitions of "non-resolving" RPRA. We defined non-resolving RPRA as patients whose fraction of normal lung did not increase between CT scans 1 and 2. The two lung transplanted patients did not have serial CT scans prior to date of transplant and were therefore excluded from this analysis. Resolving RPRA was defined as patients whose fraction of normal lung increased between CT scans 1 and 2. We have updated the results section (Page 11), methods (Page 20), and Supplemental Data File 1, containing information about study subjects and their demographic and clinical characteristics.

We agree with the reviewer that the timeline of our study precludes us from assessing whether fibrosis resolution in those patients who showed evidence of radiographic improvement was eventually complete or remained incomplete. Therefore, we avoid making any conclusions about complete fibrosis resolution in the text. We added this to the limitations of our study (Page 19).

C2: BALF cells may only represent the tip of the iceberg in fibrotic disorders, which mostly occur in the tissue. While the authors point out that monocyte-derived alveolar macrophages have been repeatedly reported to play a pathogenic role in fibrosis / after injury, other lung monocyte-derived macrophages, which are likely not found in the BALF, have been shown to play beneficial roles after injury in different contexts (PMID: 35624205, 32750316, 28506464). This should be better discussed in the manuscript. One possible scenario is that monocyte-derived alveolar macrophages indeed upregulate a stereotypical pro-fibrotic program in response to injury, regardless of the trigger and outcome, but that the response and functional diversity of tissue monocyte-derived macrophages, rather than the one of monocyte-derived alveolar macrophages, critically influence the development of fibrosis and would eventually dictate disease severity / outcome.

R2: We thank the reviewer for this excellent discussion point. Indeed, in the discussion of our revised manuscript,

we have listed our sampling technique as a limitation that may preclude the detection of other cell types involved in the persistence or progression of fibrosis (Page 18). Per the reviewer's suggestion, we have modified our discussion to indicate that other types of macrophages may mediate tissue repair and resolution of fibrosis.

Version 2:

Decision Letter:

Our ref: NI-A36352B

25th Jul 2024

Dear Dr. Misharin,

Thank you for submitting your revised manuscript "Expansion of profibrotic monocyte-derived alveolar macrophages in patients with persistent respiratory symptoms and radiographic abnormalities after COVID-19" (NI-A36352B). I have discussed your revisions and note that we'll be happy in principle to publish it in Nature Immunology, pending minor revisions to comply with our editorial and formatting guidelines.

We will now perform detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

If you had not uploaded a Word file for the current version of the manuscript, we will need one before beginning the editing process; please email that to immunology@us.nature.com at your earliest convenience.

Thank you again for your interest in Nature Immunology Please do not hesitate to contact me if you have any questions.

Kind regards,

Laurie

Laurie A. Dempsey, Ph.D. (for Ioana Staicu, Ph.D.)
Senior Editor
Nature Immunology
l.dempsey@us.nature.com
ORCID: 0000-0002-3304-796X

Version 3:

Decision Letter:

In reply please quote: NI-A36352C

Dear Dr. Misharin,

I am delighted to accept your manuscript entitled "Profibrotic monocyte-derived alveolar macrophages are expanded in patients with persistent respiratory symptoms and radiographic abnormalities after COVID-19" for publication in an upcoming issue of Nature Immunology.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Immunology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

After the grant of rights is completed, you will receive a link to your electronic proof via email with a request to make any corrections within 48 hours. If, when you receive your proof, you cannot meet this deadline, please inform us at rjsproduction@springernature.com immediately.

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Sincerely,

Ioana Staicu, Ph.D.

Senior Editor
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