# Alveolar Macrophage Heterogeneity Goes up in Smoke?

The lungs are constantly exposed to potentially harmful environmental gases, particles, toxins, allergens, and pathogens. Our immune system has been ascribed the crucial role of protection against these insults. Alveolar macrophages (AMs) perform the duties of frontline soldiers tasked with airway surveillance, immune regulation, and surfactant homeostasis. Recent interest has focused on the heterogeneity of AMs, in which healthy individuals possess distinct subsets of resident macrophages but also monocyte-like cells (1). Of intense interest is whether distinct subsets of resident macrophages and monocyte-like cells within the airspaces are also found or altered in conditions such as smoking and chronic obstructive pulmonary disease (COPD), a disease characterized by irreversible airway obstruction and ongoing inflammatory response. In this issue of the *Journal*, Liégeois and colleagues (pp. 241–252) report on their examinations of the identity and heterogeneity of AMs comparing healthy nonsmokers, non-COPD smokers, and COPD smokers using a combination of approaches that include flow cytometry and bulk and single-cell RNA sequencing (scRNA-seq) of BAL cells (2) (Figure 1).

AMs were identified by expression of CD206, a C-type lectin mannose receptor, and autofluorescence that allowed separation into two discrete populations: autofluorescent$^{high}$ (AF$^{hi}$) and autofluorescent$^{low}$ (AF$^{lo}$) AM subsets. By bulk RNA-seq, they compared CD14$^{+}$ blood monocytes and fluorescence-activated cell-sorted AF$^{hi}$ and AF$^{lo}$ AMs. While both AF$^{hi}$ and AF$^{lo}$ AMs share core macrophage-associated genes, including *FABP4*, *MARCO*, *MRC1*, and *PPARG*, AF$^{lo}$ AMs also exhibit a gene signature similar to CD14$^{+}$ blood monocytes including *CCR2*, *CX3CR1*, and *ADAM19*. When AF$^{lo}$ AMs were cultured *in vitro*, these macrophages were capable of secreting IL-10 more readily when compared with AF$^{hi}$ macrophages. These findings collectively suggest that AF$^{lo}$ AMs are distinct from AF$^{hi}$ AMs, possessing a transcriptional profile that is in-between resident AMs and blood monocytes and a plasticity that enables immunoregulatory function. Notably, these cell types were present across healthy nonsmokers, non-COPD smokers, and COPD smokers.

To examine AM heterogeneity, the authors used scRNA-seq and found four clusters of AMs. While all four clusters expressed "classical" or core macrophage-associated genes, including *MRC1*, *CD68*, *MARCO*, *LYZ*, *FCGR3A*, and *PPARG*, these clusters also exhibited gene signatures distinct from one another. Most notably, "Cluster 2" was found to be more prominent in smokers (both non-COPD and COPD current smokers) when compared with healthy nonsmokers. This Cluster 2 was highly enriched for genes involved in oxidative stress, detoxification pathways, and proinflammatory response. The authors suggest that Cluster 2 represents a "classical" AM subset responding to the toxic effects of cigarette smoke. Whether this is an adaptive or maladaptive response remains to be determined.

On the basis of prior work in mice, the prevailing concept has been that AMs self-maintain with minimal contribution from blood monocytes during homeostasis (3–5). However, recent studies, including this one, suggest that monocytes contribute to the diversity of the AM pool (1, 2, 6, 7). For example, Cluster 3 expressed both classical macrophage-associated genes in addition to monocyte-associated gene signatures. Given the heterogeneity of the Cluster 3 subset, the authors go on to recluster this subset, which yielded four new subclusters. Interestingly, Subcluster 1 was more prominent in smokers and exhibited genes associated with monocytes, including *CCR2* and *FCN1*, as well as *CLEC5A* and *VCAN*. CLEC5A, a C-type lectin receptor, and versican, an extracellular matrix protein, have previously been implicated in inflammation and COPD pathogenesis (8, 9). The authors suggest that Subcluster 1 represents recently recruited monocytes with an activated profile. Subclusters 2 and 3 were enriched in healthy nonsmokers, with Subcluster 2 displaying a chemokine gene signature and Subcluster 3 exhibiting a matrix-associated gene profile.

A major strength and novelty of the study are the scRNA-seq findings of distinct macrophage subsets that are enriched in non-COPD and COPD smokers when compared with healthy subjects. The transcriptional fingerprint of smoking appears to be one of oxidative stress and inflammation, inviting speculation that the altered landscape might be involved in the initiation or progression of disease. Another strength of the study is the comprehensive approach by which the authors profile the diversity of human AM populations using both transcriptional and phenotypic assessments. Comparing the techniques used, the Cluster 3 subset of monocyte-like macrophages identified by scRNA-seq bear some similarities to the bulk RNA-seq signature of AF$^{lo}$ AMs. Interestingly, AF$^{lo}$ AMs are capable of secreting IL-10 ex vivo and are reminiscent of regulatory interstitial macrophages and recruited monocytes found in murine lungs (10–12). The immunoregulatory potential and functional plasticity of these AF$^{lo}$ AMs may serve as useful markers of health and disease in the lung.

One limitation of the study reflects the shortcomings of scRNA-seq technology in its current state. The numbers of human individuals in the studies are small (three in each group) given the costs associated with scRNA-seq. The numbers of cells analyzed with reclustering are also small, making definitive conclusions about discrete subsets challenging. Reclustering of Cluster 2, the prominent cluster in smokers, was not performed because sufficient numbers of cells are required in each group to permit further analysis. Nevertheless, scRNA-seq is a powerful tool that enables the discovery of the diversity of the AM pool. Findings here await validation by future studies.



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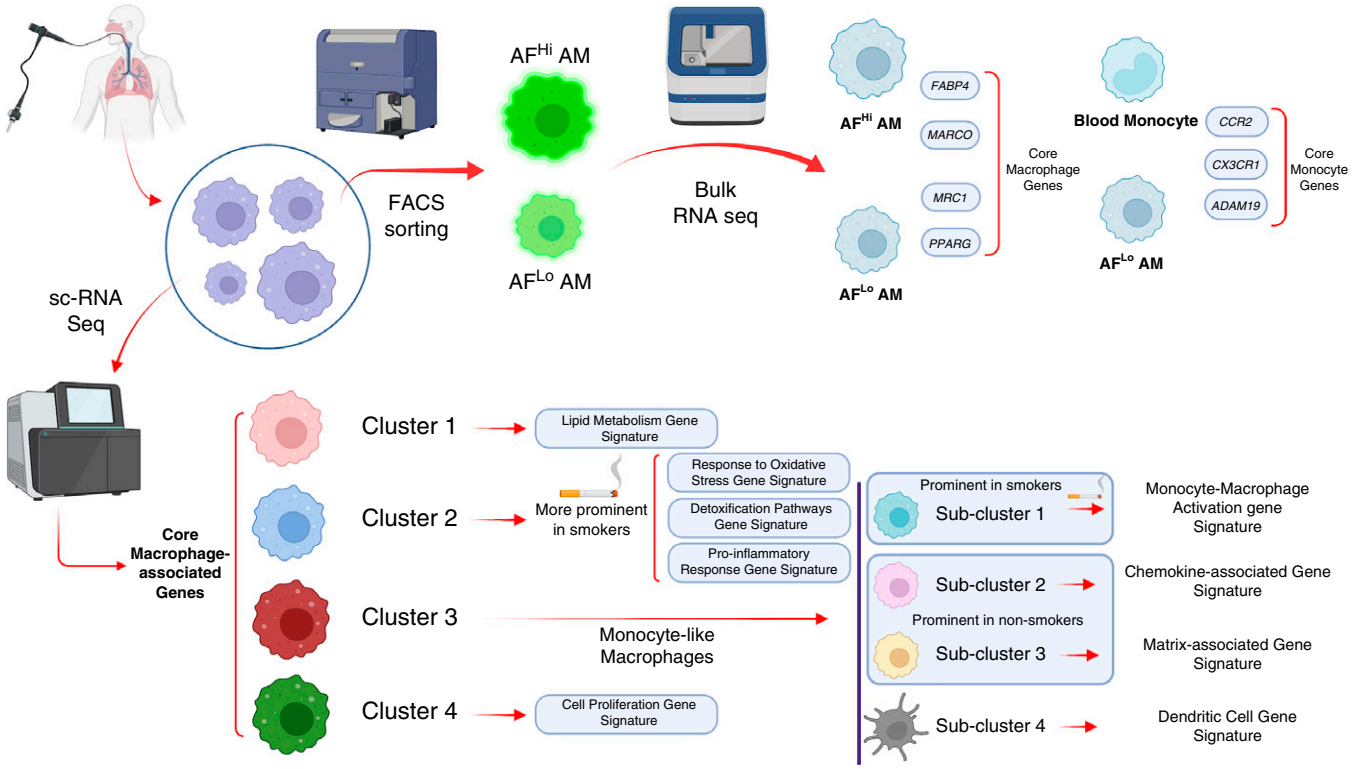


**Figure 1.** Overview of the experimental approach and findings in the alveolar macrophage (AM) pool (2). The identity and heterogeneity of alveolar macrophages were assessed among healthy nonsmokers, non-COPD smokers, and COPD smokers using a combination of approaches, including flow cytometry and bulk and single-cell RNA-seq of BAL cells. Illustration created with BioRender.com. $AF^{Hi}$ = autofluorescent$^{hi}$; $AF^{Lo}$ = autofluorescent$^{low}$; COPD = chronic obstructive pulmonary disease; FACS = fluorescence-activated cell sorting; sc-RNA Seq = small conditional RNA sequencing.

## Conclusions

Liégeois and colleagues advance our knowledge of the heterogeneity of AMs and how the transcriptional landscape is altered by conditions such as cigarette smoking and COPD. Like all good studies, the findings raise additional questions. For example, what is the contribution of cells representing Cluster 2 and Subcluster 1 to the pathogenesis of COPD and accompanying lung destruction? Is there any association between the overall increase in the number of these cells and the decline in lung function over time in smokers? What is the exact mechanism by which smoking might induce these cells and change their transcriptional profile? Are Subclusters 2 and 3 that are enriched in healthy nonsmokers protective against noxious environmental stimuli, or, when arising in excess, do these subsets contribute to profibrotic events? Further research is needed to answer the many questions that emerge from this important work. ■

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