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Human airspace macrophage signatures are conserved during sterile lung injury and repair

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Abstract

Rationale: Airspace macrophages (AM) are implicated in both persistent inflammation and tissue repair following acute lung injury. Distinct subsets of AM are associated with lung pathology in humans, but whether unique AM signatures are specific to disease states or represent a conserved response to lung inflammation is unknown.
Objectives: We sought to test the hypothesis that conserved subsets of inflammatory and reparative AM could be identified by transcriptional programming in a human model of self-resolving acute lung injury.
Methods: A total of 15 participants underwent BAL before and at a pre-assigned time point after endobronchial exposure to bacterial endotoxin. Bronchoalveolar lavage cells were subjected to single cell RNA sequencing, and longitudinal assessment of AM programming during resolution of inflammation and lung repair was performed.
Measurements and Main Results: We identify transcriptionally distinct subsets of tissue resident and recruited AM present at all time points, in all participants. Two recruited AM populations increase following inflammation: one that aligns with classical monocytes and one with interstitial macrophages (IAM). Airspace macrophage subsets display unique patterns of gene expression throughout the time course. Comparison of subset-specific markers to those identified in disease states reveals that IAM express many so-called pathogenic markers during normal lung repair.
Conclusions: By applying a uniform inflammatory stimulus to healthy adults and examining BAL cells obtained at precise time points thereafter, we construct a time-resolved kinetic of AM transcriptional programming during typical lung repair. Our data demonstrate that IAM share transcriptional similarity to AM identified in disease states and suggest they may reflect a conserved cellular response to tissue injury.

Keywords alveolar macrophage, single cell RNA sequencing, endotoxin challenge

Introduction

Bronchoalveolar lavage allows for minimally invasive sampling of the distal airspaces and study of immune cells during inflammation and repair of acute lung injury. Macrophages are the predominant cell type in BAL fluid during health and are thought to be key determinants of outcome after injury—they have been causally linked to persistent inflammation and fibrosis but are also deemed necessary for alveolar repair and return to homeostasis. The specific programming states and molecular drivers of macrophages during these disparate outcomes have not been identified.

One key determinant of airspace macrophages (AM) function is cellular origin–tissue resident AM versus those recruited to the lung during inflammation display unique programming states despite their existence in a shared microenvironment.[1,2] An evolving literature suggests that recruited lung macrophages are essential drivers of tissue repair.[3-5] Therefore, identification of recruited AM populations in humans has important implications for predicting outcomes after lung injury and identifying therapeutic targets for dysregulated repair. However, lack of shared markers across species and dynamic programming over time have hampered such studies.

Unique AM subsets have been identified and associated with outcomes in parenchymal lung diseases in humans including severe COVID-19,[6-10] acute respiratory distress syndrome (ARDS),[11-13] and pulmonary fibrosis.[14-16] It remains unclear whether the markers ascribed to these subsets are restricted to cells of a particular origin or are stably expressed over time. Further, it is not known whether these molecular markers are implicated as causal drivers of pathology or represent a nonspecific AM response to parenchymal lung damage.

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At a Glance Commentary

Scientific Knowledge on the Subject: Single cell RNA sequencing has resulted in the description of numerous transcriptionally distinct populations of lung macrophages. In disease states such as pulmonary fibrosis, COVID-19 pneumonia, and acute respiratory distress syndrome, increased numbers of unique macrophage subsets have been observed. The causal links between these subsets and pathologic mechanisms of disease are lacking.

What This Study Adds to the Field: By examining the transcriptional programming of BAL monocytes and macrophages in a self-resolving model of endotoxin-induced lung inflammation, stable populations of macrophages are identified during sterile inflammation and normal lung repair. Cellular markers of these populations are then compared to prior studies of lung disease to highlight potentially conserved cell types and unique programming states across conditions.

We sought to address these knowledge gaps by characterizing the AM repertoire during the normal process of lung repair. Using a sterile, self-resolving lung injury model we identify multiple distinct subsets of resident and recruited AM in humans and observe that each subset persists during tissue repair. By analyzing the unique markers of these subsets, we reveal overlapping signatures with cells identified in disease states, suggestive of a conserved AM response to lung injury.

Methods

Participant selection and BAL procedure

Healthy research participants aged 18-50 years were enrolled as previously described.[17,18] Briefly, participants underwent fiberoptic bronchoscopy with lavage using serial aliquots of 60 ml normal saline. Bronchoalveolar lavage fluid was filtered through a 100-micron nylon filter to remove debris and centrifuged at 300 g for 5 minutes to pellet cells. Supernatant was stored at −8°C. A small aliquot of cells was set aside for cytocentrifugation and cell counts. Remaining cells were cryopreserved. In all cases, less than 1 hour elapsed between the start of the BAL procedure and cryopreservation of biospecimens. Additional details regarding the study intervention and measurement of BAL components are available in the online supplement.

Single cell capture and RNA sequencing

A total of 30 BAL samples from 15 participants, before and after lipopolysaccharide (LPS) exposure, were submitted for single cell RNA sequencing (scRNAseq). Single-cell isolation and RNA sequencing were performed using the Chromium Box (10x Genomics, Pleasanton, CA, USA). Libraries were sequenced with the Illumina NovaSQ6000 sequencer (Illumina, Inc., San Diego, CA, USA) using an S4 flow cell. A total of 5000 cells per sample were targeted and sequenced to a depth of 100 000 reads per cell. Bioinformatic analysis methods can be found in the online supplement.

Ethical approval

The study was approved and monitored by the National Jewish Health Institutional Review Board and the Biomedical Research Alliance of New York (BRANY, FWA00000778). Clinical Center Reference Endotoxin (CCRE) administration was regulated by the Food and Drug Administration IND 18580. Written informed consent was obtained from all participants.

Results

A total of 15 unique participants each underwent two bronchoscopic lavage procedures, first during health, then at a pre-assigned time point after exposure to endobronchial endotoxin. All participants tolerated the protocol with no adverse events. Participant characteristics and lavage yields are listed in Table 1. Data from pre-exposure time points of some participants have previously been reported.[17,18]

We began by characterizing lung injury and cellular kinetics in our model. Measurement of total protein in the lavage fluid and enumeration of BAL cells and differentials were performed. Total protein levels were elevated at day 1, indicating injury of the alveolar-capillary barrier, and returned to levels measured during health by day 5 post-LPS exposure (Figure 1A, Table E1). Absolute numbers of cells obtained from bronchoscopy were variable but increased in all participants after exposure to LPS (Figure E1). Neutrophils were present in BAL samples at day 1 and in 1 of 3 samples at day 2 after exposure to LPS. Few neutrophils were detected on days 3-5 (Figure 1B, Figure E1). These data confirm that endobronchial endotoxin challenge provides a reliable model of self-resolving lung injury, characterized by acute neutrophilic lung inflammation, capillary leak of proteins, and increased numbers of total cells in BAL fluid lasting 1-2 days with restoration of alveolar-capillary barrier integrity by day 5 after challenge.

To investigate the transcriptional heterogeneity of BAL cells during acute and resolving inflammation, we next performed scRNAseq of cells from 30 paired samples (pre- and post-LPS). After application of strict quality control standards (Figure E2), 123 085 cells, with a median of 4263 (range = 1521-6708) from each participant at each time point, were partitioned into clusters using the 3000 most informative genes and annotated based on cell lineage markers[19] (Figure E3A and B, Table E2). Of note, cryopreservation and quality control methods effectively excluded neutrophils, platelets, and red blood cells from analysis. Of the remaining cells, macrophages comprised the largest number of clusters. Lymphocyte, dendritic cell (DC), or cycling cell markers were identified in smaller clusters (Figure 1C, Figure E3B). Except for the four smallest clusters (comprised of rare DC, mast, and B cells), each leukocyte type was detected in all 30 BAL samples. Interestingly, except for neutrophils, the global cellular composition of BAL

Table 1 Characteristics of participants and BAL yield.

Participant ID	Sex	Age, y	Pre-exposure		Postexposure		
			BAL efficiency (%)	BAL cell count	BAL efficiency (%)	BAL cell count	BAL interval, days postexposure
1	M	35	74	1.46E+07	52	1.65E+08	1
2	M	27	54	1.26E+07	68	2.33E+08	1
3	M	29	72	7.75E+06	63	6.80E+07	1
4	F	25	72	1.70E+06	75	4.40E+07	1
5	M	27	56	1.10E+07	66	6.70E+07	2
6	M	30	69	4.50E+06	75	1.50E+08	2
7	M	25	60	8.30E+06	57	7.60E+07	2
8	F	27	31	3.00E+06	44	2.00E+07	3
9	F	46	55	3.40E+06	54	2.60E+07	3
10	M	28	60	8.95E+06	70	2.33E+07	3
11	M	50	19	6.10E+06	36	1.10E+07	4
12	F	39	54	1.13E+07	63	3.44E+07	4
13	M	26	65	1.61E+07	70	3.19E+07	5
14	F	29	66	1.86E+07	69	2.43E+07	5
15	F	26	67	1.00E+06	68	7.20E+06	5

Self-reported racial or ethnic identifiers: White ($n=10$), Hispanic ($n=3$), Asian ($n=2$).
Abbreviations: F, female; M, male.

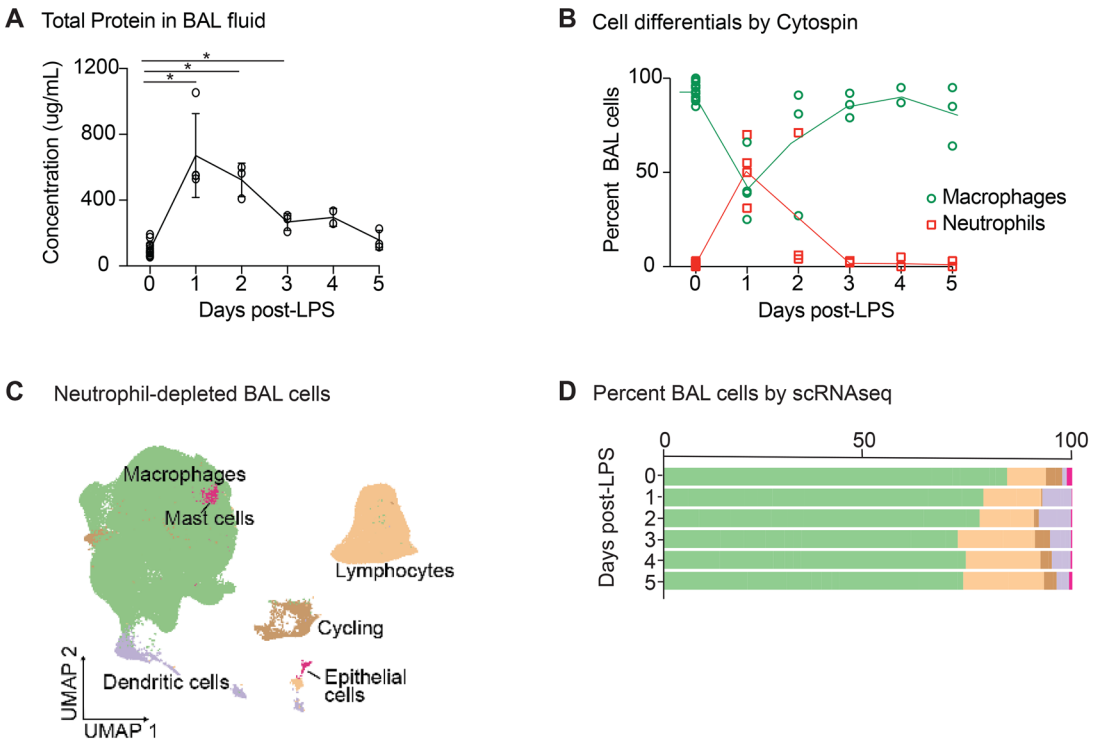


Figure 1 Human endobronchial endotoxin challenge model of acute lung inflammation. Fifteen healthy participants underwent BAL before and after instillation of bacterial lipopolysaccharide (LPS). (A) Cell differentials were determined by microscopic evaluation of cytocentrifuged BAL cells. Macrophage and neutrophil percentages are shown. Line represents mean values. (B) Total protein was measured in each BAL sample by bicinchoninic acid (BCA) assay. Protein levels were compared between time points using a linear mixed effects model, adjusting for sex; an F-test was used to test for any differences between time points before performing pairwise comparisons between each follow-up time point and baseline (*$P<.001$, Table E1). (C) Uniform manifold approximation and projection (UMAP) plot shows clustering of BAL cells based on gene expression as determined by single cell RNA sequencing overlaid by cell lineage inferred from expression of marker gene signatures. (D) Percentage of annotated cell populations at each time point (Table E3).

leukocytes changed only slightly over the course of inflammation, with small but significant increases in the percent of lymphocytes and DC and relative decreases in AM and cycling cells after exposure to LPS (Figure 1D, Figures E3C and D, E4, Table E3).

We next sought to characterize the composition and relative abundance of macrophage subpopulations during health, inflammation, and resolution of lung injury. We re-analyzed clusters annotated as macrophages to a resolution at which the smallest clusters were defined by specific gene expression patterns. This analysis yielded 14 AM subsets (Figure 2A, Figures E5-E7, Table E4). Of 14 clusters, 6 expressed markers of tissue resident AM (RAM) such as *FABP4* and *INHBA* and, as expected, constituted the greatest percentage of cells obtained during health (Figure 2B and C). Overall, RAM clusters were defined by small but significant differences in expression of gene markers but shared core identities, as we have previously reported.[17]

Four AM clusters lacked markers of mature alveolar macrophages and could be divided into two groups—one reminiscent of circulating monocytes (eg, *VCAN*, *FCN1*, *CCR2*, and *CX3CR1*) and one with gene signatures similar to interstitial macrophages (eg, *MERTK, LGMN*, and *PLA2G7*).[20] We termed these monocyte-like airspace macrophages (MoAM) or interstitial macrophages (IAM),

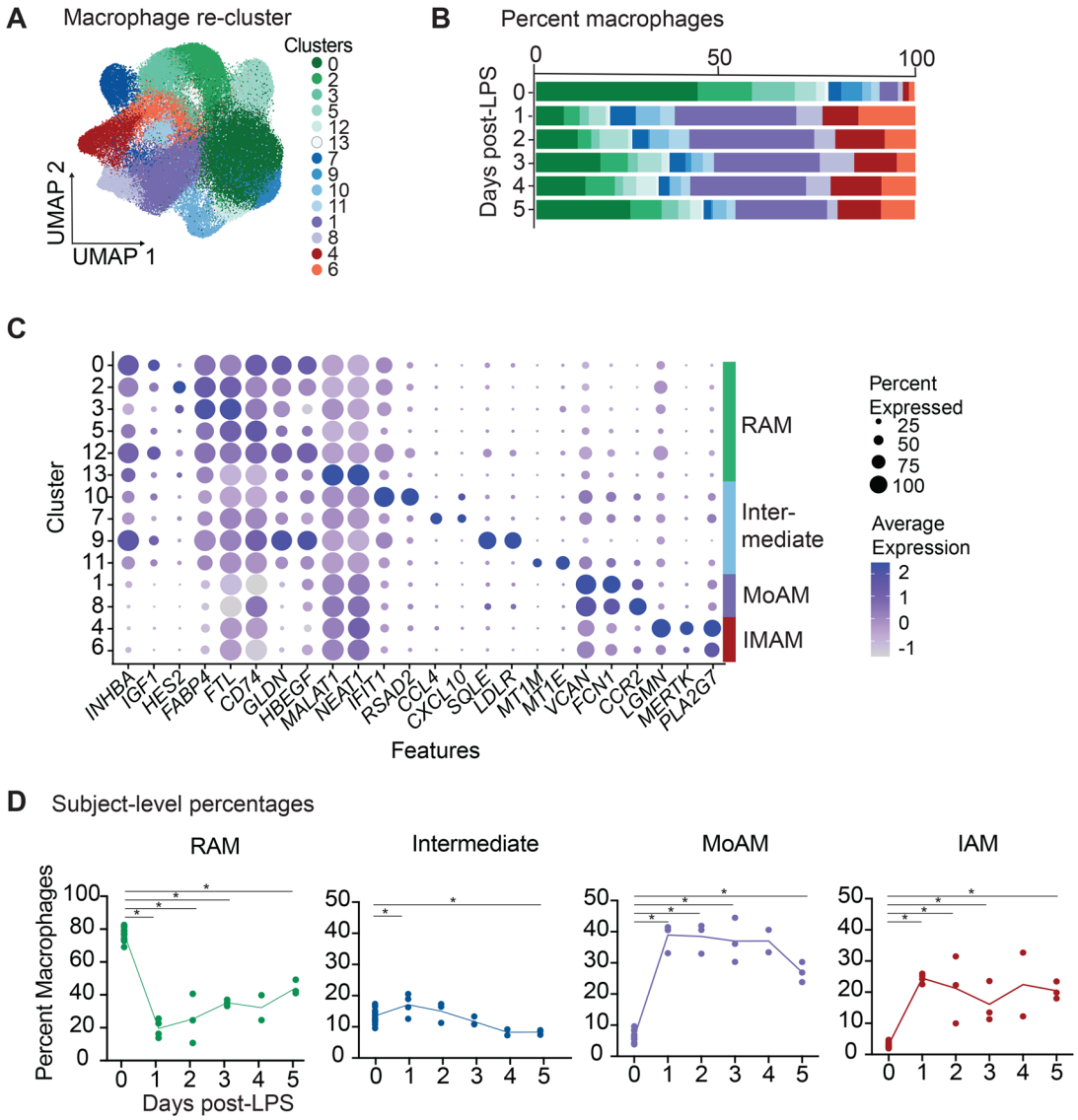


Figure 2 Distinct populations of AM persist in the lung following inflammation. (A) Cells annotated as macrophages were reclustered into 14 distinct subsets. (B) Percentage of cells within each cluster at each time point. (C) Dot plot comparing expression of marker genes across clusters where *x*-axis denotes marker genes and the *y*-axis refers to clusters in (A). Colored bars across the top of the figure denote annotation of each cluster within AM subset. Dot size is proportional to the percentage of cells expressing the gene in each cluster. Color intensity is proportional to averaged scaled log-normalized expression within a cluster. (D) Percentage of AM subset within BAL samples over time by individual participant. Differences in AM subset composition were compared between time points while controlling for sex and pairing of samples using the propeller method. An overall F-test was performed to test for any differences between time points before performing pairwise comparisons (all F-test FDR adjusted $P < 0.005$). *P*-values were adjusted using the Benjamini–Hochberg method across the four cell types in the composition (Table E5). *Pairwise comparison FDR < 0.05. Abbreviation: AM, airspace macrophage; FDR, false discovery rate; IAM, interstitial macrophage-like airspace macrophage; MoAM, monocyte-like airspace macrophage; RAM, resident airspace macrophage; UMAP, uniform manifold approximation and projection.

respectively. All four clusters increased in relative abundance during inflammation and persisted in the BAL throughout resolution of lung injury, suggesting they were selectively recruited to the airspaces (Figure 2B and C, Figure E6C, Table E5).

Lastly, four clusters were comprised of mixed cell populations—some cells expressed RAM markers and others expressed MoAM or IAM markers—and were, therefore, termed intermediate AM. Within the intermediate AM group were four subsets defined by specific gene signatures including metallothionein, pro-inflammatory cytokine (eg, *CCL4*, *CXCL10*, *IL6*, *TNF*), cholesterol metabolism (*SQLE*, *LDLR*) or interferon-responsive (*IFIT1*, *RSAD2*) genes and have previously been described by our group and others.[17,21-23] Intermediate AM remained a minor percentage of BAL cells at all time points (Figure 2B, Figure E6D).

We have previously characterized the conserved transcriptional heterogeneity of AM in healthy adults during homeostasis.[17] To understand the effect of acute inflammation on the AM repertoire, we compared the relative proportion of AM subsets in the BAL of each participant at baseline and after LPS exposure. In all participants, the relative percentage of RAM subsets decreased while recruited AM subsets increased following exposure to LPS (Figure 2D, Figure E7). Within recruited AM populations, percent MoAM at a given time point was less variable between participants than percent IAM.

Within each cluster, the vastly different microenvironments of health, acute inflammation, and lung repair resulted in altered cell states, with dynamic patterns of differentially expressed genes (DEG) over time (Figure E8, Table E6). In all clusters, patterns of expression were most distinct between health and acute inflammation (days 0 and 1 post-LPS), with subsequent time points increasingly resembling patterns noted during health. To understand the main determinants of cellular programming between subsets we performed differential gene expression analysis between RAM, MoAM, and IAM followed by pathway analysis and noted numerous unique functional domains, including phagocytosis, programmed cell death, production of growth factors, cytokines and chemokines, and signaling through cognate receptors, that were differentially enriched between AM subsets (Tables E7-E8). We curated lists of genes implicated in each of these functions based on a broad review of the literature and compared their relative expression across the three most abundant AM subgroups—RAM, MoAM, and IAM. Distinct patterns of expression were noted in all domains (Figure 3, Figures E9, E10). As previously reported, cell ontogeny appeared to be the main determinant of growth factor, cellular receptor, and phagocytic gene repertoires, since in general, RAM expression patterns were most distinct from recruited MoAM and IAM. Likewise, and in line with recent reports of macrophages obtained from murine lung homogenates,[24] we noted unique chemokine and cytokine expression profiles between populations, potentially signifying distinct roles in cellular trafficking and response to environmental stimuli. Airspace macrophages subsets also had unique expression of pro- and anti-apoptotic genes, with distinct profiles of intrinsic and extrinsic apoptosis genes expressed in each cluster, portending different determinants of cell death or viability in each population. Though expression patterns did vary over time, unique signatures were noted in specific cellular subsets (eg, inflammatory cytokine signature of intermediate cluster 7 and phagocytic signature of IAM cluster 4) that were highly conserved over time and between individuals (Figures E9, E10). Likewise, global patterns of growth factor, cellular receptor, and cell death gene expression were largely restricted to resident versus recruited cell types, confirming that intrinsic cell identities are major determinants of AM programming.

Because heterogeneity within the recruited AM population in humans has not been extensively characterized, we next sought to identify key drivers of IAM and MoAM identity. Based on myriad evidence in preclinical and translational studies, and our prior comparisons of MoAM to peripheral blood,[17] we presume that MoAM derive from circulating monocytes and are specifically recruited to the lung during acute inflammation. However, the source of IAM in our model is less clear. To our knowledge, no direct comparison of IM obtained from lung parenchymal tissue to IM-like cells lavaged from the airspaces of healthy humans is available. Further, a corollary of IAM has not been consistently identified within the airspaces or BAL of mice. We sought to compare markers of interstitial macrophage subsets isolated via enzymatic digestion of human and murine lungs to recruited AM subsets in our model. We identified gene markers of IM[20,25] (some of which are shared across species) and compared their expression between MoAM and IAM subclusters in our dataset (Figure 4A, Figure E11A, Tables E9-E10). We found that IAM (especially cluster 4) have high overlap of gene signatures with human IM isolated from tissue. Interstitial macrophage-like AM from human BAL also express markers of murine IM, specifically tissue-resident IM, which reside within the bronchovascular bundle, proliferate within lung tissue, and turn over slowly compared to monocyte-derived IM populations.[25] Tissue-resident IM identity is thought to be imprinted via maturation and residence within the lung tissue and may be induced via Notch signaling.[26] Therefore, we also compared Notch2-induced gene signatures in IAM and MoAM. Our analysis revealed high and consistent expression of Notch2-induced genes in IAM but not MoAM (Figure 4B, Figure E11B). Together these analyses suggest that IAM may originate in the lung interstitium, be induced by Notch2 signaling, and maintain their identity despite recruitment and persistence within the airspaces five days following acute inflammation.

Given both IAM and MoAM populations increase in the airspaces following inflammation, we tested an alternative hypothesis that these populations may derive from unique precursors in the circulation, such as recently described granulocyte-monocyte (GMP) and monocyte-dendritic cell progenitor (MDP)–derived monocytes in mice.[27] Because transcriptional markers of monocytes derived from these precursors have not been described in humans,[28,29] we identified human orthologues of murine markers of GMP and MDP-derived monocytes and compared their expression between AM subsets (Figure 4C, Figure E11C). Marker genes of MDP-derived cells, including *SLAMF7* and *CD209*, were expressed highly in IAM, while *SELL* and *BST1*, markers of GMP-derived cells, were more highly expressed in MoAM. Other gene markers of MDP- and GMP-derived cells did not align between AM subsets or were not consistently expressed over time. As such, we deemed this analysis unconvincing that IAM and MoAM derive from unique cellular precursors.

We also performed trajectory analysis using CellRank 2[30] with pseudo-time calculated via Monocle[31] to assess whether MoAM

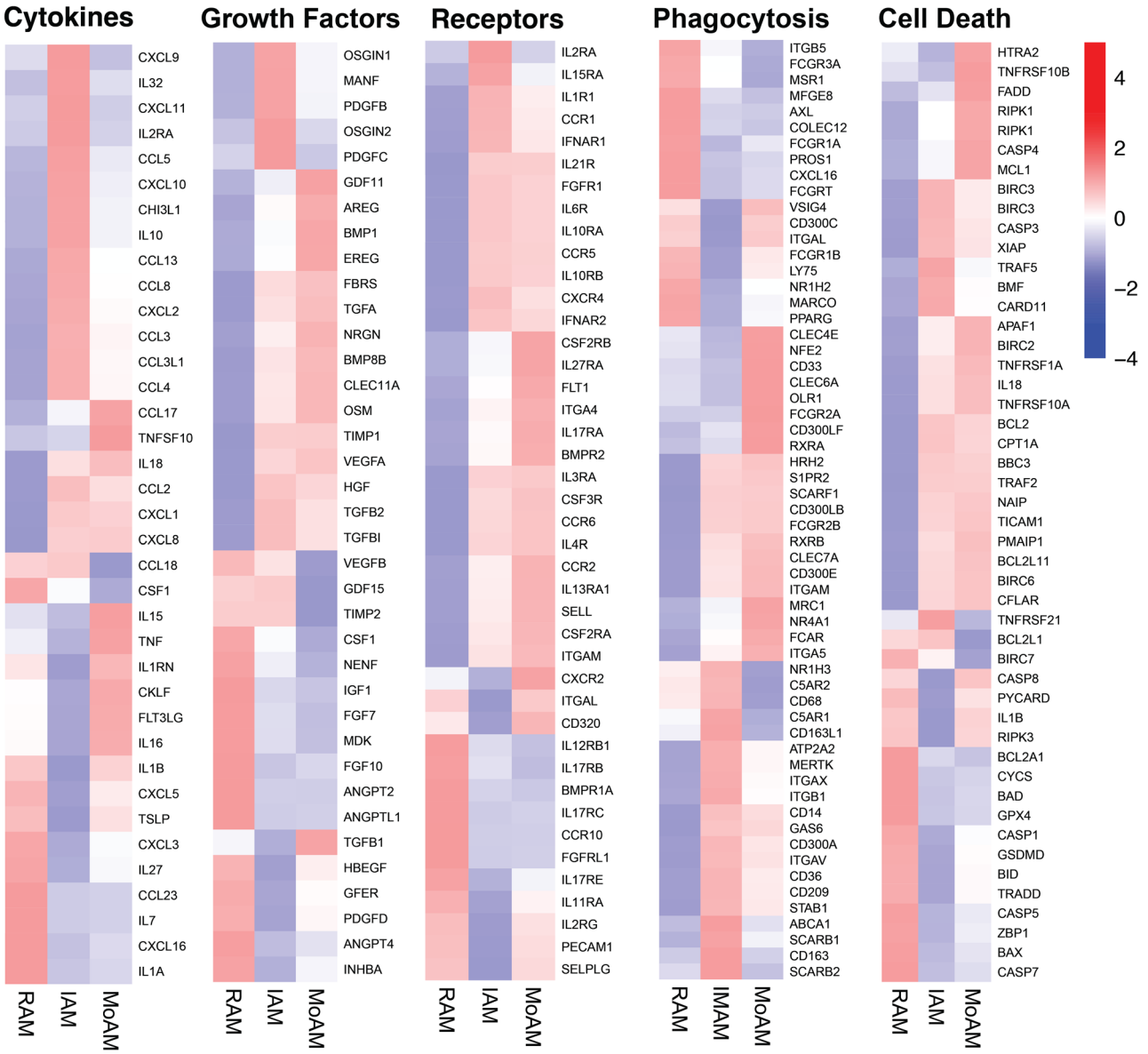


Figure 3 Putative functions of AM subsets are ascribed by unique gene expression patterns. Heatmaps demonstrate expression across curated gene lists. Heat maps are colored by average scaled log-normalized expression in each annotated macrophage subtype (from Figure 2A). All genes depicted are differentially expressed (FDR < 0.05) between at least 2 AM subsets (Table E7). Abbreviations: AM, airspace macrophage; FDR, false discovery rate; IAM, interstitial macrophage-like airspace macrophage; MoAM, monocyte-like airspace macrophage; RAM, resident airspace macrophage.

and IAM identity may reflect different phases of monocyte maturation or AM differentiation during inflammation. Analyses suggest that IAM are closely related to MoAM but did not predict a trajectory toward RAM or other AM subtypes (Figure E12).

To determine whether IAM identified in our model of sterile lung injury align with those identified in patients with lung pathology, we examined expression of so-called pathogenic AM marker genes identified from a broad literature[14,15,32-37] (Figure 5A, Figure E13). We observed expression of these genes at all time points, including in cells obtained during health. We note that *LGMN*, *FOLR2*, and *IL10* expression is largely restricted to IAM (especially cluster 4). Conversely, *SPP1* and *CCL2* expression is present in multiple AM subsets, especially at day 1 following LPS exposure, suggesting these genes may be induced by the inflammatory microenvironment. Likewise, expression of *TREM2* was noted in several subsets, particularly during resolution of lung injury. The analysis suggests that *LGMN*, *FOLR2*, and *IL10* may represent stable markers of IAM identity, while *SPP1*, *CCL2*, and *TREM2* expression are not restricted to specific AM subsets and may represent a cell state induced by environmental cues.

Finally, we compared the AM signatures from published scRNAseq studies of diseased lung tissues to IAM markers in our dataset. We found overlapping expression of IAM markers in macrophages from patients with pulmonary fibrosis and severe COVID-19[7] and less similarity to AM subsets from BAL of participants with ARDS[11] (Figure 5B). In each case, the highest enrichment was noted in AM subsets associated with the pathologic condition (Idiopathic Pulmonary Fibrosis (IPF): cluster 1; COVID-19: MoAM3; ARDS: CD163/LGMN AM). Together these data suggest that macrophages isolated in abundance from diseased lungs are closely

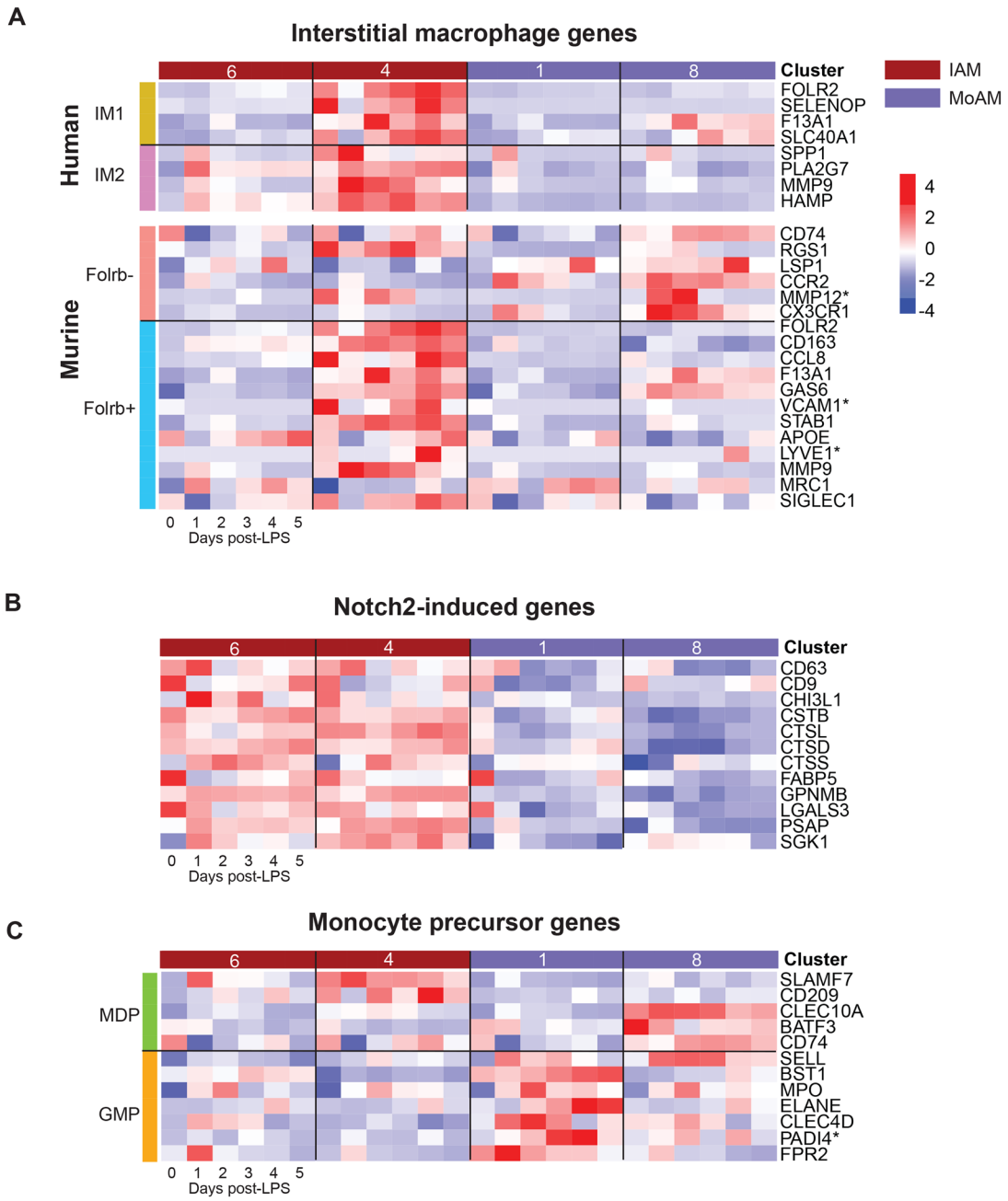


Figure 4 Enrichment of IM, notch-induced, and monocyte precursor genes in AM subsets. Heatmaps demonstrate expression across curated gene lists. Heat maps are colored by average scaled log-normalized expression in each cluster. Numbered columns refer to macrophage cluster (from Figure 2A), and color reflects annotated AM subset. All genes depicted are differentially expressed (FDR < 0.05) between at least 2 macrophage clusters except as indicated by * (Table E9). Abbreviations: AM, airspace macrophage; IAM, interstitial macrophage-like airspace macrophage; MoAM, monocyte-like airspace macrophage.

related to those present in the alveoli during self-resolving lung injury, and shared markers may represent a conserved response to tissue injury rather than a disease-specific signature.

Discussion

In this study, we characterized the time-resolved immune response and AM repertoire following sterile lung injury in healthy adults. Our protocol reliably induced neutrophilic inflammation and lung injury that resolved five days after exposure and allowed for identification of conserved RAM populations and two main groups of recruited AM: one that closely resembles classical monocytes and another that aligns with interstitial macrophages. Each subset is characterized by unique DEG and putative roles in known macrophage functions including phagocytosis, cytokine production, growth factor signaling, and cell death. Using these unique gene signatures, we test hypotheses regarding the origins of AM heterogeneity, including maturation from distinct monocyte precursors and influences of cell-cell interactions in lung

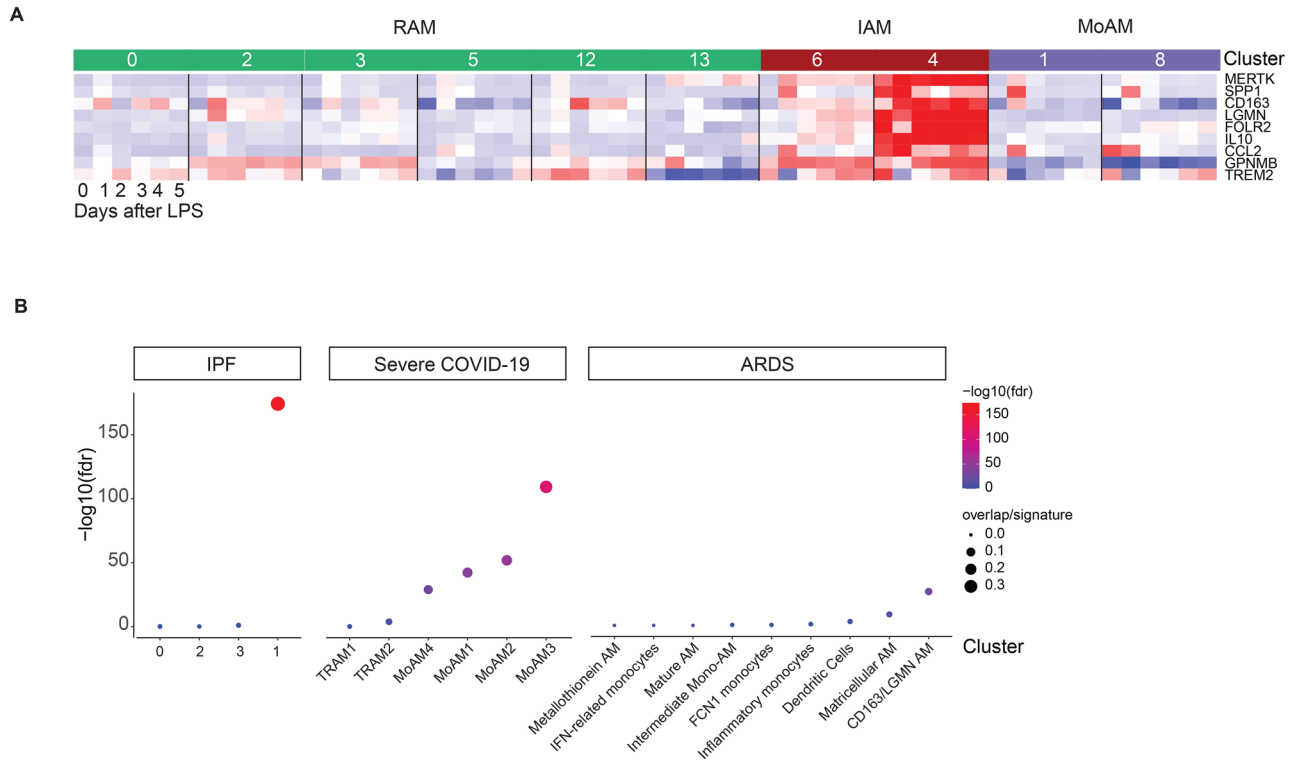


Figure 5 Pathologic AM align with IAM identified during normal lung repair. (A) Heatmaps of pathologic macrophage markers. Heatmaps demonstrate expression across curated gene lists. Heat maps are colored by average scaled log-normalized expression in each cluster. Numbered columns refer to macrophage cluster (from Figure 2A), and color reflects annotated AM subset. All genes depicted are differentially expressed (FDR < 0.05) between at least 2 macrophage clusters (Table E9). (B) Enrichment analysis demonstrates specific clusters of AM in published single cell RNA sequencing datasets of disease lungs overrepresented among markers of cluster 4 IAM in our dataset. The *x*-axis depicts macrophage clusters identified in published Idiopathic Pulmonary Fibrosis,[16] COVID-19,[24] and ARDS[13] datasets, and the *y*-axis represents FDR adjusted *P*-value (adjustment applied across macrophage clusters within each dataset: 4, 6, and 9 clusters, respectively). Abbreviations: AM, airspace macrophage; ARDS, acute respiratory distress syndrome; FDR, false discovery rate; IAM, interstitial macrophage-like airspace macrophage; MoAM, monocyte-like airspace macrophage; RAM, resident airspace macrophage.

tissues. We compare the gene signatures of each population to those identified in patients with various lung diseases. Our data support the possibility that recruited AM subsets may derive from cells in distinctive anatomic niches. Moreover, our data suggest so-called pathologic markers of AM may reflect a mere increase in numbers of IAM in response to lung injury rather than molecular signaling pathway or cell states specific to a particular disease.

Our study did not allow for tracking of individual cells within lung tissue over the time course of inflammation. As such, we are unable to definitively determine the origin or fate of specific AM subsets in our model. While our data show an association of MoAM markers with monocytes and IAM markers with those of interstitial macrophages, shifts in cellular programming of AM subsets could produce similar results. While we do note programming shifts within AM clusters over time, the high conservation of cell markers and relative abundances of each AM subset across inflammatory time points support the idea that core transcriptional identities of AM populations are cell-intrinsic factors despite wide variations in the microenvironmental milieu of health, inflammation, and lung repair. Further, despite our low sample size, we note similar proportions and shared marker expression of AM subsets across individuals, suggesting that despite differences in age, sex, and presumed environmental exposures of our participants, the cellular immune response to endobronchial LPS is largely conserved in healthy adults. This observation may have important significance for our understanding of macrophage-driven lung diseases and raises a potential concern that cell-specific targeting of AM subsets for pathologic inflammatory conditions may influence normal mechanisms of lung repair.

While many studies support the idea that cell ontogeny (tissue resident versus bone marrow–derived origin) is a main determinant of global AM programming, to our knowledge, our study is the first to highlight heterogeneity within the recruited AM population in humans during a sterile, self-limited inflammatory response. In murine models, we and others have identified heterogeneous macrophage populations in the lung interstitium, but using careful techniques to isolate cells by tissue compartment (eg, intratracheal labeling of BAL cells) has not revealed IM-like cells within the airspaces.[25,38-40] It is possible that the BAL procedure in humans results in mechanical disruption and passive expulsion of cells from interstitial tissues during the procedure rather than active migration of IAM into the alveolar space. However, given enrichment of genes suggestive of interaction with the extracellular matrix (eg, *MMP9*) and persistence of IAM in the BAL for at least five days after LPS exposure, it may be more likely that IAM represent a migratory or loosely adherent phenotype unique to IM in humans. Indeed, several markers of IAM overlap with those described as intraepithelial macrophages in human distal airways.[41] The expression of IL-10 in IAM may suggest that these cells enter the airspace

in response to commensal bacteria and may be important in prevention of chronic inflammation.[42] Similarly, given IAM share many markers of IM located in bronchovascular bundles, they may be important in controlling viral infections[43,44] and may be long-lived in lung tissues. Studies aimed at understanding their origin, fate, and function are critical to advancing our understanding of tissue homeostasis and repair in humans.

Interstitial AM identified in our analyses share many markers with so-called pathologic macrophages that have been identified in parenchymal lung diseases. By comparing the expression of these genes over time, our analysis allows for inferences about the factors that may induce the expression of these markers. For instance, *SPP1* is a marker of pathologic macrophages in various organs and disease states.[45] In our analysis, *SPP1* is most highly expressed during acute inflammation across several AM subsets. This finding implies that its expression is induced by an environmental factor and may not be useful in identifying a stable population of AM. Further insights into the induction and function of *SPP1* will be essential to understand its role in AM programming and its relevance to lung disease.

Multiple animal models suggest that targeting monocyte recruitment to the lung may attenuate lung injury and fibrosis, however no such advantage has been appreciated in humans.[46] Likewise, recent evidence suggests that expansion of Notch2-induced IM signatures are protective in models of lung injury and fibrosis[26] suggesting that the mere presence of IAM are not sufficient to drive fibrosis. The task of understanding the causal relationship between each AM subset and outcomes following lung injury will require a return to molecular techniques and careful attention to the unique signatures of human versus murine myeloid subsets.

Our study was focused on transcriptional programming of AM. Evolving evidence suggests that protein expression, metabolic and epigenetic programming are incompletely reflected by the transcriptome, which limits the comparison of our data to other 'omic platforms. Participants of this study were similar in age and lived in a single geographic region (Denver metro area), and sample size at each time point after LPS exposure limited longitudinal analyses. Our data have not been replicated in an independent population and may not be applicable to individuals of different age groups or residing in different environments. Lipopolysaccharide is an uncommon cause of clinical lung disease, may induce different levels of injury in some participants,[47] and may not closely mimic bacterial pneumonia. Lavage volumes were larger than those commonly used in clinical bronchoscopies, and return volumes varied among participants, which may have impacted cell yields. Despite these shortcomings, our data clearly demonstrate that endobronchial endotoxin challenge is an effective and reliable tool for study of the inflammatory response in the healthy human lung and may provide important insights into disease-specific versus conserved responses to lung inflammation in humans.

Conclusions

Using a sterile, self-limited model of acute lung inflammation in humans, we identify conserved AM subsets and highlight their unique putative functions. Our data suggest that in humans, recruited AM can be divided into two main groups. One group, which is closely related to tissue-resident interstitial macrophages, expresses many genes associated with clinical lung diseases. Further research into the source, function, and fate of these cells may be key to understanding mechanisms of normal lung repair and identifying therapeutic targets of dysfunctional repair or pulmonary fibrosis.

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Author contributions

C.M.M. designed the study, performed biostatistical and computational analyses, prepared figures, interpreted data, and contributed to the manuscript. S.A.M. and J.D.M. processed samples and generated data. E.A.W. performed biostatistical and computational analyses, prepared figures, and contributed to the manuscript. W.J.J. designed the study, screened subjects, interpreted data, and contributed to the manuscript. K.J.M. designed and supervised the study, screened participants, performed bronchoscopies, processed samples, analyzed and interpreted data, prepared figures, and wrote the manuscript.

Supplementary material

Supplementary material is available at *American Journal of Respiratory and Critical Care Medicine* online.

Conflicts of interest

Please see the ICMJE disclosure forms, which have been provided as supplementary material. C.M.M., S.A.M., E.A.W., J.D.M., W.J.J., and K.J.M. report no relevant financial relationships.

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Data availability

The RNA sequencing data underlying this article are available at Gene Expression Omnibus at https://www.ncbi.nlm.nih.gov/geo/ under accession codes GSE151928 and GSE300946. Code to reproduce scRNA-seq analyses is available on GitHub at https://github.com/camillemmoore/BAL_scRNAseq.

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