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Original Article

Spatial Transcriptomic Analysis Reveals Dysregulated Pro-Inflammatory Signaling in the Aged Lung

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ABSTRACT: Older individuals are more susceptible to viral and bacterial respiratory infections than younger people. While structural lung changes and a hyperinflammatory milieu have been proposed to contribute to worsened clinical outcomes, exact mechanisms remain elusive. Clinical specimens are challenging to obtain and although rodents are valuable models of human disease, their specific pathogen free status and inbred genetics limit translation of findings obtained from these models. We sought to address this question by analyzing the transcriptional landscape of lung tissue obtained from young and aged rhesus macaque using Visium spatial transcriptomics. Unlike rodents, captive rhesus macaques are genetically outbred and exposed to natural particulates and respiratory microbes that modulate their lung immunity. Analysis identified immune and structural cells clusters. Differential gene expression revealed a strong pro-inflammatory bias in aged animals while transcriptional signatures in young animals were consistent with a regulatory phenotype. Moreover, cellular signaling important for adhesion, tissue maintenance, and cell migration was significantly reduced in the aged compared to young lungs. These data indicate that aged lungs are skewed towards hyperinflammatory responses which are likely to result in higher immune mediated damage following challenge.

Keywords: Aging, Visium spatial transcriptomics, lung, rhesus macaque.

INTRODUCTION

The anticipated doubling of the global population aged 65 years and older by 2050 underscores the urgent need to uncover the mechanistic underpinnings of age-associated susceptibility to infections and related morbidity [1]. Respiratory infections such as Influenza, SARS-CoV-2, and *S. pneumoniae* [2-4], as well as reactivation of latent pathogens like *M. tuberculosis* [5, 6] substantially increase in incidence and severity with advanced age. This heightened vulnerability of older adults arises from a complex interplay between progressive structural alterations of the lung parenchyma, frequently manifesting as conditions such as chronic obstructive pulmonary disease (COPD) [7], and widespread perturbations in immune competence, commonly termed immune senescence [8, 9]. With advancing age, the lung undergoes distinct remodeling, typified by increased connective tissue deposition, decreased elastin content, reduced chest wall compliance, diminished respiratory muscle strength, and enlargement of alveolar spaces [10]. These structural changes, coupled with impaired mucociliary clearance, lead to reduced mechanical elimination of respiratory pathogens, thereby facilitating persistent microbial infection and colonization [11, 12].

Alveolar macrophages (AMs) are the dominant lung-resident immune cell population and are pivotal for orchestrating host response against inhaled pathogens and

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for participating in tissue surveillance, repair, and homeostasis [13]. Under physiological conditions, AMs efficiently phagocytose apoptotic and necrotic cells, limiting the propagation of inflammation and maintaining alveolar integrity [14]. Upon infection, they rapidly transition to a pro-inflammatory state, facilitating pathogen recognition and clearance through the coordinated release of cytokines and the recruitment of both innate and adaptive immune effectors [14, 15]. In younger individuals, macrophages exhibit remarkable plasticity, transitioning between pro-inflammatory and anti-inflammatory (regulatory) phenotypes, thereby ensuring robust host defense while mitigating collateral tissue damage [16-18]. However, with aging, this functional plasticity is progressively impaired: aged AMs display both a diminished capacity for regulatory function and a chronic, low-grade pro-inflammatory phenotype [16-19].

This low level inflammation, driven by cumulative cellular stress and the persistent accrual of senescent cells, is marked by the sustained production of pro-inflammatory cytokines and chemokines, collectively referred to as the senescence-associated secretory phenotype (SASP) [19, 20]. Prominent SASP factors (e.g. IL-6, CXCL8, CCL8, CCL16) recruit and activate immune cells as well as stimulate macrophages to engulf and remove damaged and senescent cells; however, the accumulation of damaged cells with age drive a maladaptive feedback loop of chronic inflammation [20] ultimately exceeding the clearance capacity of the innate immune system [21, 22]. This immunological overload both maintains a chronic inflammatory microenvironment and blunts capacity to respond to secondary infectious exposures, a phenomenon dubbed as innate immune tolerance [19] and immunosenescence [23-25].

Emergent evidence suggests that age-driven alterations in the alveolar microenvironment not only hinder the phagocytic and antimicrobial functions of airway-resident macrophages but also disrupt their intricate crosstalk with pulmonary epithelial and endothelial cells [13]. This dysregulation reduces the effectiveness of reparative and immunomodulatory programs required following pathogen insult or tissue injury. Structural lung changes further exacerbate these deficits, leading to impaired clearance of pathogens and a heightened risk of secondary infections, complications, and exacerbations in chronic lung disease [10-12]. Furthermore, diminished T- and B-cell diversity, and continuous antigenic stimulation reduce vaccine efficacy and impede effective immunologic recall responses [23-25].

Given this convergence of age-associated anatomical and immunological decline, there remains a critical knowledge gap regarding how these processes influence the tissue- and cell-specific transcriptional programming of the aging lung [8, 9]. While human studies are ideal, obtaining lung tissue samples is a major challenge. To address this knowledge gap and overcome the limitations of rodent models and human clinical sampling, we leveraged spatial transcriptomics to profile the transcriptional landscape of young (5-7 years) and aged (19-21 years) macaque lungs [26, 27]. By integrating transcriptional signatures with localization, our study uncovers distinct immune and structural cell clusters in the aged lung that exhibit a pronounced pro-inflammatory bias, in contrast to the reparative and wound-healing phenotypes predominant in youth. These findings provide a comprehensive resource for understanding age-associated immune dysfunction and highlight the urgent need to develop interventions tailored to the unique vulnerabilities of the aged pulmonary microenvironment.

MATERIALS AND METHODS

Ethics statement

Animal work was performed in accordance with the recommendations described in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, the Office of Animal Welfare and the Animal Welfare Act, United States Department of Agriculture. The studies were approved by the Institutional Animal Care and Use Committees (IACUC) at the Oregon National Primate Research Center.

Cohort description, animal infection, and sample collection

Samples used in the studies presented herein were obtained from rhesus macaques raised at the Oregon National Primate Research Center. All animals receive a semi-annual tuberculin skin test, viral screenings (for simian viruses SRV, SIV, STLV, Macacine herpesvirus 1, and measles virus), and prophylactic anthelminthic administration. Sentinel animals from each breeding group are tested for SRV via PCR. Monkeys imported from other National Primate Research Centers or USDA-approved vendors receive these tests and screenings along with serum chemistry and CBC (Complete Blood Count) sample collection, and rectal fecal culture for Shigella and Salmonella. Once at the ONPRC, animals undergo a minimum 31-day quarantine during which they receive 3 tuberculin skin tests, receive prophylactic treatment for intestinal parasites, and are serologically screened for SRV, SIV, STLV, and MHV-1. Imported animals also receive three-view thoracic screening radiographs. Post-mortem left cranial lung tissue samples obtained from 2 young (5-7 years; 1 female, 1 male) and 2 aged (19-21

years; 2 males), colony-bred Indian-origin rhesus macaques (*Macaca mulatta*) were subjected to Visium spatial transcriptomics. These animals were intrabronchially inoculated in the right lung with *M. avium* subsp. *hominissuis* strain 101 (MAH) 310 days prior to necropsy. MAH levels were undetectable in the BAL at necropsy by both culture and quantitative PCR and no radiological changes indicative of inflammation were ever detected in the left lung [28]. Leveraging these samples honors the 3 Rs of animal research (reduce, re-use, replace) while providing a unique opportunity to examine the impact of aging on the lung and taking advantage of access to tissues from a scare resource, aged macaques.

Visium spatial transcriptomics library preparation and analysis

Left cranial lung tissue from two young (Y1, Y2) and two aged (A1, A2) rhesus macaques was placed in cassettes then was formalin-fixed paraffin-embedded (FFPE). 5-micron thick sections of FFPE macaque lung tissue were placed onto histology slides for H&E staining, immunostaining, and Visium spatial transcriptomics. The impact of aging on lung architecture was assessed by first generation Visium spatial transcriptomics (10x Genomics) with CytAssist using Demonstrated Protocol CG000520. Briefly, a 20µm scroll from an FFPE block was deparaffinized and fixed RNA was extracted using the Qiagen FFPE RNA kit per manufacturers protocol. RNA integrity and DV200 values were first collected to ensure high quality of initial samples (Supplementary Table 1). Extracted RNA was ran on the Agilent Bioanalyzer using the pico RNA chip for DV200 determination. A serial section from blocks were mounted on a microscope slide and stained with hematoxylin and eosin. Following imaging, slides were de-crosslinked and immediately hybridized with the Visium Human Transcriptome Probe Kit v2 (PN-1000466, 10x Genomics) as per manufacturer instructions (10x Genomics, User Guide CG000495). Ligated probes were captured on Visium slides using a CytAssist instrument (10x Genomics) and probe libraries were amplified as per kit instructions. Libraries were sequenced by Novogene using the Illumina NovaSeqX with a sequencing target of 25,000 paired reads per covered spot.

For Visium spatial transcriptomic library data preprocessing, raw reads were aligned and quantified using the SpaceRanger Software Suite (Version 3.1.1, 10X Genomics) against the GRCh38 human reference genome and probe set V2. Downstream processing of aligned reads was performed using Seurat (Version 5.1.0) in R (Version 4.1.1). Data normalization and variance stabilization were performed on the integrated object using the NormalizeData and ScaleData functions in Seurat, before merging the data using Harmony. Dimensional reduction was performed using the RunPCA function to obtain the first 30 principal components and clusters visualized using Seurat's RunUMAP function with a resolution of 0.5. Cell types were assigned to individual clusters using FindAllMarkers function with a log2 fold change cutoff of at least 0.4, FDR<0.05, and using the EnrichR cell type database [29-31] (Supplementary Table 2 found on Mendeley). Differential gene expression analysis was performed using QLF pseudobulk function in EdgeR and differentially expressed genes were defined as having an FDR<0.05 and a log2 fold change ± 0.4. Functional enrichment was performed using Metascape [32] (Supplementary Table 3, Mendeley). The CellChat package was used to investigate possible differential ligand-receptor pair interactions between tissue from the left and right lung sections [33] (Supplementary Table 4). Deconvolution was performed using a reference of canonical cell types in the lung from control healthy lungs constructed from a well annotated single cell RNA sequencing data set (GSE220797) [34], the Robust Cell Type Decomposition (RCTD) analysis in the Spacexr package, and run.RCTD function was executed with doublet mode set to full [35, 36] to identify the frequency of canonical cell types in each spot of the Visium assay.

Immunohistochemistry and immunofluorescence analysis

Immunohistochemistry and immunofluorescent staining for immune cells was done as described in [28]. Immunofluorescent staining for structural cells was done on 5 µm thick lung tissue. Briefly, tissues were deparaffinized and antigen retrieval was done by heating to 95°C. Tissues were incubated overnight at 4°C in primary antibodies rat anti-KRT8 (DSHB MABT329), rabbit anti-prosurfactant protein C (Millipore AB3786), and mouse anti-alpha-smooth muscle actin (eBioscience 14-9760-82) for stain 1. Rat anti-Vimentin (Biotechne MAB2105), rabbit anti-KRT5 (Biolegend 905501), and mouse anti-acetylated tubulin (Sigma T7451) were used as primary antibodies for stain 2. Donkey anti-rat Alexa Fluor 488 (Invitrogen A-21208), donkey anti-rabbit Alexa Fluor 594 (Invitrogen R37119), and donkey anti-mouse Alexa Fluor 647 (Invitrogen A-31571) were used as secondary antibodies. Whole slide multispectral digital images were processed utilizing the HALO Image Analysis platform (version X, Indica Labs). Image analysis algorithms were constructed using the High-Plex FL module (v4.1.3). DAPI was utilized for nuclear segmentation, and unique thresholds for cytoplasmic positivity were set for each antibody.

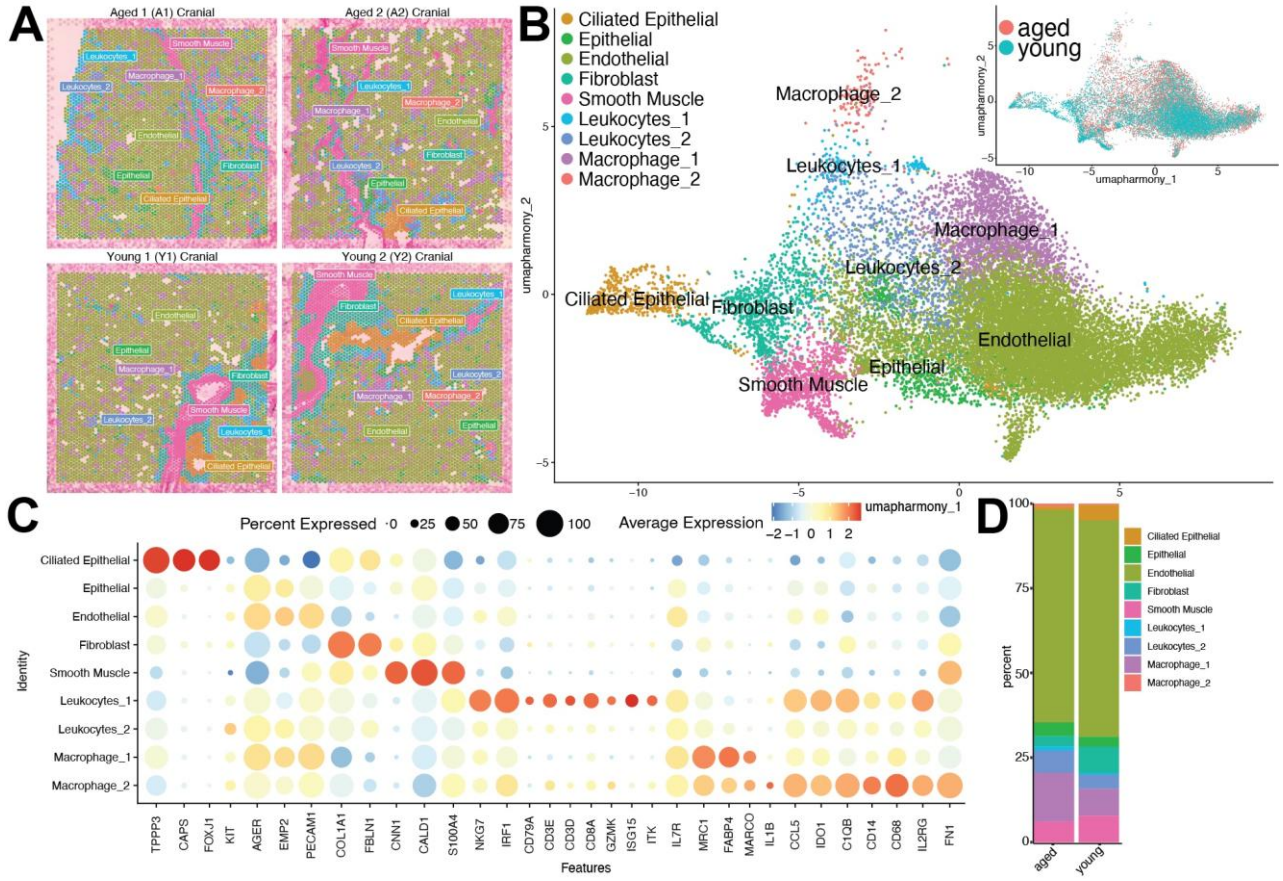

Figure 1. Distribution of immune and non-immune cells in lungs of aged and young rhesus macaques. (A) Representative image of H&E-stained young and aged lung tissue sections overlayed with cluster identity spots. **(B)** UMAP of 19106 spots from left lungs of young and aged animals. **(C)** Bubble plot of key marker genes used to identify cell populations in panel A. The size of the bubble denotes the percentage of cells expressing the marker and color denotes the average expression level of the marker. **(D)** Stacked bar plot of the percentage of each cell cluster from the total cells in young and aged lungs.

RESULTS

Spatial transcriptomics reveal a hyperinflammatory and hypoxic signature in aged lungs

We performed Visium spatial transcriptomics to uncover transcriptional differences with age. Uniform Manifold Approximation and Projection (UMAP) of 19106 spots revealed that cell clusters were equally represented from young and aged lung tissues (Fig. 1A-B). We identified 9 individual clusters: 1] ciliated epithelial (*TPPP3, CAPS, FOXJ1*); 2] epithelial (*KIT, AGER, EMP2*); 3] endothelial (*KIT, AGER, EMP2, PECAM1*); 4] fibroblasts (*COL1A1, FBLN1*); 5] smooth muscle (*CNN1, CALD1*); 6] leukocytes_1; 7] leukocytes_2 (lower levels of *NKG7, CD3D/E*, *CD8A, GZMK, CD79A* compared to leukocytes_1); 8] macrophages_1 (*MRC1, FABP4, MARCO, CD68*); and 9] macrophages_2 (*IL1B, CCL5, C1QB, CD14, CD68, FN1*) (Fig. 1B-C).

Since first generation Visium has 55µm spot sizes that can capture multiple adjacent cells at the same time, we performed a deconvolution analysis to identify the frequency of canonical cell types in each spot using an annotated single cell RNA sequencing reference [34]. We observed that all spots contained a small background of epithelial and endothelial cells, and that the localization of immune and non-immune cells aligned with our annotation of the clusters (Supplementary Fig. 1A). No significant differences in relative frequency of the various clusters were noted (Fig. 1D). H&E and immunostaining showed that the lung tissue of both young and aged animals had thin epithelial membranes forming alveolar spaces and minimal evidence of tissue damage along with comparable distribution of immune cells (Supplementary Fig. 1B, S1C). We also performed immunofluorescence to assess the distribution of several structural cells such as club cells, alveolar type 2 cells, smooth muscle cells, mesenchymal cells, epithelial cells, and ciliated epithelial cells. No differences were observed between the young or aged tissue samples (Supplementary Fig. 2).

We first used module scoring analysis to identify global differences in biological function between young

and aged lung tissue (Supplementary Fig. 3A, Supplementary Table 5 found on Mendeley). Aged lungs exhibited elevated expression of genes linked to chronic inflammation, apoptosis, hypoxia, and wound healing across all cell clusters (Supplementary Fig. 3A). In contrast, module scoring of the VEGF pathway, an important growth factor for tissue regeneration, was decreased in aged lungs (Supplementary Fig. 3A). Cluster-specific differences between young and aged lungs were further delineated by performing differential gene expression analysis for each immune cell population (Supplementary Fig. 2B-E). First, among immune cell clusters, we identified the most differentially expressed genes (DEGs) among leukocyte_2 and macrophage_1 populations (Fig. 2A-D, Supplementary Table 6), while leukocyte_1 and macrophage_2 clusters exhibited minimal differential gene expression (Supplementary Table 6). The leukocyte_2 population in aged animals expressed higher levels of genes that enriched to gene ontology (GO) terms indicative of a pro-inflammatory signature, including "response to bacterium" and "cellular response to cytokine stimulus" (*CTSH, FOS*, *SYK, DOCK2, IKZF3*). In contrast, genes upregulated in the leukocyte_2 population from young animals reflected a reparative phenotype, with enrichment of signatures such as "response to growth factor" and "response to wounding" (*PDGFB, EFGL7, COL1A1*) (Fig. 2A-B). Similarly, the macrophage_1 population of aged lungs exhibited increased expression of genes associated with innate immune responses and inflammatory responses (*C1QB, LYZ, S100A9, LAMP3*), whereas young macrophage_1 cells upregulated genes linked to wound healing and vascular development (*PDGFB, TGFB2, ACTIN1*) (Fig. 2C-D).

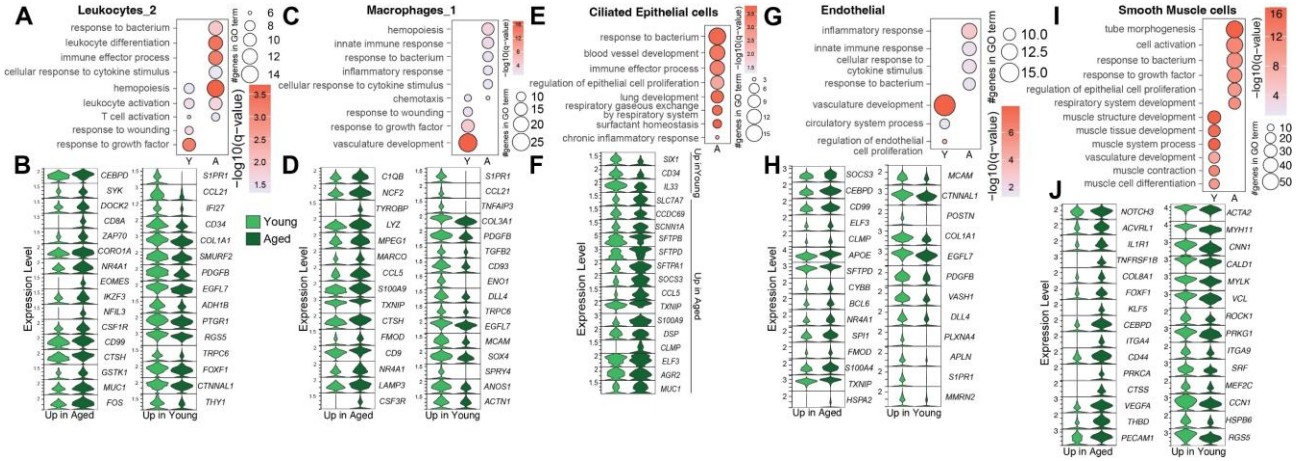

Figure 2. Immune and structural cells from aged lungs are more inflammatory than those from young lungs. Bubble plots representing Gene Ontology (GO) terms and violin plots depicting differentially expressed genes (DEG) between the left lungs from young and aged animals in **(A, B)** leukocytes_2, **(C, D)** macrophages_1, **(E, F)** ciliated epithelial cells, **(G, H)** endothelial, or **(I, J)** smooth muscle cells. For bubble plots, the size of the bubble indicates the number of genes that enriched to that GO term and color indicates the significance compared to aged samples. Violin plots represent gene expression in each cell.

The heightened inflammatory signatures extended to structural populations. In ciliated epithelial cells, aging was associated with increased expression of genes linked to immune effector functions, angiogenesis, and lung development (*CCL5, TXNIP, S100A9, MUC1*, *SCNN1A, SFTP* genes*, DSP,* and *AGR2*) (Fig. 2E-F). By contrast, epithelial cells from young animals expressed higher levels of genes involved in wound repair and lung development pathways, including multiple collagen genes (COL), as well as *CDH5*, *ITGA1, PDGFB,* and *EGFL7* (Supplementary Fig. 3B-C). Similarly, genes upregulated with age in endothelial and fibroblast populations were significantly enriched for pro-inflammatory pathways, including "cellular response to cytokine", "innate immunity", and "immune effector processes" (Figure 2G-H, Supplementary Fig. 3D-E). In contrast, genes upregulated in young animals mapped to GO terms associated with angiogenesis, including circulatory system and blood vessel development as well as growth factor signaling (*COL1A1, EGFL7, PDGFB, IGFBP5, SFRP2*) (Figure 2G-H, Supplementary Fig. 3D-E). Lastly, in smooth muscle cells, aging enhanced expression of genes related to "tube morphogenesis" (*ACVRL1, COL8A1, FOXF1, KLF5*) and "cell activation" (*IL1R1, TNFRSF1B, CD44*). Conversely, genes enriched in young animals corresponded to muscle structure development and differentiation (*ACTA2, MYLK, ITGA9*) (Fig. 2I-J). Overall, these transcriptional differences indicate age-associated shifts in lung parenchymal cells towards a pro-inflammatory, immune-activated, and fibrotic transcriptional state away from genes linked to tissue repair and homeostasis.

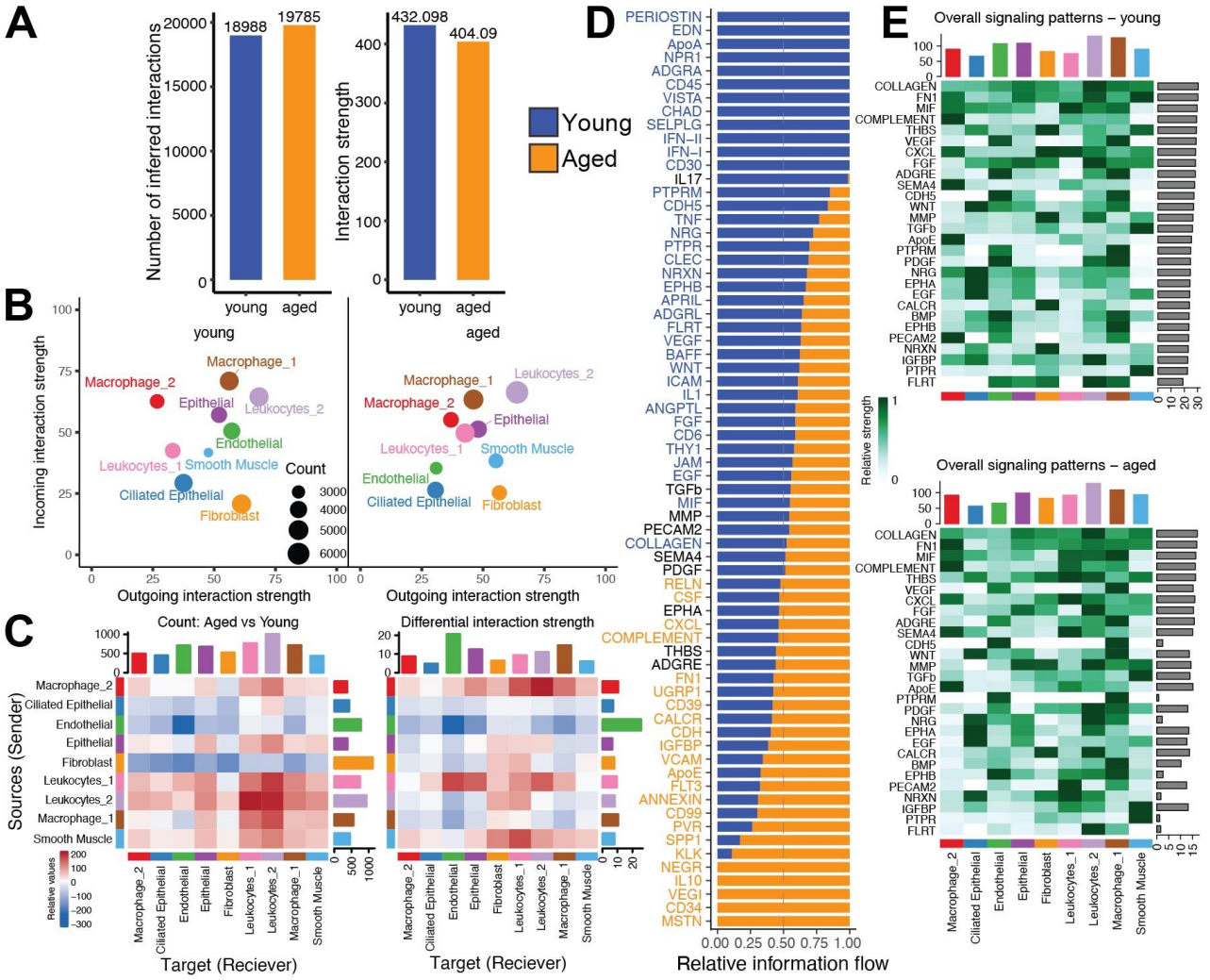

Figure 3. Aged lungs lose signaling patterns crucial for cell adhesion, communication, and immune regulation. (A) Bar plot of the inferred number (left) and strength (right) of ligand-receptor pair interactions in the young and aged lungs. **(B)** Scatter plot of incoming and outgoing interaction strength for the indicated cluster. Size of the bubble indicates the number of interactions for the indicated cluster. **(C)** Heatmap demonstrating the differential number of interactions (left) and the differential interaction strength (right) of the indicated cell clusters between young and aged lungs. Red and blue colors indicate increased or decreased interactions, respectively, in the aged relative to young tissue. Bar plot on top indicates the sum of interactions sent by the indicated cluster. Bar plot on side indicates the sum of interactions received by the indicated cluster. **(D)** Bar plot of signaling pathways ranked by relative information flow (aggregate probability of communication) in the young and aged animals. Bars predominantly blue denote pathways dominant in young lungs whereas bars predominantly orange highlight pathways dominant in aged lungs. **(E)** Heatmaps of the overall outgoing and incoming signaling strength for the cell clusters in the indicated signaling pathways. Bar plot on top indicates the total signal strength for the cell group. Bar plot on side indicates the total signal strength for the pathway.

Aged lung tissue exhibits altered intercellular communication compared to young lungs

To gain a deeper understanding on how age-associated gene expression changes intercellular communication within the lung microenvironment, we employed CellChat [33]. While the overall number of predicted cell-cell interactions remained similar between young and aged groups, the interaction strength was diminished in aged lungs (Fig. 3A). Notably, our results show a decline in the number and strength of outgoing interactions from ciliated epithelial, endothelial, and fibroblast populations (Fig. 3B-C). Furthermore, while the number of outgoing signals from immune populations such as leukocyte_2 and macrophage_1 increased in the aged lung, our results show that the strength of these signals was generally weaker (Fig. 3B-C). Overall cell-cell communication patterns across the 9 clusters revealed increased communication between macrophage_1 and 2, leukocytes_1 and 2, epithelial cells, and smooth muscle

cells with leukocytes_1 and 2, macrophage_1, and smooth muscle cells in the aged lungs (Fig. 3C, left). The number of interactions was significantly lower in ciliated epithelial, endothelial, and fibroblast cells from aged lungs (Fig. 3C, left). Macrophage_2, leukocytes_1, and smooth muscle cells generally had stronger interactions with other cell types in the aged lungs, while the interaction strengths of other cells were diminished (Fig. 3C, right).

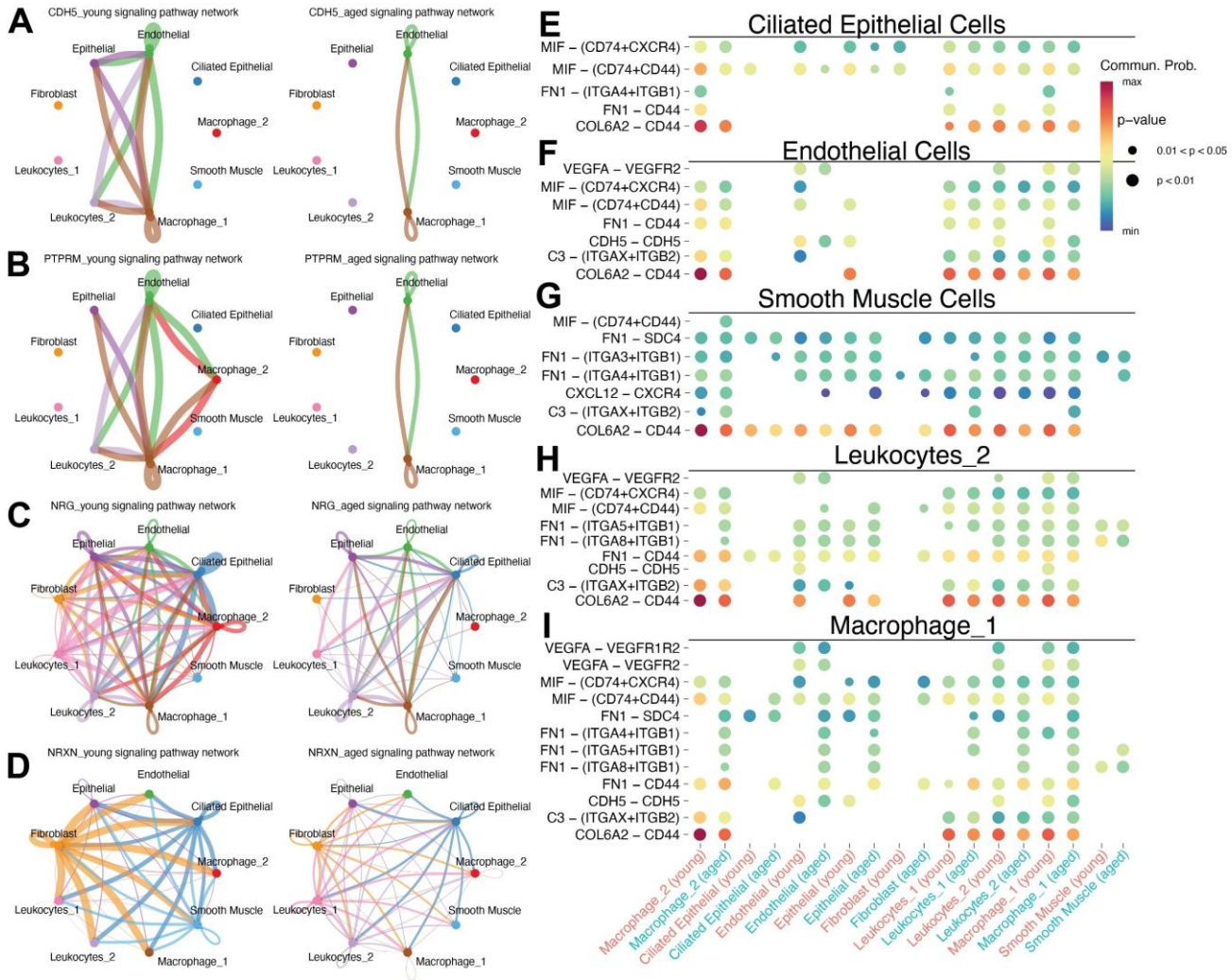

Figure 4. Loss of cell adhesion and communication networks in aged cells. Network signaling diagrams for the **(A)** CDH5, **(B)** PTPRM, **(C)** NRG, and **(D)** NRXN signaling pathways among cell subsets in the young group (on left) and the aged group (on right). Nodes represent cell subsets and edge width is proportional to the CellChat communication probability. Bubble plot of differential ligand-receptor pair signaling in young or aged lungs from **(E)** the ciliated epithelial, **(F)** endothelial, **(G)** smooth muscle, **(H)** leukocytes_2, and **(I)** macrophage_1 cells to the other indicated cell types. Color indicates the communication probability and size indicates the p-value.

We next compared information flow and strength of signaling pathways between young and aged conditions (Fig. 3D). Stronger signaling pathways in the young group were involved in tissue repair (Periostin, FLRT, VEGF, ANGPTL, FGF, EGF, TGFβ, PECAM2, Collagen, PDGF), inflammation (EDN, CD45, SELPIG, IFN-II, IFN-I, CD30, IL17, TNF, CLEC, APRIL, BAFF, ICAM, IL-1, CD6, MIF), immune regulation (ApoA, VISTA, EPHB), anti-fibrosis (NPR1), cell proliferation (WNT), and tissue remodeling (MMP) as well as cell adhesion and communication (ADGRA, CHAD, PTPRM, CDH5, NRG, PTPR, NRXN, ADGRL, THY1). Signaling pathways involved in cell adhesion (EPHA, ADGRE, FN1, CDH, PVR, NEGR, CD34), pro-inflammation (CSF, CXCL, Complement, CD39, FLT3, CD99, SPP1, KLK) and anti-inflammation (UGRP1, ApoE, Annexin, IL-10), tissue growth and development (RELN, THBS, IGFBP, MSTN), calcium and phosphate metabolism (CALCR), leukocyte trafficking (VCAM), and inhibiting angiogenesis (VEGI) were preferentially utilized in the

aged lung (Fig. 3D). Further analysis revealed strong signaling patterns in both young and aged lungs that are important for lung maintenance and repair such as Collagen, TGF-β, FN1, MMP, PDGF, and VEGF as well as inflammatory responses such as complement, MIF, and CXCL (Fig. 3E). Signaling through CDH5, NRXN, and FLRT, which are important for cell adhesion [37-40], was predicted to be weaker with age (Fig. 3E). Similarly, signaling via NRG and EPHB, which is important for tissue development [41, 42], and via PTPR and PTPRM, protein tyrosine phosphatase receptors important for cell maintenance and migration [43], was also predicted to be decreased with age (Fig. 3E).

Network signaling analysis highlights the loss of CDH5, PTPRM, NRG, and NRXN signaling in the aged compared to young lungs (Fig. 4A-D). Particularly, epithelial cells and leukocytes_2 fail to receive or send CDH5 signals in aged lungs (Fig. 4A). These subsets along with macrophage_2 lose their PTPRM signaling in aged lungs (Fig. 4B). NRG signaling is reduced in all cell types in the aged lung (Fig. 4C). NRXN signaling in fibroblasts is greatly diminished in the aged lungs (Fig. 4D). Additionally, EPHB, FLRT, and VEGF signaling patterns were also lost in the aged lung (Supplementary Fig. 4A-C). Epithelial, endothelial, and fibroblast cells from aged lungs were predicted to stop sending FN1 (fibronectin), MIF (macrophage survival), COL1A ligands (collagen), CDH5 (cell adhesion), and VEGFA (tissue development and repair) signals to other cell types (Fig. 4E-F, Supplementary Fig. 4D-E). CDH5 and VEGFA signaling was predicted to be lost in immune cell populations of the aged lungs as well (Fig. 4H-I, Supplementary Fig. 4F-G). In contrast, increased probability of CXCL12, MIF, FN1, and C3 signaling was predicted for the smooth muscle cells (Fig. 4G), leukocytes, and macrophages in the aged lungs (Fig. 4H-I, Supplementary Fig. 4F-G).

DISCUSSION

Here we show that both structural and immune cells have a hyperinflammatory transcriptional signature in the aged compared to young lungs. Immunofluorescent staining shows colocalization of pro-surfactant protein C and cytokeratin 8, indicating that there is presence of aberrant alveolar type 2 cells, which are in fibrotic lungs [44-48]. Our results predicted increased signaling between leukocytes and macrophages as well as between smooth muscle and immune cells with age. The chronic low-grade inflammation observed in aging tissue [49, 50] is likely inducing the pro-inflammatory transcriptional phenotype we observed in aged lung cells. Indeed, immune cells from aged lungs increase their signaling through FN1, MIF, COL1A, CXCL12 and C3 ligands. CXCL12 is a canonical SASP factor, which attracts immune cells to sites of inflammation [51]. FN1 and COL1A1 signaling enhance immune cell infiltration into tissues [52-54] while MIF signaling ensures immune cell survival [55, 56], which could lead to persistent inflammation. Finally, C3 signaling is crucial for pathogen eradication during the early disease response [57] and is upregulated in the aged lung. However, a persistent pro-inflammatory profile can lead to immune tolerance [58, 59].

Chronic inflammation can also decrease lung elasticity as lung tissue can develop structural impairments [60, 61]. Indeed, we also observed significant changes in signaling patterns between immune and structural cells and within structural cells in the lung with age. Specifically, signaling between endothelial, epithelial, fibroblasts and other lung resident cells was decreased. The signaling loss in aged endothelial cells may arise from disruption of junction proteins [62, 63]. CellChat analysis predicted a loss of cadherin 5 (CDH5) interactions with its receptors across multiple cell types. CDH5 is a cell adhesion molecule that maintains endothelial junctions [37]. Two other genes that encode for cell adhesion molecules, *NRXN* and *FLRT*, were also downregulated in aged lungs. Loss of these cell adhesion molecules can disrupt cellular signaling [38-40]. In addition, *NRG* and *EPHB* were also downregulated in aged lungs and are important for tissue development [41, 42]. Impaired ability to repair damaged tissue can lead to accumulation of damage-associated molecular patterns (DAMPs) which exacerbate inflammation [64, 65]. Furthermore, expression of *PTPRM* and *PTPR* which encode for protein tyrosine phosphatase receptors that control cell growth, differentiation, and migration [43] was also decreased in aged lungs. The loss of FN1, MIF, and COL1A signaling in structural cells of aged lungs may be an attempt to limit further inflammation from structural cells [66]. VEGF signaling was drastically reduced or missing from every cell type in aged lungs, but not young lungs. VEGF signaling is crucial for initiating repair processes in tissues and the loss of this signaling in aged but not young lungs explains why young lungs were undergoing wound healing processes more so than aged lungs [67]. Overall, the predicted loss of these signaling patterns indicates that aged lung cells are unable to preserve cell adherence and cannot send developmental signals to maintain the lung tissue architecture, barrier function against pathogens, and facilitate repair mechanisms necessary for healthy lung function.

Additionally, aging is associated with reduced mucociliary escalator function, which combined with loss of elasticity results in decreased pathogen clearance with age [68]. We show that while ciliated epithelial cells have minimal DEGs in the young lungs, many upregulated genes from the aged lungs indicate hyperinflammation,

which could damage the structure and function of cilia [69]. Aged ciliated epithelial cells also have reduced incoming and outgoing NRG, NRXN, and EPHB signals. NRG signaling is important for ciliated epithelial cells as it regulates epithelial barrier function, permeability, and repair [70]. NRXN has classically been understood as a cell adhesion molecule in neuronal tissues that induces junction formation [71]. It likely plays a similar role in lung tissue by supporting tissue integrity and cilia organization. EPHB signaling plays a role in cell-cell communication and maintaining proper cell polarity [72]. Loss of these three components may cause dysfunctional coordination of ciliary beats through loss of proper cell alignment and communication resulting in loss of beat synchronization and inefficient lung clearance.

This study is not without limitations. The small sample size limits our ability to assess biological variability and sex as a biological variable. Future studies with larger sample sizes and assessment of multiple lung sections are required to validate these findings. Additionally, gene expression changes were not validated by protein expression. However, our findings aligned with data from prior studies. For instance, The CCAAT enhancer binding protein delta (*CEBPD*) gene was upregulated in several cell types from aged lungs. Protein levels of CEBPD are increased in human lungs after aspergillus infection [73] and in the astrocytes of patients with Alzheimer's disease [74], in line with the heightened inflammatory state of aged lungs in our study. Cathepsin genes were also upregulated in several cell types and has been shown to have increased protein expression in aged mice brains [75]. Conversely, reduced expression of *S1PR1* aligns with the reduced S1P signaling in aged mouse endothelial lung and kidney cells [76]. Decreased expression of *RGS5*, aligns with the loss of RGS5 with age in mouse pericytes [77]. This study is also limited by investigating tissue from animals previously infected with MAH. While histology revealed no signs of inflammation and no bacteria were detected, the prior infection, albeit a year before the samples were collected, could have impacted the data. However, it should be noted that humans do not live under SPF conditions, therefore the history of a distant infection is biologically relevant. The chronic inflammatory signature across all cell types indicates that poor outcomes following infection may be mediated by immune tolerance which impedes pathogen clearance and inability to rewire the cellular transcriptome towards tissue repair which exacerbates tissue damage. Understanding the differences in these signaling pathways may provide an avenue for new therapeutic targets to help aged individuals resolve lung damage after a pathogenic assault.

Acknowledgments

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Author Contributions

B.M.D. – performed experiments and analyzed the data. E.G.N. – collected samples, analyzed the data, interpreted the results, and wrote the manuscript. M.E. – analyzed the data. M.H.D. – identified suitable animals and collected samples. C.F.B. – provided reagents and expertise for immunofluorescent staining of structural lung cells. E.R.S. – conceived and designed the experiments, identified suitable animals, and collected samples. I.M. – conceived and designed the experiments, supervised study, obtained funding, interpreted the results, and edited the manuscript.

Data availability

All data supporting the conclusions made in this study are public in the Sequence Read Archive (SRA) or Mendeley (https://data.mendeley.com/preview/p5xpj4v73r?a=56623784-6280-409d-9ce0-f70f588c7222). SRA: (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1247551) for Visium spatial transcriptomics.

Disclosures

The authors have declared that no conflict of interest exists.

Supplementary Materials

The Supplementary data are available online at: www.aginganddisease.org/EN/10.14336/AD.2025.1317.

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