



ORIGINAL ARTICLE OPEN ACCESS

Inflammation Promotes Aging-Associated Oncogenesis in the Lung

Catherine Pham-Danis¹ | Shi B. Chia¹  | Hannah A. Scarborough¹ | Etienne Danis^{2,3} | Travis Nemkov¹ | Vadym Zaberezhnyy¹ | Jessica L. Christenson⁴ | Emily K. Kleczko⁵ | Andre Navarro⁵ | Andrew Goodspeed^{2,3} | Elizabeth A. Bonney⁶ | Charles A. Dinarello⁵ | Carlo Marchetti⁵ | Raphael A. Nemenoff⁵ | Kirk C. Hansen¹ | James DeGregori^{1,7,5} 

¹Department of Biochemistry and Molecular Genetics, University of Colorado Anschutz Medical Campus, Aurora, Colorado, USA | ²Department of Biomedical Informatics, University of Colorado Anschutz Medical Campus, University of Colorado Cancer Center, Aurora, Colorado, USA | ³University of Colorado Cancer Center, University of Colorado Anschutz Medical Campus, Aurora, Colorado, USA | ⁴Department of Pathology, University of Colorado Anschutz Medical Campus, University of Colorado Cancer Center, Aurora, Colorado, USA | ⁵Department of Medicine, University of Colorado Anschutz Medical Campus, University of Colorado Cancer Center, Aurora, Colorado, USA | ⁶Department of Obstetrics, Gynecology and Reproductive Sciences, Larner College of Medicine, University of Vermont, Burlington, Vermont, USA | ⁷Department of Immunology and Microbiology, University of Colorado Anschutz Medical Campus, University of Colorado Cancer Center, Aurora, Colorado, USA

Correspondence: James DeGregori (james.degregori@cuanschutz.edu)

Received: 4 March 2024 | **Revised:** 6 July 2024 | **Accepted:** 13 August 2024

Funding: This study received funding from Veteran's Administration (1 I01 BX004495), University of Vermont College of Medicine, and National Cancer Institute (T32CA174648 and P30CA046934).

Keywords: aging | inflammation | lung cancer | microenvironment | alpha 1 antitrypsin

ABSTRACT

Background: Lung cancer is the leading cause of cancer death in the world. While cigarette smoking is the major preventable factor for cancers in general and lung cancer in particular, old age is also a major risk factor. Aging-related chronic, low-level inflammation, termed inflammaging, has been widely documented; however, it remains unclear how inflammaging contributes to increased lung cancer incidence.

Aim: The aim of this study was to establish connections between aging-associated changes in the lungs and cancer risk.

Methods: We analyzed public databases of gene expression for normal and cancerous human lungs and used mouse models to understand which changes were dependent on inflammation, as well as to assess the impact on oncogenesis.

Results: Analyses of GTEx and TCGA databases comparing gene expression profiles from normal lungs, lung adenocarcinoma, and lung squamous cell carcinoma of subjects across age groups revealed upregulated pathways such as inflammatory response, TNFA signaling via NF κ B, and interferon-gamma response. Similar pathways were identified comparing the gene expression profiles of young and old mouse lungs. Transgenic expression of alpha 1 antitrypsin (AAT) partially reverses increases in markers of aging-associated inflammation and immune deregulation. Using an orthotopic model of lung cancer using cells derived from EML4-ALK fusion-induced adenomas, we demonstrated an increased tumor outgrowth in lungs of old mice while NLRP3 knockout in old mice decreased tumor volumes, suggesting that inflammation contributes to increased lung cancer development in aging organisms.

Catherine Pham-Danis and Shi B. Chia contributed equally to this study.

This is an open access article under the terms of the [Creative Commons Attribution-NonCommercial-NoDerivs](https://creativecommons.org/licenses/by-nc-nd/4.0/) License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2024 The Author(s). *Aging and Cancer* published by Wiley Periodicals LLC.

Conclusions: These studies reveal how expression of an anti-inflammatory mediator (AAT) can reduce some but not all aging-associated changes in mRNA and protein expression in the lungs. We further show that aging is associated with increased tumor outgrowth in the lungs, which may relate to an increased inflammatory microenvironment.

1 | Introduction

Modern society has seen a steady increase in life expectancy in the last two-hundred years [1]. Thus, it is not surprising that elderly people (>65 years old) are expected to comprise greater than 20% of the world's population by 2050 [2]. This increase poses a forthcoming challenge for the public healthcare systems, particularly in the management and treatment of cancer patients. Lung cancer is the leading cause of cancer-related deaths in the world. Smoking has been known to be the major risk factor in lung cancer. Still, ~25% of lung cancers occur in never-smokers [3]. While the etiology of lung cancer in never-smokers is complex, aging is one of the most prominent prognostic factors with the median age of lung cancer diagnosis at 70 years [4].

Aging is a complex process due to stochastic, environmental, and genetic events that affect multiple cell types, which ultimately results in declining tissue function. There are a number of aging-associated alterations that occur in the lungs of mice and humans, including inflamm-aging [2, 5], declining adaptive immunity [6, 7], weakening of muscles, and declining lung function [8]. Inflammation has long been connected to the promotion of cancer [9]. In fact, chronic obstructive pulmonary disease (COPD), a chronic inflammatory lung disease, is an independent risk factor for lung cancer [10], and smokers susceptible to COPD are five times more likely to develop lung cancer compared to those with normal lung function [11]. In fact, COPD is highly associated with lung cancer risk even in never-smokers [12], although there are many complicating factors that prevent a clear causative link [13]. Interestingly, COPD usually manifests late in life and shares many similarities seen in the aging lung [10, 14].

It is widely accepted that tumorigenesis is a result of clonal evolution, where genetic variability brought about by mutations and epigenetic changes fuels selection for cell clones with progressively more malignant phenotypes. Much of the field has focused on mutations as the limiting factor in tumor development by conferring a fitness advantage in comparison to non-mutated cells. Based on this notion, cancer incidence is widely believed to be mostly limited by the time-dependent accumulation of driver mutations. The relationship to aging is thought to mainly derive from the time required for sufficient driver mutations to occur within a single cellular clone [15–17]. Previous work from our lab has demonstrated that while mutations are crucial, they are not sufficient for cancer development and that the impact of aging or other insults on selective pressures needs to be considered [18–20]. The Adaptive Oncogenesis model encompasses natural selection and microenvironmental changes to describe how selective pressure brought upon by changes in the tissue microenvironment may impinge on the selection of mutations that become adaptive in the evolved microenvironment.

Given the multitude of changes with advanced age, including inflammaging in the lungs, we hypothesize that aging-associated inflammation contributes to the selection of adaptive oncogene-initiated clones. To study how the tissue microenvironment may influence the outgrowth of lung cancers, we use an orthotopic model of lung cancer whereby cells derived from EML4-ALK fusion-induced adenomas are implanted directly into lungs of mice. EML4-ALK fusions are prevalent in lung cancers of never-smokers, with a smaller fraction in those of smokers [21]. We have previously shown that reducing inflammation in an aged background via transgenic alpha1 anti-trypsin (AAT) can prevent oncogenic adaptation in B-cell progenitors, coinciding with reduced systemic inflammation [18]. Notably, patients with AAT deficiency, which results in degradation of the lung extracellular matrix, prematurely develop COPD and exhibit a four- to fivefold increased risk of lung cancer independent of smoking status [22]. Here, we show that both human and mouse lungs and lung adenocarcinomas/squamous cell adenomas exhibit hallmarks of inflammation in older ages, as well as alterations in extracellular matrix-related gene expression. We further show that reducing age-associated inflammation can dampen the outgrowth of EML4-ALK-driven tumors in the lung.

2 | Methods

2.1 | Mouse Studies

Animals utilized were the following: human-AAT transgenic (AAT) mice [23], *Nlrp3*^{-/-} mice [24], and young and aged C57BL/6 mice. Young and aged C57BL/6 mice were provided by the National Institute of Aging (NIA). The *Nlrp3*^{-/-} and AAT mice were maintained on the C57BL/6 genetic background. These strains of mice are designated “young” (2–5 months) or “old” (20–28 months) throughout this document. Both sexes of each of these strains were used for experiments. Oncogenic events were introduced by direct implantations of cancer cells derived from EML4-ALK fusion-induced lung adenomas [25]. The University of Colorado Institutional Animal Care and Use Committee (IACUC) reviewed and approved all animal experiments. All animal experiments were conducted in accordance with the NIH Guidelines of Care and Use of Laboratory Animals.

2.2 | Genotyping Mice

The following PCR conditions were used to genotype each mouse strain:

AAT: 95°C for 2 min, 35 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s, followed by 72°C for 5 minutes. The AAT gene product produces a 450-bp fragment, and ApoB, which serves

TABLE 1 | Primer sequences for genotyping.

Mouse strain	Primer	Sequence
AAT	AAT_FW	ACTCCTCCGTACCCTCAACC
	AAT_RV	GCATTGCCCGAGGTATTTTCAT
	ApoB_FW	CTA GGC CAC AGA ATT GAA AGA TCT
	ApoB_RV	GTA GGT GGA AAT TCT AGC ATC ATC C
	AAT nested_FW	ACTGTCAACTTCGGGGACAC
	AAT nested_RV	CATGCCTAAACGCTTCATCA

as an internal control, produces a 324-bp fragment. A secondary confirmation using AAT nested primers was also performed, producing a 249 bp fragment.

Primer sequences for genotyping are listed in Table 1.

2.3 | Preparation of Slides and Immunohistochemistry

Mouse lungs were evaluated through three serial 10- μ m sections obtained 50 μ m apart by hematoxylin and eosin (H&E) staining using the standard method. Images were acquired using an Aperio Image Slide Scanner (Deer Park, IL, USA) or BZ-X810 inverted microscope (Keyence, Itasca, IL, USA). Images were analyzed using the FIJI Image J software [26].

2.4 | Lung Tissue Proteomics and Analysis

Lung tissues from young, old, and old AAT mice were perfused with PBS and were pulverized. Lung tissues were then homogenized in freshly prepared high-salt buffer (50 mM Tris-HCl, 3 M NaCl, 25 mM EDTA, 0.25% w/v CHAPS, pH 7.5) containing 1 \times protease inhibitor (Halt Protease Inhibitor, Thermofisher Scientific) at a concentration of 10 mg/mL. Homogenization was performed using a bead beater (Bullet Blender Storm 24, Next Advance, 1 mm glass beads) for 3 min at 4°C. Samples were then sequentially extracted using CHAPS buffer to obtain cellular and urea to obtain soluble fractions. The remaining pellet was then digested with either CNBr or NH₂OH followed by tryptic digestion with QconCATs [27]. Samples were then analyzed by LC-MS/MS. MS/MS spectra were extracted from raw data files and converted into .mgf files using MS Convert (ProteoWizard, Ver. 3.0). Peptide spectral matching was performed with Mascot (Ver. 2.5) against the Uniprot mouse database (release 201701). Significant proteins were analyzed using Metascope (metascope.org).

2.5 | Lung Epithelial Cell Gene Analysis

Lung epithelial cell isolation, transcriptomic profiling, and analysis have been described elsewhere [28]. Briefly, lung tissues were

homogenized using the MACS (Miltenyi Biotec, Germany) tissue dissociation system, and CD45⁺ cells were removed using anti-CD45 (Miltenyi Biotec, Germany) beads. Affymetrix GeneChip system (Mouse Gene 2.0 ST Array) was used for transcriptomic profiling. Analysis of array data was carried out using Partek Genomics Suite 6.6, Beta Analysis comparing groups with the number of probe sets passing a false discovery rate (FDR) of 0.05 or a binary filter ($p < 0.05$ and 2 \times fold change).

2.6 | RNA Seq Lung Preparation and Analysis

RNA from lungs of young, old, and old-AAT mice was extracted from whole lungs using Qiagen RNeasy mini kit according to the manufacturer's protocol. cDNA library preparations were performed on the extracted RNA and were then run on the Illumina HiSeq at 50 cycles with 12 million read depth and FASTQ files generated. Low-quality sequencing bases were trimmed before the alignment step. Trimmed sequencing reads were mapped against the mouse genome (mm10) using the Hisat2/cufflinks workflow. Cufflinks (version 2.2.1) were employed to assemble the transcripts using the RefSeq annotation as the guide and computed the transcripts' RPKM values by using the merged assembly as the guide. On average, 40.4 million (28.8–59.2 million) reads were sequenced per sample and were aligned to the human genome. Gene set enrichment analysis (GSEA) was used to perform pathways enriched in the RNA-seq as previously described. KEGG pathways were used as the gene set, and 1000 gene-set permutations were performed to determine p -value. Pathways with $p < 0.05$ and $\log_{2}FC > |1|$ were considered significant from the analysis.

2.7 | GTEx and TCGA Analyses

RNA-seq data were obtained from The Genotype-Tissue Expression (GTEx) Project GTEx portal on 08/04/2020. xCell [29] analyses were done as described elsewhere. RSEM expected counts for LUSC and LUAD TCGA data sets (illuminahiseq_rnaseqv2-RSEM_genes) were downloaded from Firehose in January, 2022. Clinical data were downloaded as Firehose Legacy from cBioPortal [30]. The limma R package was used to calculate differential expression between the indicated groups [31]. Gene set enrichment analysis was performed using the fgsea R package on fold change using gene sets from the Molecular Signatures Database [32].

2.8 | Flow Cytometric Analyses

Whole lungs were digested with 1.5 mg/mL collagenase A (Sigmaaldrich cat# COLLA-RO; St. Louis, MO), 0.4 mg/mL Deoxyribonuclease (Worthington cat# LS002139; Lakewood, NJ), 10 mM HEPES pH7.2, 5% FBS injected directed into the lungs through cannulae and incubated at 37°C for 30 min. Digested lungs were passed through 50- μ m cell strainers. Hemolytic buffer (150 mM NH₄Cl, 1 mM NaHCO₃, 1.1 mM Na₂EDTA) was used to lyse red blood cells, and single cells were resuspended in FACS buffer and were stained with antibodies (CD45-Pacific Blue, Biolegend 103126; CD4-BUV395, BD Biosciences 563790; CD8-BUV737, BD Biosciences 612759; PD1-APC, Biolegend 135210). LSR

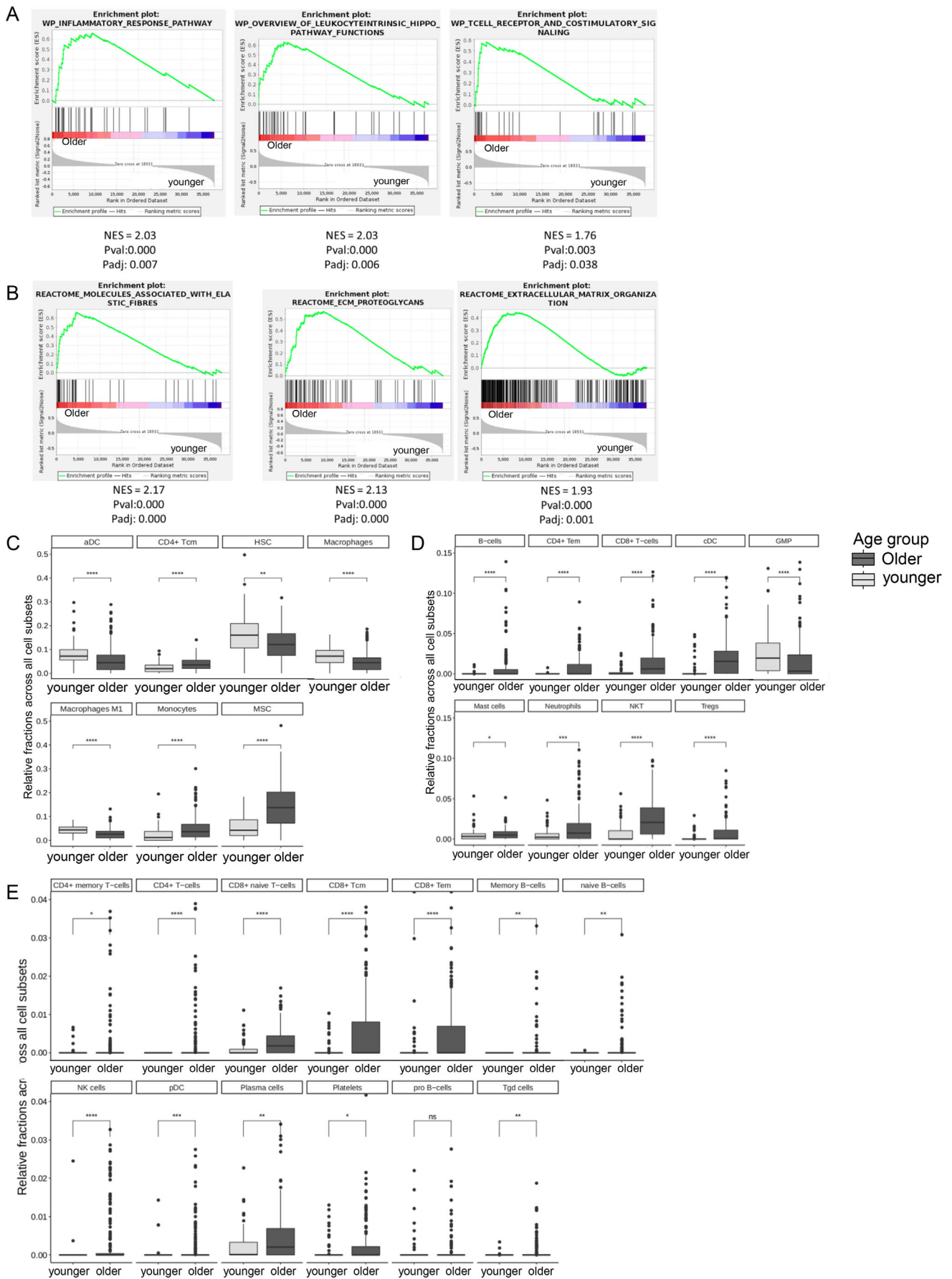


FIGURE 1 | Advanced age leads to dramatic changes in the immune landscape of human lungs. RNA-seq data from the Genotype-Tissue Expression (GTEx) project was analyzed to determine gene expression changes in non-diseased lung tissue from various ages. Dataset from “younger” (subgroups

II flow cytometer (BD Biosciences) was used for flow cytometry, and data were analyzed with the FlowJo software v10.

2.9 | Orthotopic Lung Tumor Model

EML4-ALK (EA) cell line was established by Kleczko et al., and the transplantation of EA cells into lungs of mice was described elsewhere [25]. Briefly, a 1-mm incision was made to the left side of the anesthetized mice and 2×10^5 EA cells resuspended in 40 μ l of Matrigel were injected directly into the left lobe of the lungs using 30-gauge needles, and the incision was closed with staples afterward.

2.10 | Tumor Volume Quantification

μ CT imaging was performed by the Small-Animal IGRT Core at the University of Colorado Anschutz Medical Campus using the Precision X-Ray X-Rad 225Cx MicroIGRT and SmART Systems (Precision X-Ray, Madison, CT, USA). The ITK-SNAP software was used to quantify tumor volumes from μ CT images [33].

2.11 | Statistical Analysis

For in vitro experiments, standard deviation is shown for all error and is based on biological replicates. Unless otherwise indicated, one-way analysis of variance (ANOVA) with Tukey post-test was used to compute *p*-values using GraphPad Prism. *p*-value designations for all figures were as follows: * <0.05 , ** <0.01 , *** <0.001 , and **** <0.0001 .

2.12 | Data Availability

All array, RNAseq, and proteomics data will be deposited to GEO (in progress).

3 | Results

3.1 | Advanced Age Leads to Dramatic Changes in the Immune Landscape of Human Lungs

While a dysregulation of the immune system has been reported to be a characteristic of old age [34], we sought to evaluate the molecular and cellular changes that occur with advancing age in the lung. We analyzed the publicly available RNA-seq data from the Genotype-Tissue Expression (GTEx) project to determine gene expression changes in over 500 non-diseased lung tissue from individuals of various ages. We performed gene set enrichment analysis (GSEA) comparing the expression profiles

of lung tissue from young (20–49 years old) to older ages (50–79 years old) individuals to identify significant signatures (nominal *p*-value < 0.05 , *p*-value adjusted < 0.2 , and NES > 1.5 or < -1.5) (Figure S1A). We found that several pathways related to immune activation such as inflammatory response pathways, leukocyte intrinsic hippo pathway, T-cell receptor, and costimulatory signaling were enriched in the lungs from older compared to the young individuals (Figure 1A). Additionally, we found that signaling pathways involved in inflammation including interferon gamma response and IL2/STAT5 signaling were also upregulated in the older samples relative to young (Figure S1B). Extracellular matrix (ECM) remodeling pathways were also enriched in the lungs of older individuals suggesting alterations in the tissue architecture of old lungs (Figure 1B). Interestingly, we identified Myc targets and oxidative phosphorylation gene sets enriched in lungs of younger compared to older individuals (Figure S1C), consistent with numerous studies showing a decline in Myc signaling [35] and mitochondrial metabolism [36] with advancing age.

Focusing on the young and old lung transcriptomes, we used a deconvolution method xCell [29] to enumerate different cell types based on the gene expression profiles. Of the most abundant cell types represented, we identified that CD4 memory T cells, monocytes, and mesenchymal stem cells were significantly increased in the older compared to the young lungs, while total macrophages and activated dendritic cells (aDCs) were decreased in the old lungs (Figure 1C). Of the medium abundant cell types, CD4⁺ T cells, CD8⁺ T cells, and regulatory T cells (Tregs), as well as B cells and neutrophils, were over-represented in the older lungs relative to the young (Figure 1D). Of the low abundant cell types, lymphocytes, natural killer (NK) cells, gamma-delta T cells, and platelets were also significantly up in the older lungs (Figure 1E). Note that we only show cell types that demonstrated significant aging-dependent differences. Based on these analyses of the transcriptomes from human lung tissue, there are substantial alterations in the tissue microenvironment marked by remodeling of the ECM and differences in the immune landscape with old age.

3.2 | Aging Contributes to Cellular and Phenotypic Changes in Mouse Lungs

We next asked whether similar age-dependent transcriptional changes occur in the lungs of young compared to aged mice. We analyzed gene expression using Affymetrix 5 arrays of purified lung epithelial tissue isolated from 3-month- and 18-month-old mice (data from the 3-month-old mice were previously reported [28]). Pathway analyses revealed numerous pathways under-represented (Figure S2A) and over-represented (Figure S2B) in old lung epithelium compared to young. Of the top 20 most significant pathways, alterations in the extracellular matrix

20–29, 30–39, 40–49) was compared with “older” (subgroups 50–59, 60–69, 70–79) individuals. (A) Gene set enrichment analysis (GSEA) of inflammatory pathways enriched in lung expression data from older relative to younger. (B) GSEA of ECM related/remodeling pathways over-represented in elderly compared to young data sets. (C) xCell analysis comparing young and elderly data sets showing the most abundantly expressed cell types. (D) xCell analysis comparing data sets from younger and older individuals showing cell types of medium expression. (E) xCell analysis comparing data sets from younger and older individuals showing levels of cell types with the lowest expression. Pairwise comparison with unpaired two-samples Wilcoxon test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

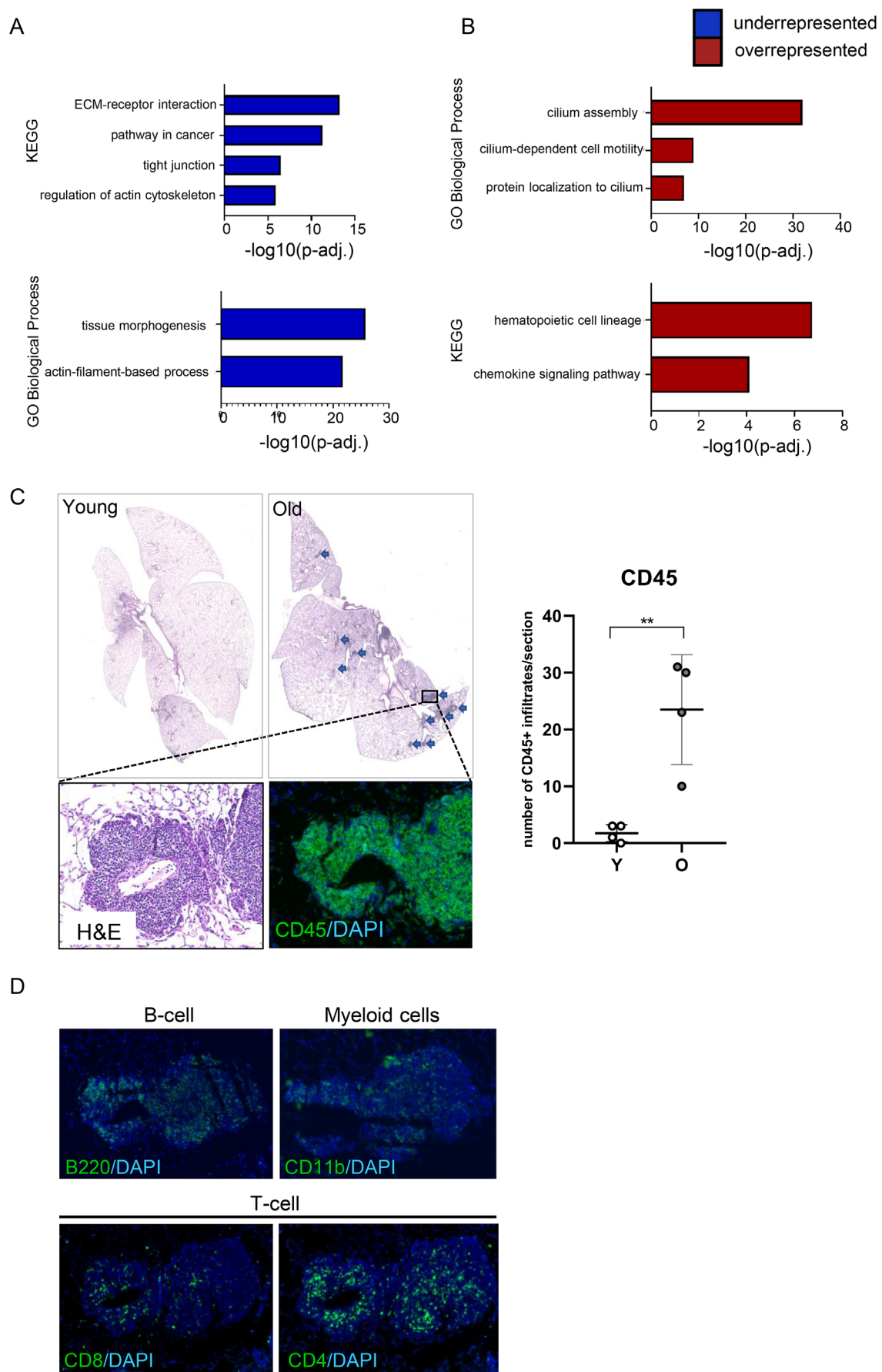


FIGURE 2 | Aging contributes to cellular and phenotypic changes in mouse lungs. Gene expression data sets analyzed via Affymetrix of lung epithelial tissue isolated from 3-month- and 18-month-old mice were mined. Metascape analysis was performed on the most significantly differentiated

and tissue structure such as ECM-receptor interaction, tight junction, regulation of cytoskeleton, tissue morphogenesis, and actin filament-based process were significantly down-regulated in old lung epithelium compared to young (Figure 2A). These results suggest there are structural changes and remodeling of the lung tissue epithelium which occur with old age. Conversely, when we analyzed pathways upregulated in aged epithelium, we found an upregulation of cilium-related pathways including cilium assembly, cilium dependent cell mobility, and protein localization to cilium included in the top 20 most significant pathways (Figure 2B). Previous single-cell RNA-seq studies have demonstrated an increase in the frequency of ciliated cells in the lungs of aged mice compared to young [37]. Additionally, we observed an increase in immune and inflammation related pathways such as hematopoietic cell lineage and chemokine signaling pathway in lung epithelium of aged mice (Figure 2B).

To determine whether there were cellular and structural changes associated with old age in the lungs, we harvested lungs from young mice (2 months) and old mice (20 months). Unlike lungs from young mice, lungs from old mice exhibited an increase in the number of immune aggregates as indicated by staining of pan-leukocyte marker CD45 compared to the lungs of young counterparts (Figure 2C). These immune aggregates were observed in close proximity to the bronchial space as well as throughout the lung parenchyma. In order to identify the type of immune cells that make up the immune lesions present in old lungs, we performed immunofluorescence and stained for CD11b (myeloid marker), B220 (B-cell marker), and CD4/8 (T-cell markers). Cell expressing B220, CD11b, CD4, and CD8 were all evident within the immune aggregates (Figure 2D). Since these immune lesions are virtually absent from young mouse lungs, we could only characterize the immune cell types present in the old lungs. It has been reported that the adaptive immune system exhibits several age-associated changes including an increase in the number of effector and memory CD4⁺ T-cells [38]. These immune aggregates identified in aged lungs resemble immune lesions such as inducible bronchus-associated lymphoid structures (iBALTs) found in the lungs of cigarette smokers [39]. Based on the mouse and human data, our results suggest that there is an age-associated increase in immune infiltrates in the lung.

3.3 | Expression of AAT Reduces Inflammatory Phenotype and Partially Reverse Age-Dependent Gene Expression Alterations in Aged Lung

Given that we observed an increase in immune infiltrates and an enrichment in inflammatory pathways in our gene expression analysis in the lungs of aged mice, we sought out a model that could possibly dampen these effects of aging in the lung. We have previously shown that aged AAT transgenic mice

reversed increased TNF- α in serum and bone marrow and IL-6 in bone marrow of old mice [40]. Additionally, AAT mice have been reported to be resistant to several inflammatory challenges [41]. The human AAT transgene is expressed from the surfactant protein C (SPC) promoter [23]; while a secreted protein, we expect higher levels in the lungs. Older individuals have decreased expression of AAT (*SERPINA1*) in lungs compared to younger individuals (Figure 3A). In contrast, there is no change in AAT expression in lungs of old mice compared to young mice, while the expression was increased in old-AAT mice, as expected (Figure S3A). Interestingly, we detected fewer immune aggregates in old-AAT mice, similarly to the lungs of young mice (Figure 3B). To gain mechanistic insight into how AAT expression may be impacting age-dependent alterations in the lungs, we performed RNA sequencing analysis of whole lungs to evaluate gene expression profiles among the different groups. We observed a significant proportion of genes altered in young compared to old lungs, and old compared to old-AAT lungs (Tables S1 and S2; Figure S3B,C). Interestingly, AAT expression in old mice was able to partially reverse some of the aging-dependent changes. We identified 153 genes down and 342 genes up in old mice relative to young. We also observed 53 genes down and 298 genes up in old-AAT lungs old relative to old control lungs. Note that AAT expression does more than simply “rejuvenate” lungs of old mice, as we observe significant differences in gene expression comparing old-AAT lungs to young lungs (Figure S3C).

We next performed GSEA on the significant genes identified in young, old, and old-AAT groups. From the Hallmark database, we identified inflammatory response and IL-6/JAK/STAT3 signaling enriched in old relative to young, with the opposite enrichment with AAT expression, indicating reversal of this aging-dependent pathway upregulation (Figure 3C,D). We also enrichment for the T-cell receptor signaling and chemokine signaling KEGG pathways in old lungs, with reversal (downregulation) of these pathways evident with AAT expression (Figure 3E,F). Lastly, we identified PD-1 signaling pathway as being more activated, with AAT expression reversing the activation (Figure S3D,E). Thus, expression of AAT restores expression of these immune-related pathway genes to more youthful levels. Flow cytometric analyses of CD4 and CD8 T cells similarly revealed an increased PD1⁺CD4⁺ and PD1⁺CD8⁺ T cells in old lungs compared to young ones, with a reversal with AAT overexpression (Figure S3F). Nonetheless, we did not observe reductions in the aging-associated increased adenoma outgrowth upon injection of anti-PD1 (Figure S3G), previously shown to promote immune reactivation [42].

3.4 | Expression of AAT Partially Restores Tissue Microenvironmental Alterations in Aged Lung

We next employed a proteomics approach to ask how aging may impact the architecture of the tissue microenvironment and how

genes in epithelial cells of lungs from young and old mice. (A) Pathways under-represented in old epithelium compared to young. (B) Pathways over-represented in old epithelium compared to young. (C) Lungs from young (2-month-old) and old (20-month-old) mice were harvested and inflated for histology. Leukocytic marker CD45 was stained using immunofluorescence to quantify immune lesions in the lungs of young and old mice. (D) Additional immunofluorescence analysis was performed to probe for B220 (B-cell marker), CD11b (myeloid marker), CD4 (helper T-cell marker), and CD8 (cytotoxic T-cell marker).

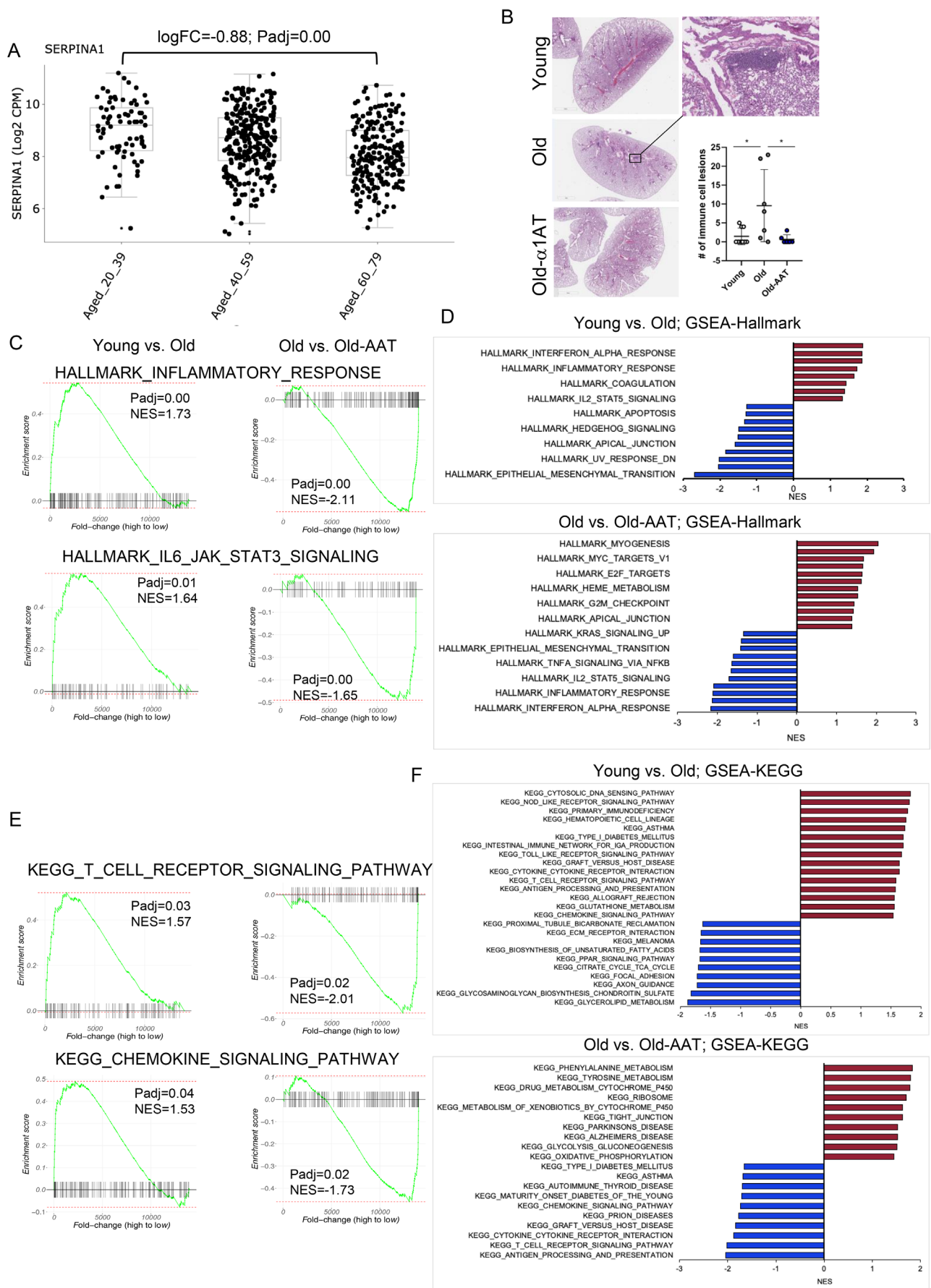


FIGURE 3 | Expression of AAT reduces inflammatory phenotype and partially reverses age-dependent gene expression alterations in aged lung. (A) AAT (*SERPINA1*) expression level in lungs of older individuals (60–79) compared to younger individuals (20–39). (B) The number of distinct immune

AAT expression may affect these changes in the lungs. To address this, we performed mass spectrometry (MS)-based proteomic analyses of lung tissue from young, old, and old-AAT mice. We were able to evaluate changes from the whole proteome, as well as the extracellular matrix (ECM) that contributes substantially to the tissue architecture of the microenvironment [27]. Lung samples from young, old, and old-AAT mice were separated into high salt (HS, enriched for cellular proteins), soluble ECM (sECM), and insoluble fractions (iECM) (Figure 4A). We assessed global proteomic changes and found dramatic alterations between young and old lungs. Interestingly, AAT was able to mitigate a subset of those changes among the different fractions, and also resulted in changes that diverged from both the young and old states (Figure 4B). Both of the tight junction proteins ZO-1 and ZO-3 were down in old lungs and effectively restored in old lungs expressing AAT in the iECM and sECM, respectively. Furthermore, the distribution of ZO-1 was changed substantially, with reduced presence in the iECM and increases in HS fraction in the lungs of old mice, suggesting age-associated tissue remodeling. In addition, these analyses revealed significant increases in the levels of neutrophilic proteases (e.g., MMP9 and ELNE/elastase) and other inflammatory markers (e.g., S100A8/A9) in the old lungs, as well as changes in key factors dictating cell fate (e.g., NEST/Nestin), which were effectively reversed by AAT overexpression (Figure 4C). Immunofluorescence detection of S100A and MMP9 is shown in Figure S4A–C, although differences were not significant likely due to variability in the old mouse lungs. Pathway analysis of all significant protein changes further showed an increase in ECM remodeling (i.e., cell–cell adhesion and actin cytoskeleton remodeling) and inflammation (i.e. inflammatory response, leukocyte activation, IL-17 signaling) enriched in old lungs and restored to youthful levels with AAT expression (Figure 4D). Additionally, indications of ECM remodeling were also evident by over-representation of protein folding and actomyosin structure organization, and tight junction pathways that were under-represented in old lungs and enriched in old-AAT lungs (Figure 4E). Nonetheless, it is also clear that AAT expression results in some protein changes in the lungs that are distinct from those observed in both young and old control lungs. In all, the proteomic analyses reveal substantial changes in the tissue microenvironment with age, which can partially be restored with AAT expression.

3.5 | Age-Dependent Increased Inflammatory Parameters Are Also Observed in Lung Adenocarcinomas and Squamous Cell Carcinomas

Given the changes observed in aging lungs, we wondered if similar changes occur in lung cancer tissue dependent on patient age. Previous studies have shown how age influences the characteristics of cancers, from mutational profiles to

gene expression (e.g., [43, 44]). We thus analyzed the publicly available RNA-seq data from The Cancer Genome Atlas (TCGA) of over 400 lung adenocarcinomas or lung squamous cell carcinomas. We similarly performed GSEA analyses comparing gene expression profiles of lung cancer tissues from young (40–49) and old (70–79) patients with lung adenocarcinoma and from young (50–59) and old (70–79) patients with lung squamous cell carcinoma. Similar to normal aging lungs (Figure 1A, Figure S1A), pathways related to inflammation, including inflammatory response, TNFA signaling via NF κ B, and interferon gamma response, were shown to be upregulated in elderly patients with both lung adenocarcinoma and lung squamous cell carcinoma (Figure 5A–D), suggesting that old age confers its inflammatory nature to cancers that develop within the lungs. Myc targets and oxidative phosphorylation gene sets were also identified to be downregulated in both cancer types with age (Figure 5E,F), similar to our observations in normal aging lungs (Figure S1B), suggestive of a common force at play in lung cancer development.

3.6 | Modeling Age-Dependent Tumor Outgrowth in Mice

To determine whether aging-associated alterations in the tissue microenvironment could be conducive to cancer progression, we used an orthotopic model whereby cells derived from EML4-ALK fusion-induced adenomas (EA cells) are directly implanted into lungs of immunocompetent young and old mice [25]. Note that the EA cells were generated in young mice, and thus we are only assessing the impact of the lung environment of the recipient mice. Using this model, we demonstrated an increased tumor growth in lungs of old mice compared to young mice (Figure 6A,B). NLR family pyrin domain containing 3 (NLRP3) inflammasome is a protein complex that mediates the activation of inflammatory mediators [45]. NLRP3 is required for the production of mature IL-1 β and IL-18 [45]. Given that transgenic AAT expression reversed some of the aging-dependent changes in lungs, including increased inflammatory response (Figure 1A, Figure S1A) and that both AAT deficiency and NLRP3 inflammasome contribute to lung inflammation through similar pathways [46, 47], we asked whether NLRP3 deficiency could prevent increased EA cells outgrowth in old mice. We implanted EA cells directly into lungs of young, old, and old *Nlrp3*^{−/−} mice, and observed a slightly subdued EA cells outgrowth in old *Nlrp3*^{−/−} mice compared to wild-type old mice (Figure 6B,C). While we no longer observed increased tumorigenesis in the aged *Nlrp3*^{−/−} mice compared to young mice ($p = 0.78$), we note that the difference between old wild-type and old *Nlrp3*^{−/−} mice was not significant ($p = 0.28$). Thus, the aged lung microenvironment promotes tumor outgrowth, and this effect appears to be potentially dampened by the loss of NLRP3 inflammasome.

areas (as shown) was quantified in young, old, and old AAT mice (ANOVA; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). Functional analysis of RNA-sequencing of lungs from young, old, and old AAT mice using GSEA. Pathways identified as being over-represented in old and restored to youthful levels with AAT expression. (C and D) Hallmark pathway analysis showed upregulated inflammatory response and IL6/JAK-STAT3 signaling in old mouse lungs, and a reversal with AAT overexpression. (E and F) KEGG pathways analysis showed upregulated T-cell receptor signaling pathway and chemokine signaling pathway in old mouse lungs, and a reversal with AAT overexpression.

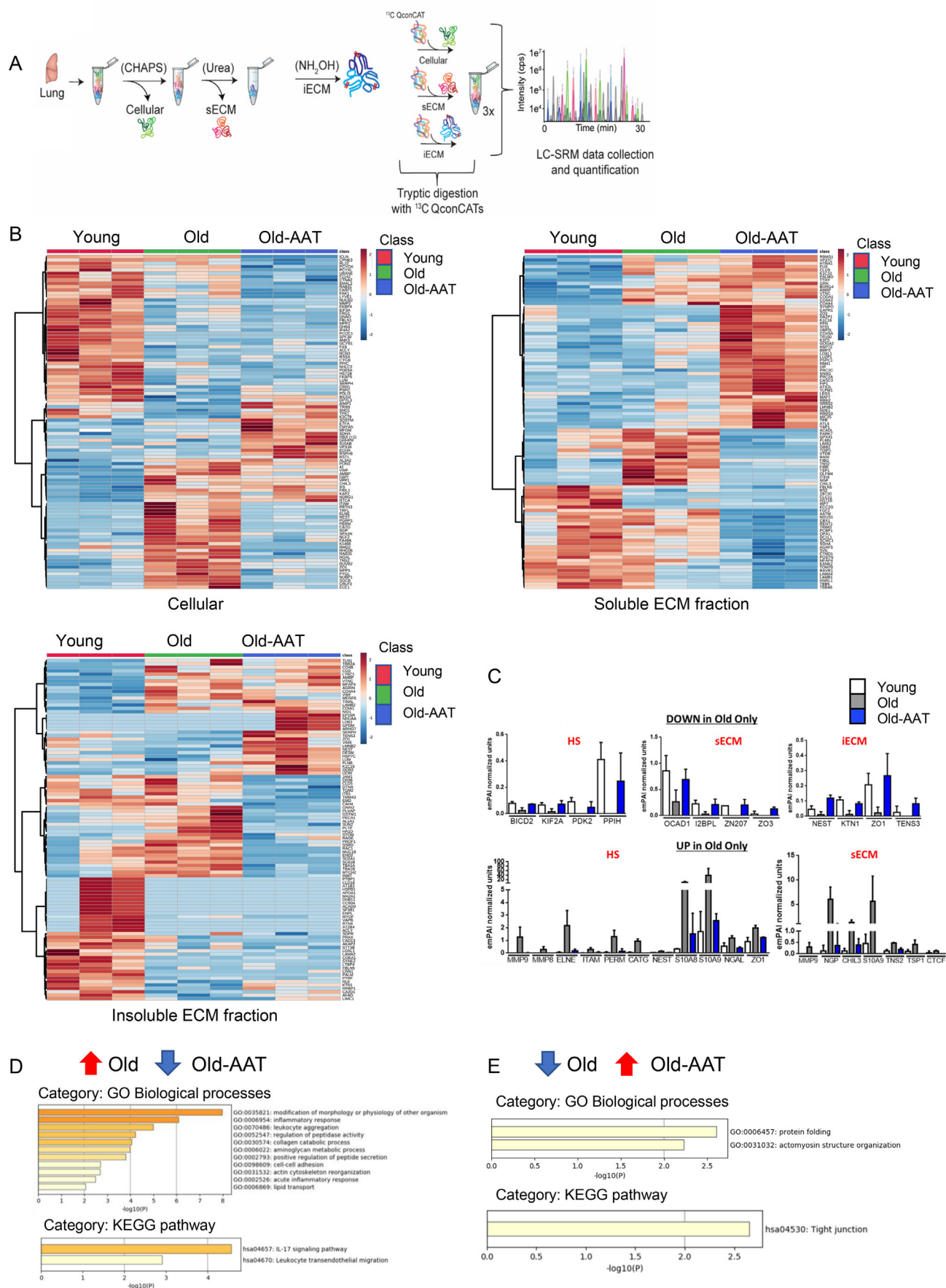


FIGURE 4 | Expression of AAT partially restores tissue microenvironment alterations in aged lung. (A) Schematic of extraction of high salt (HS), soluble ECM (sECM), and insoluble ECM (iECM) fractions for mass spectrometry analysis. Lung tissues from young (3 months), old (24 months), and

4 | Discussion

While it is widely recognized that mutations are crucial for the development of cancer, the role of the microenvironment in dictating the evolutionary trajectories of cancer progression is becoming more apparent. Previous work has demonstrated that under the contexts of microenvironmental insults such as radiation and aging can promote oncogenic selection in the hematopoietic system [18, 48, 49]. The results presented here further demonstrate that aging-associated inflammation can impact tumor progression driven by oncogenic EML4-ALK in the lungs.

Lung cancer, like most other cancers, is an aging-related disease. Aging results in an inflammaging phenotype in the lungs marked by increases of immune cell infiltrates, predominately but not limited to cells involved in the innate immune system [50]. It has previously been shown that in a conditional mouse model of oncogenic Kras^{G12D}, activation of Kras^{G12D} in the lungs [51] led to higher numbers of adenomas in old mice compared to their young counterparts [52]. Based on gene expression analyses of initiated tumors in young and old mice, the authors speculated that tumor formation in the aged lung was in part attributed to attenuated DNA damage and p53 tumor repressor responses, as well as an increased inflammatory milieu. Our data provide evidence for a causative link between the aged microenvironment, increased inflammation, and cancer susceptibility.

Still, recent studies using engineered mouse models of KRas^{G12D} dependent lung tumorigenesis [51, 53] (posted as preprints) demonstrate that old age can actually reduce tumor initiation and growth. These results are consistent with other studies of human lung cancer genomics that suggest that initiating events can occur at very young ages [54]. These oncogenically initiated cells may lie dormant until promoted later in life by the aged tissue microenvironment.

Our studies characterized how aging changes the immune landscape of lungs. We also observed changes in the immune landscape of aged lungs compared to young human lungs through mining the GTEx database. Inflammatory pathways including pathways related to T-cell activation and signaling are enriched in aged lungs relative to young. Using xCell, we observed significant increases in the number of CD4⁺ and CD8⁺ T-cell subtypes, notably an increase in memory T cells. Moreover, aged lungs displayed increases in Tregs and neutrophils, alterations in macrophage subsets, and decreases in dendritic cells. The dysregulation of both innate and adaptive immunity has shown in the lungs of aged individuals [55]. In the elderly, pro-inflammatory cytokines have been shown to be significantly elevated with an increase in immune cell infiltrates involved in innate immunity [56]. Specifically, increases in IL-1 β , IL-6, and TNF- α have been observed [57, 58]. This phenomenon occurs in the apparent

absence of an immunologic threat or infection, which may contribute to the serum proteinase-mediated weakening of the lung parenchyma with old age. In addition, adaptive immunity has also been shown to decline with old age including alterations in the number of naïve and memory CD4⁺ and CD8⁺ T-cells as well as B cells [59, 60]. Systemic increases of Tregs have also been reported with aging [61], further contributing to enhanced susceptibility to infection in the elderly.

In comparing mouse to human lung tissue, we observed similar age-dependent changes in hallmarks of inflammation. Through analyses of microarray data from young and old mouse epithelium, inflammatory (hematopoietic cell lineage, chemokine signaling pathway) and ECM remodeling-related pathways (tight junction, regulation of actin cytoskeleton) were significantly changed in young versus old epithelium, suggesting that the epithelial cells have intrinsic alterations, like the bulk lung tissue. Notably, when we perform immunofluorescence, we observe an increase in immune aggregates in aged lungs, with a major component of T cells. These immune lesions are reminiscent of tertiary lymphoid structures, which have been reported to positively correlate with immunotherapy in cancers [62, 63]; however, the role of these immune aggregates with old age and cancer initiation has yet to be elucidated. Due to the important role of inflammation in cancers [64] and from the inflammatory phenotype in aged lungs observed from our studies, we asked whether aging-associated inflammation may be contributing to oncogenic selection. To this end, we utilized an anti-inflammatory mouse model with transgenic expression of human AAT. Expression of AAT in aged lungs significantly reduced the number of immune aggregates relative to aged, similar to that of young lungs.

Through RNA sequencing, we identified pathways related to inflammation that were upregulated in old lungs, but restored or partially restored to more youthful levels in old lungs expressing AAT. These pathways included inflammatory response, innate immune response, cytokine activity, and chemokine signaling pathways. Upon closer inspection, we found that chemokines including CCL8 and CCL5 were up in old and restored to levels observed in young in the lungs of old-AAT mice. Interestingly, CCL5 has been shown to play a role in the recruitment of neutrophils specifically in the lungs [65]. Additionally, CCL5 and CCL8 have been reported to be chemoattractants for many immune cell types including monocytes, eosinophils and lymphocytes [66]. Future studies will need to explore the possible roles for these and other chemokines in promoting the aging-associated immune cell influx in the lungs of older individuals (whether mouse or human). The ability of AAT expression to prevent some of these immune changes opens up the possibility of developing interventions to mitigate some of these aging-associated changes, with the potential to lessen lung cancer risk.

old-AAT (24 months) mice were analyzed. (B) Heat map of HS, sECM, and iECM of proteomic changes with young, old, and old AAT lung tissue. (C) The top row shows proteins that are significantly reduced in aged lungs but not in young or old-AAT. The bottom row shows proteins that are significantly increased in old lungs. All graphed changes were significant by ANOVA ($p < 0.05$). EmPAI units normalized the number of detected peptides to the total number of detectable peptides per protein, and then to total protein content per sample. (D and E) Metascape analysis was performed using lists of significantly upregulated proteins in old lungs which were significantly downregulated with AAT expression (D) and lists of significantly downregulated proteins in old lungs which were significantly upregulated with AAT expression (E).

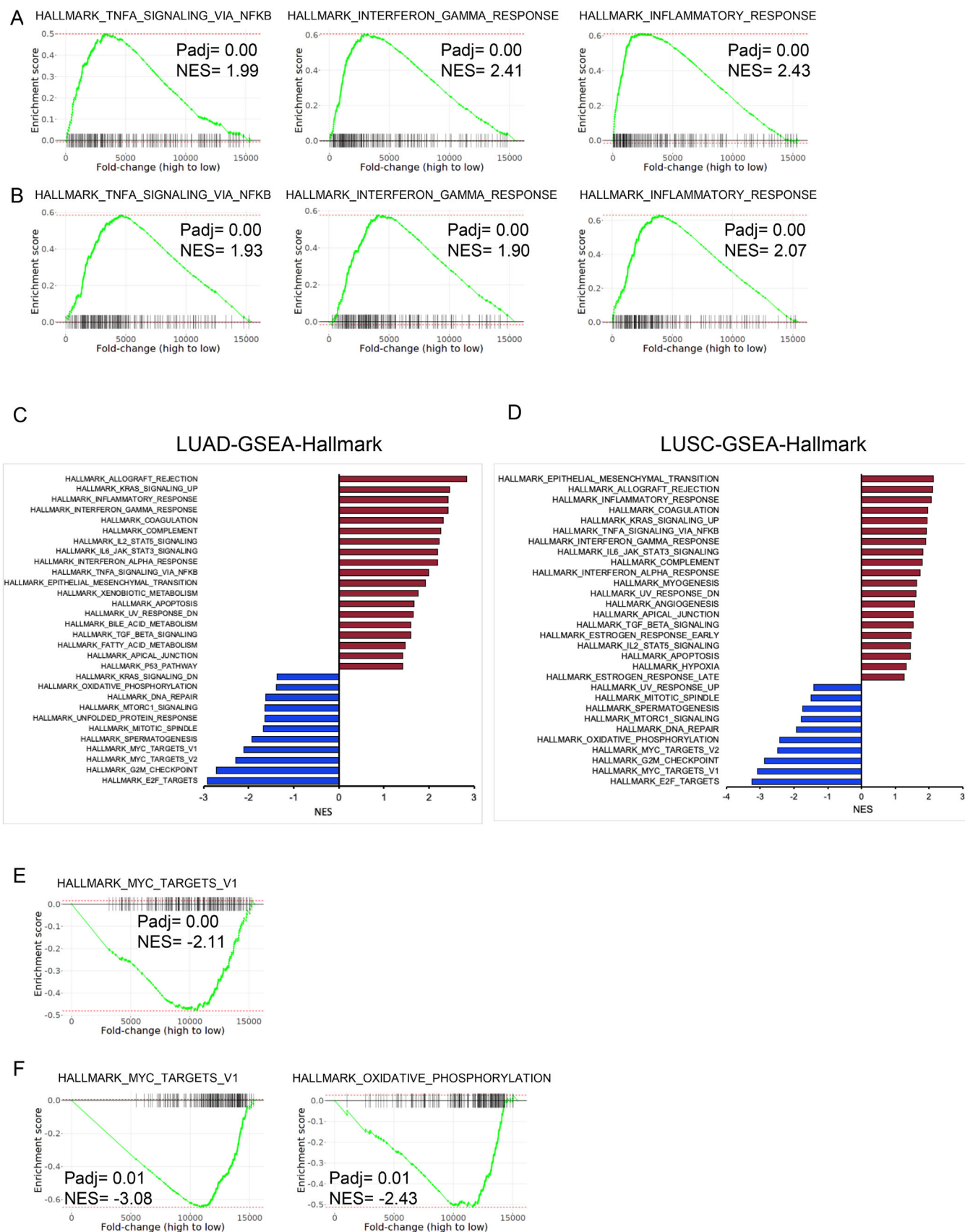


FIGURE 5 | GSEA analyses of the Genome Atlas (TCGA) RNA-seq data from patient-derived lung adenocarcinomas and squamous cell carcinomas revealed a more inflamed microenvironment in elderly patients. (A and C) Hallmark pathway analyses showed upregulated TNFA_Signaling_Via_NFκB

We also performed proteomics to assess alterations in the ECM of the lung tissue microenvironment. We observed dynamic changes in the ECM as seen through the redistribution of tight junction protein, ZO-1, in old lungs which was prevented with

the expression of AAT. The ECM serves not only as a scaffold for the overall tissue architecture but also as a niche that is crucial for determining cell fate [67]. Additionally, key proteases such as MMP9 and ELNE were also found to be elevated in the

pathway, Interferon_Gamma_Response, and Inflammatory_Response in lung adenocarcinomas from patients aged 70–79 compared to those of 40–49. (B and D) Hallmark pathway analyses showed upregulated TNFA_Signaling_Via_NFκB pathway, Interferon_Gamma_Response, and Inflammatory_Response in lung squamous cell carcinomas from patients aged 70–79 compared to those of 50–59. (E and F) Hallmark pathway analyses showed downregulated MYC_TARGETS_V1 in lung adenocarcinoma patients, MYC_TARGETS_V1, and OXIDATIVE_PHOSPHORYLATION in lung squamous cell carcinoma patients with the same age-group comparison in (A) and (B).

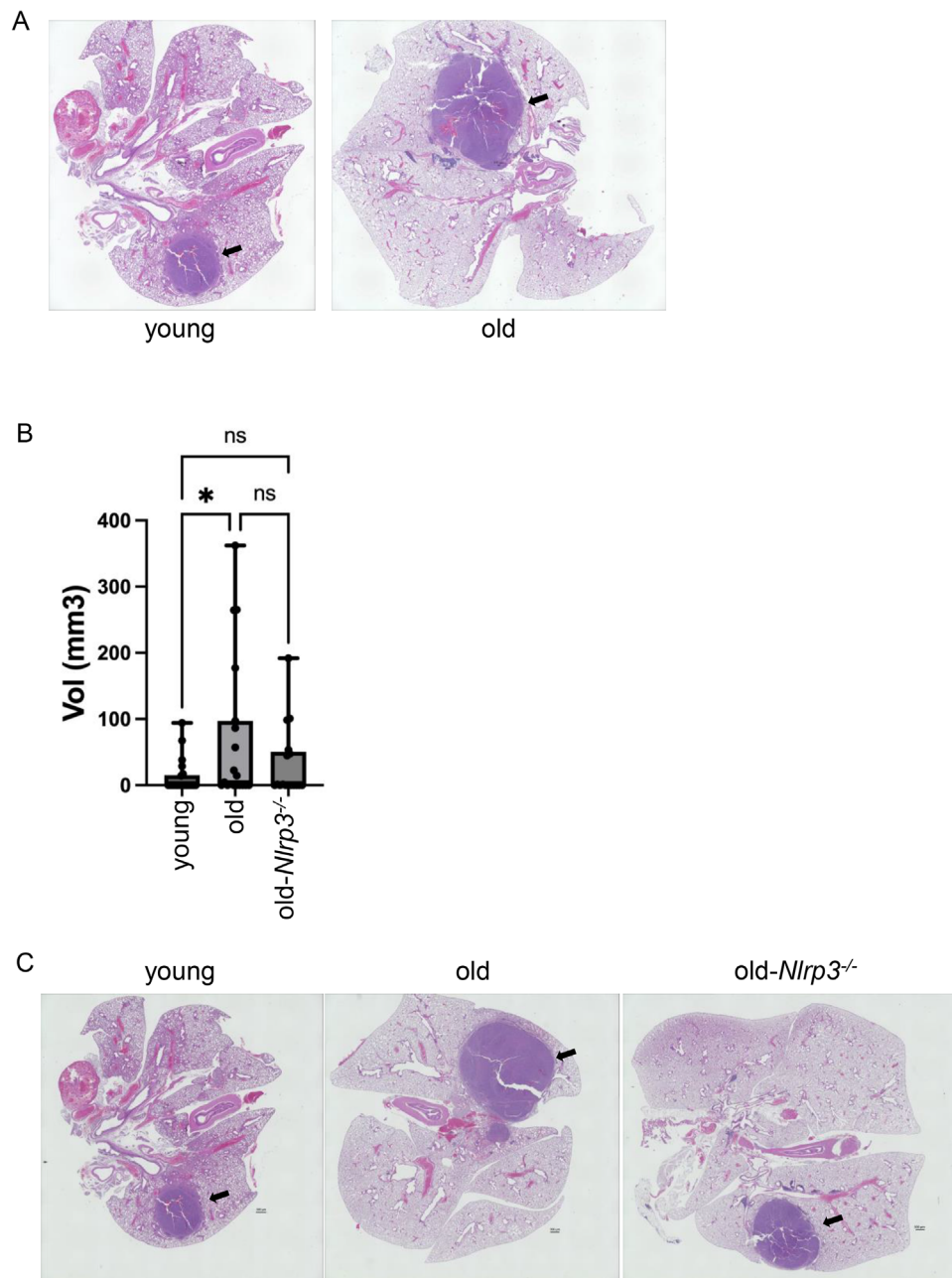


FIGURE 6 | Old age promotes the outgrowth of lung adenoma cells. 2×10^5 EML4-ALK cells per mouse were implanted into the left lungs. Orthotopic tumors established for 4 weeks where mice were imaged using μ CT imaging to determine tumor volumes before harvesting. (A) Images of orthotopic tumors from young and old mouse lungs 4 weeks after the initial implantation. (B and C) Tumor volumes (mm³) and images comparing tumor growth from lungs of young, old, and old *Nlrp3*^{-/-} mice.

HS fraction containing cellular proteins specifically in old lungs and restored to youthful levels with AAT expression. A previous study showed notable changes in inflammatory genes (*Il6*, *Il1b*, *Tnf*, *Il21*, *Il5*) at single cell resolution in the mouse lung [37]. In accordance with these observations, we observed significant differences in inflammatory markers (e.g., S100A8/A9) in old lungs compared to both young and old-AAT indicating that inflammation is a prominent phenotype in aged lungs. Despite being able to separate the HS fraction containing cellular proteins and both the insoluble and soluble ECM fractions, the ECM is complex in its composition and posttranslational modifications. Future studies will be required to determine whether there are differences in protein crosslinking with age and whether the expression of AAT impacts this crosslinking. It is notable that only a subset of the mRNA and protein changes observed in old lungs were reversed by AAT expression. Taken together, our data demonstrate that there is considerable age-dependent remodeling of the tissue architecture of the lung. Furthermore, expression of AAT was able to prevent some of these age-dependent changes.

Future studies will be required to investigate the role of key mediators during inflammation, such as IL-1 α , TNF- α , and IL-6 and whether inhibition of these cytokines in aged mice is sufficient to limit oncogenesis in the lungs. The availability of senolytic therapies (i.e., ABT-263) [68] or anti-inflammatory agents (NLRP3 inhibitor OLT1177) [69] could also potentially serve as therapeutics to modulate the tissue microenvironment to dampen oncogenic adaptation. Since the process of aging is complex, it is unlikely that changing one aspect of aging could completely prevent oncogenesis; however, it is possible to identify significant contributors involved in oncogenic adaptation. In all, our study has revealed marked changes in lung ECM remodeling and inflammation that accompany aging and demonstrates how these changes persist in lung cancers and likely contribute to lung cancer evolution. Importantly, while we are unlikely to be able to prevent many of the mutations that accumulate in our tissues during aging, thus contributing to cancer evolution, we do have the means to manipulate tissue microenvironments to alter cancer evolutionary trajectories.

Author Contributions

Catherine Pham-Danis and Shi B. Chia: Conceptualization (lead); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); project administration (lead); software (equal); supervision (lead); writing—original draft (lead); writing—review and editing (equal). **Hannah A. Scarborough:** Investigation (equal); methodology (equal). **Etienne Danis:** Formal analysis (equal); methodology (equal); software (equal). **Travis Nemkov:** Formal analysis (equal); methodology (equal); software (equal). **Vadym Zaberezhnyy:** mouse colony maintenance and mouse treatments. **Jessica L. Christenson:** Investigation (equal); methodology (equal); project administration (equal). **Emily K. Kleczko:** Methodology (supporting); resources (supporting). **Andre Navarro:** Methodology (equal); resources (equal). **Andrew Goodspeed:** Formal analysis (equal); methodology (equal); software (equal). **Carlo Marchetti:** Resources (supporting). **Kirk Hansen:** Formal analysis (equal); methodology (equal); resources (supporting). **James DeGregori:** Conceptualization (equal); funding acquisition (equal); investigation (equal); resources (equal); supervision (equal); writing—review and editing (equal).

Acknowledgments

The study was supported by grants from the Veteran's Administration to J.D. (1 I01 BX004495). H.A.S. was supported by National Cancer Institute grant T32CA174648. E.A.B. was supported by University of Vermont College of Medicine New Research Initiative Grant. The authors thank the Bioinformatics and Biostatistics, Genomics, Mass Spectrometry and Flow Cytometry Shared Resources supported by National Cancer Institute grant P30CA046934 to the University of Colorado Cancer Center.

Conflicts of Interest

James DeGregori is Editor-in-Chief of the journal and co-author of this article. He was excluded from the peer-review process and all editorial decisions related to the acceptance and publication of this article. Peer-review was handled independently by the other editor-in-chief to minimize bias.

Data Availability Statement

Data availability statement has been included in the Method Sections. Ethics statement has been included in the Method Sections, Mouse Studies subsection.

References

1. J. Oeppen and J. W. Vaupel, "Demography. Broken Limits to Life Expectancy," *Science* 296 (2002): 1029–1031, <https://doi.org/10.1126/science.1069675>.
2. M. Pinti, V. Appay, J. Campisi, et al., "Aging of the Immune System: Focus on Inflammation and Vaccination," *European Journal of Immunology* 46 (2016): 2286–2301, <https://doi.org/10.1002/eji.201546178>.
3. B. Smith, M. A. K. Ryan, D. L. Wingard, T. L. Patterson, D. J. Slymen, and C. A. Macera, "Cigarette Smoking and Military Deployment: A Prospective Evaluation," *American Journal of Preventive Medicine* 35 (2008): 539–546, <https://doi.org/10.1016/j.amepre.2008.07.009>.
4. R. L. Siegel, K. D. Miller, and A. Jemal, "Cancer Statistics, 2018," *CA: A Cancer Journal for Clinicians* 68 (2018): 7–30, <https://doi.org/10.3322/caac.21442>.
5. U. Fagiolo, A. Cossarizza, E. Scala, et al., "Increased Cytokine Production in Mononuclear Cells of Healthy Elderly People," *European Journal of Immunology* 23 (1993): 2375–2378, <https://doi.org/10.1002/eji.1830230950>.
6. G. Sharma and J. Goodwin, "Effect of Aging on Respiratory System Physiology and Immunology," *CIA* 1 (2006): 253–260, <https://doi.org/10.2147/cia.2006.1.3.253>.
7. S. Meiners, O. Eickelberg, and M. Königshoff, "Hallmarks of the Ageing Lung," *European Respiratory Journal* 45 (2015): 807–827, <https://doi.org/10.1183/09031936.00186914>.
8. K. C. Meyer, N. S. Rosenthal, P. Soergel, and K. Peterson, "Neutrophils and Low-Grade Inflammation in the Seemingly Normal Aging Human Lung," *Mechanisms of Ageing and Development* 104 (1998): 169–181, [https://doi.org/10.1016/s0047-6374\(98\)00065-7](https://doi.org/10.1016/s0047-6374(98)00065-7).
9. C. Franceschi, P. Garagnani, P. Parini, C. Giuliani, and A. Santoro, "Inflammaging: A New Immune-Metabolic Viewpoint for Age-Related Diseases," *Nature Reviews Endocrinology* 14 (2018): 576–590, <https://doi.org/10.1038/s41574-018-0059-4>.
10. Y. Sekine, A. Hata, E. Koh, and K. Hiroshima, "Lung Carcinogenesis From Chronic Obstructive Pulmonary Disease: Characteristics of Lung Cancer From COPD and Contribution of Signal Transducers and Lung Stem Cells in the Inflammatory Microenvironment," *General Thoracic and Cardiovascular Surgery* 62 (2014): 415–421, <https://doi.org/10.1007/s11748-014-0386-x>.
11. R. P. Young and R. J. Hopkins, "Link Between COPD and Lung Cancer," *Respiratory Medicine* 104 (2010): 758–759, <https://doi.org/10.1016/j.rmed.2009.11.025>.

12. H. Y. Park, D. Kang, S. H. Shin, et al., "Chronic Obstructive Pulmonary Disease and Lung Cancer Incidence in Never Smokers: A Cohort Study," *Thorax* 75 (2020): 506–509, <https://doi.org/10.1136/thoraxjnl-2019-213732>.
13. L. M. Seijo and J. J. Zulueta *A Key to More Effective Treatment and Screening* (2017), <https://www.cancernetwork.com/view/understanding-links-between-lung-cancer-copd-and-emphysema-key-more-effective-treatment-and>.
14. R. M. Tudor and I. Petrache, "Pathogenesis of Chronic Obstructive Pulmonary Disease," *Journal of Clinical Investigation* 122 (2012): 2749–2755, <https://doi.org/10.1172/JCI60324>.
15. C. Tomasetti and B. Vogelstein, "Cancer Etiology. Variation in Cancer Risk Among Tissues Can be Explained by the Number of Stem Cell Divisions," *Science* 347 (2015): 78–81, <https://doi.org/10.1126/science.1260825>.
16. D. L. Gibbons, L. A. Byers, and J. M. Kurie, "Smoking, p53 Mutation, and Lung Cancer," *Molecular Cancer Research* 12 (2014): 3–13, <https://doi.org/10.1158/1541-7786.MCR-13-0539>.
17. Z. X. Luo, "Transformation and Diversification in Early Mammalian Evolution," *Nature* 450 (2007): 1011–1019, <https://doi.org/10.1038/nature06277>.
18. C. J. Henry, A. Marusyk, and J. DeGregori, "Aging-Associated Changes in Hematopoiesis and Leukemogenesis: What's the Connection?" *Aging (Albany NY)* 3 (2011): 643–656, doi:10.18632/aging.100351.
19. A. I. Rozhok and J. DeGregori, "Toward an Evolutionary Model of Cancer: Considering the Mechanisms That Govern the Fate of Somatic Mutations," *PNAS* 112 (2015): 8914–8921, <https://doi.org/10.1073/pnas.1501713112>.
20. A. Marusyk, C. C. Porter, V. Zaberezhnyy, and J. DeGregori, "Irradiation Selects for p53-Deficient Hematopoietic Progenitors," *PLoS Biology* 8 (2010): e1000324, <https://doi.org/10.1371/journal.pbio.1000324>.
21. Y. Lei, Y. Lei, X. Shi, and J. Wang, "EML4-ALK Fusion Gene in Non-Small Cell Lung Cancer," *Oncology Letters* 24 (2022): 277, <https://doi.org/10.3892/ol.2022.13397>.
22. M. Torres-Durán, A. Ruano-Ravina, I. Parente-Lamelas, et al., "Alpha-1 Antitrypsin Deficiency and Lung Cancer Risk: A Case-Control Study in Never-Smokers," *Journal of Thoracic Oncology* 10 (2015): 1279–1284, <https://doi.org/10.1097/JTO.0000000000000609>.
23. R. Dhami, K. Zay, B. Gilks, S. Porter, J. L. Wright, and A. Churg, "Pulmonary Epithelial Expression of Human Alpha1-Antitrypsin in Transgenic Mice Results in Delivery of Alpha1-Antitrypsin Protein to the Interstitium," *Journal of Molecular Medicine (Berl)* 77 (1999): 377–385, <https://doi.org/10.1007/s001090050364>.
24. M. Kovarova, P. R. Hesker, L. Jania, et al., "NLRP1-Dependent Pyroptosis Leads to Acute Lung Injury and Morbidity in Mice," *Journal of Immunology* 189 (2012): 2006–2016, <https://doi.org/10.4049/jimmunol.1201065>.
25. E. K. Kleczko, T. K. Hinz, T. T. Nguyen, et al., "Durable Responses to Alectinib in Murine Models of EML4-ALK Lung Cancer Requires Adaptive Immunity," *NPI Precision Oncology* 7 (2023): 15, <https://doi.org/10.1038/s41698-023-00355-2>.
26. J. Schindelin, I. Arganda-Carreras, E. Frise, et al., "Fiji: An Open-Source Platform for Biological-Image Analysis," *Nature Methods* 9 (2012): 676–682, <https://doi.org/10.1038/nmeth.2019>.
27. A. S. Barrett, M. J. Wither, R. C. Hill, et al., "Hydroxylamine Chemical Digestion for Insoluble Extracellular Matrix Characterization," *Journal of Proteome Research* 16 (2017): 4177–4184, <https://doi.org/10.1021/acs.jproteome.7b00527>.
28. E. A. Bonney, K. Krebs, J. Kim, et al., "Protective Intranasal Immunization Against Influenza Virus in Infant Mice Is Dependent on IL-6," *Frontiers in Immunology* 11 (2020): 568978, <https://doi.org/10.3389/fimmu.2020.568978>.
29. D. Aran, Z. Hu, and A. J. Butte, "xCell: Digitally Portraying the Tissue Cellular Heterogeneity Landscape," *Genome Biology* 18 (2017): 220, <https://doi.org/10.1186/s13059-017-1349-1>.
30. E. Cerami, J. Gao, U. Dogrusoz, et al., "The cBio Cancer Genomics Portal: An Open Platform for Exploring Multidimensional Cancer Genomics Data," *Cancer Discovery* 2 (2012): 401–404, <https://doi.org/10.1158/2159-8290.CD-12-0095>.
31. M. E. Ritchie, B. Phipson, D. i Wu, et al., "limma Powers Differential Expression Analyses for RNA-Sequencing and Microarray Studies," *Nucleic Acids Research* 43 (2015): e47, <https://doi.org/10.1093/nar/gkv007>.
32. A. Liberzon, A. Subramanian, R. Pinchback, H. Thorvaldsdóttir, P. Tamayo, and J. P. Mesirov, "Molecular Signatures Database (MSigDB) 3.0," *Bioinformatics* 27 (2011): 1739–1740, <https://doi.org/10.1093/bioinformatics/btr260>.
33. P. A. Yushkevich, J. Piven, H. C. Hazlett, et al., "User-Guided 3D Active Contour Segmentation of Anatomical Structures: Significantly Improved Efficiency and Reliability," *NeuroImage* 31 (2006): 1116–1128, <https://doi.org/10.1016/j.neuroimage.2006.01.015>.
34. Y. Zhao, M. Simon, A. Seluanov, and V. Gorbunova, "DNA Damage and Repair in Age-related Inflammation," *Nature Reviews Immunology* 2 (2022): 75–89, <https://doi.org/10.1038/s41577-022-00751-y>.
35. P. Hydbring and L. G. Larsson, "Cdk2: A Key Regulator of the Senescence Control Function of Myc," *Aging (Albany NY)* 2 (2010): 244–250, doi:10.18632/aging.100140.
36. D. R. Green, L. Galluzzi, and G. Kroemer, "Mitochondria and the Autophagy-Inflammation-Cell Death Axis in Organismal Aging," *Science* 333 (2011): 1109–1112, <https://doi.org/10.1126/science.1201940>.
37. I. Angelidis, L. M. Simon, I. E. Fernandez, et al., "An Atlas of the Aging Lung Mapped by Single Cell Transcriptomics and Deep Tissue Proteomics," *Nature Communications* 10 (2019): 963, <https://doi.org/10.1038/s41467-019-08831-9>.
38. D. L. Woodland and M. A. Blackman, "Immunity and Age: Living in the Past?," *Trends in Immunology* 27 (2006): 303–307, <https://doi.org/10.1016/j.it.2006.05.002>.
39. G. John-Schuster, K. Hager, T. M. Conlon, et al., "Cigarette Smoke-Induced iBALT Mediates Macrophage Activation in a B Cell-Dependent Manner in COPD," *American Journal of Physiology Lung Cellular and Molecular Physiology* 307 (2014): L692–706, <https://doi.org/10.1152/ajplung.00092.2014>.
40. C. J. Henry, M. Casás-Selves, J. Kim, et al., "Aging-Associated Inflammation Promotes Selection for Adaptive Oncogenic Events in B Cell Progenitors," *Journal of Clinical Investigation* 125 (2015): 4666–4680, <https://doi.org/10.1172/JCI83024>.
41. E. C. Lewis, "Expanding the Clinical Indications for Alpha(1)-Antitrypsin Therapy," *Molecular Medicine* 18 (2012): 957–970, <https://doi.org/10.2119/molmed.2011.00196>.
42. D. L. Barber, E. J. Wherry, D. Masopust, et al., "Restoring Function in Exhausted CD8 T Cells During Chronic Viral Infection," *Nature* 439 (2006): 682–687, <https://doi.org/10.1038/nature04444>.
43. C. H. Li, S. Haider, and P. C. Boutros, "Age Influences on the Molecular Presentation of Tumours," *Nature Communications* 13 (2022): 208, <https://doi.org/10.1038/s41467-021-27889-y>.
44. K. Chatsirisupachai, T. Lesluyes, L. Paraoan, P. Van Loo, and J. P. De Magalhães, "An Integrative Analysis of the Age-Associated Multi-Omic Landscape Across Cancers," *Nature Communications* 12 (2021): 2345, <https://doi.org/10.1038/s41467-021-22560-y>.
45. Z. Wang, S. Zhang, Y. Xiao, et al., "NLRP3 Inflammasome and Inflammatory Diseases," *Oxidative Medicine and Cellular Longevity* 2020 (2020): 4063562, <https://doi.org/10.1155/2020/4063562>.
46. K. Leszczyńska, D. Jakubczyk, and S. Górka, "The NLRP3 Inflammasome as a New Target in Respiratory Disorders Treatment," *Frontiers*

- in *Immunology* 13 (2022): 1006654, <https://doi.org/10.3389/fimmu.2022.1006654>.
47. N. Kokturk, N. Khodayari, J. Lascano, E. L. Riley, and M. L. Brantly, "Lung Inflammation in Alpha-1-Antitrypsin Deficient Individuals With Normal Lung Function," *Respiratory Research* 24 (2023): 40, <https://doi.org/10.1186/s12931-023-02343-3>.
 48. C. J. Fleenor, A. I. Rozhok, V. Zaberezhnyy, et al., "Contrasting Roles for C/EBPalpha and Notch in Irradiation-Induced Multipotent Hematopoietic Progenitor Cell Defects," *Stem Cells* 33 (2015): 1345–1358, <https://doi.org/10.1002/stem.1936>.
 49. C. J. Henry, A. Marusyk, V. Zaberezhnyy, B. Adane, and J. DeGregori, "Declining Lymphoid Progenitor Fitness Promotes Aging-Associated Leukemogenesis," *PNAS* 107 (2010): 21713–21718, <https://doi.org/10.1073/pnas.1005486107>.
 50. D. A. Mogilenko, I. Shchukina, and M. N. Artyomov, "Immune Ageing at Single-Cell Resolution," *Nature Reviews Immunology* 22 (2022): 484–498, <https://doi.org/10.1038/s41577-021-00646-4>.
 51. E. G. Shuldiner, S. Karmakar, M. K. Tsai, et al., "Aging Represses Lung Tumorigenesis and Alters Tumor Suppression," *BioRxiv* (2024), <https://doi.org/10.1101/2024.05.28.596319>.
 52. N. Parikh, R. L. Shuck, M. Gagea, L. Shen, and L. A. Donehower, "Enhanced Inflammation and Attenuated Tumor Suppressor Pathways Are Associated With Oncogene-Induced Lung Tumors in Aged Mice," *Aging Cell* 17 (2018), <https://doi.org/10.1111/acer.12691>.
 53. X. Zhuang, Q. Wang, S. Joost, et al., "Aging Limits Stemness and Tumorigenesis in the Lung by Reprogramming Iron Homeostasis," *BioRxiv* (2024), <https://doi.org/10.1101/2024.06.23.600305>.
 54. J. J.-K. Lee, S. Park, H. Park, et al., "Tracing Oncogene Rearrangements in the Mutational History of Lung Adenocarcinoma," *Cell* 177 (2019): 1842–1857, <https://doi.org/10.1016/j.cell.2019.05.013>. e1821.
 55. K. C. Meyer, "Aging," *Proceedings of the American Thoracic Society* 2 (2005): 433–439, <https://doi.org/10.1513/pats.200508-081JS>.
 56. J. F. Gradowski, G. S. Mantha, J. L. Hunt, and S. Dacic, "Molecular Alterations in Atypical Adenomatous Hyperplasia Occurring in Benign and Cancer-Bearing Lungs," *Diagnostic Molecular Pathology* 16 (2007): 87–90, <https://doi.org/10.1097/PDM.0b013e318030afde>.
 57. E. J. Kovacs, K. A. Grabowski, L. A. Duffner, T. P. Plackett, and M. S. Gregory, "Survival and Cell Mediated Immunity After Burn Injury in Aged Mice," *Journal of the American Aging Association* 25 (2002): 3–9, <https://doi.org/10.1007/s11357-002-0001-4>.
 58. W. B. Ershler and E. T. Keller, "Age-Associated Increased Interleukin-6 Gene Expression, Late-Life Diseases, and Frailty," *Annual Review of Medicine* 51 (2000): 245–270, <https://doi.org/10.1146/annurev.med.51.1.245>.
 59. M. Provinciali, R. Moresi, A. Donnini, and R. M. Lisa, "Reference Values for CD4+ and CD8+ T Lymphocytes With Naive or Memory Phenotype and Their Association With Mortality in the Elderly," *Gerontology* 55 (2009): 314–321, <https://doi.org/10.1159/000199451>.
 60. H. Song, P. W. Price, and J. Cerny, "Age-Related Changes in Antibody Repertoire: Contribution From T Cells," *Immunological Reviews* 160 (1997): 55–62, <https://doi.org/10.1111/j.1600-065x.1997.tb01027.x>.
 61. S. K. Garg, C. Delaney, T. Toubai, et al., "Aging Is Associated With Increased Regulatory T-Cell Function," *Aging Cell* 13 (2014): 441–448, <https://doi.org/10.1111/acer.12191>.
 62. R. Cabrita, M. Lauss, A. Sanna, et al., "Tertiary Lymphoid Structures Improve Immunotherapy and Survival in Melanoma," *Nature* 577 (2020): 561–565, <https://doi.org/10.1038/s41586-019-1914-8>.
 63. L. Munoz-Erazo, J. L. Rhodes, V. C. Marion, and R. A. Kemp, "Tertiary Lymphoid Structures in Cancer—Considerations for Patient Prognosis," *Cellular & Molecular Immunology* 17 (2020): 570–575, <https://doi.org/10.1038/s41423-020-0457-0>.
 64. L. M. Coussens and Z. Werb, "Inflammation and Cancer," *Nature* 420 (2002): 860–867, <https://doi.org/10.1038/nature01322>.
 65. J. Faget, S. Groeneveld, G. Boivin, et al., "Neutrophils and Snail Orchestrate the Establishment of a Pro-Tumor Microenvironment in Lung Cancer," *Cell Reports* 21 (2017): 3190–3204, <https://doi.org/10.1016/j.celrep.2017.11.052>.
 66. N. Nagarsheth, M. S. Wicha, and W. Zou, "Chemokines in the Cancer Microenvironment and Their Relevance in Cancer Immunotherapy," *Nature Reviews Immunology* 17 (2017): 559–572, <https://doi.org/10.1038/nri.2017.49>.
 67. G. Burgstaller, B. Oehrle, M. Gerckens, E. S. White, H. B. Schiller, and O. Eickelberg, "The Instructive Extracellular Matrix of the Lung: Basic Composition and Alterations in Chronic Lung Disease," *European Respiratory Journal* 50 (2017), <https://doi.org/10.1183/13993003.01805-2016>.
 68. J. Chang, Y. Wang, L. Shao, et al., "Clearance of Senescent Cells by ABT263 Rejuvenates Aged Hematopoietic Stem Cells in Mice," *Nature Medicine* 22 (2016): 78–83, <https://doi.org/10.1038/nm.4010>.
 69. C. Marchetti, B. Swartzwelter, F. Gamboni, et al., "OLT1177, a Beta-Sulfonyl Nitrile Compound, Safe in Humans, Inhibits the NLRP3 Inflammasome and Reverses the Metabolic Cost of Inflammation," *PNAS* 115 (2018): E1530–E1539, <https://doi.org/10.1073/pnas.1716095115>.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.