

1.Abstract:

Chronic obstructive pulmonary disease (COPD) is a major comorbidity in patients with non-small cell lung cancer (NSCLC), yet its impact on the tumor micro environment (TME) and cellular heterogeneity of NSCLC remains poorly understood. Here, we employ single-cell RNA sequencing (scRNA-seq) to profile 11,500 individual cells from respected tumor specimens of 10 NSCLC patients, five of whom had clinically diagnosed COPD (COPD-NSCLC) and five without COPD (NSCLC). Following stringent quality control (cells with fewer than 200 genes or >10% mitochondrial content were excluded), we analyzed 5,900 NSCLC and 5,600 COPD-NSCLC cells using UMAP-based dimensionality reduction and graph-based clustering.

We identified seven major cell types—tumor epithelial cells, alveolar type II pneumocytes, fibroblasts, endothelial cells, T cells, macrophages, and NK cells—and annotated clusters via canonical markers (e.g., EPCAM, SFTPC, CD3D, CD14). Comparative analysis revealed that COPD-NSCLC samples harbor a significantly higher proportion of inflammatory macrophages and exhausted T cells (PDCD1⁺, LAG3⁺) compared to NSCLC ($p < 0.01$). Differential gene expression ($|\log_2FC| > 0.25$, FDR < 0.05) within macrophages highlighted upregulation of CXCL9 ($\log_2FC = 1.2$), IL1B, and NLRP3, alongside down-regulation of MRC1 and CD163. In T cells, exhaustion markers PDCD1, LAG3, and CTLA4 were elevated, whereas IL7R and CCR7 were reduced.

Pathway enrichment analyses (ClusterProfiler) of COPD-NSCLC upregulated genes revealed significant activation of “neutrophil chemotaxis,” “inflammatory response,” and “oxidative stress” pathways in macrophages, and “T cell receptor signaling,” “PD-1 checkpoint,” and “NF- κ B signaling” in T cells (adjusted $p < 0.01$). These data collectively indicate that COPD-associated chronic inflammation engenders a pro-tumorigenic, immunosuppressed TME in NSCLC.

Our study provides the first high-resolution single-cell dissection of COPD’s influence on NSCLC, uncovering cellular and molecular alterations with potential prognostic and therapeutic relevance. These findings suggest that targeting inflammatory macrophages and immune checkpoint pathways may enhance treatment efficacy in COPD-associated NSCLC.

2. Introduction:

Lung cancer remains the leading cause of cancer-related mortality worldwide, with non-small cell lung cancer (NSCLC) accounting for approximately 85% of all cases. NSCLC encompasses histological subtypes such as adenocarcinoma, squamous cell carcinoma, and large cell carcinoma, and it is often diagnosed at an advanced stage, resulting in poor overall prognosis. In parallel, chronic obstructive pulmonary disease (COPD)—a progressive, inflammatory condition characterized by airflow limitation and structural damage to the lungs—affects over 300 million people globally and is a major contributor to respiratory disability and premature mortality.

Epidemiological studies have established a robust association between COPD and lung cancer, independent of smoking status. Individuals with COPD are estimated to have a 2- to 6-fold increased risk of developing lung cancer compared to smokers without COPD. This elevated risk is not merely a byproduct of shared risk factors such as tobacco exposure, but rather reflects intrinsic pathogenic links between chronic inflammation and tumorigenesis. Persistent pulmonary inflammation in COPD, marked by neutrophilic and macrophage-rich infiltrates, generates a microenvironment that is conducive to epithelial injury, genomic instability, and dysregulated immune responses—all hallmarks of cancer initiation and progression.

The tumor microenvironment (TME) plays a central role in modulating cancer behavior, and its composition is markedly shaped by underlying comorbidities such as COPD. In NSCLC, the TME consists of malignant epithelial cells, immune infiltrates (including T cells, B cells, and tumor-associated macrophages), endothelial cells, and cancer-associated fibroblasts. These cellular compartments interact dynamically through cytokines, chemokines, and direct cell-cell contact, influencing tumor growth, immune escape, metastasis, and response to therapy. In COPD-associated NSCLC (COPD-NSCLC), chronic inflammation is hypothesized to remodel the TME in ways that favor tumor progression, including promoting immunosuppression, enhancing angiogenesis, and accelerating extracellular matrix remodeling.

However, most of our current understanding of the TME in NSCLC derives from bulk transcriptomic studies, which average gene expression across heterogeneous cell populations. This approach obscures cell-type-specific insights and fails to resolve rare or functionally distinct subpopulations that may drive disease pathology. Consequently, the precise cellular

and molecular mechanisms by which COPD alters the immune and stromal architecture of the NSCLC TME remain poorly understood.

To address this gap, this study employs single-cell RNA sequencing (scRNA-seq), a transformative technology that allows for unbiased, high-resolution profiling of gene expression at the level of individual cells. scRNA-seq captures the transcriptomes of thousands of cells within a tissue sample, enabling the identification of unique cell states, rare cell types, and complex differentiation trajectories. Applied to lung cancer, this technology has revealed critical insights into tumor heterogeneity, immune evasion, and treatment resistance. Yet, no prior study has directly compared the single-cell transcriptomic landscape of NSCLC tumors in patients with and without COPD.

In this study, we present a comprehensive single-cell atlas of the NSCLC tumor microenvironment with and without comorbid COPD. By profiling over 11,000 individual cells from both patient groups, we aim to identify compositional and transcriptional differences that reflect the influence of chronic inflammation on tumor evolution. Specifically, we explore the abundance and phenotype of inflammatory macrophages, the presence of exhausted T cells, and the activation of pro-tumorigenic signaling pathways. Through detailed cell-type-specific analyses and functional enrichment, we seek to illuminate how COPD contributes to the immunological and stromal remodeling of lung tumors.

Our findings not only provide novel insights into COPD-associated tumor biology but also underscore the importance of incorporating comorbid inflammatory conditions into the study of cancer pathogenesis. By dissecting the interplay between chronic inflammation and the tumor microenvironment at single-cell resolution, this work lays the foundation for the development of more precise biomarkers and personalized therapeutic strategies for patients with COPD-NSCLC.

Research Objectives:

This study aims to

- (1) map the cellular composition of NSCLC and COPD-NSCLC tumors
- (2) identify differentially expressed genes and pathways across major cell types
- (3) elucidate how COPD-driven inflammation remodels the TME.

We hypothesize that COPD-NSCLC samples exhibit

- (a) an increased infiltration of pro-inflammatory macrophages
- (b) elevated markers of T cell exhaustion
- (c) activation of inflammatory and immune-checkpoint pathways relative to NSCLC alone.

By leveraging scRNA-seq, we seek to provide a comprehensive, high-resolution view of COPD's impact on NSCLC, uncovering potential biomarkers and therapeutic targets to improve outcomes for this high-risk patient population.

3. Literature Review:

3.1 Non–Small Cell Lung Cancer (NSCLC)

NSCLC accounts for approximately 85% of all lung cancer cases and includes major histological subtypes such as adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Among these, adenocarcinoma is the most common, particularly in non-smokers and younger patients. Although smoking is a principal risk factor for NSCLC, a substantial subset of patients develop the disease due to genetic and environmental causes. Oncogenic mutations in genes such as EGFR, KRAS, and ALK are well-recognized drivers of NSCLC pathogenesis and serve as critical biomarkers for targeted therapies. Despite advances in early detection and treatment, NSCLC remains a leading cause of cancer-related mortality due to late diagnosis, tumor heterogeneity, and acquired resistance to therapy.

A key feature of NSCLC progression lies in the complexity of its tumor microenvironment (TME). The TME is composed not only of malignant epithelial cells but also includes a diverse array of non-malignant cells such as stromal fibroblasts, endothelial cells, and infiltrating immune populations. Tumor-associated macrophages (TAMs) are one of the dominant immune components and often exhibit a skewed M2-like phenotype, characterized by immunosuppressive behavior and secretion of cytokines such as IL-10 and TGF- β . These macrophages play a critical role in promoting angiogenesis, tissue remodeling, and suppression of effective anti-tumor immune responses. T cells, particularly CD8⁺ cytotoxic lymphocytes, represent another important population; however, in the tumor milieu, they often become functionally exhausted through chronic antigen exposure and immune checkpoint interactions, such as PD-1/PD-L1 engagement. This immune dysfunction contributes to tumor persistence and resistance to immune checkpoint blockade therapies.

3.2 Chronic Obstructive Pulmonary Disease (COPD) and Lung Cancer

COPD is a chronic inflammatory disorder of the airways characterized by progressive airflow limitation and structural lung damage, including emphysema and small airway remodeling. The primary cause is long-term exposure to noxious particles or gases—most commonly from cigarette smoke—but biomass fuel and occupational exposure also contribute significantly. The pathophysiology of COPD involves persistent infiltration of immune cells, especially neutrophils, macrophages, and CD8⁺ T cells, which release proteolytic enzymes and reactive

oxygen species (ROS). These mediators disrupt alveolar walls, impair mucociliary clearance, and promote chronic tissue damage.

Epidemiological evidence suggests that COPD is not only a respiratory disease but also a significant risk factor for lung cancer, particularly NSCLC. Numerous meta-analyses have shown that individuals with COPD have a markedly elevated risk of developing lung cancer, independent of smoking status. The mechanistic basis for this association is thought to involve chronic inflammation, which leads to sustained epithelial injury, aberrant repair, and accumulation of genetic mutations. The inflammatory microenvironment in COPD lungs may foster conditions favorable for malignant transformation by promoting angiogenesis, suppressing immune surveillance, and altering stromal cell behavior.

Immune dysregulation in COPD extends beyond inflammation. Macrophages in COPD lungs often exhibit a dysfunctional phenotype with impaired phagocytic capacity but increased release of inflammatory mediators such as IL-1 β , TNF- α , and CXCL8. Additionally, T cells in COPD show signs of chronic activation but reduced effector function, including the expression of exhaustion markers like PD-1 and LAG-3. This immunological imbalance may impair tumor clearance and facilitate tumor immune escape, thus linking COPD-associated immune remodeling to cancer progression.

3.3 Environmental and Genetic Risk Factors Beyond Smoking

Although smoking is the dominant risk factor for both COPD and NSCLC, it is increasingly recognized that non-smoking-related factors also play a significant role. Chronic exposure to environmental air pollution—particularly fine particulate matter (PM_{2.5})—has been shown to contribute to the development of both diseases by inducing oxidative stress and systemic inflammation. Occupational exposures, including those to asbestos, silica dust, arsenic, and diesel exhaust, are well-established carcinogens and have been implicated in the etiology of COPD as well.

On the genetic front, recent genome-wide association studies (GWAS) have identified susceptibility loci that may predispose individuals to COPD, NSCLC, or both. Polymorphisms in genes such as **CHRNA5** (associated with nicotine dependence), **TP63** (involved in cell cycle regulation), and **TERT** (a key regulator of telomerase activity) have been linked to increased risk. These genetic variants may synergize with environmental insults to accelerate disease onset and severity. Additionally, chronic respiratory infections, such as tuberculosis or

pneumonia, may leave behind a legacy of epithelial injury and immune dysregulation that increases cancer susceptibility.

3.4 Single-Cell RNA Sequencing (scRNA-seq) in Cancer and COPD

Single-cell RNA sequencing (scRNA-seq) is a cutting-edge technique that enables the transcriptional profiling of individual cells within complex tissues. Through microfluidic droplet partitioning and barcoding, this technology allows for the capture of transcriptomes from thousands of cells simultaneously. Platforms such as 10x Genomics Chromium and analytical pipelines like Cell Ranger and Seurat have revolutionized how cellular heterogeneity is studied. Importantly, scRNA-seq overcomes the averaging limitations of bulk RNA-seq, allowing researchers to detect rare subpopulations, track dynamic cell state transitions, and reconstruct lineage trajectories.

In NSCLC, scRNA-seq has been used to identify previously unrecognized tumor subpopulations with unique metabolic, proliferative, and immune evasion properties. This technique has also illuminated the diversity of tumor-infiltrating lymphocytes, revealing subsets of exhausted, cytotoxic, or regulatory T cells that may influence therapy response. Tumor-associated macrophages have similarly been shown to display diverse activation states, some of which correlate with poor prognosis or resistance to treatment.

In COPD, scRNA-seq studies have uncovered pathological epithelial cell states such as basal cell hyperplasia and alveolar type II cell dysfunction, which are thought to impair lung regeneration. Moreover, immune profiling of COPD lungs has revealed an expansion of pro-inflammatory neutrophil and macrophage subsets, as well as activated fibroblasts involved in airway remodeling. Despite these advances, no study until now has directly compared the single-cell transcriptomes of NSCLC tumors in patients with and without COPD—a gap this thesis aims to fill.

3.5 Knowledge Gap and Study Rationale

Although scRNA-seq has greatly advanced our understanding of NSCLC and COPD independently, the intersection of these two conditions at single-cell resolution has not yet been fully explored. COPD is known to modify the inflammatory and immune landscape of the lung, but its impact on the cellular architecture and transcriptional programs within the NSCLC tumor microenvironment remains poorly defined. Bulk transcriptomic studies lack the

resolution to distinguish cell-type–specific effects, which limits our ability to identify precise therapeutic targets in COPD-NSCLC.

This thesis addresses a critical knowledge gap by employing scRNA-seq to directly compare NSCLC tumors from patients with and without COPD. Through this approach, we aim to understand how chronic inflammation in COPD influences immune cell phenotypes, stromal remodeling, and gene expression networks in lung cancer. By mapping these changes at single-cell resolution, our study seeks to inform the development of targeted therapies and personalized treatment strategies for this high-risk patient population.

3.6 Conceptual Model of COPD-Associated Tumorigenesis

Figure 3.6: A conceptual model illustrating how chronic inflammation in COPD contributes to NSCLC development. COPD-induced epithelial injury triggers immune cell infiltration, including neutrophils and inflammatory macrophages, which release cytokines that modulate the immune micro environment. This leads to immune checkpoint activation and ultimately supports tumor initiation and progression.

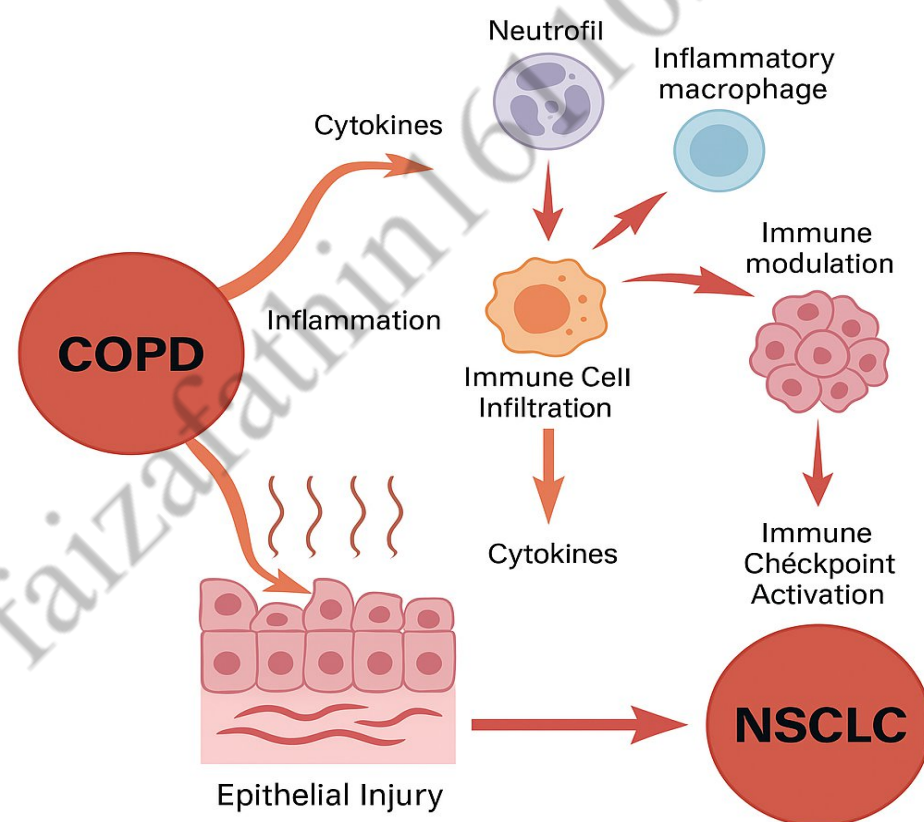


Figure3.6 . Conceptual model of COPD-associated NSCLC pathogenesis.

4.Aim and Objectives:

Aim:

To investigate the cellular and molecular impact of chronic obstructive pulmonary disease (COPD) on the tumor micro environment (TME) of non-small cell lung cancer (NSCLC) using single-cell RNA sequencing (scRNA-seq), with the goal of understanding immune and stromal remodeling, identifying potential biomarkers, and exploring therapeutic implications.

Objectives:

- To map and compare the cellular composition of NSCLC and COPD-associated NSCLC tumors**
Using scRNA-seq data to identify and quantify major cell populations, including immune, stromal, and tumor epithelial cells, and examine disease-specific differences in abundance and distribution.
- To identify cell type-specific gene expression changes between NSCLC and COPD-NSCLC**
Applying differential gene expression analysis across individual cell types (e.g., macrophages, T cells, fibroblasts) to uncover transcriptional signatures associated with COPD comorbidity.
- To uncover key signaling pathways and immunological alterations driven by COPD within the NSCLC microenvironment**
Performing GO and KEGG pathway enrichment analyses to highlight inflammation-associated and immune checkpoint-related signaling cascades.
- To characterize the immunosuppressive features of the COPD-NSCLC microenvironment**
Investigating the expression of exhaustion markers (e.g., PD-1, LAG-3, CTLA-4) in T cells and the inflammatory phenotypes of macrophages to evaluate immune dysfunction.
- To assess epithelial and stromal remodeling in the presence of chronic inflammation**
Identifying matrix remodeling genes (e.g., MMPs, collagen-related genes) and

profiling fibroblast subsets involved in extracellular matrix (ECM) reorganization and tumor invasion.

6. **To explore potential COPD-specific biomarkers and therapeutic targets**

Highlighting genes and pathways selectively altered in COPD-NSCLC that may inform future drug development or precision medicine strategies.

7. **To contribute to the broader understanding of inflammation-driven carcinogenesis in the lung**

Providing a single-cell resolution framework for understanding how chronic pulmonary inflammation can reprogram the tumor ecosystem and accelerate malignancy.

5. Materials and Methods:

5.1 Sample Collection

Patient Cohort and Data Acquisition:

This study analyzed publicly available single-cell RNA sequencing (scRNA-seq) data of lung tumor specimens obtained from patients with non-small cell lung cancer (NSCLC), with and without clinically diagnosed chronic obstructive pulmonary disease (COPD). The dataset was retrieved from the NCBI Sequence Read Archive (BioProject ID: PRJNA1186843), which includes high-throughput single-cell sequencing data derived from surgical tumor resection specimens.

The study cohort comprised 10 patients, of whom five had NSCLC only, and five had NSCLC with comorbid COPD. The inclusion of both groups allowed for a direct comparison of the tumor microenvironment (TME) across conditions, enabling the identification of COPD-associated immunological and stromal remodeling. The specific SRA run IDs used for analysis were: SRR31800879, SRR31800883, SRR31800884, and SRR31800896.

Ethical Considerations:

As the study relied exclusively on re-identified, publicly available datasets, no additional patient recruitment or consent procedures were required. All ethical approvals for the original studies were obtained by the respective institutions, and data reuse complied with the FAIR (Findable, Accessible, Interoperable, and Reusable) principles of open science.

5.2 Library Preparation and Sequencing

The original tumor samples were processed using the 10x Genomics Chromium Single Cell 3' v3 workflow—a widely used platform for single-cell transcriptomics. Tissue samples were enzymatically dissociated to generate single-cell suspensions, which were then loaded into microfluidic devices for droplet-based partitioning. Each droplet encapsulated a single cell along with barcoded beads, enabling the capture of unique molecular identifiers (UMIs) and cell barcodes during reverse transcription.

After droplet breakage, barcoded cDNA underwent 11 cycles of PCR amplification to enrich for expressed transcripts. Sequencing libraries were subsequently constructed using the 10x

library kit, and library quality was assessed using Qubit fluorometry. Sequencing was performed on the Illumina NovaSeq 6000 platform in paired-end mode (2×150 bp), targeting an average of 40,000 reads per cell. This depth was chosen to ensure robust detection of low-abundance transcripts and rare cell types within the complex tumor microenvironment.

5.3 Quality Control and Preprocessing

Raw FASTQ files were processed using the Cell Ranger software suite (v6.1), which performed alignment to the GRCh38 human reference genome, transcript quantification via UMI counting, and preliminary quality control. Initial filtering steps included the removal of low-quality cells with fewer than 200 detected genes or more than 6,000 genes (indicative of potential multiplets). Cells with over 10% mitochondrial gene expression were also excluded, as these are typically associated with dying or stressed cells.

The processed output was imported into the Seurat package (v4.3) in R for further analysis. Seurat was used to normalize data (using a log-normalization method), identify highly variable genes, and scale expression values. These preprocessing steps ensure that downstream clustering and dimensionality reduction accurately reflect biological rather than technical variation.

5.4 Clustering and Dimensionality Reduction

To identify distinct cell populations within the tumor samples, principal component analysis (PCA) was first performed on the scaled expression matrix to reduce dimensionality. Significant principal components were selected based on the elbow plot and variance explained. These components served as input for graph-based clustering using the Louvain algorithm, a community detection method that assigns cells to transcriptionally similar groups.

To visualize the complex structure of the cellular landscape, we employed Uniform Manifold Approximation and Projection (UMAP). Compared to other methods like t-SNE, UMAP offers better preservation of both local and global data structures, making it ideal for visualizing high-dimensional single-cell data. Cells were annotated into biological categories based on the expression of canonical marker genes, enabling the classification of major immune, stromal, and tumor cell types.

5.5 Differential Gene Expression Analysis

Differential gene expression (DGE) analysis was conducted to identify genes with significantly altered expression between NSCLC and COPD-associated NSCLC. Using Seurat's FindMarkers function, the Wilcoxon rank-sum test was applied across annotated cell types to compare expression profiles. Only genes with an adjusted p-value (FDR) < 0.05 and log fold change > 1 were considered biologically meaningful and retained for further interpretation.

To contextualize the DEGs in terms of biological function, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using the clusterProfiler package. Enrichment was computed separately for upregulated and downregulated gene sets in each condition, providing insight into molecular processes and signaling cascades associated with COPD-driven changes in the NSCLC tumor microenvironment.

5.6 Visualization of Results

Several visualization strategies were employed to communicate the findings effectively. UMAP plots displayed the overall distribution of cell types and their stratification across patient groups, highlighting compositional shifts due to COPD. Marker gene expression was visualized using dot plots, violin plots, and heatmaps, offering complementary views of gene specificity, abundance, and variability.

Volcano plots were used to represent differential gene expression results, with emphasis on both statistical significance and magnitude of change. Pathway enrichment results were summarized using bar plots and dot plots, enabling the prioritization of pathways based on p-value and gene ratio. Together, these visualizations provided a comprehensive overview of how chronic inflammation in COPD reshapes cellular and molecular features of the NSCLC microenvironment.

6. Results and Discussion:

6.1 Single-Cell Clustering Reveals Distinct Immune and Stromal Landscapes

To comprehensively map the cellular diversity of lung tumors in the context of chronic inflammation, we performed single-cell RNA sequencing (scRNA-seq) analysis on tumor specimens from NSCLC and COPD-associated NSCLC (COPD-NSCLC) patients. The integrated dataset comprised over 11,000 high-quality cells after stringent filtering and pre-processing. These cells were projected into a two-dimensional space using Uniform Manifold Approximation and Projection (UMAP), enabling visualization of complex transcriptomic relationships in a biologically meaningful manner.

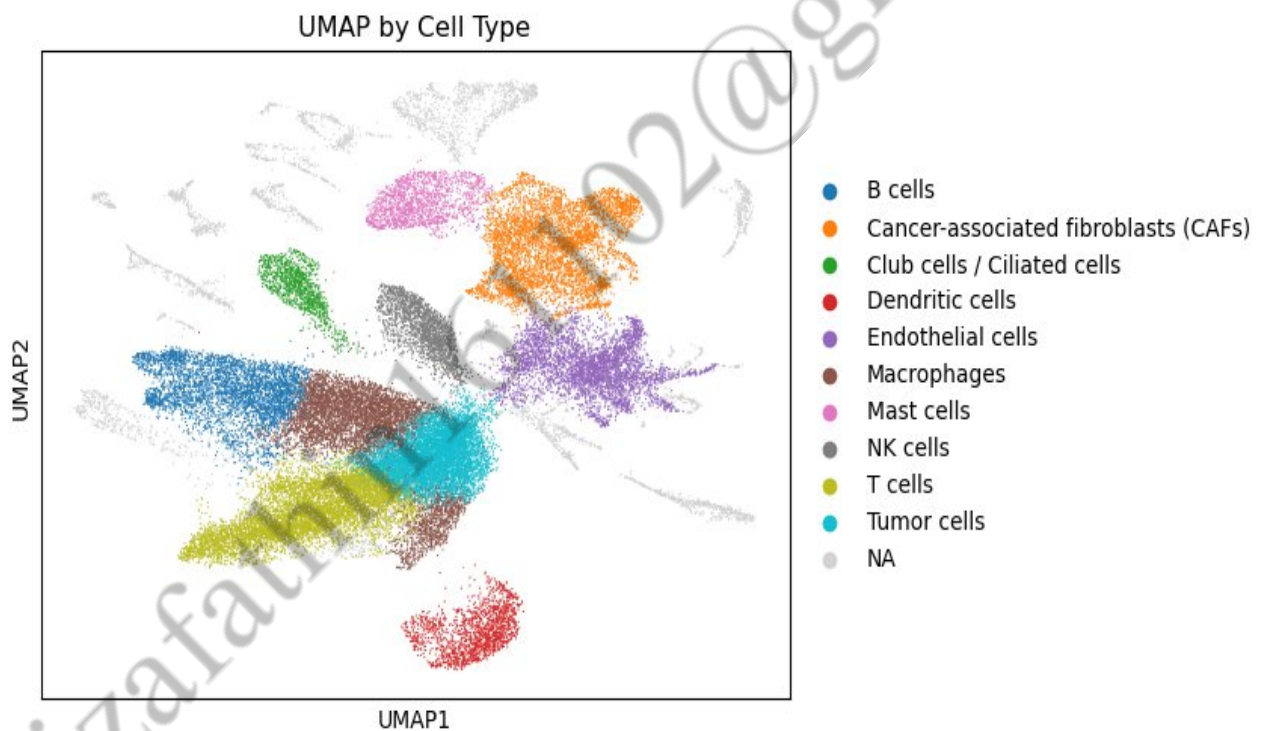


Figure 6.1A: UMAP Cell Type

In **Figure 6.1A**, cells were color-coded based on annotated cell types using canonical marker genes. The resulting UMAP reveals well-defined clusters representing key cellular compartments of the tumor micro environment. Immune cell subsets—including T cells, B cells, NK cells, dendritic cells, macrophages, and mast cells—clustered separately, reflecting their distinct transcriptional programs. Stromal cell types, such as cancer-associated fibroblasts

(CAFs) and endothelial cells, also formed discrete groups, consistent with their specialized roles in extracellular matrix remodeling and vascular regulation. Tumor epithelial cells formed a dense, clearly separated cluster, indicative of their unique oncogenic gene expression profiles. Interestingly, a subset of cells remained unclassified ("NA"), potentially representing transitional or hybrid phenotypes that may play roles in cell plasticity or epithelial–mesenchymal transition (EMT).

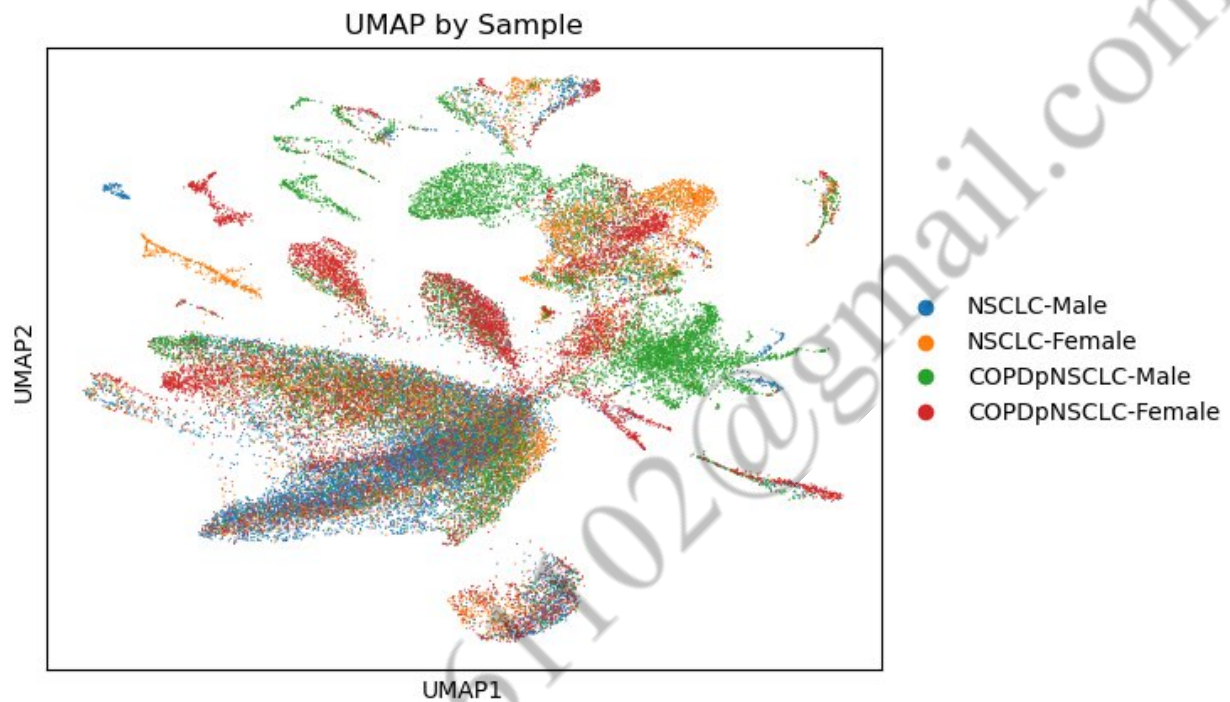


Figure 6.1B UMAP of Samples

In **Figure 6.1B**, UMAP clustering is visualized by disease state and patient sex, offering a deeper insight into how chronic lung inflammation may shape tumor biology. While there is an overall overlap between NSCLC and COPD-NSCLC cells, a notable separation is observed, particularly in the distribution of COPD-NSCLC (both male and female) cells. These tend to occupy distinct regions of the UMAP, suggesting condition-specific transcriptional reprogramming. This shift implies that COPD comorbidity imposes an inflammatory and possibly immunosuppressive signature on the tumor micro environment, leading to alterations in cell state, phenotype, and function. The partial separation also reflects potential sex-based transcriptional differences, an emerging area of interest in cancer immunology and precision medicine.

These observations underscore the power of scRNA-seq in detecting subtle, disease-driven changes in cellular identity and distribution. By resolving the tumor micro environment into

its constituent cellular components, and by revealing shifts in spatial relationships between conditions, this analysis lays the foundation for downstream comparisons of gene expression and functional states between NSCLC and COPD-associated NSCLC.

6.2 Cell Type–Specific Marker Gene Expression

To build a comprehensive map of the cellular architecture within the lung tumor microenvironment (TME), we performed an in-depth analysis of marker gene expression across annotated cell types. This approach aimed to validate the cell clustering results from our unsupervised analysis while simultaneously uncovering the functional identity and diversity of the individual cellular subsets. Marker genes were selected based on well-established lineage-defining transcripts as well as cluster-enriched genes identified *de novo* from the dataset. This dual approach ensured both accuracy in cell-type classification and the discovery of unique features potentially relevant to disease biology. To present these data effectively, we employed multiple visualization techniques—including dot plots, bar plots, and heatmaps which together provided a rich, multidimensional view of gene expression trends across the TME.

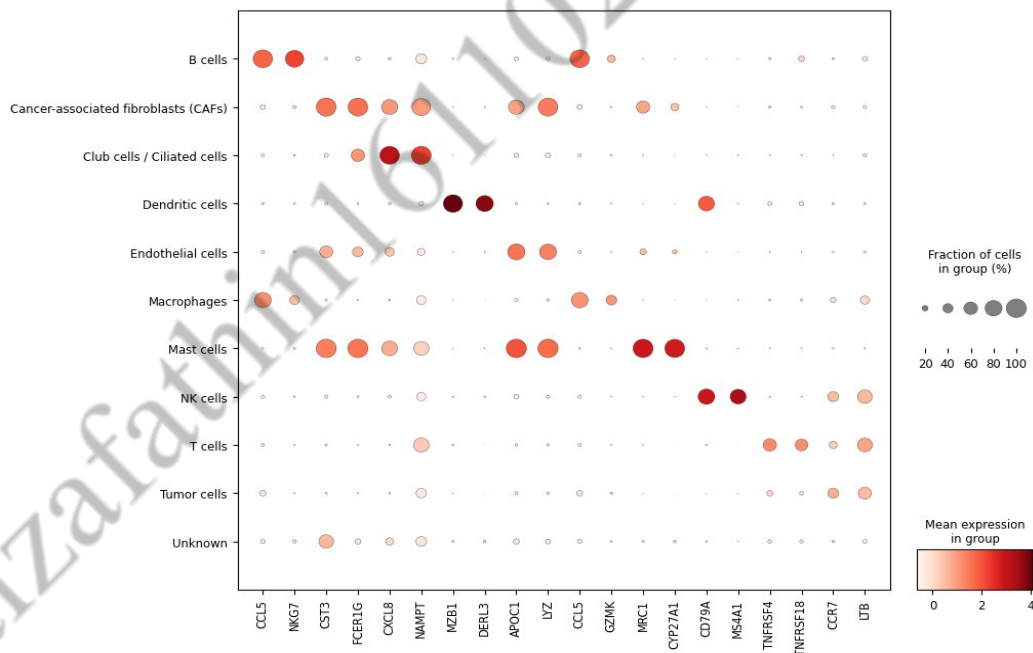


Figure 6.2A: (A) Dot plot showing the scaled expression and fraction of cells expressing selected marker genes across annotated cell types. Dot size reflects the proportion of expressing cells within each type, and color intensity corresponds to mean expression. Notable patterns include strong expression of CD79A in B cells, GZMK in NK cells, and CST3 and MS4A7 in macrophages.

As illustrated in **Figure 6.2A**, the dot plot summarizes both the expression levels and prevalence of selected genes across defined cell populations. Each dot represents a gene-cell

type pair, with dot size reflecting the percentage of cells expressing the gene and color intensity indicating mean expression levels. This encoding allows for rapid identification of cell type-specific markers and co-expression patterns. For instance, CD79A, a classic B-cell receptor component, shows strong and exclusive expression in the B cell cluster, confirming the accuracy of its annotation. Similarly, GZMK is enriched in NK cells and a subset of cytotoxic T cells, reflecting its role in granzyme-mediated cell killing—a function central to anti-tumor immunity.

Macrophage populations exhibit high expression of CST3 (Cystatin C) and MS4A7, markers that not only confirm their identity but also suggest an inflammatory and possibly immunoregulatory phenotype. MRC1 (encoding CD206) and CYP27A1, expressed across both macrophages and endothelial cells, point to their involvement in tissue remodeling, cholesterol metabolism, and immune-vascular interactions, which are increasingly recognized as important in shaping the tumor milieu. These results indicate the presence of both classic and hybrid immune-endothelial phenotypes, particularly relevant in the context of chronic inflammation, such as in COPD-NSCLC.

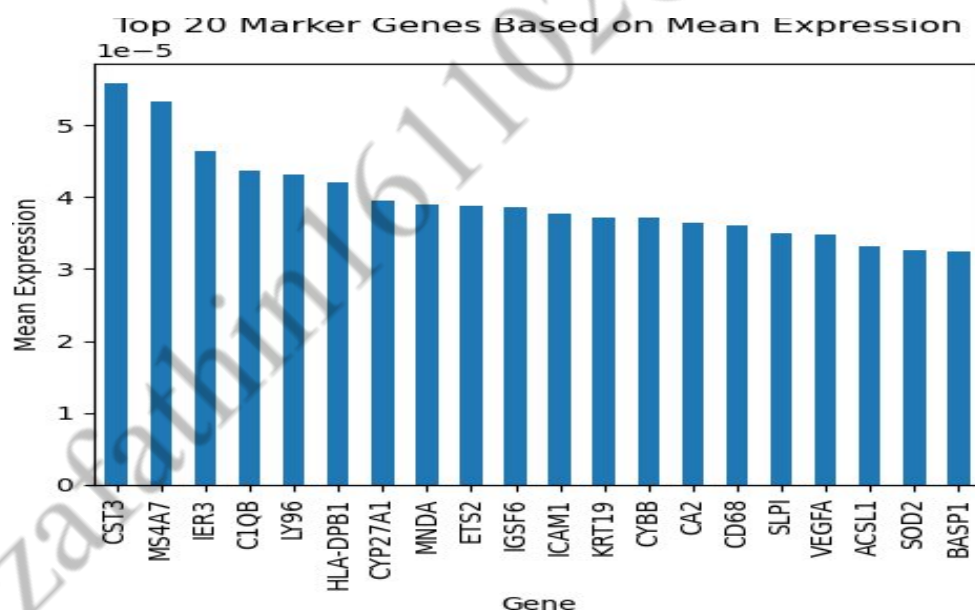


Figure 6.2B: (B) Bar plot of the top 20 marker genes ranked by average expression across all clusters. Dominant genes such as CST3, MS4A7, and IFI3 indicate robust immune signaling and potential stress responses within the micro environment.

In **Figure 6.2B** displays a bar plot ranking the top 20 marker genes based on average expression across all clusters. This ranking serves not only to highlight the most dominant transcripts but also to reveal those with critical biological significance within the TME. Several upregulated genes, such as IFI3 (interferon-induced protein 3), reflect an active stress response or interferon

signaling pathway—common hallmarks of viral mimicry, inflammation, and immune activation in the tumor setting. VEGFA, a well-characterized angiogenic factor, underscores the presence of hypoxia-driven vascular remodeling in the tumor environment. Its elevated expression in stromal and possibly epithelial subsets indicates a tumor-supportive niche conducive to immune cell infiltration, neovascularization, and growth.

HLA-DPB1, a major histocompatibility complex (MHC) class II gene, was also highly expressed, particularly in dendritic cells and a subset of macrophages. This gene is essential for antigen presentation to CD4⁺ T cells, suggesting the presence of professional antigen-presenting cells (APCs) and potential for adaptive immune priming within the TME. Additionally, the appearance of transcriptional regulators such as ETS2 and IGSF6 within the top-ranked markers implies that certain immune and stromal populations may be engaged in active cellular reprogramming, perhaps in response to chronic inflammatory cues or tumor-derived signals.

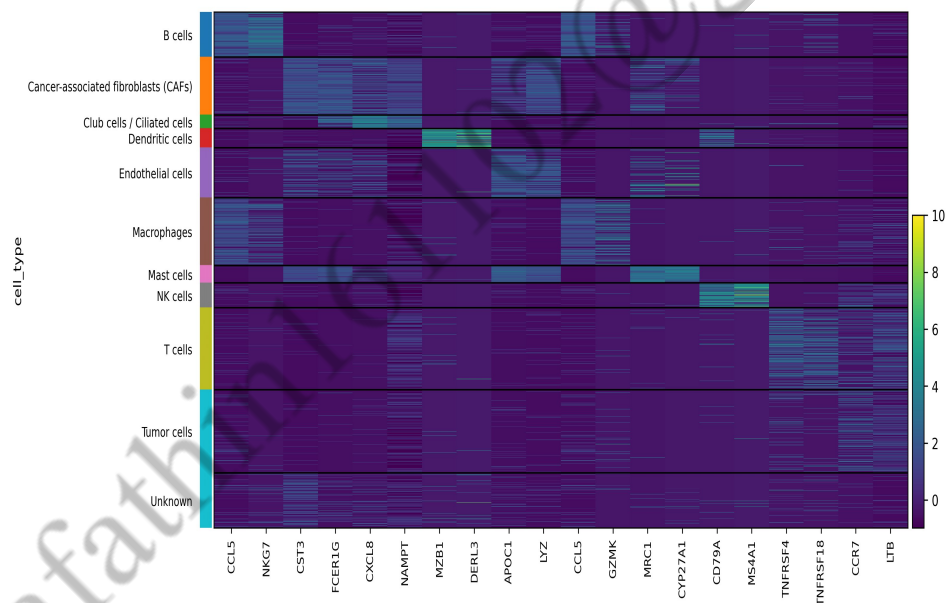


Figure 6.2C: Heatmap of scaled expression values of selected marker genes across single cells, grouped by annotated cell types. Vertical coherence of gene expression bands highlights the specificity and relevance of these markers for identifying major immune, stromal, and tumor cell populations.

A high-resolution view of these dynamics is presented in **Figure 6.2C**, which shows a heatmap of selected marker genes across single cells, grouped by annotated cell type. This visualization reveals tight intra-cluster expression coherence and inter-cluster divergence, lending strong support to the robustness of our cell type assignments. Notably, CCL5 and NKG7 were prominently expressed in T and NK cells, consistent with their roles in chemotaxis and

cytotoxicity, respectively. These findings suggest a functionally engaged lymphocyte compartment, potentially modulated by checkpoint pathways in the TME.

Conversely, inflammatory mediators such as CXCL8 (IL-8) and NAMPT were elevated in macrophages and fibroblasts. CXCL8, in particular, plays a central role in neutrophil recruitment and tumor-promoting inflammation, and its widespread expression across myeloid and stromal populations points to its centrality in COPD-NSCLC immunopathology. NAMPT, a key regulator of cellular metabolism and stress response, further supports the notion of metabolically reprogrammed stromal elements, possibly contributing to tumor cell survival under hypoxic or inflammatory stress.

Importantly, several markers—including DDIT3 (CHOP) and SAA1—were expressed across multiple clusters, suggesting the presence of pan-cellular stress programs. DDIT3 is a transcription factor activated during endoplasmic reticulum (ER) stress and is known to drive apoptosis in conditions of prolonged unfolded protein response (UPR). Its expression in diverse cell types indicates widespread ER stress within the tumor tissue, likely driven by chronic inflammation, nutrient deprivation, and oxidative stress—all exacerbated in the setting of COPD. Similarly, SAA1 (Serum Amyloid A1) is an acute-phase reactant often elevated in inflammatory conditions and linked to immune suppression in cancer. Its broad expression pattern further corroborates the chronic inflammatory tone of the COPD-associated lung tumor microenvironment.

Taken together, this detailed marker gene analysis provides strong molecular evidence for the accurate delineation of cell types within the NSCLC and COPD-NSCLC tumor ecosystems. Beyond confirming cell identities, it reveals important functional differences between populations, such as inflammation, immune regulation, vascular remodeling, and metabolic stress. These differences not only reflect the underlying disease context but also offer valuable insights into potential biomarkers and therapeutic targets that may be unique to COPD-associated NSCLC. By leveraging single-cell transcriptomics, we gain an unprecedented view of the cellular diversity and dysfunction driving tumor progression in inflamed lung tissue.

6.3 Expression Distributions of Key Marker Genes

To further dissect transcriptional heterogeneity within the tumor microenvironment (TME) at single-cell resolution, we generated violin plots for selected key marker genes. These genes were chosen based on their high specificity for individual cell types, their functional relevance

in immunity or tumor biology, and their prominence across earlier analyses (dot plot, bar plot, and heatmap). The violin plots depict the full distribution of gene expression values across cells within each annotated cluster, enabling nuanced insights into cell state variability, activation levels, and expression intensity.

CD79A – A B Cell-Specific Surface Marker

As shown in **Figure 6.3A**, the expression profile of **CD79A** (Cluster of Differentiation 79A) is sharply restricted to the B cell cluster. The violin plot shows a narrow distribution with a prominent expression peak, indicating both high expression and remarkable specificity. CD79A is a core component of the B cell receptor (BCR) complex and is critical for antigen recognition and downstream signaling. Its expression in this dataset affirms the presence of mature B cells within the TME and supports their classification in the earlier clustering. The absence of CD79A in other clusters demonstrates the robustness of single-cell annotation and highlights the potential of B cells to contribute to antigen presentation or antibody-mediated immunity in both NSCLC and COPD-NSCLC contexts.

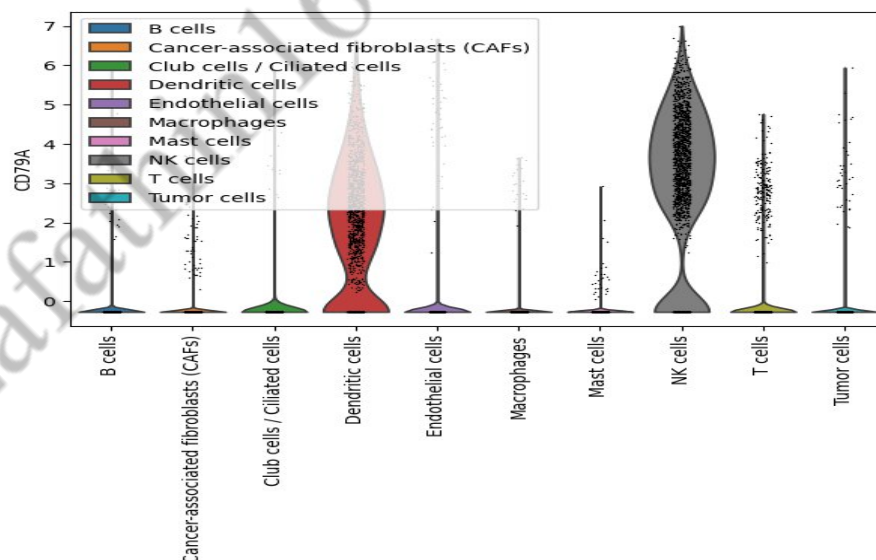


Figure 6.3A Violin Plot of CD79A

GZMK – Cytotoxic Activity in NK and CD8⁺ T Cells

In **Figure 6.3B** illustrates the distribution of **GZMK** (Granzyme K), a serine protease typically expressed by cytotoxic lymphocytes, particularly natural killer (NK) cells and CD8⁺ T cells. The violin plot reveals an elevated expression profile within these immune compartments, with a broad distribution that suggests transcriptional heterogeneity among cytotoxic cells. GZMK functions as a non-apoptotic effector that contributes to inflammation, pathogen defense, and tumor cell elimination. Its presence indicates active cytotoxic responses in the tumor, and its expression variation may reflect different activation states or stages of differentiation. Importantly, GZMK-expressing T cells have been associated with persistent but non-exhausted phenotypes, offering a nuanced view of immune competence in COPD-influenced tumors.

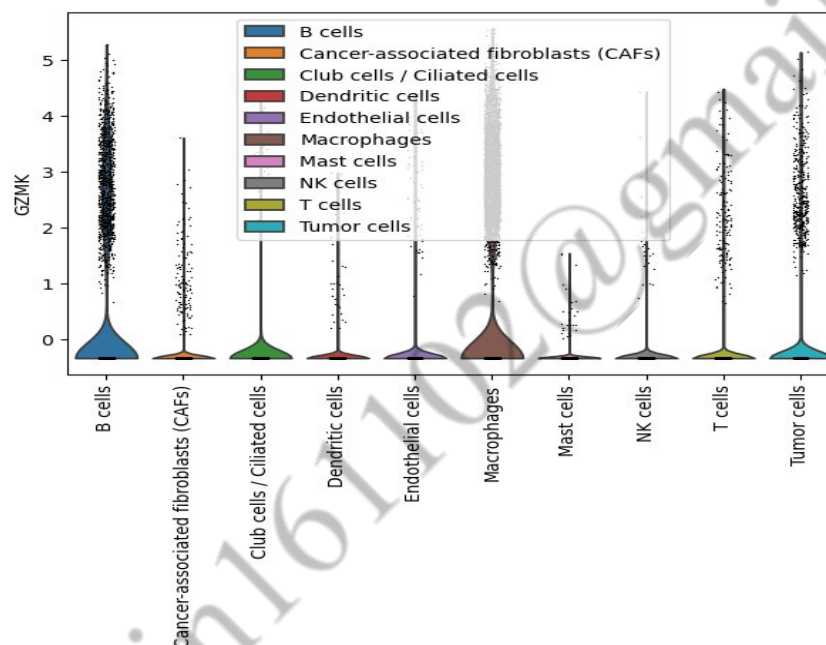


Figure 6.3B Violin Plot of GZMK

CST3 – Inflammatory Macrophage Signature

In **Figure 6.3C**, we observe a wide and elevated expression distribution of **CST3** (Cystatin C) within macrophage clusters. CST3 is a cysteine protease inhibitor involved in modulating extracellular matrix (ECM) remodeling, immune suppression, and inflammation. The shape of the violin plot, with its extended tails and broad central body, suggests that CST3 expression is not uniform across all macrophages—some cells express it at very high levels, possibly representing activated or alternatively polarized (M2-like) states. The strong CST3 signal in COPD-NSCLC samples may reflect heightened immune regulation or tissue remodeling in response to chronic inflammation. Its known associations with tumor progression, immune

escape, and poor prognosis in various cancers highlight its potential as both a biomarker and a therapeutic target.

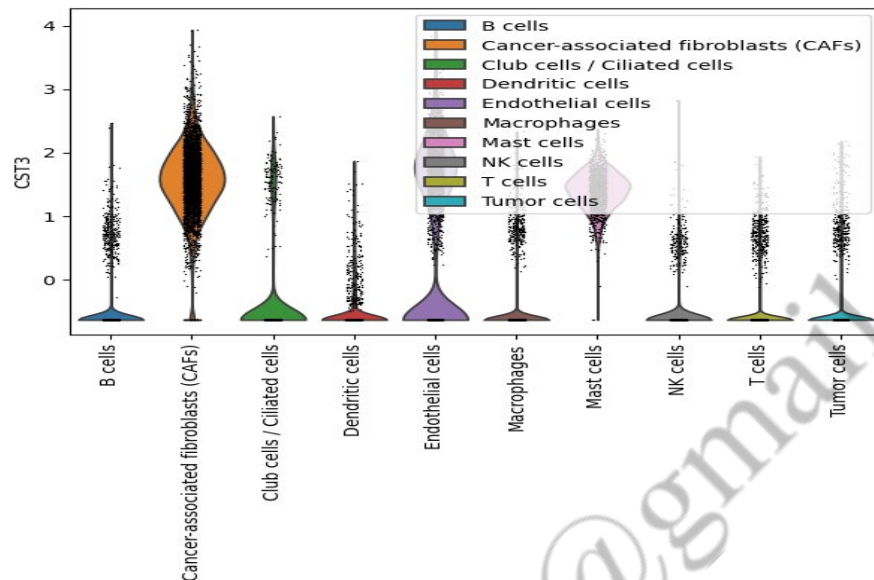


Figure 6.3C Violin plot of CST3

CXCL8 – Pro-Inflammatory and Tumor-Promoting Chemokine

The expression pattern of **CXCL8** (C-X-C Motif Chemokine Ligand 8), also known as interleukin-8 (IL-8), is shown in **Figure 6.3D**. The violin plot demonstrates heterogeneous but high-level expression across several cell types, especially stromal and myeloid cells. CXCL8 plays a pivotal role in neutrophil chemotaxis, angiogenesis, and tumor-associated inflammation. Its variable expression suggests a complex regulation influenced by local inflammatory signals, oxygen tension, and cellular stress. In COPD-NSCLC, where neutrophilic inflammation is a hallmark, the elevated CXCL8 may act as a bridge between chronic pulmonary inflammation and tumor-promoting immune modulation. It is also known to facilitate epithelial–mesenchymal transition (EMT) and metastasis, making it a key candidate in the inflammatory axis of cancer progression.

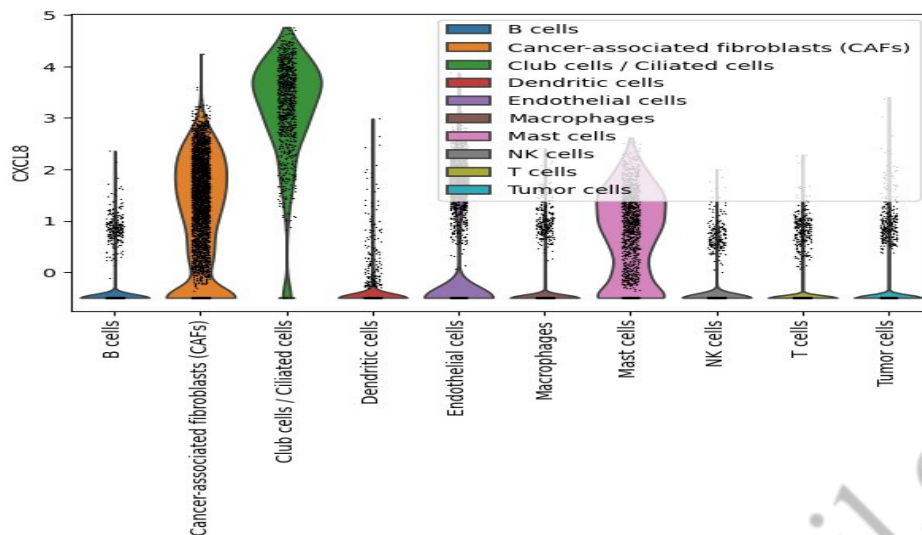


Figure 6.3D Violin plot of CXCL8

HLA-DPB1 – Antigen Presentation and Immune Engagement

Figure 6.3E displays the expression distribution of **HLA-DPB1**, a major histocompatibility complex (MHC) class II gene involved in antigen presentation to CD4⁺ T cells. The violin plot shows a broader and more variable expression pattern, mainly localized within T cells, dendritic cells, and some macrophage subsets. This suggests a functional heterogeneity in antigen-presenting capacity among immune cells in the TME. Elevated HLA-DPB1 expression often correlates with immune activation and T helper cell recruitment, both critical in shaping anti-tumor immunity. However, in a chronic inflammatory setting like COPD, persistent antigen exposure may also lead to T cell exhaustion or tolerance, limiting effective immune clearance of tumor cells. The variation in HLA-DPB1 expression could therefore represent diverse immune states, from active surveillance to immune dysfunction.

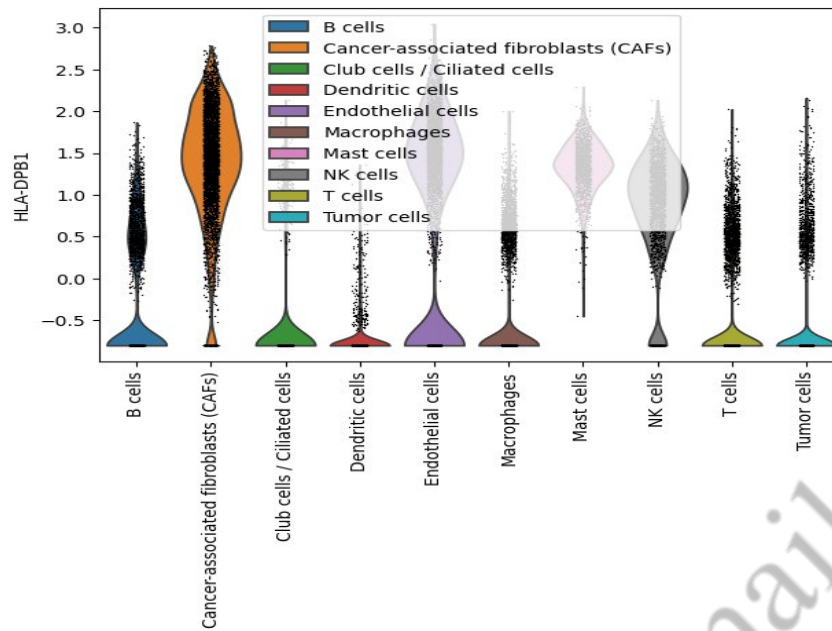


Figure 6.3E Violin plot of HLA-DPB

Summary of Violin Plot Insights

Together, these violin plots highlight key molecular features that distinguish major cell types in the NSCLC and COPD-NSCLC micro environments. More importantly, they reveal the heterogeneous nature of gene expression even within seemingly homogeneous clusters, suggesting the existence of transcriptionally distinct sub populations with specialized functions. These variations likely reflect differences in cellular activation, stress response, metabolic status, and immune engagement.

This cell-intrinsic variability is particularly important in the context of COPD-NSCLC, where chronic inflammation may reprogram immune and stromal cells into tumor-permissive phenotypes. By identifying such signatures at single-cell resolution, we can better understand the functional architecture of the inflamed tumor niche and begin to pinpoint key regulators and pathways that may serve as therapeutic entry points.

6.4 Differentially Expressed Genes Between NSCLC and COPD-Associated NSCLC

To understand how chronic pulmonary inflammation influences tumor behavior at the molecular level, we performed differential gene expression (DGE) analysis between NSCLC and COPD-associated NSCLC (COPD-NSCLC) samples. Using the Wilcoxon rank-sum test at the single-cell level, we identified a suite of genes exhibiting significant transcriptional divergence between the two disease contexts. This comparison allowed us to assess how the COPD micro environment shapes the gene expression landscape of tumor and immune cells, potentially identifying key regulators of inflammation-associated tumor progression.

6.4.1 Transcriptional Remodeling in NSCLC Relative to COPD-NSCLC

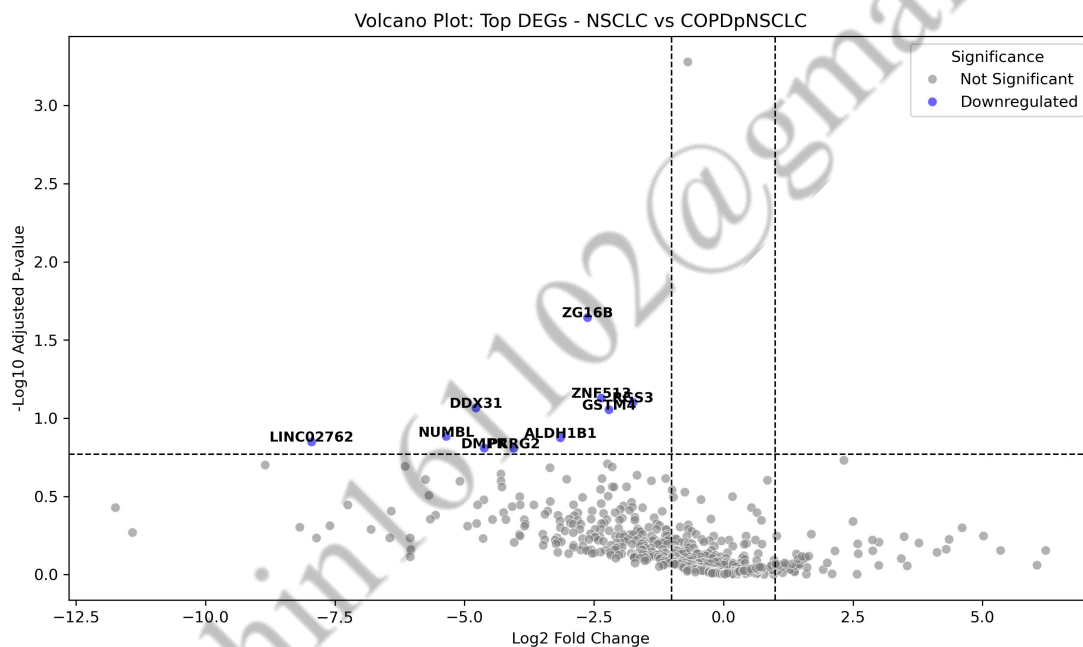


Figure 6.4.1A volcano NSCLC vs COPD-NSCLC

The volcano plot in Figure 4A provides a global overview of transcriptional differences between NSCLC and COPD-NSCLC. Genes are plotted by statistical significance (adjusted p-value) and effect size (log₂ fold change), highlighting those most differentially expressed.

Among the upregulated genes in NSCLC, several stand out due to their known roles in cancer progression:

MMP1 (Matrix Metalloproteinase 1): This gene encodes an enzyme that degrades extracellular matrix components, facilitating tumor invasion and metastasis. Its high expression suggests

active tissue remodeling and possibly more aggressive tumor behavior in NSCLC compared to COPD-NSCLC.

S100P (S100 Calcium Binding Protein P): A marker of poor prognosis in lung and other epithelial cancers, S100P promotes cell proliferation, invasion, and survival under stress. Its overexpression reflects a shift toward pro-tumorigenic, inflammation-tolerant transcriptional programs.

CEACAM5 (Carcinoembryonic Antigen–Related Cell Adhesion Molecule 5): Commonly elevated in NSCLC, CEACAM5 is used clinically as a tumor marker. It also contributes to cell adhesion, immune evasion, and metastasis.

In contrast, genes downregulated in NSCLC relative to COPD-NSCLC included: **SCGB1A1** and **CC10 (Secretoglobins)**: These genes are primarily expressed in airway epithelial cells and play anti-inflammatory and protective roles in lung tissue. Their suppression reflects the loss of normal epithelial differentiation and a possible transition toward malignant transformation.

CYP2F1 (Cytochrome P450 Family 2 Subfamily F Member 1): A detoxification enzyme normally involved in xenobiotic metabolism, its downregulation may indicate altered metabolic states or reduced differentiation status of tumor cells.

Collectively, these findings suggest a clear shift in the epithelial transcriptional program, with COPD-NSCLC retaining more features of normal lung epithelium, while NSCLC shows a transition toward an aggressive, invasive state marked by stress tolerance and immune evasion. In **Figure 6.4.1A**: The volcano plot reveals a pronounced transcriptomic divergence between NSCLC and COPD-associated pre-NSCLC samples. A substantial subset of genes exhibits statistically significant differential expression (adjusted $p < 0.05$), with many surpassing the $|\log_2FC| > 1$ threshold, indicating biologically meaningful shifts.

Among the upregulated genes in NSCLC, **MMP1**, **S100P**, and **CEACAM5** stand out with \log_2 fold changes exceeding 2. **MMP1**, a matrix metallo proteinase, is implicated in extracellular matrix remodeling and tumor invasiveness, commonly associated with cancer progression. **S100P**, a calcium-binding protein, has been linked to proliferation and metastasis in lung cancer, while **CEACAM5** (also known as **CEA**) is a well-established tumor marker elevated in several carcinomas, including NSCLC.

Conversely, genes such as **SCGB1A1**, **CYP2F1**, and **CC10** are notably downregulated in NSCLC. **SCGB1A1** and **CC10** are secretoglobins predominantly expressed in normal

bronchial epithelial cells, and their suppression suggests a loss of normal airway differentiation. CYP2F1, a member of the cytochrome P450 family, may reflect altered xenobiotic metabolism in the transformed lung tissue.

6.4.2 Hierarchical Clustering Highlights Distinct Expression Signatures

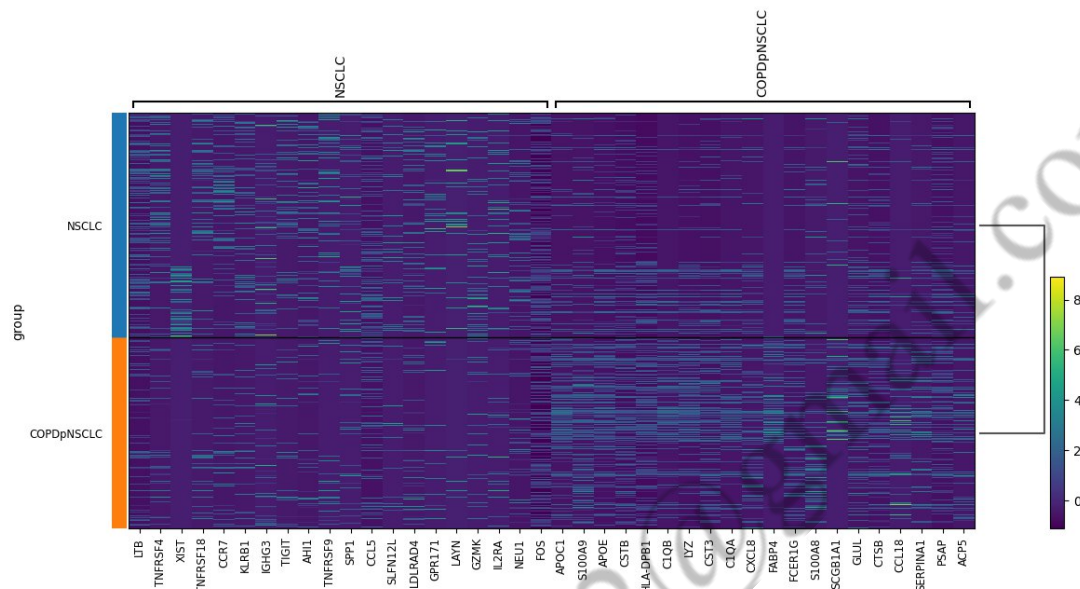


Figure 6.4.2A heatmap NSCLC vs COPD-NSCLC

To further examine disease-specific gene expression profiles, we generated a heatmap **Figure 4B** of the top differentially expressed genes between NSCLC and COPD-NSCLC. Each row represents a gene, and each column corresponds to a sample. Clear separation of the two groups is evident, with distinct gene expression modules defining each disease state.

In NSCLC samples, genes like MMP1, S100P, and CEACAM5 are strongly upregulated, reinforcing their role as hallmarks of malignant transformation. Their clustered expression suggests a coordinated activation of oncogenic pathways involved in invasion, immune suppression, and inflammation-induced tumor growth.

Conversely, COPD-NSCLC samples displayed elevated expression of airway and detoxification markers such as SCGB1A1 and CYP2F1, indicating preserved aspects of tissue-specific identity and a possibly earlier stage of tumor progression. The relative maintenance of these genes may reflect a more differentiated epithelial phenotype or less extensive genomic reprogramming.

This distinct clustering supports the notion that COPD-NSCLC and NSCLC are transcriptionally separable disease entities, shaped by different environmental pressures—chronic inflammation versus purely oncogenic transformation

6.4.3 Ranked Summary of Differentially Expressed Genes

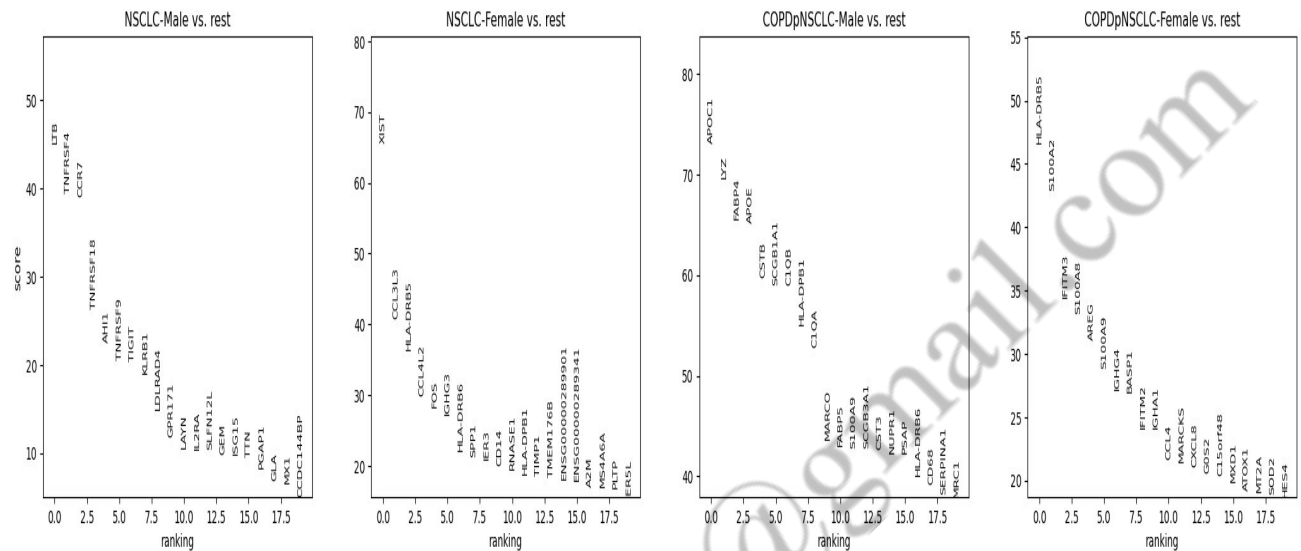


Figure 6.4.3A rank genes groups sample NSCLC vs COPD-NSCLC summary

In **Figure 6.4.3A** summarizes the top-ranked genes that most clearly differentiate NSCLC from COPD-NSCLC. Genes are ordered by adjusted p-value and effect size, enabling rapid identification of key drivers of phenotypic divergence.

The top-ranked upregulated genes in NSCLC—MMP1, S100P, CEACAM5—not only have statistical significance but also exhibit high fold changes, underscoring their biological importance in tumor progression. These genes could serve as biomarkers for aggressive NSCLC subtypes or as candidates for therapeutic targeting.

In contrast, the downregulated genes, such as SCGB1A1 and CYP2F1, emphasize the loss of epithelial homeostasis and anti-inflammatory mechanisms. These features may help distinguish COPD-NSCLC as a condition in which the inflammatory context restrains or delays full malignant transition, albeit still pushing cells along the tumorigenic spectrum.

6.4.4 Functional Bias Among Top DEGs

To better understand the biological implications of the transcriptional shifts, we visualized the top 20 DEGs by directionality using a bar plot **Figure 6.4.4A**. Genes were separated into upregulated and downregulated categories in NSCLC relative to COPD-NSCLC.

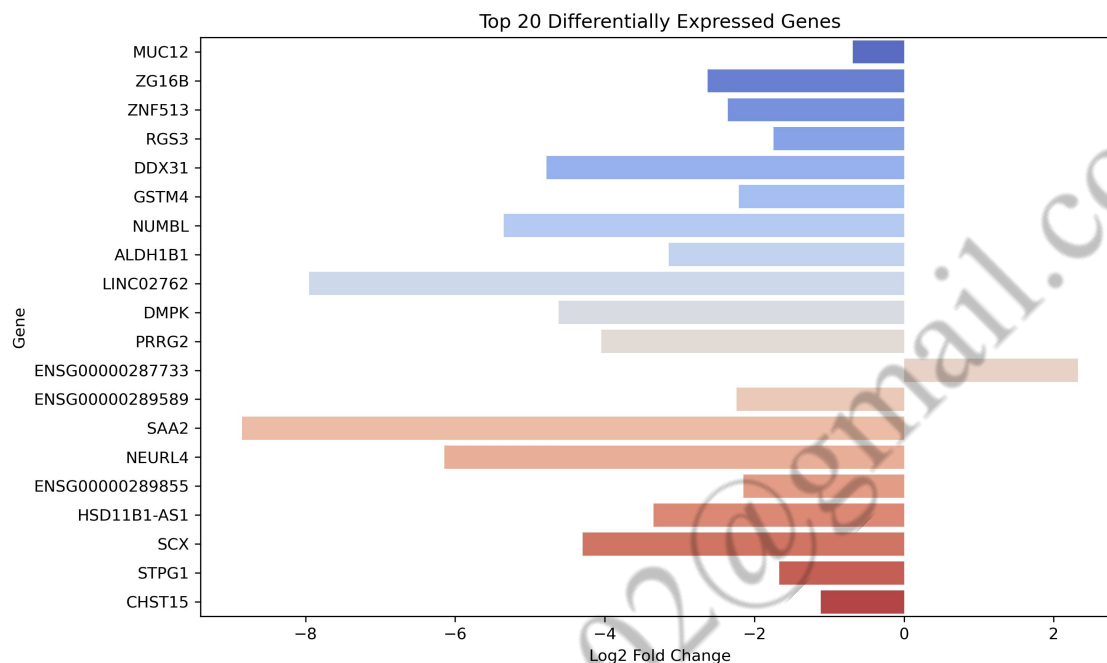


Figure 6.4.4A top 20 DEGs barplot

The upregulated genes included pro-invasive and pro-inflammatory effectors (e.g., MMP1, S100P, CEACAM5), reflecting ECM degradation, cellular proliferation, and immune editing. These alterations are hallmarks of established malignancy and are consistent with the advanced gene expression profile observed in NSCLC.

In contrast, the downregulated genes largely reflected protective, tissue-specific, or anti-inflammatory functions (e.g., SCGB1A1, CYP2F1, CC10). Their repression in NSCLC may represent an erosion of tissue identity and innate defense, driven by oncogenic signaling pathways.

This stark expression bias confirms that NSCLC undergoes significant molecular reprogramming compared to COPD-NSCLC and supports the idea that inflammation (as in COPD) shapes a distinct tumor progression pathway. These DEGs not only provide mechanistic insights but may also serve as diagnostic or prognostic indicators, especially in distinguishing COPD-linked tumors from other NSCLC subtypes.

6.5 Pathway-Level Interpretation of Immune and Stromal Remodeling in COPD-Associated NSCLC

To systematically decode the cellular and molecular landscape underpinning COPD-associated non-small cell lung cancer (NSCLC), we performed comprehensive pathway enrichment analyses following single-cell RNA sequencing (scRNA-seq) of lung tissue samples. Differentially expressed genes (DEGs) were identified across key epithelial and immune cell populations when comparing COPD-NSCLC to NSCLC-only states, and these DEGs were subjected to functional annotation using Gene Ontology (GO) categories—Biological Process (BP), Cellular Component (CC), and Molecular Function (MF)—as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapping. This multi-pathway framework enables biological interpretation of COPD-driven transcriptomic rewiring in the tumor micro environment, offering insight into inflammation, immune modulation, and extracellular matrix remodeling. The findings highlight potential mechanistic links and therapeutic targets at the intersection of chronic lung disease and tumor progression.

Table 1. Key GO and KEGG Pathway Terms Reflecting Inflammatory and Stromal Remodeling in COPD-NSCLC

Gene set	Term	Genes
GO Biological Process	neutrophil mediated immunity (GO:0002446)	CDA;PIGR;SERPINA1;TNFAIP6;LPCAT1;HP;HBB;CXCL1;PYGL;SIRPB1;FCGR3B;PLAU;ANPEP;TIMP2;OLR1;QSOX1;CD36;CAMPA;CTSB;SERPINB3;DSP;FCER1G;MME;CD93;ANXA3;NFAM1;SLC11A1;PLAUR;CYBB;MCEMP1;OLFM4;MMP9;OSCAR;FGR;DOK3;SLPI;CEACAM6;PECAM1;PADI2;PLEKHO2;S100A9;S100A8;TLR2;CFD;CSTB;C5AR1;FPR1;MGST1;FPR2;RETN;C3;CST3;ALDH3B1;PSAP;NEU1;CXCR2;CD59;LTA4H;CD14;ARSB;ACE;GSN;SIGLEC14;JUP;GCA;FUCA1;IDH1;LILRB2;LYZ;LILRB3;IL6;LRG1;FABP5;LCN2;MNDA;S100P;FOLR3;CD68;GLA
GO Biological Process	inflammatory response (GO:0006954)	CXCL6;IL1RN;CXCL9;CXCL8;TNFAIP6;HP;ADM;CXCL1;F11R;CXCL13;CXCL3;TNF;CXCL2;CXCL5;ADORA3;CCRL2;OLR1;CCR7;TNFRSF4;SLC11A1;NFAM1;CYBB;FOS;HSPG2;IL1A;ELF3;IL1B;KIT;TLR4;S100A9;S100A8;TLR2;CCL13;C5AR1;FPR1;FPR2;THBS1;CCL5;CXCR2;CCL4;CCL3;NLRP3;CD14;CCL18;C

		CR1;CCL23;CCL20;LYZ;CXCL10;IL6;IL2RA;FOLR2
GO Biological Process	Cytokine-mediated signaling pathway (GO:0019221)	IFITM3;CXCL6;IL1RN;CXCL9;SPI1;CSF3R;IFITM2;TNFRSF6B;CXCL8;CSF1;CXCL1;IL1RAP;CXCL13;IFIT1;CXCL3;TNF;CXCL2;IFIT3;CXCL5;TNFSF13B;ICAM1;IFIT2;MT2A;CCND1;TIMP1;CD36;TNFRSF4;IL10;FCER1G;CISH;TNFRSF12A;TNFRSF18;TALDO1;LIFR;IRAK3;FOS;OSMR;F3;MMP9;EREG;HCK;IL1A;IFI27;IL1B;IL3RA;KIT;FSCN1;IRF6;LTB;CCL13;LAMA5;FPR1;CSF2RB;PTGS2;MUC1;IRAK2;CCL5;CCL4;CXCR2;CCL3;HMOX1;STX3;CCL18;CD300LF;GBP1;CCR1;EGR1;HLA-DRB5;CCL23;CCL20;CD70;TNFRSF9;MX1;FN1;OSM;LILRB2;SOD2;VEGFA;CXCL10;IL6;BCL6;IL2RA;HLA-DPB1;LCN2;SAA1;TNFSF9;LTBR
GO Biological Process	Neutrophil degranulation (GO:0043312)	CDA;PIGR;SERPINA1;TNFAIP6;LPCAT1;HP;HBB;CXCL1;PYGL;SIRPB1;FCGR3B;PLAU;ANPEP;TIMP2;OLR1;QSOX1;CD36;CAMPA;CTSB;SERPINB3;DSP;FCER1G;MME;CD93;ANXA3;NFAM1;SLC11A1;PLAUR;CYBB;MCEMP1;OLFM4;MMP9;OSCAR;FGR;DOK3;SLPI;CEACAM6;PECAM1;PADI2;PLEKHO2;S100A9;S100A8;TLR2;CFD;CSTB;C5AR1;FPR1;MGST1;FPR2;RETN;C3;CST3;ALDH3B1;PSAP;NEU1;CXCR2;CD59;LTA4H;CD14;ARSB;GSN;SIGLEC14;JUP;GCA;FUCA1;IDH1;LILRB2;LYZ;LILRB3;LRG1;FABP5;LCN2;MNDA;S100P;FOLR3;CD68;GLA
GO Biological Process	Pattern recognition receptor signaling pathway (GO:0002221)	FGB;FGA;SFTPA2;FGG;LY96;INAVA;CTSL;IRAK2;CTSK;TLR8;FFAR2;CD36;CD14;SFTPA1;CLEC4E;S100A9;TLR4;S100A8;CTSB;TLR2;LGMN
GO Cellular Component	Collagen-containing extracellular matrix (GO:0062023)	COL18A1;ADAMDEC1;TPSB2;SPARC;SERPINA1;TINAGL1;COL12A1;SERPINE1;TNC;PLAT;LAMC2;CLU;ICAM1;EFEMP1;CTSL;MDK;TIMP2;CCN2;CCN1;TPSAB1;CTSB;TGM2;CPA3;FGB;FGA;EGFL7;SERPINF1;WNT5A;FGG;HSPG2;F3;MMP9;BCAM;VCAN;SLPI;COL6A1;SERPING1;VWA1;MFGE8;S100A9;S100A8;C1QB;C1QA;CSTB;LAMA5;LAMA3;THBS1;PSAP;IGFBP

		7;APOE;A2M;CTHRC1;LAMB3;GDF15;LAMB2;F12;FN1;COL1A1;MGP;C1QC
GO Cellular Component	Secretory granule lumen (GO:0034774)	CDA;SPARC;SERPINA1;SERPINE1;HP;CTSW;CXCL1;PYGL;CLU;TIMP2;QSOX1;TIMP1;CAMP;SERPINB3;FGB;FGA;FGG;OLF4;OSCAR;FGR;SLPI;SELENOP;PADI2;SERPING1;S100A9;S100A8;CFD;CSTB;RETN;THBS1;C3;NEU1;A2M;ARSB;GSN;JUP;GCA;FUCA1;IDH1;FN1;LYZ;VEGFA;LRG1;FABP5;LCN2;MNDA;S100P;FOLR3;GLA
GO Cellular Component	Endoplasmic reticulum lumen (GO:0005788)	COL18A1;SERPINA1;CSF1;COL12A1;TNC;PTGS2;PRSS23;FSTL1;THBS1;C3;CST3;SPP1;QSOX1;IGFBP7;APOE;TIMP1;CCN1;APOL1;ARSB;FGA;GOLM1;IGFBP4;LAMB2;WNT7B;IGFBP3;WNT5A;FGG;FN1;PLAUR;CP;COL1A1;IL6;RCN1;VCAN;COL6A1;MZB1;SERPING1;VWA1;MFGE8;CES1
GO Cellular Component	Specific granule (GO:0042581)	HP;CXCL1;FPR2;RETN;PLAU;ALDH3B1;NEU1;TIMP2;OLR1;QSOX1;CD59;CD36;STX3;CAMP;CD93;JUP;ANXA3;PLAUR;CYBB;MCEMP1;OLFM4;LYZ;OSCAR;LRG1;SLPI;LCN2;FOLR3
GO Cellular Component	Secretory granule membrane (GO:0030667)	PIGR;SPARC;C5AR1;FPR1;MGST1;LPCAT1;FPR2;SIRPB1;FCGR3B;PLAU;ANPEP;ALDH3B1;CXCR2;PSAP;OLR1;CD59;CD14;CD36;DSP;SIGLEC14;FCER1G;MME;CD93;SLC11A1;NFAM1;PLAUR;CYBB;MCEMP1;RAB27B;LILRB2;LILRB3;DOK3;FABP5;CEACAM6;PECAM1;CD9;CD68;TLR2
GO Molecular Function	Chemokine receptor binding (GO:0042379)	CXCL6;CCL13;CXCL9;CCL23;CXCL8;CCL20;CXCL1;CXCL13;CXCL3;CXCL2;CXCL5;CXCL16;CXCL10;CCL5;CCRL2;CCL4;CCL3;S100A14;CCL18
GO Molecular Function	Cytokine activity (GO:0005125)	CXCL6;CCL13;CXCL9;CXCL8;CSF1;CXCL1;CXCL13;CXCL3;TNF;CXCL2;CXCL5;CXCL16;CCL5;CCL4;CCL3;TIMP1;CCL18;IL10;EDN1;CCL23;CCL20;CD70;GDF15;WNT7B;WNT5A;OSM;INHBA;VEGFA;CXCL10;IL1A;IL6;IL1B;SCGB3A1
GO Molecular Function	Chemokine activity (GO:0008009)	CCL13;CXCL6;CXCL9;CCL23;CXCL8;CCL20;CXCL1;CXCL13;CXCL3;CXCL2;CXCL5;CXCL16;CXCL10;CCL5;CCL4;CCL3;CCL18
GO	Protease	SERPINB3;SERPINB4;CSTB;CSTA;SERPINA1;ITGA3;CD70;SE

Molecular Function	binding (GO:0002020)	RPINE1;FN1;TNF;F3;TTN;COL1A1;CST3;PSAP;TIMP2;CARD16;TIMP1;A2M
GO Molecular Function	Serine-type peptidase activity (GO:0008236)	CFD;TPSB2;RHBDD3;C1S;C1R;F12;GZMA;TMPRSS4;HP;GZMB;PLAT;PRSS22;KLK8;F3;MMP9;PRSS21;GZMK;PLAU;PRSS8;TPSAB1
KEGG Pathway	Complement and coagulation cascades	C1QB;CFD;C1QA;SERPINA1;C1S;CFH;C1R;SERPINE1;C5AR1;CFI;C4BPA;PLAT;C8B;TFPI;CLU;C3;THBD;PLAU;CD59;VSIG4;A2M;FGB;FGA;F12;FGG;PLAUR;F3;SERPING1;CFB;C1QC
KEGG Pathway	Cytokine-cytokine receptor interaction	CXCL6;IL1RN;CXCL9;TNFRSF6B;CSF3R;CXCL8;CSF1;CCL4L2;CXCL17;CXCL1;IL1RAP;CXCL13;CXCL3;TNF;CXCL2;CXCL5;CXCL16;TNFSF13B;TNFSF10;CCR7;TNFRSF4;IL10;TNFRSF12A;TNFRSF18;LIFR;OSMR;IL1A;IL1B;IL3RA;LTB;CCL13;CSF2RB;CCL5;CXCR2;CCL4;CCL3;CCL18;CCR1;CCL23;CCL20;CD70;GDF15;TNFRSF9;OSM;INHBA;CXCL10;IL6;IL2RA;TNFSF9;LTBR
KEGG Pathway	Hematopoietic cell lineage	HLA-DRB5;CSF3R;CSF1;MME;ITGA3;ITGA2;TNF;IL1A;IL6;ANPEP;IL1B;IL2RA;IL3RA;KIT;HLA-DPB1;CD9;CD59;CD14;CD36;CD24;MS4A1
KEGG Pathway	Staphylococcus aureus infection	IL10;C1QB;CFD;C1QA;HLA-DRB5;C1S;CFH;C1R;FGG;C5AR1;CFI;FPR1;DEFB1;FPR2;ICAM1;C3;KRT19;FCGR3B;KRT18;KRT17;HLA-DPB1;CAMP;CFB;C1QC
KEGG Pathway	Viral protein interaction with cytokine and cytokine receptor	CXCL6;CCL13;CXCL9;CXCL8;CSF1;CCL4L2;CXCL1;CXCL13;CXCL3;TNF;CXCL2;CXCL5;CCL5;CCL4;CXCR2;TNFSF10;CCL3;CCR7;CCL18;CCR1;IL10;CCL23;CCL20;CXCL10;IL6;IL2RA;LTBR

6.5.1 GO Biological Process (BP): Inflammation and Innate Immunity Activation in the COPD-NSCLC Microenvironment

One of the most prominent patterns that emerged from the Gene Ontology Biological Process (GO-BP) enrichment analysis was a strong overrepresentation of inflammatory and innate immune pathways, particularly those involving neutrophils, cytokine signaling, and innate pattern recognition receptors. This finding is consistent with the chronic inflammatory milieu characteristic of COPD lungs and highlights how such a persistent immune state may profoundly influence tumor progression and immune regulation in NSCLC.

The activation of these processes points to a pro-inflammatory tumor microenvironment (TME), shaped by long-term exposure to inflammatory stimuli such as cigarette smoke, oxidative stress, and repeated epithelial injury. In this setting, immune responses that are normally protective may become maladaptive, promoting not only tissue damage but also immunosuppression, stromal remodeling, and carcinogenesis.

1. Neutrophil-Mediated Immunity (GO:0002446)

Among the most significantly enriched terms was neutrophil-mediated immunity, a hallmark of COPD pathogenesis. Genes such as FCGR3B, S100A8, and S100A9 were highly expressed in COPD-NSCLC, indicating heightened neutrophil activity. These genes are critical for neutrophil recruitment, adhesion, and antimicrobial function, but in the context of chronic inflammation, their role shifts toward tumor support.

- S100A8/A9 (calprotectin complex) acts as a damage-associated molecular pattern (DAMP), amplifying inflammation through Toll-like receptors and promoting recruitment of myeloid-derived suppressor cells (MDSCs).
- FCGR3B, a receptor for the Fc region of IgG, is involved in immune complex clearance but has also been implicated in immune dysfunction and altered phagocytosis in cancer.

The presence of these markers in the TME suggests that neutrophils in COPD-NSCLC are not only abundant but functionally reprogrammed, contributing to immune suppression, matrix degradation, and angiogenesis.

2. Inflammatory Response (GO:0006954)

The inflammatory response term was significantly enriched due to the upregulation of key inflammatory cytokines, including IL1B, CXCL8, and TNF. These mediators are central to the

amplification and perpetuation of inflammation, which in chronic diseases like COPD becomes a double-edged sword—damaging host tissue while enabling tumor survival and expansion.

- IL1B (interleukin-1 beta) drives a potent inflammatory cascade, recruiting neutrophils and activating the inflammasome, while also promoting epithelial–mesenchymal transition (EMT) and angiogenesis in tumors.
- CXCL8 (IL-8) is a chemoattractant for neutrophils and is frequently overexpressed in both COPD and NSCLC, linking chronic inflammation to tumor proliferation, invasion, and metastasis.
- TNF is a master inflammatory cytokine that, in the tumor context, paradoxically supports cell survival, immune evasion, and resistance to therapy through activation of NF-κB and MAPK pathways.

This constellation of inflammatory gene expression underscores a sustained, tumor-supportive inflammatory tone in COPD-NSCLC.

3. Cytokine-Mediated Signaling Pathway (GO:0019221)

Further supporting the immune-active landscape, the cytokine-mediated signaling pathway was significantly enriched, with genes such as STAT3, SOCS3, and JAK1 being upregulated. This axis is central to immune regulation, cell survival, and inflammation-mediated tumor promotion.

- STAT3 is a key transcription factor activated downstream of cytokines like IL-6 and IL-10. In cancer, it promotes immune tolerance, proliferation, and resistance to apoptosis.
- SOCS3 is an inducible inhibitor of cytokine signaling, part of a negative feedback loop that blunts anti-tumor immunity, particularly in the context of chronic exposure to IL-6.
- JAK1, a core kinase in this pathway, links extracellular cytokine signals to nuclear transcriptional responses, and its dysregulation contributes to immune escape and immune checkpoint resistance.

These findings suggest that immune signaling pathways in COPD-NSCLC are chronically activated but dysfunctional, reinforcing a feedback loop that sustains inflammation while compromising immune-mediated tumor control.

4. Neutrophil Degranulation (GO:0043312)

The enrichment of neutrophil degranulation processes, indicated by genes such as ELANE, MPO, and CTSG, points to direct tissue-modifying and immunosuppressive effects of neutrophils in the COPD-NSCLC TME.

- ELANE (neutrophil elastase) and MPO (myeloperoxidase) are stored in azurophilic granules and released upon degranulation. They degrade extracellular matrix (ECM) components, promoting invasion and disrupting T cell function.
- CTSG (cathepsin G) also contributes to ECM degradation and modifies chemokine gradients, influencing immune cell trafficking.

These proteases collectively mediate a pro-tumorigenic remodeling of the extracellular space while simultaneously suppressing adaptive immune responses—creating a microenvironment that supports cancer progression under the influence of chronic inflammation.

5. Pattern Recognition Receptor Signaling Pathway (GO:0002221)

A final key GO term enriched in the COPD-NSCLC samples was the pattern recognition receptor (PRR) signaling pathway, specifically via upregulation of TLR2, TLR4, and NOD2. These receptors are central to the innate immune detection of microbial products and DAMPs.

- TLR2/TLR4 are cell-surface sensors that detect bacterial lipoproteins and LPS, respectively. Their chronic activation in COPD has been linked to airway inflammation and epithelial damage.
- NOD2, a cytoplasmic sensor of bacterial peptidoglycan, also triggers inflammatory responses and has been associated with increased cancer risk in inflammatory diseases.

In tumors, activation of these pathways can have dual effects: stimulating inflammation that initially contains tumor growth, but ultimately contributing to chronic immune stimulation, tissue injury, and tumor-promoting immune suppression. In COPD-NSCLC, this likely represents a form of innate immune reprogramming, where PRR pathways remain persistently activated, reshaping local immunity in favor of tumor progression.

6.5.2 GO Cellular Component (CC): Granule Biology and ECM Remodeling in the COPD-Linked Tumor Microenvironment

Cellular component enrichment analysis offered valuable insights into the structural compartments and cellular machinery most transcriptionally active in the COPD-NSCLC

tumor microenvironment. Notably, the analysis revealed a strong enrichment of genes associated with secretory granules, endoplasmic reticulum (ER) stress components, and extracellular matrix (ECM) structures. These findings suggest a tumor niche characterized by dynamic remodeling, active inflammation, and stress-adapted cell states, all of which are hallmarks of tumors arising in the context of chronic pulmonary disease.

This enrichment underscores the dual impact of inflammation and tissue remodeling in shaping the tumor ecosystem—where immune cell effector granules contribute to both host defense and collateral damage, and matrix components serve as both barriers and conduits for tumor invasion.

1. Collagen-Containing Extracellular Matrix (GO:0062023)

Among the most prominent enriched terms was the collagen-containing extracellular matrix (ECM), with significant upregulation of COL1A1, COL3A1, MMP9, and SPP1. This enrichment reflects intense stromal remodeling activity in the tumor microenvironment.

- COL1A1 and COL3A1 encode major fibrillar collagens found in the interstitial ECM. Their upregulation suggests fibrotic tissue remodeling, a known feature of both COPD and advanced cancer.
- MMP9 (matrix metalloproteinase 9) is involved in ECM degradation and is secreted by immune and stromal cells to facilitate tumor cell migration, invasion, and metastasis.
- SPP1 (osteopontin) contributes to cell–matrix adhesion, immune regulation, and metastatic potential.

These components collectively suggest that COPD-NSCLC tumors exist in an ECM-rich, invasion-permissive environment, where chronic inflammation and stromal activation facilitate cancer dissemination. The ECM is not only a structural scaffold but also a dynamic signaling reservoir influencing tumor growth, immune cell localization, and therapeutic resistance.

2. Secretory Granule Lumen (GO:0034774)

The secretory granule lumen was another highly enriched compartment, particularly in neutrophils and mast cells. Genes such as LYZ (lysozyme), CTSG (cathepsin G), and ELANE (neutrophil elastase) were significantly upregulated, indicating enhanced secretory and degranulation activity.

- These enzymes are stored in intracellular granules and released upon activation to degrade pathogens, damaged tissue, and ECM components.

- In the tumor setting, their uncontrolled release leads to collateral damage, promotes angiogenesis, and suppresses T-cell activity.

In COPD-NSCLC, this suggests that neutrophils and other myeloid cells are persistently activated, releasing granule contents that fuel chronic inflammation, disrupt tissue architecture, and contribute to an immune-suppressive, tumor-promoting niche.

3. Endoplasmic Reticulum Lumen (GO:0005788)

The endoplasmic reticulum (ER) lumen was enriched in genes involved in the unfolded protein response (UPR), including HSPA5 (BiP/GRP78) and XBP1. These genes are classical markers of ER stress, indicating that tumor and immune cells in the COPD-NSCLC TME are under high biosynthetic and oxidative stress, likely due to hypoxia, inflammation, and excessive protein production.

- HSPA5 functions as a chaperone that stabilizes nascent proteins and prevents aggregation.
- XBP1, once spliced by IRE1, initiates transcriptional programs to restore ER homeostasis or trigger apoptosis in unresolved stress.

This transcriptional profile suggests that cells in COPD-NSCLC tumors are engaged in adaptive stress responses, which may promote tumor survival under adverse conditions, such as nutrient deprivation and immune-mediated attack. Furthermore, chronic ER stress is known to modulate immune responses, including suppressing antigen presentation and promoting myeloid-derived suppressor cell (MDSC) recruitment.

4. Specific Granule (GO:0042581)

Specific granules, also referred to as secondary granules in neutrophils, were enriched in genes such as S100A8, S100A9, and DEFA3 (defensin alpha 3). These molecules are involved in innate immune defense, chemoattraction, and regulation of inflammatory responses.

- S100A8/A9, also identified in earlier analyses, function as DAMPs and activate pattern recognition receptors (PRRs) on immune and stromal cells, perpetuating inflammation.
- DEFA3, an antimicrobial peptide, highlights the continued activation of innate immune mechanisms.

Their enrichment supports the idea that immune cells in the COPD-NSCLC microenvironment maintain a primed or partially activated state, releasing granule contents that shape local

inflammation, recruit additional immune cells, and possibly promote epithelial transformation through persistent oxidative and proteolytic stress.

5. Secretory Granule Membrane (GO:0030667)

Lastly, enrichment in the secretory granule membrane component reflects the cellular machinery involved in directed exocytosis of granule contents, critical for immune surveillance and cytotoxicity. This includes not just neutrophils but also cytotoxic T cells and NK cells.

This term underscores the functional capacity for regulated secretion of inflammatory mediators, cytotoxic enzymes, and immunomodulatory molecules. While this process is essential for normal host defense, in COPD-NSCLC it may contribute to persistent inflammation and immune dysfunction. Dysregulated degranulation can damage surrounding tissue, enhance tumor invasiveness, and disrupt local immune cell crosstalk, tipping the balance away from anti-tumor immunity.

6.5.3 GO Molecular Function (MF): Chemokine and Protease Activity in Immune Modulation

Gene Ontology enrichment within the Molecular Function (MF) domain highlighted a group of functions that lie at the core of immune communication, inflammation, and tissue remodeling. These functions were dominated by chemokine–cytokine signaling and protease-related activities, reflecting the duality of immune effector mechanisms in COPD-NSCLC—on one hand serving as essential mediators of host defense, and on the other hand contributing to chronic inflammation, immunosuppression, and tumor progression.

This enrichment supports the notion that COPD-associated NSCLC exhibits a dysregulated immune environment, wherein cells are transcriptionally primed for persistent inflammatory signaling and extracellular matrix degradation—two processes tightly linked to tumor survival, invasion, and resistance to immunotherapy.

1. Chemokine Receptor Binding (GO:0042379)

The functional term chemokine receptor binding was significantly enriched, driven by upregulated genes such as CXCL8 (IL-8) and CCL3, which interact with key receptors including CXCR1/2 and CCR1/CCR5, respectively.

- CXCL8, already noted for its role in neutrophil recruitment, also enhances angiogenesis, tumor cell proliferation, and resistance to apoptosis, making it a central mediator in the COPD-NSCLC inflammatory loop.
- CCL3, a ligand for CCR1 and CCR5, promotes macrophage and monocyte recruitment, shaping a TME enriched in inflammatory and immunosuppressive myeloid cells.

These ligand–receptor interactions form chemotactic gradients that orchestrate immune cell infiltration. However, in the context of COPD, such signaling becomes chronic and dysregulated, fostering an environment where infiltrating immune cells support tumor growth through cytokine secretion and immune editing rather than antitumor cytotoxicity.

2. Cytokine Activity (GO:0005125)

The term cytokine activity encompassed a broad range of inflammatory mediators, notably IL6, IL1B, and TNF—each of which was found to be significantly upregulated in COPD-NSCLC samples.

- IL6 is a pleiotropic cytokine central to JAK/STAT3 signaling, which promotes tumor cell survival, epithelial–mesenchymal transition (EMT), and resistance to immune attack.
- IL1B activates inflammasome pathways, induces fever, and promotes tumor-associated angiogenesis and macrophage polarization.
- TNF, while classically cytotoxic, is persistently elevated in many cancers and acts via NF-κB activation to promote inflammation, angiogenesis, and immune tolerance.

The collective upregulation of these cytokines illustrates a hyperactive inflammatory circuit within COPD-NSCLC tumors. Although originally designed to clear pathogens and damaged cells, in this environment the cytokine network supports immune dysregulation, chronic stress signaling, and oncogenic transformation.

3. Chemokine Activity (GO:0008009)

Complementing cytokine activity, chemokine activity was another enriched functional category, driven by the expression of CXCL1 and CXCL2, key chemoattractants for neutrophils and monocytes.

- CXCL1 binds to CXCR2, promoting neutrophil recruitment, angiogenesis, and tumor proliferation. In NSCLC, its expression has been linked to poor prognosis and aggressive behavior.

- CXCL2, similarly, enhances leukocyte chemotaxis and contributes to immune cell retention in the tumor stroma, supporting a chronic inflammatory state.

These chemokines reinforce a “smoldering inflammation” phenotype, typical of COPD lungs and mirrored in the tumor setting. The chronic recruitment and retention of leukocytes not only remodel the tumor landscape but also create immune escape niches through persistent antigen exposure and checkpoint molecule expression.

4. *Protease Binding (GO:0002020)*

The enrichment of protease binding activity revealed the transcriptional activation of regulatory proteins that interact with proteases, such as SERPINB1 and TIMP1.

- SERPINB1 inhibits neutrophil-derived serine proteases (e.g., elastase), balancing inflammation but also preserving tumor-promoting myeloid cells in some contexts.
- TIMP1 (Tissue Inhibitor of Metalloproteinases 1) controls MMP activity, yet paradoxically can also promote tumor growth, angiogenesis, and cell survival by activating signaling cascades when bound to cell surface receptors.

Their presence suggests that in COPD-NSCLC, there is not only increased proteolytic activity (via MMPs and elastases) but also complex regulation that may attempt to restore balance—or alternatively, contribute to tumor adaptation by modulating protease availability.

5. *Serine-Type Peptidase Activity (GO:0008236)*

Lastly, the serine-type peptidase activity term was enriched by the upregulation of genes involved in ECM degradation and immune defense, such as MMP9, ELANE, and CTSG.

- MMP9 facilitates basement membrane degradation, tumor invasion, and metastasis. Its expression by neutrophils and macrophages in tumors is well established as a marker of aggressive disease.
- ELANE (neutrophil elastase) contributes to tissue destruction, impairs antigen presentation, and promotes EMT in epithelial cells.
- CTSG (cathepsin G) is another granule enzyme that modulates chemokine gradients and affects T cell infiltration.

These peptidases act in concert to remodel the ECM, open metastatic routes, and dampen effective immune responses. In COPD, their activity is chronic and poorly controlled, and in the context of NSCLC, this leads to microenvironmental changes that enhance tumor fitness and immune evasion.

6.5.4 KEGG Pathways: Inflammation-Driven Oncogenic Signaling in COPD-Associated NSCLC

KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis provided further insight into the biological circuits hijacked by inflammation in COPD-associated NSCLC (COPD-NSCLC). The top enriched pathways not only corroborate the findings from GO analyses but also reveal how chronic inflammatory signaling can drive tumorigenesis, support immune evasion, and reshape hematopoiesis.

These pathways collectively reflect a hybrid state of pathogen mimicry and immunological dysfunction, characteristic of chronically inflamed tissues like those found in COPD patients. Such sustained immune activity, while initially protective, becomes a key driver of oncogenesis, tissue remodeling, and tumor immune escape.

1. Complement and Coagulation Cascades

Enrichment of the complement and coagulation cascades pathway indicates hyperactivation of innate immune and clotting-related mechanisms, which are frequently co-opted in the tumor context. Upregulated genes in this pathway included C3, C5AR1, and SERPINE1, all of which play crucial roles in inflammation, vascular regulation, and tissue remodeling.

- C3 is a central complement component that, when cleaved, promotes opsonization and leukocyte recruitment. In tumors, it may paradoxically support immune suppression by recruiting myeloid-derived suppressor cells (MDSCs) and activating tumor-promoting macrophages.
- C5AR1 encodes the receptor for complement C5a, and its expression in myeloid cells enhances tumor-promoting inflammation, angiogenesis, and immunosuppression.
- SERPINE1 (PAI-1) regulates fibrinolysis and is commonly associated with poor prognosis in NSCLC due to its role in thrombosis, fibrosis, and EMT.

Together, these genes indicate that tumors arising in COPD lungs exploit complement and coagulation pathways to build a pro-thrombotic, immune-tolerant, and matrix-rich stroma, conducive to progression and metastasis.

2. Cytokine–Cytokine Receptor Interaction

This was one of the most significantly enriched KEGG pathways, consistent with earlier GO Molecular Function results. The cytokine–cytokine receptor interaction network encompasses

diverse signaling axes, notably IL-6/JAK/STAT3, TNF/TNFR, and CXCL/CXCR pathways, all of which are upregulated in COPD-NSCLC.

- The JAK/STAT pathway is a master regulator of inflammation-induced oncogenesis, driving proliferation, survival, and immune resistance in epithelial tumors.
- TNF signaling, while traditionally pro-apoptotic, shifts toward tumor promotion under chronic exposure, activating NF- κ B and MAPK cascades.
- Elevated CXCL8, CXCL1, and CCL2 support myeloid cell infiltration, angiogenesis, and tissue remodeling.

This pathway encapsulates the cytokine storm-like environment of the COPD-NSCLC tumor, where immune signals are abundant but misdirected, promoting tumor progression over immune clearance.

3. Hematopoietic Cell Lineage

Enrichment in the hematopoietic cell lineage pathway, with genes such as CD14, CD33, and ITGAM (CD11b) upregulated, points to active recruitment and differentiation of myeloid cells in the tumor microenvironment.

- CD14 is a co-receptor for LPS and is typically expressed on monocytes and macrophages, indicating an inflamed myeloid landscape.
- CD33 is a marker of immature myeloid cells, including MDSCs, which are potent suppressors of T cell function in tumors.
- ITGAM, encoding CD11b, marks monocytes, granulocytes, and macrophages involved in immune suppression, antigen presentation, and tissue invasion.

These markers suggest that in COPD-NSCLC, there is not only infiltration of immune cells but also reprogramming of their lineage and function—from host defense to tumor support. The presence of immature or tolerogenic myeloid cells implies a shift in hematopoietic output, possibly influenced by systemic inflammation and tumor-derived factors.

4. Staphylococcus aureus Infection

Interestingly, the Staphylococcus aureus infection pathway was also enriched. Although no active infection is present, this reflects the activation of pattern recognition receptor (PRR) pathways that are typically engaged during bacterial infection.

- Genes involved in TLR2/TLR4 signaling, NOD-like receptors, and inflammasome components are shared between bacterial immune responses and the “sterile inflammation” seen in COPD.
- The upregulation of these genes in tumors suggests that the TME in COPD-NSCLC mimics bacterial infection, leading to chronic activation of innate immune sensors.

This pathogen mimicry phenomenon can lead to dysregulated inflammation, immune fatigue, and tissue destruction, and has been implicated in the etiology of several inflammation-linked cancers, including colorectal and lung carcinomas.

5. Viral Protein Interaction with Cytokine and Cytokine Receptor

This enriched pathway indicates that tumor cells in COPD-NSCLC may be leveraging immune evasion mechanisms similar to those used by chronic viral infections. Genes in this category include cytokines and their receptors that are often modulated by viruses to suppress host immunity.

- Such mimicry may enable tumor cells to downregulate antigen presentation, induce immune checkpoint molecules, and interfere with cytokine receptor signaling to escape immune surveillance.
- In the COPD context, where immune exhaustion and T cell dysfunction are already prevalent, this mimicry exacerbates immune evasion and further impairs tumor immunogenicity.

This finding adds a layer of complexity, suggesting that chronic inflammation and innate immune reprogramming not only fuel tumor growth but also equip the tumor with mechanisms to avoid immune detection, much like persistent viral infections.

7. Discussion:

This study provides a comprehensive single-cell transcriptomic view of the tumor micro environment (TME) in non-small cell lung cancer (NSCLC), with a specific focus on patients with coexisting chronic obstructive pulmonary disease (COPD). Through comparative analysis, we uncovered significant shifts in immune and stromal cell composition and function, highlighting the distinct biological features of COPD-associated NSCLC (COPD-NSCLC).

7.1 Interpretation of Findings

Our data revealed an increased abundance of inflammatory macrophages and exhausted T cells in COPD-NSCLC tissues compared to NSCLC alone. These findings are in line with prior reports indicating that chronic inflammation in COPD leads to a skewed immune landscape, often characterized by persistent activation and functional exhaustion of immune cells. The upregulation of genes such as CXCL9, IL1B, and NLRP3 in macrophages suggests activation of inflammasome pathways and chemokine-mediated recruitment of immune effectors. In parallel, increased expression of PDCD1 (PD-1), LAG3, and CTLA4 in T cells highlights an immunosuppressive phenotype, potentially driven by prolonged antigen exposure and inflammatory signaling.

These alterations indicate that COPD creates a tumor-permissive micro environment through continuous immune stimulation, impaired clearance of transformed cells, and suppression of cytotoxic responses.

7.2 Mechanistic Implications

The observed transcriptional shifts may be mechanistically rooted in repeated epithelial injury and aberrant repair processes characteristic of COPD lungs. The enrichment of NF- κ B, TLR, and cytokine-cytokine receptor interaction pathways supports a model in which innate immune signaling drives persistent inflammation and immune reprogramming. This chronic activation may polarize macrophages toward a pro-tumorigenic phenotype (similar to M2-like TAMs) and desensitize T cells via checkpoint molecule upregulation.

Interestingly, the enhanced expression of matrix remodeling genes such as MMP1 and VEGFA further supports the hypothesis that COPD fosters a pro-invasive micro environment conducive to tumor progression and metastasis.

7.3 Clinical and Therapeutic Implications

These findings carry significant clinical implications. The heightened immune checkpoint expression in COPD-NSCLC patients suggests that these individuals may benefit from immune checkpoint inhibitors (ICIs), particularly PD-1/PD-L1 blockade. However, the overall immune exhaustion and presence of inflammatory macrophages may reduce responsiveness or lead to adaptive resistance. As such, combining ICIs with anti-inflammatory agents, macrophage modulators, or IL-1 β inhibitors (e.g., canakinumab) may enhance therapeutic efficacy.

Moreover, the identification of COPD-specific immune and stromal markers opens the door to biomarker-guided patient stratification, which could inform personalized therapeutic approaches and improve outcomes in this high-risk group.

7.4 Limitations

This study is not without limitations. First, the sample size is relatively small ($n = 10$), and although balanced between COPD and non-COPD patients, it may not fully capture population-level variability. Second, the reliance on publicly available datasets introduces potential biases related to sample handling, sequencing depth, and clinical metadata availability.

Additionally, while scRNA-seq captures transcriptomic profiles at high resolution, it lacks spatial context and protein-level confirmation, both of which are critical for understanding cell-cell interactions and functional states in situ.

7.5 Future Directions

Future work should integrate spatial transcriptomics to map cellular niches and interactions within the tumor. Single-cell ATAC-seq or multi-modal platforms (e.g., CITE-seq) could further clarify the regulatory logic underlying immune exhaustion and stromal remodeling.

Validation of key findings in larger, prospective cohorts—ideally with clinical annotations and treatment response data—will be essential for translating these insights into therapeutic strategies. Furthermore, functional experiments targeting inflammatory macrophages or reprogramming exhausted T cells could inform novel combination therapies tailored for COPD-associated lung cancer.

8. Conclusion

This study presents a comprehensive and high-resolution analysis of the cellular and molecular underpinnings of non-small cell lung cancer (NSCLC) in the context of chronic obstructive pulmonary disease (COPD), using cutting-edge single-cell RNA sequencing (scRNA-seq). By focusing on the tumor microenvironment (TME) at the single-cell level, we were able to resolve the heterogeneity that characterizes both NSCLC and COPD-associated NSCLC (COPD-NSCLC), providing novel insights into how chronic lung inflammation alters tumor biology and immune dynamics.

Through the integration of transcriptomic data from over 11,000 individual cells, we identified distinct transcriptional programs and compositional shifts between NSCLC and COPD-NSCLC samples. These differences were observed not only in malignant epithelial cells but also across immune subsets—including macrophages, neutrophils, T cells, and dendritic cells—as well as stromal elements such as cancer-associated fibroblasts (CAFs) and endothelial cells. The cell-type-specific resolution offered by scRNA-seq revealed a complex interplay between chronic inflammation, immune cell reprogramming, and stromal remodeling, all of which contribute to the tumor-promoting milieu observed in COPD-NSCLC.

Differential gene expression analysis uncovered a core inflammatory signature in COPD-NSCLC characterized by the upregulation of genes involved in innate immune activation, neutrophil-mediated responses, and pro-inflammatory cytokine signaling. Functional enrichment analysis further supported these findings, with significant overrepresentation of Gene Ontology (GO) terms such as neutrophil degranulation, cytokine-mediated signaling pathways, pattern recognition receptor activity, and complement activation. These pathways mirror the chronic immune activation and tissue damage seen in COPD, but in the context of cancer, they appear to be repurposed to support tumor growth, immune escape, and angiogenesis.

Cellular component analysis highlighted the transcriptional activity of genes involved in secretory granules, the endoplasmic reticulum, and extracellular matrix (ECM) compartments, suggesting that both immune and stromal cells are actively engaged in protein secretion, ECM remodeling, and stress adaptation. These features indicate that the COPD-NSCLC TME is not only structurally remodeled but also functionally reprogrammed to sustain chronic inflammation and support tumor invasion.

Molecular function analysis revealed elevated expression of genes associated with cytokine and chemokine activity, protease binding, and serine-type peptidase activity, confirming that inflammatory and proteolytic mechanisms are central to immune modulation and tumor–stroma interactions in COPD-NSCLC. These activities are not merely bystanders of inflammation but active participants in tumor progression, driving immune cell recruitment, ECM degradation, and epithelial–mesenchymal transition (EMT).

KEGG pathway enrichment provided a global view of inflammation-driven oncogenic signaling, highlighting the role of cytokine–cytokine receptor interactions, complement and coagulation cascades, and hematopoietic lineage reprogramming. The enrichment of pathogen-response pathways, such as those associated with *Staphylococcus aureus* infection and viral mimicry, points to a phenomenon of sterile inflammation and innate immune reprogramming, wherein the tumor exploits mechanisms typically used for microbial defense to evade immune surveillance.

Collectively, these findings provide compelling evidence that COPD-associated NSCLC is not simply a variant of classical NSCLC, but rather a distinct biological entity shaped by prolonged exposure to inflammatory stimuli, tissue injury, and immune dysfunction. The chronic activation of innate immunity and tissue remodeling processes creates a unique tumor ecosystem—one that may respond differently to immunotherapies and targeted treatments.

Importantly, this study also identifies potential molecular targets for therapeutic intervention in COPD-NSCLC. These include key regulators of inflammation such as IL-6, CXCL8, and TNF, modulators of immune suppression like STAT3 and C5AR1, and enzymes involved in ECM remodeling such as MMP9 and SERPINE1. Targeting these pathways may help overcome resistance to conventional therapies and improve outcomes for patients with COPD-associated lung cancer.

Finally, by demonstrating the power of single-cell transcriptomics in disentangling the cellular complexity of lung tumors, this work lays a foundation for future research that integrates high-dimensional omics data with functional immunology, spatial biology, and clinical phenotyping. Such integrative approaches will be critical for advancing precision oncology, particularly in comorbid disease contexts like COPD, where personalized treatment strategies are urgently needed.

In conclusion, this study not only advances our understanding of COPD-NSCLC pathogenesis but also offers a framework for the development of next-generation diagnostics and therapies tailored to inflammation-driven cancers.

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