



Spatial single-cell profiling reveals macrophage niches in severe COVID-19 lungs

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Abstract

Aims: Severe coronavirus disease 2019 (COVID-19) is characterized by marked heterogeneity in lung pathology, including diffuse alveolar damage, inflammation, and fibrosis. However, how spatially organized cellular interactions contribute to these divergent pathological outcomes remains incompletely understood. We aimed to define the spatial organization of cellular microenvironments in severe COVID-19 lungs and identify niche-specific cellular interactions associated with distinct histopathological states.

Methods: We applied imaging-based single-cell spatial transcriptomics to post-mortem lung tissues from patients with severe COVID-19 and control subjects, enabling high-resolution mapping of cellular composition, spatial neighborhoods, and predicted ligand-receptor interactions within intact tissue architecture.

Results: Analysis of 76,973 cells identified 20 distinct cell populations and revealed marked heterogeneity between COVID-19 samples, corresponding to different histopathological states. Spatial niche analysis identified discrete macrophage-associated microenvironments. Fibrotic niches were enriched in chitinase 3-like 1 (*CHI3L1*)/matrix metalloproteinase 9 (*MMP9*^{hi}) macrophages spatially associated with collagen type I alpha 1 chain (*COL1A1*⁺) fibroblasts and basal cells. Ligand-receptor inference predicted macrophage-derived transforming growth factor beta (TGF- β) and galectin-3 signaling as drivers of pathogenic fibroblast activation and extracellular matrix remodeling. In contrast, alveolar niches contained secreted phosphoprotein 1 (*SPP1*^{hi}) macrophages exposed to epithelial-derived cues, including WNT ligands and granulocyte-macrophage colony-stimulating factor (GM-CSF), consistent with differentiation toward an alveolar macrophage-like state.

Conclusion: Severe COVID-19 lungs are organized into distinct spatial niches associated with divergent immunopathological trajectories. Imaging-based spatial transcriptomics provides a platform to link histopathological states with localized cellular interactions and macrophage-driven tissue remodeling programs.

Keywords: Macrophage, single-cell spatial transcriptomics, COVID-19

1. Introduction

Since its emergence in late 2019, infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has resulted in nearly 800 million confirmed cases worldwide and approximately 7 million deaths^[1]. While most individuals develop mild, self-limiting respiratory symptoms resembling the common cold^[2], severe coronavirus disease 2019 (COVID-19) is characterized by acute lung injury, often progressing to acute respiratory distress syndrome (ARDS) and other life-threatening complications requiring hospitalization, intensive care unit admission, and mechanical ventilation^[3,4]. Moreover, survivors may develop long-term post-acute sequelae following primary infection, including diffuse pulmonary fibrosis^[5]. A defining feature of severe COVID-19 is the remarkable heterogeneity of pulmonary pathology, with co-existing patterns of diffuse alveolar damage (DAD), inflammation, and fibrosis^[6–8]. DAD is the main COVID-19-associated histological pattern, reflecting evolving stages of lung injury^[7] and often associated with capillary congestion and microthrombi^[9,10]. Early SARS-CoV-2 pneumonia is characterized by exudative DAD with hyaline membranes and



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inflammatory infiltrates^[6,11], followed by an epithelial proliferative phase and, in some patients, progression to fibrosis^[12]. This heterogeneity reflects, at least in part, dysregulated immune responses^[13,14]. Severe disease is indeed associated with impaired antiviral signaling, excessive myeloid-driven inflammation, and altered macrophage composition^[15-17]. In particular, the accumulation of monocyte-derived macrophages and the persistence of secreted phosphoprotein 1 (*SPP1*⁺) macrophages have been linked to disease severity, tissue remodeling, and fibrosis^[18,19]. Such dysregulated immune responses can be orchestrated by^[20] and result in^[21,22] modifications in structural cell activation. Indeed, structural cells such as epithelial cells, fibroblasts, and endothelial cells undergo profound changes, contributing to tissue damage and repair processes. However, how these diverse cell populations interact within the lung microenvironment to drive distinct pathological outcomes remains poorly understood.

Emerging evidence suggests that the functional behavior of immune and structural cells is critically shaped by their spatial organization within tissues. Recent studies combining single-cell and spatial transcriptomics have begun to map the cellular landscape of COVID-19 lungs, revealing associations between specific cell populations and disease severity^[11,12,23,24]. Imaging-based spatial transcriptomics (ST) further enables high-resolution, single-cell mapping of gene expression within intact tissue sections, providing insights into tissue architecture, cell-cell interactions, and local cellular states within their native microenvironment.

Here, we hypothesized that severe COVID-19 lungs are organized into distinct spatial niches that integrate specific cellular compositions and intercellular signaling networks, thereby modulating divergent pathological trajectories. We applied the imaging-based ST method CosMx (Nanostring)^[25] to post-mortem lung tissues from patients with severe COVID-19 and control subjects. Our analyses revealed that severe COVID-19 lungs comprise discrete, macrophage-centered niches associated with specific histopathological states. Pro-fibrotic niches contained chitinase 3-like 1 (*CHI3L1*)/matrix metalloproteinase 9 (*MMP9*^{hi}) macrophages spatially coupled to pathogenic fibroblasts and predicted to promote their activation through transforming growth factor beta (*TGF-β*) signaling. In contrast, alveolar niches were enriched in *SPP1*^{hi} macrophages exposed to epithelial-derived signals that may promote an alveolar macrophage-like state. Together, these findings highlight how localized cellular interactions may contribute to divergent immunopathological outcomes within the same organ.

2. Methods

2.1 Sample collection

Lung samples from six subjects were collected post-mortem at the University Hospital of Liege (Belgium), including three patients who died of severe COVID-19^[45] and three subjects who died from unrelated, non-pulmonary causes (Table 1). Samples were collected within 5 minutes after death by the same dedicated pulmonologist following a standardized procedure. For COVID-19 cases, tissue was collected from macroscopically identified lesional areas, without systematic preference for a particular pulmonary lobe. Non-COVID-19 control samples consisted of archived tissues selected from the available biobank material to provide the best possible match for age and sex. All tissues were provided by the Biobank of the Liege University Hospital. Lung samples were formalin-fixed, paraffin-embedded (FFPE), and 4-μm-thick serial tissue sections were prepared.

Table 1. Characteristics of human subjects from which lung samples originate.

Patient number	Gender	Age (years)	Cause of death	SARS-CoV-2 (PCR)
1	M	39	Cardiac death	No
2	F	39	Cardiac death	No
3	M	63	Cardiac death	No
4	M	63	Pneumonia	Yes
5	M	67	ARDS	Yes
6	M	56	ARDS	Yes

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; ARDS: acute respiratory distress syndrome; F: female; M: male; PCR: polymerase chain reaction.

2.2 Histopathological evaluation

Hematoxylin & eosin (H&E)-stained serial sections were evaluated by a trained pathologist. Histopathological features were assessed using descriptive and semi-quantitative criteria. Fibrosis was graded based on the extent of alveolar septal thickening due to fibrotic tissue deposition using a 4-point scale: 0, absent/minimal; 1, mild; 2, moderate; and 3, severe, as previously described^[26]. Inflammatory infiltration was graded as mild, moderate, or severe according to the extent and density of lymphoplasmacytic and histiocytic infiltrates. Other histopathological features, including pulmonary congestion, edema, air trapping, emphysematous changes, hemorrhage, and bronchiolar remodeling, were recorded qualitatively.

2.3 Single cell spatial transcriptomic analysis with CosMx

FFPE tissue section preparation for CosMx spatial molecular imager (CosMx SMI) and *in situ* hybridization were performed according to the manufacturer's instructions. Briefly, 4- μ m tissue sections were mounted on VWR Superfrost Plus Micro slides. Precisely, within each slide, tissue sections measuring 5 $\times$ 12 mm were arranged side by side to fit within the designated "Tissue Scan Area" of the slide, which allows a maximum space of 15 $\times$ 20 mm. The slides were subsequently baked overnight at 60 °C. *In situ* hybridization probes (from Human Universal Cell Characterization RNA Panel-1,000 plex) from Nanostring were used for hybridization. RNA target readout was performed on the CosMx SMI instrument and 40 imaging sections (i.e., fields of view (FOV), 0.5 $\times$ 0.5 mm each) were randomly selected for imaging in each whole-slide scan. Transcript localization was achieved with an approximate spatial resolution of 50 nm. After RNA readout, the tissue samples were incubated with a 4-fluorophore-conjugated antibody cocktail against CD298/B2M, PanCK, CD45, and CD68 proteins and DAPI stain in the CosMx SMI instrument. Images for the 5 channels (4 antibodies and DAPI) were captured. The imaging results were subsequently automatically exported to the cloud-based AtoMx™ Spatial Informatics Platform. Single cells were segmented post-acquisition to obtain cell boundaries, transcripts were assigned to their corresponding cell based on the segmentation map and further localized to a specific cellular compartment (e.g., nucleus, cytoplasm), and a transcript-by-cell count matrix was obtained.

2.4 Data analysis

Count matrix, transcripts positions, and segmentation data were exported from the AtoMx™ Spatial Informatics Platform and were loaded with the Seurat^[27] package (5.2.1) using the LoadNanostring function. The gene counts were normalized and scaled with the SCTransform method. After merging cells from the 2 slides, 39 clusters were identified using the FindClusters function (30 Principal Components (PC) included and a resolution of 1.25 was selected) and the differentially expressed genes (DEG) were calculated using the FindAllMarkers function. A filtered version of the data was obtained by keeping a subset of cells with a gene count higher than 100 and an RNA count superior to 250. A total of 29 clusters were identified in the filtered object. These clusters were subsequently annotated based on marker gene expression. The annotated cell identities were then mapped back onto the unfiltered dataset to assess the cellular composition of the original clusters. Clusters that could not be confidently annotated were labeled as "unidentified". The bioinformatic workflow for data analysis can be found in [Figure S1](#).

Some observations were validated in an independent CosMx dataset of a COVID-19 lung cohort^[28]. The corresponding Seurat object was downloaded, and autopsy sample #6, which contained the macrophage population of interest, was selected for separate analysis. Cells from this sample were clustered using FindClusters with 25 PCs and a resolution of 1.25, resulting in 20 clusters. Clusters were subsequently annotated based on marker gene expression and the original annotations provided by the authors.

2.5 Automatic cell annotations by Robust Cell Type Decomposition

Cells were annotated using Robust Cell Type Decomposition (RCTD)^[29] using the spacexr package (version 2.2.1). A reference dataset was required for the annotation, and a single-cell transcriptomics dataset was obtained from the Human Lung Cell Atlas (HLCA)^[30]. The reference consisted of cells from both normal and COVID-19 conditions using the most granular level of annotation called "ann_finetest_level". This dataset was then subsampled and only a maximum of 2,000 cells per "finetest annotation" was kept (7.6 $\times$ 10⁴ cells in total). An RCTD reference was created using the create.RCTD function and the Seurat object was annotated using the run.RCTD function in doublets mode. When double annotations were obtained for a cell, we only considered the first one.

2.6 Gene Ontology

The DEG lists for enrichment analyses were calculated using the Seurat function FindMarkers; only positively regulated genes were included, and p-values were adjusted with a Bonferroni correction using all features in the dataset. Thresholds of an average log₂ fold change of 1 and an adjusted P value of 0.05 were applied to filter the gene lists. Gene Ontology (GO) enrichment analyses were made using enrichGO functions from the clusterProfiler^[31] package with default arguments. Ontology gene sets from the Molecular Signatures Database (MSigDB) were used to test for enrichment. Gene Ratio is the proportion of DEG overlapping with a given gene set. It was calculated as the number of enriched genes in a gene set divided by the total number of genes in that gene set.

2.7 Cell-based niche analysis

After cell annotation, niche annotation was performed using the BuildNicheAssay function (Seurat). The function works by first creating a single-cell neighborhood matrix based on the cell types that compose the neighborhood, then by clustering the cells based on the composition of their neighborhood. The neighborhood of a cell was defined as its 30 nearest neighboring cells in physical space. The niche analysis was performed on both slides jointly. The K number of niches to be returned by the function was fixed to 10. This number was decided with the objective of having the highest number of niches while keeping a specific cell composition for each of them. The number of cells from each annotated cell population within each niche was placed in a count matrix. A z-score normalization was applied to the matrix for each cell type and the matrix was plotted in a heatmap using ComplexHeatmap^[32].

2.8 Ligand-receptor analysis

Cell-cell interactions were assessed using NicheNet (version 2.2.0)^[33]. For the initial analysis, *CHI3L1*/*MMP9*^{hi} macrophages were designated as sender cells, while fibroblasts were defined as receiver cells. For the second analysis, AT2 were defined as sender cells and macrophages were defined as receiver cells. Genes were considered as potential ligands if they were expressed in at least 5% of the sender cell population. In receiver cells, 2 gene sets of interest were defined by identifying DEGs between alveolar fibroblasts and *COL1A1*⁺ fibroblasts and between *SPP1*⁺ macrophages and alveolar macrophages (AM). Genes were included in the target gene set if they had an average log₂ fold change greater than 1 and an adjusted *p*-value below 0.05.

Ligand prioritization was then performed using NicheNet's scoring framework, and the top 30 ligands were selected based on their area under the precision-recall curve. A ligand-target gene heatmap was generated to visualize the predicted regulatory potential of each ligand on its target genes. The top ligand-target interactions were further visualized using a circos plot. ST outputs from CosMx SMI were imported into Giotto^[34] (version 4.2.1) using the createGiottoCosMxObject function. Spatial density plots of gene markers were then generated with the spatInSituPlotDensity function to visualize the localization patterns of relevant cell populations and marker gene expression.

3. Results

3.1 Single cell spatial transcriptomic profiling of COVID-19 and control lung tissue

Lung tissue sections from 3 severe COVID-19 patients^[15] and three subjects who died from non-pulmonary causes were analyzed using CosMx SMI (Table 1 and Figure 1a). Tissue blocks from COVID-19 patients and control subjects were mounted on separate slides, referred to as the "COVID-19" and "Control" slides, respectively. All samples were processed according to the experimental workflow outlined in Figure 1a and as explained in the Methods section.

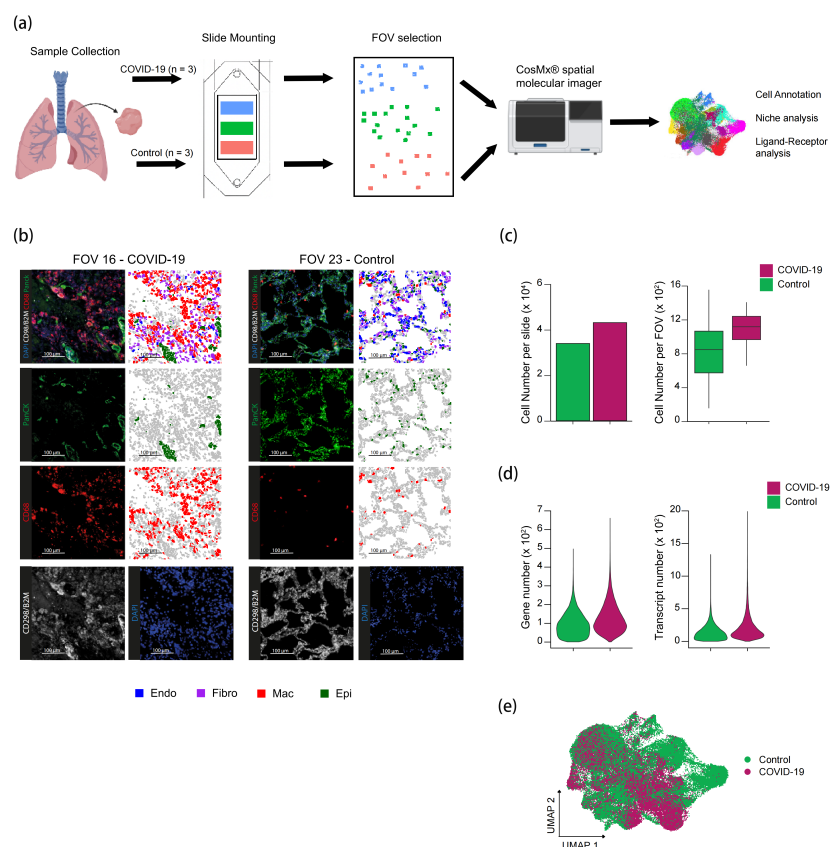


Figure 1. Overview of the experimental workflow, cell segmentation and QC metrics. (a) Schematic representation of the technical workflow, from post-mortem lung tissue collection to downstream analyses. All steps were carried out separately for COVID-19 and control samples before pooling of the data during downstream analyses; (b) Representative immunofluorescence images of FOV 16 from COVID-19 slide (left) and FOV 23 from Control slide (right), of nuclear stain DAPI (blue), cytoplasmic stain CD298/B2M (white), CD68⁺ macrophages (Mac, red) and pan-cytokeratin⁺ epithelial cells (Epi, green) present alongside the subsequent cellular annotation of FOV; (c) Cell numbers per condition represented by a barplot and cell numbers per FOVs within each condition represented by a boxplot; (d) Violin plots depicting gene and transcript numbers of each sample (width = abundance of cells); (e) UMAP plot of CosMx output. Endo: endothelial cells; Fibro: fibroblasts. FOV: field of view; COVID-19: coronavirus disease 2019; UMAP: Uniform Manifold Approximation and Projection; DAPI: 4',6'-diamidino-2-phenylindole.

Briefly, cyclic RNA readout using the SMI was performed as described^[25] and the 1,000-plex CosMx Human Universal Cell Characterization Panel (NanoString) was used. For each slide, a total of 40 FOVs were randomly selected with the aim of maximizing spatial coverage across the slide. Within each FOV, cells were identified using a machine learning-based preprocessing pipeline, followed by segmentation with a pre-trained Cellpose neural network model (Figure 1b), and each detected transcript was assigned to its corresponding cell based on the segmentation map.

We detected a total of 33,925 Control and 43,048 COVID-19 cells, corresponding to 848 ± 351 and $1,076 \pm 200$ (mean $\pm$ SD) cells per FOV, respectively (Figure 1c). An average of 142 ± 112 and 214 ± 204 transcripts per cell were detected in Control and COVID-19 samples, respectively (Figure 1d). Following processing in Seurat (Figure S1), the 76,973 cells across 80 FOVs were integrated and visualized by Uniform Manifold Approximation and Projection (UMAP) (Figure 1e).

3.2 Cellular landscape of COVID-19 and control lung samples

Graph-based clustering identified 20 cell populations, which were annotated based on marker gene expression, reference mapping to the HLCA^[30] and, when required, GO analysis (Figure 2a,b,c and Figures S1,S2,S3). To facilitate manual cluster annotation, we first filtered and subset cells with the highest amounts of transcripts (> 250 transcripts) and genes (> 100 genes), which were then re-integrated to the global dataset (Figures S1,S2).

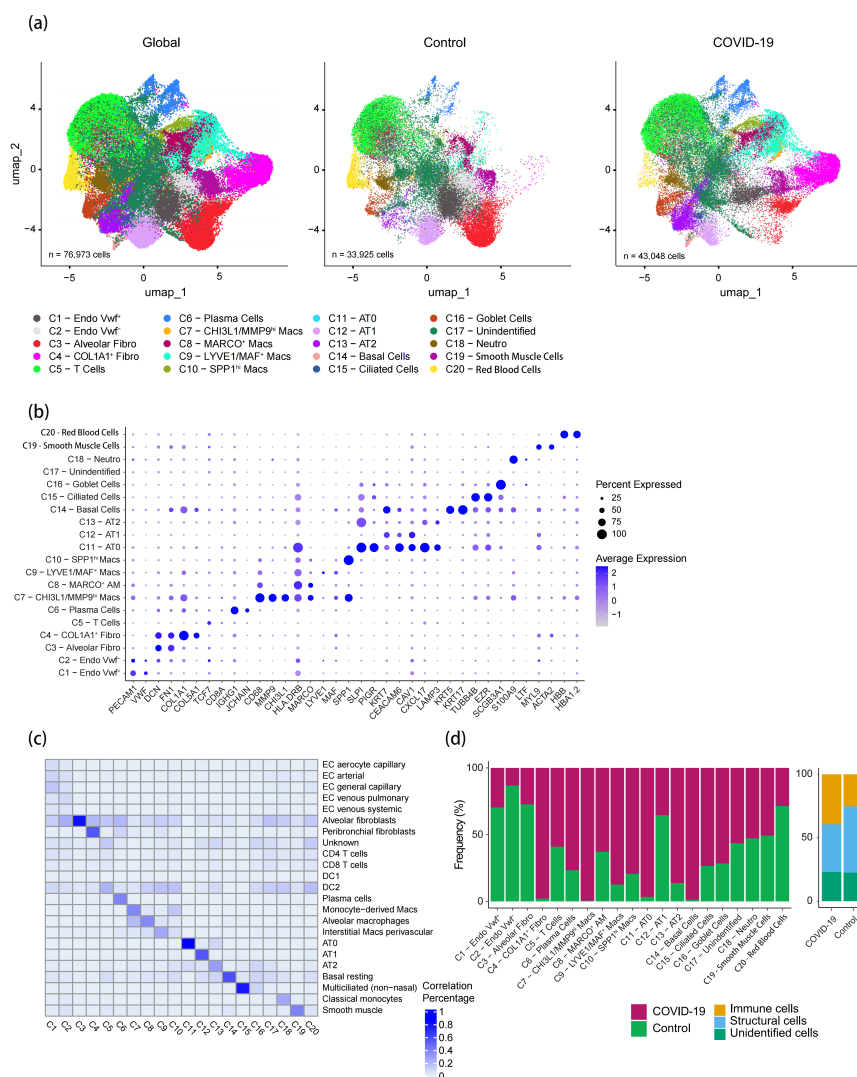


Figure 2. Annotation of cell populations. (a) UMAP plot of CosMX SMI output including cells from COVID-19 and Control samples. Cells are colored according to their annotated identity; (b) Dotplot showing average expression of the indicated genes and the percentage of cells expressing the genes within each cluster; (c) Correlation matrix showing the percentage of cells of manually annotated clusters overlapping with each automatic reference-based annotation; (d) Histogram showing the frequency of COVID-19 and Control condition within each annotated cell population (left) and showing the frequency of cell categories in each condition (right). COVID-19: coronavirus disease 2019; CosMX SMI: CosMx spatial molecular imager; UMAP: Uniform Manifold Approximation and Projection; Endo: endothelial cells; *COL1A1*⁺: collagen type I alpha 1 chain; *MMP9*^{hi}: matrix metalloproteinase 9; *CH3L1*: chitinase 3-like 1; *MARCO*⁺: macrophage receptor with collagenous structure.

Clusters 1 and 2 (C1 and C2) expressed *PECAM1* and were annotated as endothelial cells (Endos). Notably, C1 showed higher expression of *VWF* compared to C2. C3 and C4 were identified as fibroblasts (Fibros) based on the expression of canonical markers such as *DCN* and *FN1* (Figure 2b). Most cells in C3 were annotated as alveolar Fibros by reference-based annotation (Figure 2c). In contrast, Fibros in C4 overexpressed collagen-related genes, including *COL1A1* and *COL5A1*, and were annotated as *COL1A1*⁺ Fibros. C5 was annotated as T lymphocytes (T cells) based on the expression of lineage-specific genes such as *TCF7* and *CD8A* (Figure 2b), associated with the enrichment of T cells-associated responses (Figure S3). C6 was identified as plasma cells based on *IGHG1* and *JCHAIN* expression, which was confirmed by reference-based annotation. Cells in C7-C10 were annotated as macrophages (Macs) based on expression of the canonical macrophage marker *CD68* (Figure 2b). C7 was characterized by high levels of *CHI3L1* and *MMP9* and was labelled *CHI3L1/MMP9*^{hi} Macs. C8 was annotated as macrophage receptor with collagenous structure (MARCO⁺) AM, given the expression of *MARCO*, an AM-specific gene^[35]. Macs in C9 expressed high levels of *MAF* and *LYVE1*, markers classically associated with interstitial Macs (IMs)^[36,37]. C10 was characterized by elevated expression of *SPP1* and was labeled *SPP1*^{hi} Macs (Figure 2b). Both C7 and C10 were mapped to monocyte-derived Macs (Figure 2c), suggesting a hematopoietic origin. C11-C13 exhibited high expression of epithelial markers such as *SLPI* and *CAV1* and were annotated as AT0, AT1, and AT2 cells, respectively, based on reference-based classification (Figure 2c). C14 was identified as basal cells (*KRT5* and *KRT17*). C15 was identified as ciliated cells based on *EZR* and *TUBB4B* expression^[38,39]. C16 was identified as *SCGB3A1*-expressing goblet cells. C18 was labelled as neutrophils based on *LTF* and *S100A9* expression^[40,41], which was confirmed by GO analysis (Figure 2b and Figure S3). C19 encompassed smooth muscle cells (*ACTA2* and *MYL9*) and C20 expressed high levels of hemoglobin-related genes (*HBB*, *HBA1* and *HBA2*). Hence, these cells were considered as red blood cells (RBC) (Figure 2a). The remaining cells in C17 were considered unidentified, as they did not express any markers specific for a given cell type and were not annotated by reference-based annotation.

Cellular composition differed markedly between COVID-19 and Control samples (Figure 2d). COVID-19 lungs showed an enrichment of immune cells (39% in COVID-19 vs. 25% in Control), together with expansion of several epithelial populations and *COL1A1*⁺ fibroblasts (Figure 2d). Overall, 33.2% of cells were immune cells and 44.1% were structural cells, while 22.7% remained unannotated.

3.3 Histopathology and spatial transcriptomics reveal distinct pathological states

Given the limited cohort size and the spatial heterogeneity of COVID-19 lung pathology^[42,43], we first characterized the histopathological features of each analyzed tissue section rather than performing broad comparisons between COVID-19 and Control groups.

H&E-stained serial sections were evaluated by a trained pathologist (Table 2). Control samples already displayed some histological alterations, including interstitial pneumonia, alveolar edema and air trapping (Table 3 and Figure S4).

Table 2. Histopathological features of COVID-19 lung sections.

Histological Features	COVID-19 #1	COVID-19 #2	COVID-19 #3
Type of Pneumonia	Interstitial, moderate, chiefly lymphoplasmacytic and histiocytic	Interstitial, moderate, uneven, chiefly lymphoplasmacytic	Interstitial, moderate, chiefly lymphoplasmacytic and histiocytic
Fibrosis	Mild, diffuse. Score 1	Severe, diffuse, at different stages, mostly active. Score 3	Mild, interstitial, uneven in distribution. Score 1
Hemorrhages	Multiple foci of hemorrhage	Severe congestion with multifocal hemorrhages	Small multifocal hemorrhages
Alveolar compartment	Diffuse alveolar damage	-	Type II cell hyperplasia and/or bronchiolization
Bronchiolar Remodeling	-	Present, with frequent squamous differentiation	Fibrin/mucus bronchiolar plugs
Immune infiltration	Severe lymphoplasmacytic and histiocytic Moderate hyperplasia of alveolar macrophages	Moderate Lymphoplasmacytic Moderate hyperplasia of alveolar macrophages, giant cell formation	Severe lymphoplasmacytic and histiocytic Severe hyperplasia of alveolar macrophages

COVID-19: coronavirus disease 2019.

enriched in basal, goblet, and ciliated cells and localized around airway structures, whereas N6, enriched in smooth muscle cells, was mainly associated with airways and blood vessels (Figure 4). N8 was enriched in immune cells, including T cells, neutrophils, and plasma cells, and was markedly expanded in COVID-19 #1, consistent with its acute inflammatory phenotype (Figure 4a,b). Niche distributions also varied among Control samples, further highlighting inter-individual tissue heterogeneity (Figure 4b).

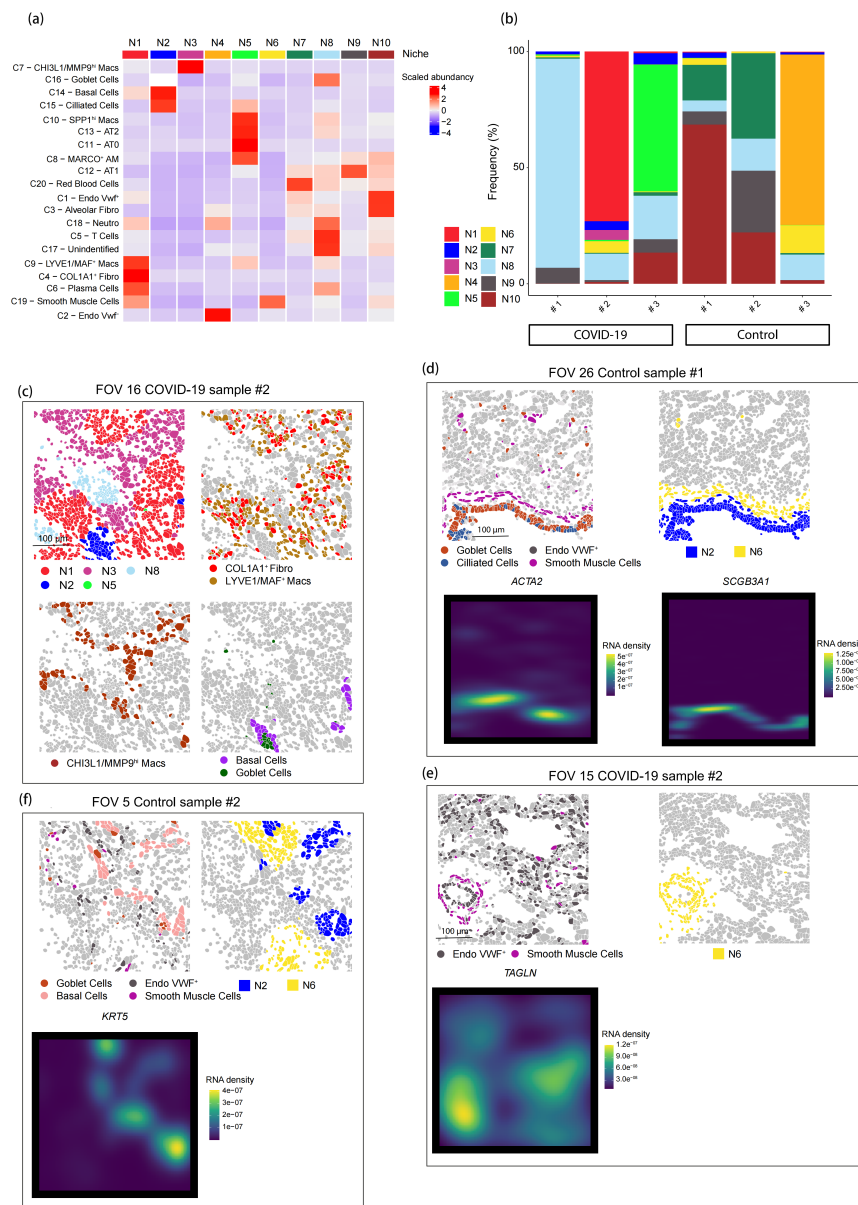


Figure 4. Cellular niche identification in COVID-19 and Control samples. (a) Scaled abundance matrix representing the count of each annotated cell population across niches. Z-score normalization was applied by row; (b) Histogram showing the frequency of each niche per sample; (c) Representative dimension plot of FOV 16 from COVID-19 sample #2 where cells are colored by niche and cell types; (d-f) Dimension plot of FOVs representing (d) FOV Control 26, (e) FOV Covid 15 and (f) FOV Control 5, with niche N2 and N6 highlighted (upper right), annotated cell type highlighted (upper left) and density plots representing RNA density (lower) of genes specific of smooth muscle cells (*ACTA2*, *TAGLN*), goblet cells (*SCGB3A1*) and basal cells (*KRT5*). COVID-19: coronavirus disease 2019; FOV: fields of view; *COL1A1*⁺: collagen type I alpha 1 chain; Endo: endothelial cells; *SPP1*^{hi}: secreted phosphoprotein 1; *MARCO*⁺: macrophage receptor with collagenous structure; *CHI3L1*: chitinase 3-like 1; *MMP9*^{hi}: matrix metalloproteinase 9; Fibro: fibroblasts; AM: alveolar macrophages.

In contrast, niches N1, N3, and N5 were detected almost exclusively in COVID-19 samples (Figure 4a,b). N1 displayed features of fibrotic remodeling, including an abundance of plasma cells^[44], and *COL1A1*⁺ Fibros^[45], together with numerous endothelial and smooth muscle cells^[46] (Figure 4a,c). N3 was primarily defined by *CHI3L1/MMP9*^{hi} Macs (Figure 4a,b), while otherwise sharing a cellular composition similar to N1 (Figure S6). Hence, the identification of this distinct niche suggests a spatial clustering of *CHI3L1/MMP9*^{hi} Macs (Figure 4c). Even though N1, N2 and N3 are three separated niches, we observed that such niches tended to be spatially adjacent inside COVID-19 #2 (Figure 4c). N5, in contrast, was predominantly composed of alveolar-associated cells, including

AT0 and AT2 cells, and MARCO⁺ an AM, together with SPP1^{hi} macrophages. Importantly, these niches segregated by samples: N1 and N3 were detected exclusively in COVID-19 sample #2, while N5 was specific to COVID-19 sample #3, and COVID-19 sample #1 was characterized by the expansion of the immune-rich N8 niche (Figure 4b).

Together, these findings support the conclusion that each COVID-19 sample represents a distinct pathological state, associated with a unique constellation of cellular niches. These data are consistent with a model in which distinct histopathological contexts are linked to specific tissue architectures and cellular compositions, ultimately reflecting divergent underlying mechanisms of immunopathology.

3.5 Macrophage-derived TGF- β signaling is predicted in pro-fibrotic niches

Next, we focused on the fibrotic COVID-19 sample #2 and its associated niches N1 and N3 to identify candidate cell-cell interactions underlying fibrotic remodeling. COL1A1⁺ Fibros exhibited a pathological phenotype characterized by high expression of ECM genes (COL1A1, COL5A1, COL6A1) (Figure 3d), reminiscent of pathogenic CTHRC1⁺ Fibros differentiating from alveolar Fibros^[47], although CTHRC1 was not included in the gene panel. Notably, CHI3L1/MMP9^{hi} Macs, which expressed pro-fibrotic genes (SPP1, CHI3L1, MMP9)^[24], were spatially adjacent to COL1A1⁺ Fibros (Figure 5a,b), suggesting local interactions between these two cell types. NicheNet analysis^[33], using CHI3L1/MMP9^{hi} Macs as sender cells and fibroblasts as receivers, identified TGFB1 and TGFB2 as top ligands predicted to regulate fibrosis-associated gene programs in receiver cells, including ECM components (COL1A1, COL2A1, COL5A1) as well as the myofibroblast-related gene ACTA2 (Figure 5c,d). In addition, LGALS3, encoding for GALECTIN-3, which can potentiate TGF- β -mediated fibrosis^[48], was also identified as a high-priority ligand. Consistently, CHI3L1/MMP9^{hi} Macs exhibited elevated and selective expression of both TGFB1 and LGALS3 (Figure 5e). Of note, the proximity between these macrophages and COL1A1⁺ Fibros was also observed upon reanalysis of an independent CosMx dataset from COVID-19 lungs^[28] (Figure S7).

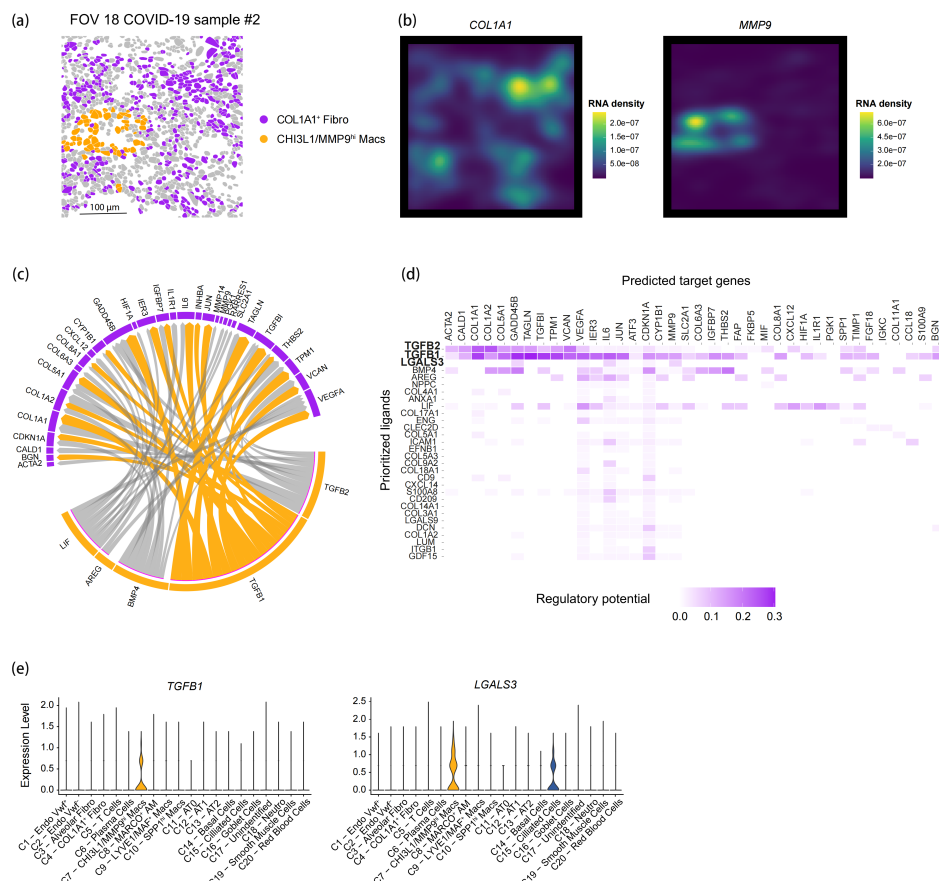


Figure 5. Ligand-receptor analysis supports pro-fibrotic macrophages driving pathogenic fibroblasts within fibrotic niches N1 & N3. (a) Dimension plot of FOV 18 from COVID-19 sample #2 representing the segmentations of cells; (b) Density plots of FOV 18, as in (A), representing RNA densities for the genes COL1A1 and MMP9; (c) Circoplot representing the putative interactions between CHI3L1/MMP9^{hi} Macs (orange) and Fibros (purple) from a NicheNet analysis. Interaction between TGFB1 and target genes in Fibros identified by NicheNet are highlighted; (d) Heatmap depicting predicted ligand activity within CHI3L1/MMP9^{hi} Macs and fibroblasts target gene activation during a potential evolution of alveolar Fibros to COL1A1⁺ Fibros on the basis of NicheNet analyses as in (c); (e) Expression levels of TGFB1 and LGALS3 in the indicated cell types. COVID-19: coronavirus disease 2019; CHI3L1: chitinase 3-like 1; MMP9^{hi}: matrix metalloproteinase 9; Fibros: fibroblasts; FOV: fields of view; COL1A1: collagen type I alpha 1 chain; Fibro: fibroblasts; Endo: endothelial cells; MARCO⁺: macrophage receptor with collagenous structure; AM: alveolar macrophage.

3.6 Alveolar niche-derived signals may shape *SPP1*^{hi} macrophage identity

(a)

FOV Covid 40

FOV Covid 26

100 μ m

AT2
SPP1^{hi} Macs
MARCO⁺ AMs
N5

(b)

Ligand activity

Prioritized ligands

Predicted target genes

AUPR

Regulatory potential

Figure 2 consists of two panels, (a) and (b). Panel (a) shows two spatial maps of predicted target genes in FOV Covid 40 and FOV Covid 26. The maps are color-coded: AT2 (purple), SPP1^{hi} Macs (olive), MARCO⁺ AMs (red), and N5 (green). A scale bar indicates 100 μ m. Panel (b) shows two heatmaps: Ligand activity (AUPR) and Regulatory potential. The rows represent prioritized ligands, and the columns represent predicted target genes. The AUPR heatmap shows activity levels from 0.01 to 0.05, and the Regulatory potential heatmap shows levels from 0.00 to 0.15.

Ligand activity	Prioritized ligands	Predicted target genes
CSF1	CDH1	CSF1
WNT5A	IL23A	CDH1
WNT3	WNT9A	WNT5A
WNT9A	WNT7B	WNT3
CSF2	MIF	WNT9A
ICAM1	IL6	WNT7B
TIMP1	PSAP	CSF2
PSAP	APOA1	MIF
APOA1	TNFSF9	ICAM1
TNFSF9	ANXA2	IL6
CTSD	CTSD	TIMP1
APP	APP	PSAP
TNFSF14	SPP1	APOA1
IL11	BMP3	TNFSF9
BMP3	OSM	ANXA2
OSM	ANGPT2	CTSD
ANGPT2	COL1A1	APP
COL1A1		TNFSF14
		SPP1
		IL11
		BMP3
		OSM
		ANGPT2
		COL1A1

Figure 6. Ligand-receptor analysis of macrophages inside alveolar niches. (a) Representative dimension plot of FOV 26 and 40 from the COVID-19 slide where cells are colored first by annotated cell types (left) and then where N5 is highlighted (right); (b) Heatmap depicting predicted ligand activity on AM-intrinsic target gene activation during macrophage differentiation, evaluated by NicheNet. COVID-19: coronavirus disease 2019; FOV: fields of view; *SPP1*^{hi}: secreted phosphoprotein 1; *MARCO*⁺: macrophage receptor with collagenous structure; AM: alveolar macrophage.

Together, these findings are consistent with the hypothesis that *SPP1*^{hi} macrophages within alveolar niches are exposed to local signals, including WNT ligands, M-CSF, and GM-CSF, which may contribute to their adaptation toward an alveolar macrophage-like state.

4. Discussion

In this study, we applied imaging-based ST using the CosMx platform to post-mortem human lung samples and provide a framework for the processing and interpretation of imaging-based ST data in the context of severe COVID-19. Rather than aiming to define broadly applicable mechanisms of COVID-19 pathology, this proof-of-concept study was designed to identify spatially organized

cellular patterns and generate biologically testable hypotheses from human tissue.

Our findings highlight the value of imaging-based ST for the in-depth characterization of pathological processes in archived tissue samples. This approach enables mapping of cellular composition and spatial organization while capturing phenotypic changes within specific cell populations. Importantly, it reveals spatial associations between cell types and thereby identifies tissue niches from which candidate cell-cell interactions can be inferred. The concordance between ST-defined niches and the histopathological features of corresponding tissue sections illustrates how spatial molecular information can complement conventional histopathological assessment.

Several limitations should be acknowledged. First, the small cohort size, analysis of a single anatomical region per subject, and marked spatial and inter-individual heterogeneity of COVID-19 lung pathology preclude generalization of the identified niches to the broader population of patients with severe COVID-19^[42,43]. Accordingly, the niches described here should be considered as representative of the pathological states captured in these particular tissue sections rather than as universal features of severe COVID-19. The use of post-mortem control tissues with pre-existing histopathological alterations represents an additional limitation. Although samples were collected according to a standardized procedure, the potential impact of fixation and FFPE processing on transcript abundance and spatial organization cannot be directly assessed in this study. Furthermore, the restricted gene panel and the relatively low number of detected RNA molecules per cell limit the depth of transcriptomic characterization. Integrating imaging-based ST with high-throughput approaches such as single-cell RNA sequencing may help overcome these limitations by improving both cell-type resolution and transcriptomic coverage.

Despite these constraints, several observations are consistent with previous studies. The increased proportion of immune cells, particularly macrophages, in COVID-19 samples aligns with previous reports^[53]. We identified cell populations corresponding to previously described subsets, including *CHI3L1*/*MMP9*^{hi} Macs, which resemble profibrotic macrophages described in severe COVID-19^[21], and *COL1A1*⁺ Fibros, which are reminiscent of pathogenic *CTHRC1*⁺ Fibros derived from alveolar fibroblasts^[45,47]. In addition, the presence of basal cells in fibrotic samples may reflect dysplastic repair processes associated with chronic lung injury in COVID-19^[53]. Importantly, our spatial approach places these cellular states within defined histopathological contexts rather than considering them independently of tissue architecture. Profibrotic niches corresponded to regions of fibrosis evaluated by histopathology and contained specific cell populations organized into spatially defined microenvironments. Similar observations have been reported using complementary spatial transcriptomic approaches. A sequencing-based GeoMx study of COVID-19 lungs identified fibrosis- and TGF- β -associated transcriptional programs in regions of organizing DAD, whereas *SPP1* expression was enriched in regions corresponding to earlier stages of DAD^[11]. Imaging-based Xenium analysis of fibrotic human lungs has likewise identified profibrotic niches containing macrophages, activated fibroblasts, and aberrant epithelial cells within remodeled distal lung regions^[54]. Together, these observations support the concept that distinct histopathological contexts are associated with specific spatial cellular organizations. Whether these niches represent sequential stages of lung injury and repair or distinct context-dependent responses remains to be determined.

Within these niches, our ligand-receptor analyses generated candidate mechanisms of intercellular communication that will require functional validation. In fibrotic niches, *CHI3L1*/*MMP9*^{hi} Macs expressing *TGFB1* and *LGALS3* were spatially adjacent to *COL1A1*⁺ Fibros, supporting a model in which macrophage-derived signals contribute to pathogenic fibroblast reprogramming through the TGF- β axis. Importantly, reanalysis of an independent COVID-19 CosMx dataset^[28] reproduced the spatial proximity between *CHI3L1*/*MMP9*^{hi} macrophages and *COL1A1*⁺ fibroblasts, indicating that this cellular association is not restricted to the original sample. However, our data do not directly demonstrate that macrophage-derived TGF- β drives fibroblast activation, and dedicated functional studies will be required to establish causality. Thus, TGF- β should be considered a candidate signaling axis emerging from the spatial analysis rather than a demonstrated mechanism.

In contrast, alveolar-associated niches displayed distinct environments, including WNT ligands and M-CSF/GM-CSF-related pathways. Notably, *SPP1*⁺ macrophages, often described as pro-fibrotic^[18,21], were predominantly localized within alveolar niches and were largely absent from severely fibrotic regions. Together with the reported enrichment of *SPP1* in earlier stages of DAD^[11], this suggests that the phenotype and potential functions of *SPP1*^{hi} macrophages may depend strongly on their local tissue environment and stage of injury. The corresponding *SPP1*^{hi} macrophage-associated alveolar niche could not be identified in the independent CosMx dataset^[28], which may reflect differences in the nature or stage of the lesions captured rather than absence of this interaction in other patients. Consistent with another report^[55,56], this observation suggests that *SPP1*⁺ macrophages may represent an early or context-dependent response to lung injury, potentially contributing to alveolar remodeling or repair rather than exclusively to advanced fibrosis.

Overall, our study should therefore be viewed as hypothesis-generating: it identifies spatially-restricted macrophage states and candidate interaction networks associated with distinct histopathological contexts, providing specific cellular and molecular relationships that could be tested experimentally. Larger cohorts encompassing different disease stages and anatomical regions, together with functional and longitudinal approaches, will be required to determine the recurrence, temporal dynamics, and biological significance of these niches.

5. Conclusion

Our study provides a proof-of-concept supporting the utility of imaging-based ST for investigating the spatial organization of immunopathology in severe COVID-19 and other lung disorders. By integrating histopathology with single-cell spatial information, we identified distinct macrophage-associated niches and generated candidate mechanisms linking local cellular interactions to fibrotic or alveolar tissue states. The recurrence of *CHI3L1/MMP9^{hi}* macrophage-*COL1A1⁺* fibroblast proximity in an independent dataset, together with concordant observations from other spatial studies, supports the biological relevance of this association while emphasizing the need for validation in larger cohorts. Future studies integrating spatial transcriptomics with functional approaches will be required to establish causality, particularly for the predicted TGF- β axis, and to determine whether these niche-specific macrophage programs contribute directly to disease progression or repair.

Supplementary materials

The supplementary material for this article is available at: [Supplementary materials](#).

Declarations

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Authors contribution

Abinet J: Methodology, formal analysis, visualization, writing-original draft.

Ruscitti C: Methodology, investigation, writing-review & editing.

Garigliany M: Investigation.

Liégeois M: Formal analysis, writing-review & editing.

Radermecker C, Marichal T: Conceptualization, supervision, funding acquisition, writing-review & editing.

All authors provided feedback on the manuscript.

Conflicts of interest

Cecilia Ruscitti and Maude Liégeois are postdoctoral fellows of the F.R.S.-FNRS. Coraline Radermecker is a Research Associate of the F.R.S.-FNRS. The other authors declare no conflicts of interest.

Ethical approval

The samples were collected with the approval of the Ethics Reviewing Board of the University Hospital of Liege, Belgium (Ref. 2020/119).

Consent to participate

Informed consent was obtained from all participants included in the study or, where applicable, from their legal representatives.

Consent for publication

Not applicable.

Availability of data and materials

The processed data files can be accessed in the Expression Omnibus database under the accession number GSE327879. The scripts used to analyze these data have been uploaded to GitHub (https://github.com/JoanAbinet/COVID19_niche).

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