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LETTER TO EDITOR

Persistent viral activity, cytokine storm, and lung fibrosis in a case of severe COVID-19

To the Editor:

The pandemic of coronavirus disease 2019 (COVID-19) caused by SARS-CoV-2 has posed a great threat to public health.[1] Although the majority of COVID-19 patients have mild diseases, some develop acute respiratory distress syndrome.[2–4] Understanding the mechanism of COVID-19 severity is a prerequisite for the development of effective vaccines or therapies.

Here, we report a particular case with severe COVID-19 to reveal some mechanistic insights. A 66-year-old man with pre-existing hypertension, developed symptoms, including fever, dry cough, myalgia, and joint pain, and traveled from Wuhan to Shenzhen in China. His infection was diagnosed on day 4 postadmission, based on his airway sample being positive for SARS-CoV-2. The clinical condition of this patient progressively deteriorated after hospitalization, and he eventually succumbed despite of all medical intervention attempts to save his life, including experimental antiviral therapy, extracorporeal membrane oxygenation, convalescent plasma transfusion, and lung transplantation (Table 1). Dynamic monitoring of the SARS-CoV-2 showed viral presence in bronchoalveolar lavage fluid (BALF) or the upper airway samples for more than 23 days. Although the SARS-CoV-2 is undetectable 17 days later in multiple samplings (Table 1), we identified the viral RNA in his lung tissues by qPCR (Figure 1A). Viral presence was further confirmed by in situ RNA hybridization, and some were colocalized with the *ACE2* RNA probes

TABLE 1 The timeline of this patient's clinical course from his admission to death

		1.11	1.12	1.13	1.14	1.15	1.16	1.17	1.18	1.19	1.20	1.21	1.22	1.23	1.24	1.25	1.26	1.27	1.28	1.29	1.30	1.31	2.1	2.2	2.3	2.4	2.5	2.6	2.7	2.8	2.9	2.10	2.11	2.12	2.13	2.14	2.15	2.16
	Hospital stay (days)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
	Fever	■	■	■	■					■	■	■	■	■		■	■		■	■	■																	Death
Treatment	Anti-virals: Interferon					Interferon → → → → → → → → → → → → → → →												Ganciclovir (cols 2.9–2.12)									
	Anti-virals: Oseltamivir / Ribavirin / Lopinavir		Oseltamivir	Oseltamivir	Oseltamivir	Ribavirin → → → → → → → → → → → → →								Lopinavir → → → → → → → → → → → → →												
	Antibiotics	Moxifloxacin	Moxifloxacin	Moxifloxacin	Moxifloxacin		Meropenem/Voriconazole →																		
	Methylprednisolone		20	20	60	60	60	40	40						20	20									Hydrocortisone	Hydrocortisone								160	80	500		
	O₂ Supplement	Low-flow	Low-flow		High-flow																																	
	Ventilation				No-invasive	No-invasive	No-invasive	No-invasive	No-invasive	No-invasive					Invasive →																	
	ECMO										ECMO → → → → → → → → → → → → → → → → → →																		
	Transfusion										Plasma-intermittent							Plasma-intermittent+Erythrocyte-intermittent → → → → → → → → → →													
	LW Heparin						√																															
	Convalescent plasma															√																						
	Tocilizumab																																	√				
	Tacrolimus																																			√		
	CRRT																						■	■	■	■	■		■	■	■						■	
Ct value	Airway samples					+				26	30	-	-	-		-				-			33.5		-		-		-			-			-			-
	BALF													-	+			20.3					-		-		-		-			-			-			
Plasma	CRP (mg/L)	45	64	36	101	40	46	46	71	155	190	163	147	150	158	185	115	107	156	189	178	121	67	34	23	38	65	60	73	104	87	154	237	185	93			
	IL-6 (pg/ml)					36	24			299	109	149	88	151	150	28	34	94	278	353	19	8	7	10	43	74	70	48	42	49	5000	414	1449	498	945			

BALF CBA (↑ ~1.19) · BALF CBA (↑ ~1.29) · BALF CBA (↑ ~2.3) · scRNA-seq/pathology, qPCR/WB/RNA Scope (↑ 2.14)

FIGURE 1 Persistent of SARS-COV-2 in the patient's lung. A, The presence of SARS-CoV-2 RNA was examined by qPCR with two probes targeting ORF1ab and nucleocapsid protein (NP), respectively. B, One cross-section of paraffin-embedded lung tissue from COVID-19 patient and healthy control was hybridized by SARS-COV-2-specific chromogenic probe (green) and ACE2-specific probe (red). C, Lung tissue lysates of a control donor and this patient were analyzed by immunoblot using anti-Spike and anti-NP antibody. D, Thin-layer electron microscopic photograph shows the presence of coronavirus particles (around 100 nm in diameter) in the patient's lung. Right panel is the magnification of the marked section of the left panel

(Figure 1B). Moreover, viral nucleocapsid protein (NP) and spike proteins in the lung were detected by immunoblotting (Figure 1C). Finally, coronavirus-like particles were identified by electron microscopy (Figure 1D). It is intriguing to observe the SARS-CoV-2 persistence in the lung, while the upper respiratory samples remained virus negative, suggesting a viral reservoir and ongoing viral activity deep in the SARS-CoV-2-infected lung. Therefore, effective antiviral treatment would be a necessary part of the therapy for patients with severe COVID-19 even if SARS-CoV-2 RNA is undetectable in airway samples.

To further understand the pathogenesis, the patient's lung was taken for histopathological examination. Significant pathological changes included extensive alveolar damage with alveolar epithelium disorganization, necro-sis, shedding, atresia, and abundant mucus with bleeding in the dilated small bronchi, bronchioles, and terminal bronchioles (Figure S1A). Unlike previous reports on pathological changes in COVID-19 patients,[5,6] obvious lung fibrosis was observed in this patient as indicated by Sirius Red, Masson staining, and collagen IHC (Figure 2A), suggesting that severe COVID-19 caused pulmonary fibrosis in this case.

To dissect the lung microenvironment, four different regions of the lungs were taken for scRNA-seq study. We integrated 32 191 cells from the patient and 81 447 cells from eight controls from a publicly available source (GSE122960)[7] for analysis. Major cell types were identified by known markers, including epithelial cells (AT1, AT2, club, and ciliated cells), NK and T lymphocytes

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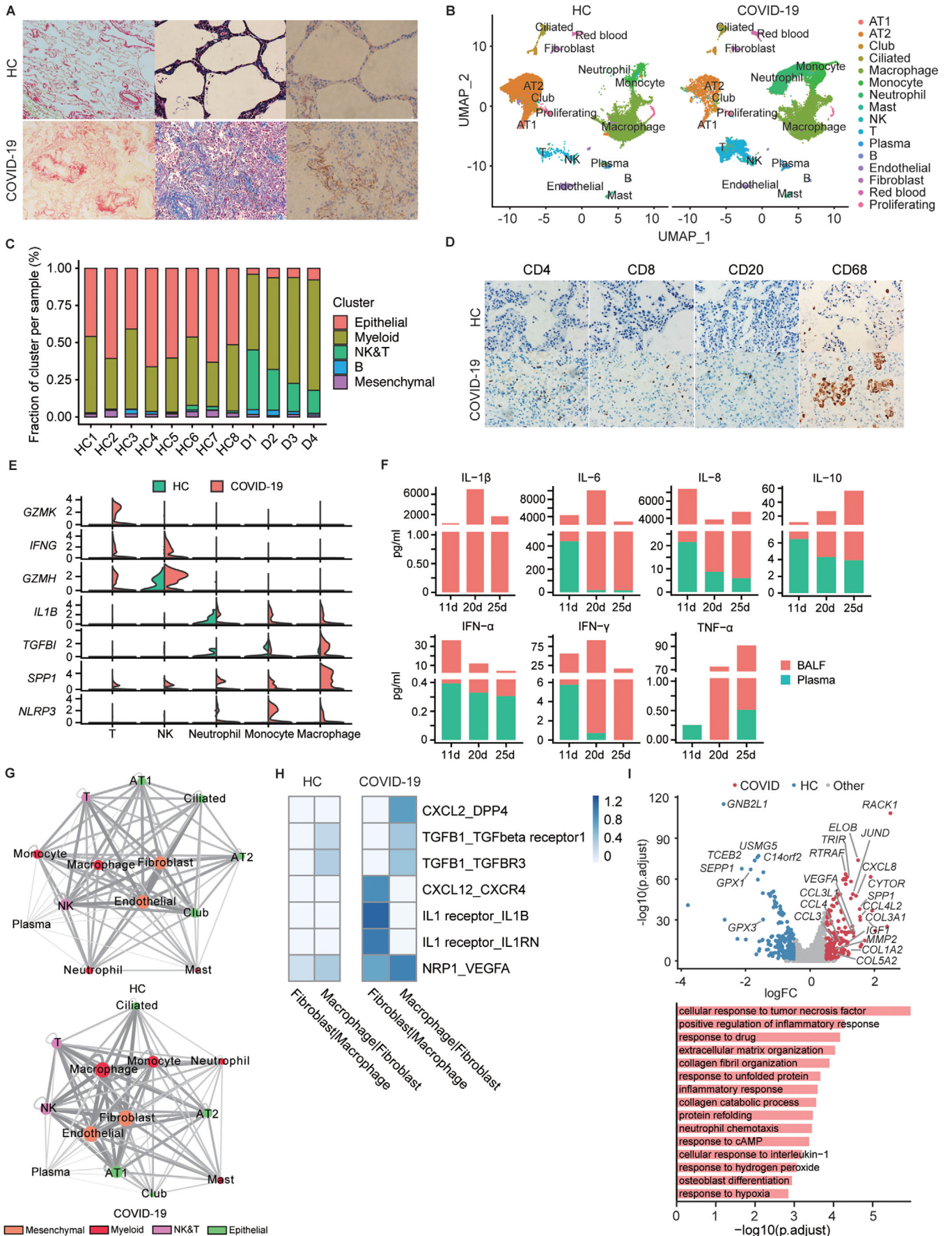
(NK, CD4, and CD8 T), mesenchymal cells (fibroblast and endothelial), myeloid cells (macrophage, monocyte, and neutrophil), B lymphocytes, and erythrocytes (Figure 2B and Figure S1B). Indeed, we observed a substantial reduction of parenchymal cells in the patient's lungs, especially epithelial cells. In contrast, the pool of immune cells, including T cells, B cells, and myeloid cells, was markedly expanded (Figure 2C). Extensive immune cell infiltration was also revealed by immunohistochemistry staining of CD4, CD8, CD20, and CD68 (Figure 2D). Gene expression analysis showed that many of those infiltrating immune cell subsets were highly activated. Compared with control, effector molecules *GZMK*, GZMH, *IFNG* were highly expressed in T cells, and *IL1B*, *NLRP3, SPP1*, and *TGFB1* were highly expressed in lung macrophages from the patients (Figure 2E). In addition, monitoring of multiple inflammatory factors showed that the patient was undergoing an inflammatory cytokine storm. The levels of inflammatory cytokines in BALF were much higher (Figure 2F). Long-term monitoring of IL6 and CRP in plasma showed that these two inflammatory factors fluctuated but maintained at high levels despite of receiving glucocorticoid treatment (Table 1). Thus, effort to relieve the cytokine storm is likely a key component of successful treatment for severe COVID-19 patients. This is consistent with the fact that recent clinical trials targeting the inflammation are showing promising results.[8]

To explore the potential molecular mechanism underlying lung fibrosis in severe COVID-19, we mapped receptor-ligand pairs among different cell subsets using the scRNA-seq data to construct a putative cell-cell interaction network. The center of interaction network in controls were endothelial cells and fibroblasts, likely a requirement for normal lung homeostasis, whereas macrophages and fibroblasts moved to the center in the patient (Figure 2G), suggesting that macrophage-derived signals might play major roles in shaping the patient's lung microenvironment. The analysis further showed that *CXCL12* highly expressed in the patient's fibroblasts might enhance the recruitment of multiple immune populations (expressing *CXCR4*), including macrophages, T cells, and NK cells, while the patient's macrophages produced more *IL1B* and *TGFB* to modulate fibroblast functions in this patient (Figure 2H). Indeed, the patient's fibroblasts showed activated phenotype and increased expression of extracellular matrix molecules, such as collagens and matrix metalloproteinases (Figure 2I). Taken together, our data highlight the cross-talk between macrophages and fibroblasts through well-known IL-1β and TGF-β signaling pathways[9] as an underlying drive for the lung fibrosis in severe COVID-19.

In summary, this case reveals pathological mechanism of severe COVID-19, including pulmonary SARS-CoV-2 persistence, robust inflammatory responses, and extensive lung fibrosis, which likely constitute the basis for the grave clinical outcomes and difficult treatment of patients with COVID-19.

CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS
Guangde Zhou, Shuye Zhang, Lei Liu, and Zheng Zhang designed this study and wrote the paper. Gang Xu performed this study and wrote the paper. Mingfeng Liao,

FIGURE 2 Pathological manifestations and single-cell transcriptomes of the patient's lung. A, Sirius Red staining (left), Masson staining (middle), and immunohistochemical staining of type IV collagen (right) in healthy and COVID-19 patient's lung revealed extensive lung fibrosis in the case. B, The uniform manifold approximation and projection (UMAP) presentation of lung single-cell transcriptomes showing the clusters of major cell types. The first 50 principle components were loaded as input to UMAP for visualization and clustered cells using a graph-based method. C, Bar plot showing the proportion of major cell types in each sample. D1, D2, D3, and D4 are samples collected from four different regions of the patient's lung. D, Immunohistochemical staining of patient's lung tissues with CD4, CD8, CD20, and CD68 antibody. E, The violin plots display the expression of selected immune genes across different immune subsets in healthy versus patient's lung. F, The levels of selected cytokines and chemokines in serial bronchoalveolar lavage fluid (BALF) and plasma samples (collected at day 11, 20, and 25 during the hospitalization) are displayed. G, Cell-cell interaction networks estimated in HC (left) and COVID-19 patient (right). The thickness of the lines represents the number of interactions between the cell types. H, Selected ligand-receptor pairs between fibroblasts and macrophages in control versus patient's lung. The color represents total mean of the individual partner average expression values in the corresponding interacting pairs of cell types. I, Volcano plot (top) showing differentially expressed genes of lung fibroblasts in patients and healthy controls. A gene is considered significant with adjust $P < 0.05$ and logFC > 0.5. (Bottom) Selected GO terms of the significantly upregulated genes of the patient's fibroblasts

Jizhou Gou, and Weilong Liu processed the lung tissue and performed immunohistochemistry experiments. Yang Liu performed bioinformatics analysis. Jing Yuan provided patient's clinical information. Gang Xu and Xin Wang constructed the single cell library. All authors approved the final manuscript.

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