NETosis promotes chronic inflammation and fibrosis in systemic lupus erythematosus and COVID-19

Running title: NETosis promotes pulmonary fibrosis

Huiqing Lin [1 †], Jiejie Liu [2 †], Ning Li [1], Birong Zhang [3, 4], Van Dien Nguyen[3, 4], Peipei Yao [2,5], Jiangpeng Feng [2], Qianyun Liu [1], Yu Chen [2], Guang Li [6 *], You Zhou [3, 4 *], Li Zhou [2, 5 *]

[1]State Key Laboratory of Virology, Department of Thoracic Surgery, Renmin Hospital, Wuhan University, Wuhan 430060, China

[2]State Key Laboratory of Virology, Modern Virology Research Center, RNA Institute, College of Life Sciences, Wuhan University, Wuhan 430072, China

[3]Systems Immunity Research Institute, Cardiff University, Cardiff, CF14 4XN UK

[4]Division of Infection and Immunity, School of Medicine, Cardiff University, Cardiff, CF14 4XN UK

[5] Institute for Vaccine Research, Animal Bio-Safety Level III Laboratory at Center for Animal Experiments, Wuhan University School of Medicine, Wuhan 430071, China

[6] Department of Critical Care Medicine, Renmin Hospital, Wuhan University, Wuhan 430060, China

† **Authors contributed equally:** Huiqing Lin and Jiejie Liu

*** Correspondence:**

Li Zhou, Ph.D, College of Life Sciences, Wuhan University, Wuhan, Hubei Province, China. Email: zhouli_jerry@whu.edu.cn

You Zhou, Ph.D, Systems Immunity Research Institute, Cardiff University, Cardiff. Email: zhouy58@cardiff.ac.uk

Guang Li, M.D, Department of Critical Care Medicine, Renmin Hospital, Wuhan University, Wuhan, Hubei Province, China. Email: guangli@whu.edu.cn

Abstract

Pulmonary fibrosis, a serious complication of systemic lupus erythematosus (SLE) and coronavirus disease 2019 (COVID-19), leads to irreversible lung damage. However, the underlying mechanism of this condition remains unclear. In this study, we revealed the landscape of transcriptional changes in lung biopsies from individuals with SLE, COVID-19-induced pulmonary fibrosis, and idiopathic pulmonary fibrosis (IPF) using histopathology and RNA sequencing, respectively. Despite the diverse etiologies of these diseases, lung expression of matrix metalloproteinase genes in these diseases showed similar patterns. Particularly, the differentially expressed genes were significantly enriched in the pathway of neutrophil extracellular trap formation, showing similar enrichment signature between SLE and COVID-19. The abundance of Neutrophil extracellular traps (NETs) was much higher in the lungs of individuals with SLE and COVID-19 compared to those with IPF. In-depth transcriptome analyses revealed that NETs formation pathway promotes epithelial-mesenchymal transition (EMT). Furthermore, stimulation with NETs significantly up-regulated α-SMA, Twist, Snail protein expression, while decreasing the expression of E-cadherin protein *in vitro*. This indicates that NETosis promotes EMT in lung epithelial cells. Given drugs that are efficacious in degrading damaged NETs or inhibiting NETs production, we identified a few drug targets that were aberrantly expressed in both SLE and COVID-19. Among these targets, the JAK2 inhibitor Tofacitinib could effectively disrupted the process of NETs and reversed NET-induced EMT in lung epithelial cells. These findings support that the NETs/EMT axis, activated by SLE and COVID-19, contributes to the progression of pulmonary fibrosis. Our study also highlights that JAK2 as a potential target for the treatment of fibrosis in these diseases.

Keywords: pulmonary fibrosis, COVID-19, neutrophil extracellular traps (NETs), systemic lupus erythematosus, epithelial to mesenchymal transition (EMT).

1 Introduction

Characterised by excessive deposition of extracellular matrix (ECM) and destruction of the normal parenchymal structure, pulmonary fibrosis leads to progressive loss of pulmonary function of alveolar gas exchange. It is a life-threatening disease with a poor prognosis. The disease has many risk factors including environmental exposures (hypersensitivity pneumonitis), occupational exposures (silica, asbestos) (1), chemicals, radiation therapy, pathogen infection and immune response (2) as well as genetic factors associated with inflammatory micro-environment. Because of the profound impact of those factors on the development of lung parenchyma injury, pulmonary fibrosis is often considered as an idiopathic disease (3).

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has led to over 6.8 million deaths by Jan 2023. Recent findings suggest that patients with COVID-19 develop pulmonary fibrosis. The clinical spectrum of COVID-19 is broad, ranging from asymptomatic infection, acute respiratory distress syndrome (ARDS) to multiorgan dysfunction (4). Over 15% of hospitalised patients with COVID-19 developed ARDS (5), which caused a sharp rise in the incidence of pulmonary fibrosis (6). In COVID-19 patients, interstitial pneumonia was observed in the early phase, along with further damage on diffuse alveolar (7), suggesting a profound role of lung fibrosis in development of ARDS (8-11). After recovering from COVID-19, approximately one-third of people still had fibrous abnormalities at time of hospital discharge as reported in several studies (12, 13). Some of them were also at risk for long-term fibrous remodelling that would dramatically reduce their quality of life (12, 13). Therefore, pulmonary fibrosis may be a long term sequelae of COVID-19 developed ARDS(2). However, the mechanism(s) of COVID-19 causing pulmonary fibrosis is not well understood.

Dysregulated host immune response in the lung is the key feature of COVID-19 pathophysiology. During the acute phase of COVID-19 pneumonia, abnormal immune-induced cytokine storms occur accompanied by a significant recruiting of neutrophils (14). Neutrophils migrate to sites of tissue injury, leading to generation of neutrophil extracellular traps (NETs) through a regulated cell death process termed NETosis. NETs are part of host defense against microbes, which are web-like chromatin fibers with microbicidal proteins and granule enzymes released by neutrophils. Recently, NETs are also found to be abundant in respiratory secretions and lung tissue from patients with COVID-19, suggesting that NETs are involved in the development of SARS-CoV-2-induced lung injury (15-17). Despite NETs have a protective role through entrapping microbes, inappropriate release of NETs causes tissue damage and inflammation (18, 19). NETs can also promote activation and differentiation of fibroblasts (20). However, the role of NETs in COVID-19 induced lung fibrosis is unknown.

Systemic lupus erythematosus (SLE) is a chronic multisystem autoimmune disease characterized by defective immune tolerance mechanisms (21). In patients with SLE, the presence of robust type I interferon activities is a common observation (22). These activities could either act as a defence mechanism against viral infections or lead to a hyperinflammatory response that may have detrimental effects during viral infection. The gene expression data from whole blood samples of SLE patients demonstrate an enrichment of the COVID-19 pathway (23). Furthermore, SLE and COVID-19 share similarities in terms of macrophage-driven inflammation observed in various inflamed tissues associated with inflammatory diseases (24). Importantly, it is worth noting that glucocorticoids and immunosuppressants, which are frequently administered to patients with SLE, may also exhibit efficacy in the treatment of COVID-19. These therapeutic approaches have already demonstrated their ability to effectively manage immune system dysfunction, which is a common

characteristic of both SLE and severe cases of COVID-19. These finding suggest that the immunopathogenic mechanisms of SLE may overlap with those underlying COVID-19.

Manifestation of SLE has been shown in pulmonary injury (25). Current publications reported that significant inflammation and fibrotic interstitial lung disease (ILD) occur in about 15% of SLE patients (26), and present dysregulated host immune response in the lung (25-28). There was a cell-mediated immune response in the respiratory system of 34 patients with SLE. And serum levels of pro-inflammatory cytokines in patients with pulmonary fibrosis were higher than those without pulmonary fibrosis (29). Fen Qiu found that CX3CR1 was an important up-regulated gene in peripheral blood mononuclear cells of SLE patients with pulmonary fibrosis compared with those without pulmonary fibrosis (30). Intriguingly, NETs are also proposed to play a fundamental role in SLE pathophysiology: acting as a primary driver of increased pulmonary fibrosis. Eleni Frangou showed that REDD1/autophagy pathway promotes fibrosis in SLE through NETs decorated with tissue factor (TF) and interleukin-17A (IL-17A) (31, 32). All these data suggest that NETs in SLE are likely to be one of the key causes of pulmonary fibrosis. However, the mechanism behind it needs to be fully investigated.

Several studies employed diverse sample types and techniques to gain insights into the connections between SARS-CoV-2 infection and autoimmune diseases. For instance, a comprehensive analysis involving SARS-CoV-2 lung biopsies, inflammatory bowel disease (IBD) intestinal tissue samples, rheumatoid arthritis (RA) tissue samples, SLE peripheral blood mononuclear cell samples, and type 1 diabetes (T1D) pancreatic islet cell samples revealed genetic connections between SARS-CoV-2 and autoimmune diseases (AD) (33). Another study integrated multiple single-cell transcriptome datasets that were derived from lung tissues affected by COVID-19, as well as tissues from inflammatory diseases such as RA, Crohn's disease (CD), ulcerative colitis (UC), SLE, and ILD. Notably, these samples exhibited a significant presence of $CXCL10^{+}$ $CCL2^{+}$ inflammatory macrophages induced by the combined stimulation of IFN-γ and TNF- α, suggesting shared cellular states and commonalities between COVID-19 and other inflammatory diseases (34).

In the present study, we employed histopathology (including immunofluorescence staining) and RNA sequencing (RNA-Seq) techniques to uncover the transcriptional changes in lung tissue biopsies from clinical patients in response to pulmonary fibrosis caused by COVID-19, SLE, and idiopathic pulmonary fibrosis (IPF) respectively (Fig. 1A). Despite the different disease etiologies, we found that the pulmonary expression of matrix metalloproteinases genes exerted a similar pattern across those diseases. Particularly, the differential expressed genes were significantly enriched in NETs formation signalling pathway in lung samples, revealing similar enrichment characteristics between SLE and COVID-19. In contrast to relatively little detection of NETs in IPF–injured lungs, we found that NETs are much abundant in SLE and COVID-19–injured lungs. Our study supports the hypothesis that NETs' formation associates with development of SLE and COVID-19-caused pulmonary fibrosis, suggesting it is a potential diagnostic biomarker for pulmonary fibrosis triggered by SLE and COVID-19.

2 Materials and methods

2.1 Ethics statement

This study was approved by the Ethics Committee of the Renmin Hospital of Wuhan University (WDRY2020-K224) and was conducted in full accordance with ethical principles (World Medical Association Declaration of Helsinks, and the Declaration of Istanbul). The lung biopsies were collected from patients undergoing standard lung transplantation.

2.2 RNA-seq library construction and sequencing

Total RNA was isolated from lungs with TRIzol reagents respectively under the instruction of the manufacturer (Ambion). mRNA was extracted from total RNA using the VAHTS mRNA-Seq V2 Library Prep Kit (Vazyme NR611-02). The rRNA-depleted RNA samples were degraded by high temperature in a buffer containing divalent cations. First-strand of cDNA was then synthesized by random primer (TAKARA). Then the second-strand of cDNA was synthesized, and dTTP in dNTP was replaced by dUTP in the reaction system. DNA library was constructed through end-repair, adaptor-ligation and PCR amplification. And it was qualified using Quant Studio 3. Qualified DNA library was sequenced with Illumina NovaSeq platform for lung samples (SLE1-2, CoV1-2 and CTRL1-2). The data for IPF samples were downloaded from the NCBI GEO database (accession no.: GSE150910) (35).

2.3 Lung histologic sectioning and staining

The fresh lung tissue samples were fixed with 10% formalin for more than 24 hours. After dehydradation and paraffin-embedding, the formalin-fixed paraffin-embedded (FFPE) lung tissue sections slides were deparaffinized and rehydrated. Slides were stained with hematoxylin and eosin, or Masson trichrome solution set (Servicebio).

To identify NETs in lung biopsies, tissue slides (4-μm-thick sections) were stained for immunofluorescence. After deparaffinization and rehydration, tissue sections were boiled with citric acid antigen repair buffer (PH6.0) for 20 min. The slides were incubated at 4℃ overnight with 1:200 diluted rabbit monoclonal antibody against human neutrophils elastase (CY6646, Abways), and then incubated with goat anti-rabbit secondary antibody (horseradish peroxidase [HRP]; DAKO) in dark at room temperature for 50 mins. After an incubation with 488-TSA for 30 mins, the slides were placed in a repair box filled with citric acid antigen repair buffer (PH6.0) and heated by microwave for 10 minutes. The sections were incubated at 4℃ overnight with 1:200 diluted rabbit anti-human rabbit anti-myelperoxidase antibody (GB11224, Servicebio) and then incubated with 1:500 diluted goat anti-rabbit secondary antibody CY3 (111-165-003, Jackson) at room temperature for 50 mins. DAPI was used to stain DNA. Images were scanned using a Pannoramic MIDI (3DHISTECH; Budapest, Hungary).

2.4 CCK-8 assay

The survival rate of A549 cells was analyzed using the Cell Counting Kit-8 (CCK-8, Vazyme, Nanjing, China) following the manufacturer's protocols. The cells were seeded and cultured in medium containing 10% FBS in 96-well microplates. Subsequently, the cells were treated with different concentrations of Tofacitinib (0, 5, 25, and 50 μmol/L). After 24 or 48 hours of treatment, a working solution of CCK-8 reagent was prepared by adding 10 μL of the reagent to 90 μL of medium. Next, 100 μL of the working solution was added to each well and incubated for 1 hour. All experiments were performed in triplicate. The absorbance at 450 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). Wells with 0 μmol/L Tofacitinib were used as the negative control. Cell proliferation was determined based on the relative absorbance.

2.5 Isolation of human neutrophils and formation of NETs

Neutrophils were isolated from freshly collected whole blood obtained from healthy individuals following a previously established protocol (36). In detail, leukocytes were separated from erythrocytes on a separating medium containing Dextran T500® (5%) in normal saline by sedimentation. Then neutrophils in peripheral blood were separated from mononuclear cells by Ficoll

centrifugation. After that, hypotonic lysis was used to remove contaminating erythrocytes, followed by the resuspension of neutrophils in Hank's buffered salt solution containing fetal calf serum (0.05%) and Hepes (10 nM).

To induce NETs, isolated neutrophils were seeded in a 96-well black plate. The cells were treated individually with PBS, Tofacitinib (Macklin, shanghai, China), Minocycline (Macklin, shanghai, China), Metformin (Macklin, shanghai, China), Bosentan (MedChemExpress, shanghai, China), Belimumab (MedChemExpress, shanghai, China), and Cannabidiol (AbMole, Shanghai, China) for 1 hour. After staining with the non-cell-permeant DNA binding dye (Sytox Green), the plate was incubated with the calcium ionophore A23187 (25 μM) with 5% CO_2 at 37°C in dark for 3 hours. NETs were then detected and quantified via green fluorescence as a result of extracellular DNA release in a microplate fluorescence reader at the wavelength of 485 nm and 527 nm. NETs were added to stimulate lung epithelial cells. After 24 hours of stimulation, the lung epithelial cells were harvested, RNA and protein were extracted from these cells for further analysis.

2.6 Western blot

Lung epithelial cells were washed with cold PBS, lysed with Radio Immunoprecipitation Assay (RIPA) lysis buffer, mixed with 5% SDS loading buffer and heated for 5 minutes. The samples were then electrophoresed with SDS PAGE and subsequently transferred to nitrocellulose membrane (GE healthcare). Membrane was blocked with 5% skim milk having Tween 20 (0.1 %) and probed overnight with the antibodies against GAPDH (#ab8245), α-SMA (#ab7817), Twist (#ab50887), Snail (#ab31787), and E-Cadherin (#ab241676) that were purchased from Abcam (UK). Enhanced chemiluminescence (ECL) substrates (Millipore, Billerica, MA) were used for detecting blotting signals.

2.7 RNA isolation and quantitative reverse-transcription polymerase chain reaction

Total RNA was extracted from the A549 cell lines using TRIzol reagent (Ambion, Austin, TX, USA), following the manufacturer's instructions. The extracted RNA was then reverse transcribed into complementary DNA (cDNA) using NovoScript®Plus All-in-one 1st Strand cDNA Synthesis SuperMix (gDNA Purge) (Novoprotein, Suzhou, China), according to the manufacturer's protocols. The cDNA obtained from the reverse transcription step was used as a template for quantitative reverse-transcription polymerase chain reaction (qRT-PCR) to detect the expression levels of GAPDH, α-SMA, Twist, Snail, and E-Cadherin. SYBR Green Master mix (Yeasen, Shanghai, China) was used for qRT-PCR amplification, following the manufacturer's instructions. The qRT-PCR experiments were independently repeated three times to ensure accuracy and reproducibility. The mRNA expression levels were normalized to the expression of GAPDH mRNA and calculated using the 2-ΔΔCt method, which allows for relative quantification of gene expression. The specific PCR primers used for amplification can be found in Supplementary Table 8.

2.8 Data availability

The raw sequencing data from this study have been deposited in the Genome Sequence Archive (37) in BIG Data Center (https://bigd.big.ac.cn/), Beijing Institute of Genomics (BIG), Chinese Academy of Sciences, under the accession number: HRA002538 (https://ngdc.cncb.ac.cn/gsa-human/s/CyYA3ze4).

2.9 Bioinformatics Analysis

RNA-seq reads were mapped to the human genome (hg38) with GENCODE gene annotation (v32) using STAR (v2.7.6A) (38). Gene expression was calculated by using featureCounts in SubReads package (v2.0.1) (39). Differentially expressed genes were called by using DESeq2 package (v1.32.0) (40) with the following criteria: BH adjusted p-value < 0.05 and fold change > 8. Functional enrichment analysis was performed using the clusterProfiler package (v 4.0.0) (41) to determine whether the genes are enriched in specific pathways. Pathway/functional enrichment calculations and PPI module analysis for genes with the same trend in SLE and COVID-19 were performed by Metascape (http://metascape.org) (42). Protein interactions were analysed using STRING database (v11.5) (https://string-db.org) (43). The interactions with a combined score >0.7 were selected to construct the PPI networks using the Cytoscape (v3.8.2).

To better investigate the association between NETs and pulmonary fibrosis, we searched for drugs that reduce the number of NETs *in vivo* from CLUE (https://clue.io/repurposing) and DRUGBANK (https://go.drugbank.com) database. 1 peptidylargynine deiminase 4 (PAD4) inhibitor and 5 DNase1 inhibitors with their corresponding gene targets were further used to explore their potential in the treatment of pulmonary fibrosis. The normalised expression of candidate drug targets among SLE, COVID-19 and normal status was calculated by variance stabilizing transformation (VST) function in DESeq2 (PMID: 25516281). The heatmap was constructed by ComplexHeatmap R package (PMID: 27207943). The source codes for the analysis are available at the https://github.com/chenyulab4126/PF.

3 Results

3.1 Clinical, Radiological and Pathological Features

We collected lung biopsies from patients with lung fibrosis from SLE, COVID-19, IPF or health donors. Patients' characteristics are summarised in Table 1. Chest X-ray (Fig.1B left) and CT images (Fig.1B right) of those patients show bilateral localized multi strip and/or patchy clouding opacity. Microscopically, some alveolar structures were destroyed, and interstitial uneven fibrosis and smooth muscle hyperplasia were observed (Fig.1C). Focal areas are marked by fibrotic honeycomb lung changes with lymphocytes and plasma cells infiltrating, some are deposited with foam cells and a large amount of cholesterol crystal. Combined with clinical manifestation, the change of Usual Interstitial Pneumonia was consistent (SLE 1) (Fig.1C). The alveolar septum was significantly widened diffusely, and the interstitium was infiltrated by lymphocytes and plasma cells. Chronic peribronchial inflammation, alveolar type II epithelial hyperplasia, and patchy intra-alveolar macrophage aggregation were observed. Moderate chronic pleuritis and pleural fibrosis, bronchiolitis, and local alveolar metaplasia were also discovered. Combined with clinical menifestation, lesions are in consistent with secondary interstitial pneumonia (SLE 2). For COVID-19 patient, histological examination indicated diffuse alveolar damage with fibrosis and the septa of the remained alveoli were slightly thickened, and marked fibrin exudation was observed (Fig.1C). CT images of the idiopathic pulmonary fibrosis lungs showed ground-glass opacities, septal thickening, and traction bronchiectasis which indicated diffuse fibrosis of both lungs (Fig.1C). Hematoxylin-eosin staining showed abundant inflammatory cell infiltration, interstitial fibrosis and vascular proliferation in the explanted lungs (Fig.1C).

Collectively, our clinical, pathological and histological analysis of lung tissues with fibrosis showed many similarities between patients with end-stage SLE, COVID-19 and IPF (Fig. 1). However, the relationship among those diseases regarding transcriptomic changes of pulmonary fibrosis currently remains poorly understood.

3.2 Transcriptional analysis of lung transplant samples

To investigate the mechanism of pulmonary fibrosis in patients with COVID-19 or SLE, we performed RNA-Seq of the lung samples from two SLE patients (SLE1-2), one COVID-19 patient with two parts of lung tissue (COVID-19 1-2), and normal lung tissues from two donors as controls (Control 1-2). We also analysed the transcriptomic data from lung samples of 67 IPF patients from a previous study (35).

The data illustrated high consistency within control or patients' groups as demonstrated by the correlation analysis of lung samples with SLE and COVID-19, respectively (Supplementary Fig. S1A). We then detected a total of 19682 expressed genes (Supplementary Table 1) in SLE patients (Fig.2A), in which 171 genes were up-regulated and the 129 were down-regulated. We identified more expressed genes (31643), in which 3641 genes were up-regulated and the 1014 genes were down-regulated (Supplementary Table 2) in COVID-19 patient compared with controls (Fig.2B). For IPF samples, we found 18266 genes with RNA expression signals, in which 1347 genes were significantly up-regulated while 604 were down-regulated (Fig.2C). Among these affected genes, there are a number of immune related genes as highlighted in Fig. 2A-C. We thus further examined infiltrating immune cells in SLE, COVID-19 (Supplementary Fig. S1B) and IPF (Supplementary Fig. S1C) using CIBERSORT (44). We identified that the activity of macrophages M0 and mast cells resting was much higher in COVID-19, SLE (Supplementary Fig. S1B) as well as IPF (Supplementary Fig. S1C) patients than controls. Noticeably, elevated T cells CD4 memory resting, along with reduced T cells CD8, were observed in COVID-19 patients (Supplementary Fig. S1B).

Matrix metalloproteinases (MMPs) are Zn^{2+} and Ca^{2+} dependent proteolytic enzymes, which have a conservative zinc-binding motif in the catalytic active site (45). MMPs regulate not only the protein activity of ECM, but also the release or activation of chemokines, cytokines, growth factors, antibiotic peptides and other bioactive molecules. Evidently, they participate in a number of physiological processes, including innate and adaptive immunity, inflammation, angiogenesis, and wound healing (46). The activity of MMPs are controlled by the members of the tissue inhibitor of metalloproteinase (TIMP) family (TIMP-1, 2, 3, 4) are prominently appreciated as natural inhibitors of metalloproteinases (47). Most MMPs promote the development of pulmonary fibrosis through diverse mechanisms (48), including activating cell proliferation and inducing epithelial-mesenchymal transition (EMT) in epithelial cells (49, 50). Upregulation of MMP1 in alveolar epithelial cells inhibited mitochondrial respiration and oxidative stress, while promoting cell proliferation and migration (50). MMP3 and MMP7 promotes fibrosis by inducing epithelial cells to undergo an EMT to generate cells that are myofibroblast-like in function (48, 49). Previous studies have shown that MMP8 can drive the development of pulmonary fibrosis (51). However, the role of MMP13 in experimental models of lung fibrosis remains uncertain, and the current findings are contradictory (50).

When we analysed MMPs and TIMPs associated gene expression, we found that several MMPs gene expression in COVID-19 rise significantly, including MMP1, MMP3, MMP7, MMP8, MMP9, MMP12, MMP13, MMP16 (Fig.2D). Similarly, the up-regulation of many MMPs associated gene expression, including MMP1, MMP7, MMP9 in SLE patients; and MMP3, MMP7, MMP9, MMP12 and MMP13 in IPF group were also observed. Therefore, abnormal MMPs expression may have a profound impact on the pathological mechanisms involved in the development of pulmonary fibrosis in COVID-19, SLE and IPF. In general, an imbalance between MMPs and TIMPs is the direct cause of fibrosis and tissue scarring. We then identified that the expression of TIMP1-3 was reduced in COVID-19, while the TIMP1 decreased both in SLE and IPF samples (Fig.2D). These results suggested that the function of TIMPs to protect the alveolar-capillary barrier by inhibiting protein

degradation of intercellular junctions or alveolar basement membrane is inhibited in pulmonary fibrosis in SLE, COVID-19 and IPF.

We further analysed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the DEGs to monitor the changes in the pulmonary fibrosis tissue from SLE, COVID-19 and IPF patients. In terms of biological processes, a large proportion DEGs were enriched in humoral response in both SLE, COVID-19 and IPF patients (Fig. 2E-G, and Supplementary Table 3-5). In SLE, we found that these DEGs were significantly enriched for the coronavirus disease - COVID-19, systemic lupus erythematosus and necroptosis pathway (Fig.2E). Some pathways, such as cytokine activity, immunoglobulin complex, neutrophil extracellular trap formation and several immune-related processes, were significantly altered in response to severe infection of SARS-CoV-2 (Fig.2F). Other modules, including extracellular matrix binding, collagen-containing extracellular matrix, were all associated with ECM components that promote fibrosis. Surprisingly, the top one enriched KEGG-term in COVID-19 was systemic lupus erythematosus signalling pathway (Fig. 2F). That means they have overlapping characteristics. Not surprisingly, most of the pathways enriched in IPF are closely associated with the progression of pulmonary fibrosis, such as extracellular structure organization, collagen-containing extracellular matrix (Fig.2G).

3.3 Comparing transcriptome data of lung samples from SLE and COVID-19 patients

Based on the enrichment analysis of differentially expressed genes, we found that SLE and COVID-19 had similar enrichment characteristics, which may be due to the late stage of pulmonary fibrosis in both diseases. To illustrate the similarity between the two diseases, we classified differentially expressed genes with the same trend between the COVID-19 sample and SLE. 84 genes were up-regulated, while 72 genes were downregulated (Fig.3A). After interrogating those genes based on their function, we identified that many of them (e.g., MMP7, MUC5B, and COL17A1) were implicated in the pathogenesis of pulmonary fibrosis (Fig.3B). The top one hit of enrichment analyses of both diseases (SLE and COVID-19) was neutrophil extracellular trap formation (Fig. 3C, Supplementary Table 6). Protein-protein interaction enrichment analysis has been carried out to identify the interaction between the same trend genes (Fig.3D). And we used the Molecular Complex Detection (MCODE) algorithm to identify four densely connected network components. Pathway and process enrichment analysis has been applied to each MCODE component independently. The largest module (MCODE1) enriched in neutrophil extracellular trap formation, a finding that is consistent with the enrichment analysis. We detected that the MCODE4 were enriched in Activation of Matrix Metalloproteinases. Additionally, proteins in MCODE2 were mainly enriched in the cytosolic ribosome, and proteins in MCODE3 were enriched in intermediate filament organization.

Neutrophils are able to exert an antibacterial activity in the extracellular space by the formation of so-called neutrophil extracellular traps (NETs), a regulated form of neutrophil death called NETosis (18, 19). In recent years, studies have shown that the persistent presence of NETs *in vivo* can constitute an autoantigen to enhance the autoimmune response (52). In our analysis, Gene Set Enrichment Analysis (GSEA) showed that the neutrophil extracellular trap formation pathway was activated in both SLE and COVID-19 samples (Fig.3E). Despite the clinical manifestations of pulmonary fibrosis in SLE and COVID-19 patients were similar to those in IPF patients, neutrophil extracellular trap formation pathway was not activated in IPF. We found that the similar transcriptome characteristics between SLE and COVID-19 were associated with the neutrophil extracellular trap formation.

3.4 Nets are important pathogenesis of SLE and COVID-19 pulmonary fibrosis

Since the largest module of genes with the same trend of SLE and COVID-19 was enriched in the neutrophil extracellular trap formation pathway, we suspected that NETs play an important role in the development of pulmonary fibrosis. NETs were then characterized by measuring the concentration of myeloperoxidase (MPO) and neutrophil elastase (NE). And myeloperoxidase (MPO), neutrophil elastase (NE), and cytoplasmic DNA were colocalized well as shown by immunofluorescence (Fig.4A). We found the presence of NETs in the lungs of COVID-19, SLE and IPF patients compared to those in Controls. And we also identified the proportion of NETs was much higher in SLE and COVID-19 than that in IPF (Fig.4B and Supplementary Fig. S2). In addition, the proportion of neutrophils in the lungs of IPF patients was significantly reduced of the infiltrating immune cells analysis results (Supplementary Fig. S1B).

Histone deacetylases (HDACs) drives NETs formation in human and mouse neutrophils (53). Dysregulation of HDACs activity has been reported in a number of diseases and is therefore an attractive option for the treatment of a variety of diseases including tissue fibrosis and inflammatory diseases (54). The persistence of NETs in the lung causes epithelial and endothelial cell damage, mainly driven by histones(55). Thus, we listed the NETs related genes and noticed that several histone genes were also up-expressed both in SLE and COVID-19 samples (Supplementary Table 7).

The epithelial cell marker genes KRT5, KRT14, KRT16, KRT17 and KRT23 were significantly upregulated in COVID-19 and SLE (Fig. 3B). We hypothesized that NETs contribute to the progression of pulmonary fibrosis in SLE and COVID-19 by promoting epithelial to mesenchymal transition (EMT). An airway *in vitro* model has been established to connect SARS-CoV2 infection, production of NETs and EMT trigger, but has not been further investigated in SLE(56). By bioinformatics and pathological analysis of clinical samples, we found NETs in both COVID-19 and SLE. To further confirm the role of NETs in pulmonary fibrosis of SLE and COVID-19, we next isolated neutrophils from human peripheral blood, which were stimulated with Calcium Ionophore A23187 to form NETs *in vitro*. Subsequently, NETs were used to incubate lung EMT which were detected via western blot. The results (Fig. 4C-G) showed that NETs stimulation significantly upregulated the protein levels of α-SMA, Snail, and Twist and decreased the protein level of E-Cadherin, suggesting that NETs could promote EMT of lung epithelial cells *in vitro*.

3.5 Key genes targeted by drugs that modulate NETs in SLE and COVID-19 patients' pulmonary fibrosis

Dyshomeostasis of NETs has a serious impact on the progression of pulmonary fibrosis. Drugs used to reduce amounts of NETs can suppress their accumulation in lung, further improving fibrosis. We compared the expression profiles of SLE and COVID-19 to identify the key target genes of several drugs that ameliorate impaired NETs degradation or inhibit NETs generation (Fig.5B). The expression of *TNFSF13B* (target gene of Belimumab), *ETFDH* (target gene of Metformin), *JAK2* (target gene of Tofacitinib), *VEGFA* and *MMP9* (target gene of Minocycline), *EDNRA* (target gene of Bosentan), *TRPV4* and *ACAT1* (target gene of Cannabidiol) were up-regulated in both SLE and COVID-19. Belimumab has been reported to reduce the formation of autoantibodies and significantly improve clinical symptoms in patients with severe SLE (57). Metformin could improve impaired NETs clearance, thereby reducing lung inflammation, inhibiting alveolar injury, and slowing down the progression of pulmonary fibrosis in patients with acute respiratory distress syndrome (ARDS) (58). Tofacitinib can simultaneously regulate the formation and degradation of NETs, and significantly increase endothelium-dependent vasodilation and endothelial differentiation (59). PAD4 deficiency reduces the content of NETs in the lung and inhibits the expression of fibrotic genes. As a reversible PAD4 inhibitor, Minocycline could be used to alleviate pulmonary fibrosis (60, 61). Bosentan could disrupt the upstream pathway of NETosis (32). Cannabidiol inhibits pro-oxidative

enzymes, such as NADPH oxidase and MPO, leading to a reduction in NETosis (62).To further investigate the inhibitory effects of Belimumab, Metformin, Tofacitinib, Minocycline, Bosentan and Cannabidiol on NETs generation and the capacity to reverse the development of pulmonary fibrosis, neutrophils were isolated and co-cultured with each of these drugs to evaluate their impact on NETs formation (Fig.5C). The results demonstrated a significant inhibition of NETs formation by Tofacitinib in a dose-dependent manner (Fig.5D). We also confirmed that Tofacitinib had no toxic effect on lung epithelial cells at different dosages (Fig.5E). Subsequently, our study revealed that Tofacitinib effectively suppressed the upregulation of α-SMA, Snail, and Twist induced by NETs, while also inhibiting the downregulation of E-Cadherin (Fig.5F). These findings indicate that Tofacitinib could effectively abrogate the process of NETs-induced EMT in lung epithelial cells *in vitro*.

4 Discussion

Pulmonary fibrosis is either a serious sequela of SLE disease or due to pulmonary infection by SARS-CoV-2, resulting irreversible damage to the lungs of patients (12, 13, 25). As the mechanisms by which COVID-19 or SLE contributes to pulmonary fibrosis is little known, there is currently no effective drugs for pulmonary fibrosis. Lung transplantation is a feasible treatment for patients with advanced pulmonary fibrosis. And we have successfully performed lung transplantation in several patients with SLE or COVID-19 (Table 1) (63). However, the availability of lung transplants is limited, and extrapulmonary disease or severe comorbidities, particularly connective tissue disease, may prevent some patients from being candidates for transplantation. Therefore, it is necessary to understand the related mechanisms and investigate appropriate therapeutic targets to delay the progression of the disease.

The pathological damage to lung tissue could be caused by different factors with overlapped characteristics, which are characterized by different degrees of inflammation and fibrosis (64). We performed a thorough radiographic evaluation of the pulmonary fibrosis features caused by COVID-19, SLE, and IPF. The results showed that lung tissues from COVID-19, SLE and IPF had similar fibrotic features (Fig. 1B). In addition to the radiographic observations in COVID-19, SLE, and IPF lung tissues, histological findings of fibrosis were also observed in HE and Masson staining (Fig. 1C). Pathology results showed an abnormal lung tissue structure with alveolar structure disappeared and a large number of fibrous tissue hyperplasia (Fig. 1C).

We then provided an in-depth profile of pulmonary fibrosis lung transcriptome in those diseases using next generation sequencing. By comparing COVID-19, SLE, and IPF with control lung tissues, we found that some highly expressed MMPs genes presented in pulmonary fibrotic tissues across all three diseases (Fig. 2D, Supplementary Fig. 1). Previous studies have shown that MMP3 and MMP7 promote pulmonary fibrosis by inducing epithelial cells to undergo EMT (48, 49). Up-regulation of MMP1 in alveolar epithelial cells inhibits mitochondrial respiration and oxidative stress, and promotes cell proliferation and migration (48-50). In addition, MMP8 can drive the development of pulmonary fibrosis (51). While MMP13 gene expression was significantly increased across three diseases as shown in our data, its role in lung fibrosis is still unclear with conflicting evidence (50). As TIMPs are endogenous inhibitors of MMPs, the balance between MMPs and TIMPs are critical for the eventual ECM remodelling in the tissue. For instance, TIMP1, a key regulator of MMP9, blocks MMP9 proteolytic activity (65). After GO and KEGG enrichment analysis of DEGs, we found that those changes including abnormal regulation of vasculature development, ECM binding and oxidative stress (Fig.2E-G, Supplementary Table 3-5). Such alterations in the alveolar microenvironment result in epithelial cell damage and accumulation of fibroblasts and

myofibroblasts, leading to persistent and progressive fibrosis. As we know, progressive fibrosis is the result of dysfunctional repair and ECM remodelling in response to lung injury (66). MMPs and TIMPs therefore may be the potential targets for preventing or treating pulmonary fibrosis. Clinical manifestations of pulmonary fibrosis in SLE and COVID-19 patients were similar to those in IPF patients. But the phenotypes of SLE, COVID-19 and IPF were extremely different. SLE and COVID-19 shared almost the same immune risk factors, particularly including persistent inflammatory responses. By comparing these two radically different but similarly devastating lung diseases, SLE and COVID-19, with healthy and normal lungs, we also discovered that bronchial epithelial cell markers KRT5, KRT14, KRT16, KRT17and KRT23 are all highly expressed in lung tissues from both SLE and COVID-19 patients (Fig.3B). We hence hypothesized that the chronic injury of SLE and COVID-19 may change the fate of certain cellular populations, inducing epithelial cells to generate fibroblasts via EMT, to promote pulmonary fibrosis. In addition, NETs formation pathway was also common activated in these two diseases (Fig.3B). Furthermore, we found a significant increasing of NETs in lung histopathological specimens of SLE and COVID-19 (Fig.4 A-B and Supplementary Fig. S2). Taken together these findings suggest NETs induced EMT, and is specifically correlated with the progression of pulmonary fibrosis in SLE and COVID-19.

The most common host reaction in patients with severe COVID-19 is abnormal activation of peripheral blood neutrophils, which may be the primary source of NETs (67). NETs contribute to inflammation-associated lung damage, thrombosis and fibrosis. Neutrophil-rich inflammatory areas in the interstitium of the lungs of patients with COVID-19 and neutrophils with NETosis are mostly present in arteriolar thrombosis (68). A study has confirmed that ribonucleoprotein immune complexes (RNP ICs) in the serum of SLE patients can induce NETosis, leading to a significant increase in inflammatory cytokines (69). Recent studies have shown that the persistent presence of NETs *in vivo* can constitute an autoantigen to enhance the autoimmune response (52). These findings further prove the reliability of our study, indicating that NETs are continuously activated in the lungs of both SLE and COVID-19 patients during the development of pulmonary disease, leading to the process of pulmonary fibrosis. This new finding shed important light on the underlying core pathway that initiates chronic lung remodelling in response to immune injury. Importantly, through in-depth transcriptome analysis, we identified that neutrophil extracellular trap formation pathway promotes EMT transformation in the lung epithelial cells (Fig.4 C-G). The role of NETs in epithelial cells was further reflected by its induction of α-SMA, Snail and Twist and reduction of E-Cadherin (Fig.4 C-G), indicating that NETs/EMT axis promotes the progression of pulmonary fibrosis. Our results support the hypothesis that NETs may represent drivers of severe pulmonary complications of SLE and COVID-19. It also suggests that blocking NETs formation is a potential therapeutic intervention for uncontrolled lung tissue damaging and thrombotic responses.

To discover key therapeutic targets that are highly relevant to SLE and/or COVID-19 pulmonary fibrotic diseases, gene sets with drug profiles targeting NETs were mapped to the expression data of lung tissues from SLE and COVID-19 patients (Fig.5B). Significantly, we observed pronounced inhibitory effects of Tofacitinib on NETs generation. Our findings demonstrated that NETs stimulation led to alterations in the gene expression profile of lung epithelial cells, exhibiting characteristic features of EMT. However, the application of Tofacitinib effectively reversed this process and suppressed EMT in lung epithelial cell. Tofacitinib has shown promise in targeting key signalling pathways involved in fibrotic lung diseases and other conditions. Studies have highlighted its potential in targeting IL-17A/IL-17RA signalling in fibrotic lung diseases (70), inhibiting the JAK-STAT pathway in systemic sclerosis (71), attenuating Dupuytren's fibrosis by modulating the STAT1-mediated IL-13Rα1 response (72), and demonstrating potential in targeting tissue-resident memory T cells in chronic COVID-19 lung injury (73). Furthermore, a study comparing three JAK-STAT inhibitors, including Tofacitinib, highlighted the combined anti-fibrotic and anti-inflammatory

properties of these inhibitors on macrophages in the context of scleroderma-associated interstitial lung disease (74). Our experimental results further support the efficacy of Tofacitinib by demonstrating its ability to reduce NETs production and inhibit the release of extracellular DNA, possibly through modulation of the JAK-STAT pathway. These discoveries hold crucial clinical significance, providing a theoretical foundation for the development of novel therapeutic strategies aimed at pulmonary fibrosis. Further investigations will contribute to a deeper understanding of the intricate regulatory mechanisms of NETs in the development of pulmonary fibrosis and provide robust support for drug development targeting pulmonary fibrosis. Due to limited samples from lung transplantation, there was an insufficient number of fibrosis samples from SLE and COVID-19 for direct intra-patient comparison. A further larger multi-column analysis is thus needed to validate our results in the future. Suitable animal fibrosis models are also needed to conclusively test causality, and to demonstrate the efficacy and safety of agents targeting NETs.

In conclusion, our study has translational potential for maintaining health that NETs formation pathway promotes epithelial-mesenchymal transition in lung epithelial cells (Fig.5A). In addition, RNA-seq datasets from pulmonary fibrotic tissue of SLE and COVID-19 patients offer a useful resource for further analysis by the community. Importantly, by analysing multiple fibrotic tissue in the lung from different diseases in parallel, we found a shared transcriptomics pattern between SLE and COVID-19 patients, which is a highlight of this article. Extending this approach to other lung diseases and to their co-mobilities will allow us to discover shared or distinct molecular pathways leading to similar clinical and pathological phenotypes. Through utilization of a variety of bioinformatics and experimental methods, we have elucidated a molecular mechanism by which SLE and COVID-19 activates NETs/EMT axis, thereby promoting the progression of pulmonary fibrosis. Our study underscore the significant inhibitory effects of Tofacitinib on NETs generation and its capacity to reverse lung epithelial cell EMT induced by NETs. These findings hold crucial clinical implications and provide a theoretical basis for the development of new treatment strategies aimed at disrupting the interplay between NETs and pulmonary fibrosis. Further research endeavors will enhance our understanding of the intricate regulatory mechanisms of NETs in the development of pulmonary fibrosis and provide robust support for the development of therapeutic interventions targeting pulmonary fibrosis.

DATA AVAILABILITY STATEMENT

All data obtained for this study is included in the article.

AUTHORS CONTRIBUTION

G.L., Y.Z., and L.Z. conceived the study and designed the experiments. H.L., J.L. P.Y., and Q. L. performed experiments and analysed the figures. N.L., B. Z., V.D. N., J.F. and Y.C. assisted with the experiments. J.L., and Y.Z., L.Z. wrote the initial draft of the manuscript. J.L., Y.Z., and L.Z. revised the manuscript. All authors read and commented on the manuscript.

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Conflict of Interests

Authors declare no competing interest from any funding agency.

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Legends

Figure 1. Radiological and histopathological characteristics of lung samples from SLE and COVID-19 patients. (A) Schematic of the experiment. Fibrotic lungs were performed for radiological, histopathological and RNA sequencing examinations. (B) Plain and contrast-enhanced chest CT scans (reconstruction) manifests pulmonary interstitial fibrosis and interstitial pneumonia. HRCT revealed intensification of pulmonary grains, and extensive uneven thickening of the interlobular septa and fissures and widespread ground-glass opacities, especially on the outer zone of lungs. The main abnormal CT finding was bilateral localized multi stripe and/ or patchy clouding opacity, especially in the lower lobes. (C) Lung tissues were subjected to HE staining and Masson staining from SLE, COVID-19, IPF patients and health donor. Histopathological characteristics shows: Microscopically, some alveolar structures were destroyed, interstitial uneven fibrosis and smooth muscle hyperplasia were observed. Focal areas are marked by fibrotic honeycomb lung changes with lymphocytes and plasma cells infiltrating, some are deposited with foam cells and a large amount of cholesterol crystal. Combined with clinical, the change of Usual Interstitial Pneumonia was consistent in SLE and COVID-19.

Figure 2. Differentially expressed genes in SLE and COVID-19 patients' lung samples. (A-C) Volcano-plot represents differential gene expression (DGE) between COVID-19 (A) and control samples, SLE (B) as well as IPF (C) and control samples. In SLE and COVID-19, genes with adjusted p value < 0.05 and log2 fold-change > 3 were described as orange spots. Genes with adjusted p value < 0.05 and log2 fold-change < -3 were described as cyan spots. Genes having absolute log2 fold-change < 3 or adjusted p value > 0.05 were described as grey. In IPF, genes with adjusted p value < 0.05 and log2 fold-change > 2 were described as orange spots. Genes with adjusted p value < 0.05 and log2 fold-change < -2 were described as cyan spots. Genes having absolute log2 fold-change < 2 or adjusted p value > 0.05 were described as grey. Labelled red spots are the immune relevant significantly differential expressed genes, respectively. (D) PPI network for MMPs and TIMPs for COVID-19 (left), SLE (middle) and IPF (right). The size of the nodes is positively correlated with the significates of genes expression changes for up-regulated and down-regulated genes in COVID-19 (E), SLE (F) and IPF (G).

Figure 3. Comparative analysis of transcriptome data from SLE and COVID-19 patients' lung samples. (A) The Venn diagram shows the genes that transcriptome data of SLE and COVID-19 samples changed together. (B) Heatmap depicting the scaled gene expression changes with same trends between SLE and COVID-19 samples. (C) GO enrichment analyses of genes expression changes with same trends between SLE and COVID-19 samples. (D) The PPI network was constructed for the genes expression changes with same trend between SLE and COVID-19 samples. Four densely connected network components were identified using MCODE algorithm and labelled with different colours. (E) GSEA showing enrichment of neutrophil extracellular trap formation pathway in SLE, COVID-19, and IPF (left to right).

Figure 4. Neutrophil extracellular traps in SLE and COVID-19 patients' lung samples. (A) Immunofluorescence microscopy of lung sections of COVID-19, SLE, IPF and control samples stained with DAPI (blue) and antibodies against myeloperoxidase (green) and neutrophil elastase (red). (B) NET frequency (NETs defined by the triple colocalization events of DNA, myeloperoxidase [MPO], and neutrophil elastase [NE]). N = 3 random fields from each sample. *P < 0.05, **P < 0.01, ***P < 0.001, vs. control group. (C-G) Representative western blots and relative quantitative results of proteins of α-SMA, Snail, Twist, and E-Cadherin in lung epithelial cells treated with NETs (N=6). *P < 0.05, **P < 0.01, ***P < 0.001, vs. PBS group.

Figure 5. Modulation of neutrophil extracellular trap formation pathway during the process of pulmonary fibrosis. (A) Schematic of the function of neutrophil extracellular trap formation pathway during the process of pulmonary fibrosis. Created with BioRender. (B) Heatmap illustrates 6 NETs targeted candidates and the expression profiles of their target genes in SLE and COVID-19 patients compared with controls. The heatmap colour represents the expression levels of corresponding genes (VST normalised counts); The "Blue-white-red" pattern was respectively mapped to the minimum-mean-maximum pattern of expression value. The text colour indicates the groups of drug candidates. (C-D) Relative fluorescence intensity of Sytox Green-stained NETs under different experimental condition: control (no treatment, only A23187), Tofacitinib, Minocycline, Metformin, Bosentan, Belimumab, and Cannabidiol. (n=3). *P < 0.05, **P < 0.01, ***P < 0.001, vs. control group. (E) Relative survival rate of human lung epithelial cells treated with Tofacitinib at concentrations of 5 μM, 25 μM, and 50 μM (n=3). *P < 0.05, **P < 0.01, ***P < 0.001, vs. control group. (F) Representative qPCR analysis and relative quantitative results of mRNA expression levels of α-SMA, Twist, Snail1, and E-Cadherin in lung epithelial cells treated with NETs collected from different Tofacitinib concentration groups (5 μM, 25 μM) (N=3). *P < 0.05, **P < 0.01, ***P < 0.001, vs. PBS group.

Table1. Clinical characteristics of all participants

Clinical characteristics of all participants

	SLE1	SLE2	COVID-19	IPF	Control1	Control2
Sex	male	female	male	male	male	female
Age (year)	39	48	65	64	73	39
Height (cm)	165	159	177	167	159	167
Weight (kg)	55	61	70	65	66	65
BMI	20.2	24.1	22.3	23.3	26.1	23.3
Smoking History	non smoker	non smoker	current smoker	current smoker	current smoker	non smoker
Pack Year	/	/	/	1200	400	/
pO_2 (mmHg)	67	61	/	73	96	91
pCO_2 (mmHg)	44	69	/	48	35	43
HCO_3 (mmol/L)	31.3	40.8	/	31.9	22.2	25.4
BE(mmol/L)	6.6	14.3	/	5.8	-1.9	0

BMI: Body Mass Index

pO_2: Partial pressure of oxygen

pCO_2 (mmHg): The partial pressure of carbon dioxide

HCO_3 (mmol/L): Hydrogen carbonate