

Original Research

CD5L Deficiency Protects Mice Against Bleomycin-Induced Pulmonary Fibrosis

Yang Guo¹, Mengyan Zhu¹, Ruling Shen^{1,*}

¹Shanghai Laboratory Animal Research Center, 201203 Shanghai, China

*Correspondence: shenruling@slarc.org.cn (Ruling Shen)

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Abstract

Background: Pulmonary fibrosis (PF), the most common clinical type of irreversible interstitial lung disease with one of the worse prognoses, has a largely unknown molecular mechanisms that underlies its progression. CD5 molecule-like (CD5L) functions in an indispensable role during inflammatory responses; however, whether CD5L functions in regulating bleomycin (BLM)-induced lung fibrosis is less clear. **Methods:** Herein, we describe the engineering of *Cd5l* knockout mice using CRISPR/Cas9 gene editing technology. The BLM-induced model of acute lung injury represents the most widely used experimental rodent model for PF. **Results:** Taking advantage of this model, we demonstrated that both CD5L mRNA and protein were enriched in the lungs of mice following BLM-induced pulmonary fibrosis. Inhibition of CD5L prevented mice from BLM-induced lung fibrosis and injury. In particular, a lack of CD5L significantly attenuated inflammatory response and promoted M2 polarization in the lung of this pulmonary fibrosis model as well as suppressing macrophage apoptosis. **Conclusions:** Collectively, our data support that CD5L deficiency can suppress the development of pulmonary fibrosis, and also provides new molecular targets for the use of immunotherapy to treat lung fibrosis.

Keywords: CD5L; pulmonary fibrosis; macrophage

1. Introduction

Pulmonary fibrosis (PF) has a poor prognosis and represents a severe health problem worldwide [1]. PF is a chronically progressive lung disease characterized by the disruption of the pulmonary parenchyma and lung architecture, and accumulation of extracellular matrix (ECM) that leads to respiratory failure [2–4]. Despite intensive studies, the complex pathogenesis and variable progression result in a median survival of only 3–5 years after diagnosis [5]. The bleomycin (BLM)-induced model of acute lung injury represents the most widely used experimental rodent model for this disease, and simulates inflammatory and fibrotic events similar to pulmonary fibrosis symptoms seen clinically [6–9]. BLM exposure results in release of inflammatory mediators and promotes initial inflow from neutrophils and, subsequently macrophages to sites of injury, with commensurate rises in ECM modulation and cytokine production including collagen, fibronectin, and hyaluronan [10].

During damage and fibrotic progression, abundant infiltration of macrophages occurs and portends poor prognosis for PF patients [11]. In particular, macrophages play a crucial role in both inflammation and fibrotic phases of the disease where they adopt diverse phenotypes and differentiation in two distinct subsets. Classically, M1-like macrophages are activated by interferon-gamma (IFN- γ) and lipopolysaccharides (LPS), and promote proinflammatory cytokines [12]. In contrast, macrophages may adopt an alternate M2-like phenotype and are stimulated by IL-4 and IL-13 to participate in wound healing and tissue remodeling

[13]. Notably, patients with idiopathic pulmonary fibrosis (IPF) were shown accumulate more M2-like macrophages during disease development and progression [14]. Animal studies also indicate that M2-like polarization is considered a promising target for both prevention and therapy of pulmonary fibrosis [15–17].

CD5 molecule-like (CD5L), also known as apoptosis inhibitor of macrophage (AIM), has been reported to function in response to inflammation and microorganisms, as well as during fat metabolism. CD5L is a broad IgM-binding secreted protein [18,19]. CD5L is principally expressed in macrophages and alveolar epithelial cells [20–22], and has been reported to protect against pro-apoptotic stimuli in macrophages, T cells, and NKT cells [19,23,24]. CD5L has also been considered to be a pro-inflammatory factor in the pathogenesis of colitis [25], atherosclerosis [26], bacterial infection [27], and chronic obstructive pulmonary disease (COPD). Current evidence shows that higher circulating CD5L levels were associated with poor liver function in patients with liver cirrhosis [28,29]. Moreover, CD5L is associated with lipid synthesis and function, for example, fatty acid synthesis, subcellular membranes [21,30], extracellular vesicles [31], LPSs and lipoteichoic acid [32]. CD5L is potentially a pattern identification molecule [33]; however, whether it can be regarded as a potential treatment target for PF has not been explored.

In this study, we hypothesized that CD5L mediates macrophage apoptosis during lung fibrosis. We took advantage of the BLM-induced lung fibrosis mouse model



to investigate: (1) Whether *Cd5l* expression would respond to the development of PF; (2) Whether *Cd5l* knockout (*Cd5l*^{-/-}) mice will exhibit physiologic differences compared to wild-type mice; (3) Whether deletion of *Cd5l* would reduce expression of fibrogenic genes and attenuate collagen deposition in early disease stages in a BLM-induced lung fibrosis mouse model; and (4) Whether deletion of *Cd5l* would regulate macrophage apoptosis during early stages of BLM-induced lung fibrosis. The results of our study provide empirical evidence that inhibition CD5L could be a beneficial preventative and therapeutic strategy for pulmonary fibrosis.

2. Method

2.1 Laboratory Mice

C57BL/6 (wild-type-WT) mice were bred in specific pathogen-free animal conditions, and all procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Laboratory Animal Research Center (Authorization numbers: 202202002).

2.2 Generation of C57BL/6-*Cd5l* Knockout (*Cd5l*^{-/-}) Mice

C57BL/6-*Cd5l* knockout (*Cd5l*^{-/-}) mice, were generated using CRISPR/Cas9 strategy in Shanghai Model Organisms Center, Inc. (SMOC, Shanghai, China). Specifically, we designed *Cd5l*-specific single guide RNAs (*sgRNAs*) to target *Cd5l* 2–4 exons sequences (Fig. 1a). The *sgRNAs* target sequence is shown in Table 1. The HiScribe™ T7 High Yield RNA Synthesis Kit (E2040S, New England Biolabs, Beijing, China) was used for transcribing *sgRNAs* *in vitro*. The mMACHINE T7 Ultra Transcription Kit (AM1345, Thermo Fisher Scientific, Waltham, MA, United States) was used for transcribing *Cas9* mRNA *in vitro*. *SgRNAs* and *Cas9* mRNA were co-injected into C57BL/6 zygotes, which were subsequently transferred into the oviducts of pseudo-pregnant ICR females to obtain F0 generation mice. The primers sequences used for PCR analysis were P1 (sequence: 5'-TCGTGGCCTGTTGATCTGAAGTAA-3') and P2 (sequence: 5'-AATCCCCGTGGCTAGGTCTGTTTT-3'). The reaction specificity and products were further performed by electrophoretic testing and sequencing (Table 2). Through the positive F0 mice were crossed with C57BL/6 mice, the F1 mice were obtained. The genotype of F1 mice were affirmed as F0 generation mice. Genomic DNA from tail biopsy were used for genotyping pups. The primer locations and the PCR outcomes are shown in Fig. 1a,b.

2.3 BLM Induction of Pulmonary Fibrosis

WT and *Cd5l*^{-/-} females were randomly assigned to BLM-treated pulmonary fibrotic group (8–10 weeks, n = 15) or saline group (8–10 weeks, n = 10). Animals were anesthetized with 1% pentobarbital sodium and given one intratracheal injection of 3 mg/kg body-

Table 1. sgRNA sequences used for *Cd5l* knockout.

<i>gRNAs</i>	Sequence (5'-3')
<i>gRNA1</i>	GGAAGGCACGAAGCCTCCAAGGG
<i>gRNA2</i>	TGTATCGTGCAATCATACTATGG

Table 2. Primer sequences.

Primer	Sequence (5'-3')
Cd5l-Com-F	TGCCCACCAGTGACCGTTCC
WT-R	CAAGGCTGATGGGCTGATGTAGAC
KO-R	AATCCCCGTGGCTAGGTCTGTTTT

weight (bw) bleomycin (HY-108345, MedChemExpress Monmouth Junction, NJ, USA) dissolved in 50 µL of sterile saline to induce an inflammatory and fibrosis response as reported [34]. No animals died during this process. The saline group mice (8–10 weeks, n = 10) received same volume of sterile saline. At day 21 post-BLM instillation mice were sacrificed.

2.4 Collection of Bronchoalveolar Lavage Fluid and Lung Sample

The lungs were prepared for lavage by cannulating the trachea with a catheter attached by a syringe, the bronchoalveolar lavage fluid (BALF) was collected by flashing the lung three times with 1 mL of sterile phosphate buffer saline (PBS) [35]. Approximately 0.6 mL of BALF was routinely harvested from each mouse. After BALF recovery, the lungs were removed and stored at -80 °C until further evaluation.

2.5 Apoptosis Assessment

To determine fibroblast apoptosis following bleomycin, an Annexin V/propidium iodide (PI) apoptosis detection kit (Thermo Fisher Scientific, United States) was used to co-stain BALF [36] to discriminate between live cells and those in early and late apoptotic stages [37]. Briefly, BALF cells were incubated with Annexin V and PI solutions for 15 min at room temperature in the dark. Fluorescence intensity was detected using a CytoFLEX Flow Cytometer (B5R3V5, Sadina, California, United States). For this evaluation by flow cytometry, a higher FITC-AnnexinV/lower PI value (termed Q3 region) defined early apoptotic cells whereas higher FITC-Annexin-V/higher PI (termed Q2 region) defined late apoptotic cells. Live cells were defined as lower FITC-Annexin-V/lower PI (termed Q4 region). The percentage of Annexin V- or PI-positive cells quantified the extent of apoptosis.

2.6 Macrophage Phenotyping

For classification of macrophages cells, the BALF was centrifuged for 10 min at 1500 rpm and immunostained. Lung macrophages cells were stained with anti-mouse CD16/CD32 (553140, Franklin Lakes, New Jer-

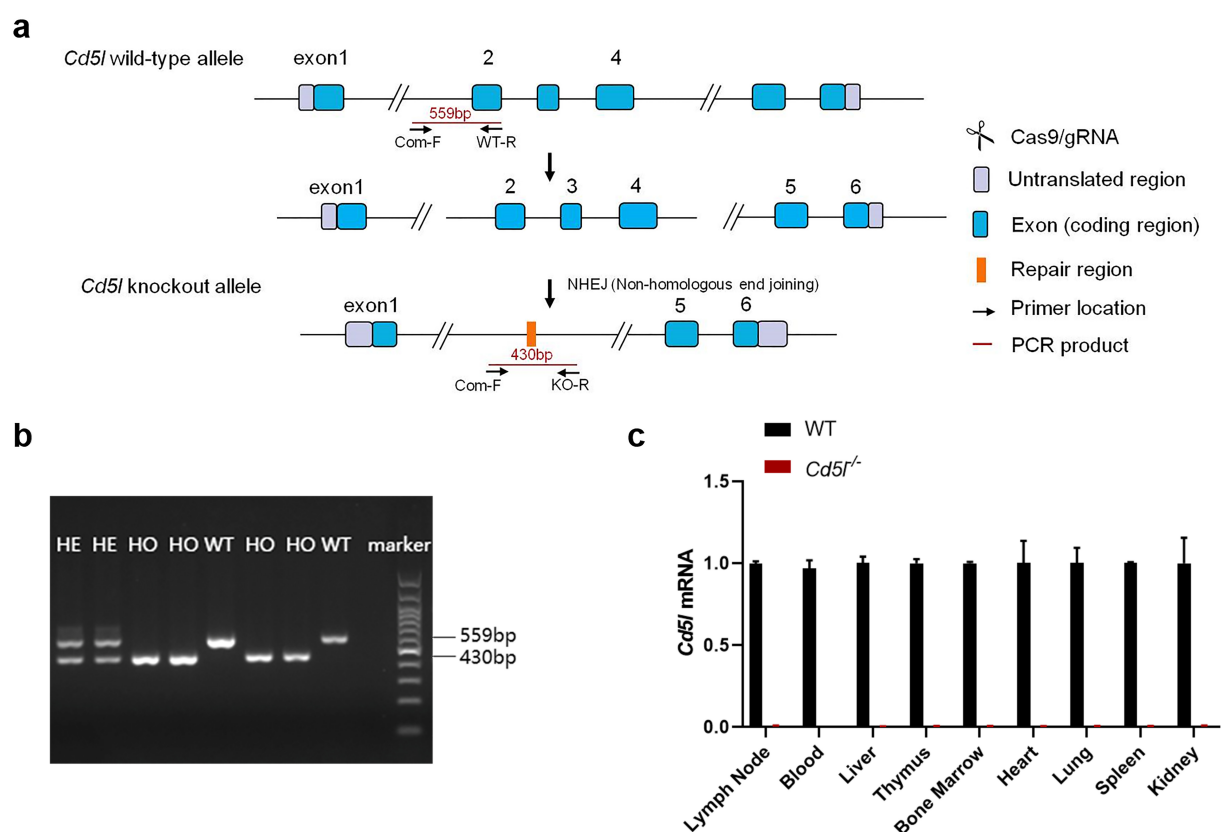


Fig. 1. Construction strategy of *Cd5l* knockout mice and *Cd5l* expression analysis. (a) Exons 2–4 were chosen as the region for gene knockout, and *sgRNAs* designed to target sites upstream and downstream of exons 2 and 4, CRISPR-Cas9 technology was used to produce a reading frame shifting mutation within the *Cd5l*. (b) PCR gel electrophoresis results of heterozygous and homozygous mice, HE-heterozygous mice, HO-homozygous mice, WT-wild type mice. (c) Statistics of *Cd5l* mRNA expression.

sey, United States) and Zombie (423101, BioLegend, San Diego, CA, United States), anti-mouse CD11b-APC (BioLegend, San Diego, CA, United States) and anti-mouse F4/80-BV421 (123131, BioLegend, San Diego, CA, United States). Subsequently, after staining with anti-mouse MHCII-PE (107608, BioLegend, San Diego, CA, United States) and anti-mouse CD206-FITC (BioLegend, United States), cells were analyzed using a CytoFLEX Flow Cytometer (B5R3V5, Beckman, California, United States). Data analysis was performed using FlowJo software (v10, FlowJo LLC, Ashland, OR, USA).

2.7 Histological Analysis

The right lung tissue was excised and immersed in 4% neutral paraformaldehyde overnight at room temperature, and subsequently embedded in paraffin. Tissue was sectioned cut into 5 μ m-thick sections for histological analysis using established protocols. After dewaxing, the alveolitis and fibrosis were investigated using hematoxylin and eosin (H&E) staining. Sirius red and Masson's trichrome staining method were conducted to reveal collagen deposition (blue staining). The severity of fibrosis changes was scored using the Ashcroft scoring system scoring from 0–8 [38,39]

in a double-blind evaluation. Each object, such as aerated lung area (an indicator of lung consolidation extent) and sum or mean collagen volume area (an indicator of collagen extent), were determined using ImagePro-Plus version 6.0 (Media Cybernetics, Rockville, MD, USA). Mean values from all fields of view analyzed in for each mouse lung were calculated.

2.8 ELISA

Whole blood (approx. 1 mL) was collected into 1.5 mL tubes and allow to stand for 30 min at room temperature. The samples were subsequently centrifuged for 15 min at 350 g to obtain serum. Serum concentrations of TGF β , IFN γ , and CCL2/MCP-1 in the serum were determined by enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's protocol (Biolegend, United States).

2.9 Western Blot Analysis

The protein in lung homogenate was extracted used by RIPA lysis buffer (Takara, Dalian, China). Western blot was analyzed using established procedures with indicated primary antibodies. Briefly, protein was separated on 10% polyacrylamide gel and electro-transferred to PVDF

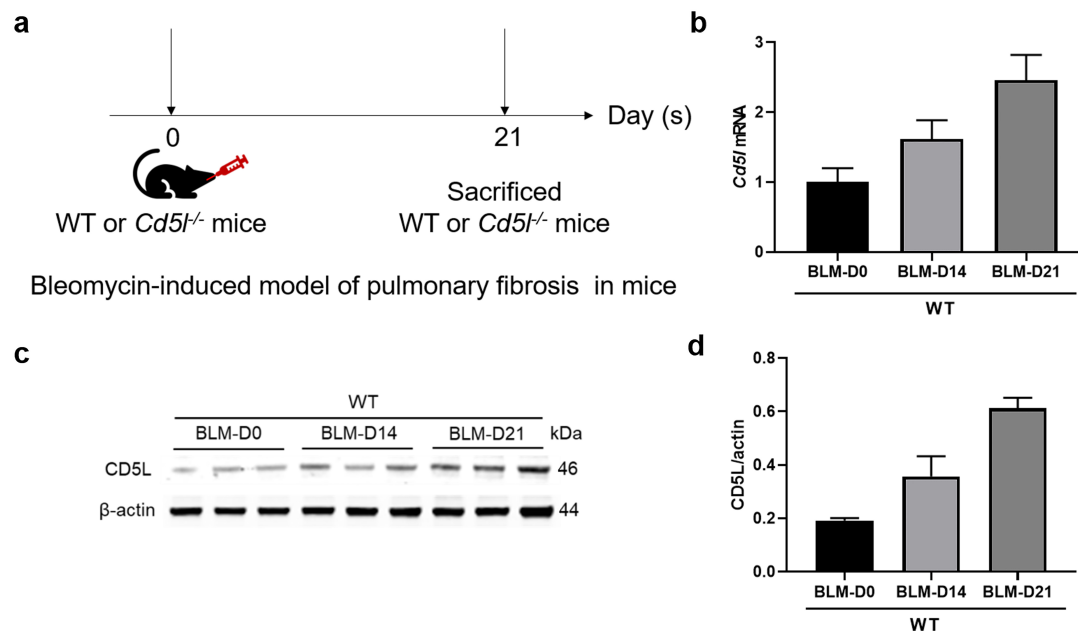


Fig. 2. Expression of *Cd5l* in bleomycin-induced pulmonary fibrosis in mice. (a) A schematic diagram for BLM-induction is shown. (b) *Cd5l* mRNA expression in the lung of WT mice treated with saline or BLM. (c,d) Western blot analysis of *Cd5l* expression in the lung of WT mice following 21 days of BLM induction. Left panel: representative western blots are shown. Right panel: Immunoblot signals were quantified and a bar graph illustrating results is shown. Statistical testing used was a Student's *t* test. BLM, bleomycin.

membrane. 5% non-fat milk was used to block PVDF membranes for 1 h and then incubated overnight at 4 °C with primary antibody to α SMA (ab232784, Abcam, Cambridge, UK). After three washes with PBST (5 min each), Donkey anti-Rabbit secondary antibodies was applied from Odyssey (LI-COR, Lincoln, Nebraska, United States) and then visualized using LICOR (Odyssey, Shenzhen, China).

2.10 Quantitative RT-PCR Analysis

Quantitative RT-PCR analysis was performed using SYBR Premix Ex Taq (AQ131, Transgen, Beijing, China). The primer sequences used for each target gene: *Tgf- β 1*, 5'-AAC CAA GGA GAC GGA ATA-3' and 5'-GTG GAG TAC ATT ATC TTT GCT-3'; *Mcp-1*, 5'-TTA AAA ACC TGG ATC GGA ACC AA-3' and 5'-GCA TTA GCT TCA GAT TTA CGG GT-3'; *Ifn γ* , 5'- ATG AAC GCT ACA CAC TGC ATC-3' and 5'-CCA TCC TTT TGC CAG TTC CTC-3'; *Colla1*, 5'-GCT CCT CTT AGG GGC CAC T-3' and 5'-CCA CGT CTC ACC ATT GGG G-3'. *Eln*, 5'-TTG CTG ATC CTC TTG CTC AAC-3' and 5'-GCC CCT GGA TAA TAG ACT CCA C-3'; The results of each target gene was normalized to housekeeping gene encoding β -actin, 5'-GGC TGT ATT CCC CTC CAT CG-3' and 5'-CCA GTT GGT AAC AAT GCC ATG T-3'.

2.11 Statistical Analysis

Differences between groups were analyzed using GraphPad Prism (version 9.0) software (GraphPad Software, Santiago, MN, USA). Data were expressed as the

mean $\pm$ SEM. Statistical significance was compared by paired *t*-test or one-way ANOVA. A *p* value < 0.05 was considered significant. **p* < 0.05 ; ***p* < 0.01 ; ****p* < 0.001 , *****p* < 0.0001 .

3. Results

3.1 Generation of C57BL/6-*Cd5l* Knockout (*Cd5l*^{-/-}) Mice

To generate *Cd5l*^{-/-} mice, CRISPR/Cas9-mediated genome editing was performed to generate *Cd5l*^{-/-} mice (Fig. 1a). Two *sgRNAs* were designed to target the exons 2–4 according to the strategy indicated in Fig. 1a. *Cas9* mRNA and *sgRNAs* were co-injected into the fertilized oocytes resulting in double-strand breaks and subsequent homologous recombination within the genomic targeted locus. PCR was performed to identify mice with *Cd5l* alteration. F0 mice with predict recombination were crossed with wild-type mice to generate 7 F1 mice, and the identification results are shown in Fig. 1b,c. As predicted, genome sequencing identified that the 7323 bp fragment encoded a knockout of the *Cd5l* gene with the mouse genome (Table 3). The *Cd5l*^{-/-} mice were generated following inbreeding of F1 positive mice for at least four generations. Real-time PCR quantified the expression of *Cd5l* mRNA level. *Cd5l* was detected in tissues of wild-type mice, while this transcript was nearly undetectable in *Cd5l*^{-/-} mice (Fig. 1c). Taken together, the *Cd5l* were knockout successfully through CRISPR/Cas9.

Table 3. Sequence analysis of the *Cd5l* locus in *Cd5l*^{-/-} and WT mice.

Genotype	Sequence (5'-3')	Mutation
<i>Cd5l</i> ^{-/-}	GGCTAGTGCCAAGAGAAGGTGGCCCTGAAAGGATGCC...(7318 bp) ...CTATGGACTATGACAGAGAATGCCGTAA...(-1 bp) ...T... (-4 bp) ...TATAACTTCTGAGTTCTTCCACC	-7323 bp
WT	GGCTAGTGCCAAGAGAAGGTGGCCCTGAAAGGATGCCCTTGGG GGCTTCGTGCCTTCCACTTTCCTTAAGAAGTTTGGC..... ACTTAAAAATTGTGTATCGTGCAATCATACTATGGACTATG ACAGAGAATGCCGTAAATACGGTATAACTTCTGAGTTCTTCCACC	

3.2 Pulmonary Fibrosis Manifests Altered *Cd5l* Expression

As *Cd5l* is associated with inflammation during the development of lung fibrosis [40]. We first reasoned that *Cd5l* may be involved in the pathophysiology of pulmonary fibrosis and to test this, a 3 mg/kg dose of bleomycin was given once intratracheally to WT and *Cd5l*^{-/-} mice to establish bleomycin-induced pulmonary fibrosis model (Fig. 2a).

We next sought to examine the expression of *Cd5l* in the lung of mice with BLM-induced pulmonary fibrosis. A 2.5-fold higher *Cd5l* RNA and 3-fold higher protein expression were detected in BLM-induced WT mice 21 days after BLM administration as compared with 0 day (Fig. 2b–d). Collectively, these data indicate that CD5L is involved in the development of pulmonary fibrosis.

3.3 Loss of *Cd5l* Attenuates BLM-Induced Lung Injury and Fibrosis as well as Reducing Lung Profibrotic Gene Expression

Based on the above observations, the effect of *Cd5l* on inflammation and fibrotic in the lung of *Cd5l*^{-/-} and WT mice treated by bleomycin were subsequently evaluated. We first sought to analyze weight loss of WT and *Cd5l*^{-/-} mice with bleomycin-treated. Saline administration to WT and *Cd5l*^{-/-} mice did not alter body weight (BW). Following day 3 of BLM induction, both *Cd5l*^{-/-} and WT mice presented with a severe loss of weight, a commonly observed effect during pulmonary fibrosis [41,42], although a temporal recovery is commonly observed. Importantly, *Cd5l*^{-/-} mice exhibited a mild weight loss throughout BLM induction when compared with WT mice (Fig. 3a). Moreover, the body weight of BLM-treated WT mice was lower than that measured in saline groups. Histopathological examination and relative lung weight confirmed no significant difference in lung morphology in untreated WT or *Cd5l*^{-/-} mice; however, marked inflammatory cell infiltration with damage and fibrosis in the lungs were observed in BLM-treated animals at day 21 (Fig. 3b). Hematoxylin and eosin (H&E) stained lung sections of *Cd5l*^{-/-} mice showed less inflammatory cell migration than WT mice, indicative of a weakened inflammatory response in *Cd5l*^{-/-} mice. Reduced collagen deposition in *Cd5l*^{-/-} mice was also noted using Masson and Sirius red staining

compared to WT mice after BLM administration (Fig. 3c–e, left panel). In particular, lower Ashcroft scores indicated that the severity of pulmonary fibrosis was substantially reduced (Fig. 3c, right panel). Collagen volume in blue-stained areas (Fig. 3d) and aerated lung area was analyzed by Image-Pro Plus software and similar results were obtained (Fig. 3e, right panel). Based on both weight changes and histopathological staining, we conclude that *Cd5l* deficiency confers considerable protection against the development pulmonary fibrosis.

3.4 *CD5L* Reduced the Expression of Fibrosis Specific Indicators Induced by BLM

Next, the levels of fibrosis-specific markers were examined. *Acta2* (α SMA), *Col1a1* (collagen I), and *Elm* (Elastin) mRNA in lung were measured by qPCR (Fig. 4a–c). As expected, the levels of α SMA, collagen I and Elastin were noticeably upregulated in the BLM groups compared with un-induced groups.

It has been have previously shown that the bleomycin-treated respiratory distress is associated with an early inflammatory reaction [42,43], thus, we next examined lung inflammation. The inflammation-linked genes *Tgf β* , *Mcp1* and *Ifn γ* were measured on day 21 after BLM challenge. As expected, results showed significant increases in expression of *Mcp1* and *Tgf β* as a result of bleomycin-induced lung injury in WT mice, whereas expression of these genes was suppressed by deletion of *Cd5l* (Fig. 4d–f). *Ifn γ* expression was higher in BLM-induced lung of *Cd5l*^{-/-} mice compared to saline *Cd5l*^{-/-} mice. ELISA detection further proved similar levels of TGF β , MCP-1 and IFN γ expression of lung between two BLM groups (Fig. 4g–i). Knock-out of *Cd5l* did not alter either of the *Tnf α* or *Il6* expression when compared with the BLM-WT group (data not shown).

3.5 *Cd5l* Deficiency Influence Macrophages Polarization

We characterized proportion of differential cells in the BALF obtained from various groups on day 21 after BLM-treatment. Flow cytometry showed that both WT and *Cd5l*^{-/-} mice displayed enhanced CD45⁺ expression as a percentage of total live cells. Compared with controls, results showed a 2.8-fold (65%) increase in CD45⁺ cells in WT mice 21 days after BLM-induced damage. *Cd5l*^{-/-} mice had a slightly inflammatory response to BLM (35%),

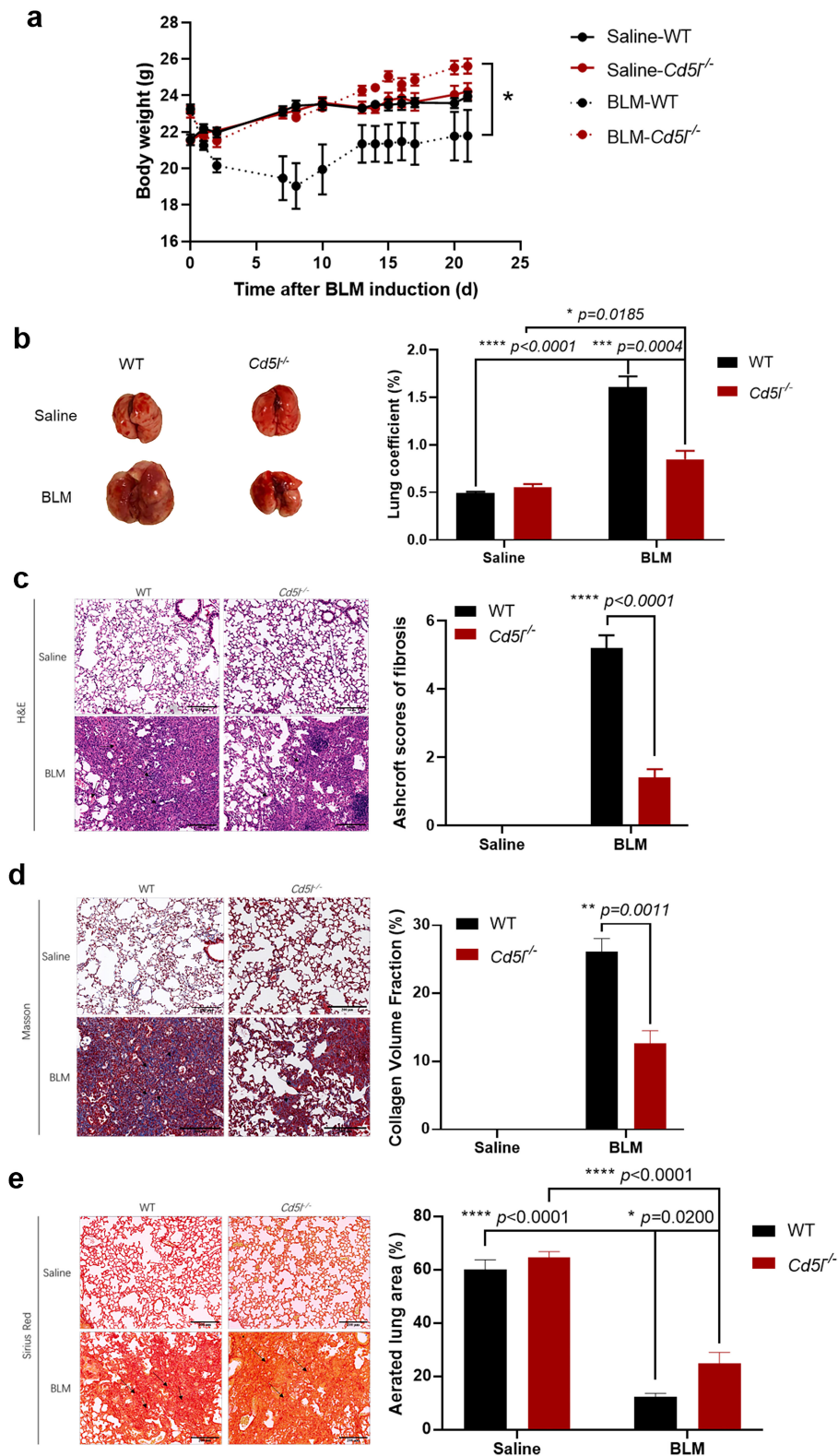


Fig. 3. Loss of *Cd5l* attenuates lung injury and fibrosis. (a) Weights of mice treated with BLM. Values are means $\pm$ SEM. (b) Lung tissue and relative weight in mice. (c–e) Histological examination of the extent of lung fibrosis in mice induced by BLM. Left panel: H&E (c), Masson (d), and Sirius red (e) staining. Collagen fibers are stained blue and muscle fibers are stained red. Black arrows indicate injury position of lung induced by BLM. Right panel: A bar graph depicting semiquantitative Ashcroft scores, collagen volume fraction (CVF), and aerated lung area fraction for the severity of fibrosis is given. Scale bar = 200 μ m, error bars represent mean $\pm$ SEM (n = 5). * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

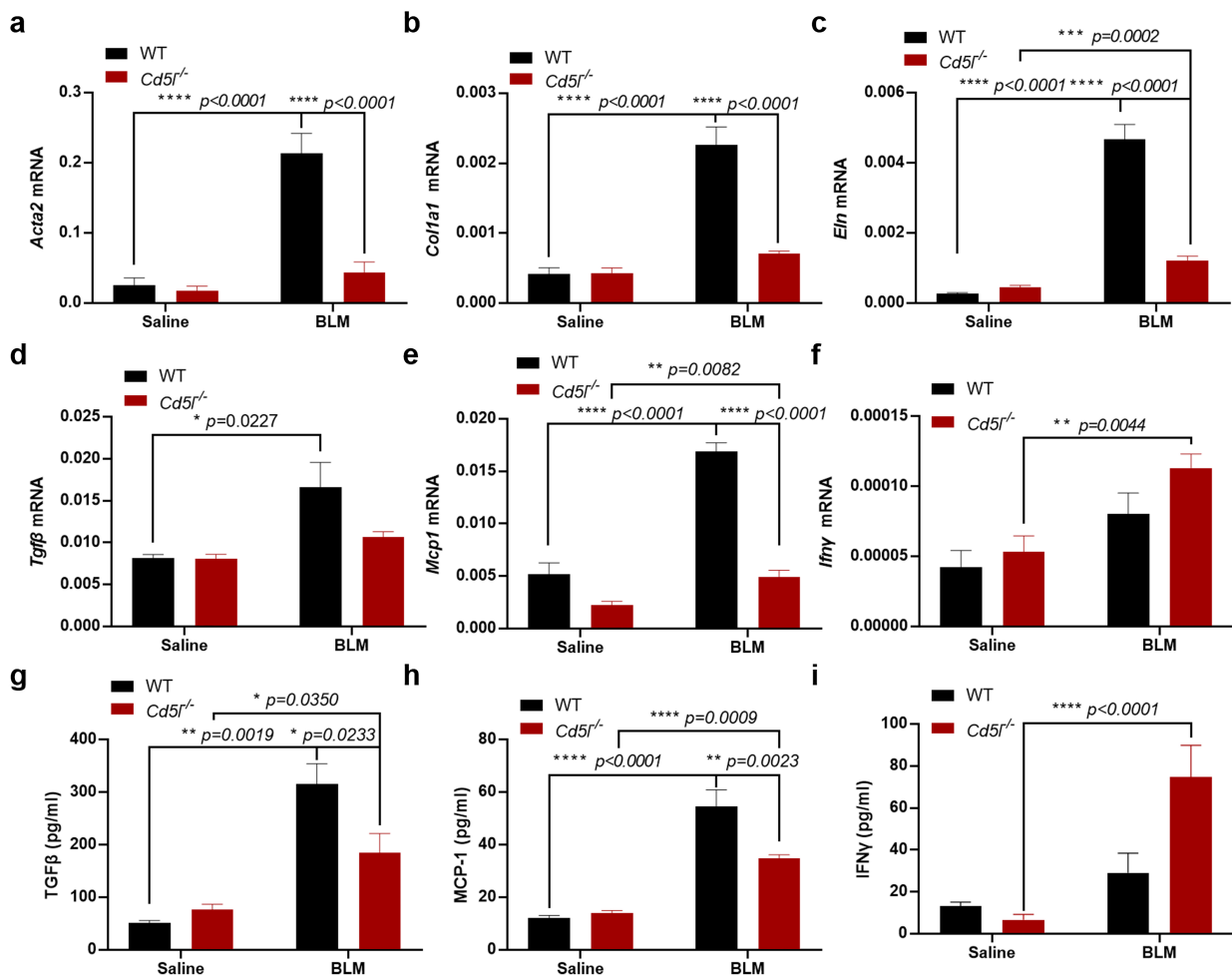


Fig. 4. Measurement of fibrogenic and proinflammatory factors in mouse lung. (a–c) Expression of the fibrogenic genes: *Acta2* (a) *Col1a1* (b) and *Eln* (c) in the lung of BLM-treated mice at 21 days. Five mice were analyzed in each group. (d–f) Inflammation markers: *Tgfβ* (d) *Mcp1* (e) and *Ifnγ* (f) were assessed by quantitative real-time PCR. (g–i) ELISA analysis for TGFβ, MCP-1 and IFNγ. Five mice were analyzed in each group. Data presented as mean ± SEM of groups. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

and a modest 30% upregulation in CD45⁺ cells compared with WT mice (Fig. 5a). CD11b⁺/F4/80⁺ macrophages in both groups showed no change after BLM-induction (Fig. 5b). We further analyzed the effect of *Cd5l* depletion on macrophage typing by examining the specific markers of M1 and M2 macrophages. M1 macrophage surface marker F4/80/MHCII confirmed BLM promoted a 1.5-fold induction of M1 macrophages in *Cd5l*^{-/-} mice, but this effect was not observed in WT mice (Fig. 5c). In contrast, the accumulation of M2 macrophages, as measured by CD206 expression, increased 3-fold in BALF from BLM-WT mice. However, a 7-fold increase in the M2 phenotype was observed in *Cd5l*^{-/-} mice after BLM challenge (Fig. 5d). Overall, these data suggest that CD5L may modulate the macrophage subtypes.

3.6 *Cd5l* Deficiency Reduced Macrophages Apoptosis

We next analyzed apoptosis in macrophages within BALF taken from BLM-treated mice by flow cytometry. An untreated control group were included in this analysis, so the death detected here may be conferred by treatment with TE buffer, and during assay preparation and centrifugation death may have occurred [44]. Annexin V/PI staining showed that the proportions of early apoptotic cells in both models were found as significantly increased compared over controls, but after bleomycin treatment, apoptotic cells in BALF of *Cd5l*^{-/-} mice displayed a remarkable reduction compared that in WT mice (Saline/WT: 0.664 ± 0.218 ; Saline/*Cd5l*^{-/-}: 0.694 ± 0.199 , BLM/WT: 11.6202 ± 1.493 ; BLM-*Cd5l*^{-/-}: 2.77 ± 0.361 , *p* = 0.0004). Flow cytometry revealed that *Cd5l*-deficiency repressed macrophage apoptosis after bleomycin stimulation (Fig. 6).

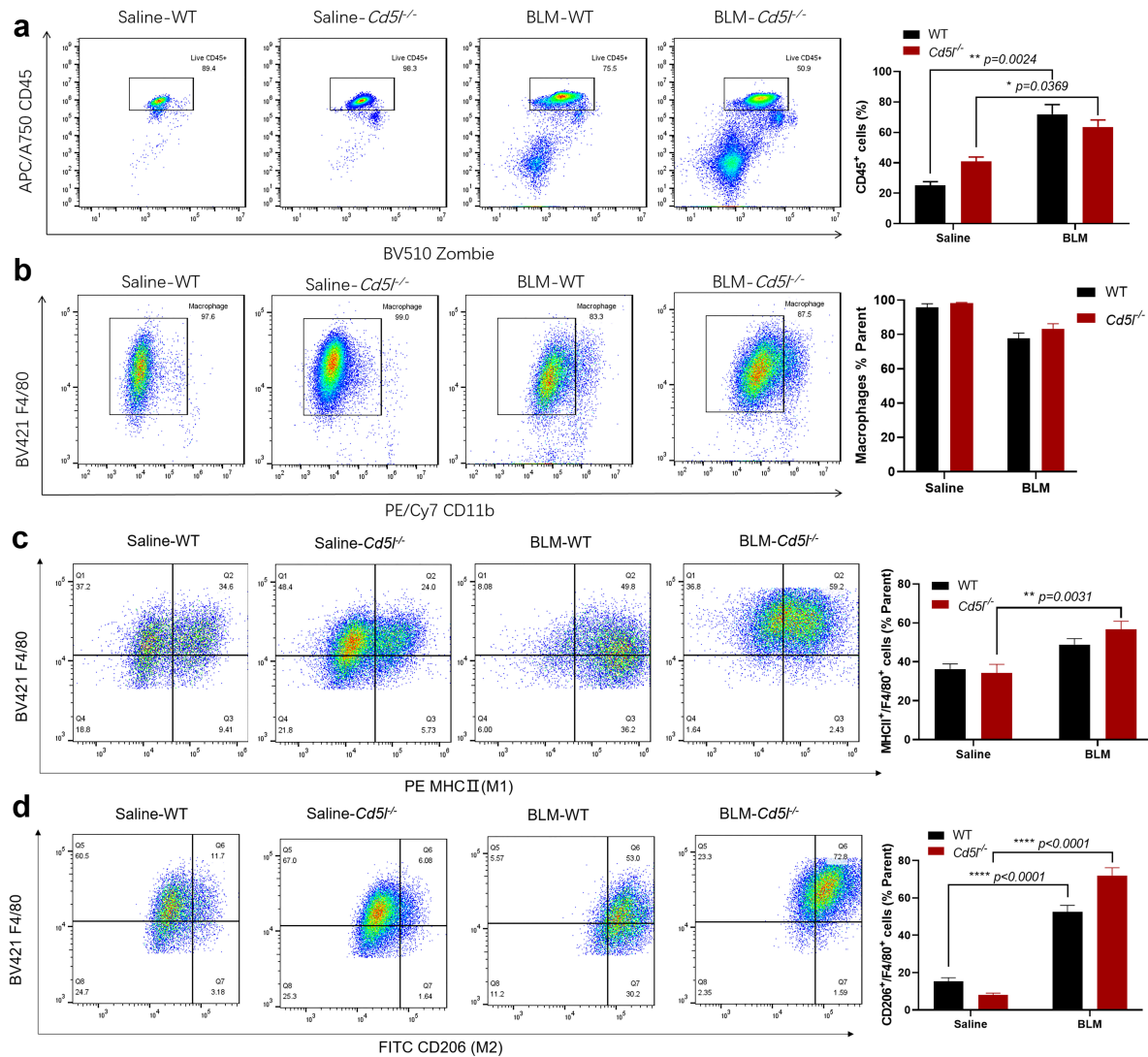


Fig. 5. CD5L predominantly induce M2 polarization in lung following BLM treatment. Left panel: Representative scatter diagram generated during FACS analysis. Right panel: A bar graph indicating the data obtained. (a) Flow cytometry analysis of CD45⁺ and macrophage differentiated cells following stimulation with or without BLM. (b) Percent of macrophages in the BALF obtained both BLM mice from both groups. The cells were first gated in zombie and CD45, and then to analyzed for CD11b and F4/80 expression. (c,d) BALF were stained with F4/80-BV421/MHCII-PE and F4/80-BV421/CD206FITC and cellular phenotype analyzed by FACS. The cells were initially gated using CD11b. Mean values from 10 replicates with mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

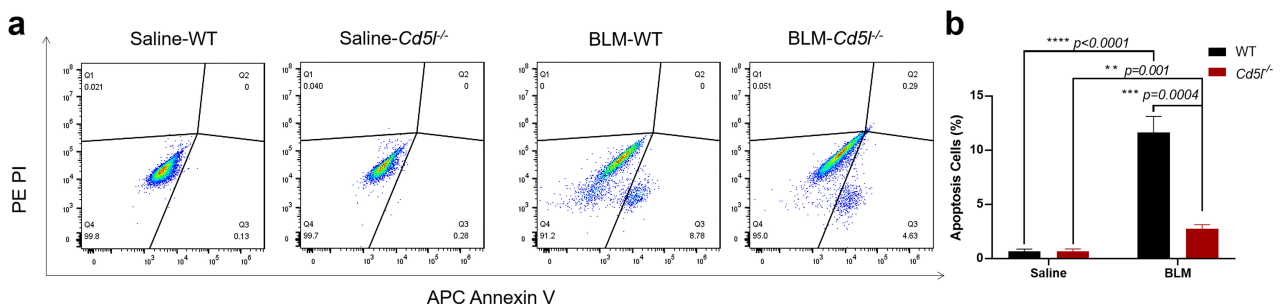


Fig. 6. *Cd5l* deficiency represses apoptosis. (a) Annexin-V/PI flow cytometry analysis of BALF following BLM stimulation. (b) Bar graph of the flow cytometry analysis results. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4. Discussion

The clinical role for CD5L in acute respiratory distress syndrome (ARDS), and trauma victims diagnosed with ARDS display a higher overall CD5L level, compared with healthy people. Further, serum CD5L was upregulated within 24 hours after trauma in patients, and severe trauma patients have higher CD5L values compared to more than mild trauma patients [45]. The serum free state of CD5L had been shown to have increased response and expression during LPS-induced lung injury in a mouse model [46].

Based on previous reports, we first examined Cd5l expression in the BLM-induced fibrotic lung of WT mice, and found that Cd5l expression was gradually elevated in the lung of BLM-treated mice at 7 and 21 days after BLM-treatment compared to controls. Consistent with mRNA, Cd5l protein was enriched in the lung of treated mice (Fig. 2). A novel *Cd5l* knockout mouse model was generated and an *in vivo* model of moderate fibrosis was established. First, we examined a potential suppressive role for Cd5l played in pulmonary fibrosis has been verified by pathological assessment. *Cd5l* knockout results in tissue inflammation and collagen accumulation. Additionally, both the mRNA and protein levels of α SMA, collagen I, and elastin, the three most specific fibrotic indicators, were consistently and significantly reduced in *Cd5l*^{-/-} mice following BLM-induced pulmonary fibrosis compared to BLM-treated WT mice (Fig. 4a–c). We also verified that Cd5l could effectively repress the progression of PF and provide more potential effective treatment for PF. Previously it had been demonstrated that CD5L blockade downregulated the expression of inflammatory cytokines and chemokines, and upregulated the production of anti-inflammatory factors, as well as the infiltration of leukocytes including macrophages, lymphocytes and neutrophils in peritoneal lavage fluid after CLP-treated sepsis [47]. In this study, the level of inflammatory cytokines (Fig. 4d–i) and the infiltration of lymphocytes were also found to be suppressed by *Cd5l* depletion in pulmonary fibrosis (Fig. 5a). In the pathogenesis of inflammatory processes, CD5L is thought to control lipid metabolism by inhibiting macrophage apoptosis and also responses to TLR activation by promoting an anti-inflammatory cytokine profile [21]. Initially, macrophages are divided into two functional subtypes. Classical active macrophages, the M1-like phenotype with representative markers iNOS or MHC II, are characterized by their association with several cytotoxic cytokines. M1 macrophages can be repolarized and transfer their phenotype toward M2 macrophages, which express the representative marker CD206, and which are defined by pro-regenerative and anti-inflammatory properties [48]. There is emerging evidence that CD5L may function as a driver of M2 macrophage polarization [49].

One could hypothesize that the rise in CD5L during PF is an adaptive response to lung injury and fibrosis. To answer these questions, flow cytometry performed on BALF

showed that M1 macrophages in *Cd5l*^{-/-} mice are similar to WT mice at baseline. However, M1 macrophages from *Cd5l*^{-/-} mice increased in the BALF after bleomycin exposure (Fig. 5c). According to previous studies [50], M2-like phenotype are crucial pathogenic factors during the course of BLM-induced pulmonary fibrosis, and the accumulated macrophage M2 program is generally related to collagen remodeling of internal tissues including the heart, kidneys, liver, and lungs. CD5L influences M1/M2 profiles and has positive feedback [49]. We thus then carried out studies and provided evidence that M2 macrophage levels were higher in BLM-treated mice. In particular, we measured a 7-fold increase in M2 macrophages in BALF obtained from *Cd5l*^{-/-} mice, which consistent with previous findings (Fig. 5d).

CD5L has previously been reported to have an anti-apoptotic effect [19,24,51]. We verified the effects of Cd5l on macrophage apoptosis in our mouse model. The proportion of apoptotic cells were significantly lower in *Cd5l*^{-/-} mice than that measured in WT mice after BLM exposure (Fig. 6). AIM is associated with insulin-like growth factor-binding protein-4 [52], and it was reported that there is cross-talk between lipid mediators including SPMs [53], DHA [54], and PGE2 [55,56] that accelerate apoptosis relative to cell type and physiological conditions. Therefore, the pathogenic/inflammatory conditions promote apoptosis the regulatory function in apoptotic cell death may enable illumination of the mechanism during apoptosis.

We have shown that *Cd5l* protects mice from pulmonary fibrosis, but its regulatory signaling mechanisms remain unclear as does its function in inhibiting apoptosis. In future work, we plan to dissect the common signaling crosstalk as more details concerning the previously mentioned pathways and targeted factors need to be uncovered.

5. Conclusions

In conclusion, our findings demonstrate that CD5 molecule-like (CD5L)-deficiency protected mice from bleomycin-induced lung injury and fibrosis by attenuating the release of inflammatory mediators, regulating macrophages polarization and apoptosis, which could be a viable strategy for the prevention and treatment of pulmonary fibrosis in clinical settings. These findings may provide a sufficient theoretical basis for the clinical application of CD5L and development of novel therapeutics for treating pulmonary fibrosis.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

MZ carried out the molecular genetic studies. YG participated in the design of the study, performed the statistical analysis and drafted the manuscript. RS participated in its design and coordination and helped to draft the manuscript. All authors have participated sufficiently in the work and agreed to be responsible for all aspects of this work. All authors have contributed to editorial changes in the manuscript and have read and approved the final manuscript.

Ethics Approval and Consent to Participate

All procedures involved animal research in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Laboratory Animal Research Center (Authorization numbers: 202202002).

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Conflict of Interest

The authors declare no conflict of interest.

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