



Quercetin Alleviates Pulmonary Fibrosis in Mice Exposed to Silica by Inhibiting Macrophage Senescence

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Quercetin exerts anti-inflammatory, anti-oxidant and other protective effects. Previous studies have shown that senescent cells, such as fibroblasts and type II airway epithelial cells, are strongly implicated in the development of pulmonary fibrosis pathology. However, the role of senescent macrophages during silicosis remains unclear. We investigated the effects of quercetin on macrophage senescence and pulmonary fibrosis, and explored underlying mechanisms. Mice were randomized to six model groups. Vitro model was also established by culturing RAW264.7 macrophages with silica (SiO₂). We examined the effects of quercetin on fibrosis, senescence-associated β-galactosidase (SA-β-Gal) activity, and senescence-specific genes (p16, p21, and p53). We showed that quercetin reduced pulmonary fibrosis and inhibited extracellular matrix (ECM) formation. Quercetin also attenuated macrophage senescence induced by SiO₂ both *in vitro* and *in vivo*. In addition, quercetin significantly decreased the expressions of the senescence-associated secretory phenotype (SASP), including proinflammatory factors (interleukin-1α (Il-1α), Il-6, tumor necrosis factor-α (TNF-α), and transforming growth factor-β1 (TGF-β1)) and matrix metalloproteinases (MMP2, MMP9, and MMP12). In conclusion, quercetin mediated its anti-fibrotic effects by inhibiting macrophage senescence, possibly via SASP.

Keywords: silicosis, quercetin, macrophage, senescence, SASP

INTRODUCTION

Silicosis is a chronic and progressive disease mainly caused by prolonged exposure to free silica dust, and is characterized by excessive nodular fibrosis in the lung (Hoy and Chambers, 2020; Kimberly and John, 2021). The precise underlying pathogenesis remains unclear, therefore, comprehensive molecular investigations are required to identify treatment strategies for this disease.

Alveolar macrophages are a major cell type residing in alveoli, and function as first line defenses against inhaled infectious agents and environmental pollutants (Kim and Nair, 2019; Chen et al., 2020; Wen et al., 2021). Recent studies reported that senescent macrophages reduced proliferation, elevated senescence-associated β-galactosidase (SA-β-Gal) activity,

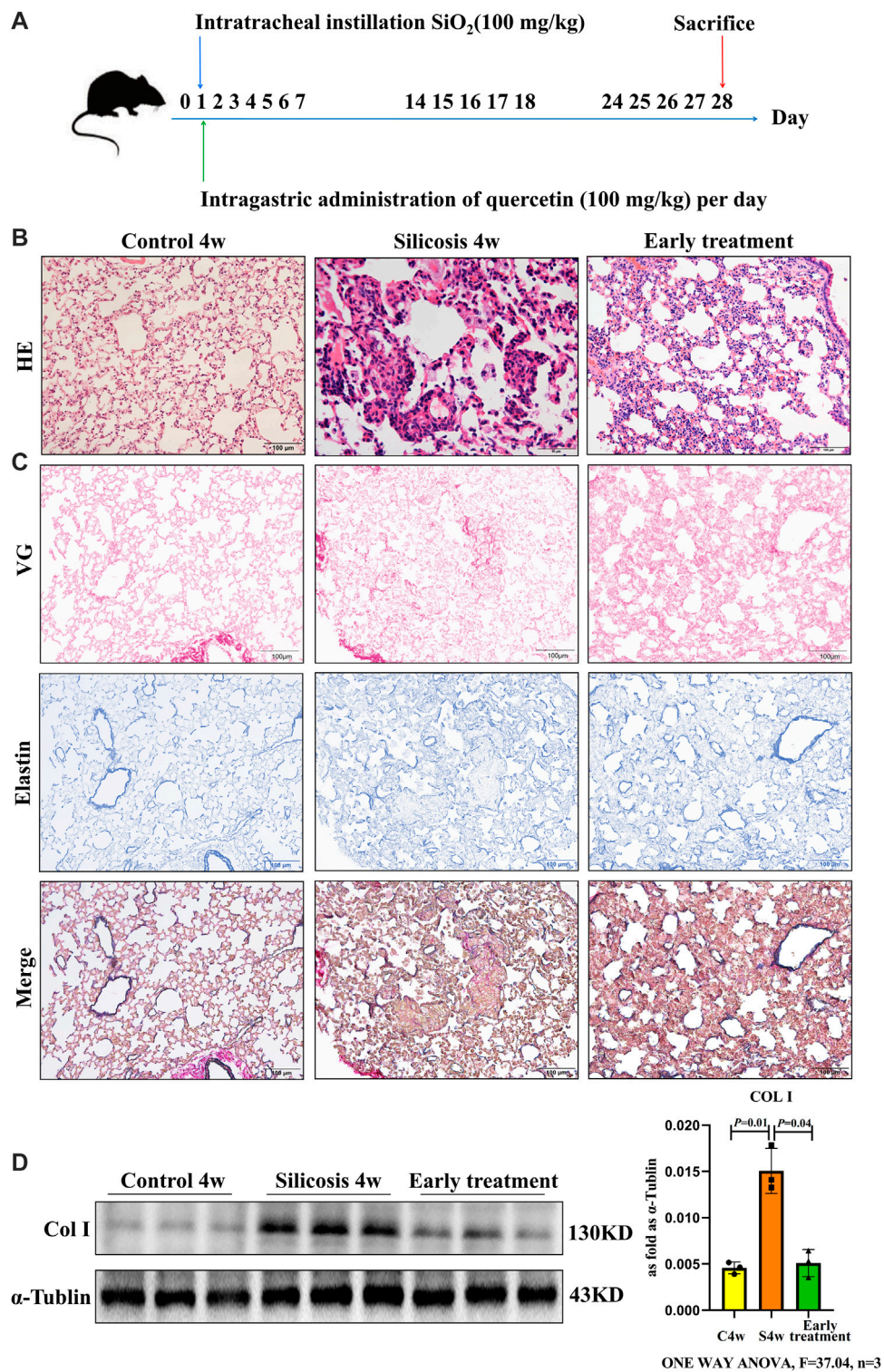


FIGURE 1 | Quercetin attenuates lung injury in an early therapeutic silicosis model. **(A)** Schematic of quercetin administration in an early therapeutic silicosis model. **(B)** Representative micrographs showing lung injury by HE staining. **(C)** VG and elastin staining of lung tissue sections. **(D)** Col I expression by western blot. Data are presented as the mean \pm SD. $n = 3$ per group.

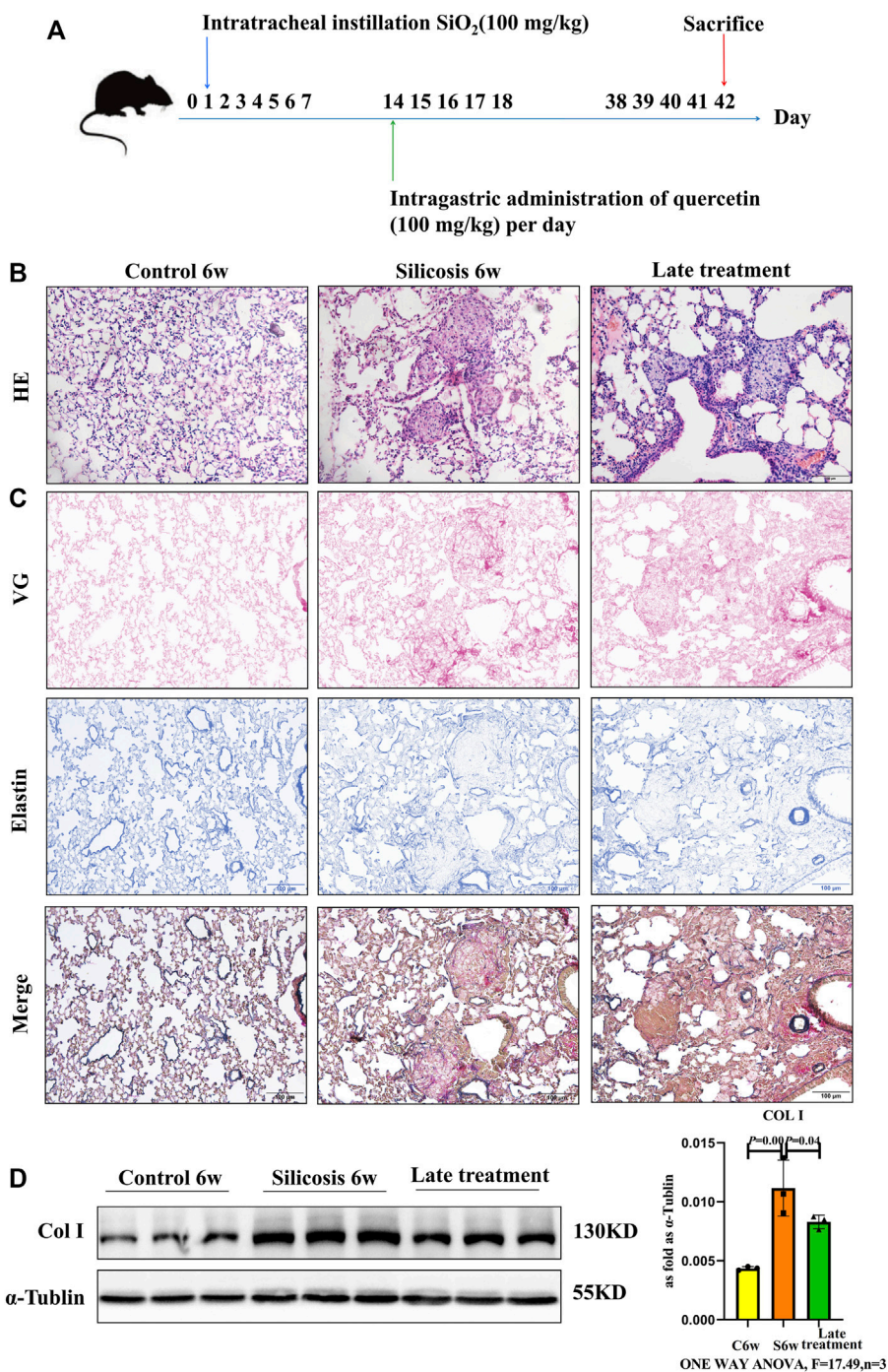
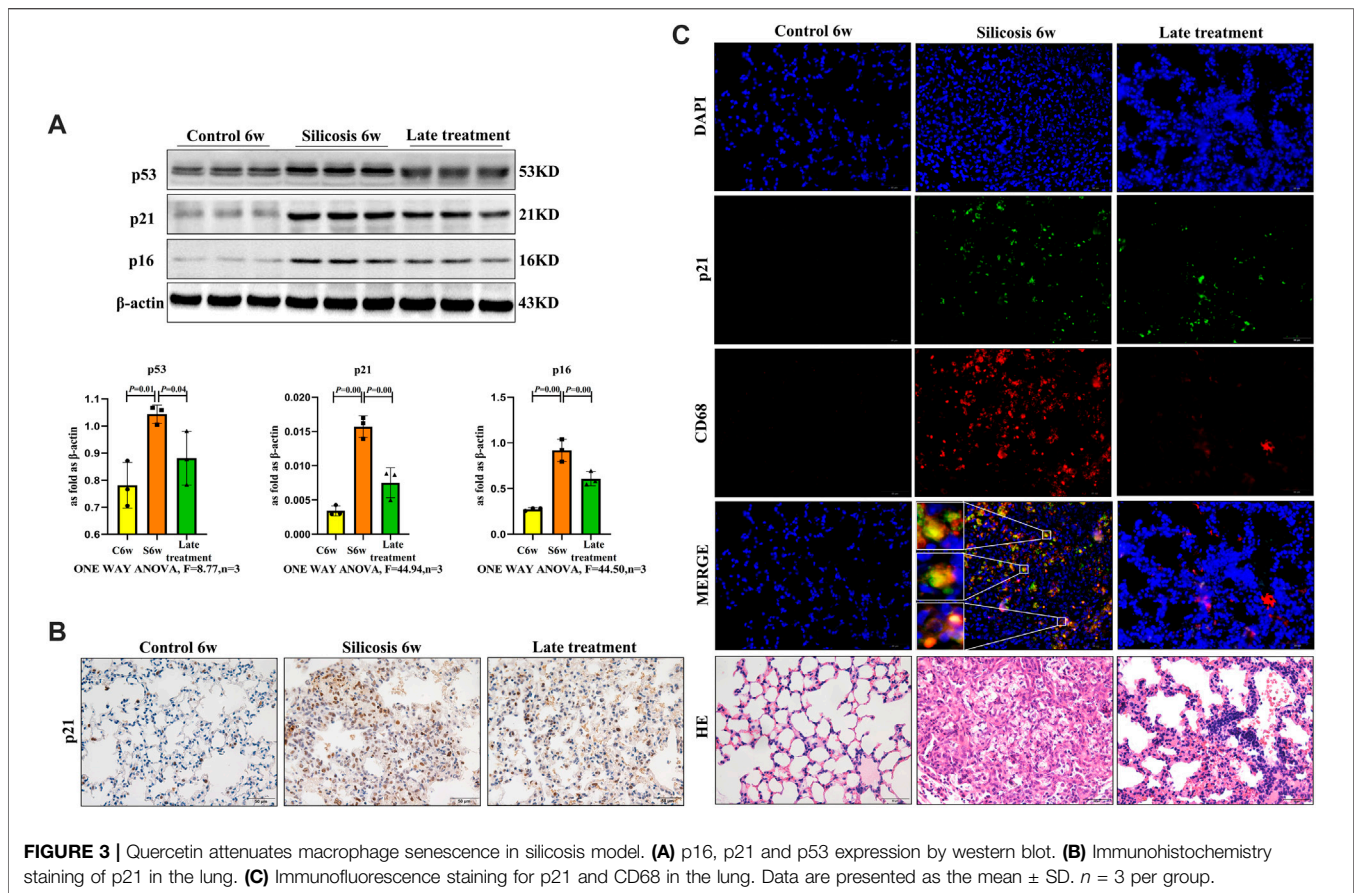


FIGURE 2 | Quercetin attenuates lung injury in a late therapeutic silicosis model. **(A)** Schematic of quercetin administration in a late therapeutic silicosis model. **(B)** Representative micrographs showing lung injury by HE staining. **(C)** VG and elastin staining of lung tissue sections. **(D)** Col I expression by western blot. Data are presented as the mean \pm SD. $n = 3$ per group.

increased senescence-associated secretory phenotype (SASP) mRNA expression, and contributed to aged disease development such as fibrosis (Sonia and Elaine, 2020; Jacques and Jesús, 2021; Su et al., 2021).

Quercetin exerts anti-oxidant, anti-inflammatory, and other protective effects (Li et al., 2016; Liang et al., 2018).

Previous studies reported that quercetin reduced senescent fibroblasts and lung fibrosis in bleomycin treated mice (Liu et al., 2020; Martel et al., 2020; Li et al., 2021). Therefore, the impact of quercetin on senescence macrophages may be a key research strategy to identify mechanisms of fibrosis.



RESULTS

Quercetin Treatment Effectively Alleviates Silicosis in a Mouse Model

To establish an early therapeutic model of quercetin administration, mice were intragastric administration of quercetin for 4 weeks at the time of silica (SiO_2) via one-off non-invasive instillation (Figure 1A). From hematoxylin and eosin (HE) staining, more infiltrating inflammatory cells and silicotic nodules were observed in silicosis group at 4 weeks. These alterations were ameliorated after treatment with quercetin (Figure 1B). After silica exposure, the expression of the fibrotic indicator, collagen was examined in lung tissues. Van Gieson (VG) and elastin staining showed excessive collagen deposition in pulmonary mesenchyme in silicosis group. Nonetheless, collagen deposition areas were diminished after quercetin administration (Figure 1C). Western blot showed that collagen I (Col I) levels were significantly higher in silicosis group compared with control group. In quercetin treatment group, a marked decrease of Col I levels was observed (Figure 1D). Thus, early therapeutic administration of quercetin ameliorated silicosis *in vivo*.

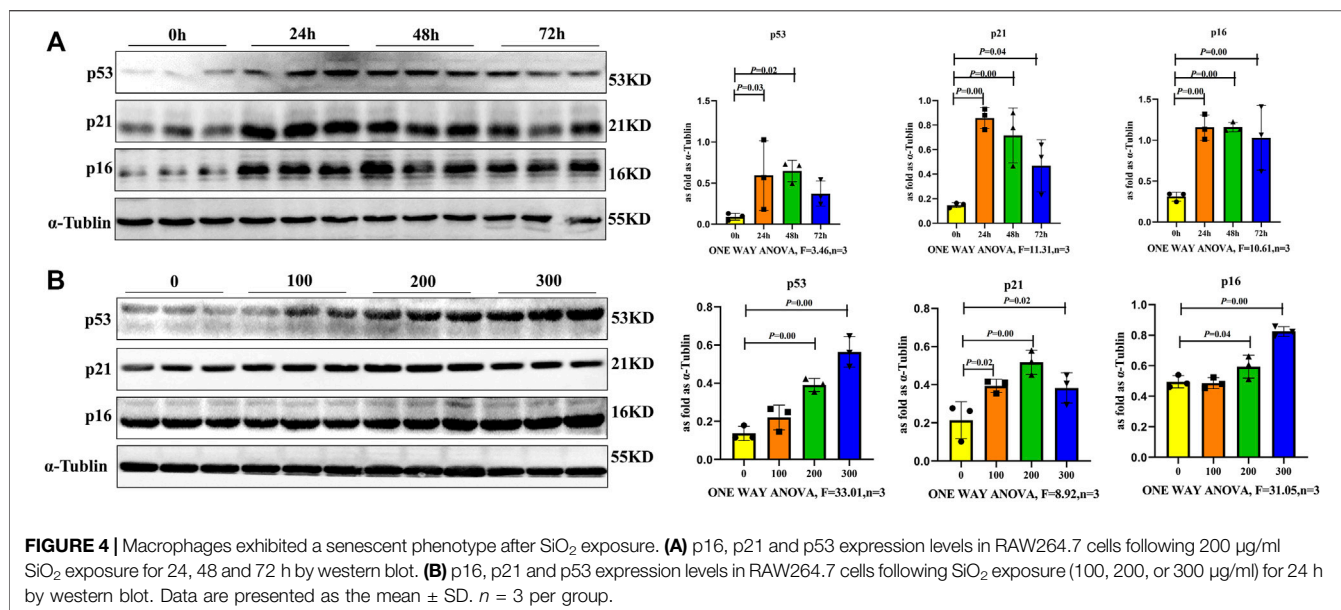
We next investigated the late therapeutic model of quercetin administration after the formation of silicotic nodules in silicosis group, and mice were also intragastric administration of

quercetin for 4 weeks beginning at 2 weeks after SiO_2 exposure (Figure 2A). HE staining showed the anti-fibrotic effects of quercetin treatment for the onset of silicotic nodules (Figure 2B). In addition, reduced Col I levels by western blot were consistent with the results using VG and elastin staining (Figures 2C,D). Collectively, these data suggest that quercetin has improved effects on pulmonary injury when administered after onset insilicosis mice also, and subsequent mechanism experiments were also conducted in this late therapeutic silicosis model.

Quercetin Ameliorates Macrophage Senescence in Mice With Silicosis

Western blot showed that quercetin administration downregulated the higher level of senescence markers, p16, p21 and p53 in silicosis group (Figure 3A).

While previous studies showed that mice exposed to SiO_2 induced a significant increase in senescent cells and contributed to silicosis, the role of senescent macrophages requires clarification. To further assess if quercetin was associated with reduced macrophage senescence and mediated in silicosis, the senescence marker-p21 and macrophage marker-CD68 were used to examine macrophages senescence in silicosis. We observed increased numbers of $\text{p21}^+\text{CD68}^+$ cells after SiO_2



administration, thereby supporting the role of macrophages in senescence-associated silicosis. The percentage of p21⁺ macrophages was significantly decreased in the quercetin group compared with the silicosis group (Figures 3B,C). Thus, quercetin treatment inhibited SiO₂-induced macrophage senescence.

SiO₂ Induces Macrophage Senescence in a Time- and Dose-Dependent Manner

To further verify if SiO₂ induced macrophage senescence, RAW264.7 cells were examined at different times after 100, 200 or 300 µg/ml SiO₂ exposure. Senescence-associated proteins of p16, p21 and p53 were gradually upregulated in a time- and dose-dependent manner. The levels of p16, p21 and p53 induced by the 200 µg/ml SiO₂ displayed higher expression in 24 and 48 h groups compared with control (Figure 4A). In addition, the levels of p16, p21 and p53 for 24 h displayed higher expressions in 200 µg/ml and 300 µg/ml groups compared with control (Figure 4B). Thus, macrophages could be induced senescent by 200 µg/ml SiO₂ after 24 h, suggesting senescent macrophages may be involved in silicosis development.

To investigate the effect of quercetin on SiO₂ induced macrophage senescence, RAW264.7 cells were treated with SiO₂ (200 µg/ml) and/or quercetin (25 µmol/L) for 24 h. As shown in Figure 5A, the expressions of p16, p21, and p53 were significantly increased by SiO₂ stimulated for 24 h, however, p16, p21, and p53 expressions were decreased after quercetin treatment. Cytoplasmic SA-β-gal activity, which is a marker of cell senescence, was examined by SA-β-gal staining. These assays showed that positive staining cell numbers in Quercetin + SiO₂ group were significantly lower than SiO₂ group (Figure 5B), confirming our western blot findings.

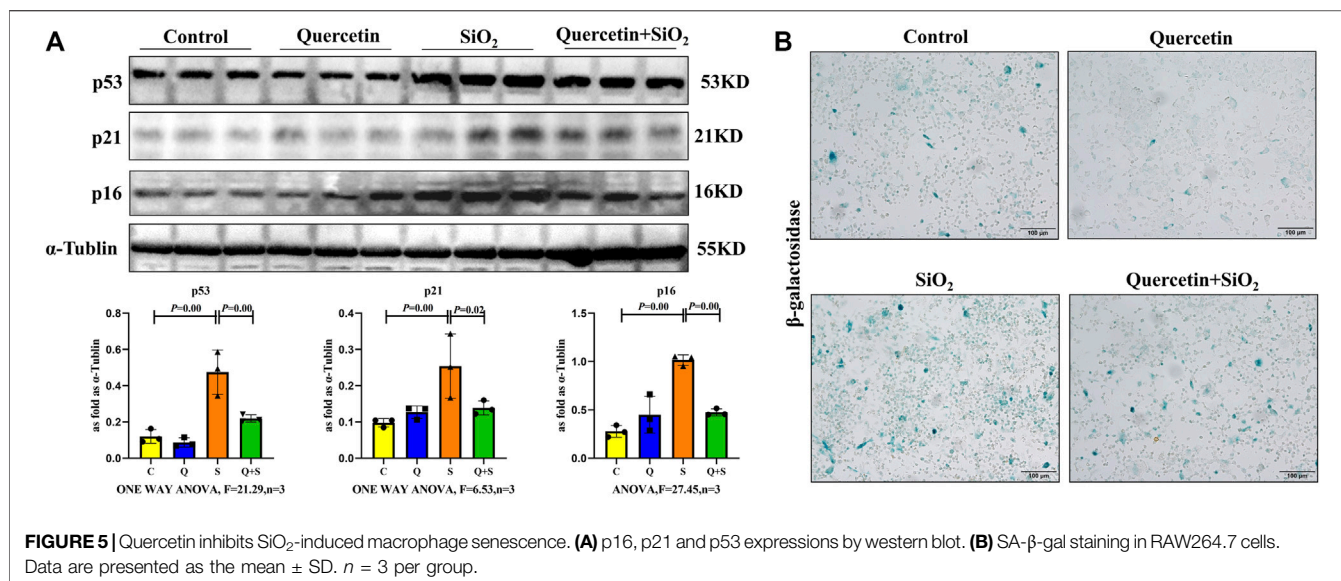
Thus, quercetin administration significantly delayed macrophage senescence induced by SiO₂.

Network Pharmacology Analysis of a Quercetin-Senescence-Silicosis Intersection Target Network

To clarify quercetin mechanisms related to senescence, we performed a network pharmacology analysis. In total, 223 potential quercetin targets were identified using Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) and Swiss Target Prediction Database. We also identified 251 silicosis disease targets in GeneCards and Diogene. The GeneCards Database revealed 4,780 potential targets for senescence. Using a Venn diagram of quercetin, silicosis and senescence target intersections, 38 overlapping targets were identified (Figure 6A).

These 38 targets were then imported into the STRING Database to establish a protein-protein interaction (PPI) network. Next, cytoscape software was used to generate a quercetin-senescence-silicosis target network diagram, showing the top 10 molecular targets: interleukin-6 (IL-6), protein kinase B (AKT1), tumor necrosis factor (TNF), prostaglandin-endoperoxide synthase 2 (PTGS2), peroxisome proliferator activated receptor gamma (PPARG), IL-1β, transforming growth factor-β1 (TGF-β1), matrix metalloproteinases-9 (MMP9), TP53, and c-Jun N-terminal kinase (JUN) (Figure 6B).

After gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses, 343 biological processes, 34 molecular functions, 26 cell components and 28 signal pathways were identified (Figures 6C,D). According to adjusted *p* values, the top ten pathways were selected, including pathways in cancer, TNF signaling pathway, Toll-like receptor signaling pathway, Estrogen signaling pathway,



Mitogen-activated protein kinase (MAPK) signaling pathway, Forkhead box protein O (FoxO) signaling pathway, Hypoxia inducible factor 1(HIF-1) signaling pathway, oxytocin signaling pathway, T cell receptor signaling pathway, and thyroid hormone signaling pathway.

The Senescent Macrophage Secretome Is Profibrotic and Proinflammatory

To explore the role of senescent macrophages during silicosis, dynamic changes in SASP components were determined after macrophages were exposed to SiO₂, including the analysis of growth and matrix remodeling factors. Firstly, the SASP proinflammatory factors, including IL-1β, IL-6, TNF-α, and TGF-β1 were significantly upregulated in senescent macrophages at 24 h after 200 μg/ml SiO₂ exposure (Figures 7A,C). Secondly, MMPs including MMP2, MMP9 and MMP12 of the SASP matrix in macrophages were increased after macrophages exposure to SiO₂ (Figures 7B,D). Taken together, our results supported the notion that SiO₂ promoted SASP secretion in senescent macrophages.

Importantly, SASP proinflammatory factor secretion and MMPs in the SASP matrix were decreased after quercetin treatment. (Figure 7).

Quercetin Alleviates Silicosis by Reducing SASP Levels of Senescent Macrophages in a Silicosis Mouse Model

Fibrotic and SASP protein expression levels in the lungs of the silicosis model were analyzed. Western blot showed that the expression levels of IL-1β, IL-6, TNF-α and TGF-β1 in lung tissue from the silicosis group were significantly elevated (Figures 8A,C). Additionally, increased MMP2, MMP9 and MMP12 expression levels were also detected after SiO₂ exposure (Figures 8B,D).

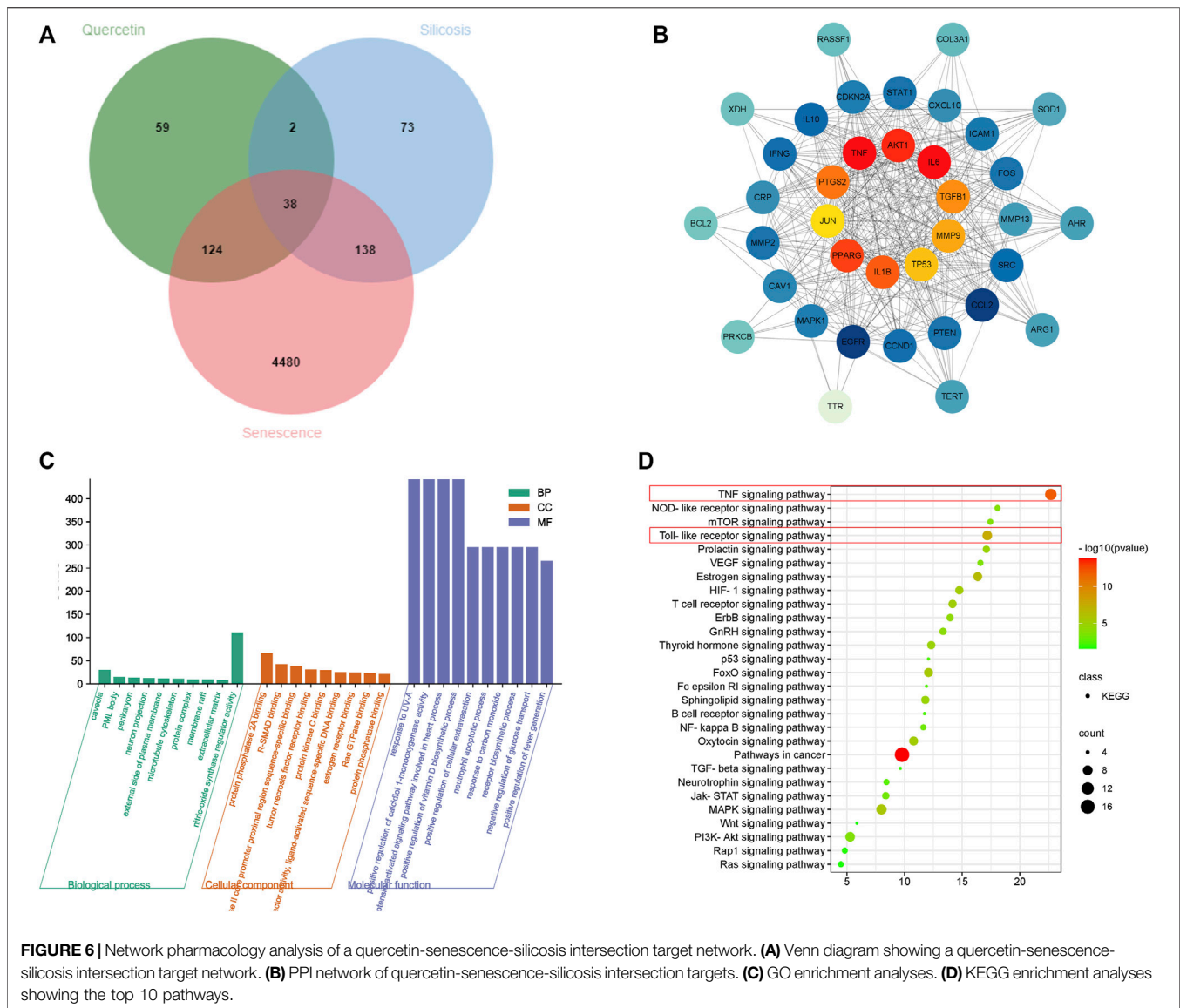
Quercetin treatment significantly decreased fibrotic and SASP protein expression levels *in vivo*. Thus, quercetin administration inhibited silicosis activity by attenuating SASP levels in senescent macrophages in a silicosis model.

DISCUSSION

Silicosis has a chronic, progressive and ultimately fatal fibrotic course, thus effective preventative and therapeutic measures are required. The disease is characterized by fibroblast proliferation, ECM accumulation, and ultimately leading to respiratory failure (Wei et al., 2019; Li et al., 2020). Although the disease pathogenesis has been widely investigated, detailed molecular and cellular pathogenic mechanisms are unclear with current therapeutic strategies largely ineffective.

Quercetin is a natural plant flavonoid in many fruits, vegetables and grains, which displays numerous beneficial biological activities including anti-inflammatory, anti-oxidant, immune regulation and other effects (Fontana and Partridge, 2015). Quercetin suppressed bleomycin induced epithelial-mesenchymal transition by suppressing reactive oxygen species via Smad and β-catenin signaling pathways (Takano et al., 2020). Quercetin also exerted anti-fibrogenic and anti-inflammatory effects, possibly by modulating the redox balance via nuclear erythroid factor 2-related factor 2 (Nrf2) induction (Boots et al., 2020). Quercetin (100 mg/kg) markedly reduced the extent of tissue damage in bile duct ligation (BDL) - and carbon tetrachloride (CCl₄) -liver fibrosis model mice, confirming its protective effect (Wu et al., 2017). In our study, we showed that both early and late therapeutic administration of quercetin (100 mg/kg) protected against SiO₂-induced silicosis in mice.

Cellular senescence involves DNA damage responses which activate the tumor suppressors including p16, p21 and p53, and the subsequent induction of SA-β-gal, which has key roles in embryogenesis, wound healing and protection against cancer (Birch et al., 2017). Previous studies reported that senescent cells



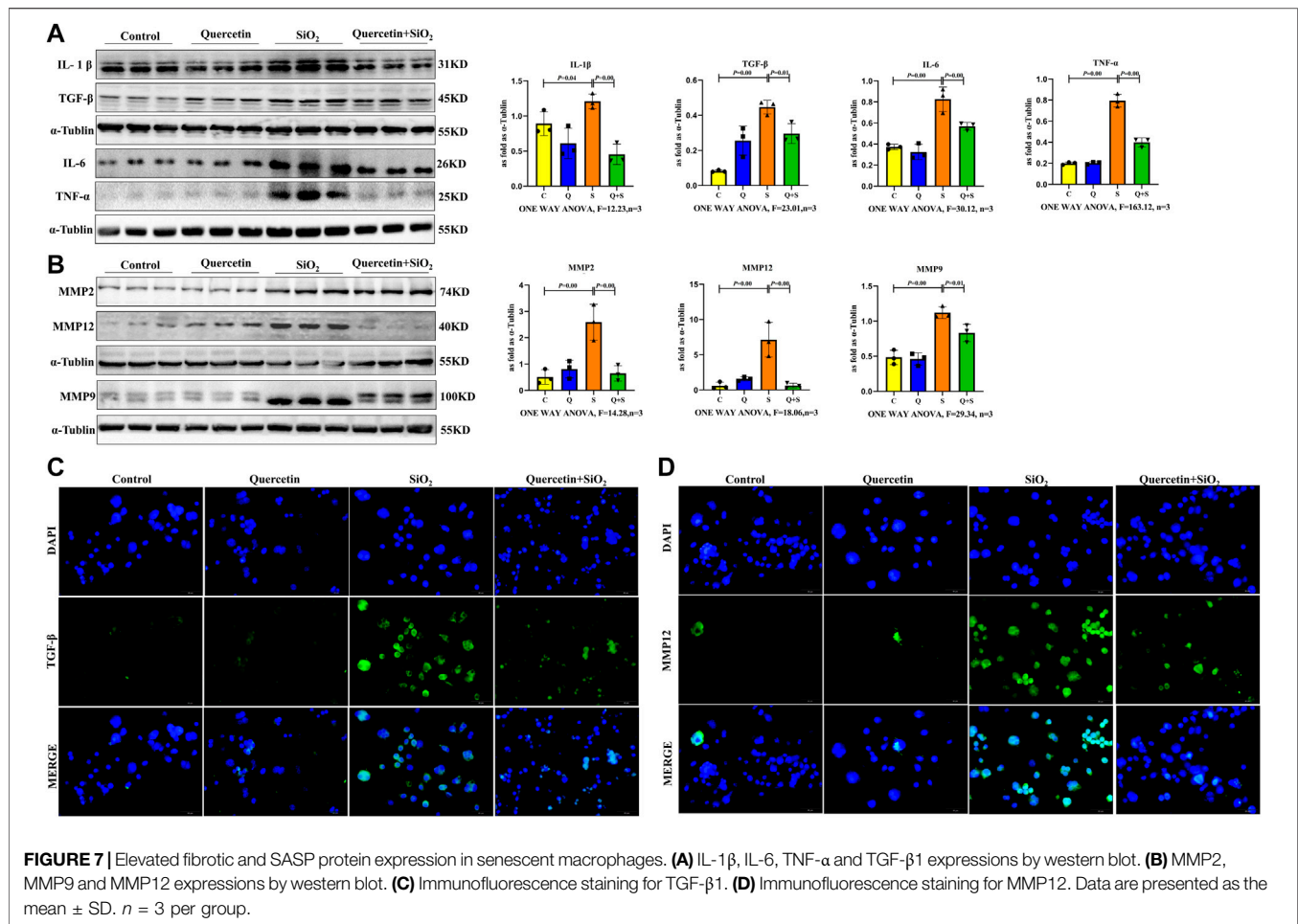
including senescent fibroblasts and type II airway epithelial cells are contributed to the development of pulmonary fibrosis and (Schafer et al., 2017). Pulmonary macrophages are predominant effector cells with vital roles in lung fibrosis pathogenic mechanisms. However, the precise role of these macrophages remains largely unexplored. Here, we showed that similar trait combinations were acquired by macrophages following SiO₂ exposure both *in vivo* and *in vitro*. In the silicosis model, SiO₂ increased the expressions of the senescence-specific proteins, p16, p21 and p53 in lung tissue, and numbers of p21-expressing macrophages by immunohistochemistry staining. *In vitro*, RAW264.7 cell elevated SA-β-Gal activity after SiO₂ exposure, suggesting pulmonary macrophages exhibited senescence characteristics during silicosis.

Quercetin inhibited phosphatidylinositol-3-hydroxykinase (PI3K) and Akt to reduce senescent cells and delay aging as indicated by improved cardiac function and exercise capacity (Zhu et al., 2015). Quercetin (20 μmol/L) also alleviated

kidney fibrosis by reducing renal tubular epithelial cell senescence via mitophagy, possibly by activating the Sirtuin-1(SIRT1)/PTEN-induced kinase 1 (PINK1)/Parkin axis (Liu et al., 2020). 25 μmol/L of quercetin inhibited the formation of foam cells induced by oxidized low density lipoprotein (ox-LDL) and delayed RAW264.7 cell senescence (Cao et al., 2019). Our findings showed that quercetin (25 μmol/L) effectively delayed macrophage senescence after SiO₂ exposure, thereby confirming the anti-silicosis and anti-aging effects of quercetin.

Generally, senescent cells contribute to tissue dysfunction via SASP, which comprises pro-inflammatory cytokines (e.g., IL-1β and IL-6) and MMPs (Daniel and Manuel, 2014). Senescent cells and SASP may fuel increasingly fibrotic responses, driving lung deterioration and severe functional decline during silicosis.

To screen for key quercetin targets against senescence and silicosis, our network pharmacology approach identified 38



overlapping targets, with 10 hub genes identified: IL-6, AKT1, TNF, PTGS2, PPARG, IL-1 β , TGF- β 1, MMP9, TP53 and JUN. Based on KEGG pathway analyses of target proteins, both TNF and Toll-like receptor signaling pathways were related to senescence responses and silicosis. Thus, TNF, IL-6, IL-1 β , TGF- β 1 and MMP9 modulation may be key pharmacological mechanisms underpinning quercetin action against senescence. Our findings support the hypothesis that SiO₂ induced macrophage senescence and promoted the SASP secretion of profibrotic factors, chemokines and MMPs leading to irreversible lung fibrosis.

Also, SiO₂ induced macrophage senescence and silicosis in lung tissue, both of which were accompanied by increased fibrosis-associated factors (IL-1 β , IL-6, TNF, and TGF- β 1) and MMPs (MMP2, MMP9 and MMP12). Importantly, quercetin appeared to restrain SASP secretion.

MATERIALS AND METHODS

Animal Studies

Animal studies were approved by the Institutional Animal Care and Use Committee of the North China University of Science and Technology. Animals received *ad libitum* access to food and

water according to the guidelines of the National Institutes of Health (NIH, MD, United States).

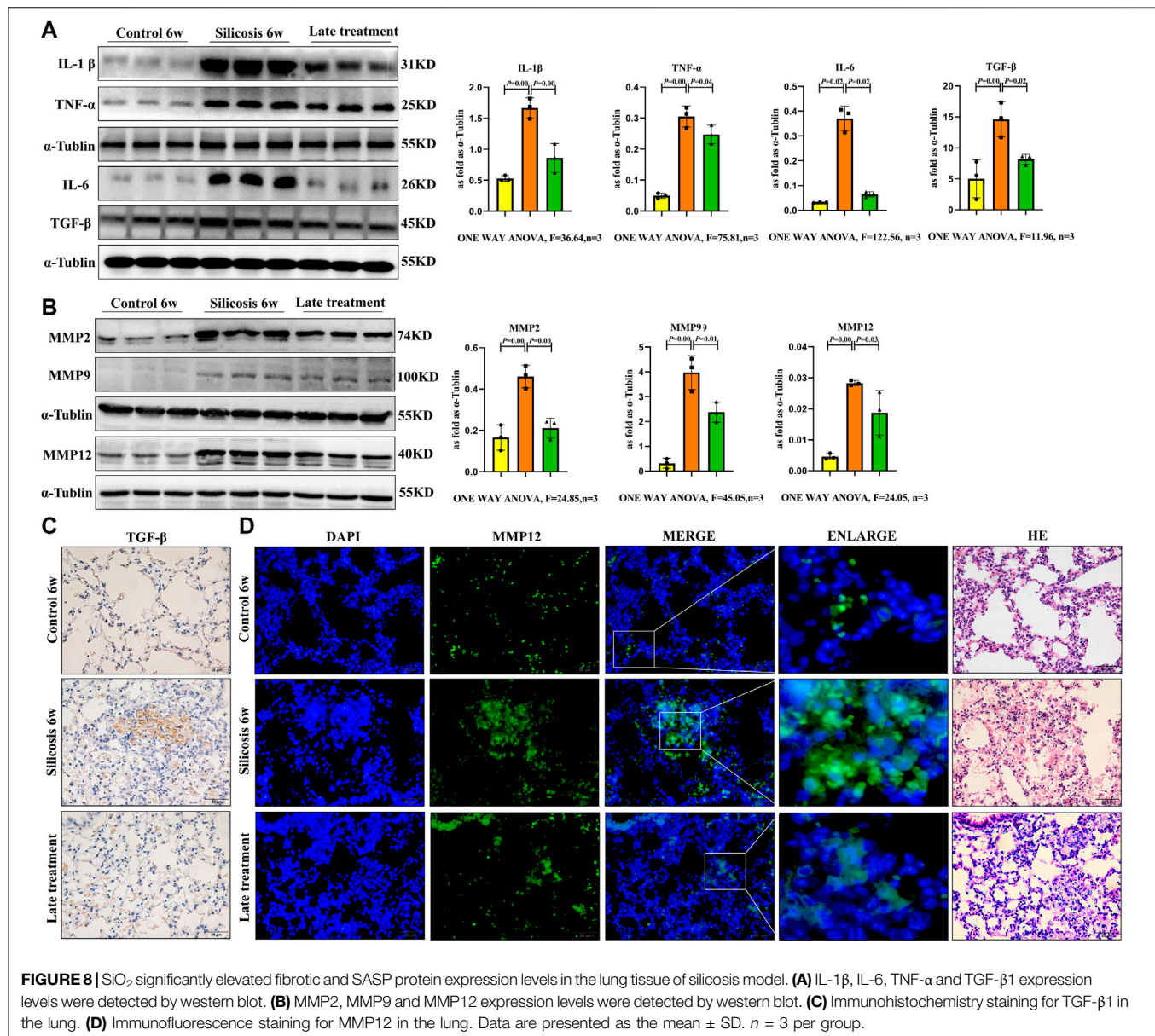
Female C57BL/6 mice (6–8 weeks old) were purchased from Beijing HFK Bioscience Co. Ltd. (Beijing, China). The silicotic model was established by given silica (SiO₂) (100 mg/kg) *via* one-off non-invasive intratracheal instillation. The quercetin therapeutic group was intragastric administration of quercetin (100 mg/kg) once a day.

To establish both early and late therapeutic model of quercetin administration, sixty mice were randomly divided into six groups: 1) Control 4w group (placebo treatment for 4 weeks); 2) Silicosis 4w group (given SiO₂ for 4 weeks); 3) Quercetin 4w groups (given SiO₂ and simultaneously administration of quercetin for 4 weeks); 4) Control 6w group (placebo treatment for 6 weeks); 5) Silicosis 6w group (given SiO₂ for 6 weeks); 3) Quercetin 6w groups (given SiO₂ and administration of quercetin for 4 weeks beginning at 2 weeks after silica exposure).

Mice were humanely anaesthetized, and sera and lung samples collected and frozen at -80°C until required.

Cell Culture

RAW264.7 mouse macrophages were purchased from the Chinese Academy of Sciences Cell Library (Shanghai, China). Cells were cultured in F12K media (BOSTER; California,



United States) containing 10% fetal bovine serum (Bovogen Biologicals; Melbourne, Australia) at 37°C in 5% CO₂. After 8 h starvation in serum-free F12K, cells were randomly divided into four groups: Control; Quercetin (25 μ mol/L); SiO₂ (200 μ g/ml); Quercetin + SiO₂ (added of quercetin (25 μ mol/L) for 2 h before SiO₂ (200 μ g/ml) stimulation).

Histology

Briefly, lung tissue slides were stained in HE to assess silicosis. VG and elastin stains were used to assess collagen fiber deposition.

Western Blot

Total protein was extracted from lung tissue or cells using RIPA buffer (Cat. No. JFR0150; JFBioTech, Shanghai, China) containing

a protease inhibitor (Cat. No. RP-WA0102; Report Biotech, China). Protein concentrations were measured using the Bradford assay (Cat. No. RP-WA0201; Report Biotech). Then, 15 μ g protein was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels. Proteins were transferred to polyvinylidene difluoride membranes (Sigma-Aldrich; Shanghai, China) and membranes blocked in 5% non-fat milk at room temperature for 1 h. Then, membranes were incubated overnight at 4°C with primary antibodies (anti-collagen type I [Cat. No. Ab34710; Abcam, Cambridge, United Kingdom], anti-p16 [Cat. No. A0262; Abclonal, Wuhan, China], anti-p21 [Cat. No. 5556431; BD Pharmingen, Guangzhou, China], anti-p53 [Cat. No. SC-6243; Santa Cruz Biotechnology, CA, United States], anti-interleukin-1 α (anti-IL-

1 β) [Cat. No. DF6251; Affinity, Cincinnati, OH, United States], anti-IL-6 [Cat. No. A0286; Abclonal], anti-tumor necrosis factor- α (anti-TNF- α) [Cat. No. A11534; Abclonal], anti-transforming growth factor- β 1 (anti-TGF- β 1) [Cat. No. ARG56429; Arigo, Taiwan, China], anti-matrix metalloproteinase 2 (anti-MMP2) [Cat. No. AF0577; Affinity], anti-MMP9 [Cat. No. AF5228; Affinity], and anti-MMP12 [Cat. No. ET1602-42; HUABIO, Hangzhou, China].

Membranes were washed three times in TBS-T and incubated with diluted (1:5,000) peroxidase-labelled affinity-purified antibodies targeting rabbit/mouse IgG (H + L) (Cat. No. 5220-0336(074-1506) and Cat. No. 5220-0341(074-1806); Seracare, United States) at 37°C for 1 h. After washing three times in TBS-T, membranes were visualized by adding enhanced chemiluminescent Prime Western Blot Detection Reagent (Cat. No. ZD310A-1; ZOMANBIO, China). Grey values were analyzed using ImageJ software (NIH). α -Tubulin and β -actin was used as a loading control.

Immunocytochemistry and Immunohistochemistry

These techniques were performed on lung tissue sections and RAW264.7 cells. Non-specific binding sites were blocked for 30 min in blocking solution (phosphate-buffered saline (PBS) plus 1% bovine serum albumin). Samples were incubated overnight at 4°C with primary antibodies against p21 and TGF- β 1, and then incubated with secondary antibodies (Cat. No. PV-6000; ZSGB-BIO, Beijing, China) at 37°C for 30 min. Immunoreactivity was visualized with diaminobenzidine (Cat. No. ZLI-9018; ZSGB-BIO). Brown staining was considered a positive result.

SA- β -Gal Staining

SA- β -gal activity staining was performed as per manufacturer's instructions (Cat. No. C0602; Beyotime, Shanghai, China). Briefly, cells were washed in PBS (pH 7.2), fixed in fixative solution (provided by the kit), and stained in a 5-bromo-4-Chloro-3-Indolyl β -D-Galactopyranoside (X-gal) solution for 48 h at 37°C. Imaging was performed using bright-field microscopy where senescent cells stained blue.

Network Pharmacologic Analysis

Identify protein targets of quercetin were used TCMSP (<http://tcmsp.com/>) and Swiss Target Prediction (<http://www.swisstargetprediction.ch/>). Targets for silicosis and senescence were collected from Gene Cards database (<https://www.genecards.org/>) and Disgene (<http://www.disgenet.org/>). The PPI network of these targets was obtained from the String (<https://string-db.org/>). Finally, we used Cytoscape software to open the results, created quercetin-silicosis-senescence targets network.

Statistical Analysis

Statistical analyses were conducted in SPSS statistical software and performed with one-way analysis of variance,

followed by *post-hoc* analysis with Bonferroni's tests for multiple comparisons. Data were presented as the mean \pm standard deviation. *p* values <0.05 were statistically significant.

CONCLUSION

In summary, we suggest that senescent macrophages and SASP mechanisms may be treatment targets for silicosis, with quercetin functioning to eliminate senescent macrophages and block SASP activity, thereby acting as a novel therapeutic approach for silicosis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by North China University of Science and Technology. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

Conceptualization, FG, MX, ZW, and FY; methodology, FG and MX; software, FG, LZ, HZ, and FJ; validation, TL, YL, and XY; formal analysis, FG and XY; investigation, YL; resources, FY and HX; data curation, MX and JL; writing—original draft preparation, FG; writing—review and editing, ZW and FY; visualization, XG, NM, and WC; supervision, YS and HL; project administration, ZW; funding acquisition, SL and ZW. All authors have read and agreed to the published version of the manuscript.

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