Journal of Inflammation Research

Dovepress
Taylor & Francis Group

Open Access Full Text Article

ORIGINAL RESEARCH

Fu Tu Sheng Jin Rehabilitation Formula Mitigate Airway Inflammation, Mucus Secretion and Immune Dysfunction Induced by SARS-CoV-2 Spike Protein

Bo-Han Wang[1,*], Ke-Yao Yu[2,*], Xiao-Na Zhang[3,*], Xian-Hong Sun[2], Ling-Ling Tang[4], Xiao-Lu Shi[2]

[1]NanJing JiangNing Hospital of Chinese Medicine/Affiliated jiangning Hospital of Chinese Medicine, China Pharmaceutical University, Nanjing, Jiangsu, 210029, People's Republic of China; [2]Affiliated Hospital of Nanjing University of Chinese Medicine/ Jiangsu Provincial Hospital of Traditional Chinese Medicine, Nanjing, Jiangsu, 210029, People's Republic of China; [3]Nanjing Hospital of Chinese Medicine, Nanjing, Jiangsu, 210029, People's Republic of China; [4]School of Chinese Medicine & School of Integrated Chinese and Western Medicine, Nanjing University of Chinese Medicine, Nanjing, Jiangsu, 210023, People's Republic of China

*These authors contributed equally to this work

Correspondence: Xiao-Lu Shi; Ling-Ling Tang, Email fsyy00721@njucm.edu.cn; 290602@njucm.edu.cn

Objective: To evaluate the effects of Fu Tu Sheng Jin Rehabilitation Formula (FTSJRF) on airway inflammation, mucus secretion, and immunoreaction in a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike protein-induced mouse model.

Methods: Forty-two mice were randomly divided into seven groups: normal, D1, D3, D10, D10H, D10M and D10L, according to the days of modeling and different dosages of FTSJRF. D1, D3, D10, D10H, D10M and D10L group mice were intratracheally administered with 15 μg SARS-CoV-2 spike protein; mice in the D10H, D10M, and D10L groups were intragastrically administered FTSJRF (46, 23 and 11.5 g/kg, respectively). Observe the pathological changes in lung tissues, expression of inflammatory factors, and mucins in different groups of mice using HE and PAS staining methods, as well as ELISA and RT-qPCR. Flow cytometry was used to detect T helper 17 (Th17)/regulatory T (Treg) cells and T helper 1(Th1)/T helper 2 (Th2) lymphocyte ratios and the proportions of conventional myeloid dendritic cells (cDCs), plasma cell-like DCs, CD80 and CD86 cells in mouse spleens.

Results: HE and PAS staining showed that, compared to that in the normal group, the lung tissue of the D1 group mice showed a significant inflammatory damage response, whereas the D3 and D10 groups showed a gradual recovery trend. Groups D1 and D3 showed mild mucus secretion, whereas the D10 group had excessive mucus secretion. The D10 group of mice displayed increased levels of IL-4, TNF-α, IL-33 and mucin genes such as MUC1, MUC4, etc, and FTSJRF inhibited the expression of these molecules, mucus secretion and lung damage in SARS-CoV-2 spike protein-induced mouse model. Flow cytometry results showed a decrease in the number of cDCs and an abnormal recovery of DC mature cells in the D10 group. FTSJRF increased the number of cDCs and promoted DC maturation. A higher Th17/Treg ratio was observed in the D3 and D10 groups than in the normal group, whereas this ratio decreases under the effect of FTSJRF. D10 had significantly lower Th1/Th2 ratio than normal, D1 and D3 groups, and high doses of FTSJRF increased it.

Conclusion: FTSJRF mitigates airway inflammation and mucus secretion induced by SARS-CoV-2 spike protein. Additionally, FTSJRF regulates immune functions by promoting DC maturation and Th17/Treg and Th1/Th2 cell homeostasis.

Keywords: COVID-19, SARS-CoV-2 spike protein, Fu Tu ShengJin Rehabilitation Formula, airway inflammation, mucus secretion, immune dysfunction

Introduction

The COVID-19 pandemic, caused by SARS-CoV-2, has resulted in hundreds of millions of infections and deaths worldwide and continues to pose an enormous global health threat.[1,2] SARS-CoV-2 can infect the lung, heart, liver, and kidney tissues, and T lymphocytes and macrophages.[3] Although most patients recover from acute infections, SARS-CoV-2 infection can cause damage to the human body, which may last for several months, and physical recovery after discharge can be a lengthy

Received: 25 July 2024
Accepted: 27 November 2024
Published: 22 January 2025

process.[4,5] Initially called long-COVID, post-COVID-19 syndrome is a condition characterized by symptoms that persist after (above 12 weeks) a SARS-CoV-2 infection.[6] Patients with COVID-19 have reported varying levels of sequelae, including fatigue, breathlessness, psychological disturbances, declining lung function, and an overall reduced quality of life.[7,8] Symptoms may appear new, recur, or persist for more than 4 weeks following infection onset, even one year after a patient with COVID-19 has recovered and been discharged from the hospital, their health status has not returned to baseline.[9] Several studies have observed that timely and effective rehabilitation with traditional Chinese medicine (TCM) in patients with COVID-19 sequelae can improve the complications, sequelae, and quality of life.[10,11]

Host immune disorders, characterized by an inflammatory cytokine storm and lymphocyte depletion, are prominent manifestations of the SARS-CoV-2 infection.[12] Viral infection causes an antiviral immune response and stimulates immune cell activation and inflammatory responses. The inflammatory response helps the organism to eliminate the pathogen in most cases.[13] However, excessive inflammation can result in multiple organ failure and systemic inflammatory response syndrome.[14] The SARS-CoV-2 virus activates GM-CSF, which further stimulates inflammatory monocytes to produce IL-6, TNF-α and other cytokines.[15] Depletion and dysfunction of lymphocytes can occur with excessive release of pro-inflammatory cytokines. There have been reports of a series of immune response disorders observed in patients with COVID-19 in the acute phase, including decreased lymphocytes and elevated inflammatory factors.[16] In patients who have recovered from COVID-19, lymphocyte depletion can be observed, particularly manifested as sustained and significant reductions in CD4+T and CD8+T lymphocytes.[17] Previous studies have shown that the Fu Tu Sheng Jin Rehabilitation Formula (FTSJRF) can effectively improve the clinical symptoms of patients with post-COVID-19 syndrome and, to a certain extent, restore their immune function, but the specific mechanism is not yet clear.[18]

Several structural proteins are present in SARS-CoV-2, including spikes, envelopes, membrane proteins, and nucleocapsids.[19] In order to enter the human body, SARS-CoV-2 binds to ACE2 via the spike protein, which has a stronger viral attachment. In addition to releasing and replicating viral genomes within cells, it produces viral proteins that trigger immune responses through biosynthesis.[20,21] During the pathogenesis and vaccine development of SARS and MERS, the spike protein is used as an allergen to infect mice, inducing SARS-CoV lung injury and immune disorders.[22,23] Despite SARS-CoV-2 not using mouse ACE2 as a receptor, the N501Y mutated spike protein increases its binding affinity for mouse ACE2. Gu et al showed that BALB/c mice received nasal injections of N501Y mutated spike protein for 3 days, and their lung pathological response and viral RNA load reached their peak, with attenuation on days 5 and 7, indicating a self-recovery process in mice after spike protein infection.[24] In this study, we used the N501Y mutated Spike protein to simulate the recovery period after COVID-19 infection on day 10 post-infection, which recapitulate post-COVID-19 syndrome in mice, and further explore the pathological and immune injury characteristics of post-COVID-19 syndrome. Moreover, we investigated the effects of FTSJRF on SARS-CoV-2 spike protein-induced mouse model and observed that FTSJRF reduced lung damage, improved mucus secretion, promoted the maturation of DCs, and maintained Th17/Treg and Th1/Th2 cell homeostasis. Altogether, this study revealed that FTSJRF is a potential treatment for post-COVID-19 syndrome.

Materials and Methods

Preparation of FTSJRF

We purchased raw FTSJRF medica from the Affiliated Hospital of Nanjing University of Chinese Medicine (Nanjing, Jiangsu, China). FTSJRF comprises 15 raw medica: 20 g *Codonopsis pilosula* (Franch). Nannf., 20 g *Astragalus membranaceus* (Fisch). Bge., 10 g *Atractylodes macrocephala* Koidz., 10 g *Poria cocos* (Schw). Wolf, 10 g *Pinellia ternate* (Thunb). Breit., 6 g *Citrµs reticµlata* Blanco, 15 g *Juglans regia* L., 15 g *Dioscorea opposita* Thunb., 6 g *Inula japonica* Thunb., 5 g *Rubia cordifolia* L., 10 g *Albizia julibrissin* Durazz., 15 g *Setaria italica* (L). Beauv., and 5 g *Glycyrrhiza glabra* L. FTSJRF decoction was prepared conventionally. Herbs were soaked in 1000 mL of water for 30 minutes before being decocted for 45 minutes. After adding 500 mL of water, the herbs were decocted for 45 minutes more. Finally, we filtered the two extracts, concentrated them to 3 g/mL, and stored them at 4°C. Qualitative analysis of FTSJRF were shown in the Supplementary Table 2 and Supplementary Figure.

Animals and Experimental Protocols

We obtained 42 male BALB/c mice (6–8 weeks old, 18–22 g) from Shanghai Bikai Keyi Biotechnology Co., Ltd. (Shanghai, China) and housed them at the Affiliated Hospital of Nanjing University of Chinese Medicine's Animal Laboratory (Nanjing, China). The production license and license for the experimental animals were SCXK(Hu)2018–0006 and SYXK(Su)2022–0070, respectively. The Animal Care and Use Committee of Nanjing University of Chinese Medicine approved the animal protocol (2023DW-032-01, Nanjing, China).

Adaptive feeding was performed on 42 mice for one week, then seven groups were randomly selected: normal, D1, D3, D10, D10H, D10M, and D10L, according to the days of modeling and different dosages of FTSJRF. On day 0, all mice, except those in the normal group, were anesthetized via intraperitoneal injection with pentobarbital sodium (40 mg/kg). After the trachea was exposed by separating the underlying muscles and glands, mice were intratracheally administered with 15 μg of N501Y-mutated SARS-CoV-2 spike protein (Beyotime, China). Between days 3 and 10, mice in the D10H (High dose 46 g/kg), D10M (Medium dose 23 g/kg), and D10L (Low dose 11.5 g/kg) groups were intragastrically administered FTSJRF once daily, and the D10 group received normal saline (0.2 mL). Mice in groups D1, D3, and D10 were sacrificed on days 1, 3, and 10, respectively, after infection with the SARS-CoV-2 spike protein. Mice in the normal, D10H, D10M, and D10L groups were sacrificed on day 10.

Lung Histopathology

Formalin-fixed lung tissue sections were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS). Histopathological changes in the different lung tissues were observed under an optical microscope after neutral balsam was used to seal the sections.

Immunofluorescence Staining

Sections of lung tissue were cut from formaldehyde-fixed, dehydrated, paraffin-embedded tissue that had been fixed in formaldehyde. The tissue sections were dewaxed and hydrated, and then sealed with goat serum for two hours at room temperature. The sections were incubated overnight at 4°C with SARS-CoV-2 spike protein (1:100; CST, USA) and ACE2 antibody (1:100; Proteintech, China). The fluorescence secondary antibody was stained with AlexaFluor-488 antibody (green) and CY3-labeled antibody (red) incubated at 37°C in the dark for 1.5 h. After washing, nuclei were stained with DAPI (blue). After sealing, cells were observed under a fluorescence microscope (NIKON, Japan).

Enzyme-Linked Immunosorbent Assay (ELISA)

We weighed mouse lung tissue and added phosphate-buffered saline at a 1:9 mass/volume ratio. The tissue was ground thoroughly using a grinder and centrifuged at 5000 rpm at 4°C for 10 min. Supernatants were added to an ELISA-coated plate, antibodies to IL-4, IL-5, and IL-6 were added, and the plate was incubated for 2 h. Next, the wells were washed three times, a biotinylated antibody working solution was added, and the plate was incubated at 37°C for 1 h. After three washes, streptavidin HRP working solution was added, and the plate was incubated at 37°C for 30 min. Finally, the plate was washed three times, and substrate for color development was added as a termination solution. Microplate readers (Bio-Tek, USA) were used to measure optical density and substituted into the standard curve.

Quantitative Reverse Transcription PCR (RT-qPCR)

FreeZol Reagent was used to extract total RNA from mouse lung lobes (Vazyme Biotech Co., Ltd., China). Reverse transcription was performed using a kit for reverse transcription of RNA into cDNA (ABclonal Technology Co., Ltd., China). Real-time PCR Master Mix (SYBR Green; ABclonal Technology Co., Ltd., China) was used for RT-qPCR. Supplementary Table 1 lists the forward and reverse primer sequences. A total of 40 cycles of 94°C for 10 min, 94°C for 30s, 60°C for 1 min were used to analyze gene expression. Using β-actin as an internal reference, the $2^{-\Delta\Delta CT}$ method was used to analyze relative gene expression.

Flow Cytometry Analysis

Mouse spleens were separated and ground into a cell suspension. Next, DC (CD11b+CD11c+CD317+CD80+CD86+CD3), Th1 (CD4+IFNγ+), Th2 (CD4+IL-4+), Th17 (CD4+IL-17+) and Treg (CD25+Foxp3+) fluorescent antibodies were added to each 100 μL cell suspension as instructed by the manufacturer. The mixture was incubated at room temperature in the dark. After washing and centrifugation, the samples were resuspended and fixed. The proportions of cDCs, pDCs, CD80+, and CD86+ cells in DCs and the proportions of Th1, Th2, Th17 and Treg cells in CD4+ cells in each group were determined using flow cytometry.

Statistical Analysis

Statistical analyses were performed with SPSS software (ver. 21.0) using mean ± standard deviation for all experiments. Data were analyzed using single-factor analysis of variance, or Student's *t*-test, and statistical significance was set at $p < 0.05$.

Results

FTSJRF Reduced Lung Damage Induced by SARS-CoV-2 Spike Protein in Mice

In order to verify FTSJRF's protective effects against the pathological reactions associated with post-COVID-19 syndrome, SARS-CoV-2 spike protein-induced mouse model was established. The SARS-CoV-2 spike extracellular domain protein interacts with ACE2 to mediate infection. An immunofluorescence staining confirmed colocalization of mouse ACE2 and SARS-CoV-2 spike protein in mouse lungs, indicating that the SARS-CoV-2 spike protein bound successfully to the mouse ACE2 receptor (Figure 1A). One day after infection with the SARS-CoV-2 spike protein, mice showed severe lung injury, characterized by alveolar structural destruction, epithelial cell degeneration and collapse, inflammatory cell infiltration, focal exudation, and bleeding (Figure 1B). Lung injury was slightly alleviated 3 days after inoculation, and lung injury was much lighter 10 days after inoculation than after 3 days, suggesting a self-recovery process and indicating that it was in the recovery period of COVID-19. However, FTSJRF treatment improved the lung tissue damage caused by the SARS-CoV-2 spike protein, and the curative effects were obvious in the D10M group. As illustrated in Figure 1C, compared to that in the normal group, the D1 and D3 groups secreted mucus slightly, whereas the D10 group of mice showed excessive secretion of mucus. In contrast, treatment with medium and high doses of FTSJRF alleviated mucus secretion, but the curative effects were not evident in the D10L group.

FTSJRF Inhibited the Level of Inflammatory Factors in Mice Induced by SARS-CoV-2 Spike Protein

Inflammatory cytokines (including IL-6, IL-5, IL-4, TNF-α, IL-33 and IL-1β) in mouse lung tissue were raised upon SARS-CoV-2 spike protein challenge. D1 mice showed increased levels of IL-6, whereas D3 mice showed increased levels of IL-5 in lung tissue homogenates, IL-6, IL-4 and IL-5 levels in lung tissue homogenates of D10 mice increased, as shown in Figures 2A–2C. Compared to D10, D10M showed a decrease in IL-6 and IL-5 levels, while D10L showed a decrease in IL-4 levels. Compared to that in the normal group, IL-1β levels in the lung tissue of the D1 and D3 groups of mice were elevated, and the levels of TNF-α, IL-33 and IL-1β in the lung tissue of the D10 group (Figures 2D–F). The levels of TNF-α and IL-1β in the lung tissue of mice in the D10M group were found to be lower compared to those in the D10 group, while the levels of TNF-α and IL-33 in the lung tissue of the D10L group were also reduced. TNF-α levels were higher in the D10 group compared to the D1 and D3 groups, and IL-6 levels were higher in the D10 group compared to the D3 group. These results indicate that the levels of inflammatory cytokines increase in the acute phase of COVID-19 and persist in the recovery phase. FTSJRF inhibited inflammatory cytokine levels in mice with post-COVID-19 syndrome induced by the SARS-CoV-2 spike protein.

FTSJRF Inhibited Mucus Secretion Levels in Mice Induced by SARS-CoV-2 Spike Protein

Mucin, a significant macromolecular constituent of mucus, serves a crucial function in the respiratory defense mechanism, regulated by distinct genetic sequences. As of the present time, a total of 21 human mucin genes have been characterized.

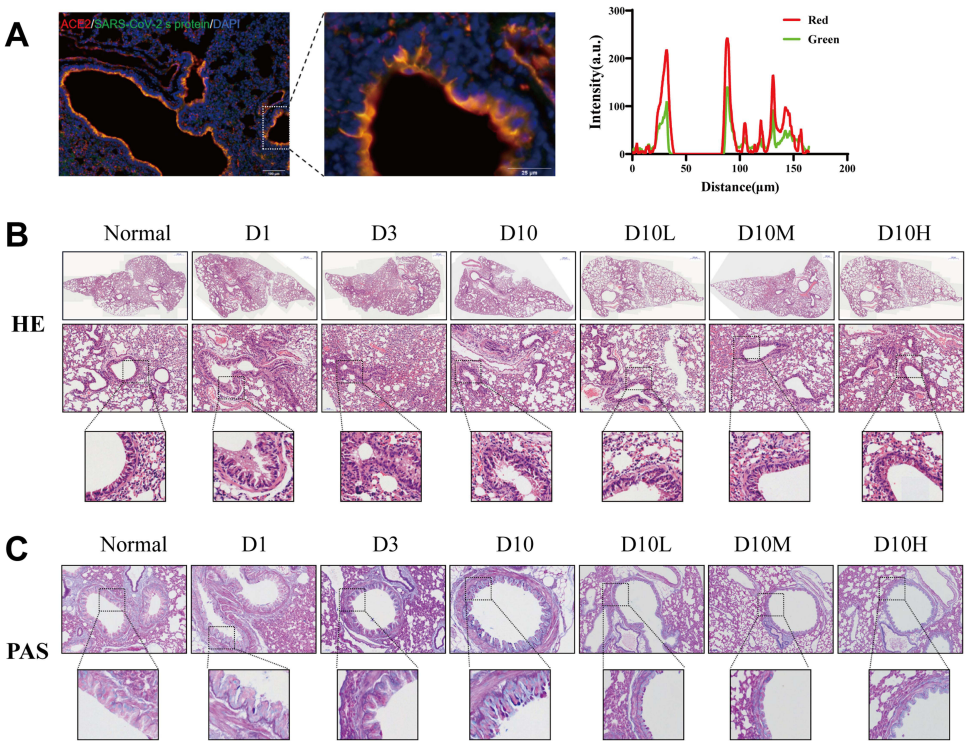

Figure 1 FTSJRF reduced lung damage induced by SARS-CoV-2 spike protein in mice. (**A**) Colocalization of SARS-CoV-2 spike protein (green) and mouse ACE2 (red) in the lungs. (**B**) Representative images of the mouse lung tissue using HE staining. Scale bar: 50 μm (n = 6). (**C**) Representative images of mouse lung tissue stained using PAS staining. Scale bar: 50 μm (n = 3).

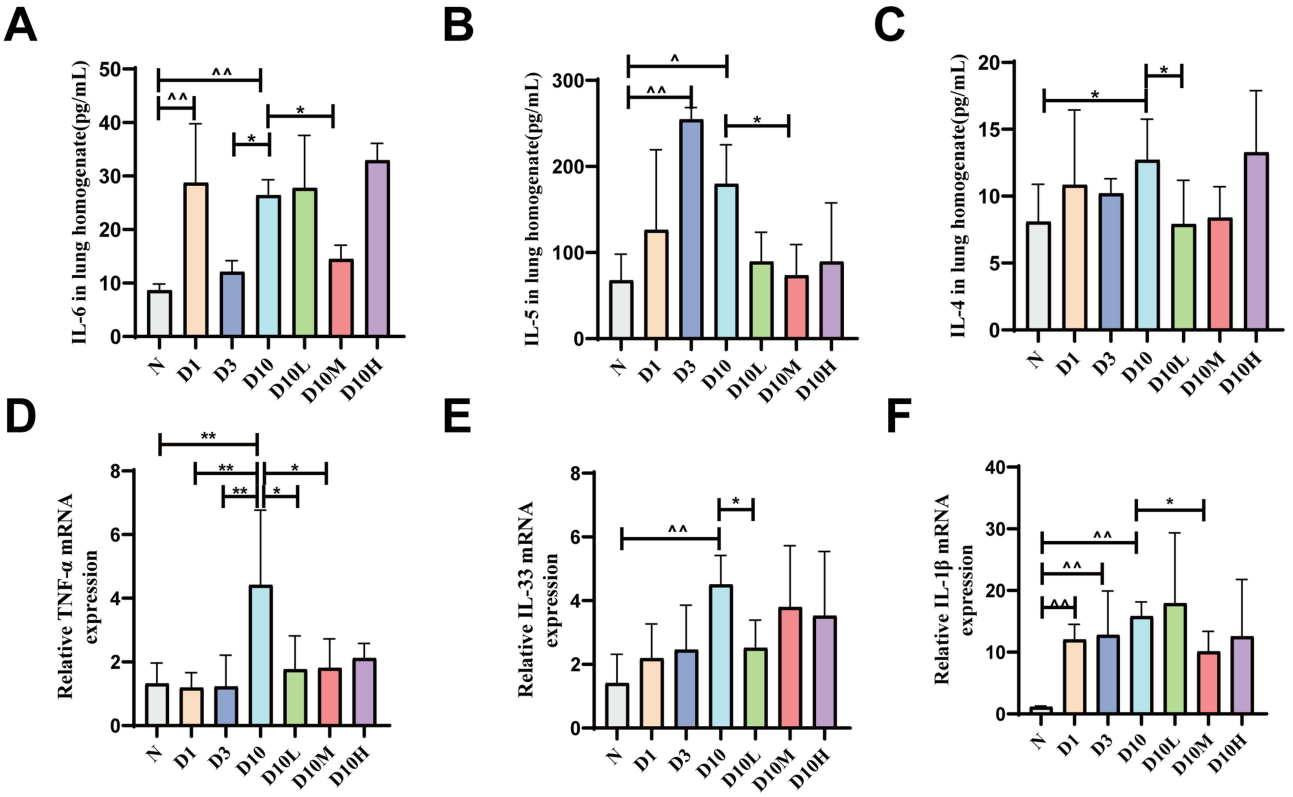

Figure 2 FTSJRF inhibited the level of inflammatory factors in mice induced by SARS-CoV-2 spike protein (n = 4) (**A**–**C**) ELISA for serum levels of IL-6, IL-5 and IL-4 in mouse lung tissues. (**D**–**F**) Expression levels of TNF-α, IL-33 and IL-1β in mouse lung tissues were assessed using RT-qPCR. All data are represented as means ± SD. ^*p* <0.05, ^^*p* <0.01 vs normal group; **p* <0.05, ***p* <0.01 vs D10 group.

https://doi.org/10.2147/JIR.S480112

Among these, the predominant mucins synthesized in the respiratory tract are the secreted polymeric mucins MUC5AC and MUC5B[25] and the cell-tethered mucins MUC1, MUC4, MUC16 and MUC20.[26] Compared to the normal group, the D1 group had higher MUC4 levels (Figures 3A–C). The levels of MUC1, MUC4, and MUC5B in the D10 group were found to be significantly elevated compared to those in the normal group. Additionally, MUC5B levels were observed to be higher in the D10 group compared to the D1 and D3 groups, while MUC1 levels were higher in the D10 group compared to the D1 group, and MUC4 levels were higher in the D10 group compared to the D3 group. Compared to those in the D10 group, FTSJRF inhibited the levels of MUC1, MUC4 and MUC5B. As shown in Figures 3D–F, Levels of MUC20 in the D1 group, as well as levels of MUC4 and MUC20 in the D3 group, exhibited increases relative to the normal group. Additionally, levels of MUC5AC, MUC16, and MUC20 in the D10 group surpassed those in the normal group, with MUC5AC levels exceeding those in the D1 group, and both MUC5AC and MUC16 levels surpassing those in the D1 group. Furthermore, MUC16 and MUC20 levels were higher in the D3 group compared to the normal group. Compared to those in the D10 group, FTSJRF inhibited the levels of MUC5AC, MUC16 and MUC20. Mucus secretion in mice during the acute attack period of COVID-19 was low, whereas mucus secretion in mice induced by SARS-CoV-2 spike protein was significantly increased, and FTSJRF effectively inhibited mucus secretion.

FTSJRF Promoted DC Maturation in Mice Induced by SARS-CoV-2 Spike Protein

DCs are immune cells with the strongest antigen presentation ability, which are capable of absorbing, processing, and presenting antigens, as well as enhancing T and B lymphocyte proliferation.[27] Mature DCs (expressing CD80+ and CD86+) can activate primitive T cells to initiate immune responses by secreting cytokines that play an important role in viral invasion. Compared to those in the normal group, the proportion of CD80+ and CD86+ cells in the D10 group decreased, whereas the proportion of CD86+ cells in the D1 group significantly decreased (Figure 4A and C). Compared to those in the D10 group, FTSJRF increased

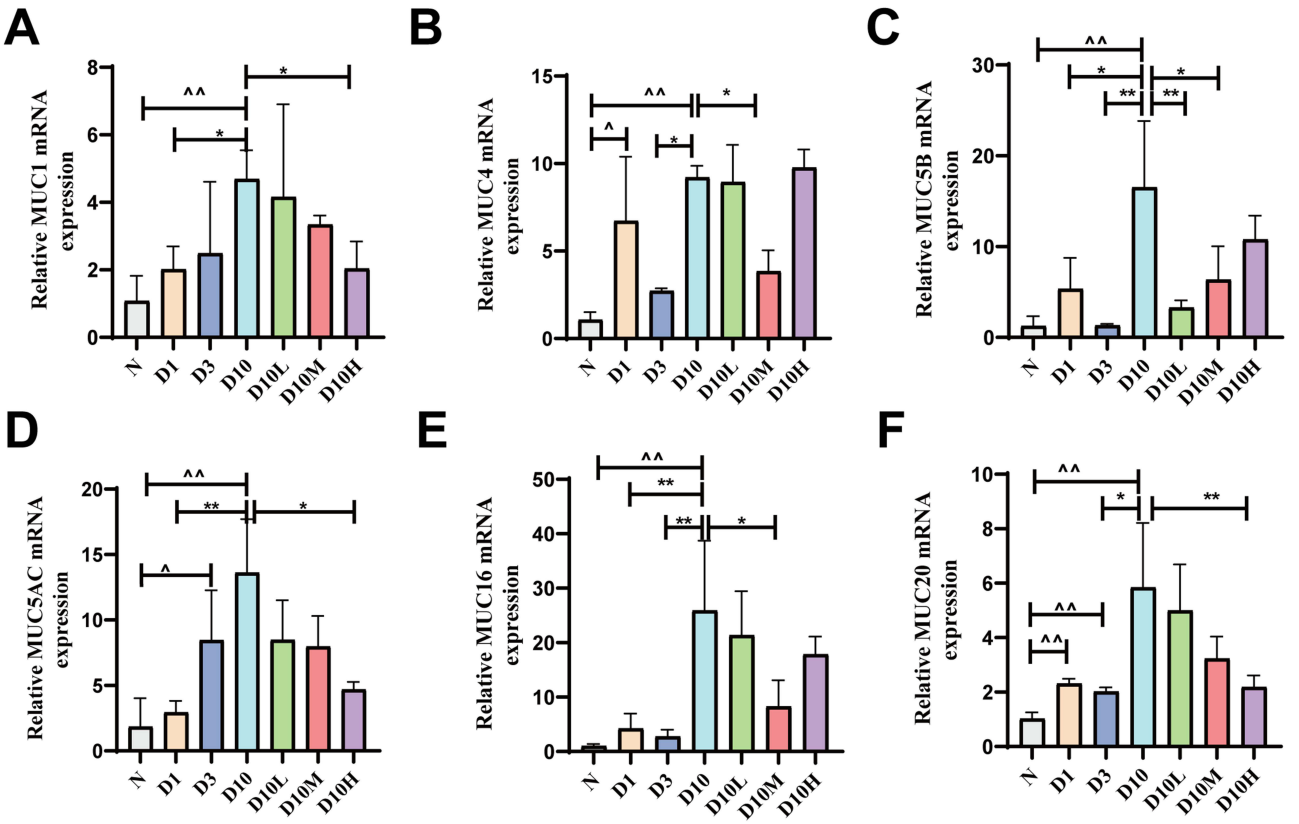

Figure 3 FTSJRF inhibited mucus secretion levels in mice induced by SARS-CoV-2 spike protein (n = 3). (**A**–**C**) expression of MUC1, MUC4, and MUC5B in mouse lung tissues was assessed using RT-qPCR. (**D**–**F**) Expression levels of MUC5AC, MUC16 and MUC20 in mouse lung tissue were assessed using RT-qPCR. All data are represented as means ± SD. ^*p* <0.05, ^^*p* <0.01 vs normal group; **p* <0.05, ***p* <0.01 vs D10 group.

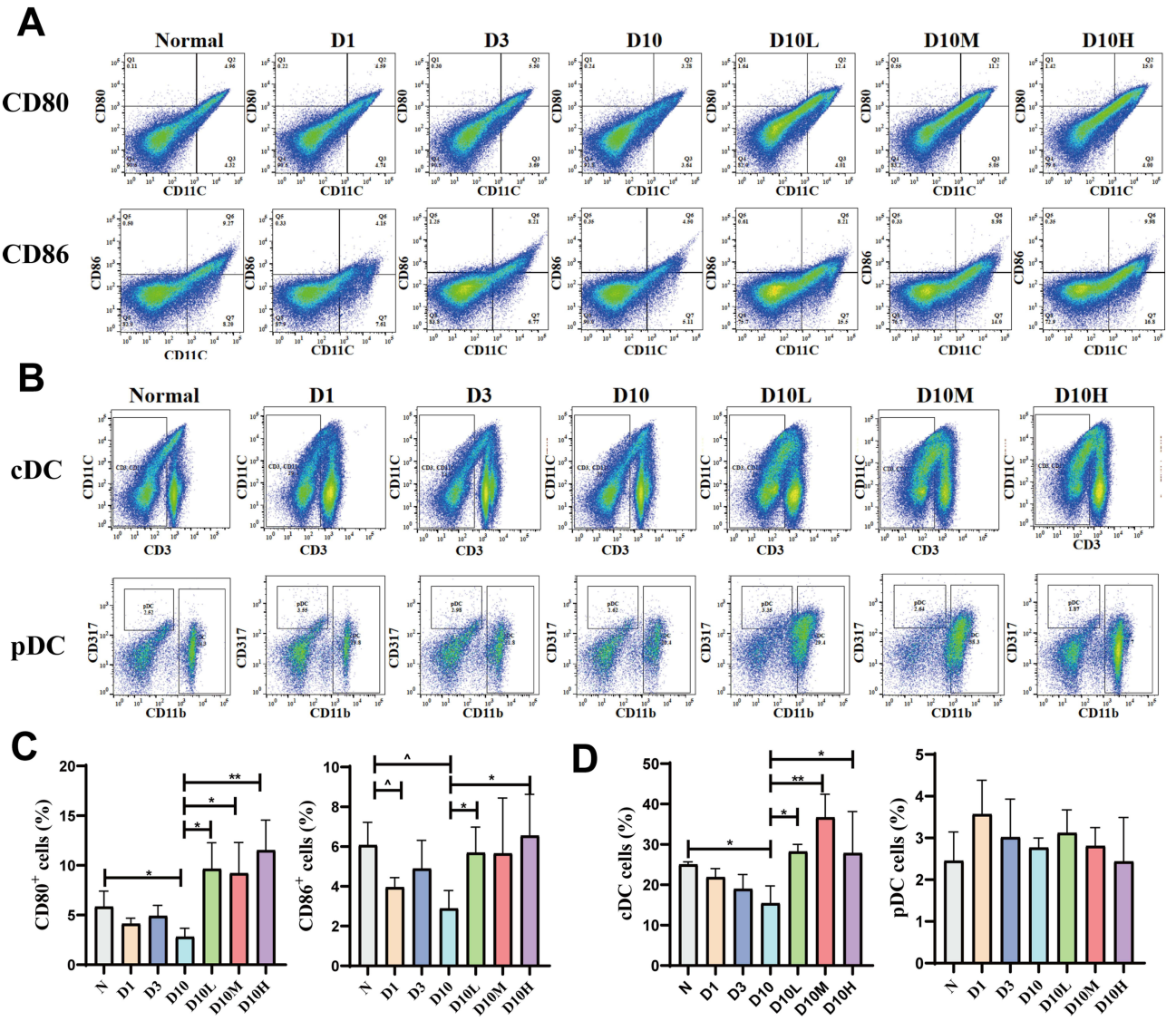

Figure 4 FTSJRF promoted DC maturation in mice induced by SARS-CoV-2 spike protein (n = 3). (**A**) The proportion of CD80+ and CD86+ cells in the spleen DCs of mice was assessed using flow cytometry. (**B**) Proportion of cDCs and pDCs in the spleen of mice was assessed using flow cytometry. (**C** and **D**) Proportion of various types of DCs in the spleen of mice. All data are represented as means ± SD. ^p <0.05 vs normal group; *p <0.05, **p <0.01 vs D10 group.

the proportion of CD80+ and CD86+ cells. DCs can be divided into cDCs and pDCs. Our results showed that, compared to that in the normal group, the proportion of cDCs decreased in the D10 group, whereas FTSJRF increased the proportion of cDCs, and the D10M group had the highest proportion of cDCs (Figure 4B and D). These results indicate that there is a decrease in the number of cDCs and abnormal recovery of mature DCs in our mouse model induced by SARS-CoV-2 spike protein and that FTSJRF can increase the number of cDCs and promote DC maturation.

FTSJRF Maintained Th17/Treg Cell Homeostasis in Mice Induced by SARS-CoV-2 Spike Protein

The analysis of Th17/Treg homeostasis is essential for immune response regulation in pneumonia. The proportions of Th17 and Treg cells were examined in mice treated with FTSJRF. It was observed that the proportion of Th17 cells decreased in the D10L and D10M groups compared to the D10 group, suggesting that FTSJRF suppressed the Th17 cell response (Figures 5A and D). However, the proportion of Treg cells did not show significant changes (Figure 5B and C). As illustrated in Figure 5E, the Th17/

https://doi.org/10.2147/JIR.S480112

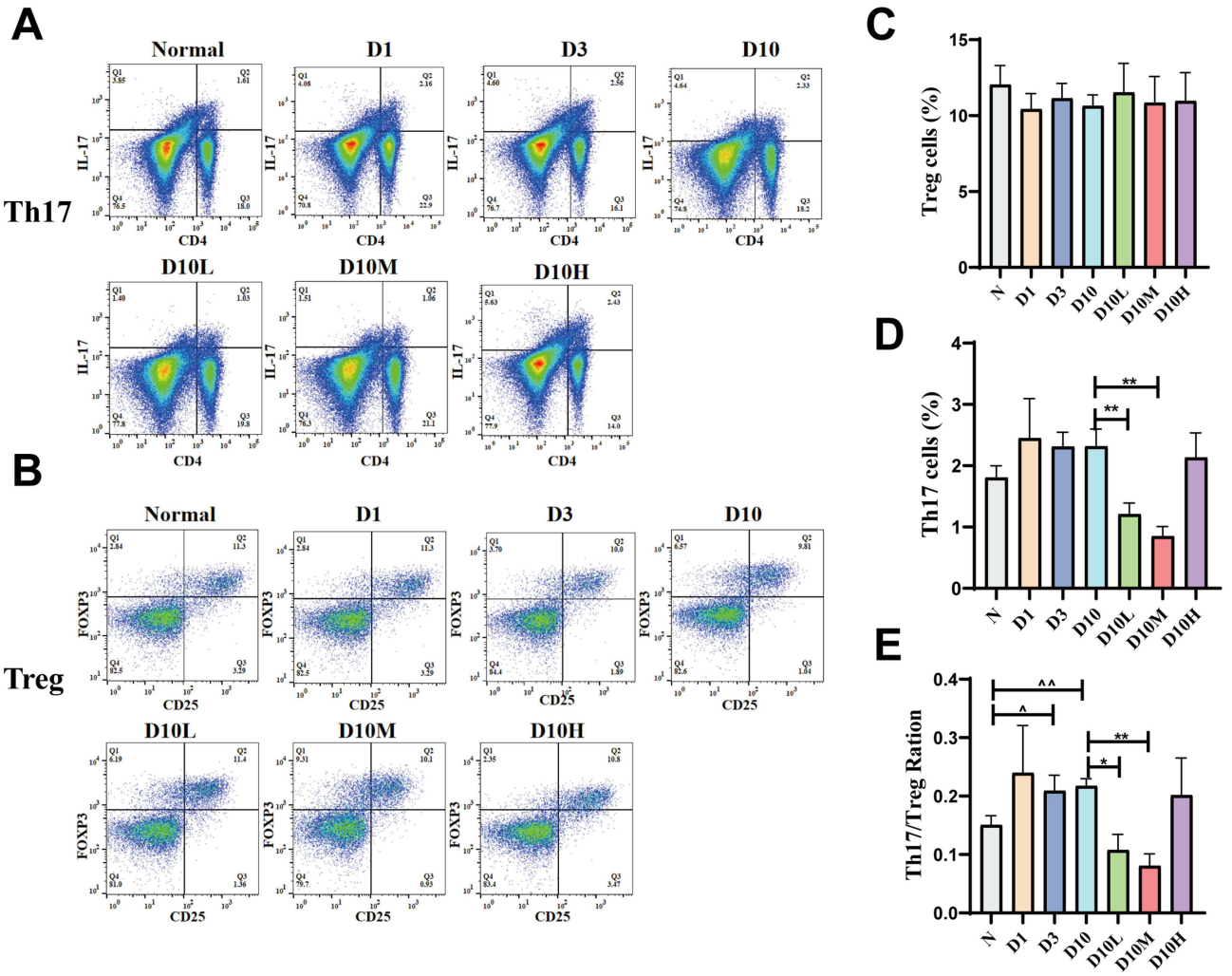

Figure 5 FTSJRF maintained Th17/Treg cell homeostasis in mice induced by SARS-CoV-2 spike protein (n = 3). (**A**) Proportion of Th17 cells in the spleen of mice was assessed using flow cytometry. (**B**) Proportion of Tregs in the spleen of mice was assessed using flow cytometry. (**C** and **D**) Ratio of Th17 and Treg cells in the spleen of mice. (**E**) Ratio of Th17/Treg cells in the spleen of mice. All data are represented as means ± SD. ^p <0.05, ^^p <0.01 vs normal group; *p <0.05, **p <0.01 vs D10 group.

Treg ratio exhibited an increase in the D3 and D10 groups in comparison to the normal group, while a decrease was observed in the Th17/Treg ratio in the D10L and D10M groups relative to the D10 group. These results indicate that the Th17/Treg ratio in post-COVID-19 syndrome is imbalanced, and FTSJRF regulates the differentiation of T cells to maintain Th17/Treg homeostasis and reduce pulmonary inflammation.

FTSJRF Maintained Th1/Th2 Cell Homeostasis in Mice Induced by SARS-CoV-2 Spike Protein

Under normal circumstances, Th1 and Th2 cells mutually constrain each other and maintain a balance in the body's immune function. According to the data presented in Figures 6A–D, the D10 group exhibited a decrease in the proportion of Th1 cells and an increase in the proportion of Th2 cells. Administration of high doses of FTSJRF resulted in an increase in Th1 cells, while medium and low doses led to a decrease in Th2 cells. Furthermore, the Th1/Th2 ratio in the D10 group was significantly lower compared to the normal, D1, and D3 groups. High doses of FTSJRF increased the Th1/Th2 ratio, leading to a balance in the Th1/Th2 ratio (Figure 6E).

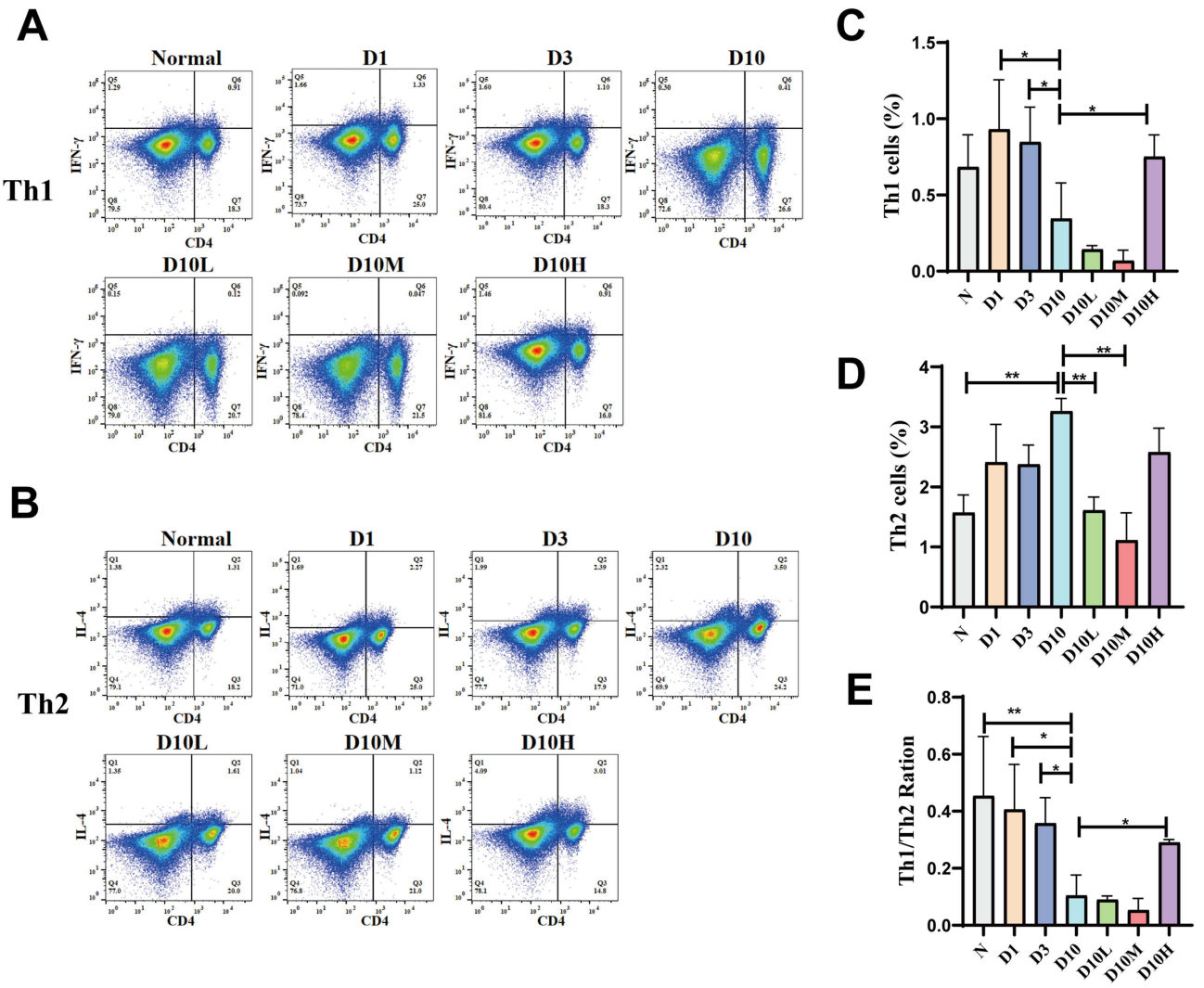

Figure 6 FTSJRF maintained Th1/Th2 cell homeostasis in mice induced by SARS-CoV-2 spike protein (n = 3). (**A**) Proportion of Th1 cells in the spleen of mice was assessed using flow cytometry. (**B**) Proportion of Th2 cells in the spleen of mice was assessed using flow cytometry. (**C** and **D**) Ratio of Th1 and Th2 cells in the spleen of mice. (**E**) Ratio of Th1/Th2 cells in the spleen of mice. All data are represented as means ± SD. *p <0.05, **p <0.01 vs D10 group.

Discussion

With the emergence of various health conditions after COVID-19 recovery, post-COVID-19 healthcare has become a global issue. At present, a reliable COVID-19 recovery period model for investigating its potential pathogenesis and treatment targets does not exist. Given the heightened infectivity and pathogenicity of SARS-CoV-2, experiments involving this virus should be carried out in a biosafety level 3 facility, with limitations on antiviral research. The spike protein, crucial for the interaction between SARS-CoV-2 and host cell surface ACE2 receptors, may serve as an allergen for inducing SARS-CoV-2 lung injury and immune disorders in mice.[28,29] Wild-type mice are considered to be less susceptible to SARS-CoV-2 because it does not use mouse ACE2 as a receptor. However, relevant reports have shown that the N501Y-mutated spike protein increases its binding affinity for mouse ACE2.[24] In this study, we administered N501Y-mutated spike protein via airway instillation to BALB/c mice, observed changes in spike protein infection on days 1, 3, and 10, and administered FTSJRF. Clinical research[30,31] shows that although the chest CT inflammatory manifestations of many patients in the recovery period after COVID-19 infection are absorbed to varying degrees compared to acute infection, there are still slight ground glass-like density shadows or consolidation. Pathological staining of lung tissue showed that acute severe lung injury appeared on the first day of spike protein infection and gradually subsided on days 3 and 10, which was consistent with the clinical manifestations of COVID-19. FTSJRF protects against SARS-CoV-2 spike protein-induced lung tissue damage in mice.

https://doi.org/10.2147/JIR.S480112

The functional characteristics of mucus depend on its main macromolecular component, mucin. Excessive mucin can interact with platelets or form aggregates, leading to pulmonary airway obstruction. In critically ill COVID-19 patients, abnormal secretion of airway mucus is a leading cause of death.[32] The primary mucin proteins produced in the airways are MUC5AC, MUC5B, MUC1, MUC4, MUC16 and MUC20. Several studies have shown that factors, such as MUC5AC, MUC5B and MUC1, are overexpressed in patients with COVID-19.[33] These changes may cause respiratory distress owing to mucus adhesion and airway obstruction. Studies have shown that the expression levels of MUC5B and MUC5AC RNA in the airway region of COVID-19 autopsied lungs increase, especially in the subacute or chronic disease stages after SARS-CoV-2 clearance.[34] Furthermore, it was observed that human bronchial epithelial (HBE) cells infected with SARS-CoV-2 exhibited peak titers at 3 days post-inoculation, while the expression of MUC5B/MUC5AC peaked at 7–14 days post-inoculation.[34] These results indicate that mucus secretion is not significant in the acute phase of COVID-19 but is significant in the subacute or chronic phase. This is consistent with our findings. In this study, PAS staining showed that 10 days after mice were infected with the S protein, mucus secretion was the most significant, and the levels of MUC1, MUC4, MUC5B, MUC5AC, MUC16 and MUC20 increased. After FTSJRF treatment, the expression levels of these mucins decreased, indicating that FTSJRF could effectively inhibit mucus secretion in the spike protein-induced mouse model.

A study examining cytokine profiles in individuals with acute COVID-19 and long-term COVID-19 syndrome indicates that levels of TNF-α and IL-17 are elevated in patients during the recovery phase compared to those in the acute phase, while levels of IL-6 are higher in patients during the acute phase compared to those in the recovery phase.[35] IL-6 has been posited as a potential mediator of prolonged COVID-19 symptoms, potentially attributable to its sustained presence.[36] In the context of SARS-CoV-2 spike protein-induced lung injury, inflammatory mediators including IL-1β, IL-6, and TNF-α play a significant role. Our experimental approach involved infecting mice with the SARS-CoV-2 spike protein for a duration of 1 day, resulting in a notable increase in IL-6 and IL-1β levels. While TNF-α levels did not show a significant change initially, by day 10 post-infection, all three inflammatory mediators remained elevated. Our mouse model showed an inflammatory reaction consistent with the clinical manifestations of COVID-19 convalescence. FTSJRF reduced the levels of these inflammatory factors and improved inflammatory reactions. In our study, IL-33 levels in the lung tissue in the D10 group significantly increased, and FTSJRF inhibited IL-33 levels. IL-33 plays a pivotal role as a cytokine in both the innate and adaptive immune responses of mucosal organs, leading to heightened airway inflammation, mucus secretion, and Th2 cytokine production in the lungs following respiratory infections.[35] This may be one of the reasons why FTSJRF inhibits airway inflammation and mucus secretion.

Both cDCs and pDCs have the ability to activate immature T cells. cDCs are potent antigen-presenting cells, while pDCs are effective producers of type I interferon.[37] Prior research has demonstrated that patients with COVID-19 exhibit impaired T cell activation capacity in cDCs and diminished production of type I interferon (IFN) by pDCs. Additionally, reports indicate a reduction in the frequency and absolute count of both pDCs and cDCs in the peripheral blood of individuals with COVID-19, with a more pronounced decrease observed in cDCs and pDCs.[38,39] This phenomenon may be attributed to the severity of the illness. Prolonged deficiency and impairment of DCs can persist for an extended period following infection with SARS-CoV-2, causing tissue injury and the persistence of viral antigens that are challenging to eradicate, consequently giving rise to a range of sequelae in the recovery from COVID-19.[39,40] Flow cytometry was used to detect DCs in our mouse model, and the results were consistent with those of previous studies. The flow cytometry results showed that our mouse model had a decrease in the number of cDCs and an abnormal recovery of mature DCs. FTSJRF increased the number of cDCs and promoted DC maturation.

Th17 cells, a pro-inflammatory subgroup, are recruited to sites of inflammation upon activation, potentially leading to autoimmune tissue damage. Tregs function as inhibitory subgroups that modulate immune responses by suppressing Th17 cell activity. The equilibrium between Th17 and Treg cell populations can significantly impact immune response outcomes. Clinical investigations have revealed elevated Th17 cell responses and diminished Treg cell responses in patients with COVID-19 compared to healthy individuals.[41] This study demonstrates that FTSJRF has the ability to suppress the generation of Th17 cells, suggesting its potential in mitigating inflammation through modulation of the Th17/Treg equilibrium in post-COVID-19 syndrome. Prior research has indicated that SARS-CoV-2 induces Th2 responses alongside increased production of pro-inflammatory and Th1 cytokines.[42] In addition, elevated levels of Th2 cytokines, specifically IL-4 and IL-5, have been shown to suppress the protective Th1 antiviral responses in individuals

with COVID-19. This suggests that inadequate regulation and stability of the immune response following SARS-CoV-2 infection may play a role in the immune dysregulation observed in the progression of COVID-19.[43,44] In our study, FTSJRF increased the proportion of Th1 cells while decreasing the proportion of Th2 cells, thus bringing Th1/Th2 ratios towards equilibrium.

Traditional Chinese medicine (TCM) is well recognized for its effectiveness in preventing and treating COVID-19.[45] Our results showed that FTSJRF effectively alleviated the inflammatory response, lung tissue injury and immune dysfunction in SARS-CoV-2 spike protein induced mouse model, providing experimental evidence for the use of FTSJRF in the treatment of COVID-19. However, this study has several limitations. Although we used spike protein to simulate some manifestations of post-COVID-19 syndrome, this is not a mimic for virus infection, which cannot fully represent the post-COVID-19 syndrome model. In addition, although the therapeutic effect of FTSJRF on spike protein induced damage has been determined, the specific active ingredient has not been determined. Future studies should aim to isolate and identify these active ingredients in order to achieve more targeted therapeutic approaches. And further studies to verify the related pathways and molecular targets mediated by FTSJRF.

Conclusion

In summary, FTSJRF inhibited lung tissue injury, mucus secretion and inflammation induced by SARS-CoV-2 spike protein. Additionally, FTSJRF regulates immune functions by promoting DC maturation and Th17/Treg and Th1/Th2 cell homeostasis. Our study provides new insights into the molecular mechanism and targets of FTSJRF for the treatment of post-COVID-19 syndrome, and provides a new avenue for the development of drugs. In future studies, we will conduct related clinical studies to broaden the scope of use of this prescription.

Abbreviations

COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TCM, traditional Chinese medicine; FTSJRF, Fu Tu Sheng Jin Rehabilitation Formula; ACE2, angiotensin-converting enzyme-2; HE, hematoxylin and eosin; PAS, periodic acid-Schiff; Treg, regulatory T cell; cDCs, conventional myeloid dendritic cells; pDCs, plasma cell-like DCs.

Data Sharing Statement

Data will be made available from the corresponding author.

Author Contributions

All authors made significant contributions to the work reported, whether in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas, took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; agreed on the journal to which the article has been submitted; and agreed to be accountable for all aspects of the work.

Funding

This study was supported by funding from the Natural Science Foundation of Jiangsu Province (BK20211391) and National Administration of Traditional Chinese Medicine (2021ZYLCYJ05-3).

Disclosure

All of the authors have no conflicts of interest to declare for this work.

References

1. Zhou P, Yang XL, Wang XG, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature*. 2020;579(7798):270–273. doi:10.1038/s41586-020-2012-7
2. Goel RR, Apostolidis SA, Painter MM, et al. Distinct antibody and memory B cell responses in SARS-CoV-2 naive and recovered individuals following mRNA vaccination. *Sci Immunol*. 2021;6(58):eabi6950. doi:10.1126/sciimmunol.abi6950

3. Fenrich M, Mrdenovic S, Balog M, et al. SARS-CoV-2 dissemination through peripheral nerves explains multiple organ injury. *Front Cell Neurosci*. 2020;14:229. doi:10.3389/fncel.2020.00229
4. Huang C, Huang L, Wang Y, et al. 6-month consequences of COVID-19 in patients discharged from hospital: a cohort study. *Lancet*. 2023;401 (10393):e21–e33. doi:10.1016/S0140-6736(23)00810-3
5. McAuley H, Evans RA, Bolton CE, et al. Prevalence of physical frailty, including risk factors, up to 1 year after hospitalisation for COVID-19 in the UK: a multicentre, longitudinal cohort study. *EClinicalMedicine*. 2023;57:101896. doi:10.1016/j.eclinm.2023.101896
6. Najafi MB, Javanmard SH. Post-COVID-19 syndrome mechanisms, prevention and management. *Int J Prev Med*. 2023;14:59. doi:10.4103/ijpvm.ijpvm_508_21
7. Willi S, Luthold R, Hunt A, et al. COVID-19 sequelae in adults aged less than 50 years: a systematic review. *Travel Med Infect Dis*. 2021;40:101995. doi:10.1016/j.tmaid.2021.101995
8. Martin C, Obadeyi O, Yeo E, Tran D, Pak E. Pain complaints and intubation risk in COVID-19: a retrospective cohort study. *Cureus*. 2023;15(1): e33851. doi:10.7759/cureus.33851
9. Huang L, Yao Q, Gu X, et al. 1-year outcomes in hospital survivors with COVID-19: a longitudinal cohort study. *Lancet*. 2021;398 (10302):747–758. doi:10.1016/S0140-6736(21)01755-4
10. Barker-Davies RM, O'Sullivan O, Senaratne K, et al. The Stanford Hall consensus statement for post-COVID-19 rehabilitation. *Br J Sports Med*. 2020;54(16):949–959. doi:10.1136/bjsports-2020-102596
11. Chen Y, Liu C, Wang T, et al. Efficacy and safety of Bufei Huoxue capsules in the management of convalescent patients with COVID-19 infection: a multicentre, double-blind, and randomised controlled trial. *J Ethnopharmacol*. 2022;284:114830. doi:10.1016/j.jep.2021.114830
12. Ouyang Y, Yin J, Wang W, et al. Downregulated gene expression spectrum and immune responses changed during the disease progression in patients with COVID-19. *Clin Infect Dis*. 2020;71(16):2052–2060. doi:10.1093/cid/ciaa462
13. Huang TY, Huang CY, Chao CH, et al. New biscembranoids sardigitolides A-D and known cembranoid-related compounds from sarcophyton digitatum: isolation, structure elucidation, and bioactivities. *Mar Drugs*. 2020;18(9):452. doi:10.3390/md18090452
14. Togami Y, Matsumoto H, Yoshimura J, et al. Significance of interferon signaling based on mRNA-microRNA integration and plasma protein analyses in critically ill COVID-19 patients. *Mol Ther Nucleic Acids*. 2022;29:343–353. doi:10.1016/j.omtn.2022.07.005
15. Hu B, Huang S, Yin L. The cytokine storm and COVID-19. *J Med Virol*. 2021;93(1):250–256. doi:10.1002/jmv.26232
16. Quirch M, Lee J, Rehman S. Hazards of the cytokine storm and cytokine-targeted therapy in patients with COVID-19: review. *J Med Internet Res*. 2020;22(8):e20193. doi:10.2196/20193
17. Niedzwiedzka-Rystwej P, Majchrzak A, Kurkowska S, et al. Immune Signature of COVID-19: in-depth reasons and consequences of the cytokine storm. *Int J Mol Sci*. 2022;23(9):4545. doi:10.3390/ijms23094545
18. Shi SF, Fang ZY, Xiong K, et al. Clinical observation of futu shengjin convalescent prescription in treating patients with lung and spleen deficiency syndrome in convalescent period of COVID-19. *J Nanjing Univ Chin Med*. 2020;36(03):281–285.
19. Rahayu I, Timotius KH. Phytochemical analysis, antimutagenic and antiviral activity of moringa oleifera l. leaf infusion: in vitro and in silico studies. *Molecules*. 2022;27(13):4017. doi:10.3390/molecules27134017
20. Ortega JT, Pujol FH, Jastrzebska B, Rangel HR. Mutations in the SARS-CoV-2 spike protein modulate the virus affinity to the human ACE2 receptor, an in silico analysis. *EXCLI J*. 2021;20:585–600. doi:10.17179/excli2021-3471
21. Huang YX, Li NF, Li CY, et al. Clinical features and effectiveness of Chinese medicine in patients with COVID-19 from overseas: a retrospective study in Xiamen, China. *Front Public Health*. 2022;10:1038017. doi:10.3389/fpubh.2022.1038017
22. Kuba K, Imai Y, Rao S, et al. A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. *Nat Med*. 2005;11(8):875–879. doi:10.1038/nm1267
23. Kim MH, Kim HJ, Chang J. Superior immune responses induced by intranasal immunization with recombinant adenovirus-based vaccine expressing full-length spike protein of Middle East respiratory syndrome coronavirus. *PLoS One*. 2019;14(7):e0220196. doi:10.1371/journal.pone.0220196
24. Gu H, Chen Q, Yang G, et al. Adaptation of SARS-CoV-2 in BALB/c mice for testing vaccine efficacy. *Science*. 2020;369(6511):1603–1607. doi:10.1126/science.abc4730
25. Thornton DJ, Rousseau K, McGuckin MA. Structure and function of the polymeric mucins in airways mucus. *Annu Rev Physiol*. 2008;70:459–486. doi:10.1146/annurev.physiol.70.113006.100702
26. Hattrup CL, Gendler SJ. Structure and function of the cell surface (tethered) mucins. *Annu Rev Physiol*. 2008;70:431–457. doi:10.1146/annurev.physiol.70.113006.100659
27. Dong M, Zhang G, Meng J, et al. MMP9-associated tumor stem cells, CCL1-silenced dendritic cells, and cytokine-induced killer cells have a remarkable therapeutic efficacy for acute myeloid leukemia by activating t cells. *Stem Cells Int*. 2023;2023:2490943. doi:10.1155/2023/2490943
28. Cao X, Tian Y, Nguyen V, et al. Spike protein of SARS-CoV-2 activates macrophages and contributes to induction of acute lung inflammation in male mice. *FASEB J*. 2021;35(9):e21801. doi:10.1096/fj.202002742RR
29. Nan FY, Wu CJ, Su JH, Ma LQ. Potential mouse models of coronavirus-related immune injury. *Front Immunol*. 2022;13:943783. doi:10.3389/fimmu.2022.943783
30. Wu X, Liu X, Zhou Y, et al. 3-month, 6-month, 9-month, and 12-month respiratory outcomes in patients following COVID-19-related hospitalisation: a prospective study. *Lancet Respir Med*. 2021;9(7):747–754. doi:10.1016/S2213-2600(21)00174-0
31. Lei M, Lin L, Hu KY, et al. Clinical analysis of 46 cases of novel coronavirus pneumonia during convalescence. *J Guangzhou Med Univ*. 2020;48 (04):8–11.
32. Zhang Y, Wang Z, Zhang Y, et al. Potential mechanisms for Traditional Chinese Medicine in treating airway mucus hypersecretion associated with coronavirus disease 2019. *Front Mol Biosci*. 2020;7:577285. doi:10.3389/fmolb.2020.577285
33. Kumar SS, Binu A, Devan AR, Nath LR. Mucus targeting as a plausible approach to improve lung function in COVID-19 patients. *Med Hypotheses*. 2021;156:110680. doi:10.1016/j.mehy.2021.110680
34. Kato T, Asakura T, Edwards CE, et al. Prevalence and mechanisms of mucus accumulation in COVID-19 lung disease. *Am J Respir Crit Care Med*. 2022;206(11):1336–1352. doi:10.1164/rccm.202111-2606OC
35. Queiroz M, Neves P, Lima SS, et al. Cytokine profiles associated with acute COVID-19 and long COVID-19 syndrome. *Front Cell Infect Microbiol*. 2022;12:922422. doi:10.3389/fcimb.2022.922422

36. Kappelmann N, Dantzer R, Khandaker GM. Interleukin-6 as potential mediator of long-term neuropsychiatric symptoms of COVID-19. *Psychoneuroendocrinology*. 2021;131:105295. doi:10.1016/j.psyneuen.2021.105295
37. Welner RS, Pelayo R, Nagai Y, et al. Lymphoid precursors are directed to produce dendritic cells as a result of TLR9 ligation during herpes infection. *Blood*. 2008;112(9):3753–3761. doi:10.1182/blood-2008-04-151506
38. Sanchez-Cerrillo I, Landete P, Aldave B, et al. COVID-19 severity associates with pulmonary redistribution of CD1c+ DCs and inflammatory transitional and nonclassical monocytes. *J Clin Invest*. 2020;130(12):6290–6300. doi:10.1172/JCI140335
39. Perez-Gomez A, Vitalle J, Gasca-Capote C, et al. Dendritic cell deficiencies persist seven months after SARS-CoV-2 infection. *Cell Mol Immunol*. 2021;18(9):2128–2139. doi:10.1038/s41423-021-00728-2
40. Montani D, Savale L, Noel N, et al. Post-acute COVID-19 syndrome. *Eur Respir Rev*. 2022;31(163).
41. Sadeghi A, Tahmasebi S, Mahmood A, et al. Th17 and treg cells function in SARS-CoV2 patients compared with healthy controls. *J Cell Physiol*. 2021;236(4):2829–2839. doi:10.1002/jcp.30047
42. Aleebrahim-Dehkordi E, Molavi B, Mokhtari M, et al. T helper type (Th1/Th2) responses to SARS-CoV-2 and influenza A (H1N1) virus: from cytokines produced to immune responses. *Transpl Immunol*. 2022;70:101495. doi:10.1016/j.trim.2021.101495
43. Choreno-Parra JA, Jimenez-Alvarez LA, Cruz-Lagunas A, et al. Clinical and Immunological Factors That Distinguish COVID-19 from pandemic influenza A(H1N1). *Front Immunol*. 2021;12:593595. doi:10.3389/fimmu.2021.593595
44. Petersen E, Koopmans M, Go U, et al. Comparing SARS-CoV-2 with SARS-CoV and influenza pandemics. *Lancet Infect Dis*. 2020;20(9):e238–e244. doi:10.1016/S1473-3099(20)30484-9
45. Hu S, Luo D, Zhu Q, et al. An updated meta-analysis of Chinese herbal medicine for the prevention of COVID-19 based on Western-Eastern medicine. *Front Pharmacol*. 2023;14:1257345. doi:10.3389/fphar.2023.1257345