Efficacy of Fengshi Feibi Formula in Treating Rheumatoid Arthritis with Interstitial Lung Disease in TNF-Tg Mice: A Postprint

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Abstract

Background: Rheumatoid arthritis-associated interstitial lung disease (RA-ILD) constitutes a common complication of rheumatoid arthritis (RA), affecting 20%~50% of RA patients and substantially increasing mortality risk. Currently, clinically effective and safe therapeutic agents with clear targets for RA-ILD are lacking. Traditional Chinese medicine demonstrates certain advantages in RA-ILD management. Fengshi Feibi Formula (FSFBF), a commonly prescribed herbal formulation, exhibits definitive therapeutic efficacy, though its mechanism of action remains unclear and requires experimental validation.

Objective: To investigate the therapeutic efficacy and mechanism of FSFBF in tumor necrosis factor-α transgenic (TNF-Tg) mice with RA-ILD.

Methods: Between March 2024 and January 2025, female TNF-Tg mice were utilized as an RA-ILD model. Twenty-four 2.5-month-old TNF-Tg mice were randomly divided into model group (TNF-Tg group), low-dose FSFBF group (Low group), medium-dose FSFBF group (Middle group), and high-dose FSFBF group (High group), while six littermate wild-type mice were randomly selected as control group (WT group). The TNF-Tg group received normal saline, whereas the Low, Middle, and High groups received FSFBF at 5.825, 13.65, and 27.3 g · kg-1 · d-1 via gavage. After 9 weeks, body weight, ankle joint clinical scores, and forelimb grip strength were measured; ankle and lung tissues were subjected to HE staining, Masson staining, and Safranin O-Fast Green staining; serum inflammatory cytokine levels were determined by enzyme-linked immunosorbent assay (ELISA); lung macrophages were labeled by immunofluorescence staining; and lung inflammatory cytokine mRNA relative expression was quantified by real-time quantitative PCR (RT-qPCR).

Results: At 4.5 months of age, body weight in the TNF-Tg group was lower

than in the WT group, while the Low and High groups displayed higher values than the TNF-Tg group ($P<0.05$); ankle joint clinical scores in the TNF-Tg group were higher than in the WT group, whereas the Middle and High groups exhibited lower scores than the TNF-Tg group ($P<0.05$); forelimb grip strength in the TNF-Tg group was lower than in the WT group, while the High group showed higher strength than the TNF-Tg group ($P<0.05$). Pathological analysis revealed: ankle joint inflammation area, lung inflammation area, and lung type I collagen fiber area in the TNF-Tg group were greater than in the WT group; ankle joint inflammation area in the Middle and High groups was smaller than in the TNF-Tg group; joint cartilage area in the Middle group was larger than in the TNF-Tg group; lung inflammation and lung type I collagen fiber area in the Low, Middle, and High groups were smaller than in the TNF-Tg group ($P<0.05$). Immunofluorescence staining demonstrated: lung M1- and M2-type macrophage numbers in the TNF-Tg group were greater than in the WT group; M1-type macrophage numbers in the Low, Middle, and High groups were lower than in the TNF-Tg group, while M2-type macrophage numbers were higher than in the TNF-Tg group ($P<0.05$). ELISA analysis indicated: serum TNF-α, IL-1β, and IL-6 levels in the TNF-Tg group were higher than in the WT group; serum TNF-α and IL-1β levels in the Low, Middle, and High groups were lower than in the TNF-Tg group; serum IL-6 level in the High group was lower than in the TNF-Tg group ($P<0.05$). RT-qPCR analysis showed: relative expression of TNF-α and IL-1β in lungs of TNF-Tg group mice was higher than in the WT group, while the High group was lower than the TNF-Tg group ($P<0.05$); relative expression of IL-1 in lungs in the Middle and High groups was lower than in the TNF-Tg group ($P<0.05$); relative expression of TNF-α and IL-1β in ankle joints of TNF-Tg group mice was higher than in the WT group, while the Low, Middle, and High groups were lower than the TNF-Tg group ($P<0.05$).

Conclusion: FSFBF ameliorates RA-ILD manifestations in TNF-Tg mice by modulating lung macrophage polarization, attenuating joint inflammation, cartilage destruction, lung inflammation, and alveolar fibrosis, suggesting that FSFBF may represent a novel therapeutic strategy for clinical management of RA-ILD.

Full Text

Therapeutic Effects of Fengshi Feibi Decoction on Rheumatoid Arthritis-Associated Interstitial Lung Disease in TNF-Tg Mice

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Abstract

Background: Rheumatoid arthritis-associated interstitial lung disease (RA-ILD) is a common complication of rheumatoid arthritis (RA), with 20% to 50% of RA patients developing ILD, which substantially increases mortality risk. Currently, there is a lack of safe, effective, and targeted therapeutic agents for RA-ILD. Traditional Chinese medicine offers certain advantages in treating RA-ILD. Fengshi Feibi Decoction (FSFBF), as a commonly used prescription, demonstrates proven efficacy, but its mechanism of action remains unclear and requires validation through experimental research.

Objective: To investigate the therapeutic efficacy and underlying mechanisms of FSFBF in TNF-α transgenic (TNF-Tg) mice with RA-ILD.

Methods: From March 2024 to January 2025, female TNF-Tg mice were used as an RA-ILD model. Twenty-four 2.5-month-old TNF-Tg mice were randomly divided into four groups: model group (TNF-Tg), low-dose FSFBF group (Low), medium-dose FSFBF group (Middle), and high-dose FSFBF group (High). Additionally, six age-matched wild-type (WT) littermates were randomly selected as controls. The TNF-Tg group received normal saline, while the treatment groups received FSFBF via oral gavage at doses of 5.825, 13.65, and 27.3 $g \cdot kg^{-1} \cdot d^{-1}$, respectively, for 9 weeks. Body weight, ankle joint clinical scores, and forelimb grip strength were recorded. Ankle joint and lung tissues were collected for histological analysis using H&E, Masson's trichrome, and Safranin O-fast green staining. Serum levels of inflammatory cytokines were measured by enzyme-linked immunosorbent assay (ELISA). Immunofluorescence staining was performed to assess macrophage polarization in lung tissues. Quantitative real-time polymerase chain reaction (RT-qPCR) was used to evaluate the relative mRNA expression of inflammatory cytokines in ankle joints and lungs.

Results: At 4.5 months of age, body weight in the TNF-Tg group was lower than that in the WT group, while the Low and High groups had higher body weights than the TNF-Tg group ($P<0.05$). Ankle joint clinical scores in the TNF-Tg group were higher than those in the WT group, while the Middle and High groups had lower scores than the TNF-Tg group ($P<0.05$). Forelimb grip strength in the TNF-Tg group was lower than that in the WT group, while the High group had higher grip strength than the TNF-Tg group ($P<0.05$).

Pathological results showed that the TNF-Tg group had larger ankle joint inflammation areas, lung inflammation areas, and lung type I collagen fiber areas than the WT group. The Middle and High groups had smaller ankle joint inflammation areas than the TNF-Tg group. The Middle group had larger joint cartilage areas than the TNF-Tg group. The Low, Middle, and High groups had smaller lung inflammation and lung type I collagen fiber areas than the TNF-Tg group ($P<0.05$). Immunofluorescence staining results showed that the number of M1 and M2 macrophages in the lungs of the TNF-Tg group was greater than that in the WT group. The number of M1 macrophages in the Low, Middle, and High groups was lower than that in the TNF-Tg group, while the number of M2 macrophages was higher than that in the TNF-Tg group ($P<0.05$). ELISA results showed that serum TNF-α, IL-1β, and IL-6 levels in the TNF-Tg group were higher than those in the WT group. Serum TNF-α and IL-1β levels in the Low, Middle, and High groups were lower than those in the TNF-Tg group, and serum IL-6 levels in the High group were lower than that in the TNF-Tg group ($P<0.05$). RT-qPCR results showed that the relative expression levels of TNF-α and IL-1β in the lungs of mice in the TNF-Tg group were higher than those in the WT group, while the High group was lower than the TNF-Tg group ($P<0.05$). The Middle and High groups had lower relative expression levels of IL-1β in the lungs than the TNF-Tg group ($P<0.05$). The relative expression levels of TNF-α and IL-1β in the ankle joints of the TNF-Tg group were higher than those in the WT group, while the Low, Middle, and High groups were lower than the TNF-Tg group ($P<0.05$).

Conclusion: FSFBF ameliorates RA-ILD symptoms in TNF-Tg mice by modulating macrophage polarization in the lungs. It alleviates joint inflammation, cartilage degradation, pulmonary inflammation, and alveolar fibrosis. These findings suggest that FSFBF may represent a promising novel therapeutic strategy for the clinical treatment of RA-ILD.

Keywords: Rheumatoid arthritis; Interstitial lung disease; Fengshi Feibi Decoction; TNF-Tg mice; Macrophage polarization

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by synovial hyperplasia, inflammatory infiltration, cartilage destruction, and bone erosion. These pathological changes lead to joint deformity and disability in patients [1-2]. Rheumatoid arthritis-associated interstitial lung disease (RA-ILD) represents one of the most significant extra-articular manifestations of RA. Studies have demonstrated that ILD increases the risk of mortality in RA patients threefold [3]. The prevalence of RA is approximately 0.5% to 1%, with significantly higher incidence in women compared to men, and the peak onset occurs in individuals aged 30 to 50 years [4-5]. A cross-sectional study revealed that the standardized prevalence of RA in China reached 0.28%,

with notable geographic clustering in North China and economically developed coastal regions [6]. Between 20% and 50% of RA patients develop secondary ILD, and the progressive pulmonary fibrosis symptoms cause irreversible lung function damage. The acute progressive nature of this disease presents severe challenges for early intervention, making it a major public health concern worldwide [7].

The pathophysiological mechanisms of RA-ILD are complex. The deposition of autoantibodies and immune complexes and subsequent tissue inflammation constitute the fundamental pathogenesis of the disease, and suppressing inflammation can effectively improve patient symptoms [8]. Macrophages differentiate into distinct functional phenotypes in response to various microenvironmental signals. Polarized macrophages directly influence tissue inflammation progression, resolution, and tissue repair, and their dynamic balance is crucial for maintaining immune homeostasis [9]. Classically activated macrophages (M1) secrete pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β, whereas alternatively activated macrophages (M2) secrete IL-10 and transforming growth factor-β, which can suppress inflammation and promote angiogenesis and tissue repair [8,10]. The 2023 American College of Rheumatology/American Thoracic Society guidelines [11] recommend using disease-modifying antirheumatic drugs, anti-fibrotic agents, and glucocorticoids for RA-ILD treatment. However, specific medications for RA-ILD are still lacking, and patients must weigh the risks when selecting recommended drugs. Therefore, exploring the pathogenesis of RA-ILD and identifying novel therapeutic approaches holds significant clinical value.

According to Traditional Chinese Medicine theory, RA-ILD belongs to the category of "Feibi" (lung impediment), primarily caused by wind, cold, and dampness pathogens obstructing lung qi [12]. The discussion in *Suwen · Bilun* regarding "Pibi" (skin impediment) affecting the lungs and causing "Feibi" reveals its pathogenesis [13]. Fengshi Feibi Decoction (FSFBF) is a novel herbal formulation developed by national TCM master Professor Shi Qi, combining traditional Shi 氏 trauma theory with modern clinical experience. Adhering to the principle of "taking qi as the primary focus and blood as the foremost priority," the formula comprises seven herbs: raw Astragalus, Agrimonia pilosa, Zhejiang Fritillaria, ginger-processed Pinellia, Curcuma zedoaria, Sinomenium acutum, and Gentiana macrophylla. This formula possesses qi-supplementing, blood-activating, wind-dispelling, dampness-removing, and pain-relieving effects and is clinically used to treat patients with deficiency of vital qi and blood stasis, wind-dampness impediment pain, accompanied by cough, dyspnea, and chest fullness. Currently, research on the pharmacological effects and mechanisms of FSFBF remains limited. This study employed female TNF-α transgenic (TNF-Tg) mice as an RA-ILD model to investigate the therapeutic efficacy and mechanisms of FSFBF in TNF-Tg mice with RA-ILD, providing new strategies and reference bases for TCM treatment of RA-ILD.

Methods

Study Period

March 2024 to January 2025.

Experimental Materials

Experimental Animals Female TNF-Tg mice on a C57BL/6J background (line 3647) were gifted by Professor Xing Lianping from the University of Rochester. This TNF-Tg mouse line carries the human TNF gene, resulting in overexpression of human TNF-α, and is maintained as heterozygotes through backcrossing. Non-transgenic littermates were used as wild-type (WT) controls. This study utilized 24 female TNF-Tg mice at 2.5 months of age and 6 WT littermate controls. Genotyping of TNF-Tg mice was performed by Cyagen (Suzhou) Biotechnologies Co., Ltd. All animals were housed in SPF-grade facilities with normal feed and water supply, under controlled temperature and humidity with a 12-hour light/dark cycle. This study was approved by the Laboratory Animal Management and Use (Animal Welfare) Committee of Longhua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine (Approval No.: LHERAW-25021). All animal procedures complied with the *Guiding Principles for the Care and Use of Laboratory Animals* issued by the National Science and Technology Commission of China.

Drugs Fengshi Feibi Decoction consists of raw Astragalus 18 g, Agrimonia pilosa 15 g, Zhejiang Fritillaria 12 g, ginger-processed Pinellia 9 g, Curcuma zedoaria 15 g, Sinomenium acutum 12 g, and Gentiana macrophylla 9 g. All herbs were obtained from the outpatient TCM pharmacy of Longhua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine. Based on a conversion coefficient of 9.01, TCM dosages were converted between mice (approximately 0.02 kg body weight) and humans (approximately 60 kg body weight) to establish the standard dosing regimen [14]. Low, medium, and high concentration decoctions were prepared at 0.5×, 1×, and 2× the standard dose, yielding concentrations of 2.73 g/mL, 1.365 g/mL, and 0.6825 g/mL, respectively. For preparation, seven doses of the herbs were placed in a stainless steel pot, soaked in approximately 10 volumes of water (1000 mL) for 30 minutes, then boiled vigorously before simmering for 30 minutes. The solution was filtered, and the remaining herbs were subjected to a second decoction with approximately 8 volumes of water (600 mL) for another 30 minutes. The two filtrates were combined and concentrated by slow heating to obtain the FSFBF decoction, which was aliquoted and stored at -80°C until use.

Major Reagents H&E staining solution (Wuhan Servicebio Technology Co., Ltd., Cat# G1005), Modified Masson's Trichrome staining kit (Beijing Solarbio Science & Technology Co., Ltd., Cat# G1346), Modified Safranin O-Fast Green cartilage staining kit (Beijing Solarbio Science & Technology Co., Ltd., Cat# G1371), F4/80 antibody (Abcam, UK, Cat# ab6640), iNOS

antibody (Abcam, UK, Cat# ab3523), CD206 antibody (Abcam, UK, Cat# ab64693), anti-rat IgG (H+L) (Alexa Fluor® 488 Conjugate) (Cell Signaling Technology, USA, Cat# 4416), anti-rabbit IgG (H+L) (Alexa Fluor® 555 Conjugate) (Cell Signaling Technology, USA, Cat# 4413), anti-fluorescence quenching mounting medium (with DAPI) (Beyotime Biotechnology, Shanghai, Cat# P0131), One-tube genomic DNA removal and reverse transcription kit (Wuhan Servicebio Technology Co., Ltd., Cat# G3337), Universal SYBR Green qPCR Master Mix (Wuhan Servicebio Technology Co., Ltd., Cat# G3328). Primers: GADPH forward 5'-GGTGAAGGTCGGTGTGAACG-3', reverse 5'-CTCGCTCCTGGAAGATGGTG-3'; TNF-α forward 5'-AGTGACAAGCCTGTAGCCC-3', reverse 5'-GAGGTTGACTTTCTCCTGGTAT-3'; IL-1β forward 5'-TCGCTCAGGGTCACAAGAAA-3', reverse 5'-CATCAGAGGCAAGGAGGAAAAC-3'.

Major Instruments Electronic scale (Sartorius, Germany, Model BSA124S), tissue automatic dehydrator, tissue embedding machine, paraffin microtome, cryostat (Leica, Germany, Models ASP300 S, EG1150, RM2125 RTS, CM3050 S), whole-slide scanner (Olympus, Japan, Model VS200), thermal cycler (Thermo Fisher, USA, Model TCA0096), real-time fluorescence quantitative PCR instrument (Thermo Fisher, USA, Model CFX Opus 384), microplate reader (BioTek Instruments, USA, Model Cytation 5).

Experimental Methods

Animal Grouping and Administration Twenty-four 2.5-month-old TNF-Tg mice were numbered in ascending order of body weight and randomly divided into four groups (n=6 each) using a random number table. Additionally, six WT littermates were randomly selected as negative controls. The groups were: control group (WT), TNF-Tg model group (TNF-Tg), low-dose FSFBF group (Low), medium-dose FSFBF group (Middle), and high-dose FSFBF group (High). After one week of acclimatization, mice received daily oral gavage (0.2 mL per dose) for 9 weeks until 4.5 months of age. The Low, Middle, and High groups received FSFBF at doses of 6.825, 13.65, and 27.3 $g \cdot kg^{-1} \cdot d^{-1}$, respectively.

Body Weight and Ankle Joint Clinical Score Assessment Body weight was measured before and after gavage (at 2.5 and 4.5 months of age). Ankle joint clinical scores were assessed after gavage (at 4.5 months). Mice were placed on the experimental platform to acclimate freely. Body weight was measured twice using an electronic scale and averaged. Ankle joint clinical scoring was based on visual observation, with each paw scored from 0 to 4: 0 = no swelling (normal); 1 = redness and mild deformity limited to a single digit; 2 = redness and mild deformity in multiple digits; 3 = redness and moderate deformity; 4 = redness and severe deformity of the entire paw [15].

Forelimb Grip Strength Measurement Forelimb grip strength was measured after gavage (at 4.5 months). Mice were allowed to acclimate on the test platform. The grip strength meter was calibrated, and mice were gently pulled backward by the tail until their forelimbs released from the metal grid. The maximum force displayed was recorded as one valid measurement. Each mouse was measured three times with 30-second intervals, and the average value was taken as the final forelimb grip strength.

Serum and Tissue Sample Collection After intraperitoneal injection of 1% pentobarbital sodium, blood was collected via enucleation, allowed to clot, and centrifuged to obtain serum. Following euthanasia, forelimb and hindlimb ankle joints were collected. Forelimbs were snap-frozen in liquid nitrogen and stored at -80°C. Hindlimbs were fixed in 4% paraformaldehyde, decalcified in 10% EDTA for 4 weeks, dehydrated, and paraffin-embedded. Lung tissues were collected; the left lung and right upper lobe were fixed in 4% paraformaldehyde, dehydrated, and subjected to frozen and paraffin embedding, respectively. The remaining right lung was snap-frozen in liquid nitrogen and stored at -80°C.

H&E Staining of Lung and Joint Tissues Paraffin sections of lung and joint tissues were dewaxed and stained sequentially with hematoxylin for 3-5 minutes, followed by distilled water wash; hematoxylin differentiation solution for 2-5 seconds, followed by thorough rinsing; bluing solution for 5 seconds, followed by thorough rinsing; dehydration through 85% and 95% ethanol for 5 minutes each; eosin staining for 5 minutes; two rounds of absolute ethanol dehydration for 5 minutes each; and mounting with neutral balsam.

Masson Staining of Lung Tissues Paraffin sections of lung tissues were dewaxed and stained sequentially with mordant solution at room temperature for 8-12 hours; distilled water wash; celestine blue staining for 3 minutes; distilled water wash; hematoxylin staining for 3 minutes; distilled water wash; acid differentiation solution for several seconds; thorough rinsing; Ponceau fuchsin staining for 10 minutes; distilled water wash; phosphomolybdic acid treatment for 10 minutes; aniline blue staining for 5 minutes; weak acid solution wash for 2 minutes; rapid dehydration through 95% ethanol for 3-5 seconds; and mounting with neutral balsam.

Safranin O-Fast Green Staining of Joint Tissues Paraffin sections of joint tissues were dewaxed and stained sequentially with hematoxylin for 5 minutes, distilled water wash; acid differentiation solution for 15 seconds, distilled water for 10 minutes; fast green staining for 5 minutes, weak acid solution wash for 15 seconds, air-dried; Safranin O staining for 5 minutes; absolute ethanol wash; two rounds of absolute ethanol dehydration for 5 minutes each; and mounting with neutral balsam.

Immunofluorescence Staining of Lung Tissues Frozen sections of lung tissues were rehydrated, blocked with 5% BSA containing 0.3% Triton X-100 for 1 hour, and incubated overnight at 4°C with primary antibodies against F4/80, iNOS, and CD206 in PBS. Sections were then incubated for 1 hour with anti-rat IgG (H+L) and anti-rabbit IgG secondary antibodies, followed by mounting with anti-fluorescence quenching medium containing DAPI.

Real-Time Quantitative PCR (RT-qPCR) Lung and ankle joint tissues were homogenized. After RNA extraction and purification, reverse transcription was performed using reverse transcriptase. The 20 L reverse transcription reaction was conducted at 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 seconds. The cDNA was diluted 5-fold and used for qPCR with SYBR Green Mix, using glyceraldehyde-3-phosphate dehydrogenase (GADPH) as the internal reference. The 20 L PCR reaction was performed at 50°C for 2 minutes (1 cycle); 95°C for 30 seconds (1 cycle); and 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Each sample was run in triplicate, and the average value was taken as the final result.

ELISA Detection of Serum TNF-α, IL-1β, and IL-6 Levels Mouse serum was thawed to 4°C. ELISA kits were used to detect serum TNF-α, IL-1β, and IL-6 levels. After preparing protein standards, blank wells, standard wells, and sample wells were set up (each sample in triplicate). To each well, 40 L of sample, 10 L of biotinylated antibody, and 50 L of enzyme conjugate were added. Plates were sealed and incubated at 37°C for 30 minutes. After thorough washing, chromogenic substrate was added, and absorbance was measured at 450 nm. The average value was taken as the final result.

Statistical Analysis Data were analyzed using SPSS 26.0 statistical software. Normally distributed measurement data are expressed as ($\bar{x}\pm s$). Comparisons among multiple groups were performed using one-way ANOVA, with pairwise comparisons between groups conducted using LSD-t test. $P<0.05$ was considered statistically significant.

Results

Body Weight, Ankle Joint Clinical Score, and Forelimb Grip Strength

At 2.5 months of age, there were no significant differences in body weight among the groups ($P>0.05$). At 4.5 months, body weight in the TNF-Tg group was lower than that in the WT group, while the Low and High groups had higher body weights than the TNF-Tg group ($P<0.05$). At 4.5 months, ankle joint clinical scores in the TNF-Tg group were higher than those in the WT group, while the Middle and High groups had lower scores than the TNF-Tg group ($P<0.05$). At 4.5 months, forelimb grip strength in the TNF-Tg group was lower

than that in the WT group, while the High group had higher grip strength than the TNF-Tg group (P<0.05) .

Ankle Joint H&E Staining

At 4.5 months, TNF-Tg mice exhibited obvious deformities in the ankle joints of all four limbs, which improved after FSFBF treatment. Further observation of talus sagittal sections from hindlimb ankle joints was performed. H&E staining results showed that WT mice had no obvious abnormalities in ankle joints. The ankle joint inflammation area in the TNF-Tg group was larger than that in the WT group, while the Middle and High groups had smaller inflammation areas than the TNF-Tg group (P<0.05). Safranin O-fast green staining revealed no cartilage damage in WT mice. The talus cartilage area in the TNF-Tg group was lower than that in the WT group, while the Middle group had higher cartilage area than the TNF-Tg group (P<0.05) [Figure 1: see original paper], .

Lung Histopathology

At 4.5 months, TNF-Tg mice showed significantly enlarged lung volume, which decreased after FSFBF treatment. H&E staining of lung sections showed no inflammatory infiltration in WT mice. The number of inflammatory cell clusters in the TNF-Tg group was greater than that in the WT group, while the Low, Middle, and High groups had fewer clusters than the TNF-Tg group (P<0.05). Masson staining showed no fibrosis in WT mice. The type I collagen fiber area in the lungs of TNF-Tg mice was larger than that in WT mice, while the Low, Middle, and High groups had smaller collagen fiber areas than the TNF-Tg group (P<0.05) [Figure 2: see original paper], .

Lung Immunofluorescence Staining

Immunofluorescence staining of lung tissue frozen sections was performed using anti-iNOS and F4/80 antibodies to label M1 macrophages, and anti-CD206 and F4/80 antibodies to label M2 macrophages, with DAPI labeling cell nuclei. The results showed no M1 or M2 macrophages in alveoli of WT mice. The numbers of both M1 and M2 macrophages in alveoli of TNF-Tg mice were greater than those in WT mice. The Low, Middle, and High groups had fewer M1 macrophages but more M2 macrophages than the TNF-Tg group (P<0.05) [Figure 3: see original paper], .

Serum Cytokine Levels

ELISA results showed that serum TNF-α, IL-1β, and IL-6 levels in the TNF-Tg group were higher than those in the WT group. The Low, Middle, and High groups had lower serum TNF-α and IL-1β levels than the TNF-Tg group, and the High group had lower serum IL-6 levels than the TNF-Tg group (P<0.05) .

Relative mRNA Expression of Inflammatory Cytokines in Joints and Lungs

RT-qPCR results showed that relative expression levels of TNF-α and IL-1β in the lungs of TNF-Tg mice were higher than those in WT mice, while the High group had lower expression than the TNF-Tg group ($P<0.05$). The Middle and High groups had lower relative expression of IL-1β in the lungs than the TNF-Tg group ($P<0.05$). Relative expression levels of TNF-α and IL-1β in the ankle joints of the TNF-Tg group were higher than those in the WT group, while the Low, Middle, and High groups had lower expression than the TNF-Tg group ($P<0.05$) .

Discussion

RA-ILD is one of the most severe pulmonary manifestations of RA, with limited clinical treatment options and no widely recognized highly effective drugs. In recent years, Traditional Chinese Medicine has emerged as a potential new strategy for RA-ILD treatment. Fengshi Feibi Decoction (FSFBF), as a classic formula supported by TCM theory, is widely used clinically for RA-ILD, but its pharmacological mechanisms remain to be validated. A prominent pathological feature of RA-ILD is immune cell infiltration in local tissues, with synovial tissue inflammation predominating in joints and interstitial lung inflammation representing the primary manifestation in lungs [16].

This study employed female line 3647 TNF-Tg mice as an RA-ILD model to systematically evaluate the intervention effects of FSFBF on RA-ILD and preliminarily explore its mechanism of regulating inflammatory responses through macrophage polarization modulation, aiming to provide new evidence and therapeutic insights for TCM intervention in RA-ILD.

The results demonstrated that TNF-Tg model mice exhibited significant body weight loss and joint functional impairment at 4.5 months of age, manifested by ankle joint clinical deformities and decreased grip strength [17]. Medium and high doses of FSFBF significantly improved body weight loss and alleviated clinical scores, suggesting its capacity to ameliorate the systemic inflammatory state and joint function in this model. Pathological experiments indicated that FSFBF significantly improved synovial inflammatory cell infiltration and cartilage destruction in TNF-Tg mice, with particularly notable effects in the medium and high dose groups. Additionally, the formula significantly reduced pulmonary inflammation and decreased fibrotic area.

Multiple studies have shown that compared with collagen-induced arthritis models, TNF-Tg mice more authentically simulate RA patients with concurrent pulmonary disease due to continuous overexpression of human TNF-α, exhibiting more pronounced RA complication symptoms. These mice develop ankle joint deformities and inflammatory erosive arthritis beginning at 2.5 months of age,

and die from multiple organ complications by 5.5 months of age [17-19]. In the lungs, these mice exhibit inflammatory infiltration, fibrosis around pulmonary vessels and bronchioles, and increased numbers of monocytes, dendritic cells, B lymphocytes, and T lymphocytes [20-22]. Our findings are consistent with these existing studies and further validate that TCM intervention can effectively improve related symptoms.

We further investigated the mechanism of FSFBF and found significant changes in M1 and M2 macrophage numbers in the lungs after treatment, with marked reduction in M1 macrophages and increased M2 macrophages. Concurrently, inflammatory cytokine expression in serum, lungs, and ankle joints decreased significantly across all dose groups, particularly in the medium and high dose groups. Macrophages are important innate immune cells that polarize toward M1 or M2 phenotypes in response to local microenvironmental stimuli. Overactivation of M1 macrophages and dysregulation of the M1/M2 ratio are closely associated with RA disease progression. In inflammatory environments dominated by Toll-like receptor and interferon signaling, macrophages polarize toward an M1-like phenotype, enhancing expression of pro-inflammatory cytokines such as TNF-α and IL-6, leading to persistent inflammation and bone destruction. Conversely, M2-like macrophages express CD163 and CD206, promoting growth factor release, alleviating tissue inflammation, facilitating cell proliferation, wound healing, and angiogenesis [9,23]. Studies have shown that M1-like macrophages are the primary source of TNF-α in RA patient synovial tissue [24], and various TCM formulas such as Wutou Decoction and Wuweiganlu Decoction can regulate the M1/M2 macrophage balance to improve RA symptoms [10,25]. Our study is the first to validate this mechanism in the lungs of TNF-Tg model mice, suggesting that macrophage polarization may play a key role in RA-ILD pathogenesis, and confirming that FSFBF can modulate systemic and local tissue inflammation through this mechanism.

This study has several limitations. RA-ILD is a chronic disease, but our study was limited to a relatively short treatment duration, and the long-term efficacy and safety of FSFBF have not been validated. Additionally, while we identified the formula's effects on macrophage polarization, its impact on related signaling pathways and specific molecular targets remains to be elucidated. Future studies should employ transcriptomic or proteomic technologies combined with investigations of active components to further clarify the molecular mechanisms.

In summary, this study systematically evaluated the therapeutic effects of FSFBF in TNF-Tg mice, confirming its ability to modulate macrophage polarization, alleviate pulmonary fibrosis and joint damage, suggesting that FSFBF may represent a novel TCM therapeutic strategy for RA-ILD. This research enriches the theoretical basis for TCM in RA-ILD treatment and provides new clues for clinical drug development and mechanistic studies.

Author Contributions

Yang Kunru proposed the main research objectives, designed the study, conducted the experiments, wrote the manuscript, and took overall responsibility. Chen Shaohua participated in study implementation and manuscript writing. Ruan Ming and Li Xuanru collected and organized data, performed statistical analysis, and prepared figures and tables. Li Ning revised the manuscript. Liang Qianqian was responsible for quality control, review, and supervision.

Conflict of Interest

The authors declare no conflict of interest.

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