Synovial and pulmonary dysfunctions are induced by crosstalk of Smad and Erk pathways in an arthritis model

Wan Lei¹, Liu Jian†, Huang Chuanbing¹, Chen Xi¹, Liu Lei¹, Liu Tianyang¹, Ge Yao¹, Fan Haixia¹, Zhao Lei², Li Zhneg³

¹The First Affiliated Hospital of Anhui University of Chinese Medicine, Hefei 340031, China
²Anhui University of Chinese Medicine, Hefei 340031, China
³Department of Cancer Biology, City of Hope National Medical Center and Beckman Research Institute, California 91010, USA

*Correspondence to: liujianahzy@126.com
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Abstract: In the current experiment, the effects of transforming growth factor (TGF)-β1/Smad and ERK pathway crosstalk on synovial and pulmonary systems during rheumatoid arthritis have been investigated. For this purpose, rats were divided into normal control (NC) and model control (MC) groups. In the MC group, 0.1 ml Freund’s complete adjuvant was injected intradermally into the right hind paw, and the resulting inflammation represented a rheumatoid arthritis model. Joint swelling and changes in lung functions were observed in arthritis rats. Synovial and lung were observed by light and electron microscopies. Enzyme-linked immunosorbent assays were used to detect TGF-β1, interleukin (IL)-1β, IL-4, IL-10, interferon-γ (IFN-γ), connective tissue growth factor (CTGF), and fibroblast growth factor (FGF). PCR, immunohistochemistry, and immunoblotting were used to detect changes in Smad and ERK pathways of synovial and lung tissues. Compared with the NC group, toe swelling was elevated in the MC group. Pulmonary functions FEV1, FEF50, FEF75, MMF, and PEF were decreased (P<0.01). Serum cytokines IL-1β, IL-4, TGF-β1, and CTGF were increased, while IFN-γ, IL-10, Th1/Th2 cell ratio, and FGF were decreased (P<0.01 or P<0.05). Expression of TGF-β1 and Smad2/3/4 mRNAs and TGF-β1, TβRI, TβRII, Smad2/3, p-Smad2/3, and Smad4 proteins in the synovial membrane and lung tissue were increased, and expression of Smad7 mRNA and protein was decreased (P<0.01 or P<0.05). Expression of ERK2 mRNA and p-ERK1/2 protein was increased in the synovial membrane and lung tissue, and expression of ERK1/2 mRNAs and ERK1/2 and p-ERK1/2 proteins was increased in lung tissue (P<0.01 or P<0.05). Correlation analysis showed that FEV1 was negatively correlated with TGF-β1 mRNA and protein in arthritis rats, FEF25 was negatively correlated with Smad4 protein, and FEF50 was negatively correlated with the TβRII protein, and FEF75, TGF-β1 and Smad3 mRNAs. There was a negative correlation between Smad2/3 protein and a negative correlation between PEF and TGF-β1 protein (P<0.05). FEV50 and MMF were positively correlated with Smad7 mRNA (P<0.05). FEV1 was negatively correlated with ERK2 mRNA, and FEF25 was negatively correlated with p-ERK1/2 protein. FEF75 and MMF were negatively correlated with ERK1/2 and p-ERK1/2, respectively (P<0.05). ERK1 mRNA was positively correlated with Smad3 mRNA and TβRII protein, ERK2 mRNA was positively correlated with p-Smad2/3, and ERK1/2 protein was positively correlated with Smad2 mRNA, Smad4 protein, p-ERK1/2 protein, Smad4 mRNA, and p-Smad2/3 protein (P<0.05). p-ERK1/2 protein was negatively correlated with Smad7 protein (P<0.05). It is concluded that arthritic rats have synovial and systemic pulmonary damage. Smad and ERK pathway crosstalk leads to systemic lesions. Smad and ERK pathways are gradually activated by phosphorylation under the induction of the TGF-β1 promoter, and then participate in transcriptional activities, leading to the increase in synovial inflammation of arthritis, pulmonary lesions, and decreases in lung functions.

Key words: Rheumatoid arthritis; Arthritis; Lung function; TGF-β1/Smad pathway; ERK1/2 pathway.

Introduction

Rheumatoid arthritis (RA) can cause damage to articular cartilage and bone. The basic pathological manifestations of RA are synovitis and vasculitis. RA lesions occur in joints as well as other tissues and organs. Because lungs are rich in connective tissue and blood vessels, lung tissue is more likely to be affected and secondary to lung lesions. RA lung lesions include pulmonary interstitial fibrosis, pleurisy, pulmonary vasculitis, and pulmonary hypertension (1-5). Studies have found that the incidence of lung involvement in RA patients with disease duration of more than 8 years is as high as 50% (6-9). About 70.5% of patients with RA lung lesions have pulmonary interstitial lesions, causing pulmonary interstitial fibrosis (10-11). RA pulmonary interstitial fibrotic lesions are characterized by a sudden onset, rapid progression, and high mortality, which seriously affect the quality of life of RA patients. The early stage of RA pulmonary interstitial fibrosis changes to alveolitis that can progress to interstitial fibroblast proliferation, massive extracellular matrix deposition, coughing, wheezing, shortness of breath, chest tightness, and other difficulties in breathing. However, the early clinical manifestations of RA lung lesions are mild or atypical, and imaging indicators such as X-ray and CT often show no significant changes. Pulmonary function changes are advanced in the clinical manifestations of the respiratory system and chest imaging abnormalities (12-13). Therefore, because of the early or no atypical symptoms of RA lung disease, diagnosis is easily missed. Thus, it is necessary to systematically and deeply study the detrimental changes in lung functions of RA. A previous study has found that pulmonary function parameters FVC,
FEV1, MVV, FEF25, FEF50, FEF75, VC and PEF are significantly low in active RA patients (14). Therefore, it is of great importance to study RA lung lesions by observing changes in lung functions of patients.

Materials and Methods

Animals
Specific pathogen-free male Wistar rats were obtained from the Experimental Animal Center of Anhui Province (license number: SYXK (wan) 2005-02). Standard clean animal house. Freund’s complete adjuvant (FCA) was prepared from Sigma (United States). Interleukin (IL)-1β, IFN-γ, IL-4, IL-10, and TGF-β1 ELISA Kits were purchased from R&D (United States). A Trizol kit was purchased from Invitrogen (United States). Rabbit anti-TGF-β1, -Smad, and -ERK antibodies were purchased from Bioworld Biotechnology (United States).

Model establishment
Rats were divided into normal control (NC) and model control (MC) groups. MC group rats were injected with 0.1 ml FFA into the right hind paw to cause inflammation and establish the RA model.

Observation of main indicators

Pulmonary function test
Pulmonary function parameters included forced vital capacity (FVC), first-second forced expiratory volume (FEV1), the maximum expiratory flow of 25% of vital capacity (FEF25), the maximum expiratory flow of 50% of vital capacity (FEF25), 75% of vital capacity, the maximum expiratory flow (FEF75), the maximum expiratory mid-flow (MMF), and the maximum expiratory flow (PEF). A software analysis system was used for data acquisition, real-time analysis and control, curve dynamic display, trend graphing, lung function parameter data conversion, and storage.

Detection of serum IL-1β and other cytokines by ELISA
Serum IL-1β, IFN-γ, IL-4, IL-10, TGF-β1, CTGF, and FGF were determined by ELISAs, according to the ELISA Kit protocols. The ratio of IFN-γ to IL-4 indicated that of Th1 to Th2 cells (15-20).

Detection of TGF-β/Smad and ERK mRNAs in synovial and lung tissues by RT-PCR

According to the gene sequences in GenBank, TGF-β1 (NM-021578), Smad2 (NM-019191), Smad3 (NM-013095), Smad4 (NM-019275), Smad7 (NM-019275), Smad4 60 ℃, Smad7 65 ℃, ERK1 65℃, ERK2 58℃, GAPDH 60 ℃; PCR cycle from the second step cycle 30 to 35 cycles. According to the RT-PCR experimental procedure, changes in TGF-β, Smad2, Smad3, Smad4, Smad7, ERK1, and ERK2 mRNA were observed in the synovial membrane and lung tissue, and the ratio to the internal reference GAPDH was applied to determine the relative expression.

Western blotting of TGF-β/Smad and ERK pathway-associated proteins in synovial and lung tissues

After extraction of total protein from the synovial membrane and lung tissue, changes in each protein were detected by immunoblotting. The X-ray film was photographed by a scanner. Protein bands were analyzed by Band Scan software to calculate the gray value. The ratio of the gray values to the β-actin gray value as the internal reference was used to determine relative expression levels of TβRI, TβRII, Smad2/3, p-Smad2/3, Smad7, ERK1, and p-ERK1/2 proteins.

Statistics
The SPSS 18.0 software package was used for statistical analyses. The experimental data were expressed. Datasets were tested for normality. The t-test was used for comparison between groups. Correlations were determined by Spearman's analysis. The level of significance was P< 0.05.

Results

Joint swelling and changes in lung functions
At 6–8 hours after adjuvant injection, the right hind toes of the MC group showed redness and swelling, and the skin was tight and had local thermal sensation. At 2–3 days, swelling of the toes was aggravated. At 6–7 days, the toe was partially ulcerated. Compared with the NC group, swelling of the toes in the MC group was significantly lower, P< 0.01 (Figure 1 and Table1).

Joint and lung pathologies
Articular observations revealed that the synovial

Figure 1. Changes of Joint swelling in Rats. A: MC group B: NC Group.
Table 1. Changes in lung function (n=10, \( \bar{x} \pm s \)).

<table>
<thead>
<tr>
<th>Group</th>
<th>FVC</th>
<th>FEV(_1)</th>
<th>FEF(_{25})</th>
<th>FEF(_{50})</th>
<th>FEF(_{75})</th>
<th>MMF</th>
<th>PEF</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>6.09±2.04</td>
<td>68.6±16.38</td>
<td>45.2±26.21</td>
<td>39.4±16.84</td>
<td>37.8±15.87</td>
<td>39.2±15.74</td>
<td>39.5±14.85</td>
</tr>
<tr>
<td>MC</td>
<td>5.37±1.70</td>
<td>55.7±15.76</td>
<td>40.7±18.85</td>
<td>27.0±13.07</td>
<td>20.3±12.31</td>
<td>28.5±17.96</td>
<td>29.6±12.57</td>
</tr>
</tbody>
</table>

Note: *\(P < 0.05\), **\(P < 0.01\), \(\Delta\) vs NC, FEF25, FEF50, The lung function parameter FVC unit is ml, FEV1, FEF75, MMF, PEF unit is ml/s.

Synovial and lung ultrastructural observations

Ultrastructural observations of the synovial membrane indicated that the NC group had no deformation or swelling of mitochondria, rich endoplasmic reticulum, a clear nuclear membrane boundary, uniform chromatin distribution, and regular arrangement of condyles. Synovial cells of the MC group were deformed and swollen, mitochondria are swollen and destroyed, the rough endoplasmic reticulum was reduced, the nuclear membrane was incomplete and the boundary was unclear, and chromatin was unevenly distributed.

Ultrastructural observations of type II alveolar cells showed no significant abnormalities in the NC group. The cell membrane boundary of type II alveolar cells in the MC group was unclear. Microvilli of cells were reduced, the basement membrane of the pulmonary capillaries was edematous, and nuclei were irregular or condensed. The nuclear chromatin edge was aggregated, and the lamellar body was reduced and evacuated. Mitochondria in the cytoplasm were obviously swollen, and alveolar septal fibroblasts had proliferated in individual lung tissues together with collagen fibers occasionally. The ultrastructural changes of type II alveolar cells suggested that pulmonary interstitial fibrosis was secondary in the arthritis model (Figure 4-5).

Changes in cytokines, growth factors, and Th cells

Compared with the NC group, cytokines IL-1β and IL-4 were increased significantly, cytokines IFN-γ and IL-10 were decreased, and the Th1/Th2 cell ratio was
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 decreased in the MC group, P< 0.05 or P< 0.01. In addition, compared with the NC group, the expression of TGF-β1 and CTGF was increased, and FGF was decreased in the MC group, P< 0.01 (Tables 2-3).

**Changes in TGF-β1 and Smad mRNA expression of synovial and pulmonary tissues**

RT-PCR showed that the expression of TGF-β1, Smad2, Smad3, and Smad4 mRNAs in the synovium of the MC group was higher than that in the NC group. Expression of Smad7 mRNA was decreased, P< 0.05 or P< 0.01, and that of TGF-β1, Smad2, Smad3 and Smad4 mRNAs was increased in lung tissue of the MC group. See Table 4-5.

**Changes in ERK1/2 mRNA expression of synovial and lung tissues**

Compared with the NC group, the expression of ERK1/2 mRNA was increased in the synovial membrane and expression of ERK1/2 mRNAs was increased in lung tissue of the MC group, P< 0.01 or P< 0.05 (Table 6).

**Expression of TGF-β1 and Smad proteins in synovial and pulmonary tissues**

Immunoblotting showed that the levels of TGF-β1, TβRI, TβRII, Smad2/3, p-Smad2/3, and Smad4 were higher and the Smad7 protein level was lower in the MC group than in the NC group, P< 0.05 or P< 0.01 (Figure 6).

**Changes of ERK1/2 and p-ERK1/2 proteins in the synovial membrane and lung tissue**

Compared with the NC group, p-ERK1/2 proteins were significantly increased in the synovial membrane, P< 0.01, and lung tissue of the MC group, P< 0.05 or P< 0.01.
Correlation analysis

Correlation analysis between arterial lung function parameters and TGF-β1/Smads showed that FEF75 was negatively correlated with TGF-β1 and Smad3 mRNAs. FEV1 was negatively correlated with TGF-β1 mRNA, ERK2 mRNA, and TGF-β1 protein. FEF25 was negatively correlated with Smad4 and p-ERK1/2 proteins. FEF50 was negatively correlated with TβRII protein. There was a negative correlation between FEF75 and TβRI, Smad2/3, and p-ERK1/2 proteins, P < 0.05. MMF was negatively correlated with Smad2/3 and p-ERK1/2 proteins, P < 0.05. There was a negative correlation between PEF and TGF-β1 protein, P < 0.05. FEF50 was positively correlated with Smad7 mRNA, and MMF was positively correlated with Smad7 protein, P < 0.05.

Correlation analysis between TGF-β1/Smads and ERK1/2 showed that ERK1 mRNA was positively correlated with Smad3 mRNA and TβRII protein, P < 0.05. ERK2 mRNA was positively correlated with p-Smad2/3, P < 0.05. ERK1/2 protein was positively correlated with Smad2 mRNA and Smad4 protein, P < 0.05. p-ERK1/2 proteins were positively correlated with Smad4 mRNA and p-Smad2/3 proteins, P < 0.05. p-ERK1/2 was negatively correlated with Smad7 protein, P < 0.05 (Tables 7-8).

Discussion

RA is an autoimmune disease described by joint lesions, which can affect multiple organs (21-22). Moreover, through the blood flow and internal environment, lungs can detrimentally affect the whole body because of the deterioration of the internal environment, resul-

Table 7. Relationship between lung function and TGF-β1/Smads pathway in lung tissue.

<table>
<thead>
<tr>
<th>index</th>
<th>FVC</th>
<th>FEV_1</th>
<th>FEF_25</th>
<th>FEF_50</th>
<th>FEF_75</th>
<th>MMF</th>
<th>PEF</th>
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<tr>
<td>mRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF-β1</td>
<td>-0.145</td>
<td>-0.395*</td>
<td>-0.089</td>
<td>-0.301</td>
<td>-0.383*</td>
<td>-0.217</td>
<td>0.174</td>
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<td>Smad2</td>
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<td>-0.214</td>
<td>-0.047</td>
<td>-0.278</td>
<td>-0.103</td>
<td>-0.267</td>
<td>0.115</td>
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<td>Smad3</td>
<td>0.045</td>
<td>-0.156</td>
<td>-0.217</td>
<td>-0.185</td>
<td>-0.421*</td>
<td>-0.164</td>
<td>0.086</td>
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<td>Smad4</td>
<td>-0.154</td>
<td>-0.405*</td>
<td>0.174</td>
<td>-0.242</td>
<td>-0.264</td>
<td>-0.265</td>
<td>-0.145</td>
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<tr>
<td>Smad7</td>
<td>0.186</td>
<td>0.097</td>
<td>0.135</td>
<td>0.433*</td>
<td>0.143</td>
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<td>0.412*</td>
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<tr>
<td>ERK1</td>
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<td>-0.019</td>
<td>-0.154</td>
<td>-0.078</td>
<td>-0.313</td>
<td>-0.201</td>
<td>0.007</td>
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<tr>
<td>ERK2</td>
<td>-0.187</td>
<td>-0.405*</td>
<td>0.074</td>
<td>-0.232</td>
<td>-0.095</td>
<td>-0.185</td>
<td>-0.283</td>
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<td></td>
</tr>
<tr>
<td>TGF-β1</td>
<td>-0.117</td>
<td>-0.396*</td>
<td>0.106</td>
<td>0.064</td>
<td>-0.266</td>
<td>-0.201</td>
<td>-0.391*</td>
</tr>
<tr>
<td>TβRI</td>
<td>0.074</td>
<td>-0.145</td>
<td>-0.095</td>
<td>-0.226</td>
<td>-0.406*</td>
<td>-0.184</td>
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<td>TβRII</td>
<td>-0.096</td>
<td>-0.224</td>
<td>0.083</td>
<td>-0.405*</td>
<td>-0.165</td>
<td>0.066</td>
<td>-0.195</td>
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<tr>
<td>Smad2/3</td>
<td>-0.135</td>
<td>0.033</td>
<td>0.124</td>
<td>-0.213</td>
<td>-0.445*</td>
<td>-0.442*</td>
<td>-0.209</td>
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<tr>
<td>p-Smad2/3</td>
<td>-0.168</td>
<td>-0.245</td>
<td>0.036</td>
<td>-0.069</td>
<td>-0.399*</td>
<td>-0.118</td>
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<tr>
<td>Smad4</td>
<td>0.039</td>
<td>-0.051</td>
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<td>-0.296</td>
<td>-0.059</td>
<td>-0.247</td>
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<td>0.258</td>
<td>0.217</td>
<td>0.024</td>
<td>0.407*</td>
<td>0.105</td>
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<tr>
<td>ERK1/2</td>
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<td>-0.305</td>
<td>0.006</td>
<td>0.064</td>
<td>-0.466*</td>
<td>-0.301</td>
<td>-0.091</td>
</tr>
<tr>
<td>p-ERK1/2</td>
<td>0.274</td>
<td>-0.045</td>
<td>-0.395*</td>
<td>-0.154</td>
<td>-0.168</td>
<td>-0.439*</td>
<td>0.084</td>
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</table>

Note: The abscissa is associated with the ordinate index, * P < 0.05.

Table 8. Relationship between Smads and ERK pathways in lung tissue (r).

<table>
<thead>
<tr>
<th>index</th>
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<th>protein</th>
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<tr>
<td></td>
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<td>ERK2</td>
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<tr>
<td>mRNA</td>
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<tr>
<td>TGF-β1</td>
<td>0.165</td>
<td>-0.061</td>
</tr>
<tr>
<td>Smad2</td>
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<tr>
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<tr>
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<tr>
<td>TβRI</td>
<td>-0.007</td>
<td>0.245</td>
</tr>
<tr>
<td>TβRII</td>
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</tr>
<tr>
<td>Smad2/3</td>
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<td>0.066</td>
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<tr>
<td>p-Smad2/3</td>
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<td>0.442*</td>
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<tr>
<td>Smad4</td>
<td>0.033</td>
<td>-0.052</td>
</tr>
<tr>
<td>Smad7</td>
<td>-0.169</td>
<td>-0.061</td>
</tr>
</tbody>
</table>

Note: The abscissa is associated with the ordinate index, * P < 0.05.
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The pulmonary function parameter FEV1 was associated with ERK, and activation of the ERK1/2 signaling pathway also decreased lung functions in RA rats. After phosphorylation, ERK1/2 promotes cell proliferation and differentiation. Correlation analysis showed that ERK1 mRNA was positively correlated with Smad3 mRNA and TβRII protein, ERK2 mRNA was positively correlated with p-Smad2/3, and ERK1/2 protein was positively correlated with Smad2 mRNA, Smad4 protein. When the ERK pathway is activated, ERK1/2 translocates from the cytoplasm into the nucleus, transducing the signal from the membrane surface receptor (33-35). ERK1/2 then binds to transcription factors, such as calnexin and cytoskeletal proteins, in the nucleus to induce epithelial-to-mesenchymal transition in the cells, which converts lung fibroblasts into myofibroblasts, eventually leading to a decrease in lung functions during arthritis. This study found that the mRNA expression of ERK1 and ERK2 in lung tissue was increased when lung function parameters were decreased in the arthritis model. Protein expression of ERK1/2 and p-ERK1/2 was also elevated in lung tissue. These observations indicated that the reduction of lung functions in the arthritis model was closely related to the activation of the ERK1/2 pathway. Similar to the Smad pathway, the ERK1/2 pathway also bound to transcription factors in the nucleus to exert biological effects, leading to the occurrence of pulmonary interstitial fibrosis (36).

Correlation analysis of TGF-β1/Smad and ERK1/2 pathways in lung tissue of the arthritis model showed that decreased lung functions may be related to their activation. Therefore, crosstalk of Smad and ERK1/2 pathways may have resulted in the decreased lung functions of the arthritis model. This study found that, when the lung functions of the arthritis model were reduced, expression of ERK1/2 genes was increased in the lungs and synovium, and protein expression of ERK1/2 and p-ERK1/2 was also increased, thereby verifying the RA rats. Therefore, decreased lung functions may be associated with the activation of ERK1/2.

Acknowledgments
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Conflict of Interest
The authors declare that they have no competing interests.

Author’s contribution
W.L. and L.J. conceived and designed the study. The experiment was conducted by G.Y., F.H.X., Z.L., and L.Z.
analysed the data. W.L. wrote the manuscript including a figure by a critical discussion with H.C.B, C.X., L.L. and L.T.Y. All authors contributed to the final manuscript.

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