Roflumilast Reduced the IL-18-Induced Inflammatory Response in Fibroblast-Like Synoviocytes (FLS)

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1. INTRODUCTION

Rheumatoid arthritis (RA) is a common chronic inflammatory autoimmune disease that may affect all joints of the body, especially the small joints of the extremities. The symptoms of RA include swelling and stiffness of the joint. RA is characterized by continuous arthrosynovitis, causing the destruction of cartilage, bone damage, and deformity in joints, ultimately leading to disability, loss of quality of life, and premature death. The underlying mechanism of RA is complicated. Previous studies have demonstrated that the pathogenesis of RA is related to heredity, infection, hormones, and environmental factors. Interleukin-18 (IL-18) is a pro-inflammatory cytokine that belongs to the interleukin-1 (IL-1) family. IL-18 is primarily well known as an interferon-γ (IFN-γ)-inducing factor and has been demonstrated to play an important role in the innate and adaptive immunobiology. IL-18 can be expressed by a wide range of immune and nonimmune cells. The main function of IL-18 is to stimulate lymphocytes to produce IFN-γ and regulate cell activity, thereby increasing the expression of pro-inflammatory cytokines and chemokines such as IL-6, IL-8, TNF-α, CCL5, CXCL9, and CXCL10. Moreover, IL-18 can trigger the transcription of multiple inflammatory genes via the activation of nuclear factor-κB (NF-κB) to promote an inflammatory response. In the clinic, it is observed that the overproduction of IL-18 is closely related to RA. Fibroblast-like synoviocytes (FLS) are cells derived from the mesenchyme that line the internal synovial tissues and play an important role in the development of RA. FLS proliferate aberrantly when stimulated with pro-inflammatory cytokines such as IL-1β and TNF-α. When activated, FLS release multiple factors including pro-inflammatory cytokines, chemokines, and matrix-degrading enzymes, resulting in inflammation and destruction of the extracellular matrix (ECM). Therefore, FLS have been recognized as a potential therapeutic target for the treatment of RA.

Roflumilast is a selective phosphodiesterase-4 (PDE-4) inhibitor and is distinctive to cyclic adenosine 3′,5′-monophosphate (cAMP) degradation. Roflumilast has been approved for the treatment of chronic obstructive pulmonary disease (COPD) to reduce the risk of exacerbations in patients with COPD. Previous studies showed evidence that inhibition of PDE-4 could drive the resolution of inflammation.
Actually, as a PDE-4 inhibitor, the anti-inflammation effects of roflumilast have been proven in several studies.\textsuperscript{15,16} However, whether it is therapeutically effective in RA is unknown. This study aims to investigate the protective effects of roflumilast against IL-18-induced inflammation in FLS.

2. RESULTS

2.1. Effects of Roflumilast in Cell Viability of MH7A FLS. The molecular structure of roflumilast is shown in Figure 1A. First, we tested the cytotoxicity of roflumilast in FLS. Cells were treated with six doses of roflumilast ranging from 0.5 to 100 μM for 24 h. The results in Figure 1B show that roflumilast did not affect the cell viability of FLS when its concentration was not more than 10 μM. However, 50 and 100 μM roflumilast significantly reduced the cell viability, 9 and 12%, respectively. Therefore, 5 and 10 μM roflumilast were used in subsequent experiments.

2.2. Roflumilast Reduced IL-18-Induced Oxidative Stress in MH7A FLS. To assess the effect of roflumilast on oxidative stress in FLS, the production of reactive oxygen species (ROS) and malondialdehyde (MDA) was measured. As shown in Figure 2A, treatment with IL-18 induced a 3.1-fold increase of the level of intracellular ROS, which were reduced to 2.2- and 1.5-fold by two doses of roflumilast, respectively. Similarly, the results in Figure 2B show that the expression of MDA was significantly inhibited by the same doses of roflumilast, respectively, compared with the exposure to IL-18 only. Taken together, roflumilast shows a strong inhibitory effect on oxidative stress induced by IL-18 in FLS in a dose-dependent manner.

2.3. Roflumilast Reduced IL-18-Induced Expression and Secretions of Pro-inflammatory Cytokines in MH7A FLS. Pro-inflammatory cytokines play a pivotal role in the development of an inflammatory response. We tested the expressions and secretions of IL-6, IL-8, and TNF-α. As shown in Figure 3A–C, stimulation with IL-18 induced a significant increase of these three cytokines at mRNA level; however, 5 and 10 μM roflumilast reduced their expression remarkably, in a dose-dependent manner. As expected, the results in Figure 3D–F indicate a strong inhibitory effect of roflumilast on the secretion of these pro-inflammatory cytokines.

2.4. Roflumilast Reduced IL-18-Induced Expression and Secretions of Pro-inflammatory Chemokines. As shown in Figure 4A–F, exposure to IL-18 significantly increased the production of the pro-inflammatory chemokines CCL5, CXCL9, and CXCL10 in FLS, while the presence of 5 and 10 μM roflumilast significantly reduced the IL-18-induced production of CCL5, CXCL9, and CXCL10 in a dose-dependent manner at both mRNA and protein levels, respectively.

2.5. Roflumilast Reduced IL-18-Induced Expression and Secretions of MMP-3 and MMP-13. MMP-3 and MMP-13 are recognized as the main collagens responsible for the degradation of the articular extracellular matrix (ECM). The results in Figure 5A–D reveal that treatment with IL-18 resulted in a remarkable increase in expressions of MMP-3 and MMP-13. However, two doses of roflumilast suppressed the IL-18-induced expressions of MMP-3 and MMP-13 in a dose-dependent manner at both mRNA and protein levels.

2.6. Roflumilast Reduced IL-18-Induced Activation of AP-1. To evaluate the effect of roflumilast on activation of the transcriptional factor AP-1, we tested the protein expressions of c-Jun and c-Fos and the luciferase activity of AP-1. As illustrated in Figure 6A, expressions of c-Jun and c-Fos were increased at significantly higher levels by exposure to IL-18 only, while roflumilast reduced their expression in a dose-dependent manner. Congruously, the results in Figure 6B show that roflumilast also inhibited the luciferase activity of AP-1. Our data shows that roflumilast has an inhibitory effect on the activation of AP-1.

2.7. Roflumilast Reduced IL-18-Induced Activation of NF-κB. Activation of NF-κB is considered a key mediator in an inflammatory response and has been shown to drive the development of numerous diseases including RA. To determine the effects of roflumilast on IL-18-induced
activation of NF-κB, we measured the nuclear translocation of p65 and luciferase activity of NF-κB. The results in Figure 7A,B show that two doses of roflumilast significantly inhibited the nuclear translocation of p65 as well as the luciferase activity of NF-κB, compared with the remarkable increase induced by IL-18.

3. DISCUSSION

RA is one of the most common chronic inflammatory diseases around the world and brings a critical burden to patients, families, and the society. The risks of RA include not only the destruction of cartilage and deformity in joints but also systemic complications such as rheumatoid vasculitis, atherosclerotic cardiovascular disease, and RA-associated lung disease, which increase the mortality of patients with RA.17−19 Proliferative FLS play crucial roles in joint inflammation, destruction of cartilage, and bone damage.20 Therefore, it is meaningful to find the related crucial inflammatory target molecules in FLS for the research of the RA mechanism. The current study aimed to investigate the anti-inflammatory effects of roflumilast on IL-18-stimulated FLS and to elucidate the underlying mechanism involved. Our
results revealed that roflumilast significantly suppressed IL-18-induced oxidative stress through mitigating the production of ROS and MDA, inhibiting the expressions of pro-inflammatory cytokines and chemokines. In addition, roflumilast reduced the production of MMP-3 and MMP-13, which play an important role in the degradation of cartilage. Furthermore, roflumilast suppressed the activation of the transcriptional factor AP-1 and NF-κB.

Oxidative stress is defined as an imbalance between the production of ROS and antioxidants. This imbalance directly results in the damage to cells. Furthermore, oxidative stress can lead to chronic inflammation via activating a variety of transcription factors including NF-κB and AP-1, which mediate the expression of various inflammatory factors and enzymes to promote the development of inflammation. Several research studies pointed out that increased levels of ROS production in patients with RA were observed, leading to increased levels of different markers such as MDA. Our data shows that expressions of ROS and MDA were significantly increased in IL-18-induced FLS. Meanwhile, we found that 5 and 10 μM roflumilast significantly suppressed the generation of ROS and MDA. Therefore, we demonstrated the inhibitory effect of roflumilast on oxidative stress.

Figure 5. Roflumilast reduced IL-18-induced expressions and secretions of MMP-3 and MMP-13 in MH7A FLS. Cells were stimulated with IL-18 (10 ng/mL) in the presence or absence of 5 and 10 μM roflumilast. mRNA of MMP-3 (A) and MMP-13 (B); protein levels of MMP-3 (C) and MMP-13 (D) (**P < 0.001 vs vehicle group; ***P < 0.001 vs IL-18 group).

Several relevant pro-inflammatory cytokines have been determined to play important roles in the progression and development of RA, especially IL-6, IL-8, and TNF-α. They can not only activate FLS but also induce the expression of other cytokines, chemokines, and metalloproteinases. Interestingly, a previous study found that in cultured synovial cells from patients with RA, blockade of TNF-α with antibodies reduced the expressions of IL-6 and IL-8. In our study, overexpression of these three cytokines was observed in IL-18-induced FLS. Another research showed that roflumilast has an inhibitory effect on the expression of pro-inflammatory cytokines in a murine model of chronic asthma. As expected, in our study, we confirmed that roflumilast could suppress the

Figure 6. Roflumilast reduced IL-18-induced activation of AP-1 in MH7A FLS. Cells were stimulated with IL-18 (10 ng/mL) in the presence or absence of 5 and 10 μM roflumilast. (A) Protein expression of c-Jun and c-Fos and (B) luciferase activity of AP-1 (**P < 0.01, ***P < 0.001 vs IL-18 group).
expression of pro-inflammatory cytokines at both mRNA and protein levels, in a dose-dependent manner. Additionally, roflumilast also reduced the expression of pro-inflammatory chemokines such as CCL5, CXCL9, and CXCL10, which are responsible for recruiting immune cells to the synovial tissue, promoting the development of inflammation. ECM plays an important role in maintaining the structure of cartilage tissues in the joint. It has been demonstrated that ECM can be degraded by metalloproteinases such as MMP-3 and MMP-9 in osteoarthritis.27 Similarly, in our study, we found that treatment with roflumilast significantly reduced the production of MMP-3 and MMP-9 in IL-18-induced FLS. This finding indicates that roflumilast might have a potential effect in preventing the degradation of cartilage.

NF-κB and AP-1 are important regulatory transcription factors that are associated with inflammation and proved to be relevant to RA disease. Upon activation, free NF-κB formed by a heterodimer of p50 and p65, and c-Jun and c-Fos (the subunits of AP-1), pass into the nucleus. Subsequently, they bind to the promoter regions of genes for inflammatory proteins such as cytokines, chemokines, and enzymes.30,25 Besides, in animal arthritis models, inhibition of NF-κB delayed the destruction of joints.30 Meanwhile, evidence shows that activation of AP-1 is related to the production of pro-inflammatory cytokines in RA.31 In the present study, we found that the expressions of p65, c-Jun, and c-Fos were significant in IL-18-induced FLS. Our data demonstrates that roflumilast has a strong inhibitory effect on the activation of NF-κB and AP-1. A recent study also confirmed this viewpoint in a sepsis model.12

Taken together, the present study shows that roflumilast prevents the IL-18-induced inflammatory response in FLS. More research is required to further investigate the protective effects of roflumilast on RA.

4. MATERIALS AND METHODS

4.1. Cell Culture and Treatment. MH7A fibroblast-like synoviocytes (FLS) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin−streptomycin (P/S). Cells were stimulated with IL-18 (10 ng/mL, Cat# 10119-HNCE, SinoBiological) in the presence or absence of 5 and 10 μM roflumilast (Cat# A10804, Adooq Bioscience).16,18

4.2. Cell Viability. MH7A FLS (1 × 10^4/well) were added in a 96-well plate and cultured for 6–8 h. After being treated with roflumilast at different concentrations for 24 h, cell viability was determined using the MTT assay (Cat# ab211091, Abcam). The cells were incubated with the MTT assay at 37 °C for 4 h. The absorbance of each well was then measured at 570 nm using a microplate reader (Thermo Fisher Scientific), and the percentage viability was calculated.

4.3. Dihydroethidium (DHE) Staining. ROS levels were determined using the DHE assay (Cat# ab236206, Abcam). Briefly, the cells were cultured on 4-well chamber slides for 4 h and treated with IL-18 (10 ng/mL) in the presence or absence of 5 and 10 μM roflumilast for 24 h. The cells were then incubated with prepared DHE (5 μM) reagent for 30 min at 37 °C in the dark. After capturing the fluorescent image density using a fluorescence microscope, the fluorescence density of DHE staining was calculated using ImageJ software. Briefly, we characterized the regions of interest in the images. The integrated density value (IDV) of fluorescence and total cells in the regions were recorded. Average levels of intracellular ROS = IDV/total number of cells.

4.4. mRNA Isolation and Real-Time Polymerase Chain Reaction (PCR). Cells were plated at a concentration of 2.5 × 10^5 cells/well in 6-well plates. After the required treatment, mRNA was extracted with an RNase Mini Kit (Qiagen). Then, 2 μg of total RNA was reverse-transcribed to cDNA using SuperScript II Reverse Transcriptase (Thermo Fisher Scientific) and then subjected to real-time PCR using the iTaq Universal SYBR Green Supermix (BioRad). The cDNAs were then amplified with specific primers for IL-6, IL-8, TNF-α, CCL5, CXCL9, CXCL10, MMP-13, and MMP-3. All reactions were run in triplicate. A semi-quantitative analysis was performed based on the cycle number (CT) at which the SYBR Green fluorescent signal crossed a threshold in the log-linear range of real-time PCR. The fold change of all target genes was shown, relative to the change in the expressions of GAPDH RNA that was measured as an internal control.

4.5. Enzyme-Linked Immunosorbent Assay (ELISA). The secretion levels of IL-6, IL-8, TNF-α, CCL5, CXCL9, CXCL10, MMP-3, and MMP-13 were determined using the ELISA assay. For ELISA assay, the cells were cultured in a coated 96-well plate. ELISA was performed following the protocol from the manufacturer. The following ELISA kits were used in this study: IL-6 (Cat# E-EL-R0015c, Elabscience), IL-8 (Cat# E-EL-H6008, Elabscience), TNF-α (Cat# kt99985, MSK), CCL5 (Cat# SEKRT-0136, Solarbio), CCL9 E (Cat# EK122S, Boster), MMP-3 (Cat# k90046, MSK), and MMP-13 (Cat# ab100605, Abcam).

4.6. Protein Isolation and Western Blots. Protein was isolated from the cells using the radioimmunoprecipitation assay (RIPA) buffer. The concentration of protein was determined using the Pierce BCA Protein Assay Kit (Cat# 23225, Thermo Fisher Scientific). Protein (20–40 μg) was loaded in the wells of Nu-PAGE precast 4–12% sodium dodecyl sulfate (SDS)-poly-acrylamide gels (Invitrogen) and then transferred onto poly(vinylidene fluoride) membranes by a dry electrophotograph method using an I-Blot. The membranes were then incubated with specific primary antibodies including c-Jun antibody (#9165, Cell Signaling Technology), c-Fos antibody (#2250, Cell Signaling Technology), NF-κB p65 (#3034, Cell Signaling Technology), secondary antibodies including mouse anti-rabbit IgG-HRP (Santa Cruz, sc-2357, Santa Cruz Biotechnology), and Goat Anti-Mouse IgG-HRP (SE131, Solarbio) step by step and incubated with visualized SuperSignal West Atto Ultimate Sensitive Substrate (Cat# A38555, Thermo Fisher Scientific) for 5 min before visualizing on the X-ray film (no. C500046, Sango Biotech) in the darkroom.

4.7. Luciferase Activity. The cells (2 × 10^6) cultured in a 6-well plate were transfected with AP-1 luciferase plasmid, NF-κB luciferase plasmid, or a plasmid encoding β-galactosidase following the product instructions. The cells were collected and centrifuged for 5 s at 12,000 rpm to pellet the cell debris. The supernatant was collected in a new tube. Then, 20 μL of the cell extract was mixed with 100 μL of the luciferase assay reagent at room temperature. The luciferase reporter activity was measured using a luminometer reader, and the result was normalized to the β-galactosidase activity.

4.8. Statistical Analysis. The data was displayed as mean ± standard deviation (SD). The contrast among different groups was analyzed using the analysis of variance (ANOVA), followed by the Student−Newman−Keuls post hoc test. P-
value < 0.05 was regarded as a statistically significant difference between the groups.

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**Notes**

The authors declare no competing financial interest.

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