Celastrol Inhibits Lipopolysaccharide-Stimulated Rheumatoid Fibroblast-Like Synoviocyte Invasion through Suppression of TLR4/NF-κB-Mediated Matrix Metalloproteinase-9 Expression

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Abstract

Invasion of fibroblast-like synoviocytes (FLSs) is critical in the pathogenesis of rheumatoid arthritis (RA). The metalloproteinases (MMPs) and activator of Toll-like receptor 4 (TLR4) are crucial in initiating and driving RA in concert with inflammatory populations that might participate in RA pathogenesis, FLSs are specific pathways or cell lineages. Among the inflammatory cell populations that might participate in RA pathogenesis, FLSs are crucial in initiating and driving RA in concert with inflammatory cells. They contribute to the destruction of cartilage and bone by secreting metalloproteinases (MMPs) into the synovial fluid and by direct invasion into extracellular matrix (ECM), further exacerbating joint damage [4,5]. The migration of activated FLS is partly responsible for spreading arthritis destruction to distant joints [6]. FLSs have inherent invasive qualities not observed in other fibroblasts, and initial descriptions of a tumor-like phenotype were reported by Fassbender in the early 1980s [7]. In addition, RA-FLSs share many similar biological properties with tumor cells: they undergo tumor-like proliferation, migration and invasion, as well as possessing an increased resistance to apoptosis [8]. The control of RA-FLS invasion represents an important therapeutic target.

MMPs are involved in the development and processes of RA [9] and are responsible for the invasion properties of many cell lines [10]. MMP-2 and MMP-9, also called collagensases, degrade type IV collagen, gelatin and elastin, and are induced in RA-FLSs by pro-inflammatory cytokines, through the activation of transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) [11]. It is well established that pro-inflammatory

Introduction

Rheumatoid arthritis (RA) is a progressive inflammatory autoimmune disease mainly affecting the joints, characterized by synovial hyperplasia and inflammatory cell infiltration, leading to tissue destruction and functional disability [1,2]. Its exact cause is unknown, but genetic and environmental factors are contributory. The pathobiology of RA is multifaceted and involves T cells, B cells, fibroblast-like synoviocytes (FLSs) and the complex interaction of many pro-inflammatory cytokines. Novel biologic agents that target tumor necrosis factor or interleukin (IL)-1 and IL-6, in addition to T- and B-cell inhibitors, have resulted in favorable clinical outcomes in patients with RA [3]. Despite this, at least 30% of RA patients are resistant to available therapies, suggesting novel mediators should be identified that can target other disease-specific pathways or cell lineages. Among the inflammatory cell populations that might participate in RA pathogenesis, FLSs are crucial in initiating and driving RA in concert with inflammatory cells. They contribute to the destruction of cartilage and bone by
cytokines are key mediators of RA-FLS invasion and involved in the pathogenesis of RA [4]. Lipopolysaccharide (LPS), a cell wall constituent of gram-negative bacteria, is released during bacterial lysis and exerts a direct effect on tumor cell proliferation, invasion and metastasis in vitro and in vivo. Toll-like receptor 4 (TLR4), the receptor for LPS, also expressed in FLSs, is important in the regulation of immune responses and is involved in inflammation-induced cell motility [12]. Various signal transduction pathways are related to RA pathogenesis, and the TLR4 signaling pathway especially has a crucial role in RA. The most direct evidence to date that interfering with TLR4 might have therapeutic potential in RA was shown by Abdollahi-Roodaz et al., where inhibition of TLR4 signaling with a specific TLR4 antagonist prevented the inflammatory feedback loop in a mouse model of autoimmune destructive arthritis, and suppressed both clinical and histologic characteristics of arthritis [13]. Silencing TLR4 expression also inhibited cancer cell invasion properties, indicating that TLR4 plays a significant role in connecting inflammation and cancer cell invasion [14]. However, the role and mechanism of LPS/TLR4 on FLS invasion is still not clear.

 CELASTROL, a pentacyclic-triterpene extract from Tripterygium wilfordii Hook, is used in traditional Chinese medicine as an anti-tumor agent [15,16]. In recent years, an increasing number of plant-derived herbal products have been considered for the treatment of RA [17]. However, the mechanisms of action of many potentially anti-arthritic plant products have not been fully defined. Recent reports suggest that Celastrol extract and bioactive celastrol have beneficial anti-arthritic effects in an adjuvant-induced arthritis (AIA) model [15,18,19]. Studies to define the anti-arthritic therapeutic mechanism of celastrol in an AIA model showed it suppressed key proinflammatory cytokines (IL-17, IL-6, and IFN-γ), serum levels of anti-cyclic citrullinated peptides antibodies and MMP-9 activity [15]. It also modulated immune responses rather than induced immunosuppression [20], inhibited synovial immune cell infiltration and proliferation [21], reduced inflammation-induced bone damage primarily via the deletion of receptor activator of NF-κB ligand/osteoprotegerin ratio in favor of anti-osteoelastic activity, and reduced osteoclast numbers [19]. However, the effect and molecular mechanisms underlying the effect of celastrol on LPS induced RA-FLS invasion are poorly understood. In the present study, we treated human RA-FLSs with celastrol in vitro and examined the effect of celastrol on LPS-induced cell migration and invasion. Furthermore, we investigated the mechanisms involved and evaluated the therapeutic efficacy of celastrol in an in vivo collagen-induced arthritis (CLA) rat model to determine the potential therapeutic efficacy of celastrol to treat RA-type diseases.

Materials and Methods

Reagents

The TLR4 signaling pathway inhibitor TAK-242 (TAK) and TLR4 neutralizing antibody (anti-TLR4) were from Sigma-Aldrich (St. Louis, MO, USA).

Experimental Treatment with Celastrol

Purified celastrol (Fig. 1A) isolated from Caulasus scandens was obtained from Calbiochem (La Jolla, CA, USA). The powder was dissolved in dimethyl sulfoxide (DMSO) and stored as aliquots (20 mM) at −20°C until used, following the method described previously [22]. Celastrol was dissolved in 10% DMSO, and vehicle (distilled water containing 10% DMSO) was used as a control. For simplicity, Celastrol-DMSO and ddH₂O-DMSO refer to celastrol and control, respectively, throughout the manuscript.

Ethical Declaration

FLSs were obtained from the synovium of active RA patients during knee joint arthroscopy according to the approval of the Ethics Committee of Showa University (IRB no. 20110032). Patients gave their written informed consent. All enrolled patients with active RA satisfied the American Rheumatism Association 1987 diagnostic criteria for RA [23]. Active RA was defined as ≥6 swollen joints (28-joint count), ≥6 tender joints (28-joint count), and at least 1 of the following: erythrocyte sedimentation rate greater than the upper limit of normal (ULN) for the local laboratory or C-reactive protein level greater than the ULN for the central reference laboratory (>8.0 mg/liter). Animal experiments protocols were approved by the Animal Care and Use Committee of Showa University (ACUC no. 01167).

Cell Culture and Flow Cytometry

FLSs were isolated from synovial tissues by enzymatic digestion as previously described [24]. FLSs were grown in Dulbecco’s modified Eagle’s medium/Nutrient Mixture F-12 (DMEM/F-12) (GIBCO, Grand Island, NY, USA) medium containing 10% fetal bovine serum (FBS), supplemented with antibiotics (100 mg/mL streptomycin and 100 U/mL penicillin) in a humidified incubator at 37°C under 5% CO₂, 21% O₂, and 75% N₂ (Sanyo, Osaka, Japan). Cells used for experiments were at the third to sixth passage. CD14 and CD68 are macrophage markers and CD90 is a fibroblast marker. Isolated RA-FLSs were identified by flow cytometry (FCM; BD Biosciences, San Jose, CA, USA) as a homogeneous population (phenotype: <1% CD14, CD68 and >98% CD90, data not shown). Briefly, Cells were washed three times with phosphate-buffered saline (PBS) and then were treated with fluorescein isothiocyanate (FITC)-conjugated anti-CD68, CD90 antibody (BD Biosciences, San Jose, CA, USA), PerCP-conjugated anti-CD14 antibody (R&D Systems, Minneapolis, MN, USA) or FITC-conjugated Mouse IgG1 (BD Biosciences), as a control for 20 min in the dark. Cells were washed with PBS and then analyzed by FACS Calibur FCM.

Cell Viability Assay

All viability assays were based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Briefly, FLSs were seeded in a 96-well plate at a density of 1×10⁶ cells/well. After treatment with various concentrations of celastrol (0.05, 0.1, 0.2, 0.4 and 0.8 μM) for 30 min at room temperature. Cells were pelleted and lysed in 100 μL of DMSO, and the absorbance at 550 nm was measured using a microplate reader (Thermo, Waltham, MA, USA).

Cell Cycle Determination

Cell cycle distribution was analyzed by FCM. Briefly, FLSs were plated at a density of 1×10⁶ cells per 100-mm culture dish and treated with different concentrations of celastrol (0.05, 0.1, 0.2 and 0.4 μM) in triplicate for 24 h. Subsequently, the cells were harvested, washed twice with PBS, and fixed in 70% ethanol at 4°C for 1 h and centrifuged. Fixed cells were incubated with RNase (50 μg/mL) for 30 min prior to staining nucleic acids with propidium iodide (50 μg/mL) for 30 min at room temperature. The sub G₁ value in each group was analyzed by FCM.
In vitro Migration and Invasion Assay

Cell migration in vitro was determined using 6.5 mm Transwell chambers with 8 μm pores (Corning, NY, USA). Celastrol treated-FLSs (1×10⁵ cells) were plated in the upper chambers in duplicate filters. Serum-free culture medium with or without 1 μg/mL LPS (Escherichia coli, strain 0128:B12, Sigma) was added to the lower part of the chambers. Cells were allowed to migrate for 24 h. After a 24 h incubation period at 37°C, the non-migrating cells were removed from the upper surface by cotton swabs and the filters were stained with crystal violet. Cells that migrated through the membrane to the lower surface were counted in five representative microscopic fields (×100 magnification) and photographed. Cell invasion ability was determined using Matrigel invasion chambers (BD Biosciences, Tokyo, Japan) according to the manufacturer’s instructions.

Figure 1. Effect of celastrol on LPS-induced RA-FLS migration and invasion. A: Chemical structure of celastrol. B: FLSs were incubated with the indicated concentrations of celastrol in serum containing medium for 24 h, and cell viability was measured by MTT assay. *P<0.05, **P<0.01 versus normal control group. C: FLSs were incubated with the indicated concentrations of celastrol for 24 h. Cells were harvested and the cell cycle distribution in the sub-G1 phase was determined by FCM analysis. ***P<0.01 versus normal control group. D and E: The migration and invasion abilities of FLSs were detected by cell migration and invasion assay. FLSs were pretreated with the indicated concentrations of celastrol for 24 h. Then, FLSs were allowed to migrate with or without LPS (1 μg/mL) for 24 h. The number of migrating and invasive cells in each chamber was plotted as the mean ± SD in three independent experiments. The results were analyzed by ANOVA. **P<0.01 versus normal control group, *P<0.05, ***P<0.01 versus LPS alone-treated group.
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instructions. The upper chambers were freshly coated with Matrigel and medium was added to the lower chamber as described above. RA-FLSs (5 × 10⁴ cells) were suspended in medium containing 2% FBS and seeded into Matrigel pre-coated Transwell chambers. Cell invasion was allowed to occur for 24 h and the gel and cells on the top membrane surface were removed with cotton swabs. Cells that had penetrated to the bottom were counted.

Small Interfering RNA Transfection

FLSs were transiently transfected with small interfering RNA (siRNA) that targeted MMP-9 (Applied Biosystems, Austin, TX, USA) as described in the text. Briefly, siRNA (1 mg) for human MMP-9 (GenBank accession number NM_004994) was suspended in 100 μL of Lipofectamine 2000 solution and mixed with an equal volume of serum-free DMEM medium. The mixture was added to 5 × 10⁴ FLSs cultured in 100-mm dishes. Control siRNA was used as a negative control. Silencing effects were confirmed by western blot. Samples were run in triplicate and all experiments were performed at least twice.

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using Trizol according to the manufacturer’s protocol. qRT-PCR was performed using the ABI PRISM 7900HT cycler (Applied Biosystems) and SuperScript™ III Platinum® SYBR® Green one-step qRT-PCR kit (Invitrogen, Carlsbad, CA, USA). Primer sequences are shown in Table 1. Primer specificity was assessed from monophasic dissociation curves, and all had a similar efficiency (data not shown). The forward and reverse primers were designed using Primer Express software (version 2.0-PE Applied Biosystems). Primer sequences are shown in Table 1. Primer specificity was assessed from monophasic dissociation curves, and all had a similar efficiency (data not shown). The threshold cycle (Ct) for the endogenous control GAPDH mRNA and target signals were determined, and relative RNA quantification was calculated using the comparative 2⁻ΔΔCt method where ΔΔCt = (Ct Target – Ct GAPDH) – (Ct Control – Ct GAPDH). All reactions were performed in duplicate.

Table 1. Primers used for qRT-PCR.

| Gene   | Forward Sequence (5’–3’)                  | Reverse Sequence (5’–3’)                  |
|--------|------------------------------------------|------------------------------------------|
| MMP1   | ACTCTGGAGAATGTCACACT   GTGTTCCACCTTCTCTCA |                                          |
| MMP2   | CCCTGCGGCATCACTGAGTT  GTCGTCGGGCGATCCAAA  |                                          |
| MMP3   | AGCTTTCAACTCCTGTTGCT  TCCCTGCTACCAATCC   |                                          |
| MMP9   | GGACACGGCAGACTGTCATC   TCAGTCTGCAGGATACGG  |                                          |
| GAPDH  | ATCCCGCTTTACATCAATG    GTGTTCCACACCCCAAA  |                                          |

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MMP-9 was established using known concentrations of cytokine by plotting optical density vs log of the concentration.

Western Blot Analysis

After experimental treatment, whole cell lysates from FLSs were generated using a Total Protein Extraction Kit (Millipore, Billerica, MA, USA) according to the manufacturer’s instructions. Protein concentrations were determined using a Pierce BCA Protein Assay Kit (Thermo Scientific). Equal amounts of protein (30 μg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to enhanced chemiluminescence (ECL) nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). After blocking with 5% BSA for 2 h, blots were probed with primary antibodies against 15 kDa (1:400), phospho-1xβz (p-1xβz) (1:500), TLR4 (1:500), TRIF (1:400), MyD88 (1:500), MMP-2 (1:400), MMP-9 (1:400) and β-actin (1:1000), and then incubated with secondary anti-mouse antibody (1:10000) for 2 h. All antibodies were from Santa Cruz (Santa Cruz, CA, USA). Membranes were then incubated with appropriate secondary antibodies for 2 h at room temperature. ECL reagent (GE Healthcare) was used for protein detection. β-Actin was used as an internal control. The relative expression of each protein was determined by densitometric analysis and normalized to the control. Each blot shown is representative of at least three similar independent experiments.

Gelatin Zymography

The enzymatic activities of MMP-2 and MMP-9 were determined by gelatin zymography. Briefly, cells were seeded and allowed to grow to confluence and then incubated in serum-free medium for 24 h. The supernatants were collected 24 h after stimulation, mixed with non-reducing sample buffer, and separated by 10% SDS-PAGE containing 1% gelatin. After electrophoresis, gels were renatured by washing in 2.5% Triton X-100 solution twice for 30 min to remove all SDS. The gels were then incubated in 50 mM Tris-HCl (pH 7.5), 5 mM CaCl₂ and 1 μM ZnCl₂ at 37°C overnight. Gels were then stained with 0.25% Coomassie brilliant blue R-250 for 30 min and then destained in distilled water.

Transient Transfection and Luciferase Reporter Assay

To determine promoter activity, we used a dual-luciferase reporter assay system (Promega, Madison, WI, USA). MMP-9, NF-κB and AP-1 promoter luciferase reporter plasmids and the mutants MMP-9 mutant NF-κB (mNF-κB) and MMP-9 mAP-1 were constructed using standard molecular biology techniques as previously described [25]. RA-FLSs (1 × 10⁴) were seeded into 24-well plates and incubated at 37°C. Cells at 70-80% confluence were co-transfected with reporter constructs and Renilla luciferase reporter vector using Lipofectamine 2000 (Invitrogen) for 24 h according to the manufacturer’s protocol. In the same experiment, we added an empty control plasmid to ensure that each transfection received the same amount of total DNA. The transfected cells were incubated with celastrol and then stimulated with 1 μg/mL of LPS. To assess promoter activity, cells were collected and disrupted by sonication in lysis buffer. After centrifugation, aliquots of the supernatants were assayed according to the manufacturer’s protocol (Promega) using a LuminoMeter (Turner BioSystems, Sunnyvale, CA, USA). Relative luciferase activity was normalized to Renilla luciferase activity and expressed as the mean of three independent experiments.
Figure 2. Celastrol inhibits LPS-induced RA-FLS migration and invasion by suppression of MMP-9 activity. FLSs were incubated with the indicated concentrations of celastrol for 24 h followed by LPS (1 μg/mL) stimulation. A and B: After 24 h, the mRNA and protein levels of endogenous MMP-1,2,3,9 were measured by qRT-PCR and western blot. GAPDH and β-actin were used as the internal controls, respectively. The histogram shows the mRNA levels from three independent experiments. *P<0.01 versus normal control group, **P<0.05, ***P<0.01 versus LPS.
alone-treated group. C and D: The supernatants were collected and assayed for the amount and activity of secreted MMP-9, by ELISA and gelatin zymography, respectively. The histogram shows the ELISA results from three independent experiments. *P<0.01 versus normal control group, **P<0.05, ***P<0.01 versus LPS alone-treated group. E: MMP-9 siRNA was used to study the role of MMP-9 in LPS-induced migration and invasion of FLSs. After transfection with MMP-9 siRNA or control siRNA for 48 h, and stimulation with LPS, FLSs were allowed to migrate for 24 h. Left, photomicrographs of cells that have passed through membrane (100× magnification). Right, the cell number per microscopic field was plotted (mean ± SD in three independent experiments) in the histogram. *P<0.01 versus normal control group. **P<0.05 versus LPS and control siRNA treated group.

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Electrophoretic Mobility Shift Assay (EMSA)

Cell nuclear lysates were harvested using a NucBuster™ Protein Extraction Kit (Novagen, Germany) according to the manufacturer’s instructions. Nuclear extracts (10 μg) were used to detect NF-κB and AP-1 translocation. Nuclei were resuspended in lysis buffer supplemented with 0.5 mM dithiothreitol and 0.2 mM phenylmethylsulfonyl fluoride. The AP-1 consensus oligonucleotides (5'- CGC TTG ATG AGT CAG CCG GAA -3') and NF-κB consensus oligonucleotides (5'-AGT TGA GGG GAC TTT CCC AGG C -3') labeled with 32P by T4 polynucleotide kinase (Promega) were incubated with nuclear extracts in binding buffer at 30°C for 30 min. The free DNA and DNA-protein mixtures were resolved using a 5% native polyacrylamide gels in 0.5×TBE buffer (0.4 M Tris, 0.45 M boric acid, 0.5 M EDTA, pH 8.0) by EMSA. Gels were dried and subjected to autoradiography analysis.

Chromatin Immunoprecipitation (ChIP) Assay

To detect the in vivo association of nuclear proteins with the human MMP-9 promoter, chromatin from FLSs was fixed and immunoprecipitated using the ChIP assay kit as recommended by the manufacturer (Upstate Biotechnology, NY, USA). Immune complexes were prepared using anti-NF-κB p65 antibody and anti-IgG antibody (Santa Cruz). The supernatant of an immunoprecipitated complexes were prepared using anti-NF-κB antibody (Promega) were incubated with nuclear extracts in binding buffer at 30°C for 30 min. The presence of the selected DNA sequence was assessed by PCR. After DNA purification, PCR primers for the MMP-9 promoter (373 bp including NF-κB consensus oligonucleotides (5'-AGT TGA GGG GAC TTT CCC AGG C -3') and NF-κB consensus oligonucleotides (5'-AGT TGA GGG GAC TTT CCC AGG C -3') labeled with 32P by T4 polynucleotide kinase (Promega) were incubated with nuclear extracts in binding buffer at 30°C for 30 min. The free DNA and DNA-protein mixtures were resolved using a 5% native polyacrylamide gels in 0.5×TBE buffer (0.4 M Tris, 0.45 M boric acid, 0.5 M EDTA, pH 8.0) by EMSA. Gels were dried and subjected to autoradiography analysis.

Induction and Evaluation of CIA

Female Wistar rats (5–6 weeks old, 170±10 g) were purchased from Sankyo Labo Service Co., Ltd. (Saitama, Japan). Animals were housed with free access to food and water, and acclimatized to standard laboratory conditions (23°C ±3°C, 55% ±10% humidity, and 12 h light/dark cycles) for 7 days. For the assessment of acute toxicity, female Wistar rats were randomly divided into seven groups (1 control group and 6 treated groups) of six animals each. Celastrol suspended in 10% DMSO was administered intraperitoneally at doses of 1, 5, 10, 20, 30 and 40 mg/kg. The control group received an equal volume of vehicle by the same route. The animals were observed for mortality once a day for 14 days. The toxicological effect was assessed on the basis of mortality for 14 days, which was expressed by the median lethal dose value (LD50) estimated by the log-probit analysis method using GraphPad Prism software. The LD50 was 20.5 mg/kg intraperitoneally for celastrol.

CII (Sigma-Aldrich) from bovine articular cartilage was solubilized at a concentration of 0.5 M acetic acid (5 mg/mL) and the solution was emulsified in an equal volume of incomplete Freund’s adjuvant (Sigma-Aldrich) on ice. On day 0, rats were injected in the right hind metatarsal footpad with 100 μL of the emulsified CII. Twenty-one days after the primary immunization, rats received a booster immunization of CII as described previously. Control rats were injected with an equivalent volume of normal saline at both time points. Control rats were injected with an equivalent volume of normal saline at both time points. After performing pilot experiments on the modulation of CIA with different doses of celastrol ranging from 0.5 to 1 mg/kg, the two doses finally selected for use in this study corresponded to the LD50 dose as follows: 0.5 mg/kg and 1 mg/kg. On day 20 after the primary immunization, rats were randomly divided into the following groups: (1) CIA control group; (2) celastrol (0.5 mg/kg/d) treated CIA group; (3) celastrol (1 mg/kg/d) treated CIA group. A fourth group of rats was maintained as normal controls. Each group contained eight rats. Celastrol treatment was administered for 21 days intraperitoneally. CIA model and normal control rats were given an equal volume of vehicle by the same route.

The severity of arthritis was clinically evaluated using a four point scale by direct observation of all four limbs of each animal, based on the swelling of ankle and wrist joints and small interphalangeal joints as follows [26]: 0, no redness and swelling (normal joint); 1, redness and swelling in one digit or interphalangeal joint; 2, redness and swelling of more than one digit or interphalangeal joint, or mild redness and swelling of ankle or wrist joints; 3, moderate redness and swelling of ankle or wrist joints, but able to bend and walk; 4, severe redness and swelling and deformation of ankle or wrist joints, and inability to bend and walk. All four legs are scored, so the highest possible articular index (AI) was 16. The score was determined every 3 days until 3 weeks after treatment.

On day 42, the rats were sacrificed and the synovial membrane of right hind knee joints was prepared for histopathological analysis. The rats were anesthetized with sodium pentobarbital (60 mg/kg), and the joint cavity was opened and the synovial membrane was excised together with the patella, patellar ligament and joint capsule after detachment of patellar ligament from the upper part of the tuberosity of the tibia. The synovial membrane, together with infrapatellar fat pad, was separated from the patellar ligament and joint capsule. The separate synovial membrane was dried overnight in 4% paraformaldehyde and cryoprotected with 30% sucrose in PBS for 48 h. The synovium was sectioned at 5 μm thickness using a cryostat-microtome (Yamato, Tokyo, Japan) and stained with hematoxylin and cosin (H&E). The H&E-stained sections were used to determine the degree of synovial hyperplasia and inflammatory cells infiltration. Of these features, the degree of proliferation of synovial cells was scored as previously described [27].

Statistical Analysis

All values were expressed as the mean ±SD, unless otherwise stated. Results from different groups were analyzed by one-way analysis of variance (ANOVA) with Fisher’s probable least-squares difference test or Student’s t-test. Statistical analysis was performed using SAS 9.2 software (SAS Institute Inc., NC, USA). Differences
Figure 3. Celastrol inhibits the transcriptional activity of MMP-9 by suppression of LPS stimulated NF-κB activity. A: RA-FLSs were transfected with pGL2-MMP-9WT and Renilla luciferase reporter vector plasmids. The transfected cells were treated with the indicated concentrations of celastrol and/or LPS for 24 h. The relative luciferase activity in the cell extract was normalized to Renilla luciferase activity. Each value represents the mean ± SD of triplicate experiments and is expressed relative to the control. B, C and D: FLSs were transfected with pGL2-MMP-9 mNF-κB, pGL2-MMP-9 mAP-1-U and pGL2-MMP-9 mAP-1-P. The transfected cells were treated with the indicated concentrations of celastrol and/or LPS for 24 h. The relative luciferase activity is normalized to Renilla luciferase activity and is expressed relative to the controls. E and F: FLSs were transfected with reporter plasmids containing tandem elements for NF-κB or AP-1 binding sites. After 24 h, cells were treated with or without 1 μg/mL of LPS for 24 h and the luciferase activities were determined. *P<0.05, **P<0.01 versus normal control group, *P<0.05, **P<0.01 versus LPS alone-treated group. doi:10.1371/journal.pone.0068905.g003
resulting in probability ($\textit{P}$) values less than 0.05 were considered statistically significant.

**Results**

**Effect of Celastrol on LPS-induced RA-FLS Migration and Invasion**

The cytotoxic effect of celastrol on human RA-FLSs was examined (Fig. 1B). FLSs were treated with various concentrations of celastrol in serum-containing medium for 24 h, and cell viability was determined using the MTT assay. Treatment with 0.05 to 0.2 $\mu$M celastrol had no significant effect on cell viability at 24 h. However, 0.4 $\mu$M and 0.8 $\mu$M celastrol decreased cell viability by approximately 1- and 2-fold, respectively, in comparison with the control group. Cell cycle analysis by FCM showed celastrol did not influence the cell cycle transition at low doses (0.05, 0.1 and 0.2 $\mu$M), but a high dose (0.4 $\mu$M) caused sub-G1 accumulation (Fig. 1C). Therefore, celastrol had no significant cytotoxicity in FLSs at low doses. Based on data from preliminary studies, three different concentrations of celastrol (0.05, 0.1 and 0.2 $\mu$M) were chosen for the following experiments. Whether celastrol could inhibit LPS-induced cell migration and invasion was analyzed using a Transwell chamber. As shown in Fig. 1D and E, the in vitro migration ability of FLSs was increased 3.05-fold when stimulated with LPS for 24 h. Similarly, data obtained from the invasion assay showed that LPS increased cell invasion 7.02-fold in comparison with the control group. However, LPS-induced cell migration and invasion were inhibited by celastrol in a dose-dependent manner. These results suggest that non-toxic concentrations of celastrol ranging from 0.05 $\mu$M to 0.2 $\mu$M could inhibit RA-FLS migration and invasion induced by LPS in vitro.

**Celastrol Inhibits LPS-induced RA-FLS Migration and Invasion by Suppression of MMP-9 Activity**

As celastrol inhibited RA-FLS migration and invasion, we examined the effect of celastrol on MMPs gene expression. As shown in Fig. 2A and B, LPS induced MMP-1, MMP-2 and MMP-9 mRNA expression in RA-FLSs, whereas treatment with
Celastrol suppressed LPS-induced MMP-9 expression in a dose-dependent manner. However, MMP-1, MMP-2 and MMP-3 mRNA expression were not affected by celastrol treatment. We next examined the effect of celastrol on the secretion and proteolytic activity of MMP-9 protein in conditioned medium. As shown in Fig. 2C and D, the secretion and proteolytic activity of MMP-9 in FLSs were induced when cultured in serum-free medium with 1 μg/mL LPS for 24 h. The treatment of FLSs with celastrol suppressed LPS-induced MMP-9 secretion and activity in a dose-dependent manner. These results indicated that celastrol selectively inhibited LPS-induced MMP-9 expression at both the gene and protein levels, and subsequently suppressed the enzymatic activity of MMP-9. Furthermore, to identify the role of MMP-9 in the invasiveness induced by LPS, siRNA was used to inhibit MMP-9. After siRNA knockdown of MMP-9 and stimulation with LPS (1 μg/mL), the number of cells migrating and invading the basal membrane decreased by 70% compared with the control siRNA group (Fig. 2E). These results indicate that MMP-9 might contribute to the migration and invasion induced by LPS.

Celastrol Inhibits the Transcriptional Activity of MMP-9 by Suppression of LPS Stimulated NF-κB Activity

The two principal pathways activated MMP-9 are the NF-κB and AP-1 pathways [28,29], which induce MMP-9 expression. The MMP-9 gene is regulated at the transcriptional level by interactions between NF-κB and AP-1 binding sequences in the MMP-9 promoter region [30]. Since celastrol suppresses mRNA expression of MMP-9, to test which of these transcription factors may regulate the MMP-9 gene in FLSs, the cells were transiently transfected with reporter genes that included the wild-type MMP-9 promoter or a promoter with mutations in the NF-κB site or one or both AP-1 sites. As shown in Fig. 3A–D, treatment with celastrol in the presence of LPS decreased the transcription activity of the reporter with the AP-1 mutation, but had no effect on the reporter with NF-κB mutations, suggesting that the target of celastrol is the NF-κB transcription factor. As shown in Fig. 3E and F, luciferase activity in FLSs transfected with the NF-κB reporter was dose-dependently reduced by treatment with celastrol, whereas celastrol had no statistically significant
effect on the luciferase activity of FLSs transfected with AP-1 reporters.

**TLR4 Mediates LPS-induced NF-κB/MMP-9**

Furthermore, the TLR4 pathway inhibitor TAK and TLR4 neutralizing antibody were used to examine the involvement of TLR4/NF-κB pathway in LPS-induced MMP-9 activation. FLSs were pretreated with TAK (5 μM) or anti-TLR4 (1 μg/mL) for 24 h and then stimulated with 1 μg/mL of LPS for 24 h. qRT-PCR showed that treatment of FLSs with TAK and anti-TLR4 decreased LPS-stimulated MMP-9 mRNA expression (Fig. 4A), indicating that TAK and anti-TLR4 inhibited the transcription of MMP-9 induced by LPS. Culture media were subjected to gelatin zymography and western blot analysis. As shown in Fig. 4B, TAK and anti-TLR4 inhibited LPS-induced MMP-9 protein expression and activation. The effect of TAK and anti-TLR4 on the activity of MMP-9 and NF-κB promoter expression was investigated using FLSs that were transiently transfected with a luciferase reporter gene linked to the MMP-9 or NF-κB promoter sequence. Treatment of FLSs with TAK and anti-TLR4 clearly decreased the LPS-induced luciferase activity (Fig. 4C and D).

**Celastrol Inhibits the Binding Activity of NF-κB in the MMP-9 Promoter**

We examined the inhibitory effect of celastrol on the binding of NF-κB and AP-1 isolated from LPS-stimulated FLSs to oligonucleotides that contain NF-κB and AP-1 binding sites in the MMP-9 promoter using EMSA. As shown in Fig. 5A and B, in vitro celastrol treatment suppressed LPS-induced NF-κB binding to MMP-9 promoter on EMSA, but had no effect on AP-1 binding activity. Furthermore, we used ChIP to determine whether celastrol-suppressed NF-κB complexes could bind to the MMP-9 promoter in vivo. Chromatin was extracted and immunoprecipitated using anti-p65 antibody, and the MMP-9 promoter region was amplified by PCR. As shown in Fig. 5C, in vivo binding of NF-κB to the MMP-9 promoter increased in response to LPS treatment, whereas LPS-induced NF-κB binding to the MMP-9 promoter was significantly inhibited by celastrol. These results further demonstrate that celastrol suppresses LPS-induced MMP-9 transcription through control of NF-κB. To characterize the molecules involved in the inhibitory effect of celastrol, we examined whether celastrol regulated the TLR4/NF-κB signaling pathway. FLSs were pretreated with different concentrations of celastrol and stimulated with LPS for 24 h and then levels of TLR4, MyD88, TRIF, and 1κBz were measured by western blot. As shown in Fig. 5D, LPS stimulation induced significant phosphorylation of 1κBz and 1κBz degradation. Celastrol markedly inhibited the LPS-induced phosphorylation of 1κBz, TLR4 and MyD88 expression, but celastrol did not inhibit the expression of TRIF, suggesting celastrol inhibits LPS-induced MMP-9 activation by suppressing TLR4/MyD88/NF-κB pathway in RA-FLSs.

**Celastrol Improves Clinical Outcome and Histopathology of CIA Rats**

A classic rat CIA model was used to explore the anti-arthritic effect of celastrol. The onset of arthritis in ankle joints was visually determined between days 18 and 21 post immunization. After the booster immunization, the right hind paws of rats were clearly larger because of swelling. Celastrol (0.5 mg/kg/d) or celastrol (1 mg/kg/d) was administered on day 20 after primary immunization over a 21-day treatment period. As shown in Fig. 6, an inhibitory effect of celastrol on AI was observed from day 22 until the end of the observation period. By comparison, the effect of high dose of celastrol-treated group was better than the low-dose treated group (P<0.05). As shown in Fig. 7A-D, a malignant proliferation of synoviocytes that caused apparent synovial hyperplasia and significant inflammatory cells infiltration was present in the CIA control group compared with the normal group. However, in the celastrol treated groups, histopathological analysis suggested only moderate synovial hyperplasia and inflammatory cells infiltration. As shown in Fig. 7E, statistical analysis demonstrated that the celastrol (0.5 mg/kg and 1 mg/kg) treatment groups had significantly ameliorated synovial hyperplasia and inflammatory cells infiltration compared with the CIA group (P<0.05), and the improvement of high dose of celastrol-treated group was slightly better than that of the low-dose treated group (P<0.05).
Figure 7. Celastrol improves histopathology of CIA rats. A–D: On day 20 after the primary immunization, CIA rats received celastrol (0.5 and 1 mg/kg, intraperitoneally) daily for 3 weeks. The histological features of right hind knee joint synovium from Normal control, CIA+Vehicle, CIA+Cel (0.5 mg/kg), and CIA+Cel (1 mg/kg) groups were assessed by H&E staining. E: Comparisons of histological scores among groups (n = 8 observations each group). The H&E stained sections were scored for the degree of synovial hyperplasia and inflammatory cells infiltration by grading from 0 to 3 (scale bar: 200 μm). Values are shown as mean ± SD. *P<0.05 versus CIA+Vehicle, †P<0.05 versus CIA+Cel (0.5 mg/kg).
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Discussion

Celastrol is the main active ingredient in *Celastrus* and thunder god vine and has been utilized as a medicinal herb in traditional Chinese medicine for the treatment of arthritis for many years [31]. Although several studies have shown that celastrol has anti-arthritis activities in an AIA model [19,31], the precise mechanisms by which celastrol can alleviate the clinical symptoms of experimental arthritic models are not well defined. Therefore, the present study was undertaken to examine the possible therapeutic mechanisms of celastrol on RA through RA-FLSs/CIA system *in vivo* and *in vitro*. In this study, we assessed the effect of celastrol on LPS-induced RA-FLS motility. Our results clearly showed that treatment of RA-FLSs with celastrol suppressed LPS-induced cell migration and invasion. Furthermore, it revealed that celastrol inhibited the transcriptional activity of MMP-9 by suppressing the binding activity of NF-κB in the MMP-9 promoter and inhibited TLR4/MyD88/NF-κB pathway signaling. Subsequently, celastrol alleviated the clinical outcomes, synovial hyperplasia and inflammatory cells infiltration in CIA rats.

MMP-9 is an important ECM-degrading enzyme and overexpression of MMPs is important for the invasiveness of RA-FLSs [32,33]. Previously, we demonstrated that celastrol inhibited IL-17A-induced migration and invasion by suppression of NF-κB mediated MMP-9 expression and activity in human RA-FLSs [22]. LPS can stimulate FLS to secrete MMPs, and this induction is regulated at the transcriptional and translational levels [10]. In the present study, LPS treatment of FLS caused an increase in MMP-2 and MMP-9 levels. The increase in MMP-9 expression and secretion was inhibited by celastrol. Furthermore, these effects were mimicked by MMP-9 siRNA. These results therefore indicate that the inhibition of LPS-induced FLS invasion by celastrol occurs primarily by inhibiting MMP-9 expression and activity.

The two principal pathways activated by MMP-9 are the NF-κB and mitogen-activated protein kinase pathways, and the roles of both in the pathogenesis of destructive arthritis have been studied [29,34]. The MMP-9 promoter region contains a cis-regulatory element, including one NF-κB, two AP-1 and one stimulator protein-1 (SP-1) binding sites [35]. To detect the mechanism of celastrol-induced inhibition of MMP-9 expression, we examined MMP-9 promoter activity using wild type and mutant reporter plasmids. Celastrol suppressed MMP-9 induction by repressing transcription activation of the MMP-9 promoter. Mutational analysis of the promoter revealed that the major target of celastrol was NF-κB, which was confirmed by the use of reporter plasmids containing synthetic elements specific for the transcription factors. In addition, luciferase assay indicated that celastrol regulated MMP-9 transcription by suppressing NF-κB promoter activity, but not AP-1. Treatment with TLR4 inhibitor and anti-TLR4 antibody reduced the LPS-induced expression and enzyme activity of MMP-9. In addition, TLR4 inhibition reduced the LPS-induced transcriptional activity of NF-κB.

Next, we investigated the functional significance of NF-κB transactivation of MMP-9 activation in RA-FLSs. Results from *in vitro* EMSA and *in vivo* ChIP assays showed that celastrol suppressed LPS-induced NF-κB binding to the MMP-9 promoter. Given that NF-κB regulates transcriptional activation of multiple inflammatory cytokines, we expected that celastrol might target NF-κB to suppress MMP-9 transcription by LPS. TLR4 is an important upstream signal transducer of NF-κB, which is activated by TLR ligands and cytokines. NF-κB is sequestered in the cytoplasm by binding to IκB family molecules and is activated by IκBα phosphorylation, whose subsequent degradation in the proteasome allows the NF-κB subunits, including p65 and p50, to enter the nucleus and activate target genes [36]. To address whether celastrol modulated the TLR4/NF-κB signaling pathway, we attempted to analyze the expression of TLR4/NF-κB signaling transduction proteins in the absence or presence of celastrol. We showed that LPS induced phosphorylation of IκBα and triggered degradation of IκBα in RA-FLSs and that celastrol inhibited the effect in a dose-dependent manner. Furthermore, celastrol inhibited the expression of TLR4 and MyD88, which indicated that celastrol might inhibit NF-κB activity in a MyD88 dependent way. The above findings collectively demonstrate that celastrol suppresses LPS-induced MMP-9 expression through the inhibition of the induced TLR4/NF-κB signaling pathway. The MMP-9 gene is one of many TLR4/NF-κB-regulated genes, and these data indicate that celastrol can be a potent TLR4/NF-κB inhibitor that suppresses the expression of TLR4/NF-κB-regulated genes, and thus affects various biological events.

CIA is a commonly used model of RA and has been used in numerous studies to examine the pathogenesis of arthritis and to identify potential therapeutic targets. To confirm our results from a local to a systemic level, we treated CIA rats with celastrol (0.5 and 1 mg/kg). The treatment of arthritic rats with celastrol significantly alleviated the clinical outcomes, synovial hyperplasia and inflammatory cell infiltration, as demonstrated by clinical evaluation and histomorphometry.

Taken together, our results provide evidence that celastrol exerts anti-arthritic effects by inhibiting LPS-induced RA-FLS migration and invasion, and the mechanisms may involve the suppression of TLR4/NF-κB-mediated MMP-9 expression. Although further work is needed to clarify the complicated mechanism of celastrol-induced anti-invasion of FLSs, celastrol might be used as a future anti-inflammatory drug with therapeutic efficacy in the treatment of immune-mediated inflammatory diseases such as RA.

Author Contributions

Conceived and designed the experiments: GQL, YQL. Performed the experiments: GQL, DL, YZ, YYQ, HZ. Analyzed the data: GQL, YQL. Contribution of reagents/materials/analysis tools: SYG, MS, TH. Wrote the paper: GQL.

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