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# Suppression of autoimmune arthritis by Celastrus-derived Celastrol through modulation of pro-inflammatory chemokines

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ABSTRACT

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of the synovial joints, deformities, and disability. The prolonged use of conventional anti-inflammatory drugs is associated with severe adverse effects. Therefore, there is an urgent need for safer and less expensive therapeutic products. Celastrol is a bioactive component of Celastrus, a traditional Chinese medicine, and it possesses anti-arthritic activity. However, the mechanism of action of Celastrol remains to be fully defined. In this study based on the rat adjuvant-induced arthritis (AA) model of RA, we examined the effect of Celastrol on two of the key mediators of arthritic inflammation, namely chemokines and their receptors, and related pro-inflammatory cytokines. We treated arthritic Lewis rats with Celastrol (200 μg/rat) or its vehicle by daily intraperitoneal (ip) injection beginning at the onset of AA. At the peak phase of AA, the sera, the draining lymph node cells, spleen adherent cells, and synovial-infiltrating cells of these rats were harvested and tested. Celastrol-treated rats showed a significant reduction in the levels of chemokines (RANTES, MCP-1, MIP-1α, and GRO/KC) as well as cytokines (TNF-α and IL-1β) that induce them, compared to the vehicle-treated rats. However, Celastrol did not have much effect on cellular expression of chemokine receptors except for an increase in CCR1. Further, Celastrol inhibited the migration of spleen adherent cells in vitro. Thus, Celastrol-induced suppression of various chemokines that mediate cellular infiltration into the joints might contribute to its anti-arthritic activity. Our results suggest that Celastrol might offer a promising alternative/adjunct treatment for RA.



## 1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory polyarthritis of autoimmune origin.[1] Although the precise antigen that triggers RA has not yet been identified with certainty, it is clear that T helper cells and macrophages secreting pro-inflammatory cytokines play a vital role in disease pathogenesis. These cytokines can induce the expression of adhesion molecules as well as chemoattractant cytokines (chemokines) and their receptors, and cause the endothelium to become permeable allowing the transmigration of leukocytes into the joint.[2] This cellular infiltrate and its soluble products contribute to cartilage and bone erosion in the joints.[1,2]

Chemokines are a superfamily of cytokines that are associated with cell migration and recruitment to the inflammation site.[2,3] Classified as per the location of conserved cysteine residues, chemokines are categorized into four groups, CXC, $CX_3C$, CC, and C.[4] The receptors associated with chemokines are 7-transmembrane, G-protein coupled receptors that are named based on the chemokine each receptor interacts.[5] During inflammatory conditions such as RA, the interaction of chemokines and their receptors results in leukocyte trafficking into the joints. This leads to increased inflammation, pannus formation and tissue damage in the joints.[1,2] Among the chemokines, RANTES (regulated upon activation, normally T cell expressed and secreted chemokine), MCP-1 (monocyte chemotactic protein-1), MIP-1α (Macrophage Inflammatory Protein-1 alpha) and GRO/KC (growth-related oncogene/keratinocyte chemoattractant) have been shown to play an important role in RA and experimental arthritis.[1,2,6]

Complementary and alternative medicine (CAM) products including those of traditional Chinese medicine (TCM) have been long used to treat inflammatory disorders and they are generally well tolerated by patients. Herbal TCM have been shown to reduce inflammatory mediators including chemokines.[7,8] In 2007, 61% of Americans indicated they had utilized at least one form of CAM and 17% said they used herbal remedies.[9,10] This growing use of CAM has necessitated urgent research into the mechanism of action of natural products. One TCM, *Celastrus aculeatus* Merr.

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(Celastrus), has been used in folk medicine for the treatment of a variety of inflammatory disorders including RA.[11] In our previous study in adjuvant arthritis (AA), an animal model of RA, we have shown that Celastrol, a bioactive component of Celastrus, possesses anti-arthritic activity.[12] Another study published subsequently has further validated the arthritis-suppressive effect of Celastrol.[13] However, neither of these studies has addressed the effect of celastrol on chemokines and their receptors in rats with AA. Celastrol has anti-inflammatory, anti-oxidant and anti-cancer activities. For example, Celastrol is an inhibitor of Hsp90, and the cellular targets of Celastrol include, but are not limited to, NF-κB, co-chaperone p23, Annexin II, and β-tubulin.[14–16]

AA can be induced in Lewis rats by immunization with heat-killed *Mycobacterium tuberculosis* H37Ra, and it shares many of the characteristic features associated with RA.[17,18] In this study based on AA, we have examined the effect of Celastrol on the production of pro-inflammatory chemokines and their receptors both in vivo and in vitro. Also examined were a few pro-inflammatory cytokines associated with arthritis. We observed that Celastrol inhibits the production of pro-inflammatory chemokines and cytokines by spleen adherent cells (SAC). However, Celastrol did not have much effect on the associated chemokine receptors except for CCR1. In addition, Celastrol inhibited the migration of SAC in vitro.

## 2. Materials and methods

### 2.1. Animals, antigens and other reagents

Male Lewis rats (LEW/Hsd, RT.1[1]; 5–6 weeks old) were purchased from Harlan Sprague–Dawley (Indianapolis, IN) and housed in the vivarium of the University of Maryland School of Medicine, Baltimore, MD. All experimental procedures on these rats were performed following the guidelines of the Institutional Animal Care and Use Committee. Heat-killed *Mycobacterium tuberculosis* H37Ra (Mtb) was obtained from Difco (Detroit, MI). Purified Celastrol isolated from *Celastrus scandens* was purchased from Calbiochem (La Jolla, CA). Dimethyl sulfoxide (DMSO) was from Sigma–Aldrich (St. Louis, MO). Rat recombinant IL-1β was obtained from Shenandoah Biotechnology, Inc. (Warwick, PA). All the media used for cell culture were purchased from Invitrogen (Grand Island, NY) unless stated otherwise. For preparation of Mtb sonicate, Mtb (2 mg) was suspended in 1 mL PBS and sonicated at 50 V.

### 2.2. Induction of adjuvant arthritis (AA) and treatment of rats with Celastrol

AA was induced in Lewis rats as described elsewhere.[12,18] Briefly, Lewis rats were immunized subcutaneously with Mtb (1.5 mg/rat) suspended in mineral oil. Following immunization, these rats were graded regularly for arthritic signs in each paw using the grading system as described elsewhere.[12,18] Celastrol stock solution (20 mg in 0.6 ml of DMSO) was diluted in PBS (6 μl stock in 500 μl of PBS/rat). Celastrol (200 μg/rat; final concentration) was injected intraperitoneally (ip) to Mtb-challenged rats starting from the onset of AA and then continued uninterrupted until the day before tissue harvesting.[12] Control rats received the vehicle, DMSO (1.2%) in PBS.

### 2.3. Preparation and Mtb-restimulation of lymph node cells (LNC), spleen adherent cells (SAC), synovial-infiltrating cells (SIC), and serum collection from rats

Draining lymph nodes (*para*-aortic, inguinal, and popliteal), Spleens and hind paws of Celastrol-treated and control rats were harvested on d 18 of the disease course and LNC, SAC and SIC were prepared from them as described previously.[12,19] These cells were cultured in a 6-well plate ($1.5 \times 10^6$ cells per well) in RPMI-1640 medium or DMEM supplemented with 2% FBS, 2 mM L-glutamine, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate in the presence or absence of sonicated Mtb (10 μg/ml) for 24 h, and the culture supernatants were collected and stored at −80 °C until analyzed. Blood was also collected from these rats by cardiac puncture after euthanization, and then serum was separated and stored at −80 °C until analyzed.

### 2.4. Stimulation of rat synovial fibroblasts with sonicated Mtb or IL-1β in the presence or absence of Celastrol

Rat synovial fibroblasts were harvested from the arthritic rat joints and then cultured in vitro as described previously.[12,19] Fibroblasts at passage 2 were seeded into a 6-well plate ($2 \times 10^5$ cells/well) and cultured for 24 h. Fresh DMEM containing reduced FBS (2%) was added to these cells and cells then stimulated with Mtb (10 μg/ml) or IL-1β (10 ng/ml) for 48 h in the presence or absence of different concentrations of Celastrol (0.1–0.3 μM). In a parallel set, fibroblasts were exposed to the vehicle, DMSO. The culture supernatants were then collected and stored at −80 °C until analyzed.

### 2.5. Multiplex analysis for chemokines and cytokines

Culture supernatants and serum samples were analyzed for multiple analytes (Cytokine Core Facility, University of Maryland School of Medicine) using a Multiplex assay for particular chemokines (GRO/KC, MCP-1, MIP-1α and RANTES) and cytokines (TNF-α, IL-1β, and IL-6).[8] The assays were read using the luminex 100 system following the manufacturer's recommendations.

### 2.6. Restimulation of SAC with Mtb for testing chemokine receptor expression

Spleens were harvested from rats treated with Celastrol or PBS on d 18 after Mtb immunization. A single cell suspension of spleen cells was prepared and these cells were plated in a 6-well culture plate to prepare SAC as described previously.[12,19] These SAC ($1.5–2.0 \times 10^6$ cells/well) were restimulated with sonicated Mtb (10 μg/ml) for 24 h. For in vitro studies, SAC from untreated arthritic rats were restimulated for 24 h with sonicated Mtb in the presence or absence of different concentrations of (0.1 and 0.3 μM) of Celastrol. RNA was isolated from these samples using TRIzol reagent (Invitrogen), and then cDNA was prepared using an iScript cDNA synthesis kit (Bio-Rad). The cDNA thus prepared was amplified in an ABI PRISM 7900HT cycler (Applied Biosystems, Foster City, CA) by quantitative real time PCR (qPCR) using SYBR Green PCR Master Mix (Applied Biosystems) and appropriate primers.[12,19] The primers for the detection of mRNAs for different chemokine receptors and hypoxanthine-guanine phosphoribosyltransferase (HPRT) were designed using the Primer Express 2.0 Program (Applied Biosystems). The mRNA levels of specific genes were normalized to those of the HPRT gene, and the relative gene expression levels were determined.[12,19] The results were expressed as 'relative message'.

### 2.7. Flow cytometry for chemokine receptor expression

Flow cytometry was performed using spleen adherent cells (SAC) prepared as described in detail under section 2.6. SAC that had been stimulated with Mtb for 24 h were suspended in 0.5 mL of TrypLE Express (Invitrogen) per well for 10 min. Suspended cells were washed twice with PBS. Cells were surface stained with a mouse anti-rat CD11b/c PerCP-eFlour 710 antibody (eBioscience)

and a cross-reactive mouse anti-human CCR1 Alexa Fluor 647 antibody (Biolegend) for 30 min followed by two washes with washing buffer. Cells were fixed in 1% formaldehyde. Mouse IgG2a PerCP-eFlour 710 antibody and mouse IgG2b Alexa Fluor 647 antibody isotype were used as controls for flow cytometric analysis. Flow cytometry data was analyzed using FlowJo (Tree Star).

### 2.8. Cell migration assay using a Transwell chamber

Cell migration assay was performed using Transwell containing 24 mm diameter inserts with an 8-μm pore size polycarbonate membrane (Corning, NY). Spleen adherent cells (SAC) ($1 \times 10^6$ per well) from arthritic rats were placed in the upper compartment of a Transwell chamber. Serum-free RPMI containing 50 ng/ml RANTES in the presence or absence of Celastrol (0.1 and 0.3 μM) was placed in the lower compartment. (The concentrations of Celastrol used do not induce any cell death, as reported in our previous study.[12]) Cells were allowed to migrate through the membrane for 24 h at 37 °C in the presence of 5% $CO_2$. After 24 h incubation, cells from the upper surface of the filter were removed with a cotton swab and discarded. Thereafter, cells on the lower surface of the membrane were fixed for 30 min in 4% formaldehyde and stained for 10 min with 4′,6-diamidino-2-phenylindole (DAPI). The inserts were washed twice with PBS for 10 min. Then the cells were examined using Nikon Eclipse TE2000-U microscope system and images were taken using Nikon Imaging Systems (NIS) Elements—Version 3.0. Quantification of the migrated cells was done using ImageJ software.

## 3. Results

### 3.1. Production of chemokines and cytokines in immune cells from arthritic rats in response to sonicated Mtb

We tested the draining LNC, SAC and SIC harvested from arthritic rats on d 18 to determine the optimal cell type and conditions to assess chemokine expression. These cells were restimulated for 24 h with or without Mtb. The culture supernates were tested for the indicated chemokines and cytokines (Fig. 1). The levels of the chemokines and cytokines produced varied with the type of cells. Overall, the levels of various chemokines and cytokines were highest for SAC, intermediate for SIC, and lowest for LNC. In view of these results, we used SAC for subsequent experiments for a sensitive readout of any effect of Celastrol on chemokine production, chemokine receptor expression and cell migration described below.

### 3.2. Decrease in the production of GRO/KC, MCP-1, RANTES, TNF-α and IL-1β in arthritic rats treated with Celastrol

SAC (Fig. 2A) were harvested from Celastrol- and PBS-treated rats separately after 18 d of Mtb immunization. These cells were restimulated with sonicated Mtb, and the culture supernates were tested for the indicated chemokines and cytokines. There was a significant decrease in the production of GRO/KC, MCP-1, RANTES, TNF-α and IL-1β in Celastrol-treated rats compared with the PBS-treated rats (Fig 2A). IL-6 showed a trend towards decrease but the difference was not significant statistically. In contrast, no change in MIP-1α was observed. Serum from the same rats showed a decrease in GRO/KC, RANTES, TNF-α and IL-1β (Fig 2B) in Celastrol-treated rats versus controls. However, MCP-1 showed an increase after Celastrol treatment. IL-6 showed no difference between the two groups. In contrast to SAC mentioned above, LNC and SIC each revealed comparable levels of cytokines and chemokines in Celastrol-treated and control rats (Supplementary Fig. 1).

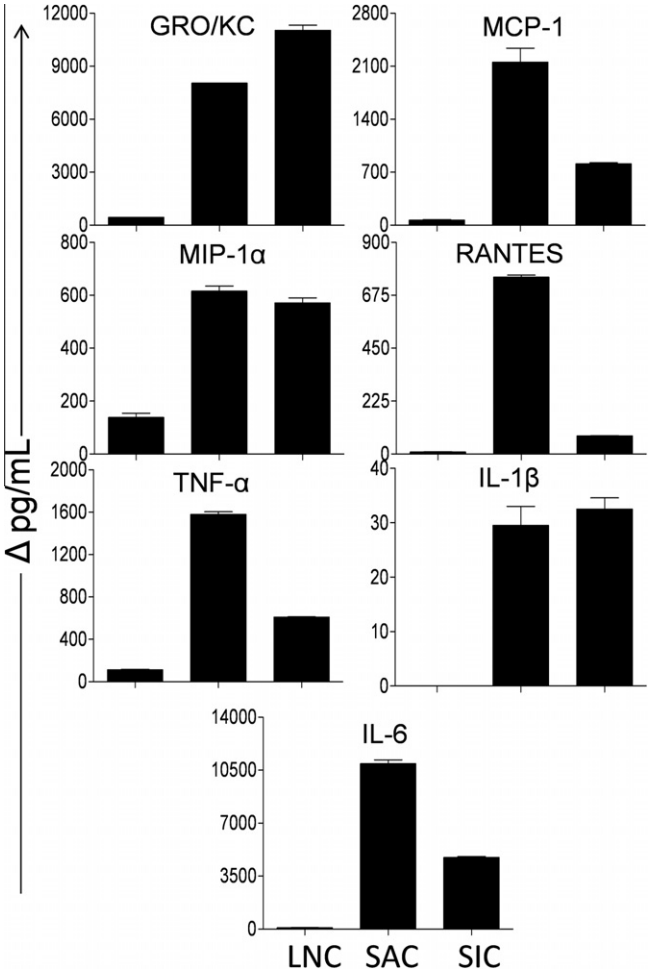


**Figure 1.** Chemokine production by lymph nodes cells (LNC), spleen adherent cells (SAC) and synovial-infiltrating cells (SIC) after Mtb re-stimulation. LNC, SAC and SIC were prepared from Mtb-immunized arthritic rats on d 18 after Mtb immunization. These cells were stimulated in vitro with Mtb (10 μg/ml) for 24 h. Thereafter, the culture supernatants collected from the wells were analyzed for the indicated chemokines and cytokines using a Multiplex assay. The levels shown represent the differences between Mtb re-stimulated and control samples. The results shown are representative of two independent experiments, each done in triplicates.

### 3.3. Celastrol treatment of SAC in vitro altered the production of chemokines and cytokines

Fibroblasts from arthritic rats were stimulated with Mtb in vitro in the presence or absence of Celastrol, and the chemokine and cytokine expression was then measured in the culture supernates. Celastrol significantly decreased Mtb-induced production of GRO/KC, MCP-1, MIP-1α, RANTES, TNF-α and IL-6 in a concentration-dependent manner when tested at concentrations of 0.1 and 0.3 μM (Fig 3A). Celastrol also decreased the IL-1β-induced production of chemokines/cytokines in a concentration-dependent manner (Fig. 3B).

### 3.4. Celastrol-treated rats do not reveal any change in chemokine receptor expression in SAC (except for baseline CCR1 expression), whereas in vitro treatment with Celastrol increased CCR1 expression in SAC

SAC from arthritic rats treated with or without Celastrol were restimulated with Mtb for 24 h. There was no significant change in the expression of various chemokine receptors including CCR2, CCR3, CCR5, CCR6, CXCR1, CXCR2, and CXCR4 following Mtb

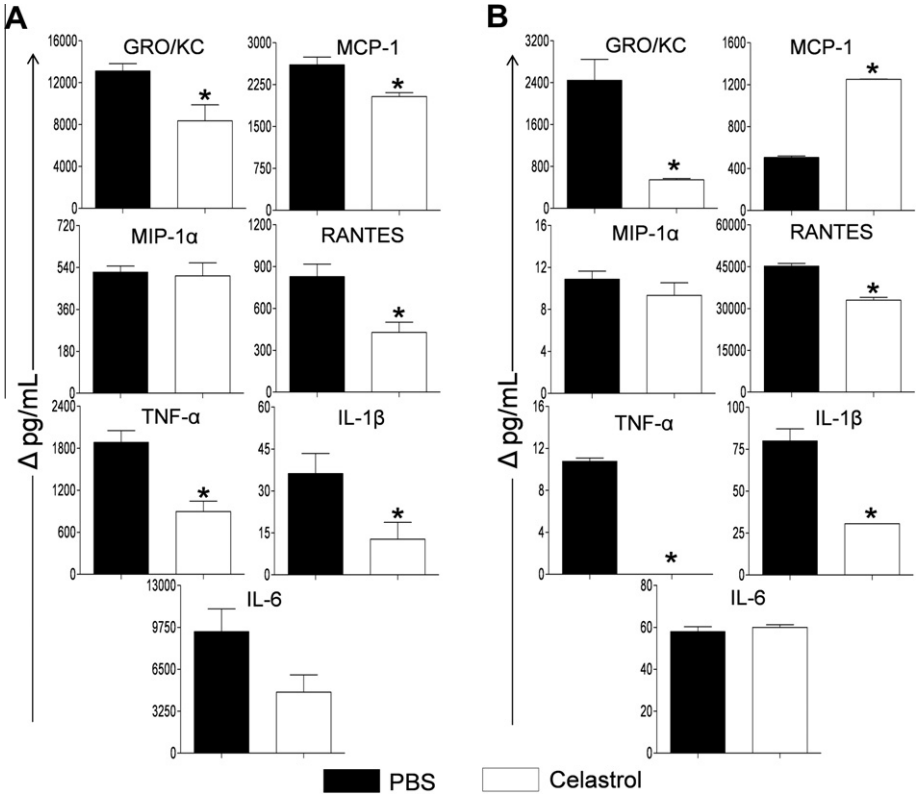


**Figure 2.** Effect of Celastrol treatment on chemokine/cytokine levels in SAC and serum: (A) SAC: Mtb-immunized Lewis rats were treated with Celastrol (in DMSO) starting from the onset of the disease. Control rats were treated with vehicle (PBS in DMSO). SAC from these rats were prepared on d 18 after Mtb immunization and then stimulated in vitro with sonicated Mtb (10 μg/ml) for 24 h. Thereafter, the culture supernatants were analyzed for the indicated chemokines/cytokines using a Multiplex assay. The mean + SD of triplicate assays from 4 to 5 rats are shown. (B) Serum: Serum was prepared from the blood samples collected from the same rats and then analyzed for various chemokines and cytokines. The results obtained from pooled serum samples from 4 rats tested in triplicate are shown. * = $P < 0.05$, versus control group.

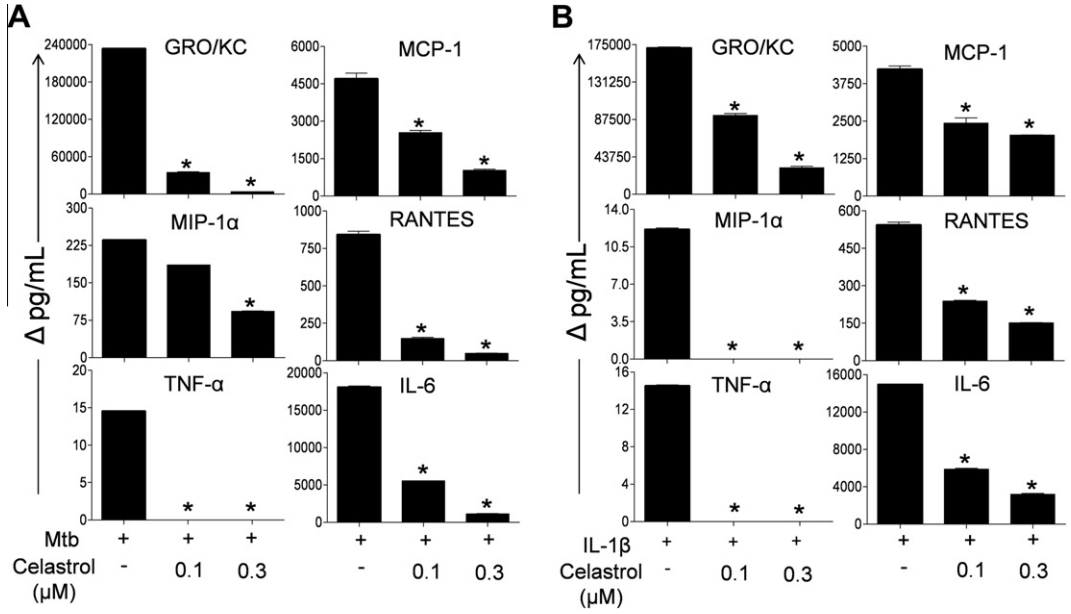


**Figure 3.** Inhibition of Mtb-/IL-1β-induced chemokine and cytokine production by Celastrol in rat synovial fibroblasts. Rat synovial fibroblasts plated in a 6-well plate ($2 \times 10^5$ cells/well) were treated with Celastrol (0.1–0.3 μM) for 30 min, followed by stimulation with sonicated-Mtb (10 μg/ml) (A) or IL-1β (10 ng/ml) (B) for 48 h. Thereafter, the culture supernatants were analyzed for chemokines and cytokines using a Multiplex assay. The graph shows the delta values (Δ = test-medium) of a representative of two independent experiments done in triplicates.

stimulation of SAC (Supplementary Fig. 2). Although there was a significant increase in CCR1 after Mtb stimulation compared to the baseline (medium) in both PBS-treated and Celastrol-treated rats (Fig. 4A), the difference in the two groups was significant only for the baseline expression (cells in medium) but not after Mtb stimulation. On the contrary, the in vitro treatment of SAC with

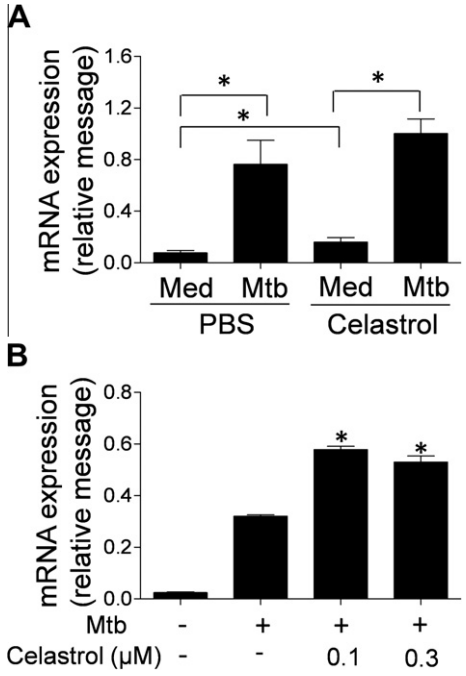


**Figure 4.** Effect of Celastrol on CCR1 expression in SAC. SAC ($1.5–2.0 \times 10^6$ cells/well) from Celastrol-treated and PBS-treated rats were restimulated with sonicated Mtb (10 μg/ml) for 24 h (A). Similarly, SAC from an untreated arthritic rat were restimulated with Mtb for 24 h in the presence of different concentrations (0.1 and 0.3 μM) of Celastrol (B). Thereafter, total RNA was isolated from SAC and the mRNA expression for CCR1 was analyzed by qPCR. Results are expressed as 'relative message' after normalization to HPRT.

Mtb in the presence or absence of Celastrol revealed a significant increase in CCR1 expression after Celastrol treatment (Fig 4B), and the effect was dose-dependent. The results of qPCR for CCR1 expression in vitro were validated by surface protein expression by flow cytometry (Supplementary Fig. 3).

### 3.5. Celastrol inhibits cellular migration of SAC

We studied the effect of Celastrol on the migration of SAC across the membrane using the Transwell assay. SAC cells from arthritic Lewis rats were allowed to migrate in response to RANTES in the presence of different concentrations of Celastrol (0.1 and 0.3 μM). The migration of cells in response to RANTES alone was taken to be 100 percent. Celastrol (0.3 μM) reduced the migration of cells by 63% ($p < 0.05$) (Fig. 5). (The concentrations of Celastrol used do not induce any cell death, as reported in our previous study.[12]) We suggest that Celastrol-induced reduction in chemokine expression (Fig. 3) contributes to this reduced cellular migration.

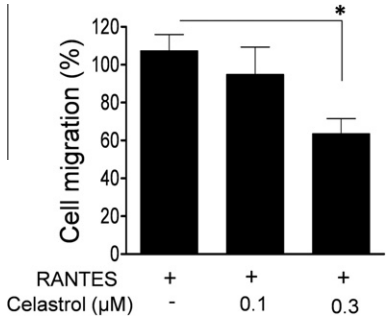


**Figure 5.** Cell migration assay. Spleen adherent cells (SAC) derived from an arthritic rat were allowed to migrate from the upper to lower surface of the Transwell membrane in response of RANTES (50 ng/ml) in the presence or absence of Celastrol. Cells that migrate to the lower surface of the membrane were stained with DAPI and then observed under Nikon Eclipse TE2000-U microscope with 10× magnification.

## 4. Discussion

The migration of lymphocytes, macrophages, and other cells form blood into various tissues to the site of inflammation is orchestrated by defined interactions mediated by chemokines and adhesion molecules.[3,20–22] For example, among the chemokines, RANTES influences the migration of mononuclear cells,[23] MCP-1 controls the migration of monocytes and T lymphocytes,[24] and GRO/KC is chemotactic for neutrophils.[25] The expression of chemokines and their receptors is influenced by cytokines and other inflammatory stimuli. We report here significantly reduced levels of chemokines (RANTES, MCP-1, MIP-1α, and GRO/KC) as well as the pro-inflammatory cytokines TNF-α and IL-1β that induce them in Celastrol-treated versus control rats.

Altered expression of chemokines and/or their receptors may lead to immune pathology and therefore, they also are attractive targets for arthritis therapy.[2,26–30] Studies on the temporal expression of chemokines during the course of arthritic inflammation have revealed the potential role of several chemokines (e.g., MCP1, MIP-1α, MIP-1β, RANTES, ENA-78, fractalkine, CXCL16, etc.) in the disease process.[2,28,31–33] Furthermore, Increased levels of RANTES and MIP-1α are found in the synovial fluid and serum of RA patients[23,34] as well in the blood and synovial tissue of rodents with experimental arthritis.[2,35–37] Similarly, GRO/KC has been shown to be increased in the synovial fluid of untreated arthritic rats.[38]

The blocking or neutralization of chemokines via antagonists or antibodies is being exploited for the treatment of inflammatory diseases, including RA.[2,26–30] Blocking CXCL16 suppressed inflammatory arthritis in the CIA model.[39] Moreover, pretreatment of LEW rats with anti-human ENA-78 antibody[28] or antihuman RANTES antibody[26] significantly suppressed the development of paw inflammation, but antibody to MIP-1α was ineffective. Interestingly, antibody to IP-10[40] or bindarit[41] can suppress inflammation. Other anti-chemokine antibodies such as anti-MCP-1,[35] anti-ENA-78 antibody,[28] and anti MIP-1α, or MIP-2 antibody[42] have been employed for treatment of arthritis in animal models. Blocking the receptor that binds MCP-1 also affords protection against AA.[43] In this regard, our results showing that Celastrol treatment suppresses the production of RANTES, MCP-1, MIP-1α, and GRO/KC are of significance. A reduction of MCP-1 and RANTES by other herbal products used for the control of arthritis has also been reported by other investigators.[44,45] Feeding rats with green tea polyphenols (GTE) attenuated the severity of arthritis, and this effect was associated with a reduction in MCP-1 and GROα.[45] Similarly, GTE-treated arthritic mice showed reduced production of TNF-α and IL-1β[46], as observed in our study based on Celastrol treatment. Further, the treatment of arthritic rats with herbal fumigation led to reduced TNF-α, IL-1β, and intercellular adhesion molecule-1 (ICAM-1).[47] In another study, feeding of a group of healthy subjects with formulations of encapsulated fruit and vegetable juice powder concentrate resulted in a significant decrease in the blood levels of RANTES, MCP-1 and MIP-1α.[44]

Enhanced expression of chemokine receptors CCR1, CCR2, CCR3, and CCR5 has been found to correlate with the level of inflammation in experimental arthritis.[31,48] In this context, a reduction in chemokine receptor expression might be anticipated following attenuation of inflammation by anti-arthritic agents. To test this possibility, we determined the expression of chemokine receptors on spleen adherent cells (SAC) derived from Celastrol-treated rats.

Our results show that there was no significant change in the expression of chemokine receptors, with the sole exception of increased expression of CCR1 following Celastrol treatment. The precise reasons for the differential effect on CCR1 receptor are not yet clear. Regardless, these results suggest that Celastrol had a predominant effect on the expression of a number of chemokines but without much effect on their corresponding chemokine receptors (except CCR1). However, as described above, treatment of arthritic rats with GTE caused increased expression of chemokine receptors CCR1, CCR2, CCR5 and CXCR1 in the rat joints.[45] Thus, different herbal products can have disparate effects on chemokine receptor expression yet lead to protection against arthritis. This can be explained by the fact that multiple chemokine–chemokine receptor interactions are involved in the disease process, and that increased chemokine receptor expression might contribute to a reduction in the concentration of its respective ligand presumably via the scavenging action of the receptor.[49] Thus, increased receptor expression may not always worsen inflammation; instead it may protect against inflammation.

On the basis of our results showing Celastrol-induced reduction in the level of chemokines, we reasoned that Celastrol might inhibit the migration of leukocytes. In fact, our results of Transwell migration assay support this proposition and suggest, albeit indirectly, that the anti-arthritic activity of Celastrol in the Lewis rat involves reduction of chemokine production by leukocytes, which are critical mediators of arthritic inflammation and tissue damage in the joints. Studies examining the biochemical/molecular basis of reduction of chemokines by Celastrol are in progress in our laboratory. We suggest that Celastrol is a promising anti-arthritic agent that can be used as an adjunct to conventionally used drugs for the treatment of RA.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.06.050.

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