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ORIGINAL ARTICLE

Cilostazol add-on therapy for celecoxib synergistically inhibits proinflammatory cytokines by activating IL-10 and SOCS3 in the synovial fibroblasts of patients with rheumatoid arthritis

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Abstract

Cilostazol (an inhibitor of phosphodiesterase type III) has potent anti-inflammatory effects, and celecoxib (a COX-2 specific inhibitor) has been reported to improve the unsatisfactory profile of NSAIDs. This study investigated the synergistic anti-arthritic potential of a multitarget-based cotreatment, in which cilostazol was used as an add-on therapy for celecoxib, using the synovial fibroblasts of RA patients (RASFs). Increased COX-2 protein expression and PGE_2 synthesis by LPS (1 μg/ml) were significantly and synergistically attenuated by cotreatment with 3 μM cilostazol and 30 μM celecoxib, whereas monotherapy with either cilostazol or celecoxib showed little effects. IL-10 mRNA levels in LPS-treated RASFs were moderately increased by pretreating cilostazol (1–10 μM) or celecoxib (10–50 μM) monotherapy, but 3 μM of cilostazol add-on for 30 μM celecoxib treatment synergistically increased IL-10 mRNA levels and IL-10 release to culture media. Cilostazol and celecoxib cotreatment similarly showed synergistic increase in SOCS3 mRNA levels. Accordingly, LPS-induced increases in IL-1β and IL-6 mRNA and TNF-α release were significantly and synergistically diminished by cilostazol and celecoxib cotreatment. Moreover, synovial cell proliferation was significantly suppressed by cotreatment. Summarizing, cotreatment with cilostazol and celecoxib exhibited a synergistic increase in IL-10 production and SOCS3 expressions, thereby resulted in synergistic decreases in IL-1β mRNA, IL-6 mRNA expression and TNF-α synthesis in association with synergistic decreases in COX-2 and PGE_2 protein expression in the RA synovial fibroblasts. In conclusion, these observations suggest low concentrations of cilostazol and celecoxib cotreatment may ensure a synergistic anti-arthritic potential.

Keywords Cotreatment · Cilostazol · Celecoxib · Synovial fibroblast · Rheumatoid arthritis

Abbreviations

COX-2	Cyclooxygenase-2
FLS	Fibroblast-like synoviocytes
IL-1β	Interleukin-1β
JNK	c-Jun NH2-terminal kinase
MMP	Matrix metalloproteinases
PGE_2	Prostaglandin E2
RASF	Synovial fibroblasts from RA patients
RA	Rheumatoid arthritis
SOCS	Suppressor of cytokine signaling
STAT	Signal transducers and activators of transcription

Introduction

Rheumatoid arthritis (RA) is characterized by the presence of hyperplastic synovium in association with immune-mediated inflammatory synovitis associated with chronic production of proinflammatory cytokines, which lead to

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cartilage and bone degradation (Goldring and Gravallese 2000; Sweeney and Firestein 2004). Rheumatoid synovial fibroblasts (RASFs) play a key role in the pathogenesis of RA synovitis because they cause fibroblast proliferation, pannus formation and produce various inflammatory mediators. It has been previously shown that inflammatory cytokines and cyclooxygenase (COX)-2 released from RASFs are crucially involved in the destruction of both articular bone and cartilage (Henderson et al. 1993; Han et al. 2003).

Cilostazol, an inhibitor of phosphodiesterase type III, has been reported to exert potent anti-inflammatory effects, and this anti-inflammatory effect was ascribed to cAMP-dependent protein kinase activation-coupled suppression of NF-κB gene transcription (Shin et al. 2004; Park et al. 2006). Park et al. (2010) showed cilostazol increases the apoptosis of synovial fibroblast in the inflamed joints of mouse collagen-induced arthritis (CIA) models. In a later study, Park et al. (2013)reported cilostazol inhibited LPS-induced increases in proinflammatory cytokine (TNF-α, IL-1β) production and suppressed IκBα degradation and the nuclear translocation of NF-κB p65.

COX has two isoforms: COX-1 is expressed constitutively and COX-2 is inducible and upregulated at inflamed sites. Increases of COX-2 induction and PGE_2 production occur in response to proinflammatory stimuli, such as IL-1β and TNF-α (Dayer et al. 1986; LeGrand et al. 2001). Cilostazol has been reported to suppress the productions of proinflammatory cytokines such as IL-1β, TNF-α, nitric oxide, and PGE_2 in LPS-stimulated BV2 microglia in a concentration (10 ~ 30 μM)-dependent manner by inactivating NF-κB and inhibiting MAPK (Jung et al. 2010). Celecoxib (a specific COX-2 inhibitor) has been reported to potently inhibit TNF-α -induced transcriptional activity and the DNA-binding activity of NF-κB (Funakoshi-Tago et al. 2008).

Interleukin-10 (IL-10) is known to function as an anti-inflammatory cytokine in rheumatoid synovium (Isomaki et al. 1996) and to inhibit cytokine synthesis by blocking NF-κB activity (Schottelius et al. 1999). IL-10 has also been reported to induce the expression of SOCS-3 (suppressor of cytokine signaling-3), which by suppressing cytokine-mediated signal transduction acts as a negative regulator of inflammation (Qin et al. 2006). Interestingly, Park et al. (2013) reported IL-10 levels were markedly increased by cilostazol in the culture media of RA macrophages, and that cilostazol pretreatment significantly reduced LPS-induced increases in TNF-α and IL-1β production.

In view of these reports, it would appear that cilostazol and celecoxib target similar sites, which suggests their cotreatment might exhibit synergistic anti-arthritic effects. In the present study, we assessed the synergistic increases in IL-10 and SOCS3, accompanied by decreased IL-1β, IL-6 and TNF-α, in association with decreases in COX-2 and PGE_2 in the synovial fibroblasts from RA patients under cotreatment with cilostazol and celecoxib.

Materials and methods

Materials

Celecoxib, cilostazol, and lipopolysaccharide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies specific for COX-2 and TNFα were from Abcam (Cambridge, UK), antibodies specific for NF-κB p65 and hnRNA A1 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), and antibodies specific for SOCS3, STAT3, and pSTAT3 were from Cell Signaling (Danvers, MA, USA).

Cell preparations

Synovial fluid samples were obtained from the knees of 10 RA patients that fulfilled the 1987 revised criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) at the time of therapeutic arthrocentesis. All patients provided informed consent and this study was approved by the Institute Research Medical Ethics Committee at Dong-A University Hospital (DAUHIRB-TEMP-16-016). All patients attended the Rheumatology Clinic at Dong-A University Hospital (Busan, Korea). After centrifuging fluid samples at 450 g for 30 min, cell pellets were resuspended in DMEM containing 10% FBS, and then incubated for 24 h at 37 °C. Non-adherent cells were washed out and the medium was changed daily for 3 days.

Cell proliferation assay

An MTT assay was used to determine the proliferation rates of synovial fibroblasts of RA patients (RASFs). Briefly, cells were treated with MTT working solution (20 μl/well, EZ-Cytox, Daeil Laboratories, Seoul, Korea) and incubated at 37 °C for 1 h. After 24 h of incubation with cilostazol and/or celecoxib, OD values of solution were measured at 450 nm using an enzyme-linked immunosorbent assay (ELISA). The results are expressed as percentages of vehicle controls.

Western analysis

Proteins were loaded into 10% polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted using antibodies against COX-2, NFκ-B p65, SOCS3, STAT3, pSTAT, hnRNA A1, and β-actin. Protein bands were

visualized using the Supersignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA), and band signals were quantified using UN-SCAN-IT gelTM software (Silk Scientific, Orem, UT, USA).

RT-qPCR analysis

For quantify gene expressions, total RNA was isolated from cells using TRIzol reagent (Invitrogen, San Diego, CA, USA) and RT-PCR were performed. mRNA levels were normalized versus human GAPDH, and data were analyzed using LightCycler 96 Software (Roche Molecular Biochemicals). The sequences of the primers were as follows:

IL-1β, F; 5′-TAC CTG TCC TGC GTG TTG AAA- 3′, R; 5′- CTG CTT GAG AGG TGC TGA TGT -3′
IL-6, F; 5′-AAT GAG GAG ACT TGC CTG GTG- 3′, R; 5′- GTT GGG TCA GGG GTG GTT ATT -3′
IL-10, F; 5′- CCT GCC TAA CAT GCT TCG AGA T- 3′, R; 5′- TGA TGT CTG GGT CTT GGT TCT C-3′
TNF-α, F; 5′- CCT GCC TAA CAT GCT TCG AGA T-3′, R; 5′- CTG CCC AGA CTC GGC AA-3′
SOCS3, F; 5′- GCC ACT CTT CAG CAT CTC TGT- 3′, R; 5′- TTA AAG CGG GGC ATC GTA CTG-3′
GAPDH, F; 5′- GGA AGG TGA AGG TCG GAG TC -3′, R; 5′- GAA GGG GTC ATT GAT GGC AA -3′

Enzyme-linked immunosorbent assay

TNFα, IL-1β, IL-6, IL-10, and PGE_2 levels in culture supernatants and mouse serum were measured using an ELISA kit (Enzo Life Sciences, Plymouth Meeting, PA, USA) according to the manufacture's protocol.

Determination of PGE_2 and IL-10 levels

PGE_2 and IL-10 levels in culture media were also determined using an ELISA kit (Enzo Life Sciences, Plymouth Meeting, PA, USA) in accordance with the instructions of the manufacturer.

Statistical analysis

The significances of differences were determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The analysis was performed using GraphPad Software (San Diego, CA, USA). Results are expressed as mean ± SEMs and p values of < 0.05 were considered significant.

Results

Synergistic attenuation of COX-2 expression and PGE_2 synthesis in RASFs by cilostazol and celecoxib cotreatment

PGE_2 is an important inflammatory product of COX-2 activity. Thus, we studied the synergistic effects of cilostazol and celecoxib cotreatment on COX-2 protein expression and PGE_2 synthesis in response to LPS in RASFs. Western blot analysis was performed with extracts of RASFs pre-treated with cilostazol and celecoxib alone or in combination for 6 h, followed by exposure to LPS for 24 h. COX-2 protein expression was markedly increased by LPS (1 μg/ml) by 1.90 ± 0.09-fold ($p < 0.001$) and this increase was significantly attenuated by 10 μM cilostazol to 1.22 ± 0.07-fold ($p < 0.001$) and by 50 μM of celecoxib to 1.01 ± 0.11-fold ($p < 0.001$) (Fig. 1a). When cotreated with 3 μM cilostazol and 30 μM celecoxib, LPS-stimulated COX-2 protein expressions were significantly and synergistically reduced to 0.97 ± 0.07-fold ($p < 0.001$), whereas COX-2 protein levels were unaffected by treatment with either 3 μM cilostazol alone or 30 μM celecoxib alone (Fig. 1b).

The significantly increased PGE_2 production (from 2.30 ± 0.07 to 5.87 ± 0.84 ng/ml, $p < 0.01$) induced by LPS (1 μg/ml) was diminished by pretreating cells with 30 μM celecoxib ($p < 0.01$), but not by pretreating with 3 μM cilostazol. However, cotreatment with cilostazol plus celecoxib synergistically suppressed LPS-induced-PGE_2 synthesis to 1.22 ± 0.14 ng/ml. This level showed significant difference from the monotherapy with either cilostazol (3 μM, $p < 0.001$) or celecoxib (30 μM, $p < 0.05$) alone (Fig. 1c).

Synergistic upregulation of IL-10 mRNA and protein levels by cilostazol and celecoxib cotreatment in RASFs

IL-10 is an anti-inflammatory cytokine and can selectively block the expressions of proinflammatory genes encoding cytokines in myeloid cells activated by LPS (Moore et al. 2001). We assessed whether cotreatment with cilostazol and celecoxib synergistically increases the IL-10 mRNA expression in the RASF. IL-10 mRNA expression was little affected by LPS (1 μg/ml) but was marginally increased by cilostazol (1 ~ 10 μM). But celecoxib (10 and 30 μM) monotherapy showed moderate increase and 50 μM of celecoxib significantly elevated IL-10 mRNA expression to 3.16 ± 0.31-fold ($p < 0.01$). Intriguingly, cotreatment with 3 μM of cilostazol and 30 μM of celecoxib synergistically

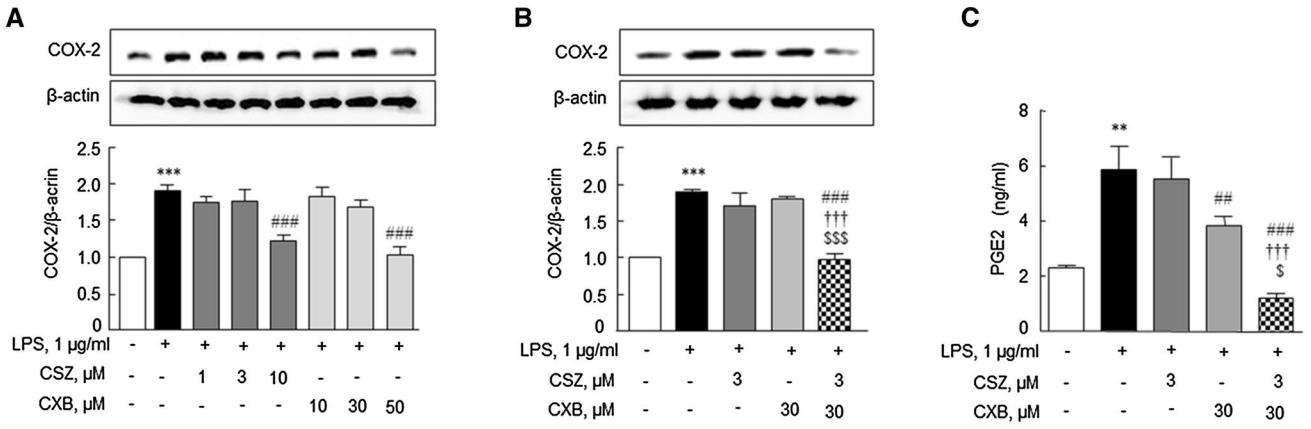

Fig. 1 Synergistic suppression of COX-2 and PGE_2 expression by treatment with cilostazol (CSZ) and celecoxib (CXB) alone and in combination in the RASF. **a** Concentration-dependent suppression of LPS (1 μg/ml)-stimulated COX-2 protein level by cilostazol (1 ~ 10 μM) and celecoxib (CXB, 10 ~ 50 μM). Synergistic inhibition of COX-2 protein expression (**b**) and PGE_2 production (**c**) by a cotreatment with cilostazol (3 μM) and celecoxib (30 μM) compared to each mono-treatment. After pretreatment with cilostazol or celecoxib for 3 h, the cells were incubated with LPS (1 μM) for 24 h. The results are presented as the mean ± SEM of four independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. none; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. LPS alone; $^{\dagger\dagger\dagger}p < 0.001$ vs. cilostazol; $^{\$}p < 0.05$, $^{\$\$\$}p < 0.001$ vs. celecoxib

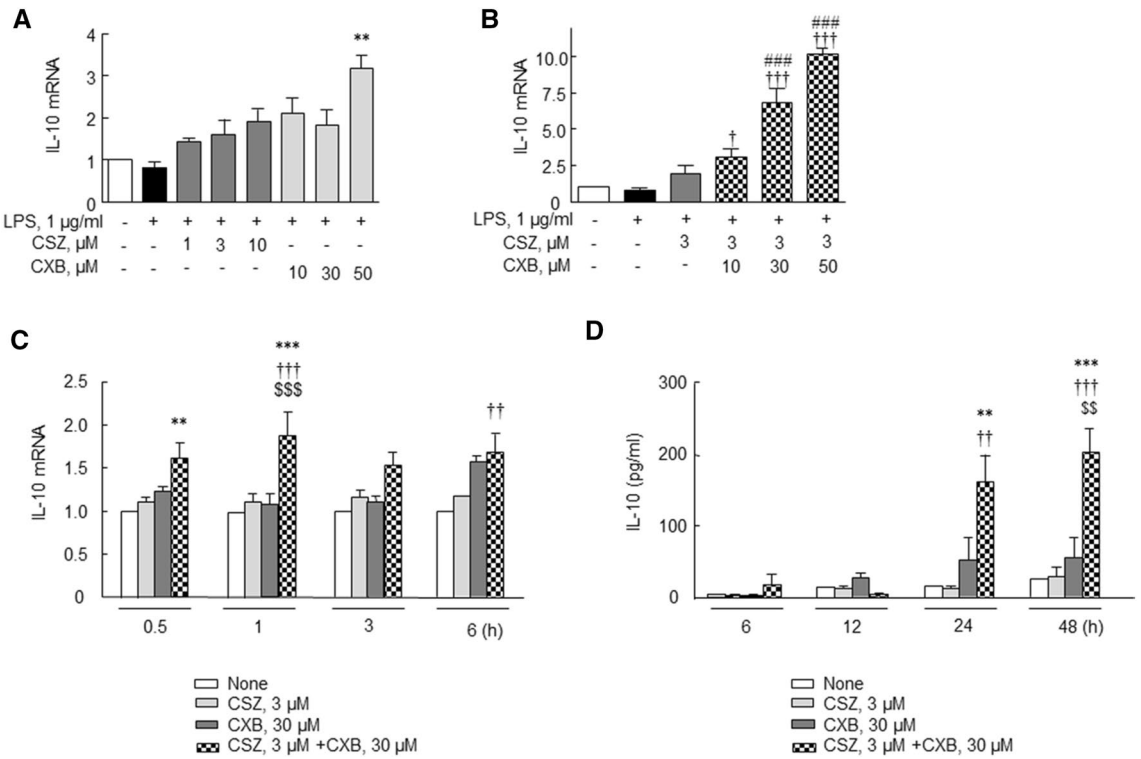

Fig. 2 Synergistic increment of IL-10 mRNA expression and IL-10 production by a cotreatment with low concentrations of cilostazol (CSZ) and celecoxib (CXB) in RASF. **a** Concentration-dependent increase in IL-10 mRNA expression in the presence of LPS (1 μg/ml) by cilostazol (1 ~ 10 μM) and celecoxib (10 ~ 50 μM). **b** Synergistic increase in IL-10 mRNA expression by 3 μM of cilostazol as an add-on treatment for celecoxib (10, 30 and 50 μM). **c** Time-course (0.5, 1, 3 and 6 h) expression of IL-10 mRNA by cilostazol (3 μM) and celecoxib (30 μM) alone and in combination. **d** Synergistic increase in IL-10 release at 24 and 48 h in the culture media by cilostazol (3 μM) and celecoxib (30 μM) alone and in combination. The results are presented as the mean ± SEM of 4 independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. none; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ vs. cilostazol; $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs. celecoxib

increased IL-10 mRNA expression to 6.84 ± 0.97-fold ($p < 0.001$ versus 3 μM cilostazol; $p < 0.05$ versus 3 μM cilostazol + 10 μM celecoxib), whereas 3 μM cilostazol alone had little effect (Fig. 2a, b).

Based on these results, the time-course (0.5, 1, 3, and 6 h) of IL-10 mRNA expression was assessed after treating RASFs with 3 μM cilostazol and 30 μM celecoxib alone and their combination in the absence of LPS. As shown in Fig. 2c, treatment with 3 μM cilostazol or 30 μM celecoxib had little effect on IL-10 mRNA expression from 0.5 to 6 h, but cilostazol and celecoxib cotreatment caused significant and synergistic increases in IL-10 mRNA expression at 0.5 and 1 h after treatment, which subsequently declined at 3 h and 6 h.

In line with these observations, this study assessed the level of IL-10 released into the culture media after a treatment with 3 μM cilostazol, 30 μM celecoxib alone and their combination time-dependently (6, 12, 24, and 48 h). The basal level of IL-10 released into the culture media (for 6 and 12 h) was 4.9 ± 2.1 pg/ml and 14.3 ± 5.2 pg/ml (each, $N = 4$), respectively. Cotreatment with 3 μM cilostazol plus 30 μM celecoxib did not produce a significant increase in IL-10 release at 6 and 12 h, but showed synergistic increases in IL-10 release at 24 h (from the control release of 16.4 ± 1.0–161.6 ± 35.4 pg/ml, $p < 0.01$ versus cilostazol alone) and at 48 h (from the control release of 25.8 ± 2.1–203.3 ± 50.1 pg/ml, $p < 0.001$ versus cilostazol alone and $p < 0.01$ versus celecoxib alone). On the other hand, neither 3 μM cilostazol nor 30 μM celecoxib alone showed any significance (Fig. 2d).

Synergistic effect of cotreatment with cilostazol and celecoxib on the increased expression of p-STAT3 (Tyr 705)

This study tested whether p-STAT3 (Tyr 705) signaling is involved in the IL-10-induction of SOCS-3 expression. The p-STAT3 (Tyr 705) levels were changed marginally by cilostazol (1 and 3 μM) and significantly increased by 10 μM cilostazol. They were significantly increased with 30 and 50 μM of celecoxib, but not with 10 μM of celecoxib (Fig. 3a). Cotreatment with 3 μM cilostazol and 30 μM celecoxib showed a time-dependent increase up to 24 h followed by a decrease at 36 h (Fig. 3b). In addition, the 3 μM cilostazol (monotherapy, 1.26 ± 0.14-fold) add-on for 30 μM of celecoxib produced a synergistic increase in the p-STAT3 (Tyr 705) level to 2.97 ± 0.44-fold ($p < 0.001$ versus 3 μM cilostazol) (Fig. 3c). In addition, LPS (1 μg/ml)-stimulated nuclear NF-κB p65 expression level (2.96 ± 0.11-fold) was attenuated by 3 μM cilostazol and 30 μM celecoxib, and more significantly by their combination to 0.29 ± 0.06-fold (Fig. 3d).

Synergistic effect of cotreatment with cilostazol and celecoxib on the increased expression of SOCS3 mRNA expression

IL-10 inhibits the expression of many proinflammatory genes through the de novo synthesis of SOCS3 in monocytes (Cianciulli et al. 2015). Therefore, this study examined whether SOCS3 gene expression is regulated by cilostazol and celecoxib. When RASF were treated with drugs for 6 h, SOCS3 mRNA expression was increased marginally by cilostazol (1, 3, 10 μM), and was elevated concentration-dependently by celecoxib [2.27 ± 0.42-fold by 10 μM ($p < 0.05$) and 2.72 ± 0.47-fold by 30 μM ($p < 0.01$)] (Fig. 4a).

The time-course changes (0.5, 1, 3, and 6 h) in SOCS3 mRNA expression were estimated under treatment with 3 μM cilostazol, 30 μM celecoxib alone and their combination. As shown in Fig. 4b, treatment with either 3 μM cilostazol or 30 μM celecoxib had little impact on SOCS3 mRNA expression from 0.5 to 6 h. On the other hand, cotreatment with both drugs in combination resulted in a significant increase in SOCS3 mRNA expression at 0.5 ~ 1 h in a synergistic manner, but showed little change between 3 and 6 h. In contrast, 3 μM cilostazol (monotherapy, 1.40 ± 0.15-fold) add-on for 30 μM celecoxib produced a synergistic increase in SOCS3 mRNA expression to 6.47 ± 1.08-fold ($p < 0.001$) at 24 h. Correspondingly, significant increases in SOCS3 protein expression showed dual phases; those were identified at 12 h (1.75 ± 0.12-fold, $p < 0.01$) and 36 h (1.86 ± 0.19-fold, $p < 0.001$) after cotreatment with 3 μM cilostazol and 30 μM celecoxib (Fig. 4c). In addition, this study examined whether SOCS3 protein expression is mediated by IL-10 and STAT3 signaling pathways. As shown in Fig. 4d, elevated SOCS3 protein expression (1.91 ± 0.22-fold, measured at 36 h) was significantly attenuated by S3I-201 (STAT3 inhibitor; Siddiquee et al. 2007) and IL-10 neutralizing antibody (NA, 2 μg/ml), but not by AG490 (JAK2 inhibitor), suggesting that SOCS3 expression was not mediated via JAK2 activation, but by STAT3 and IL-10. These results suggest that IL-10-induced STAT-3 activation plays a critical role for subsequent SOCS-3 gene expression.

Synergistic inhibition of the mRNA expression of IL-1β, IL-6 and production of TNFα

IL-10 induces the synthesis of SOCS3 in monocytes, which is closely related to its inhibitory actions in expression of many genes, such as TNF-α and IL-1β (Cianciulli et al. 2015). In this study, RT-PCR was performed to examine how cilostazol and celecoxib regulate proinflammatory cytokine expression at the transcriptional level. As shown in Fig. 5a, a marked increase in IL-1β mRNA expression (16.07 ± 1.22-fold, $p < 0.001$) was observed in RASFs after LPS (1 μg/ml

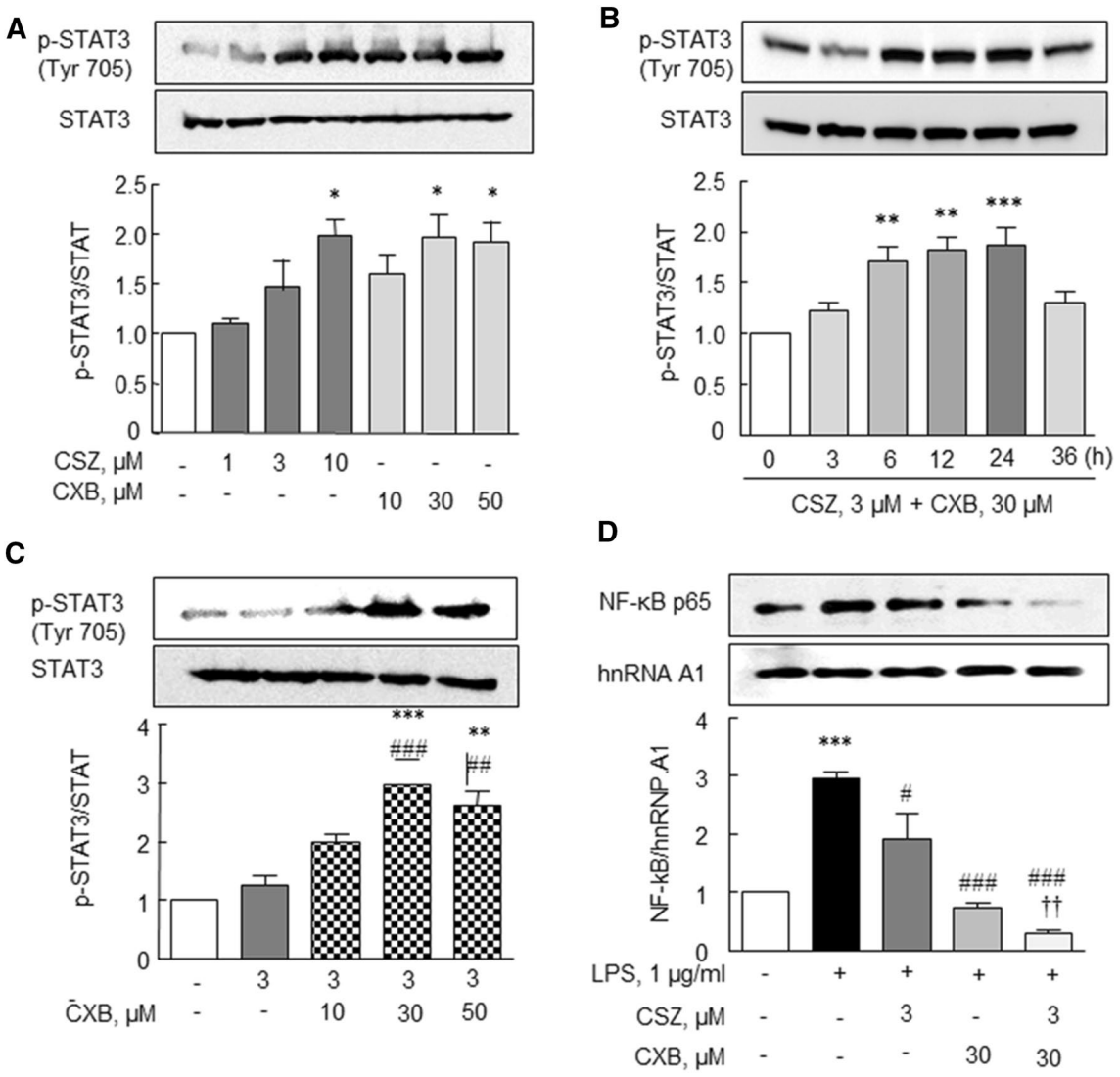

Fig. 3 Synergistic increment of p-STAT3 (Tyr 705) levels by a cotreatment with cilostazol (CSZ) and celecoxib (CXB) in RASF. **a** Concentration-dependent increase in the p-STAT3 (Tyr 705) levels by cilostazol (1 ~ 3 μM) and celecoxib (10 ~ 50 μM). **b** Time-dependent increase in the p-STAT3 (Tyr 705) levels under cotreatment with cilostazol (3 μM) and celecoxib (30 μM). **c** Synergistic increase in p-STAT3 (Tyr 705) levels by 3 μM cilostazol as an add-on therapy for celecoxib (10, 30, 50 μM). **d** Significant decrease in nuclear NF-κB p65 levels by cilostazol (3 μM) plus celecoxib (30 μM) alone and in combination. The results are presented as the mean ± SEM of four independent experiments. $^{*}p<0.05$; $^{**}p<0.01$, $^{***}p<0.001$ vs. none; $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ vs. LPS alone; $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ vs. cilostazol alone

for 6 h) exposure. This increase was significantly reduced by not only cilostazol (1 ~ 10 μM) but also by celecoxib (10 ~ 50 μM) in a concentration-dependent manner. Interestingly, 3 μM cilostazol (monotherapy 10.16 ± 0.80-fold) add-on for 10 μM celecoxib produced a markedly synergistic decrease in IL-1β mRNA expression to 2.34 ± 0.56-fold ($p<0.001$ versus 3 μM cilostazol) (Fig. 5b).

Similarly, a large increase in IL-6 mRNA expression (7.43 ± 0.62-fold, ($p<0.001$) in RASF cells was observed after 6 h of LPS (1 μg/ml) exposure. This was significantly decreased by 10 μM cilostazol ($p<0.05$) and 50 μM celecoxib ($p<0.05$), but not by 3 μM cilostazol (monotherapy 6.23 ± 0.57-fold) or 30 μM celecoxib (monotherapy 5.21 ± 0.95-fold). Nevertheless, 3 μM cilostazol add-on for 30 μM celecoxib showed significant decrease in IL-6 mRNA expression to 3.53 ± 0.15-fold ($p<0.01$ versus 3 μM cilostazol alone) (Fig. 5c, d). In addition, a large increase in TNF-α level (18.8 ± 1.14 pg/ml, $p<0.01$; determined by Elisa) in RASFs was assessed after 24 h of LPS (1 μg/ml) exposure. This increase was not decreased significantly by 3 μM cilostazol or 30 μM celecoxib, but cotreatment with 3 μM cilostazol and 30 μM celecoxib showed a significant

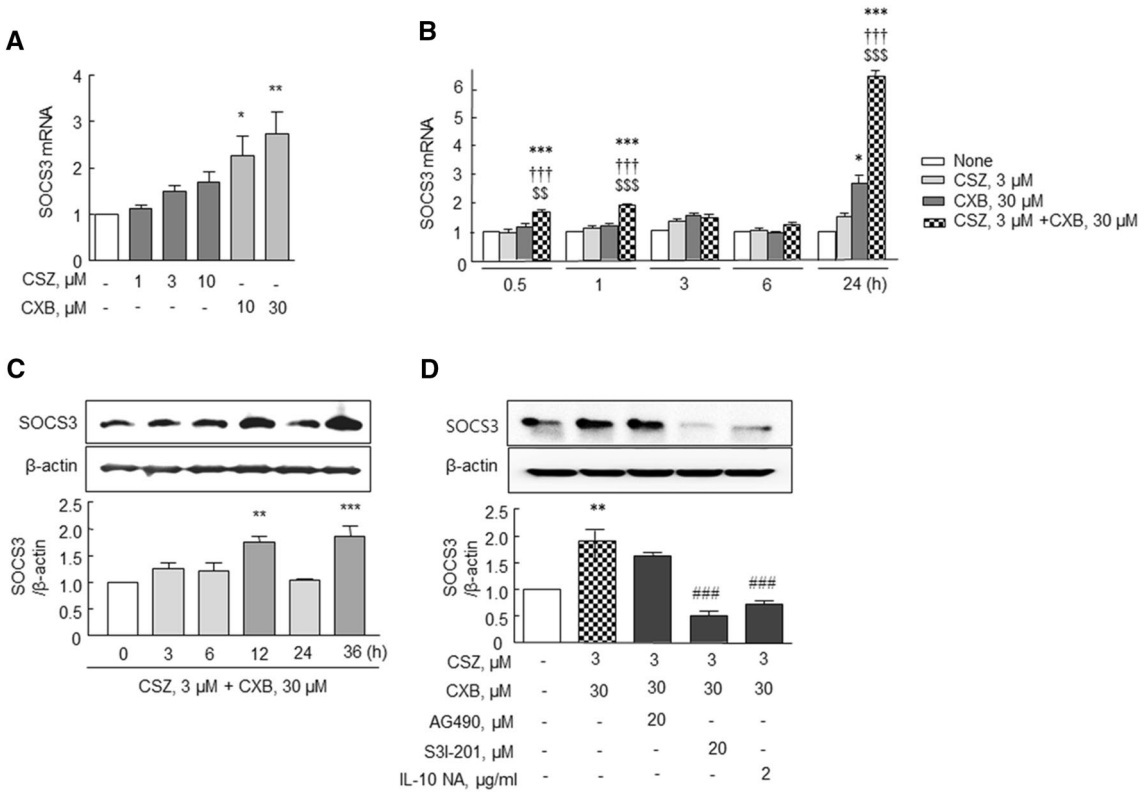

Fig. 4 Synergistic increase in SOCS3 mRNA expression by a cotreatment with cilostazol (CSZ) and celecoxib (CXB) in RASF. **a** Concentration-dependent increase in SOCS3 mRNA expression by cilostazol (1 ~ 10 μM) and celecoxib (10, 30 μM). **b** Time-course (0.5, 1, 3, 6 and 24 h) expression of SOCS3 mRNA under cilostazol (3 μM) and celecoxib (30 μM) alone and in combination. The results are represented as the mean ± SEM of five independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. none; $^{\dagger\dagger\dagger}p<0.001$ vs. cilostazol alone; $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$ vs. celecoxib alone. **c** Time-dependent increase in SOCS3 protein expression by cotreatment with cilostazol (3 μM) and celecoxib (30 μM). **d** Effects of S3I-201 (STAT3 inhibitor), IL-10 neutralizing antibody (NA) and AG490 (JAK2 inhibitor) on the elevated SOCS3 protein expression induced by a cotreatment with cilostazol and celecoxib. The results are represented as the mean ± SEM of four independent experiments. $^{**}p<0.01$, $^{***}p<0.001$ vs. none; $^{\#\#\#}p<0.001$ vs. cotreatment

decreased TNF-α level to 12.17 ± 0.76 pg/ml, $p<0.05$) (Fig. 5e).

Confirmation of the role of IL-10 in the suppression of inflammatory cytokine production using exogenous IL-10 neutralizing antibody

In this experiment, a IL-10 neutralizing antibody (IL-10 NA) (2 μg/mL) was added to the cell cultures to test the possible involvement of IL-10 in the modulation of mRNA expression of IL-1β. IL-6 and the production of TNF-α that were suppressed by cotreatment with cilostazol and celecoxib (pretreatment for 18 h) in the RASFs. As shown in Fig. 6a, the LPS (1 μg/ml for 6 h)-induced increase in IL-1β mRNA expression (11.6 ± 0.6-fold, $p<0.001$) was significantly suppressed by a cotreatment with 3 μM cilostazol and 30 μM celecoxib (pretreatment for 18 h) to 8.2 ± 0.4-fold ($p<0.001$). This suppressed IL-1β mRNA was reversed in the presence of IL-10 NA (2 μg/mL, $p<0.05$; $N=7$). Similarly, suppressed IL-6 mRNA expression by a treatment with 3 μM cilostazol and 30 μM celecoxib was similarly reversed by the application of IL-10 NA ($N=7$) (Fig. 6b). The decreased expression of the TNF-α mRNA level under cotreatment also showed a significant reverse in the presence of IL-10 NA (2 μg/mL, $p<0.05$; $N=6$) (Fig. 6c).

Accordingly, the production of IL-1β was increased markedly to 173.7 ± 30.8 pg/ml, which was significantly attenuated to 58.4 ± 6.2 pg/ml ($p<0.05$; $N=3$). This decreased level was significantly reversed by IL-10 NA (Fig. 6d). Similarly, both IL-6 and TNF-α release in the culture media were significantly elevated by LPS (1 μg/ml), those of which were significantly suppressed by a cotreatment with 3 μM cilostazol and 30 μM celecoxib ($p<0.01$). These decreased levels of IL-6 and TNF-α release were reversed in the presence of IL-10 NA (2 μg/mL) (Fig. 6e, f). Overall, these results suggest that IL-10 is strongly involved in the suppression of these cytokines by a cotreatment with cilostazol and celecoxib.

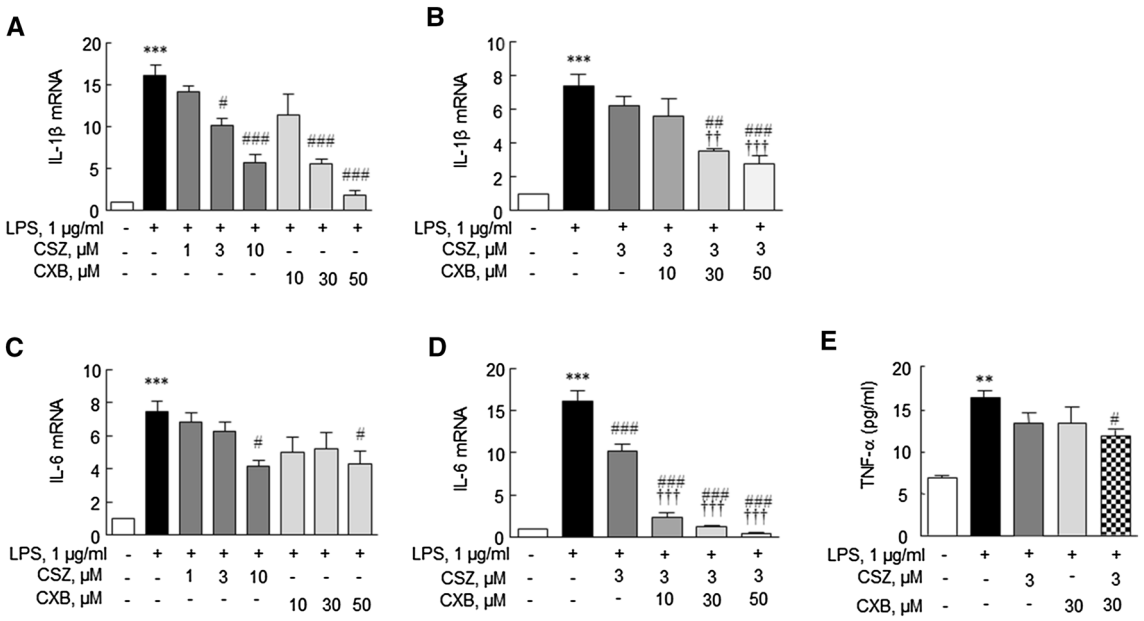

Fig. 5 Synergistic inhibition of the mRNA expression of IL-1β, IL-6 and release of TNF-α by a cotreatment with low concentrations of cilostazol (CSZ) and celecoxib (CXB) in RASF. Concentration-dependent decrease in LPS (1 μg/ml)-stimulated IL-1β mRNA (**a**) and IL-6 mRNA (**c**) expression by cilostazol (1 ~ 10 μM) and celecoxib (10 ~ 50 μM). Synergistic decrease in IL-1β mRNA (**b**), IL-6 mRNA expression (**d**) and TNF-α release (**e**) under cilostazol (3 μM) as an add-on therapy for celecoxib (10, 30, 50 μM). The results are presented as the mean ± SEM of four independent experiments. $^{**}p<0.01$, $^{***}p<0.001$ vs. none; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. LPS alone; $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ vs. cilostazol (3 μM) alone

Inhibitory effects of cilostazol, celecoxib alone and in combination on synovial cell proliferation

Synovial fibroblasts are cells that play critical roles in the pathogenesis of RA by secreting proinflammatory cytokines, such as IL-1β, IL-6 and TNF-α, which perpetuate joint destruction (Koch et al. 1995; Bartok and Firestein 2010). RASF proliferation was analyzed using a modified MTT assay. Synovial cells were treated with cilostazol (1, 3, 10 μM), celecoxib (10, 30, 50 μM), and their combination. Cell counting at 24 h revealed significant inhibition of synovial cell proliferation under treatment with cilostazol (3 and 10 μM) and celecoxib (30 and 50 μM). As shown in Fig. 7a, cilostazol (3, 10 μM) and celecoxib (30, 50 μM) alone showed significant suppression. Additionally, the monotherapy with 3 μM cilostazol showed 91.7 ± 2.1% ($N=4$, $p<0.01$). However, 3 μM cilostazol as an add-on therapy for 30 μM celecoxib further significantly decreased synovial cell proliferation to 80.6 ± 1.5% ($N=4$, $p<0.001$ vs. control and $p<0.001$ vs. 3 μM cilostazol alone) (Fig. 7b), suggesting the synergistic inhibition of synovial cell proliferation.

Discussion

The present study demonstrates that cilostazol as an add-on treatment for celecoxib significantly inhibited the LPS-induced increased COX-2 protein expression and PGE_2 production in a synergistic manner in the synovial fibers from RA patients, whereas a mono-treatment with either at the same concentration had little significant effect. Furthermore, the expression of IL-10 mRNA and IL-10 release showed a synergistic increase along with SOCS3 mRNA expression by cilostazol as an add-on treatment for celecoxib therapy. Accordingly, LPS-induced increases in the expression of IL-1β and IL-6 mRNA, and TNF-α level were significantly decreased by cotreatment with cilostazol and celecoxib in a synergistic manner. Consequently, synovial cell proliferation was significantly suppressed by a cotreatment with both drugs.

Several studies have shown that celecoxib as a selective inhibitor of COX2 strongly inhibits the proliferation of RASFs along with a decrease in PGE_2 production and causes apoptosis in the synovial fibroblasts that might be

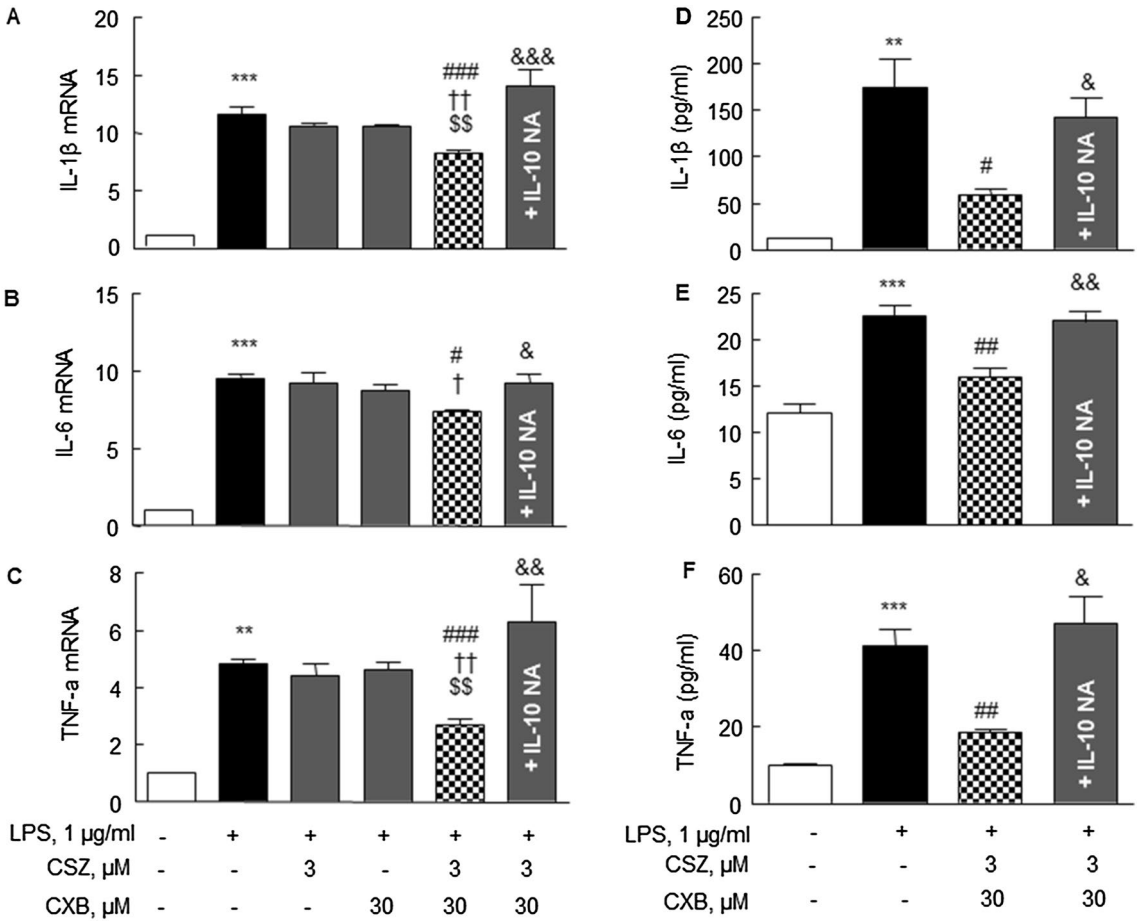

Fig. 6 Effect of cotreatment with cilostazol (CSZ, 3 μM) and celecoxib (CXB, 30 μM) on the expression of proinflammatory cytokines: IL-1β mRNA (**a**), IL-6 mRNA (**b**), TNF-α mRNA expression (**c**) and their releases into the culture media (**d**, **e** and **f**) in the absence and presence of IL-10 neutralizing antibody (NA, 2 μg/ml). RASF were pre-treated with cilostazol and celecoxib for 18 h and then exposed to LPS (1 μg/ml) for 6 h. The results are presented as the mean ± SEM of 6 ~ 7 independent experiments. $^{**}p<0.01$; $^{***}p<0.001$ vs. none; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. LPS alone; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ vs. cilostazol alone; $^{\$\$}p<0.01$ vs. celecoxib alone; $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ vs. cilostazol + celecoxib

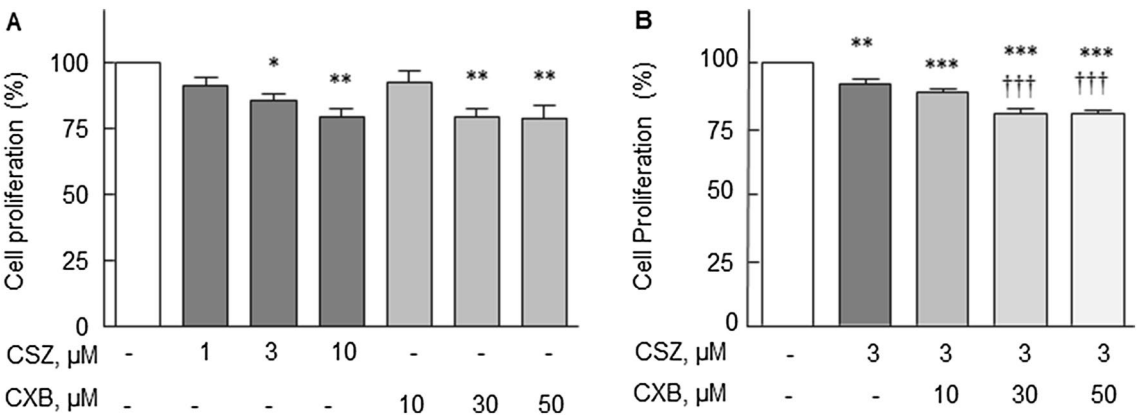

Fig. 7 **a** Concentration-dependent suppression of synovial cell proliferation by cilostazol (CSZ, 1 ~ 10 μM) and celecoxib (CXB, 10 ~ 50 μM). **b** Synergistic decrease in cell proliferation by cilostazol (3 μM) as an add-on therapy for celecoxib (10, 30, 50 μM). The results are presented as the means ± SEM of four independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. none; $^{\dagger\dagger\dagger}p<0.001$ vs. cilostazol alone

useful for the treatment of RA (Kusunoki et al. 2002; 2005; Baier et al. 2003). Celecoxib was also reported to inhibit the TNF-α-induced transcriptional activity and DNA-binding activity of NF-κB (Funakoshi-Tago et al. 2008). Overexpression of COX-2 characteristically was reported to display the enhanced growth of synovial fibroblasts and resistance to apoptosis in association with PGE_2 (Liagre et al. 2004). Unfortunately, the use of high therapeutic doses of selective COX-2 inhibitors is associated with a range of gastrointestinal and cardiovascular toxicities that have greatly limited their clinical use (McCormack 2011). Therefore, it is likely that a cotreatment with cilostazol and celecoxib at low doses will have synergistic anti-arthritic potential with few side effects for the long term.

As predicted, when cotreated with 3 μM cilostazol and 30 μM celecoxib in the RA synovial fibroblasts, LPS-stimulated COX-2 protein expression was significantly decreased in a synergistic manner, while the monotherapy with either dose of cilostazol or celecoxib had little effect. PGE_2 production was also decreased in a synergic manner by cotreatment with 3 μM cilostazol and 30 μM celecoxib, indicating a close interplay between cilostazol and celecoxib.

IL-10 is an immunosuppressive cytokine produced by macrophages, T cells, certain subsets of B cells, and dendritic cells (Saraiva and O'Garra 2010). RA patients showed significantly reduced IL-10 levels in the serum compared to healthy donors, suggesting that IL-10 synthesis is depressed in RA patients (Lapadula et al. 1995). On the other hand, the synovial fluid from patients with RA contains detectable levels of the anti-inflammatory cytokine, IL-10, but they are insufficient to counterbalance the effects of proinflammatory cytokines (Isomaki et al. 1996). Therefore, a deficit of endogenous IL-10 may perpetuate RA synovitis. Henningsson et al. (2012) showed that mice transplanted with transduced haematopoetic stem cells with lentiviral particles encoding the IL-10 gene (genetically modified to produce IL-10) developed a reduced severity of arthritis compared to the controls. On the other hand, its therapeutic efficacy for RA patients remains unsatisfactory. Therefore, enhancement of endogenous IL-10 expression and its increased levels require the use of pharmaceutical drugs.

In this study, a cotreatment with minimum concentration of cilostazol and celecoxib in the RASF resulted in a synergistic increase in IL-10 mRNA expression, whereas a monotherapy with either cilostazol or celecoxib did not elicit a significant increase. Accordingly, the release of IL-10 in the culture media resulted in a synergistic increase at 24 h and 48 h in stark contrast to each monotherapy. These results provide evidence showing that a cotreatment with minimum concentrations of cilostazol and celecoxib can promote the production of IL-10 in the synovial fibroblasts from RA patients.

IL-10-activated STAT-3 is essential for SOCS-3 gene transcription in macrophages, which inhibits LPS-induced cytokine production (Berlato et al. 2002, Williams et al. 2007). Furthermore, NF-κB is an important transcription factor involved in inflammatory responses and regulates the production of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α (Hayden and Ghosh 2004). In agreement with the postulation that IL-10 is acting as an inhibitory factor on cytokine synthesis by blocking NF-κB activity (Schottelius et al. 1999), the LPS-stimulated nuclear NF-κB p65 expression level was attenuated by monotherapy with cilostazol and celecoxib, and synergistically by their cotreatment (see Fig. 3d), indicating that this cotreatment induces the efficient blockade of the transcriptional expression COX2, PGE_2 and proinflammatory cytokines.

Interestingly, elevated SOCS3 protein expression induced by a cotreatment with cilostazol and celecoxib was attenuated significantly by S3I-201 (STAT3 inhibitor; Siddiquee et al. 2007) as well as by the IL-10 neutralizing antibody. These results suggest that IL-10-induced STAT-3 activation plays a key role in subsequent SOCS-3 gene expression. IL-10 has been reported to induce SOCS-3 expression, a negative regulator of inflammation, which is capable of suppressing cytokine-mediated signal transduction during acute inflammatory processes (Starr et al. 1997; Qin et al. 2006). Interestingly, the present study showed that low concentrations of cilostazol add-on therapy for celecoxib elicited a synergistic increase in SOCS3 mRNA expression by approximately sixfold. In agreement with Cianciulli et al. (2015), these results also showed that suppression by a cotreatment with cilostazol and celecoxib of the LPS-induced increased IL-1β, IL-6 and TNF-α expression levels were reversed significantly by an IL-10 neutralizing antibody, indicating the involvement of the anti-inflammatory effect of IL-10. Synovial fibroblasts play critical roles in the pathogenesis of RA by secreting proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, and they perpetuate joint destruction (Koch et al. 1995), and develop a unique aggressive phenotype, such as resistance to apoptosis, and exacerbating joint damage (Bartok and Firestein 2010).

In the current study, significant suppression of synovial cell proliferation was significantly decreased under cotreatment with cilostazol (3 μM) and celecoxib (30 μM). In the previous studies, cilostazol (10–30 μM) concentration-dependently suppressed the proliferation of synovial fibroblasts obtained from patients with RA (Park et al. 2010). In line with these in vitro results, we observed the in vivo effect of cilostazol (20 mg/kg/day) in the collagen-induced mice to assess suppression of osteoclast formation with reduced synovial hyperplasia (Kim et al. 2012). Considering these facts, it is anticipated that cotreatment with cilostazol (10 mg/kg/d) and celecoxib (5 mg/kg/d) may ensure the synergistic anti-arthritic potential in the animal study.

Overall, these results suggest that a cilostazol add-on for celecoxib therapy at lower concentrations has synergistic anti-arthritic potential by inhibiting proinflammatory cytokine production in synovial fibroblasts through the IL-10/SOCS3 pathways, consequently leading to the inhibition of synovial cell proliferation and overcoming the perpetuation of synovial inflammation.

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Compliance with ethical standards

Conflict of interest The authors declare no potential conflicts of interest.

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