



Original article

Beneficial anti-inflammatory effects of combined rosuvastatin and cilostazol in a TNF-driven inflammatory model

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ABSTRACT

Background: Due to anti-inflammatory and anti-thrombotic functions, statins and antiplatelets are widely used for patients with cardiovascular-related or coronary artery diseases. Patients with systemic or complex diseases are commonly prescribed multiple targeted medications; thus, a proper combination of two or more drugs for beneficial efficacy is considered in clinical therapy. Recent studies have suggested that combinational therapy with statins and other medications accelerates their single effect to suppress inflammatory responses. However, the therapeutic efficacy and underlying mechanism of combination treatment with rosuvastatin and cilostazol have been poorly studied.

Methods: Mice were administered rosuvastatin alone, cilostazol alone or rosuvastatin and cilostazol in combination, and then injected with LPS or TNF to induce acute inflammation. The serum TNF level, macrophage infiltration of the lesioned aortas and mice mortality were observed in the acute inflammation model. The phosphorylation of MAPK was analyzed in TNF-stimulated HeLa cells.

Results: Compared to the treatment with cilostazol alone, the combination treatment with rosuvastatin and cilostazol significantly reduced not only the levels of TNF in the sera but also macrophage infiltration in aortic lesions. In addition, the combination therapy decreased TNF-mediated phosphorylation of the MAPK signaling pathway and improved the survival rate in the TNF-driven inflammatory mice model.

Conclusion: Rosuvastatin combined with cilostazol therapy can greatly improve the anti-inflammatory effect of monotherapies, resulting in reduced mortality of mice; thus, we propose the potential of use of this combination therapy as anti-TNF agent.

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Introduction

Statins are mostly metabolized by the cytochrome P450 3A4 (CYP3A4); thus, they are susceptible to combination drug treatments with other drugs that inhibit the CYP3A4. Statins not only have strong lipid-lowering capabilities but also have anti-inflammatory and immunomodulatory functions, which improve endothelial dysfunction, stabilize platelet function and lead to immunological reactions [1–4]. So statins are extensively used to treat patients with acute coronary dysfunction as well as cardiovascular disease (CVD) and the central nervous system (CNS) [5]. Rosuvastatin has a low potential to be metabolized by CYP3A4 [6] and prevents ischemia-reperfusion injury and myocardial disease due to its anti-inflammation and anti-atherothrombosis functions [7,8].

Antiplatelet agents are an arterial vasodilators, inhibit platelet adhesion, aggregation and activation by blockade of membrane receptor P2Y12 or enzymes such as cyclooxygenase or phosphodiesterase type III (PDE III). Aspirin, cilostazol, clopidogrel, and prasugrel are well-known platelet-inhibiting agents that play a pivotal role in the treatment of atherosclerosis and acute coronary events [9]. Cilostazol, a selective inhibitor of PDE III, leads to rise an intracellular cAMP levels and causes vasodilation [10]. It also possesses multiple roles such as antiplatelet, antithrombus formation and anti-inflammation function by inhibiting various pro-inflammatory cytokines and adhesion molecule genes and by protecting endothelial cell activation [9]. Cilostazol decreased pro-inflammatory cytokines and mortality in mice with LPS-induced inflammatory disease [11]. Patients with systemic or complex diseases are commonly prescribed multiple targeted medications, a proper combination of two or more drugs for beneficial efficacy is considered in clinical therapy. Despite the proven anti-inflammatory effects of each rosuvastatin and cilostazol, the combination therapeutic effect against tumor necrosis

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factor- α (TNF)-mediated inflammatory diseases and its mechanisms are not clearly demonstrated.

TNF is an important cytokine for regulating immune response. However, aberrant production of TNF induces apoptosis or necrosis and causes acute and chronic disorders, and systemic inflammatory diseases such as rheumatoid arthritis (RA), inflammatory bowel disease and atherosclerosis [12,13].

Our previous study (under submission) showed that cilostazol could attenuate inflammatory responses. In this study, we investigated whether combined therapy with rosuvastatin and cilostazol provides beneficial anti-inflammatory effects and their mechanistic actions on the TNF-mediated acute inflammatory models.

Materials and methods

Reagents and materials

Cilostazol, LPS (*Escherichia coli*, 0111:B4) and α -galactosamine (D-Gal) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and rosuvastatin was kindly provided by Prof. Euichaul Oh (The Catholic University of Korea, South Korea). Recombinant human TNF (rhTNF) was expressed in *E.coli* 21 (DE3) and purified using N-NTA agarose (Qiagen, Valencia, CA, USA). Antibodies specific for p38, ERK1/2, SAPK/JNK, p-p38, p-ERK1/2 and p-SAPK/JNK were purchased from Cell Signaling Technology (Beverly, MA, USA). Mouse monoclonal antibody specific for GAPDH was purchased from GenScript (Piscataway, NJ, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG or mouse IgG were purchased from BETHYL (Montgomery, TX, USA).

Experimental animals and serum cytokine measurement

C57BL/6 mice (8 weeks old, male of 22–24 g; Orient-Bio, Korea) were maintained under pathogen-free conditions in the animal facility on a 12 h light/dark cycle environment with free access to food and water. Animal experiments conformed to internationally accepted standards and were approved (2014–020) by the Department of Laboratory Animal, Institutional Animal Care and Use Committee at Sungsim campus of The Catholic University of Korea (Bucheon, Korea). Mice in each group were orally pre-administered rosuvastatin (40 mg/kg of body weight) and/or cilostazol (40 mg/kg of body weight) once, except for the LPS and vehicle group, which was orally administered with the solvent for drugs once. All drugs were dissolved in 10% DMSO diluted in D.W with 0.05% CMC. With regard to LPS-induced inflammation experiment, mice were intraperitoneally injected with LPS (5 mg/kg of body weight) 30 min after pre-administration. After 1 h of the LPS challenge, mice (n=5) were sacrificed, and blood was collected retro-orbitally to analyze the serum TNF levels using a TNF ELISA kit (BioLegend Inc, San Diego, CA, USA) according to the manufacturer's instructions. With regard to mortality experiment, mice were challenged with intraperitoneal injections of α -Gal (700 mg/kg of body weight) and rhTNF (10 μ g/kg of body weight) (n=6–7). Mice were monitored every 3 h for 30 h, and mortality was then measured.

Histological and immunohistochemical staining

The descending aortas from mice were prepared as previously described [14]. Briefly, the harvested aortas were fixed in 4% paraformaldehyde, embedded with the ornithinecarbamoyl-transferase (OCT; CellPath Ltd, Mochdre, Newtown, UK), and the cryosections were stained by hematoxylin (YD Diagnostics, Seoul, Korea) and eosin (MUTO PURE CHEMICALS CO., Ltd,

Japan) (H&E) for observing the aortic morphology. The cryosectioned descending aortas occupied with lesions were fixed in acetone at -20°C , blocked with 10% BSA (Bovogen Biologicals, Keilor East VIC, Australia), incubated with anti-macrophage-2 (MOMA-2; Abcam, Cambridge, MA, USA) and anti-rat HRP-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) sequentially. The sections were visualized using a DAB kit (Carpentaria, CA, USA) and morphometric data were obtained using a slide scanner (APERIO CS2; Leica Biosystems, Wetzlar, Germany). Three portions of each section were randomly chosen for analysis of macrophage infiltration using ImageJ imaging software (National Institutes of Health, Bethesda, MD, USA).

Cell culture and western blot analysis

HeLa cells were grown and used for western blotting. Briefly, cells were starved and then treated with pre-mixed rhTNF (50 ng/ml) and rosuvastatin (400 nM) and/or cilostazol (1 μ m) for 15 min. After washing the cells with PBS, proteins were obtained using RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA), which included a protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific, Rockford). Approximately 5–10 μ g of protein from cell lysates were electrophoresed through 10% SDS-polyacrylamide gel and transferred onto PVDF membranes (Amersham Bioscience, Freiburg, Germany). The membranes were incubated with the appropriate primary antibodies (all specific antibodies against p38, ERK1/2, SAPK/JNK, p-p38, p-ERK1/2, and p-SAPK/JNK (at a dilution of 1:1000)) overnight at 4°C and then sequentially incubated with HRP-conjugated goat anti-mouse (at a dilution of 1:5000) or rabbit secondary antibodies (at a dilution of 1:5000) for 1 h at RT. Each protein was visualized using the ECL reagent (Thermo Fisher Scientific, USA) and the ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA, USA). The intensity of protein bands was quantified using ImageJ densitometry software (NIH, Bethesda, MD, USA), and the quantitated density was normalized to GAPDH. Bands were specifically detected at 43 and 40 kDa for phospho-p38 MAPK and p38 MAPK, at 46 and 54 kDa for phospho-SAPK/JNK and SAPK/JNK, at 42 and 44 kDa for phospho-ERK1/2 and ERK1/2.

Statistical analysis

Data values are expressed as the mean \pm SEM. The statistical analyses used were the one way analysis of variance (ANOVA) and the Log-rank test (GraphPad Prism software, GraphPad, San Diego, CA, USA). A value of $p < 0.05$ was considered as statistically significant.

Results

Combined treatment with rosuvastatin and cilostazol reduces serum TNF secretion in the LPS-driven inflammatory model

Our previous study and other groups [15] have reported that cilostazol inhibits the production of pro-inflammatory cytokines and has an anti-inflammatory function. To investigate whether the combined treatment with rosuvastatin and cilostazol can improve the anti-inflammatory effect of cilostazol, we first set up an LPS-induced inflammatory model. Mice were pre-administered rosuvastatin alone, cilostazol alone or in combination, then mice were injected with LPS (5 mg/kg of body weight) to induce an acute inflammation. Blood was collected to analyze TNF production by ELISA. As shown in Fig. 1, rosuvastatin alone did not reduce serum TNF level, but cilostazol alone did. Combined rosuvastatin and

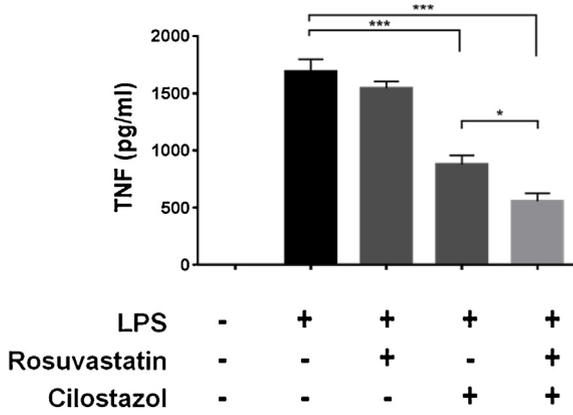


Fig. 1. Combined treatment with rosuvastatin and cilostazol reduces serum TNF secretion in the LPS-driven inflammatory model. C57BL/6 mice (n=5) were pre-administered rosuvastatin (40 mg/kg of body weight) and/or cilostazol (40 mg/kg of body weight) for 30 min and subsequently intraperitoneally injected with LPS (5 mg/kg of body weight). After 1 h, blood was collected retro-orbitally. Serum TNF levels were detected by ELISA. Data were presented as the mean ± SEM. Significance was analyzed by ANOVA. ***p < 0.001 vs. the LPS; *p < 0.05 compared between cilostazol vs. combination.

cilostazol treatment greatly inhibited (67%, $p < 0.001$) the TNF level compared to the LPS.

Combined treatment with rosuvastatin and cilostazol inhibits macrophage accumulation in lesioned aortic tissue

We then performed immunohistochemistry to confirm whether the more significant reduction in TNF by the rosuvastatin and cilostazol combination treatment is correlated to lesion attenuation of aortic tissue in LPS-driven inflammatory mice. After LPS injection, the onset of aortic endothelium lesions and necrotic lesions were observed in the mice. The serial sections of mouse descending aortas were stained with anti-MOMA-2 to monitor the monocyte/macrophage infiltration. Compared with the robust macrophage infiltration in the LPS (Fig. 2), a prominent decrease in macrophage infiltration was shown in the rosuvastatin alone (35.8%) and cilostazol alone (60.4%) treatments. Interestingly, the combination treatment inhibited macrophage infiltration at a similar level as that of the vehicle group ($p < 0.001$). These results indicate that add-on rosuvastatin synergistically enhances the anti-inflammatory properties of cilostazol in the LPS-mediated inflammation condition.

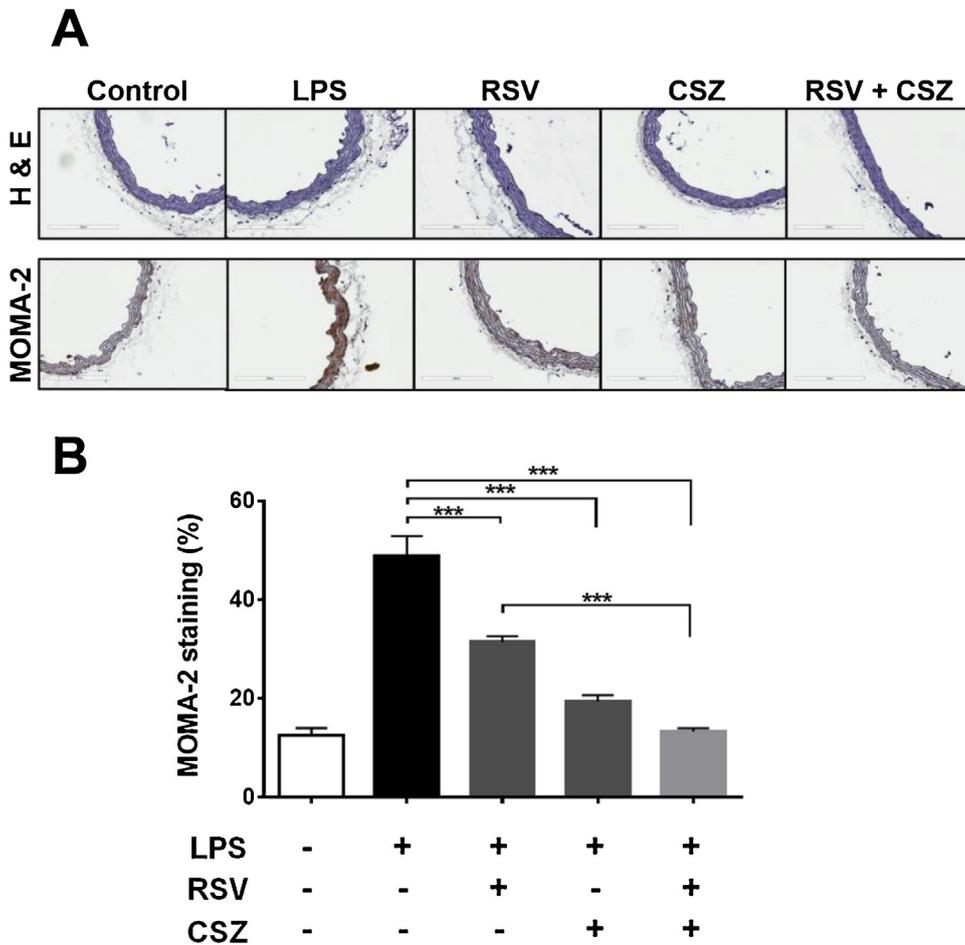


Fig. 2. Combined treatment with rosuvastatin and cilostazol suppresses monocyte/macrophage accumulation in lesioned aorta tissue. C57BL/6 mice (n=5) were pre-administered rosuvastatin (40 mg/kg of body weight) and/or cilostazol (40 mg/kg of body weight) for 30 min followed by an LPS injection. 1 h after the LPS injection, mice were sacrificed, and the aortas were extracted. Control group was orally administered with the solvent for drugs. (A) The serial sections of descending aortas were stained by H&E to observe morphology, and they were stained with anti-MOMA-2 to detect the infiltrating area of monocytes/macrophages. (B) Three portions of each aortic section were randomly chosen and quantified by densitometry. Monocyte/macrophage infiltration was expressed as the percentage of the total lesioned area comprised of the MOMA-2 positive area. Data were presented as the mean ± SEM. Significance was analyzed by ANOVA. Scale bar=200 μm, ***p < 0.001 vs. the LPS or compared between RSV vs. combination. Cilostazol; CSZ, Rosuvastatin; RSV.

Combined treatment with rosuvastatin and cilostazol attenuates TNF-mediated MAPK activation

TNF has been reported to mediate inflammatory responses and activate MAPK signaling pathways [16]. We thus examined whether the inhibition of TNF secretion by combination therapy could affect TNF-mediated MAPK activation. Cells were treated with preincubated rhTNF (50 ng/ml) and rosuvastatin and/or cilostazol for 15 min and we then performed western blotting. Etanercept, a TNF antagonist, was used as a positive control. We observed that phosphorylation of p38 MAPK, JNK and ERK in the combination treatment was significantly down-regulated to a similar level to that of etanercept treatment. These data suggest that rosuvastatin combined therapy can provide an additive benefit in the reduction of MAPK phosphorylation (Fig. 3).

Combined treatment with rosuvastatin and cilostazol improves survival of acute lethal TNF-mediated inflammation mice

Next, we examined whether the combination therapy improves the survival of mice suffering from TNF-driven acute inflammation. Mice were orally administered either rosuvastatin or cilostazol, or in combination. All groups were treated with rhTNF (10 µg/kg of body weight) except for the control, and they were then monitored

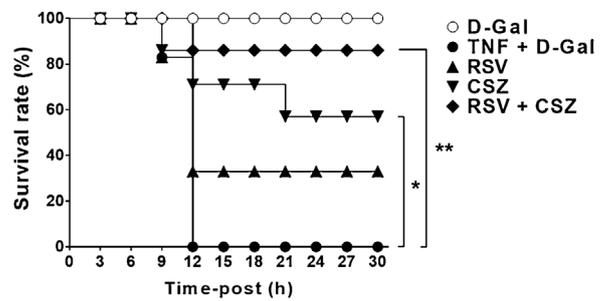


Fig. 4. Combined therapy with rosuvastatin and cilostazol improves survival of TNF-mediated acute lethal mice. To verify the beneficial effect of combination therapy, mice were orally administered rosuvastatin (100 mg/kg of body weight) and/or cilostazol (30 mg/kg of body weight) followed by an injection of rhTNF (10 µg/kg of body weight) plus D-gal (700 mg/kg of body weight). The vehicle group was treated only with D-gal. The mortality of mice (n = 6–7) was monitored every 3 h for 30 h. Significance was analyzed by the Log-rank test. *p < 0.05, **p < 0.01 vs. the TNF plus D-Gal. Cilostazol; CSZ, Rosuvastatin; RSV.

for mortality. As shown in Fig. 4, although the mortality of rosuvastatin treatment alone did not markedly improve, cilostazol alone and the combination treatment significantly improved the survival in comparison with the survival of the TNF, by 57% (p < 0.05) and 86% (p < 0.01), respectively. These results indicate that

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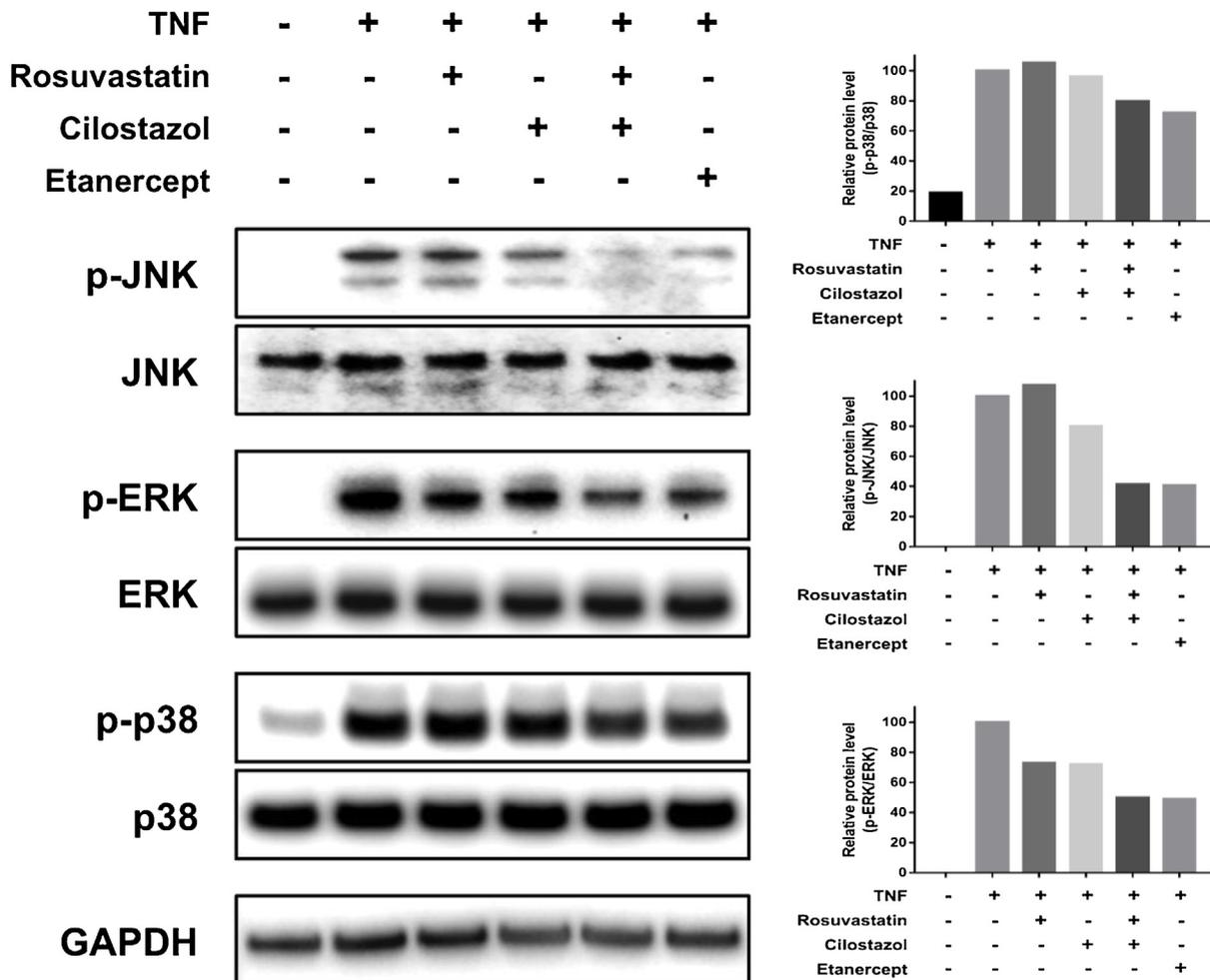


Fig. 3. Combined treatment with rosuvastatin and cilostazol attenuates TNF-mediated MAPK activation. HeLa cells were treated with rhTNF (50 ng/ml) and rosuvastatin (400 nM) and/or cilostazol (1 µM) or etanercept (30 ng/ml) for 15 min. Cell lysates were used for western blotting using antibodies against p38, ERK1/2, SAPK/JNK, p-p38, p-ERK1/2, and p-SAPK/JNK. GAPDH was used as a control. Relative values of the phosphorylated forms and the total forms were analyzed by densitometry.

combination therapy with cilostazol could enhance the anti-inflammatory effect of rosuvastatin monotherapy resulting in an improved survival rate of TNF-driven acute lethal mice (Fig. 4).

Discussion

Statins are well-described drugs, which are now widely accepted as a conventional therapy for CVD and atherosclerosis due to their pleiotropic properties [17]. Together with statins, antiplatelet agents are also applied for the prevention of CVD and acute coronary diseases [15,18]. Despite favorable evidence from clinical outcomes, monotherapy with high-dose statins or antiplatelet agents have resulted in adverse effects, and low-dose monotherapy has not led to sufficient therapeutic effects [19]. Therefore, combination therapy with statins and other agents is considered in pharmacotherapy. However, not all combinations have exhibited beneficial prevention or treatment effects; thus, an appropriate combination design is an ongoing target in the search for successful therapy.

TNF is a pleiotropic cytokine that is important in host defense and increases platelet activation elements [20]. However, the dysregulated production of TNF is associated with the development of several diseases including RA, Crohn's disease, inflammatory bowel disease, sepsis, and diabetes [13]. TNF has been studied as a therapeutic target for numerous diseases, and anti-TNF drugs such as etanercept, infliximab, and adalimumab are currently being used in clinical trials [21].

Our previous study (under submission) reported that the combination of pravastatin and cilostazol inhibited TNF-mediated inflammation *in vivo*. Because rosuvastatin has effects of anti-inflammation and cilostazol also has effects of antiplatelet and anti-inflammation [9], we chose rosuvastatin and cilostazol for combination therapy. Combined treatment of rosuvastatin and cilostazol remarkably inhibited TNF secretion into the serum of mice suffering inflammation by LPS-stimulation (Fig. 1). TNF is the first cytokine to be released into the blood after exposure to injury, stress or any infection; thus our result indicates that the combination therapy more powerfully inhibits pathogen, bacterial-derived LPS compared to monotherapy of rosuvastatin or cilostazol. As a similar result, immunohistochemical staining with anti-MOMA2 showed that although cilostazol alone inhibited macrophage infiltration, combined treatment with rosuvastatin almost completely blocked macrophage infiltration in the lesioned descending aortas (Fig. 2). Macrophages are key producers of TNF and a major defense against the invasion of pathogens. The results indicate that combination therapy synergistically enhances the anti-inflammatory properties of cilostazol, which resulted in protection from macrophage infiltration by inhibiting inflammatory responses in the LPS-mediated inflammation condition.

Rosuvastatin has been reported to have antioxidant and anti-inflammatory effects in inflammatory bowel disease, and it regulates vascular smooth muscle cell proliferation and migration, and endothelial function *via* the MAPK signaling pathway [22,23]. We therefore examined the interplay of combination treatment and MAP kinases in TNF-stimulated HeLa cells. Although the monotherapy with rosuvastatin and cilostazol did not significantly suppress the phosphorylation of JNK and p38, combination treatment of rosuvastatin and cilostazol significantly downregulated the phosphorylation of ERK1/2, JNK and p38 (Fig. 3) to similar levels to those of etanercept. The results showed that the combined rosuvastatin and cilostazol treatment provides an additive benefit in the reduction of TNF-mediated MAPK phosphorylation, and it inhibits TNF to a similar level to that of etanercept, which is an anti-TNF therapeutic agent. These results indicate that the combination treatment with rosuvastatin and cilostazol exerts

pleiotropic effects including TNF-targeted anti-inflammatory properties as well as their intrinsic properties.

Our previous study (under submission) reported that decreased MAPK phosphorylation by combination therapy affects the anti-inflammatory actions and recovery of TNF-mediated inflammatory mice. Therefore, we tried combination therapy with rosuvastatin and cilostazol on TNF-mediated acute lethal mice. We found that the survival of mice treated with combination therapy was greatly improved (86%) in comparison with survival in the TNF control group, and it was also improved by cilostazol monotherapy (57%). This result indicates that combination therapy with rosuvastatin and cilostazol could enhance the anti-inflammatory effect of cilostazol monotherapy resulting in an improved survival rate of TNF-driven acute lethal mice.

We previously demonstrated [24] that adding etanercept to pravastatin and sarpogrelate therapy exhibited advantageous effects on preventing the progression of atherosclerosis in an aging-related atherosclerosis model. The results indicated that etanercept is a key player in maximizing the anti-atherosclerosis effect. Taken together, we suggest that the combination therapy at optimal concentrations of two drugs can be a potential novel therapeutic agent for many TNF-driven diseases including RA and atherosclerosis without adding a TNF inhibitor.

In conclusion, our present results demonstrated that combination therapy with rosuvastatin and cilostazol exerts beneficial effects in inhibiting both LPS- and TNF-driven inflammatory activities and contributes to improving the anti-inflammatory effects, resulting in suppression of pro-inflammatory cytokine secretion and macrophage infiltration. In addition, we showed that the inhibition of TNF by combination therapy rescued mice from an acute inflammatory response. Our findings suggest that the combined rosuvastatin and cilostazol treatment can be used for therapeutic agent in acute phase inflammatory diseases related with TNF.

Conflict of interest

The authors confirm that there are no conflicts of interest.

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