Nanoparticle-Mediated Delivery of Pitavastatin Inhibits Atherosclerotic Plaque Destabilization/Rupture in Mice by Regulating the Recruitment of Inflammatory Monocytes

Running title: Katsuki et al.; Statin nanoparticles inhibit plaque rupture

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Abstract

Background—Preventing atherosclerotic plaque destabilization and rupture is the most reasonable therapeutic strategy for AMI. Therefore, we tested the hypotheses that (i) inflammatory monocytes play a causative role in plaque destabilization and rupture and (ii) the nanoparticle-mediated delivery of pitavastatin into circulating inflammatory monocytes inhibits plaque destabilization and rupture.

Methods and Results—We used a model of plaque destabilization and rupture in the brachiocephalic arteries of ApoE-/- mice fed a high-fat diet and infused with angiotensin II. The adoptive transfer of CCR2+/Ly-6Chigh inflammatory macrophages, but not CCR2+ leukocytes, accelerated plaque destabilization associated with increased serum MCP-1, M-CSF, and MMP-9.

We prepared poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs), which were incorporated by Ly-6G+CD11b+ monocytes and delivered into atherosclerotic plaques after intravenous administration. Intravenous treatment with pitavastatin-incorporated NPs, but not with control nanoparticles or pitavastatin alone, inhibited plaque destabilization and rupture associated with decreased monocyte infiltration and gelatinase activity in the plaque. Pitavastatin- incorporated NPs inhibited MCP-1-induced monocyte chemotaxis and the secretion of MCP-1 and MMP-9 from cultured macrophages. Furthermore, the NP-mediated anti-MCP-1 gene therapy reduced the incidence of plaque destabilization and rupture.

Conclusions—The recruitment of inflammatory monocytes is critical in the pathogenesis of plaque destabilization and rupture, and NP-mediated pitavastatin delivery is a promising therapeutic strategy to inhibit plaque destabilization and rupture by regulating MCP-1/CCR2-dependent monocyte recruitment in this model.

Key words: nanoparticle, plaque rupture, acute myocardial infarction, statin therapy, monocyte
Introduction

Coronary heart disease (CHD) is the leading cause of death worldwide, and at least 7 million patients die from this disease each year (386,324 people in the United States). Acute myocardial infarction (AMI) is the most severe type of CHD and the most frequent cause of heart failure, and it impairs the quality of life and inflates medical costs. Timely and successful revascularization therapy for AMI reduces short-term mortality, and current standard medical therapy with angiotensin-converting enzyme (ACE) inhibitors and β blockers ameliorates the development of post-MI heart failure. However, these recent advances in therapeutic intervention for AMI are associated with the increased prevalence of heart failure with high long-term mortality, which remains a serious concern. In the United States, 5.1 million people suffered from heart failure in 2010, and 274,601 people died of heart failure in 2009. Therefore, there is an urgent need for preventive treatment to avoid plaque destabilization and rupture, which directly cause AMI.

Rupture-prone unstable atherosclerotic plaques feature monocyte/macrophage infiltration, lipid core formation, and fibrous cap thinning by matrix metalloproteinases (MMPs). Recent reports suggest that monocytes are functionally polarized into at least two major subsets: inflammatory monocytes (CD14+CD16- in humans and Ly-6ChighCCR2+CX3CR1low in mice) and anti-inflammatory monocytes (CD14+CD16+ in humans and Ly-6CloｗCCR2CX3CR1high in mice). Inflammatory monocytes are found in the peripheral blood of patients with AMI, suggesting that they have pathological roles in plaque destabilization; however, substantial proof for a causative role of inflammatory monocytes in the pathogenesis of plaque destabilization and rupture is lacking.

HMG-CoA reductase inhibitors (statins) lower serum cholesterol levels and reduce
cardiovascular events and mortality by 32-37% in primary prevention trials\textsuperscript{6, 7} and 24-30% in secondary prevention trials.\textsuperscript{8, 9} Data from clinical trials indicate that although the intensive use of statins reduces serum C-reactive protein levels and cardiovascular risk and attenuates the progression of coronary artery plaque,\textsuperscript{10-13} the risk reduction caused by on-label doses of statins remains insufficient to suppress AMI.\textsuperscript{10} In animals, plaque-stabilizing effects have been observed after high doses of statins.\textsuperscript{14-18} Statins exert multiple vasculoprotective effects on endothelial cells, vascular smooth muscle cells, and monocytes.\textsuperscript{19-21} Thus, we hypothesized that the controlled delivery of statins to inflammatory monocytes and atherosclerotic plaques might optimize the plaque-stabilizing effects of statins.

Recently, we developed a novel nanoparticle (NP)-mediated drug delivery system (DDS), which is formulated from bioabsorbable poly(lactic-co-glycolic acid) (PLGA) polymer, and reported that NPs were taken up by a variety of cells, such as monocytes, vascular smooth muscle cells and endothelial cells. In addition, nanoparticle-mediated DDS showed a significant enhancement in the therapeutic effects on ischemia-induced neovascularization\textsuperscript{22, 23} and pulmonary arterial hypertension\textsuperscript{24} in animal models compared with the conventional administration. NPs are rapidly taken up by circulating monocytes and mononuclear phagocytic system after intravenous administration,\textsuperscript{25} and nano-sized contrast agents accumulate in atherosclerotic plaques.\textsuperscript{26} To our knowledge, no prior studies have addressed whether polymeric NPs accumulate in unstable atherosclerotic plaques after intravenous administration or whether the NP-mediated delivery of pitavastatin has therapeutic effects on plaque destabilization and rupture \textit{in vivo}. Therefore, we tested the hypotheses that (i) inflammatory monocytes play a causative role in plaque destabilization and rupture and (ii) the NP-mediated delivery of pitavastatin inhibits plaque destabilization and rupture by targeting inflammatory monocytes.
Materials and Methods

Experimental animals
Male ApoE−/− mice on the C57BL/6J genetic background were purchased from Jackson Laboratory (Bar Harbor, ME, USA). ApoE−/−CCR2−/− and ApoE−/−CCR2+/+ mice with the same genetic background (C57BL/6J and 129/svjae hybrids) were used.27 Animals were maintained on a 12-h light-dark cycle with free access to normal rodent chow and water.

Diet preparation
A high-fat diet (HFD) that contained 21% fat from lard and was supplemented with 0.15% (wt/wt) pure cholesterol (Oriental Yeast, Tokyo Japan) was prepared according to the formula recommended by the American Institute of Nutrition. Additional details can be found in the online Data Supplement.

Experimental protocol
The study protocol was reviewed and approved by the Committee on the Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences. The four sets of animal experiments are depicted in a schematic online (Supplementary Figure 1). Brachiocephalic arteries were isolated with the aortic arches. Isolated samples were fixed in 3.7% formaldehyde for the histological and immunohistochemical analysis or were snap-frozen in liquid nitrogen and stored at -80°C for the biochemical analysis. Additional details for each experimental protocol can be found in the online Data Supplement.

Histopathology and immunohistochemistry
Histopathological and immunohistochemical evaluations were performed to quantify atherosclerosis and examine both plaque morphology and the mechanism of plaque destabilization and rupture. Atherosclerotic plaques were stained with Oil Red O, and cross
sections of the brachiocephalic arteries and aortic root were stained with elastica van Gieson (EVG), anti-macrophage surface glycoprotein Mac3 and monocyte chemoattractant protein-1 (MCP-1). Additional details are provided in the online Data Supplement.

**Flow cytometry**

Leukocytes from peripheral blood and the spleen and peritoneal cells were obtained from mice and analyzed with a FACSCalibur cytometer (Becton-Dickinson Biosciences, San Jose, CA, USA). Additional details can be found in the online Data Supplement.

**Preparation of PLGA nanoparticles**

PLGA NPs encapsulated with FITC (FITC-NP), pitavastatin (pitavastatin-NP) and 7ND plasmid (7ND-NP) were prepared using an emulsion solvent diffusion method, as previously reported. Additional details can be found in the online Data Supplement. 22-24

**In vivo kinetics of the nanoparticles**

Excised aortas from the atherosclerotic mice were intravenously injected with or without FITC-NP and were evaluated by stereoscopic and fluorescence microscopy. Sections of the brachiocephalic artery were evaluated by fluorescence microscopy or were stained with hematoxylin-eosin (HE). Additional details can be found in the online Data Supplement.

**In situ zymography**

Gelatinase (MMP-2/gelatinase-A and MMP-9/gelatinase-B) activity was measured in unfixed frozen sections using quenched fluorescein-labeled gelatinase substrate (DQ gelatin, Invitrogen, Eugene, OR, USA). Additional details can be found in the online Data Supplement. 28

**Gelatin zymography**

The LPS-induced gelatinase activity of RAW264.7 cells was measured using a Gelatin Zymo-Electrophoresis Kit (Primary Cell, Hokkaido, Japan) according to the manufacturer’s directions.
Additional details can be found in the online Data Supplement.

**Real-time quantitative RT PCR**

Real-time PCR amplification was performed with the mouse cDNA with the use of the ABI PRISM 7000 Sequence Detection System (Applied Biosystems), as described previously. The polymerase chain reaction primers and TaqMan probes can be found in the online Data Supplement.

**Chemotaxis assay**

The chemotactic activity of THP-1 cells in response to MCP-1 was measured by a Boyden chamber method, as described previously. Additional details can be found in the online Data Supplement.

**Statistical analysis**

Data are expressed as the mean ± SE. For analysis of the number of buried/disrupted fibrous caps, differences were statistically analyzed by ANOVA followed by Dunn’s multiple comparison tests and differences between two groups were analyzed with using Mann-Whitney test. For the other analyses, differences were statistically analyzed by ANOVA followed by post-hoc Bonferroni’s or Dunnett’s multiple comparison tests. Differences between two groups were analyzed with using an unpaired t-test. P<0.05 was considered significant.

**Results**

**The adoptive transfer of inflammatory macrophages accelerated plaque destabilization and rupture**

To clarify the role of inflammatory monocyte/macrophages in plaque destabilization and rupture, we first examined whether the adoptive transfer of inflammatory macrophages accelerates plaque...
destabilization and rupture in a murine model. We collected thioglycollate-induced peritoneal leukocytes from ApoE−/−CCR2+/+ mice that contained Ly-6C^high inflammatory macrophages and from ApoE−/−CCR2−/− mice that contained <5% inflammatory macrophages (Supplementary Figure 2A, B).4,31 ApoE−/− mice were assigned to the no treatment group, the CCR2+/+ inflammatory macrophage group (1x10⁶ peritoneal leukocytes from ApoE−/−CCR2+/+ mice), and the CCR2−/− leukocyte group (1x10⁶ peritoneal leukocytes from ApoE−/−CCR2−/− mice). The adoptive transfer of CCR2+/+ inflammatory macrophages tended to increase the incidence of disrupted/buried fibrous caps. The adoptive transfer of CCR2−/− leukocyte significantly decreased the incidence of disrupted/buried fibrous caps compared with that of CCR2+/+ inflammatory macrophages (Figure 1A, Table 1). These results demonstrate that inflammatory macrophages responsible for plaque rupture are recruited from the circulation via MCP-1/CCR2 signaling, suggesting a role of peripheral Ly-6C^high monocytes as the precursor of inflammatory macrophages. Since splenic monocytes show a comparable phenotype with peripheral monocytes,32 we purified splenic monocytes from ApoE−/− mice fed a HFD and examined whether the adoptive transfer of splenic monocytes accelerated plaque destabilization and rupture in the same model. We confirmed approximately 90% purity of splenic monocytes after negative selection using magnetic cell separation, approximately 85% of which expressed high level of Ly-6C (Supplementary Figure 3A). In consistent with the data from the adoptive transfer of CCR2+/+ peritoneal macrophages, the adoptive transfer of splenic monocytes tended to increase the incidence of disrupted/buried fibrous caps (P=0.056) (Supplementary Figure 3B), confirming the detrimental role of inflammatory monocyte/macrophage lineage in atherosclerotic plaque rupture (Supplementary Figure 3B).

Immunohistochemical analysis of serial sections revealed that Mac3-positive macrophage
infiltration was frequently observed in the shoulder regions of atherosclerotic plaques, and this infiltration was increased in the CCR2\textsuperscript{+/+} inflammatory peritoneal macrophage group compared with the no treatment group (Figure 1A, Table 1). MCP-1 immunostaining colocalized with macrophages and was also observed in the media. MCP-1 expression in atherosclerotic plaques increased in the CCR2\textsuperscript{+/+} inflammatory macrophage group compared with the no treatment group, but not in the CCR2\textsuperscript{-/-} leukocyte group (Figure 1A, Table 1). In a subset of experiments, we labeled peritoneal macrophages with PKH26 before adoptive transfer and found that PKH26-labeled peritoneal macrophages were present in the atherosclerotic plaques in the brachiocephalic arteries (Supplementary Figure 2C). In the whole aorta and aortic root, the adoptive transfer of CCR2\textsuperscript{+/+} inflammatory macrophages increased the atherosclerosis area compared with the adoptive transfer of CCR2\textsuperscript{-/-} leukocytes (Figure 1B, C), although serum lipid profile was comparable among three groups (Supplementary Table 1).

Measuring serum biomarkers using multiplex immunoassay system showed that the adoptive transfer of CCR2\textsuperscript{+/+} peritoneal macrophages, but not CCR2\textsuperscript{-/-} leukocytes, increased the serum levels of MCP-1, MCP-3, and MCP-5, and monocyte-colony stimulating factor (Supplementary Table 2). Importantly, the adoptive transfer of CCR2\textsuperscript{+/+} inflammatory macrophages increased MMP-9, a metalloproteinase that may degrade the fibrous cap of plaques. Interestingly, the adoptive transfer of CCR2\textsuperscript{+/+} inflammatory macrophages did not affect the serum levels of inflammatory cytokines, such as IFN\textgreekgamma TNF\textalpha, and the interleukin family (Supplementary Table 2).

**Cellular uptake and in vitro kinetics of PLGA nanoparticles in macrophages**

To clarify the advantage of the nanoparticle-mediated drug delivery system for targeting inflammatory monocytes/macrophages, the cellular uptake and kinetics of PLGA nanoparticles
were examined in cultured macrophages. Murine peritoneal macrophages took up FITC-NPs, which distributed within the cytosol, as detected using confocal microscopy (Supplementary Figure 4A, B). Electron microscopy revealed that the PLGA nanoparticles were incorporated into the lysosomes (Supplementary Figure 4C). Cellular uptake of FITC-NP and FITC was quantified as the cellular fluorescent intensity in RAW264.7 cells after a 2-hour incubation with FITC-NP or FITC, followed by a washout period. The FITC signal intensity in cells incubated with FITC-NPs was greater than in cells incubated with FITC over a 7-day period (Supplementary Figure 4D), suggesting that FITC-NP leads to enhanced and sustained uptake in cultured macrophages.

In vivo localization of FITC-NPs after intravenous administration

Flow cytometric analysis of the blood revealed that neutrophils (36 ± 13%) and monocytes (61 ± 13%) showed FITC signal 2 hours after FITC-NP injection, suggesting that FITC-NPs were taken up through phagocytosis. Flow cytometric analysis of the spleen also revealed the uptake of FITC-NPs in neutrophils and monocytes (Figure 2A). Fluorescence microscopy analysis of the aortic arch and the brachiocephalic arteries revealed the presence of FITC signals mainly in the macrophage areas of atherosclerotic plaques in FITC-NP-injected animals (Figure 2B).

Treatment with pitavastatin-NPs inhibits plaque destabilization and rupture

A 4-week treatment with pitavastatin-NPs, but not with FITC-NPs or pitavastatin, significantly reduced the incidence of disrupted/buried fibrous caps associated with thick luminal fibrous caps (Figure 3A, Table 2) and decreased serum biomarkers, including MCP-1, CD40L, VEGF, and vWF (Supplementary Table 3). To clarify the pharmacokinetics of NP treatment, we measured plasma concentration of pitavastatin in pitavastatin and pitavastatin-NP group. Plasma concentration of pitavastatin was below the limit of detection (0.625 ng/mL) except 2 hours after
intravenous injection of pitavastatin-NP (Supplementary Table 4). There were no significant differences in lipid profiles among the 4 groups (Supplementary Table 5).

Immunohistochemical analysis revealed that treatment with pitavastatin-NPs attenuated the infiltration of Mac3-positive cells and MCP-1 expression in atherosclerotic plaques (Figure 3A, Table 2). In cultured monocytes, pretreatment with pitavastatin-NPs at 10 μM, but not pitavastatin at the same dose, inhibited IFNγ-induced gene expression of MCP-1 (Figure 4B).

Consistent with these data, treatment with pitavastatin-NPs, but not FITC-NPs or pitavastatin, decreased the atherosclerosis area in the whole aorta and aortic root (Figure 3B, C). Treatment with pitavastatin-NPs, but not FITC-NPs or pitavastatin, also attenuated the infiltration of Mac3-positive cells to the aortic root (Figure 3C).

**Treatment with pitavastatin-NPs inhibits gelatinase activity in the atherosclerotic plaque**

As previously reported, MMP secretion was upregulated in inflammatory monocytes, and focal activation of MMPs is another potential mechanism through which plaques can be destabilized.33 In situ zymography revealed that pitavastatin-NPs, but not control-NPs, reduced gelatinase activity (Figure 5A). A gelatinase activity assay with gelatin zymography in the culture medium of RAW264.7 cells showed that pitavastatin-NPs reduced MMP-9 secretion in a dose-dependent manner, while the same dose of pitavastatin showed no effects on MMP-9 secretion (Figure 5B). Both the pro- and active forms of MMP-2 were almost undetectable in the culture medium of RAW264.7 cells, even when stimulated with LPS (Figure 5B).

**Treatment with pitavastatin-NPs inhibits the recruitment of Ly-6C<sup>high</sup> monocytes to the circulating blood**

Monocytosis and increased Ly-6C<sup>high</sup> monocytes in the circulating blood critically promote the progression of atherosclerosis.31 Treatment with pitavastatin-NP reduced the percentage of
monocytes among the total leukocytes on days 7 (Figure 4A). A monocyte subset analysis showed a marked reduction of Ly-6C<sup>high</sup> monocytes in the circulating blood in the pitavastatin-NP group (Figure 4A). Although CCR2, a key chemokine receptor that promotes the migration of Ly-6C<sup>high</sup> monocytes, was not affected by pretreatment with pitavastatin-NPs (Figure 4B), MCP-1-induced chemotaxis was inhibited by pretreatment with pitavastatin-NPs in a dose-dependent manner (Figure 4C).

**NP-mediated anti-MCP-1 gene therapy inhibits plaque destabilization and rupture**

To further elucidate the impact of inhibiting MCP-1/CCR2 signaling through NP-mediated DDS on plaque destabilization and rupture, we examined the effects of NPs containing plasmids that encode 7ND, a deletion mutant of MCP-1. We previously reported that systemic gene therapy with the 7ND plasmid attenuated the development and progression of atherosclerosis in ApoE<sup>−/−</sup> mice,<sup>27,34,36</sup> and that NP-mediated transfection of the 7ND plasmid inhibited MCP-1-induced monocyte chemotaxis <em>ex vivo</em>.<sup>37</sup> Treatment with 7ND-NPs reduced macrophage infiltration into the plaques and the incidence of disrupted/buried fibrous caps associated with thick fibrous caps (Figure 6A, Table 3), although serum lipid profile was comparable (Supplementary Table 6).

In contrast, treatment with 7ND-NPs did not decrease the atherosclerosis area in the aorta (Figure 6B).

**Effects of oral treatment with pitavastatin on plaque destabilization and rupture**

Oral daily administration of pitavastatin at 0.1 mg/kg had no significant effects on plaque destabilization and rupture, but administration of pitavastatin at 1.0 mg/kg reduced the incidence of disrupted/buried fibrous caps associated with thick fibrous caps and plaque size (Supplementary Figure 5A, Table 4). Oral daily administration of pitavastatin at 1.0 mg/kg also decreased the atherosclerosis area in the whole aorta and aortic root, while administration of
pitavastatin at 0.1 mg/kg did not (Supplementary Figure 5B, C). Oral daily administration of pitavastatin at 1.0 mg/kg significantly inhibited macrophage infiltration and MCP-1 expression in the brachiocephalic artery [Mac3 immunostaining area: 49±3 (×10³ μm²) (N=9), 53±5 (×10³ μm²) (N=10) and 22±4 (×10³ μm²) (N=11) for No Treatment, pitavastatin at 0.1 mg/kg and pitavastatin at 1 mg/kg, respectively (P<0.01 in No Treatment vs. pitavastatin at 1 mg/kg), MCP-1 immunostaining area: 47±3 (×10³ μm²) (N=9), 40±5 (×10³ μm²) (N=10) and 21±2 (×10³ μm²) (N=11) for No Treatment, pitavastatin at 0.1 mg/kg and pitavastatin at 1 mg/kg, respectively (P<0.01 in No Treatment vs. pitavastatin at 1 mg/kg)] (Supplementary Figure 5A, Table 4).

The serum lipid profile was comparable among three groups (Supplementary Table 7). The cumulative effective dose of orally administered pitavastatin was approximately 20 times greater than that of the dose of pitavastatin-NPs required to achieve plaque stability (28 mg/kg versus 1.6 mg/kg, respectively).

**Discussion**

Advanced atherosclerotic plaques spontaneously rupture in the brachiocephalic arteries of ApoE⁻/⁻ or LDL-R⁻/⁻ mice, and this rupture appears to represent several key histological features of ruptured human plaques, including an increase in plaque destabilization markers (e.g., monocyte infiltration/activation, lipid accumulation, fibrous cap thinning) and evidence of disrupted and buried fibrous caps. The recruitment of Ly-6C<sup>high</sup> monocytes was observed during the development of aortic atherosclerosis in ApoE⁻/⁻ mouse; however, substantial proof for a decisive role of Ly-6C<sup>high</sup> monocytes in plaque destabilization and rupture has been lacking. In the present study, we used this murine model and found that (i) the recruitment of inflammatory monocytes into the atherosclerotic plaque is critical for accelerating plaque destabilization and
(ii) the NP-mediated delivery of pitavastatin inhibits plaque destabilization and rupture by inhibiting the recruitment of inflammatory monocytes.

Recent study reported that local macrophage proliferation rather than monocyte recruitment is a major component of atherosclerosis formation. In the present study, we demonstrated that the adoptive transfer of inflammatory monocytes accelerated plaque destabilization in a CCR2-dependent manner, suggesting a role of direct recruitment of inflammatory monocytes in this process. The different importance of monocyte recruitment and local macrophage proliferation between these studies may owe to the difference of the model, because angiotensin II infusion strongly induces MCP-1 expression in the arterial walls, which might unveil the importance of monocyte recruitment. There is a possibility that adoptive transfer of inflammatory monocytes indirectly enhanced local macrophage proliferation through an increase in serum monocyte-colony stimulating factor (M-CSF) (Supplementary Table 2) to accelerate atherosclerosis. Further study is needed to examine relative importance of different mechanisms of monocyte/macrophage contribution to atherosclerosis in different pathological settings. Adoptive transfer of CCR2⁺Ly-6C<sup>high</sup> macrophages also increased serum levels of monocyte/macrophage chemoattractants (MCP-1, -3, -5) (Supplementary Table 2), which might further accelerate monocyte/macrophage-mediated inflammation. These findings suggest that targeting CCR2⁺Ly-6C<sup>high</sup> inflammatory monocytes/macrophages with a DDS is a promising strategy to inhibit the destabilization of rupture-prone atherosclerotic plaques.

We employed a PLGA nanoparticle as a phagocyte-directed DDS in this study because PLGA is a biocompatible material that is already in clinical use. Intravenously administered FITC-NPs were rapidly taken up by circulating and splenic leukocytes, predominantly by monocytes/macrophages, and accumulated in macrophages in atherosclerotic plaques (Figure 2).
An in vitro pharmacodynamics assay using FITC-NPs showed that nanoparticulation enhanced cellular uptake and retention over a 7-day period in cultured macrophages (Supplementary Figure 4). Weekly intravenous treatment with pitavastatin-NPs inhibited plaque destabilization and rupture associated with reduced macrophage infiltration and MCP-1 expression without affecting serum cholesterol levels. We previously reported that pitavastatin-NPs inhibited LPS-induced NF-κB activation in cultured macrophages. NF-κB is a central regulator of monocyte inflammatory activation and leads to the upregulating MCP-1 and MMP expression. Indeed, pitavastatin-NPs inhibited MCP-1 expression and gelatinase activity in macrophages both in vivo and in vitro. In the present study, interfering with MCP-1/CCR2 signaling by the intravenous administration of 7ND-NP inhibited the recruitment of monocytes into the plaque, followed by plaque stabilization in the brachiocephalic artery (Figure 6), confirming the pivotal role of MCP-1/CCR2 signaling-mediated monocyte migration, which makes this signaling pathway a therapeutic target to inhibit plaque destabilization. In the present study, aortic atherosclerosis was not reduced by intravenous injection of 7ND-nanoparticle that primarily targets circulating monocytes and their microenvironment. We have previously reported that aortic atherosclerosis in ApoE−/− mice fed a HFD (without angiotensin II infusion) was attenuated by HVJ liposome-mediated 7ND gene transfer into hindlimb muscles, which maintains 7ND-mutant MCP-1 in the circulation. Since we have previously shown that angiotensin II infusion strongly induces MCP-1 expression in the smooth muscle cells (SMC) in the aorta, different source of MCP-1 (macrophages on HFD, and macrophages and SMC on HFD and angiotensin II infusion) might affect the different therapeutic effect of 7ND plasmid upon different mode of administration, although it is beyond the scope of the present study.

Importantly, pitavastatin-NPs reduced the number of circulating Ly-6C<sup>high</sup> inflammatory
monocytes, suggesting that pitavastatin-NPs inhibited the recruitment of Ly-6C<sup>high</sup> monocytes from the sites of hematopoiesis to the circulation<sup>43</sup> in addition to inhibiting the recruitment from the circulation to the atherosclerotic lesions. Because pitavastatin-NPs did not affect CCR2 expression, in contrast with MCP-1 expression (Figure 4B), pitavastatin-NPs may inhibit monocyte chemotaxis by inhibiting the geranylgeranylation of RhoA, which regulates the ERM (Ezrin/Radixin/Moesin) family of proteins.<sup>44, 45</sup>

Pitavastatin was used in this study because we previously found that this compound elicited the most potent inhibitory effects on HMG-CoA reductase activity in rodent liver microsomes compared with other statins (authors’ unpublished observation). A prior study reported that daily oral administration of pitavastatin at 1 and 10 mg/kg, which exceeds the clinical norm and could lead to serious adverse side effects, attenuates the development of aortic atherosclerosis in ApoE<sup>−/−</sup> mice.<sup>46</sup> We thus examined whether NP-mediated pitavastatin delivery is superior to daily oral administration of pitavastatin alone for the inhibition of aortic atherosclerosis, plaque destabilization and rupture, and we found that oral daily administration of pitavastatin at 0.1 mg/kg per day for 28 days (cumulative dose = 0.1 mg/body) had no therapeutic effects, but administration of pitavastatin at 1.0 mg/kg per day (cumulative dose = 1.0 mg/body) showed significant therapeutic effects. Therefore, our NP-mediated DDS (0.05 mg pitavastatin per body) potentiates therapeutic efficacy of pitavastatin (at least 20-fold). Our NP-mediated DDS may extend the usages of statin treatment while reducing potential side effects.

There are some limitations in the present study. First, we could not detect thrombus formation in the brachiocephalic artery, myocardial infarction or stroke in our murine model because mouse ruptured plaques rarely undergo thrombotic occlusion that may reflect the difference in coagulation/fibrinolytic activity between mice and humans.<sup>47</sup>
Second, we could not determine the proportion of transferred macrophage migrated into the arteries. Although substantial proportion of transferred macrophage might be trapped in reticuloendothelial system such as liver and lung, we could observe increased macrophage infiltration to the brachiocephalic artery associated with upregulated monocyte/macrophage-associated serum proteins, suggesting that transferred macrophages were biologically active in the present study.

Third, the tissue concentrations of pitavastatin could not be measured because the sample size from the brachiocephalic arteries was too small for high performance liquid chromatography. Although we examined the plasma level of pitavastatin in the pitavastatin and pitavastatin-NP group, the concentration of pitavastatin could be measured only after 2 hours after intravenous injection. Further studies are needed to clarify the in vivo pharmacokinetics of pitavastatin after pitavastatin-NP treatment in larger animals.

Finally, we only adopted the protocol of weekly intravenous administration of pitavastatin-NPs at the indicated dose. The dose of pitavastatin-NPs used in this study was selected because of its effectiveness in previous studies from our laboratory in murine and rabbit models of hindlimb ischemia. Further studies are needed to determine the optimal dose range and interval of pitavastatin-NPs for clinical application.

In conclusion, the recruitment of inflammatory monocytes is critical in the pathogenesis of plaque destabilization and rupture. NP-mediated pitavastatin delivery inhibited plaque destabilization and rupture, and regulated recruitment of inflammatory monocytes by interfering in MCP-1/CCR2 signaling in this model. This nanotechnology-based modality can be developed as a new therapeutic strategy for vulnerable patients with rupture-prone unstable plaques.
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Conflict of Interest Disclosures: Dr. Egashira holds a patent on the results reported in the present study. The remaining authors report no conflicts.

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Essential role of angiotensin ii type 1a receptors in the host vascular wall, but not the bone marrow, in the pathogenesis of angiotensin ii-induced atherosclerosis. *Hypertension Res.* 2008;31:1791-1800.


**Table 1.** Characteristics of the brachiocephalic artery plaques in the no treatment, CCR2+/+ inflammatory macrophage, and CCR2−− leukocyte groups.

<table>
<thead>
<tr>
<th></th>
<th>No Treatment (N=9)</th>
<th>CCR2+/+ Inflammatory Macrophage (N=5)</th>
<th>CCR2−− Leukocyte (N=8)</th>
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<tbody>
<tr>
<td>Ruptured Plaques per Animal (n)</td>
<td>3.3±0.2</td>
<td>5.0±0.7†</td>
<td>2.6±0.4</td>
</tr>
<tr>
<td>Plaque Area (×10³ μm²)</td>
<td>230±10</td>
<td>230±10</td>
<td>170±10*</td>
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<tr>
<td>Fibrous Cap Thickness (μm)</td>
<td>1.6±0.2</td>
<td>1.1±0.1†††</td>
<td>2.2±0.3</td>
</tr>
<tr>
<td>Lipid Core Area (%)</td>
<td>15±3</td>
<td>20±4</td>
<td>15±3</td>
</tr>
<tr>
<td>Macrophage Area (%)</td>
<td>22±2</td>
<td>32±1***†††</td>
<td>20±3</td>
</tr>
<tr>
<td>MCP-1 Area (%)</td>
<td>21±1</td>
<td>28±2*†</td>
<td>20±3</td>
</tr>
</tbody>
</table>

The data are expressed as the mean±SEM. *P<0.05 vs. the No Treatment group. **P<0.01 vs. the No Treatment group. †P<0.05 vs. the CCR2−− Leukocyte group. †††P<0.001 vs. the CCR2−− Leukocyte group. The data of ruptured plaques per animal was compared using ANOVA followed by Dunn’s multiple comparison tests. The other data were compared using ANOVA followed by Bonferroni’s multiple comparison tests.
Table 2. Characteristics of the brachiocephalic artery plaques in the no treatment, FITC-NP, pitavastatin, and pitavastatin-NP groups.

<table>
<thead>
<tr>
<th></th>
<th>No Treatment (N=9)</th>
<th>FITC-NP (N=7)</th>
<th>Pitava (N=6)</th>
<th>Pitava-NP (N=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ruptured Plaques per Animal (n)</td>
<td>3.3±0.2</td>
<td>3.4±0.5</td>
<td>3.3±0.2</td>
<td>2.0±0.4*</td>
</tr>
<tr>
<td>Plaque Area (×10^3 μm²)</td>
<td>230±10</td>
<td>170±10</td>
<td>130±40*</td>
<td>150±20*</td>
</tr>
<tr>
<td>Fibrous Cap Thickness (μm)</td>
<td>1.6±0.2</td>
<td>1.3±0.1</td>
<td>1.2±0.1</td>
<td>1.9±0.2*</td>
</tr>
<tr>
<td>Lipid Core Area (%)</td>
<td>15±3</td>
<td>12±3</td>
<td>8±2</td>
<td>11±2</td>
</tr>
<tr>
<td>Macrophage Area (%)</td>
<td>22±2</td>
<td>22±3</td>
<td>27±2</td>
<td>14±3*</td>
</tr>
<tr>
<td>MCP-1 area (%)</td>
<td>21±1</td>
<td>24±2</td>
<td>26±2</td>
<td>15±3*</td>
</tr>
</tbody>
</table>

The data are expressed as the mean±SEM. *P<0.05 vs. the No Treatment group. The data of ruptured plaques per animal was compared using ANOVA followed by Dunn’s multiple comparison tests. The other data were compared using ANOVA followed by Bonferroni’s multiple comparison tests.

Table 3. Characteristics of the brachiocephalic artery plaques in the FITC-NP and 7ND-NP groups.

<table>
<thead>
<tr>
<th></th>
<th>FITC-NP</th>
<th>7ND-NP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ruptured Plaques per Animal (n)</td>
<td>3.1±0.4 (N=12)</td>
<td>1.4±0.4* (N=10)</td>
</tr>
<tr>
<td>Plaque Area (×10^3 μm²)</td>
<td>140±15 (N=8)</td>
<td>170±30 (N=5)</td>
</tr>
<tr>
<td>Fibrous Cap Thickness (μm)</td>
<td>1.6±0.3 (N=8)</td>
<td>3.6±0.8* (N=9)</td>
</tr>
<tr>
<td>Macrophage Area (%)</td>
<td>16±1 (N=5)</td>
<td>9±1* (N=6)</td>
</tr>
<tr>
<td>MCP-1 area (%)</td>
<td>17±2 (N=6)</td>
<td>14±3 (N=6)</td>
</tr>
</tbody>
</table>

The data are expressed as the mean±SEM. The data of ruptured plaques per animal was compared using Mann-Whitney test. The other data were compared using the unpaired t-test. *P<0.05 vs. FITC-NP.

Table 4. Characteristics of the brachiocephalic artery plaques in the no treatment, pitavastatin 0.1 mg/kg, and pitavastatin 1.0 mg/kg groups.

<table>
<thead>
<tr>
<th></th>
<th>No Treatment (N=9)</th>
<th>Pitava 0.1 mg/kg (N=10)</th>
<th>Pitava 1.0 mg/kg (N=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ruptured Plaques per Animal (n)</td>
<td>3.3±0.2</td>
<td>3.2±0.7</td>
<td>1.9±0.3†</td>
</tr>
<tr>
<td>Plaque Area (×10^3 μm²)</td>
<td>230±10</td>
<td>190±20</td>
<td>130±10*</td>
</tr>
<tr>
<td>Fibrous Cap Thickness (μm)</td>
<td>1.6±0.2</td>
<td>1.5±0.1</td>
<td>2.6±0.4**</td>
</tr>
<tr>
<td>Lipid Core Area (%)</td>
<td>15±3</td>
<td>26±2</td>
<td>14±2</td>
</tr>
<tr>
<td>Macrophage Area (%)</td>
<td>22±2</td>
<td>26±3</td>
<td>18±4</td>
</tr>
<tr>
<td>MCP-1 area (%)</td>
<td>21±1</td>
<td>17±1</td>
<td>16±3</td>
</tr>
</tbody>
</table>

The data are expressed as the mean±SEM. *P<0.05 vs. the No Treatment group. **P<0.01 vs. the No Treatment group. The data of ruptured plaques per animal was compared using ANOVA followed by Dunn’s multiple comparison tests. The other data were compared using ANOVA followed by Bonferroni’s multiple comparison tests. †P<0.05 vs. the No Treatment group. The data were compared using ANOVA followed by Dunnett’s multiple comparison tests.
Figure Legends:

Figure 1. The adoptive transfer of inflammatory macrophages accelerated plaque destabilization and rupture. (A) Upper panel: Photomicrographs of atherosclerotic plaques in the brachiocephalic artery stained with elastica van Gieson (EVG), Mac3 or MCP-1 in the No Treatment (N), CCR2+/+ Inflammatory Macrophage (I) and CCR2+/- Leukocyte (L) groups. Arrowheads indicate disrupted/buried fibrous caps. The scale bar indicates 100 μm. Lower panel: Quantitation of the number of disrupted/buried fibrous caps, fibrous cap thickness, and Mac3- and MCP-1-positive areas. The data are reported as the mean±SEM. *P<0.05, **P<0.01, and ***P<0.001 versus the CCR2+/+ inflammatory macrophage group. (B) Upper panel: Photomicrographs of the intraluminal surface of the total aorta, stained with oil red O. Lower panel: Quantitation of the percentage of the oil red O-positive area compared with the total luminal surface area. The data are reported as the mean±SEM. *P<0.05 versus the CCR2+/+ inflammatory macrophage group. (C) Upper panel: Photomicrographs of atherosclerotic plaques in the aortic root stained with EVG or Mac3. Lower panel: Quantitation of plaque size and Mac3-positive areas. The scale bar indicates 200 μm. The data are reported as the mean±SEM. †P<0.05 versus the CCR2+/+ inflammatory macrophage group using one-way ANOVA followed by Dunnett’s multiple comparison tests.

Figure 2. In vivo nanoparticle-mediated drug delivery. (A) Representative flow cytometry dot plots of circulating leukocytes 2 hours after an intravenous injection of FITC-NPs. Cells were stained with lineage markers and anti-CD11b. The histograms demonstrate FITC uptake by neutrophils (i) and monocytes (ii) in the blood and spleen. Purple indicates control fluorescence...
in cells derived from uninjected mice. Green indicates fluorescence in cells derived from the FITC-NP-injected mice. The percentages of FITC-positive cells are reported as the mean±SEM (N = 3 per group). (B) Upper panel: Representative fluorescent and light stereomicrographs of isolated segments of the aortic arch 24 hours after the intravenous injection of saline (control) or FITC-NPs. The scale bar indicates 1 mm. Lower panel: Representative photomicrographs of serial sections of atherosclerotic plaques in the brachiocephalic artery (red dotted line of upper panel) examined with fluorescence microscopy or stained with hematoxylin-eosin (HE). In the fluorescent micrographs, the nuclei were counterstained with propidium iodide (red). The scale bar indicates 200 μm.

**Figure 3.** Treatment with pitavastatin-NPs inhibits atherosclerotic plaque destabilization and rupture. (A) Upper panel: Photomicrographs of atherosclerotic plaques in the brachiocephalic artery stained with elastica van Gieson (EVG), Mac3 or MCP-1 from the No Treatment (N), FITC-NP (FN), pitavastatin (P), and pitavastatin-NP (PN) groups. The scale bar indicates 100 μm. Lower panel: Quantitation of the number of disrupted/buried fibrous caps, fibrous cap thickness, and Mac3- and MCP-1-positive areas. The data are reported as the mean±SEM. *P<0.05 versus the No Treatment group. (B) Upper panel: Photomicrographs of the intraluminal surface of the total aorta stained with oil red O. Lower panel: Quantitation of the percentage of oil red O-positive area compared with the total luminal surface area. The data are reported as the mean±SEM. *P<0.05 versus the No Treatment group. (C) Upper panel: Photomicrographs of atherosclerotic plaques in the aortic root stained with EVG or Mac3. Lower panel: Quantitation of plaque size and Mac3-positive areas. The scale bar indicates 200 μm. The data are reported as the mean±SEM. †P<0.05 versus the No Treatment group using one-way ANOVA followed by
Dunnett’s multiple comparison tests. *P<0.05 versus the No Treatment group using one-way ANOVA followed by Bonferroni’s multiple comparison tests.

**Figure 4.** Effects of pitavastatin-NPs on the recruitment of Ly-6C<sup>high</sup> monocytes. (A) Left panel: Representative flow cytometry dot plots and histograms from mice injected intravenously with control (empty)-NPs or pitavastatin-NPs. Right panel: The quantitative analysis of the ratio of monocytes and Ly-6C<sup>high</sup> monocytes to total leukocytes is also presented. The data are reported as the mean±SEM (N = 3-4 per group). *P<0.005 and **P<0.001 versus the Control-NP group using unpaired t-test. (B) Effects of pitavastatin-NPs on the mRNA levels of CCR2 and MCP-1 in RAW264.7 cells. Data are reported as the mean ± SEM (N = 3 per group). The data were compared using one-way ANOVA followed by Bonferroni’s multiple comparison tests. *P<0.05 versus the IFNγ group. (C) Effects of pitavastatin-NPs on MCP-1-induced monocyte chemotaxis in THP-1 cells. Data are reported as the mean±SEM (N – 3 per group). The data were compared using one-way ANOVA followed by Bonferroni’s multiple comparison tests. *P<0.05 and **P<0.001 versus the MCP-1 group.

**Figure 5.** Effects of pitavastatin-NPs on the gelatinase activity of the atherosclerotic plaque. (A) Left panel: In situ zymography for the gelatinase activity of atherosclerotic plaque in the brachiocephalic artery. The nuclei were counterstained with DAPI. The scale bar indicates 100 µm. The addition of EDTA was utilized as a negative control for each section. Right panel: Quantitative analysis of the relative fluorescence units (RFUs) of gelatinase activity in atherosclerotic plaques treated with control-NPs or pitavastatin-NPs. Data are reported as the mean±SEM. *P<0.05 versus the Control-NP group using unpaired t-test. (B) Quantitative
analysis of the native form of MMP-9 by gelatin zymography. The data are reported as the mean±SEM (N = 3 per group) and were compared using two-way ANOVA followed by Bonferroni’s multiple comparison tests. *P<0.05 versus the LPS group using one-way ANOVA followed by Bonferroni’s multiple comparison tests.

**Figure 6.** Nanoparticle-mediated anti-MCP-1 gene therapy inhibits plaque destabilization and rupture. (A) Upper panel: Photomicrographs of atherosclerotic plaques in the brachiocephalic artery stained with elastica van Gieson (EVG) or Mac3. Arrowheads indicate disrupted/buried fibrous caps. The scale bar indicates 100 μm. Lower panel: Quantitative comparison of the number of disrupted/buried fibrous caps, fibrous cap thickness, and Mac3-positive area. The data are reported as the mean±SEM. *P<0.05 and ***P<0.001 versus the FITC-NP group. (B) Quantitation of the percentage of the plaque area compared with the total luminal surface area. The data are reported as the mean±SEM. There were no statistically significant differences between the two groups.
Figure 1

**A**

<table>
<thead>
<tr>
<th>N: No Treatment</th>
<th>I: CCR2+/+ Inflammatory Macrophage</th>
<th>L: CCR2-/- Leukocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>EVG</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>Mac3</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
</tr>
<tr>
<td>MCP-1</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Disrupted/buried fibrous caps

Fibrous Cap Thickness

**B**

En Face Analysis

Atherosclerosis of Aorta

Oil Red O

(**% of whole aorta**)

**C**

Quantitative Analysis

Atherosclerosis of Aortic Root

Plaque area

Mac3

![Image](image10.png)

![Image](image11.png)

![Image](image12.png)

![Image](image13.png)
Figure 2

A

(i) Neutrophil
(ii) Monocyte

CD11b

Lin

CD11b

No Treatment
FITC-NP

Blood

Cell count

36±13% 61±13%

(i) Neutrophil (ii) Monocyte

Spleen

20±7% 48±9%

FITC

B

FITC-NP
No Treatment

Aortic Arch

Fluorescence

Light

Brachiocephalic Artery

Fluorescence

HE
Figure 3

A. En Face Analysis

Atherosclerosis of Aorta

Oil Red O

Disrupted/buried Fibrous caps

Fibrous Cap Thickness

Mac3 MCP-1

EVG

Quantitative Analysis

Atherosclerosis of Aortic Root

Plaque area

Mac3

Disruption of the fibrous cap is observed in images labeled as "Disrupted/buried Fibrous caps." The thickness of the fibrous cap is shown in images labeled as "Fibrous Cap Thickness." Macrophage marker (Mac3) and monocyte chemotactic protein-1 (MCP-1) are visualized in images labeled as "Mac3 MCP-1."

B. En Face Analysis

Atherosclerosis of Aorta

Oil Red O

C. Quantitative Analysis

Atherosclerosis of Aortic Root

Plaque area

Mac3

Graphs showing quantitative analysis of plaque area and Mac3 expression across different treatment groups (N: No Treatment, FN: FITC-NP, P: Pitavastatin, PN: Pitava-NP).
Figure 4

A

Monocyte

Control NP  Pitava-NP

CD115

% of total leukocytes

Control Pitava NP

CD11b

% of total leukocytes

B

Ly-6C

Cell counts

Control NP  Pitava-NP

Ly-6C

% of total leukocytes

Ly-6C

% of total leukocytes

C

CCR2

Relative Expression

IFNγ  0 0.01 1 10

Pitava1NP (μM)

MCP-1

x10^4 cells/ml

MCP-1

Pitava1NP (μM)

Chemotaxis

0 0.01 1 10

Pitava1NP (μM)
Figure 5
**Figure 6**

A FITC-NP 7ND-NP

**EVG**

Disrupted/buried Fibrous Caps

**Mac3**

Fibrous Cap Thickness

Mac3

Disrupted/buried Fibrous Caps

**En Face Analysis**

Atherosclerosis Aorta

FITC-NP 7ND-NP

Oil Red O

(% of whole aorta)

(% of plaque area)

(N / animal)
Nanoparticle-Mediated Delivery of Pitavastatin Inhibits Atherosclerotic Plaque Destabilization/Rupture in Mice by Regulating the Recruitment of Inflammatory Monocytes
Shunsuke Katsuki, Tetsuya Matoba, Soichi Nakashiro, Kei Sato, Jun-ichiro Koga, Kaku Nakano, Yasuhiro Nakano, Shizuka Egusa, Kenji Sunagawa and Kensuke Egashira

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SUPPLEMENTARY MATERIAL

Supplementary Materials and Methods

Diet preparation

A high-fat diet (HFD) that contained 21% fat from lard and was supplemented with 0.15% (wt/wt) pure cholesterol (Oriental yeast, Tokyo Japan) was prepared according to the formula recommended by the American Institute of Nutrition. This diet contained the following constituents: casein, cystine, corn starch, sucrose, cholic acid, a mineral mixture, a vitamin mixture, powdered cellulose, choline bitartrate, and tert-butylhydroquinone.

Experimental protocols

At 16 to 18 weeks of age, mice began receiving the HFD. After 4 weeks of HFD, all mice were infused with angiotensin II dissolved in phosphate-buffered saline (PBS) at 1.9 mg/kg per day via an osmotic mini-pump (Alzet, Cupertino, CA, USA) for 4 weeks.\(^1,2\) Systolic blood pressure and heart rate were measured by the tail-cuff method and body weight was measured 4 weeks after the angiotensin II infusion. Mice were euthanized with intraperitoneal injection of pentobarbital at day 28 of angiotensin II infusion for analysis. Blood samples were collected via the left ventricles. Commercially available enzyme-linked immunosorbent assay kits (Wako Pure Chemical Industries, Osaka, Japan) were used to measure plasma lipid profiles (total cholesterol and triglycerides). Serum levels of various biomarkers were measured with the Luminex LabMAP instruments (Charles River Laboratories, Wilmington, MA, USA) (http://www.criver.com/en-US/ProdServ/ByType/Discovery/Pages/PlasmaBiomarkerAnalysis.aspx).

*Experimental protocol 1:* To examine whether inflammation-activated macrophages accelerate plaque destabilization and rupture, CCR2\(^{+/+}\)-inflammatory macrophages from the peritoneal cavity of ApoE\(^{-/-}\)CCR2\(^{+/+}\) mice and CCR2\(^{-/-}\)-leukocytes from the peritoneal
cavity of ApoE\(^{-/-}\)CCR2\(^{-/-}\) mice were collected three days after intraperitoneal injection of 2 ml of 0.05% thioglycollate (BD Biosciences, Franklin Lakes, NJ, USA), and these cells were directly injected intravenously three times during angiotensin II infusion. At the beginning of the angiotensin II infusion, animals were divided into 2 groups: (i) the CCR2\(^{+/-}\)-inflammatory macrophage group (1x10\(^6\) cells/ 200 µl PBS) (n=5); and (ii) the CCR2\(^{-/-}\)-leukocyte group (1x10\(^6\) cells/ 200 µl PBS) (n=8). Mice were euthanized over a several day period after 4 weeks of angiotensin II infusion for this protocol.

**Experimental protocol 2:** To examine the effect of nanoparticle-mediated delivery of pitavastatin on plaque destabilization and rupture, animals were divided into 4 groups at the beginning of angiotensin II infusion: (i) the no treatment group (n=9); (ii) the FITC-incorporated NP group (0.1 mg PLGA/ 200 µl PBS) (n=7); (iii) the pitavastatin-only group (0.012 mg pitavastatin/ 200 µl PBS) (n=6); and (iv) the pitavastatin-incorporated NP group (0.1 mg PLGA/ 0.012 mg pitavastatin/ 200 µl PBS) (n=10). FITC-NP, pitavastatin, and pitavastatin-NP were administered intravenously via the tail vein once per week.

**Experimental protocol 3:** To examine the effect of nanoparticle-mediated delivery of the 7ND plasmid (a dominant negative inhibitor of MCP-1\(^{3-6}\)) on plaque destabilization and rupture, animals were divided into 2 groups at the beginning of the angiotensin II infusion: (i) the FITC-incorporated NP group (1.3 mg PLGA/ 200 µl PBS) (n=12); and (ii) the 7ND plasmid-incorporated NP group (1.3 mg PLGA/ 5 µg 7ND plasmid/ 200 µl PBS) (n=10). NPs were administered by weekly intravenous injection.

**Experimental Protocol 4:** To examine the effect of daily oral administration of pitavastatin on plaque destabilization and rupture, animals were divided into 2 groups at the beginning of angiotensin II infusion: (i) the low pitavastatin group (lower dose: 0.1 mg/kg per day); and (ii) the high pitavastatin group (higher dose: 1.0 mg/kg per day). Pitavastatin was
administered by oral gavage every day for 4 weeks.
The no treatment group in protocol 2 was also used as the control group in protocols 1 and 4.

**Histopathology**

To quantify the extent of the atherosclerotic lesions in the whole aorta, the aortic arch and the thoracic aorta was opened longitudinally, stained with oil red O, and pinned on a black wax surface. The percentage of the plaque area stained by oil red O with respect to the total luminal surface area was quantified. To quantify the extent of the atherosclerotic lesions in the aortic root, approximately 3 serial cross sections (5 µm thick) of the aortic root were prepared according to the method described by Paigen et al., with a slight modification. In brief, atherosclerotic lesions in the aortic sinus region were examined at 3 locations, each separated by 100 µm, with the most proximal site starting after the appearance of at least two aortic valve leaflets. Serial sections were stained with elastica van Gieson (EVG). The largest plaque of the three valve leaflets was adopted for morphological analysis. The brachiocephalic arteries were embedded in paraffin or OCT compound (Sakura Finetecchnical Co. Ltd, Tokyo, Japan). Sections were cut at 3 µm for paraffin-embedded sections or 5 µm for OCT-embedded sections. Three sets of serial sections obtained at 30 µm intervals (starting from the proximal end) were stained with EVG to measure the total number of disrupted and buried fibrous caps, as previously described. Disrupted and buried fibrous caps were defined as follows. A disrupted fibrous cap was defined as a visible defect in the cap accompanied by an intrusion of erythrocytes into the plaque. A buried fibrous cap was defined as an elastin layer that was overlaid with foam cells. All morphometric analyses were made on EVG-stained sections, and three vessel cross sections were quantified per mouse by computerized image analysis. Fibrous cap thickness was determined at the thinnest part of the cap by computerized image
analysis. The analysis was necessarily restricted to those plaques that had developed sufficiently to form fibrous caps, and the average thickness of three fibrous caps per section was obtained for quantitative analysis. Plaque macrophage or MCP-1 expression areas were determined by the ratio of Mac3- or MCP-1-positive areas to the intima areas of plaques.

Immunohistochemistry

Serial brachiocephalic arterial sections adjacent to those sections that were stained with EVG were deparaffinized, and endogenous peroxidase was blocked by incubation with 0.3% H$_2$O$_2$ in methanol for 5 minutes. For antigen retrieval, sections were boiled for 20 minutes in citrate buffer (pH=6.0). After blocking with 3% skim milk, sections were incubated overnight at 4°C with the following antibodies: anti-mouse macrophage antibody (Mac3; dilution 1:100, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and anti-mouse MCP-1 antibody (dilution 1:200, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) followed by incubation with biotin-conjugated secondary antibodies. Then, the sections were washed and treated with avidin-peroxidase. The sections were developed using the DAB substrate kit (Wako Pure Chemical Industries, Osaka, Japan), and nuclei were counterstained with hematoxylin. Serial aortic root sections were also stained using anti-mouse Mac3 antibody. Multiple observers who were blinded to the experiment protocol performed the quantitative analysis. All images were captured with a Nikon microscope equipped with a digital camera (HC-2500) and analyzed using Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA, USA) and Scion Image 1.62 for Windows (Scion, Frederick, MD, USA).

Flow cytometry

Peripheral blood was drawn via a cardiac puncture, and red blood cells were lysed with
VersaLyse Lysing solution (Becton Dickinson Biosciences, San Jose, California) for 10 minutes at room temperature. Spleens were removed and triturated in HBSS at 4 °C and filtered through nylon mesh (BD Biosciences). The cell suspension was centrifuged at 300 x g for 5 minutes at 4 °C. Red blood cells were also lysed with VersaLyse Lysing solution. After blocking the Fc receptor with anti-CD16/32 mAb (BD Pharmingen, San Diego, California) for 5 minutes at 4°C, peripheral leukocytes were incubated with a cocktail of CA, USA against CD11b-APC (BD Pharmingen, San Diego, California), CD115-PE (BD Pharmingen, San Diego, California) and Ly-6C-FITC (eBioscience, San Diego, CA, USA), and peritoneal leukocytes were incubated with a cocktail of mAb against F4/80-APC (AbD Serotec, Oxford, UK), CD115-PE (BD Pharmingen) and Ly-6C-FITC (eBioscience) for 30 minutes at 4°C; all leukocytes were then analyzed with FACSCalibur (Becton Dickinson Biosciences, San Jose, CA, USA). For the cellular uptake of FITC-NPs, leukocytes were incubated with a cocktail of mAb against lineage cell marker (Lin: CD90/B220/CD49b/NK1.1/Ly-6G)-PE (BD Pharmingen, San Diego, CA, USA) and CD11b-APC. The leukocytes were also incubated with appropriate isotype controls (BD Pharmingen, San Diego, CA, USA). Macrophage subsets were identified as either Ly-6C<sup>hi</sup>F4/80<sup>-</sup>CD115<sup>+</sup> or Ly-6C<sup>lo</sup>F4/80<sup>-</sup>CD115<sup>+</sup>, as previously described. Monocyte subsets were identified as either Ly-6C<sup>hi</sup>CD11b<sup>+</sup>CD115<sup>-</sup> or Ly-6C<sup>lo</sup>CD11b<sup>+</sup>CD115<sup>+</sup>, as previously described. For the cellular uptake of FITC-NPs, neutrophils and monocytes were identified as Lin<sup>-</sup>CD11b<sup>+</sup> and Lin<sup>-</sup>CD11b<sup>+</sup>, respectively.

In vivo accumulation of inflammation-activated macrophages

Thioglycollate-elicited macrophages were labeled with PKH26 (Sigma Aldrich, St. Louis, MO, USA) ex vivo according to the manufacturer’s protocol. 24 hours after the intravenous injection of PKH26-labeled macrophages, the brachiocephalic arteries were
fixed with 3.7% formaldehyde and embedded in OCT compound. Sections were cut at 5 µm for OCT-embedded sections and evaluated by fluorescence microscopy. Nuclei were stained with DAPI (Vector Laboratories Inc., Burlingame, California).

**Splenic Monocyte/Macrophage isolation and adoptive transfer**

Splenic monocytes and macrophages were obtained by negative selection using the Mouse Monocytes Enrichment Kit (StemCell Technologies, British Columbia, Canada) according to the manufacturer’s protocol. In brief, monocytes and macrophages were isolated from an ApoE−/− mouse spleen. Cells were passed through a 100 µm nylon filter, and suspended in lysis buffer. Mouse monocytic cells were further purified from the spleens using the Purple EasySep magnet (StemCell Technologies) prior to flow cytometry analysis. These cells (0.8-1.0 x10⁶ cells/ 200 µl PBS) were directly injected intravenously via the femoral vein once to ApoE−/− mice fed a high-fat diet and infused with angiotensin II. Mice were euthanized with intraperitoneal injection of pentobarbital at day 7 of angiotensin II infusion for analysis.

**Preparation of PLGA nanoparticles**

Poly(lactic-co-glycolic acid) (PLGA) polymer with an average molecular weight of 20,000 and a lactide-to-glycolide copolymer ratio of 75:25 (Wako Pure Chemical Industries, Osaka, Japan) was used to prepare the nanoparticles. PLGA nanoparticles incorporated with fluorescein isothiocyanate (FITC; Dojindo Laboratories, Kumamoto, Japan) (FITC-NP), pitavastatin (Kowa Pharmaceutical Co Ltd, Tokyo, Japan) (pitavastatin-NP), or 7ND plasmid (7ND-NP) were prepared by a previously reported emulsion solvent diffusion method in purified water.¹¹⁻¹³ PLGA was dissolved in a mixture of acetone and methanol. Then, FITC, pitavastatin, or 7ND plasmid was added to this solution. The resultant PLGA-FITC, PLGA-pitavastatin, or PLGA-7ND solution was
emulsified in polyvinyl alcohol with stirring at 400 rpm using a propeller-type agitator with 3 blades (Heidon 600G, Shinto Scientific, Tokyo, Japan). After the system was agitated for 2 hours under reduced pressure at 40°C, the entire suspension was centrifuged (20,000 x g for 20 minutes at -20°C). After the supernatant was removed, purified water was added and mixed with the sediment. The wet mixture was then centrifuged again to remove excess polyvinyl alcohol and the unencapsulated reagent that could not adsorb onto the surfaces of the nanoparticles. After this process was repeated, the resultant dispersion was freeze-dried under the same conditions. The FITC-, pitavastatin-, or 7ND-loaded PLGA nanoparticles contained 5.0% (w/v) FITC, 12.0% (w/v) pitavastatin, or 0.40% (w/v) 7ND, respectively. The average diameters of the PLGA-NPs were 231 nm, 159 nm, and 290 nm for the FITC-NP, pitavastatin-NP, and 7ND-NP, respectively. The surface charges (zeta potential) analyzed by Zetasizer Nano (Sysmex, Hyogo, Japan) were -16.7 mV, -4 mV, and 8.1 mV, respectively.

**Cellular uptake and in vitro kinetics of the nanoparticles**

Murine peritoneal macrophages were obtained from wild-type mice that were injected intraperitoneally with 2 ml of 0.05% thioglycollate 72 hours before the extraction. The cells were centrifuged at 1,000 rpm for 5 minutes at 4°C, suspended in lysis buffer and washed twice with phosphate-buffered saline (PBS) for 5 minutes at 4°C. The cell pellets were suspended in DMEM plus 10% FBS and 1% PS at an initial concentration of 5.0 × 10^5 cells/ml in a 35 mm culture dish. FITC-NPs were added to the dish (1.0 mg PLGA/ml) and incubated at 37°C in a 5% CO₂ environment overnight. After two washes with PBS for 5 minutes at room temperature, the cells were fixed with methanol and counterstained with propidium iodide (Vector Laboratories Inc., Burlingame, CA, USA). The intracellular uptake of FITC-NP was evaluated by fluorescence microscopy (BX50,
Olympus, Tokyo, Japan) and confocal microscopy (FV1000-D, Olympus). RAW264.7, a murine macrophage cell line, was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were seeded on 6-well-chamber slides and incubated at 37°C in a 5% CO₂ environment until they were subconfluent. The growth medium was replaced with 2 ml of an OsO₄-NP suspension medium (1.0 mg PLGA/ml), incubated for 6 hours and fixed with 2.5% glutaraldehyde. The intracellular uptake of OsO₄-NPs was observed by transmission electronic microscopy (Hitachi H7000E, Tokyo, Japan). To examine the FITC kinetics of cultured macrophages, confluent RAW264.7 cells were incubated with FITC-NP or FITC only at the indicated concentrations (1, 3, 10, 30, and 100 µM). After 2 hours of incubation, extracellular FITC-NPs or FITC was washed out, and FITC with diffuse interference contrast image was observed by confocal laser microscopy (Nikon A1R, Tokyo, Japan) at the indicated time points (days 0, 1, 4, and 7). The average fluorescent intensity of FITC was analyzed using the National Institutes of Health Image Software.

In vivo kinetics of the nanoparticles

The whole aortas of ApoE⁻/⁻ mice that were fed with HFD and infused with angiotensin II were excised 24 hours after an intravenous injection of the FITC-NPs. The excised aortas were evaluated by stereoscopic and fluorescence microscopy (Nikon SMZ1500 equipped with Nikon HB-10103AF and appropriate fluorescence filter sets, Tokyo, Japan). The brachiocephalic arteries were fixed with 3.7% formaldehyde and embedded in OCT compound. Sections were cut at lengths of 5 µm and evaluated by fluorescence microscopy (BX50, Olympus). The nuclei were stained with propidium iodide (Vector Laboratories Inc., Burlingame, CA, USA). Serial sections were stained with hematoxylin-eosin.
In situ zymography

Gelatinase (MMP-2/gelatinase-A and MMP-9/gelatinase-B) activity was measured in unfixed frozen sections (6 µm thick) using quenched fluorescein-labeled gelatinase substrate (DQ gelatin, Invitrogen, Eugene, OR, USA). The fluorescent area produced by the proteolytic digestion of quenched fluorescein-labeled gelatin was recognized as combined gelatinase activity (MMP-2 + MMP-9). The brachiocephalic artery sections were incubated at 37°C for 30 minutes according to the manufacturer’s protocol. Fluorescent microscopy was used to detect gelatinase activity as green fluorescence. Negative control zymograms were incubated in the presence of 20 mM EDTA. The specific removal of essential divalent cations resulted in no detectable gelatinolytic activity.

Gelatin zymography

The RAW264.7 cells were prepared as described above. The growth medium was replaced with pitavastatin at 0.01, 0.1, or 1 µM; pitavastatin-incorporated NPs containing 0.0367, 0.367, or 3.67 µg/mL of PLGA and 0.01, 0.1, or 1 µM of pitavastatin; FITC-incorporated NPs containing 3.67 µg/mL of PLGA-NP; or the vehicle alone. When the medium was replaced, LPS was added at 25 ng/mL to each well. Twenty-four hours after the LPS stimulation, the culture supernatant was obtained. The supernatant was subjected to gelatin zymography using the Gelatin Zymo-Electrophoresis Kit (Primary Cell, Hokkaido, Japan) according to the manufacturer’s directions.

Real-time quantitative RT PCR

Real-time PCR amplification was performed with the mouse cDNA with the use of the ABI PRISM 7000 Sequence Detection System (Applied Biosystems), as described previously. For mouse MCP-1, the sense and antisense PCR primers were
5’-CCTGGATCGGA-ACCAATGA-3’ and 5’-CGGGTCAACTTCA-CATTCAAAG-3’, respectively, and the probe oligonucleotide was 5’-
AACT-GCATCTGCCCTAAGG-TCTTCAGCA-3’. For mouse CCR2, the sense and
antisense primers were 5’-CCTTGGGA-ATGAGTAACTGTGTGAT-3’ and 5’-ATGGA-
GAGATACCTTCGGAACTTCT-3’, and the probe oligonucleotide was 5’-
CACTTAGACCAGGCCATGCAGGT-GACA-3’. The GAPDH probe was purchased
from Applied Biosystems.

Chemotaxis assay

THP-1, the human monocyte cell line, was obtained from the German Collection of
Micro-organisms and Cell Cultures (DSMZ; Braunschweig, Germany). The cells were
cultured in RPMI 1640 with 10% FBS and 37°C in a 5% CO₂ environment until they were
subconfluent. The growth medium was replaced with starvation medium with
pitavastatin-NPs containing 0.00367 to 36.7 µg/mL of PLGA and 0.001 to 10 µM of
pitavastatin, FITC-NPs containing 36.7 µg/mL of PLGA, or vehicle alone for 24 hours.
The chemotactic activity of THP-1 cells in response to 10 ng/mL MCP-1 was measured in
a 96-well microchemotaxis Boyden chamber (ChemoTx; Neuroprobe), as described
previously. Monocytes that had transmigrated through the micropore were stained with
trypan blue. The number of monocytes that migrated in response to MCP-1 was counted.

Measurements of statin concentration in plasma

Pitavastatin concentrations in plasma were measured at predetermined time points by
liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS). Briefly, the
high-performance liquid chromatography (HPLC) analysis was performed using Agilent
1100 series system (Agilent Technologies, Inc, Santa Clara, CA, USA). The column
temperature was maintained at 40 °C. The flow rate was 0.3 mL/min. Pre-prepared plasma
solutions were injected from the autosampler into the HPLC system. The turbo ion spray interface was operated in the positive ion mode at 4800 V and 550 °C. The analytical data were processed using Analyst software (version 1.4, Applied Biosystems, Foster City, CA, USA).
References and Notes


deployment to inflammatory sites. *Science.* 2009;325:612-616


Supplementary Figure Legends

Supplementary Figure 1. Experimental protocols for the treatments in ApoE<sup>−/−</sup> mice. At 16-18 weeks of age, mice began receiving the HFD. After 4 weeks of the experimental diet, all mice were infused with angiotensin II dissolved in phosphate-buffered saline (PBS) at 1.9 mg/kg per day.

Protocol 1. Animals were divided into 2 groups at the beginning of angiotensin II infusion: (i) adoptively transferred CCR2<sup>+/+</sup>-inflammatory macrophages from ApoE<sup>−/−</sup> mice (1 x 10<sup>6</sup> cells/ 200 µl PBS) and (ii) adoptively transferred CCR2<sup>−/−</sup>-leukocytes from ApoE<sup>−/−</sup>/CCR2<sup>−/−</sup> mice (1 x 10<sup>6</sup> cells/ 200 µl PBS).

Protocol 2. Animals were divided into 2 groups at the beginning of angiotensin II infusion: (i) the FITC-incorporated NP group (1.3 mg PLGA/ 200 µl PBS) and (ii) the 7ND-incorporated NP group (5 µg 7ND plasmid/ 200 µl PBS). NPs were administered by weekly intravenous injection.

Protocol 3. Animals were divided into 4 groups at the beginning of angiotensin II infusion: (i) the no treatment group, (ii) the FITC-incorporated NP group (0.1 mg PLGA/ 200 µl PBS), (iii) the pitavastatin-only group (0.012 mg pitavastatin/ 200 µl PBS), and (iv) the pitavastatin-incorporated NP group (0.1 mg PLGA/ 0.012 mg pitavastatin/ 200 µl PBS). NPs were administered by weekly intravenous injection.

Protocol 4. Animals were divided into 2 groups at the beginning of angiotensin II infusion: (i) oral daily administration of pitavastatin at a low dose (0.1 mg/kg/day) and (ii) oral daily administration of pitavastatin at a high dose (1.0 mg/kg/day). Pitavastatin was daily administered by oral gavage.

The no treatment group in protocol 2 was also used as the control group in protocols 1 and 4.

Supplementary Figure 2. Characteristics and kinetics of adoptive transferred macrophages. (A) Quantitative flow cytometric analysis of the number of F4/80<sup>+</sup>/CD115<sup>+</sup> macrophages in the peritoneal cavities of ApoE<sup>−/−</sup> or ApoE<sup>−/−</sup>/CCR2<sup>−/−</sup> mice induced by intraperitoneal injection of thioglycollate (TG). (B) Quantitative analysis of the mean
fluorescence intensity (MFI) of Ly-6C expression in the F4/80\(^+\)CD115\(^+\) macrophages from the peritoneal cavities of the ApoE\(^{-/-}\) mice. The data are reported as the mean±SEM.  

(C) Left panel: A fluorescence photomicrograph of the brachiocephalic artery of an ApoE\(^{-/-}\) mouse from the no treatment group. Upper middle and right panel: PKH fluorescence photomicrographs of the brachiocephalic artery of an ApoE\(^{-/-}\) mouse transferred with PKH-labeled activated macrophages. Lower middle and right panel: FITC autofluorescence photomicrographs of the brachiocephalic artery of an ApoE\(^{-/-}\) mouse transferred with PKH-labeled activated macrophages. Right panel: An expanded image of the red square area in the middle panel. The nuclei were stained with DAPI. The scale bar indicates 100 \(\mu\)m.

**Supplementary Figure 3.** The adoptive transfer of splenic monocytes accelerates plaque destabilization and rupture in the brachiocephalic arteries. (A) Left panel: Representative flow cytometry dot plots of splenic leukocytes from ApoE\(^{-/-}\) mice. Middle panel: The Representative flow cytometry dot plots of splenic leukocytes negatively selected with antibodies against the leukocytes other than monocytes from ApoE\(^{-/-}\) mice. Right panel: The Representative histogram of Ly-6C expression on negatively selected splenic monocytes. (B) Upper panel: Photomicrographs of atherosclerotic plaques in the brachiocephalic artery stained with elastica van Gieson (EVG) in the No Treatment (N) and the Monocytes (M) groups. Arrowheads indicate disrupted/buried fibrous caps. The scale bar indicates 100 \(\mu\)m. Lower panel: Quantitation of the number of disrupted/buried fibrous caps and fibrous cap thickness. The data are reported as the mean±SEM. *\(P<0.05\) versus the No Treatment group. There were no statistically significant differences in fibrous cap thickness between the two groups.

**Supplementary Figure 4.** Cellular uptake and *in vitro* kinetics of the NPs in macrophages. (A) Fluorescence photomicrographs of murine peritoneal macrophages incubated with FITC-NPs for 24 hours. An inset depicts a photomicrograph of macrophages incubated without FITC-NPs. (B) A fluorescence confocal microscopy image of RAW264.7 cells
incubated with FITC-NPs for 24 hours. (C) Electron microscopy image of RAW264.7 cells incubated with OsO₄-NPs for 24 hours. (D) Upper panel: Time course of the FITC signal retained in RAW264.7 cells after a 2-hour incubation with FITC-NPs or FITC (0.3, 1, 3, 10, 30, 100 µM) followed by a washout period. Cells were observed at 0, 24, 72 hours, and 1 week of washout. Lower panel: Quantitative analysis of relative fluorescence units (RFUs) of RAW264.7 cells incubated with FITC-NPs (green lines) or FITC (blue lines). *P<0.01 and **P<0.001 versus FITC (N = 4 per group). Data were compared using two-way ANOVA followed by Bonferroni’s multiple comparison tests.

Supplementary Figure 5. Effects of daily oral administration of pitavastatin (0.1 or 1.0 mg/kg per day) on atherosclerotic plaque rupture in the brachiocephalic arteries. (A) Upper panel: Photomicrographs of atherosclerotic plaques stained with elastica van Gieson (EVG), Mac3 or MCP-1 in the No Treatment (N), pitavastatin 0.1 mg/kg (0.1), and pitavastatin 1.0 mg/kg (1.0) groups. Arrowheads indicate disrupted/buried fibrous caps. The scale bar indicates 100 µm. Lower panel: Quantitation of the number of disrupted/buried fibrous caps, fibrous cap thickness and Mac3- and MCP-1-positive areas. The data are reported as the mean±SEM. †P<0.05 versus the No Treatment group using one-way ANOVA followed by Dunnett’s multiple comparison tests. **P<0.01 versus the No Treatment group using one-way ANOVA followed by Bonferroni’s multiple comparison tests. (B) Upper panel: Photomicrographs of the intraluminal surface of the total aorta stained with oil red O. Lower panel: Quantitation of the percentage of the plaque area compared with the total luminal surface area. The data are reported as the mean±SEM. *P<0.05 versus the No Treatment group. (C) Upper panel: Photomicrographs of atherosclerotic plaques in the aortic root stained with EVG or Mac3. Lower panel: Quantitation of plaque size and Mac3-positive areas. The scale bar indicates 200 µm. The data are reported as the mean±SEM. †P<0.05 versus the No Treatment group using one-way ANOVA followed by Dunnett’s multiple comparison tests.
**Supplementary Tables**

**Supplementary Table 1.** Body weight, heart rate, systolic blood pressure, and lipid profiles in the no treatment, CCR2<sup>++</sup> inflammatory macrophage, and CCR2<sup>-/-</sup> leukocyte groups.

<table>
<thead>
<tr>
<th></th>
<th>No Treatment (N= 9)</th>
<th>CCR2&lt;sup&gt;++&lt;/sup&gt; Inflammatory Macrophage (N= 5)</th>
<th>CCR2&lt;sup&gt;-/-&lt;/sup&gt; Leukocyte (N= 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>33±1</td>
<td>35±3</td>
<td>34±3</td>
</tr>
<tr>
<td>Heart Rate (beat/min)</td>
<td>650±20</td>
<td>640±40</td>
<td>580±40</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mmHg)</td>
<td>120±2</td>
<td>112±8</td>
<td>115±9</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dl)</td>
<td>660±30</td>
<td>710±100</td>
<td>720±60</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>65±9</td>
<td>74±16</td>
<td>69±5</td>
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</table>

The data are expressed as the mean±SEM. The mean values were compared using ANOVA and Bonferroni’s multiple comparison tests, and there are no significant differences for any of these parameters among these groups.

**Supplementary Table 2.** Body weight, heart rate, systolic blood pressure, and lipid profiles in the no treatment, FITC-NP, pitavastatin, and pitavastatin-NP groups.

<table>
<thead>
<tr>
<th></th>
<th>No Treatment (N= 9)</th>
<th>FITC-NP (N= 7)</th>
<th>Pitava (N= 6)</th>
<th>Pitava-NP (N= 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>33±1</td>
<td>30±1</td>
<td>34±1</td>
<td>32±1</td>
</tr>
</tbody>
</table>
Heart Rate (beat/min) 650±20 630±30 650±20 590±20
Systolic Blood Pressure (mmHg) 120±0 110±10 12±10 120±0
Total Cholesterol (mg/dl) 660±30 670±50 610±50 710±40
Triglyceride (mg/dl) 65±9 60±5 53±5 70±6

The data are expressed as the mean±SEM. The mean values were compared using ANOVA and Bonferroni’s multiple comparison tests, and there are no significant differences for any of these parameters compared with the No Treatment group.

**Supplementary Table 3.** Body weight, heart rate, systolic blood pressure, and lipid profiles in the FITC-NP and 7ND-NP groups.

<table>
<thead>
<tr>
<th></th>
<th>FITC-NP (N= 9)</th>
<th>7ND-NP (N= 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>28±1</td>
<td>25±1</td>
</tr>
<tr>
<td>Heart Rate (beat/min)</td>
<td>640±30</td>
<td>680±10</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mmHg)</td>
<td>130±10</td>
<td>120±10</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dl)</td>
<td>720±60</td>
<td>730±20</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>47±11</td>
<td>53±18</td>
</tr>
</tbody>
</table>

The data are expressed as the mean±SEM. The mean values were compared using the unpaired t-test, and there are no significant differences for any of these parameters between these 2 groups.
**Supplementary Table 4.** Body weight, heart rate, systolic blood pressure, and lipid profiles in the no treatment, pitavastatin 0.1 mg/kg, and pitavastatin 1.0 mg/kg groups.

<table>
<thead>
<tr>
<th></th>
<th>No Treatment (N= 9)</th>
<th>Pitavastatin 0.1 mg/kg (N= 10)</th>
<th>Pitavastatin 1.0 mg/kg (N= 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Weight (g)</strong></td>
<td>33±1</td>
<td>30±1*</td>
<td>32±0</td>
</tr>
<tr>
<td><strong>Heart Rate (beat/min)</strong></td>
<td>650±20</td>
<td>610±20</td>
<td>630±10</td>
</tr>
<tr>
<td><strong>Systolic Blood Pressure (mmHg)</strong></td>
<td>120±0</td>
<td>110±0</td>
<td>120±0</td>
</tr>
<tr>
<td><strong>Total Cholesterol (mg/dl)</strong></td>
<td>660±30</td>
<td>780±20</td>
<td>800±50</td>
</tr>
<tr>
<td><strong>Triglyceride (mg/dl)</strong></td>
<td>65±9</td>
<td>82±15</td>
<td>43±5</td>
</tr>
</tbody>
</table>

The data are expressed as the mean±SEM. *P<0.05 versus the No Treatment group. The data were compared using ANOVA followed by Bonferroni’s multiple comparison tests.

**Supplementary Table 5.** Serum biomarkers in the no treatment, CCR2⁺⁺ inflammatory macrophage, and CCR2⁻⁻ leukocyte group.

<table>
<thead>
<tr>
<th>Apo A1</th>
<th>µg/mL</th>
<th>No Treatment (N= 7)</th>
<th>CCR2⁺⁺ Inflammatory Macrophage (N= 5)</th>
<th>CCR2⁻⁻ Leukocyte (N= 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD40</td>
<td>pg/mL</td>
<td>87±9</td>
<td>160±40</td>
<td>75±9</td>
</tr>
<tr>
<td>CD40 Ligand</td>
<td>pg/mL</td>
<td>2600±300</td>
<td>5700±400**</td>
<td>4600±700*</td>
</tr>
<tr>
<td>CRP</td>
<td>µg/mL</td>
<td>11±1</td>
<td>10±1</td>
<td>11±2</td>
</tr>
<tr>
<td>Protein</td>
<td>Unit</td>
<td>Value 1</td>
<td>Value 2</td>
<td>Value 3</td>
</tr>
<tr>
<td>------------------</td>
<td>--------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>EGF</td>
<td>pg/mL</td>
<td>16±1</td>
<td>23±1**</td>
<td>21±1**</td>
</tr>
<tr>
<td>Endothelin-1</td>
<td>pg/mL</td>
<td>18±1</td>
<td>21±2</td>
<td>17±2</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>pg/mL</td>
<td>320±20</td>
<td>330±50</td>
<td>380±30</td>
</tr>
<tr>
<td>Factor VII</td>
<td>ng/mL</td>
<td>14±1</td>
<td>19±1*</td>
<td>18±1</td>
</tr>
<tr>
<td>FGF-basic</td>
<td>ng/mL</td>
<td>7.0±0.6</td>
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<td>9.0±0.8</td>
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<tr>
<td>GCP-2</td>
<td>ng/mL</td>
<td>31±7</td>
<td>13±7</td>
<td>5±1**</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>µg/mL</td>
<td>140±20</td>
<td>190±30</td>
<td>200±20</td>
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<tr>
<td>IFN-γ</td>
<td>pg/mL</td>
<td>N.D.</td>
<td>23±8</td>
<td>N.D.</td>
</tr>
<tr>
<td>IgA</td>
<td>µg/mL</td>
<td>42±5</td>
<td>52±9</td>
<td>60±7</td>
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<tr>
<td>IL-10</td>
<td>pg/mL</td>
<td>430±20</td>
<td>N.D.</td>
<td>N.D.</td>
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<tr>
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<td>N.D.</td>
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<td>85±29</td>
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<tr>
<td>IL-17</td>
<td>ng/mL</td>
<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>IL-18</td>
<td>ng/mL</td>
<td>18±1</td>
<td>30±1***</td>
<td>27±0***</td>
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<td>IL-1α</td>
<td>pg/mL</td>
<td>260±72</td>
<td>160±45</td>
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<tr>
<td>IL-1β</td>
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<td>17±1</td>
<td>20±1</td>
<td>20±1</td>
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<tr>
<td>IL-5</td>
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<td>N.D.</td>
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<td>IL-6</td>
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<td>15±4</td>
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<tr>
<td>IL-7</td>
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<td>0.22±0.12</td>
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<td>IP-10</td>
<td>pg/mL</td>
<td>68±9</td>
<td>230±140</td>
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<tr>
<td>LIF</td>
<td>pg/mL</td>
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<td>1500±100</td>
<td>1200±100</td>
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<tr>
<td>Lymphotactin</td>
<td>pg/mL</td>
<td>120±50</td>
<td>180±40</td>
<td>100±20</td>
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<tr>
<td>MCP-1</td>
<td>pg/mL</td>
<td>130±10</td>
<td>220±30**</td>
<td>110±10</td>
</tr>
<tr>
<td>MCP-3</td>
<td>pg/mL</td>
<td>400±30</td>
<td>700±100**</td>
<td>490±40</td>
</tr>
<tr>
<td>MCP-5</td>
<td>pg/mL</td>
<td>21±2</td>
<td>49±6**</td>
<td>37±6</td>
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<tr>
<td></td>
<td>Unit</td>
<td>Original Value</td>
<td>Corrected Value</td>
<td>Significance</td>
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<td>------------------</td>
<td>--------</td>
<td>----------------</td>
<td>-----------------</td>
<td>--------------</td>
</tr>
<tr>
<td>M-CSF</td>
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<td>MDC</td>
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<td>460±20</td>
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<tr>
<td>MIP-1α</td>
<td>ng/mL</td>
<td>2.4±0.3</td>
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<tr>
<td>MIP-1β</td>
<td>pg/mL</td>
<td>190±40</td>
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<tr>
<td>MIP-1γ</td>
<td>ng/mL</td>
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<td>52±7</td>
</tr>
<tr>
<td>MIP-2</td>
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<td>28±3</td>
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</tr>
<tr>
<td>MIP-3</td>
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<td>3.5±0.3**</td>
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</tr>
<tr>
<td>MMP-9</td>
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<td>110±20</td>
<td>210±20**</td>
<td>140±20</td>
</tr>
<tr>
<td>MPO</td>
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<td>110±20</td>
<td>200±10**</td>
<td>160±10*</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>ng/mL</td>
<td>320±260</td>
<td>260±200</td>
<td>78±32</td>
</tr>
<tr>
<td>OSM</td>
<td>ng/mL</td>
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<td>N.D.</td>
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<td>RANTES</td>
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<td>N.D.</td>
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<tr>
<td>SAP</td>
<td>µg/mL</td>
<td>47±2</td>
<td>35±1*</td>
<td>38±4</td>
</tr>
<tr>
<td>SCF</td>
<td>pg/mL</td>
<td>310±40</td>
<td>230±30</td>
<td>190±20*</td>
</tr>
<tr>
<td>SGOT</td>
<td>µg/mL</td>
<td>51±9</td>
<td>50±5</td>
<td>73±4</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>ng/mL</td>
<td>4.9±0.7</td>
<td>5.2±0.9</td>
<td>4.7±0.6</td>
</tr>
<tr>
<td>Tissue Factor</td>
<td>ng/mL</td>
<td>8.6±0.3</td>
<td>11±2</td>
<td>7.5±1.0</td>
</tr>
<tr>
<td>TNF-α</td>
<td>ng/mL</td>
<td>0.11±0.02</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>TPO</td>
<td>ng/mL</td>
<td>110±10</td>
<td>150±10**</td>
<td>130±0*</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>ng/mL</td>
<td>2200±100</td>
<td>3500±700*</td>
<td>2700±100</td>
</tr>
<tr>
<td>VEGF</td>
<td>pg/mL</td>
<td>290±40</td>
<td>200±10</td>
<td>190±10*</td>
</tr>
<tr>
<td>vWF</td>
<td>ng/mL</td>
<td>150±10</td>
<td>330±130</td>
<td>160±20</td>
</tr>
</tbody>
</table>

The data are expressed as the mean±SEM. The means were compared by means of
ANOVA and Bonferroni’s multiple comparison tests. *P<0.05 versus the No Treatment group, **P<0.01 versus the No Treatment group, ***P<0.001 versus the No Treatment group. Multiplex immunoassay was performed using the Luminex LabMAP instruments by Charles River Inc. Apo A1 (Apolipoprotein A1), CD (Cluster of Differentiation), CRP (C Reactive Protein), EGF (Epidermal Growth Factor), FGF-9 (Fibroblast Growth Factor-9), FGF-basic (Fibroblast Growth Factor-basic), GCP-2 (Granulocyte Chemotactic Protein-2), GM-CSF (Granulocyte Macrophage-Colony Stimulating Factor), GST-α (Glutathione S-Transferase alpha), IFN-γ (Interferon-gamma), IgA (Immunoglobulin A), IL (Interleukin), IP-10 (Inducible Protein-10), KC/GROα (Melanoma Growth Stimulatory Activity Protein), LIF (Leukemia Inhibitory Factor), MCP (Monocyte Chemoattractant Protein), M-CSF (Macrophage Colony-Stimulating Factor), MDC (Macrophage-Derived Chemokine), MIP (Macrophage Inflammatory Protein), MMP-9 (Matrix Metalloproteinase-9), MPO (Myeloperoxidase), OSM (Oncostatin M), RANTES (Regulation Upon Activation, Normal T-Cell Expressed and Secreted), SAP (Serum Amyloid P), SCF (Stem Cell Factor), SGOT (Serum Glutamic-Oxaloacetic Transaminase), TIMP-1 (Tissue Inhibitor of Metalloproteinase Type-1), TNF-α (Tumor Necrosis Factor-alpha), TPO (Thrombopoietin), VCAM-1 (Vascular Cell Adhesion Molecule-1), VEGF (Vascular Endothelial Cell Growth Factor), vWF (von Willebrand Factor). N.D. (Not Detected).

**Supplementary Table 6.** Serum biomarkers in the FITC-NP and pitavastatin-NP groups.

<table>
<thead>
<tr>
<th></th>
<th>FITC-NP (N= 6)</th>
<th>Pitava-NP (N= 9)</th>
</tr>
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<tbody>
<tr>
<td>Apo A1</td>
<td>45±2</td>
<td>46±2</td>
</tr>
<tr>
<td></td>
<td>Unit</td>
<td>Value1</td>
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<tr>
<td>----------</td>
<td>------</td>
<td>-----------</td>
</tr>
<tr>
<td>CD40</td>
<td>pg/mL</td>
<td>110±10</td>
</tr>
<tr>
<td>CD40 Ligand</td>
<td>pg/mL</td>
<td>1900±100</td>
</tr>
<tr>
<td>CRP</td>
<td>µg/mL</td>
<td>7.6±0.8</td>
</tr>
<tr>
<td>EGF</td>
<td>pg/mL</td>
<td>26±3</td>
</tr>
<tr>
<td>Endothelin-1</td>
<td>pg/mL</td>
<td>24±2</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>pg/mL</td>
<td>370±10</td>
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<tr>
<td>Factor VII</td>
<td>ng/mL</td>
<td>28±2</td>
</tr>
<tr>
<td>FGF-basic</td>
<td>ng/mL</td>
<td>17±2</td>
</tr>
<tr>
<td>GCP-2</td>
<td>ng/mL</td>
<td>39±5</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>µg/mL</td>
<td>150±10</td>
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<tr>
<td>IgA</td>
<td>µg/mL</td>
<td>44±12</td>
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<tr>
<td>IL-10</td>
<td>pg/mL</td>
<td>N.D.</td>
</tr>
<tr>
<td>IL-11</td>
<td>pg/mL</td>
<td>120±60</td>
</tr>
<tr>
<td>IL-18</td>
<td>ng/mL</td>
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<tr>
<td>IL-1α</td>
<td>pg/mL</td>
<td>440±130</td>
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<tr>
<td>IL-1β</td>
<td>ng/mL</td>
<td>7.9±0.3</td>
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<tr>
<td>IL-4</td>
<td>pg/mL</td>
<td>71±28</td>
</tr>
<tr>
<td>IL-5</td>
<td>ng/mL</td>
<td>0.80±0.12</td>
</tr>
<tr>
<td>IL-6</td>
<td>pg/mL</td>
<td>N.D.</td>
</tr>
<tr>
<td>IL-7</td>
<td>ng/mL</td>
<td>0.082±0.018</td>
</tr>
<tr>
<td>IP-10</td>
<td>pg/mL</td>
<td>40±3</td>
</tr>
<tr>
<td>LIF</td>
<td>pg/mL</td>
<td>1900±100</td>
</tr>
<tr>
<td>Lymphotactin</td>
<td>pg/mL</td>
<td>80±9</td>
</tr>
<tr>
<td>MCP-1</td>
<td>pg/mL</td>
<td>130±10</td>
</tr>
<tr>
<td>Protein</td>
<td>Unit</td>
<td>Control</td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
<td>---------</td>
</tr>
<tr>
<td>MCP-3</td>
<td>pg/mL</td>
<td>380±30</td>
</tr>
<tr>
<td>MCP-5</td>
<td>pg/mL</td>
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<td>M-CSF</td>
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<tr>
<td>MIP-1α</td>
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<td>3.3±0.2</td>
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<tr>
<td>MIP-1β</td>
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<td>200±30</td>
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<td>MIP-1γ</td>
<td>ng/mL</td>
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<td>MIP-2</td>
<td>pg/mL</td>
<td>28±2</td>
</tr>
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<td>MIP-3</td>
<td>ng/mL</td>
<td>2.0±0.1</td>
</tr>
<tr>
<td>MMP-9</td>
<td>ng/mL</td>
<td>130±10</td>
</tr>
<tr>
<td>MPO</td>
<td>ng/mL</td>
<td>140±20</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>ng/mL</td>
<td>240±60</td>
</tr>
<tr>
<td>OSM</td>
<td>ng/mL</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td>SAP</td>
<td>µg/mL</td>
<td>32±2</td>
</tr>
<tr>
<td>SCF</td>
<td>pg/mL</td>
<td>280±10</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>ng/mL</td>
<td>5.0±0.7</td>
</tr>
<tr>
<td>Tissue Factor</td>
<td>ng/mL</td>
<td>14±1</td>
</tr>
<tr>
<td>TPO</td>
<td>ng/mL</td>
<td>30±3</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>ng/mL</td>
<td>2600±100</td>
</tr>
<tr>
<td>VEGF</td>
<td>pg/mL</td>
<td>200±20</td>
</tr>
<tr>
<td>vWF</td>
<td>ng/mL</td>
<td>180±10</td>
</tr>
</tbody>
</table>

The data are expressed as the mean±SEM. The mean values were compared using an unpaired t-test. *P<0.05 versus the FITC-NP group.
**Supplementary Table 7.** Plasma concentration of pitavastatin in the pitavastatin and pitavastatin-NP groups.

<table>
<thead>
<tr>
<th></th>
<th>2 hours</th>
<th>6 hours</th>
<th>24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pitavastatin (ng/mL)</strong></td>
<td>1.3±0.2</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td><strong>Pitavastatin-NP (ng/mL)</strong></td>
<td>2.5±0.2*</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

The data are expressed as the mean±SEM. The mean values were compared using an unpaired \( t \)-test. *\( P<0.05 \) versus the Pitavastatin group.
Experiment Protocol 1
ApoE−/−mice or ApoE+/−CCR2−/−mice
Thioglycollate-induced peritoneal macrophages
ApoE−/−mice 18 weeks of age

Treatment Group
1. CCR2+/−-Inflammatory Macrophage (1x10^6 cells/ 200 µL PBS)
2. CCR2−/−-Leukocyte (1x10^6 cells/ 200 µL PBS)

Experiment Protocol 2
ApoE−/−mice 16 weeks of age

Treatment Group
1. No treatment
2. FITC-NP (0.1 mg PLGA/ 200 µL PBS)
3. Pitavastatin (Pitavastatin 0.012 mg/ 200 µL PBS)
4. Pitavastatin-NP (0.1 mg PLGA/ Pitavastatin 0.012 mg/ 200 µL PBS)

Experiment Protocol 3
ApoE−/−mice 16 weeks of age

Treatment Group
1. FITC-NP (1.3 mg PLGA/ 200 µL PBS)
2. 7ND-NP (1.3 mg PLGA/ 5 µg 7ND plasmid/ 200 µL PBS)

Experiment Protocol 4
ApoE−/−mice 16 weeks of age

Treatment Group
1. Pitavastatin (Lower dose: 0.1 mg/kg/day)
2. Pitavastatin (Higher dose: 1.0 mg/kg/day)

Histopathological Analysis
High-Fat Diet
Adoptive transfer

Weekly intravenous Administration of Pitavastatin Encapsulated Nanoparticles (Pitava-NP)

Weekly Intravenous Administration of 7ND plasmid (5 µg) Encapsulated Nanoparticles (7ND-NP)

Supplementary Figure 1, Katsuki S et al
A

Supplementary Figure 2, Katsuki S et al

Number of F4/80^+CD115^+ cells

- No treatment: ApoE^/-, ApoE^/-CCR2^/-
- Transfer of PKH26-labeled Macrophages: ApoE^/-

B

Ly-6C expression on F4/80^+CD115^+ cells

- No treatment: ApoE^/-, ApoE^/-CCR2^/-
- Transfer of PKH26-labeled Macrophages: ApoE^/-

C

No treatment

Transfer of PKH26-labeled Macrophages

PKH

FITC
Supplementary Figure 3, Katsuki S et al

Panel A:
- **Control** vs **Negative selection**
- Scatter plots showing cell counts in the Lin vs CD11b and Ly-6C dimensions.
- Graph showing cell counts over Ly-6C.

Panel B:
- **N**: No Treatment
- **M**: Monocytes
- Images of EVG-stained sections showing disrupted/buried fibrous caps.
- Comparison of fibrous cap thickness between Control and Negative selection, with statistical significance (P=0.056) and non-significance (ns) indicated.

**Key Details**:
- Lin-CD11b-Cell counts-Ly-6C
- Fibrous Cap Thickness
- Disrupted/buried Fibrous Caps
- Control vs Negative selection
- Statistical tests: P=0.056 vs ns
**A**

![Image A](image1.png)

**B**

![Image B](image2.png)

**C**

![Image C](image3.png)

**D**

![Graphs](image4.png)

**Figure D**

*Supplementary Figure 4, Katsuki S et al.*

**Graphs**

- **FITC**
- **FITC-NP**

<table>
<thead>
<tr>
<th>Conc ($\mu$M)</th>
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<th>1</th>
<th>3</th>
<th>10</th>
<th>30</th>
<th>100</th>
</tr>
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<tbody>
<tr>
<td>Immediately after incubation</td>
<td><img src="chart1.png" alt="Graph" /></td>
<td><img src="chart2.png" alt="Graph" /></td>
<td><img src="chart3.png" alt="Graph" /></td>
<td><img src="chart4.png" alt="Graph" /></td>
<td><img src="chart5.png" alt="Graph" /></td>
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<tr>
<td>24 hrs</td>
<td><img src="chart7.png" alt="Graph" /></td>
<td><img src="chart8.png" alt="Graph" /></td>
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<td>72 hrs</td>
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<td>1 wk</td>
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<td><img src="chart23.png" alt="Graph" /></td>
<td><img src="chart24.png" alt="Graph" /></td>
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**A**

Oral administration of Pitavastatin (mg/kg)

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<th>No Treatment</th>
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<td>EVG</td>
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<td>Mac3</td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
</tr>
<tr>
<td>MCP-1</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
</tbody>
</table>

Disrupted/buried fibrous caps

Fibrous Cap Thickness

<table>
<thead>
<tr>
<th>Mac3</th>
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<tbody>
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<td>ns</td>
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</tbody>
</table>

**B**

En Face Analysis

Atherosclerosis Aorta

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<tbody>
<tr>
<td>% of whole aorta</td>
</tr>
<tr>
<td>0.1</td>
</tr>
<tr>
<td>1.0</td>
</tr>
</tbody>
</table>

**C**

Quantitative Analysis

Atherosclerosis of Aortic Root

<table>
<thead>
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<tbody>
<tr>
<td>(x10³ µm²)</td>
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<tr>
<td>0.1</td>
</tr>
<tr>
<td>1.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mac3</th>
</tr>
</thead>
<tbody>
<tr>
<td>(% of plaque area)</td>
</tr>
<tr>
<td>% animal</td>
</tr>
<tr>
<td>0.1</td>
</tr>
<tr>
<td>0.1</td>
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<tr>
<td>1.0</td>
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</table>

ns