Endothelial Dysfunction in Rheumatoid Arthritis
The Role of Monocyte Chemotactic Protein-1–Induced Protein

Ming He,* Xiao Liang,* Lan He, Wen Wen, Sijia Zhao, Liang Wen, Yan Liu, John Y.-J. Shyy, Zuyi Yuan

Objective—Patients with rheumatoid arthritis are prone to atherosclerosis. We explored the role of elevated level of monocyte chemotactic protein-1 (MCP-1)–induced protein (MCPIP) in endothelial dysfunction associated with rheumatoid arthritis.

Approach and Results—The level of MCP-1 was elevated in sera from mice with collagen-induced arthritis (CIA) and was negatively correlated with endothelium-dependent vessel dilation. Aortas from CIA mice showed increased expression of MCPIP but decreased bioavailability of endothelial NO synthase–derived NO. Administering MCP-1 neutralizing antibody to CIA mice decreased the MCPIP level in aortas and alleviated endothelial dysfunction. In vitro, treating cultured vascular endothelial cells with MCP-1 or sera from CIA mice or rheumatoid arthritis patients increased the expression of MCPIP but inhibited endothelial NO synthase phosphorylation. These detrimental effects were reproduced in endothelial cells overexpressing MCPIP, with elevated redox stress. Small interfering RNA knockdown of MCPIP restored the endothelial NO synthase–derived NO bioavailability. Administering simvastatin to CIA mice ameliorated the endothelial dysfunction, with attendant decreased aortic level of MCPIP. The beneficial effect of the statin was mediated by inhibiting nuclear factor κB binding to the MCPIP gene enhancer.

Conclusions—Increased MCPIP is found in rheumatoid arthritis leading to endothelial dysfunction. Statin treatment or MCP-1 neutralizing antibody administration antagonizes MCPIP expression, thereby attenuating the endothelial dysfunction. (Arterioscler Thromb Vasc Biol. 2013;33:1384-1391.)

Key Words: atherosclerosis ● endothelial dysfunction ● monocyte chemotactic protein-1–induced protein ● rheumatoid arthritis

A

mple evidence indicates that patients with rheumatoid arthritis (RA) can be predisposed to atherosclerosis.1,2 Clinical trial have shown a positive association of RA duration and prevalence of atherosclerosis.3 Both considered inflammation-associated diseases,4 RA and atherosclerosis exhibit similar expression profiles of cytokines and chemokines, including monocyte chemotactic protein-1 (MCP-1) and its receptor chemokine (C-C motif) receptor 2 (CCR2).5,6 One hypothesis linking RA with atherosclerosis is that the RA-increased circulation levels of proinflammatory cytokines and chemokines may cause impaired endothelium in the arterial wall, thus leading to atherosclerosis.7 Manifested by attenuated endothelial NO synthase (eNOS)–derived NO bioavailability, endothelial dysfunction precludes vascular impairment. RA patients and animal models often show decreased flow-mediated vasodilation,8,9 indicating that endothelial dysfunction is associated with the proinflammatory state of RA. However, the molecular mechanism by which endothelial dysfunction in RA leads to vascular impairments remains unclear.

The MCP-1–induced protein (MCPIP), encoded by the human ZC3H12A gene, is highly induced in monocytic lineage cells stimulated by inflammatory cytokines, such as MCP-1, interleukin-1β (IL-1β), tumor necrosis factor α, bacterial lipopolysaccharide, and phorbol 12-myristate-13-acetate.10–12 However, mice with systemic-ablated zc3h12a show increased immune response.11 Several distinct functions of MCPIP have been reported. MCPIP acts as an RNase to degrade its own mRNA and that of other cytokines, such as IL-1β.13 As well, it induces reactive oxygen species and reactive nitrogen species in cultured cardiac myoblasts, thus leading to autophagy and apoptosis.13,14 Furthermore, MCPIP can stimulate the proliferation and migration of human umbilical vein endothelial cells (HUVECs),
which is related to MCP-1–induced angiogenesis.\textsuperscript{15} Given the proinflammatory role of MCP-1, MCPIP may be involved in endothelial inflammatory responses.

The pleiotropic effect of statins involves its anti-inflammatory effect on the vasculature.\textsuperscript{16} Under increased inflammation and oxidative stress, statins can restore eNOS-derived NO bioavailability.\textsuperscript{17,18} Simvastatin, atorvastatin, and lovastatin can inhibit the transactivation of nuclear factor-κB (NF-κB) in endothelial cells (ECs).\textsuperscript{19} The Trial of Atorvastatin in Rheumatoid Arthritis study showed that statin reduces inflammation and clinical outcomes in patients with RA.\textsuperscript{20} Assessing flow-mediated dilation of the brachial artery, Mäki-Petäjä et al\textsuperscript{21} showed that both simvastatin and ezetimibe can restore the flow-mediated dilation of the brachial artery, NO bioavailability.\textsuperscript{17,18} Simvastatin, atorvastatin, and lovastatin can inhibit the transactivation of nuclear factor-κB (NF-κB) in endothelial cells (ECs).\textsuperscript{19} The Trial of Atorvastatin in Rheumatoid Arthritis study showed that statin reduces inflammation and clinical outcomes in patients with RA.\textsuperscript{20} Assessing flow-mediated dilation of the brachial artery, Mäki-Petäjä et al\textsuperscript{21} showed that both simvastatin and ezetimibe can restore the impaired endothelial function in RA patients. The beneficial effect of simvastatin on endothelial function in patients with RA is also supported by a trial conducted by Hermann et al.\textsuperscript{22}

In this study, we explored the pathogenic link between RA and endothelial dysfunction, with an emphasis on the role of MCPIP. Induction of MCPIP in the endothelium with sera from mice with collagen-induced arthritis (CIA) or patients with RA played a major role in RA-associated endothelial dysfunction. Furthermore, treatment with statins could alleviate NF-κB transactivation on MCPIP.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**Endothelial Dysfunction in CIA Mice**

In all mice receiving type II collagen, ≥1 paw ankle or wrist joint exhibited swelling 8 weeks after injection (Figure 1A). Severe redness and swelling of the entire paw were found for most mice, and the joint clinical score was 9.8±1.5. Histological sections from proximal interphalangeal and metacarpophalangeal joints of the CIA models showed an increase in synovial proliferation and pannus but decrease in articular cavity, together with bone and cartilage inflammation and destruction (Figure 1A). The mean pathological score for joints with lesions was 6.2±2.0. However, CIA and control mice had similar body weight, blood glucose, and serum levels of total cholesterol and triglyceride (Table III in the online-only Data Supplement).

We examined whether endothelial function was affected by CIA-associated inflammation by examining vascular relaxation in mice. Vascular dilation was lower with ACh (endothelial-dependent) in CIA than control mouse aortas (Figure 1B). However, sodium nitroprusside-induced relaxation (endothelial-independent) was comparable between the 2 groups of mice. CIA mice were divided into moderate and low vascular dilation response groups by maximum relaxation percentage (66±11% and 42±7%); the score for the control group was 87±16%. Phosphorylation of eNOS Ser-1177 in pooled aortic extracts was lower for CIA than control mice. Those dilated poorer had lower level of eNOS phosphorylation (Figure 1C).

To examine whether CIA-associated inflammation affected eNOS-derived NO bioavailability in the endothelium through circulation, we incubated EOMA cells with sera pooled from CIA or control mice with or without vascular endothelial growth factor (VEGF). VEGF with control sera greatly induced eNOS phosphorylation in EOMA cells (Figure 1D), which was dose-dependently decreased with CIA sera.
Elevated Serum Level of MCP-1 Inversely Correlates With Vessel Tone

Because CIA sera decreased eNOS phosphorylation in ECs, we then explored the factor(s) in the sera that might cause impaired eNOS phosphorylation. The levels of C5a, soluble intercellular adhesion molecule-1, MCP-1, tissue inhibitor of metalloproteinase 1, and monokine induced by gamma interferon in sera from the 2 groups of mice significantly differed on mouse cytokine array (Figure 2A). ELISA revealed levels of MCP-1, tissue inhibitor of metalloproteinase 1, and monokine induced by gamma interferon higher but C5a level lower in CIA than control sera (Figure 2B). We correlated concentrations of these cytokines with maximum vessel relaxation percentage induced by Ach in CIA mice and found only serum level of MCP-1 is inversely correlated ($r = -0.517; P = 0.04$; Figure 2C).

To link results from CIA mice with clinical settings, sera from patients with OA or RA were incubated with cultured HUVECs. Likewise, RA sera reduced eNOS phosphorylation responding to VEGF, as compared with osteoarthritis sera (Figure 2D). Of note, the higher the concentration of serum MCP-1, the lower the eNOS phosphorylation. We further performed a complimentary experiment with aortic rings from normal DBA/1j mice treated with recombinant mouse MCP-1 from 1 to 3 ng/mL before assessing vaso-relaxation.

MCP-1 Induces MCPIP Expression in ECs

Although MCP-1 is known to decrease eNOS-derived NO bioavailability, the exact molecular mechanism is still unknown. We speculated that MCPIP is involved in the endothelial dysfunction and thus compared the level of MCPIP in EOMA cells treated with CIA or control sera. CIA sera dose-dependently induced the expression of MCPIP in EOMA cells, which was blocked by antagonism of CCR2, the MCP-1 receptor (Figure 3A). Similar results were obtained with HUVECs treated with sera from RA patients (Figure 3B).

In line with results in Figure 3A, MCPIP and CCR2 were highly expressed in aortas of CIA mice (Figure 3C), and high mRNA levels of MCP-1 were found in the ankle and aorta but not in liver of CIA mice (Figure II in the online-only Data Supplement). To verify the specificity of MCP-1 in inducing MCPIP in CIA mice and RA patients, we incubated EOMA cells with recombinant mouse MCP-1 at 6 and 12 nmol/L for 12 or 24 hours. Like CIA or RA sera, MCP-1 dose-dependently induced the expression of MCPIP in ECs (Figure 3D), with CCR2 antagonism blocking the effect.
Elevated Level of MCPIP Decreases the eNOS-Derived NO Bioavailability
Given the commonly found MCPIP elevation in CIA sera–stimulated, RA sera–stimulated, and MCP-1–stimulated ECs, we then examined the role of MCPIP in eNOS-derived NO bioavailability. Knocking down MCPIP in EOMA cells with small group interfering RNA restored the VEGF-induced phosphorylation of Akt and eNOS, as well as NO production (Figure 4A and 4B). In vivo, administration of anti–MCP-1 antibody to CIA mice decreased the level of MCPIP but increased eNOS phosphorylation in the aorta (Figure 4C). The restoration of eNOS-derived NO bioavailability in these vessels was shown by increased endothelial-dependent dilation response to ACh (Figure 4D).

We used a gain-of-function approach with MCPIP overexpression in HUVECs to test whether elevated MCPIP can impair NO bioavailability. VEGF-induced Akt and eNOS phosphorylation was hindered in HUVECs overexpressing exogenous MCPIP as compared with mock transfection controls (Figure 5A). Elevated MCPIP in cardiomyocytes is linked to increased oxidative stress.13 We found that MCPIP overexpression increased oxidative stress in ECs, which was attenuated by coincubation with a permeable superoxide dismutase (Figure 5B). Similarly, CIA and RA sera decreased Akt and eNOS phosphorylation with attendant increased oxidative stress in EOMA cells and HUVECs, respectively (Figure 5C–5F). SOD treatment of these cells alleviated the imposed oxidative stress and partially restored the impaired Akt and eNOS phosphorylation (Figure 5A, 5C, and 5E).

Therefore, elevated MCPIP with increased oxidative stress contributes to impaired vascular tone during RA.

Statins Downregulate MCPIP Expression
Clinically, administration of statins can improve endothelial function in RA patients and reduce circulatory MCP-1 level in patients with coronary heart disease.21,22,24 Consistently, we found that simvastatin administration to CIA mice for 9 weeks improved the vaso-relaxation response to ACh but not SNP (Figure 6A and 6B). In addition, the level of MCP-1 was lower but phosphorylation of eNOS was higher in aortas of CIA mice receiving simvastatin than vehicle (Figure 6C). Treating EOMA cells with simvastatin or atorvastatin dose-dependently decreased the MCP-1–induced MCPIP protein and mRNA levels (Figure 6D; Figures III and IV in the online-only Data Supplement). Given that 4 putative NF-κB binding sites are present in the enhancer region of MCPIP,25 we performed chromatin immunoprecipitation assay and found that MCP-1 stimulation of HUVECs increased the binding of the p65 subunit of NF-κB to the enhancer of the MCPIP (Figure 6E). However, MCP-1 stimulation with simvastatin decreased the binding of NF-κB to the MCPIP gene enhancer, so the anti-inflammatory effect of statins was mediated at least in part by decreased NF-κB transactivation of MCPIP.

Discussion
Experiments involving CIA mice, as well as RA patient sera, revealed that MCP-1 induction of MCPIP plays a key role in...
endothelial dysfunction associated with RA. Elevated MCP-1 in circulation, directly or indirectly from ankle inflammation, increased the MCPIP level in the vascular endothelium. The consequent increased oxidative stress caused impaired eNOS-derived NO bioavailability (summarized in Figure 6F). Because dysfunctional endothelium initiates many vascular diseases, we suggest that MCPIP may link RA with impaired vascular functions.

Exhibiting high and low inflammation, RA and atherosclerosis share a similar expression profile of cytokines and chemokines. The proinflammatory cytokines highly expressed in inflammatory joints may have caused dysfunctional endothelium through endocrine actions. Although our data suggest that MCP-1 in both rodent and human RA plays a key role in inducing MCPIP level in endothelium, other RA-associated inflammatory cytokines, such as C-reactive protein or IL-1β, may also be involved. Such a possibility is supported by previous studies showing MCPIP level upregulated by MCP-1 or IL-1β in monocytes, macrophages, HEK293 and HepG2 cells, cardiomyocytes, and ECs. Indeed, the level of C-reactive protein was positively correlated with that of MCP-1 in patient samples involved in the current study (Figure V in the online-only Data Supplement). Elicited by multiple proinflammatory cytokines via NF-κB, MCPIP may thus be a common inflammatory mediator leading to impaired eNOS-derived NO bioavailability.

With respect to the molecular mechanism by which MCPIP exacerates endothelial dysfunction, MCPIP-increased oxidative stress may negatively regulate eNOS phosphorylation, as seen in Figure 5. In ECs, the major source of free radicals are NADPH oxidases, complex III of the mitochondrial respiratory chain, and reactive nitrogen species. MCPIP overexpression in H9c2 cardiomyoblasts induced both reactive oxygen species and reactive nitrogen species production by upregulating NADPH oxidase subunit phox47 and inducible NOS. Thus, the increased redox stress in ECs with elevated MCPIP level may be, in part, because of induction of phox47 and inducible NOS. With the structural feature of a transcription factor, MCPIP may transcriptionally activate these free-radical–producing proteins. Regarding the decreased eNOS phosphorylation under elevated redox stress, the MCPIP-increased reactive oxygen species level can inhibit the PTEN-PI3K pathway, thereby attenuating Akt activity. This notion is supported by a deactivated Akt-eNOS pathway under long-term oxidative stress. Alternatively, eNOS activity may be altered by reactive oxygen species-induced S-nitrosylation.
Our results imply that MCP-1 induction of MCPIP level in ECs under RA is proinflammatory, which is consistent with MCPIP induction of the JNK-p38-p53-PUMA pathway, tumor necrosis factor, and tumor necrosis factor–associated protein in cardiomyocytes and cardiomyoblasts.10,14,31,32 The proinflammatory role of MCPIP is further evidenced by its reduced expression in ECs and vessels from CIA mice administered with statins (Figure 6). Statins exert their anti-inflammatory effect by inhibiting NF-κB–mediated transactivation.33 The simvastatin inhibition of NF-κB binding to the MCPIP enhancer (in Figure 6E), like the action of vascular cell adhesion molecule 1 and intracellular adhesion molecule 1,34 reinforces the proinflammatory role of MCPIP in ECs. However, MCPIP may have a variety of biological effects depending on the tissue and pathophysiological condition.32,35 Containing an RNase-characteristic PIN-like domain, MCPIP can also act as an RNase to degrade its own mRNA and that of chemokines, such as IL-1, IL-6, and IL-12p40.11 Furthermore, a feedback loop between NF-κB and MCPIP seems to exist; NF-κB transcriptionally activates MCPIP expression, and MCPIP can also decrease NF-κB activity.25 When exerting these anti-inflammatory effects in ECs, MCPIP may regulate an endothelial network of inflammation or anti-inflammation. If MCPIP is secreted from inflamed ECs to affect other cells in the cardiovascular system, circulating levels of MCPIP may be a clinical marker for cardiovascular events and poor prognosis of atherosclerosis. Because MCP-1 is involved in myocarditis, ischemia–reperfusion injury, cardiac remodeling, angiogenesis, and immunosuppression,36 the clinical impact of elevated MCPIP level in these pathological conditions deserves exploration. Given that the administration of both MCP-1 neutralizing antibody and simvastatin to CIA mice could restore endothelial function, with attendant decrease in MCPIP expression in endothelium, MCPIP may be a new therapeutic target. Tumor necrosis factor α antibodies are used as first-line

Figure 5. Monocyte chemotactic protein-1 (MCP-1)–induced protein (MCPIP)–decreased endothelial NO synthase (eNOS) phosphorylation is mediated by increased redox stress. A and B, HUVECs were transiently transfected with pcDNA3 or MCPIP–pcDNA3 plasmid for 24 hours. C and D, EOMA cells were treated with 10% DBA/1j control or collagen-induced arthritis (CIA) mouse sera (described in Figure 2) for 24 hours. E and F, HUVECs were treated with 10% rheumatoid arthritis (RA) or OA sera (described in Figure 2) for 24 hours. In all groups, the transfected cells were treated with or without SOD (1.0×105 U/L) or vascular endothelial growth factor (VEGF) for 30 minutes before lysis. Western blot analysis with the indicated antibodies (A, C, and E) and reactive oxygen species (ROS) were monitored with an ROS-sensing fluorescent dye (B, D, and F). Data are mean±SD from 3 independent experiments. *P<0.05; **P<0.01 as compared with respective control group or basal level or as indicated. OA indicates osteoarthritis.
drugs for RA but have multiple side effects, including high incidence of tuberculosis and decreased anti-inflammation effects. MCPIP antagonist may be an adjuvant agent for RA. These drugs, like statins, may benefit patients with vascular diseases manifested by dysfunctional endothelium.

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Disclosures
None.

References
Patients with rheumatoid arthritis (RA) are prone to atherosclerosis and known to have endothelial dysfunction. In this study, we explore the pathogenic link between RA and endothelial dysfunction, with an emphasis on the role of monocyte chemoattractant protein-1 (MCP-1)–induced protein as a mediator of endothelial injury. As MCP-1 expression is elevated in the inflammatory lesion of mouse RA models, increased MCP-1 in the circulation binds to the chemokine (C-C motif) receptor 2 in the endothelium, leading to elevated level of MCP-1–induced protein. As a result, redox stress is increased, which impairs the endothelial NO synthase–derived NO bioavailability. Such RA–associated endothelial dysfunction can be alleviated by statin administration. Similar induction of MCP-1–induced protein is also seen in endothelium treated with sera from RA patients. **Significance**
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Materials and Methods

Reagents
Polyclonal or monoclonal antibodies against eNOS, phospho-eNOS Ser1177, Akt, and phospho-Akt Ser473 were from Cell Signaling Technology. Anti-MCP-1, anti-MCPIP, and anti-p65 were from Santa Cruz Biotechnology. Anti-GAPDH and all secondary antibodies were from Epitomics Abcam. Freund’s incomplete and complete adjuvant, bovine type II collagen, phenylephrine, acetylcholinesterase (Ach), sodium nitroprusside (SNP), vascular endothelial growth factor (VEGF), MCP-1, CCR2 antagonist (BMS CCR2 22), polyethylene glycol-superoxide dismutase (SOD), simvastatin, and atorvastatin calcium salt trihydrate were from Chondrex, Sigma-Aldrich and Tocris Bioscience.

Mouse models with CIA
The protocols for mouse feeding, sacrifice and blood and tissue harvesting were approved by the Institutional Ethics Committee for Animal Experiments of the Medical School, Xi’an Jiaotong University. DBA/1j mice were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China).

The mice were kept under a 12-hr dark/light cycle at 24°C and fed ad libitum. At 6-8 weeks of age, mice were injected intradermally into the base of the tail with 100 μL of bovine type II collagen emulsified in Freund’s complete adjuvant (containing 4 mg/mL Mycobacterium tuberculosis). The control group was given 0.05 mol/L acetic acid emulsified in Freund’s incomplete adjuvant. Arthritis developed at 3.5 to 4 weeks and the greatest incidence of arthritis was seen at 6 to 8 weeks after immunization. A qualitative scoring system was used to assess the severity of arthritis once a week by 2 different observers for the entire 8-week of experiment. The scores were 0 (normal); 1 (mild, but definite redness and swelling of the ankle or wrist, or apparent redness and swelling limited to individual digits, regardless of the number of affected digits); 2 (moderate redness and swelling of wrist ankle); 3 (severe redness and swelling of the entire paw including digits); and 4 (maximally inflamed limb with involvement of multiple joints). The paw thickness was measured once a week using a thickness gauge (Mitutoyo).

In the MCP-1 neutralizing antibody study, MCP-1 antibody (15 μg/mice) was administered by intravenous injection on days 38, 45, and 52; rat monoclonal IgG2b was used as a control. For the statin treatment study, at 1 week before immunization, simvastatin (50 mg/kg/d) was administered to mice by gastric gavage, and phosphate buffered saline (PBS) was fed to control mice. All mice were killed at week 8 after immunization.

Hind limbs of every mouse were fixed with PBS containing 4% paraformaldehyde, then decalcified in 10% ethylenediaminetetraacetate solution for 3 weeks. Paraffin-embedded limbs were sectioned and stained with hematoxylin and eosin. The specimens were scored for sub-synovial inflammation, synovial hyperplasia, pannus formation, cartilage destruction, and bone damage, with each limb receiving a maximum of 16 points.

Human subjects
In total, 30 patients who had active RA according to the 1987 American Rheumatism Association criteria were recruited from the First Affiliated Hospital of the Medical School, Xi’an Jiaotong University. Inclusion criteria included disease activity score in 28 joints (DAS-28) >3.5 and serum C-reactive protein (CRP) level >6 mg/L. We excluded patients with cardiovascular diseases, untreated hypertension (blood pressure ≥140/90 mmHg), diabetes, hypercholesterolemia (total cholesterol ≥ 6.5 mmol/L), renal disease, infection or
potential infection, current smokers, and patients receiving vaso-active drugs. Ten patients with osteoarthritis (OA) were recruited as a control group. Clinical data were recorded and blood was collected for clinical examination. The investigation conformed to the principles outlined in the Declaration of Helsinki. The study protocol was approved by the First Affiliated Hospital of the Medical College, Xi’an Jiaotong University Ethics Committee. Full informed consent was obtained from all subjects before the study. The characteristics of human subjects are described in Supplemental Table I.

Vaso-relaxation assessments

Total endothelium-dependent and -independent vaso-relaxation was analyzed by standard protocols. Briefly, aortic rings were placed in an organ bath containing 5 mL Kreb’s solution for equilibration for 1 hr, then contracted with 60 mmol/L KCl to detect vessel viability. Contraction was evoked by adding 1 μmol/L phenylephrine. At the plateau of contraction, Ach or SNP was added. Relaxation percentage was calculated as [(mN (Phe) - mN (Ach or SNP)/mN (Phe) - mN (baseline))] × 100%.

Mouse cytokine array panel and ELISA

The Proteome Profiler™ mouse cytokine array panel A (R&D Systems) was used to detect cytokine expression profile in mouse sera. Briefly, 15 μL of the antibody cocktail was added to 500 μL pooled serum samples for incubation at 4°C for 12 hr. Blots were detected by horseradish peroxidase-conjugated streptavidin with enhanced chemiluminescent substrate (Pierce). ELISA was performed with the use of mouse complement component C5a kit (R&D Systems), Quantikine mouse tissue inhibitor of metalloproteinase 1 (TIMP-1) and mouse CXCL9/monokine induced by γ interferon (MIG) immunoassay kits (R&D Systems), and mouse and human MCP-1 kits (Pierce).

Cell culture

The murine EC line (EOMA) obtained from the American Type Culture Collection was cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 15% fetal bovine serum (FBS, Invitrogen) containing 100 U/mL penicillin and 100 U/mL streptomycin. HUVECs were maintained in M199 medium supplemented with 20% FBS, 1 ng/mL recombinant human fibroblast growth factor, 90 μg/mL heparin, 100 U/mL penicillin, and 100 U/mL streptomycin.

siRNA and plasmid transfection

The primer sequences for MCPIP siRNA are described in Supplemental Table II. EOMA cells at 10⁵/mL were transfected with siRNA by use of TurboFect siRNA Transfection Reagent (Fermentas Inc). Medium was replaced with complete grown medium after 6 hr. The human MCPIP-pcDNA3.0 plasmid was a gift of Dr. Jolanta Jura at Jagiellonian University, Krakow, Poland. HUVECs were transfected with human MCPIP-pcDNA3.0 plasmid or pcDNA3.0 by use of the Cytofect HUVEC Transfection Kit (Cell Applications).

Quantitative PCR and Western blot

Total RNA was isolated by use of Trizol reagent and proteins were isolated by use of ProteoJET Mammalian Cell Lysis Reagent (Fermentas Inc). The extracted RNA was reverse transcribed and analyzed by quantitative PCR with use of the SYBR PrimeScript RT-PCR Kit (TaKaRa). GAPDH was a housekeeping gene. The primers used in quantitative PCR are in Supplemental Table I. For Western blot analysis, protein extracts were separated by SDS-PAGE and the transferred blots were incubated overnight at 4°C with
various antibodies, then horseradish peroxidase-conjugated anti-goat and anti-rat, or anti-rabbit secondary antibodies. The enhanced chemiluminescent substrate was used to detect protein bands.

**ROS detection**

The intracellular level of ROS was detected by use of a kit from Enzo Life Sciences. The cell pellets were resuspended in 500 μl of ROS detection solution for incubation for 1 hr in the dark, then analyzed by flow cytometry with excitation at 490 nm and emission 525 nm.

**ChIP assay**

ChIP analysis was performed using the Agarose Chip Kit (Pierce). Briefly, 2×10^6 cells were crosslinked and precleared with protein A/G plus agarose. For immunoprecipitation, the lysates were incubated with p65 antibody at 4°C overnight; rabbit IgG was a negative control and anti-RNA polymerase antibody a positive control. For qPCR, the primer sequences for human MCPIP were included in Supplemental Table II.

**Statistical analysis**

Data are expressed as means±SD. In parametric data, Student’s t test or ANOVA was used to analyze differences among groups if data were determined to be normal distribution. For non-parametric data, Mann-Whitney U test were used to analyze differences between 2 groups. P<0.05 was considered statistically significant.

**Reference:**

Supplemental file

Endothelial Dysfunction in Rheumatoid Arthritis: the Role of Monocyte Chemotactic Protein-1-Induced Protein

*Ming He, Xiao Liang, Lan He, Wen Wen, Sijia Zhao, Liang Wen, Yan Liu, John Y-J. Shyy, Zuyi Yuan*
Supplemental Table I. Characteristics of patients with osteoarthritis and rheumatoid arthritis

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Data are number or mean±SD. DAS-28: disease activity score in 28 joints; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; MCP-1: monocyte chemotactic protein 1. * P<0.05; ** P<0.01 compared with OA group. ζ P<0.01 compared with Male RA patients.
### Supplemental Table II. Primers used for real-time PCR

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</tr>
<tr>
<td><strong>For siRNA</strong></td>
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<td></td>
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</tr>
<tr>
<td>MCPIP</td>
<td>5’-CGAGGACACACAUCAGAUUATTUAAUAUUGUCUGUGCUCAGG</td>
<td>3’-GGACAGAAGAGAGGAGAGGAAACAGAGGGTGAATTTG</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-CACAGAAGAGCTGAUCAGAGUUGUGAAGCAGGTGAATTTG</td>
<td>3’-GGACAGAAGAGAGGAGAGGAAACAGAGGGTGAATTTG</td>
<td></td>
</tr>
</tbody>
</table>

### Supplemental Table III. Characteristics of mice used in the study

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex</th>
<th>Weight (g)</th>
<th>C-Scores</th>
<th>P-Scores</th>
<th>Glucose (mmol/L)</th>
<th>Cholesterol (mmol/L)</th>
<th>Triglyceride (mmol/L)</th>
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</thead>
<tbody>
<tr>
<td><strong>Arthritis animal model</strong></td>
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</tr>
<tr>
<td>DBA</td>
<td>8♂8♀</td>
<td>15.7±1.4</td>
<td>-</td>
<td>-</td>
<td>6.17±0.72</td>
<td>3.27±0.76</td>
<td>1.47±0.26</td>
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<tr>
<td>CIA</td>
<td>8♂8♀</td>
<td>16.8±0.8</td>
<td>9.75±1.53</td>
<td>6.21±1.95</td>
<td>6.25±0.84</td>
<td>3.36±0.67</td>
<td>1.53±0.24</td>
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<tr>
<td><strong>MCP-1 neutralizing antibody treatment</strong></td>
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<td></td>
</tr>
<tr>
<td>CIA</td>
<td>5♂5♀</td>
<td>16.7±1.9</td>
<td>9.83±1.23</td>
<td>6.46±2.02</td>
<td>5.86±0.78</td>
<td>3.31±0.76</td>
<td>1.38±0.36</td>
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<tr>
<td>CIA+Treat</td>
<td>5♂5♀</td>
<td>16.4±1.5</td>
<td>9.76±1.21</td>
<td>6.39±1.84</td>
<td>6.02±0.94</td>
<td>3.37±0.51</td>
<td>1.43±0.31</td>
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<td><strong>Simvastatin treatment</strong></td>
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</tr>
<tr>
<td>DBA</td>
<td>4♂4♀</td>
<td>16.9±1.5</td>
<td>-</td>
<td>-</td>
<td>5.96±0.82</td>
<td>3.21±0.36</td>
<td>1.46±0.30</td>
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<tr>
<td>DBA+Treat</td>
<td>4♂4♀</td>
<td>17.2±1.3</td>
<td>-</td>
<td>-</td>
<td>5.92±0.77</td>
<td>2.67±0.33*</td>
<td>1.41±0.29</td>
</tr>
<tr>
<td>CIA</td>
<td>4♂4♀</td>
<td>17.3±1.9</td>
<td>9.25±0.92</td>
<td>6.31±2.37</td>
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<td>3.13±0.41</td>
<td>1.42±0.33</td>
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<tr>
<td>CIA+Treat</td>
<td>4♂4♀</td>
<td>17.1±1.6</td>
<td>9.05±0.82</td>
<td>6.14±2.77</td>
<td>5.89±0.73</td>
<td>2.52±0.32*</td>
<td>1.37±0.29</td>
</tr>
</tbody>
</table>

CIA: collagen-induced arthritis; DBA: DBA/1j mice; C-scores: clinical scores; P-scores: pathology scores; * P<0.05 when compared with control group.
Supplemental Fig. I. MCP-1 reduces the endothelium-dependent vaso-relaxation ex vivo. (A) and (B) show the endothelium-dependent (Ach) and -independent (SNP) vaso-relaxation of aortas from DBA/1j mice with or without indicated concentrations of MCP-1 for 12 hr. * $P<0.05$ when MCP-1 (3 ng/ml) group compared with control; $\Delta P<0.05$ MCP-1 (2 ng/ml) group compared with control.
Supplemental Fig. II

MCP-1 level is increased in the ankle and aorta of CIA mice. RT-PCR analysis of MCP-1 mRNA in ankle, liver, and aorta in CIA or control mice. GAPDH level was the normalization control. The bar graphs below are mean±SD from 3 independent experiments. * P<0.05 and ** P<0.01 when compared with control group (dotted line).
Supplemental Fig. III. Atorvastatin decreases the MCPIP expression in EOMA cells. EOMA cells were pre-treated with atorvasatin at the indicated concentrations for 3 hr, then MCP-1 (12 nmol/L) for 12 hr. Western blot analysis of protein level of MCPIP. GADPH was a normalization control. Data are mean±SD from 3 independent experiments. ** P<0.01 as compared with control (dotted line).
Supplemental Fig. IV

Statin decreases the MCPIP expression in EOMA cells. EOMA cells were pre-treated with simvastatin or atorvastatin at the indicated concentrations for 3 hr, then MCP-1 (12 nmol/L) for 12 hr. RT-PCR analysis of mRNA level of MCPIP. GAPDH level was an internal reference. Data are mean±SD from 3 independent experiments. * \( P<0.05 \) and ** \( P<0.01 \) as compared with control (dotted line).
Supplemental Fig. V

Supplemental Fig. V. The correlation analysis between MCP-1 and CRP level in serum from human subject (n=40).