Journal of the American College of Cardiology Vol. 56, No. 22, 2010

ISSN 0735-1097/$36.00
doi:10.1016/j.jacc.2010.04.066

PRE-CLINICAL RESEARCH

A New Monocyte Chemotactic Protein-1/Chemokine CC Motif Ligand-2 Competitor Limiting Neointima Formation and Myocardial Ischemia/Reperfusion Injury in Mice

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Objectives A nonagonist monocyte chemotactic protein-1 (MCP-1/CCL2) mutant (PA508) with increased affinity for glycosaminoglycans and thus competing with CCL2 was evaluated as a candidate for preventing neointima formation or myocardial ischemia/reperfusion injury.

Background Myocardial infarction (MI) remains a major cause of death worldwide despite improved interventional and therapeutic options. Therefore, the discovery of drugs that limit restenosis after intervention and post-MI damage remains an important challenge.

Methods The function of PA508 was assessed in functional assays in vitro and in mouse models of wire-induced neointima formation and experimental MI.

Results PA508 was functionally inactive in CC chemokine receptor 2 (CCR2) binding and calcium influx but inhibited monocyte chemotaxis or transendothelial migration toward CCL2, suggesting that it interferes with CCL2 presentation. In wild-type but not CCR2-deficient mice, PA508 reduced inflammatory leukocyte recruitment without affecting differential leukocyte counts, CCL2 levels, organ function, or morphology, indicating that it specifically attenuates the CCL2-CCR2 axis. Compared with vehicle, daily intraperitoneal injection of PA508 significantly ($p < 0.05$, $n = 5$) reduced neointimal plaque area and mononuclear cell infiltration in carotid arteries of hyperlipidemic apolipoprotein E-deficient mice while increasing smooth muscle cell content. In C57Bl/6J mice that underwent myocardial ischemia/reperfusion, treatment with PA508 significantly reduced infarction size, monocyte infiltration, and collagen and myofibroblast content in the infarction area and preserved heart function compared with vehicle ($p < 0.05$, $n = 4$ to 8).

Conclusions Here we demonstrate that administration of a rationally designed CCL2 competitor reduced inflammatory monocyte recruitment, limited neointimal hyperplasia, and attenuated myocardial ischemia/reperfusion injury in mice and could therefore be envisioned as a combined therapeutic approach for restenosis and MI. (J Am Coll Cardiol 2010;56:1847–57) © 2010 by the American College of Cardiology Foundation

The major cause of death in Western countries, atherosclerosis and subsequent myocardial infarction (MI), triggers a complex inflammatory reaction accompanied by cytokine and chemokine release, which remains incompletely understood and not sufficiently targeted by current therapeutic strategies (1–5). Chemokines are considered major arbitrators that modulate

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Manuscript received March 9, 2010; revised manuscript received April 13, 2010, accepted April 16, 2010.

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Abbreviations and Acronyms

CCL = chemokine CC motif ligand

CCR = chemokine CC motif receptor

GAG = glycosaminoglycan

I/R = ischemia/reperfusion

LV = left ventricular

MCP = monocyte chemotactic protein

MI = myocardial infarction

this inflammatory response during the development of atherosclerosis (5,6), but are also involved in myocardial healing and scar formation after MI (1,7,8). Chemokines have highly conserved and well-defined structures tailored to their function as inflammatory mediators. In particular, most chemokines can form multimeric structures, and specific amino acid motifs confer high affinity for glycosaminoglycans (GAGs), allowing effective presentation on the surface of vascular cells. Chemokines are functionally intolerant to modifications of the N terminus, a property that has been exploited for regulated on activation, T-cell expressed, and secreted (RANTES/CCL5) to create the antagonist Met-CCL5 (9). Mutations that affect oligomerization and/or GAG interaction of chemokines have also generated chemokine variants with antagonistic properties (10,11).

The CC motif chemokine monocyte chemoattractant protein (MCP)-1/CCL2 is a potent mononuclear cell chemoattractant involved in various diseases characterized by monocyte-rich leukocyte infiltrates (12). CCL2 can directly influence wound angiogenesis by mediating endothelial cell migration (13) and can modulate fibrous tissue deposition by stimulating extracellular matrix production and differentiation of fibroblasts toward a myofibroblast-like phenotype (14,15). CCL2 is also essential in the development of restenotic changes after coronary intervention by mediating monocyte infiltration and their subsequent activation (16). Mice that lack CCR2, the receptor for CCL2, show a marked reduction in neointima formation, monocyte recruitment, and neointimal macrophage content after arterial injury (17). After ischemia/reperfusion (I/R) injury, these mice exhibit a decrease in macrophage infiltration and infarct size compared with wild-type mice (15,18). Similarly, CCL2-deficient mice display a decreased and delayed macrophage infiltration and myofibroblast accumulation associated with an improvement of left ventricular (LV) dysfunction and regional hypocontractility after MI (19,20). In line with these findings, CCL2 can induce a novel transcription factor that causes cardiac cell death and ventricular dysfunction (21). Hence, pharmacological inhibition of CCL2 might represent an attractive approach to prevent detrimental vascular remodeling and to attenuate damage after MI. Indeed, the inhibition of CCL2 by an antibody resulted in reduction of atherosclerosis and inflammation in *Apoe*$^{-/-}$ mice, inducing a stable atherosclerotic plaque phenotype (22) and thus demonstrating the feasibility of this approach. However, antibodies are large proteins difficult to be economically produced in sufficient amounts, need to be humanized, and carry the risk of undesired binding to other antigens, which may affect safety of long-term treatment.

The purpose of our study was to explore beneficial effects of a recombinant CCL2 competitor (PA508), a CCR2-activation incompetent variant of CCL2, which was engineered to have an increased affinity for GAGs, as a novel therapeutic approach for the prevention of neointimal formation and myocardial I/R injury in mice.

Methods

Generation of PA508. The amino acid mutations in human CCL2 complementary DNA were introduced by standard molecular cloning (23).

Surface plasmon resonance. Equilibrium binding of CCL2 and PA508 to heparin pentasaccharide (Sanofi-Aventis, Paris, France) was determined (24,25) and used for Scatchard plot analysis and calculation of dissociation constants (kilodaltons).

Calcium mobilization. Calcium transients in Fluo-4–labeled THP-1 cells were recorded in a microplate reader. After baseline measurement, cells were stimulated with H_2O (control), CCL2, or PA508 (50 ng/ml).

Chemotaxis and transendothelial migration. The effects of PA508 on THP-1 cell migration toward CCL2 for 2 h at 37°C were assessed using 5-μm Transwell filters seeded without or with human umbilical vein endothelial cells and analyzed by flow cytometry.

Chemokine receptor binding studies. PA508 binding studies to CCR2-transfected cells were performed using ^{125}I-CCL2 displaced by PA508 or Met-CCL2 in comparison with wtCCL2 in Hank's balanced salt solution buffer.

Pharmacokinetics of PA508. Female Balb/cOlaHsd mice (Harlan Laboratories, Horst, Belgium) were injected intraperitoneally with Met-CCL2 or PA508 20 μg per mouse. Serum concentrations of Met-CCL2 or PA508 were determined by species-specific enzyme-linked immunosorbant assay for human CCL2 (Anogen, Yes Biotech Laboratories Ltd., Mississauga, Ontario, Canada) at various time points.

PA508 treatment of mice. Mice were treated intraperitoneally with PA508 (0, 1, 5, or 10 μg in phosphate-buffered saline). All animal experiments and study protocols were approved by local authorities, complying with German animal protection laws.

Examination of PA508-treated mice. Venous blood was obtained from C57Bl/6J mice before treatment (6 mice per group) and at 1, 3, and 8 days and assayed for alanine-aminotransferase, creatinine, and CCL2 levels. Organs were harvested for histological analysis. Blood cells were analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, New Jersey).

Leukocyte recruitment in vivo. An air-pouch model (26) was performed in C57Bl/6J (8 per group) or CCR2$^{-/-}$ mice (4 per group) treated intravenously with phosphate-buffered saline, PA508 (10 μg), or heat-inactivated PA508 (HI-PA508) (10 μg, n = 4).

Wire-injury and ex vivo perfusion models. Eight-week-old *Apoe*$^{-/-}$ or *Ccr2*$^{-/-}$*Apoe*$^{-/-}$ mice underwent carotid

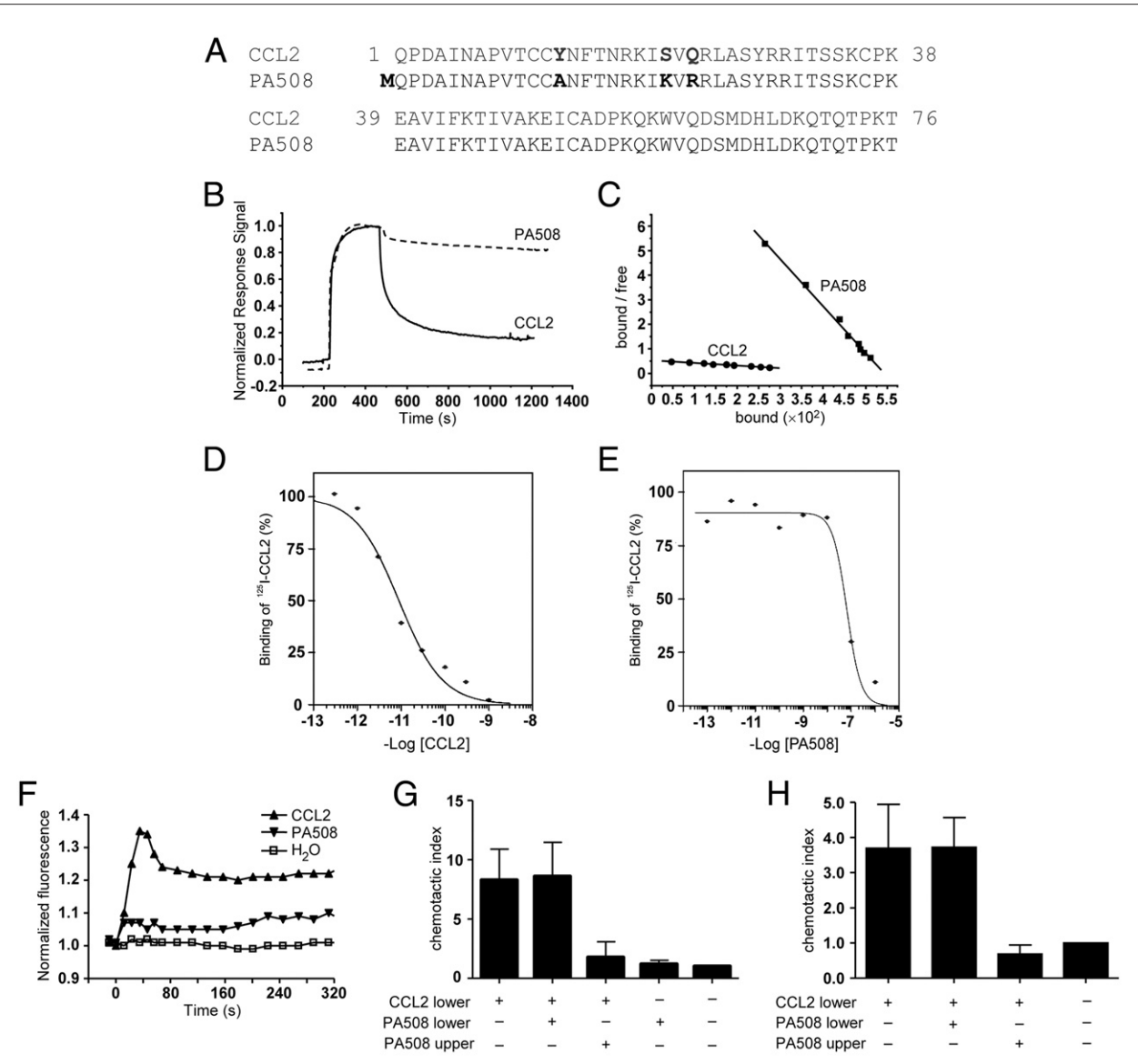

Figure 1 **Design and Functional Characterization of PA508**

(A) Sequence comparison of human CCL2 and PA508 with modified residues is shown in bold type. **(B)** Normalized surface plasmon resonance sensorgrams of 500 nM of CCL2 **(solid line)** and PA508 **(dashed line)** perfused over immobilized heparin pentasaccharide. **(C)** Scatchard analysis of CCL2 **(solid circles)** and PA508 **(solid squares)** binding to immobilized heparin pentasaccharide. Receptor binding competition of radiolabeled ^{125}I-CCL2 from CCR2-transfected cells by CCL2 **(D)** or PA508 **(E)**, compound concentrations (M) were converted to negative logarithms. **(F)** Calcium signaling of CCL2 **(triangles)**, PA508 **(inverted triangles)**, or H_2O **(open squares)** in Fluo-4–labeled THP-1 cells. Transwell filter chemotaxis **(G)** or transendothelial migration across unstimulated human umbilical vein endothelial cell monolayers **(H)** of monocytic THP-1 cells toward buffer, PA508 (100 ng/ml), or CCL2 (50 ng/ml) in the absence or presence of PA508 (100 ng/ml) in the upper or lower chamber of the Transwell filter.

wire injury (17,27,28). Mice were treated with vehicle or PA508 1 day before injury and daily for 3 weeks. Neointimal areas, reported as a percentage of external elastic lamina and cell content, were analyzed by histomorphometry. Some arteries were perfused ex vivo with MonoMac6 cells 1 day post-injury (29).

Murine model of myocardial I/R. Eight-week-old male C57Bl/6J mice underwent sham or coronary occlusion and reperfusion (19,30). Mice were treated with vehicle or PA508 10 min after ligation, 2 h after reperfusion, and daily for 7 days and analyzed for myocardial function, infarction size, and cell content by echocardiography, Millar pressure catheter, Langendorff perfusion, and histomorphometry (see Online Appendix Methods section for details).

Statistical analysis. Data represent mean ± SEM and were analyzed by Student *t* test, 1-way analysis of variance, or Kruskal-Wallis test followed by Newman-Keuls or Dunn's post-test, respectively, using Prism4 software (GraphPad, La Jolla, California), as appropriate. A p value <0.05 was considered significant.

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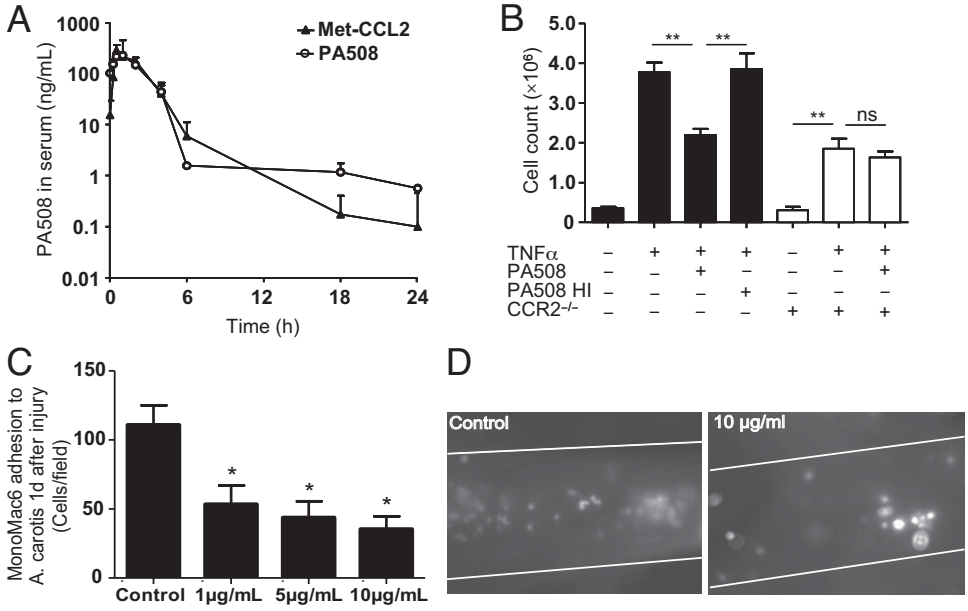

Figure 2 **Pharmacokinetics and Leukocyte Recruitment Inhibition of PA508**

Pharmacokinetic profiles of Met-CCL2 and PA508 after intraperitoneal administration in the mouse. **(A)** Serum concentrations were determined by enzyme-linked immunosorbent assay after 1, 15, and 30 min and 1, 2, 4, 18, and 24 h. Total leukocyte counts in mouse air pouch fluids after treatment with vehicle or tumor necrosis factor (TNF)-α (10 ng/ml) after intravenous pre-treatment with phosphate-buffered saline (control), PA508 (10 μg), or heat-inactivated (HI) PA508 (10 μg) in wild-type **(solid bars)** or $CCR2^{-/-}$ mice **(open bars)** **(B)** (mean ± SEM, $^{**}p < 0.001$, 3 to 6 per group) ns = not significant. Injured carotid arteries **(C)**, incubated with different doses of PA508 and perfused with monocytic MonoMac6, showed a decreased dose-dependent adhesion of the monocytes (mean ± SEM, 3 to 6 per group, $^{*}p < 0.05$ vs. control). **(D)** Representative images of adherent MonoMac6 cells in injured ex vivo perfused carotid arteries treated without or with PA508 (10 μg).

Results

Design and functional characterization of PA508. To obtain a CCL2-based decoy protein that has increased GAG binding affinity and is deficient in CCR2 activation, we introduced basic amino acids into the GAG binding site of CCL2 besides the tyrosine 13 to alanine mutation, which prevents CCR2 activation (31) (i.e., the existing GAG binding motif in the amino acids 21 to 24 region was engineered by mutating serine 21 and glutamine 23 into lysine and arginine, respectively) (Fig. 1A). This gave rise to PA508, an inactive CCL2 mutant with increased GAG binding affinity that could compete with natural CCL2 for GAG binding sites (23). Surface plasmon resonance revealed the predicted increase in affinity of PA508 for GAGs, showing a markedly prolonged retention of PA508 on immobilized heparin pentasaccharide compared with wild-type CCL2 (Fig. 1B). Scatchard analysis of these data yielded an almost 20-fold increase in affinity of PA508 for heparin with calculated dissociation constants (kilodaltons) of 5.2×10^{-8} M for PA508 and 9.4×10^{-7} M for wild-type CCL2 (Fig. 1C). Isothermal titration calorimetry confirmed increased affinity for chondroitin sulfate (data not shown). Receptor binding assays demonstrated a >7,000-fold lower affinity of PA508 for CCR2, as evident by its reduced ability to compete with a radiolabeled tracer for binding to CCR2-transfected cells, with an IC_{50} of 6.6×10^{-8} M compared with the IC_{50} value obtained for wtCCL2 of 8.9×10^{-12} M (Figs. 1D and 1E). In this setup, Met-CCL2 exhibited a similar IC_{50} value as PA508 (data not shown), suggesting that N-terminal methionylation of CCL2 strongly impairs binding to CCR2. Functional assays revealed that PA508 was inactive as a CCR2 agonist because, unlike CCL2, it neither induced calcium influx nor chemotaxis in monocytic cells (Figs. 1F to 1H). Despite impaired binding to CCR2, PA508 prevented both chemotaxis and transendothelial migration of monocytic cells toward CCL2, when added to the top chamber, implying that presentation of CCL2 on GAGs precedes activation of CCR2 and that PA508 actively interferes with this process (Figs. 1G and 1H). PA508 did not affect transendothelial migration of monocytic cells induced by other atherogenic chemokines (e.g.,

Table 1 **Pharmacokinetic Parameters of Met-CCL2 and PA508**

	Met-CCL2	PA508
C_{max}, ng/ml	314	226
T_{max}, h	1	1
$t_{1/2}$, h	2.8	2.9
$AUC_{0\text{-}24}$, ng/ml × h	774.7	660.2

$AUC_{0\text{-}24}$ = area under the curve from 0 to 24 h.

Table 2 Cell Counts of Leukocyte Populations

Cell Type and Time Point	Control (× 10,000/ml)	PA508 (10 μg) (× 10,000/ml)	p Value
Neutrophils			
Day 0	61 ± 28 (6)	73 ± 29 (6)	NS
Day 1	97 ± 53 (6)	90 ± 31 (6)	NS
Day 3	110 ± 63 (6)	95 ± 35 (6)	NS
Day 8	93 ± 30 (6)	115 ± 24 (5)	NS
Monocytes			
Day 0	15 ± 4.3 (6)	17 ± 5.1 (6)	NS
Day 1	30 ± 17 (6)	28 ± 15 (6)	NS
Day 3	25 ± 7.6 (6)	38 ± 7.8 (6)	<0.05
Day 8	17 ± 5.6 (6)	24 ± 3.3 (5)	<0.05
T cells			
Day 0	229 ± 61 (6)	243 ± 106 (6)	NS
Day 1	185 ± 65 (6)	253 ± 134 (6)	NS
Day 3	269 ± 88 (6)	260 ± 63 (6)	NS
Day 8	217 ± 45 (6)	283 ± 54 (6)	<0.05
B cells			
Day 0	280 ± 106 (6)	284 ± 120 (6)	NS
Day 1	284 ± 98 (6)	314 ± 165 (6)	NS
Day 3	367 ± 190 (6)	325 ± 75 (6)	NS
Day 8	261 ± 64 (6)	286 ± 14 (6)	NS

Values are mean ± SD (n).

CCL5 or CXCL1), indicating a remarkable specificity for CCL2 (Online Fig. 1A).

Pharmacokinetics of PA508 and effects on leukocyte recruitment. Pharmacokinetic profiles of Met-CCL2 and PA508 were monitored by antigen measurement in serum samples obtained at different time points after intraperitoneal administration (20 μg per mouse) (Fig. 2A). Met-CCL2 displayed a rapid passage to the blood circulation, with a peak level of 314 ng/ml (C_{max}) at 1 h (t_{max}) and plasma half-life of 2.8 h, similar to what has been reported for native CCL2 (11). PA508 showed a comparable profile with a peak of 226 ng/ml (C_{max}) reached at 1 h (t_{max}) and a plasma half-life of 2.9 h (Table 1). At 24 h, Met-CCL2 was detectable at 0.1 ng/ml, whereas PA508 was present at 0.6 ng/ml. During an 8-day treatment with PA508 (10 μg/day per mouse), total and differential leukocyte counts did not undergo major variations compared with baseline (Table 2). The levels of endogenous CCL2 in serum were not influenced by treatment with PA508 (Online Fig. 1B), no differences in alanine aminotransferase and creatinine levels were observed (data not shown), and histological analysis of the lungs, liver, and kidneys did not reveal abnormalities (Online Fig. 2).

To validate that PA508 acts as a CCL2 competitor in vivo, its effect was assessed in a mouse air pouch model of leukocyte recruitment. Injection of tumor necrosis factor-α resulted in a marked influx of leukocytes into the air pouch fluid that could be effectively reduced by pre-treating mice with an intravenous dose of PA508 (Fig. 2B). Administration of heat-inactivated PA508 or vehicle did not decrease leukocyte recruitment. In $CCR2^{-/-}$ mice, tumor necrosis factor-α–induced leukocyte influx was impaired compared with wild-type mice and equivalent to levels seen in PA508-treated wild-type mice (Fig. 2B). Notably, treatment with PA508 did not attenuate leukocyte recruitment in $CCR2^{-/-}$ mice, highlighting the specificity of the competitor in vivo. Injection of tumor necrosis factor-α in air pouches of wild-type mice markedly increased CCL2 levels in the pouch fluids, when mice were pre-treated with vehicle or heat-inactivated PA508 but not with PA508 (Online Fig. 1C), likely reflecting reducing effects of leukocyte influx.

Endothelial damage is followed by a local coagulatory and inflammatory response leading to deposition of platelets and adhesion of mononuclear cells. Monocyte adhesion after arterial injury is mediated by CCL2 present on activated platelets (17). To investigate an inhibitory effect of PA508 in this setting, carotid arteries were injured and excised after 1 day, incubated with varying doses of PA508 (1, 5, and 10 μg/ml), and perfused with monocytic cells ex vivo. We found a dose-dependent decrease in luminal monocyte adhesion to injured carotid arteries (Figs. 2C and 2D). Our results establish the anti-inflammatory potential of PA508 and its feasibility as a therapeutic agent in cardiovascular pathologies such as restenosis or MI.

Effects of PA508 on neointima formation after wire injury. Next, we subjected $Apoe^{-/-}$ mice treated with PA508 or vehicle to wire-induced endothelial denudation injury of the carotid artery. Compared with vehicle-treated mice, PA508 treatment resulted in a significant reduction in neointima area in wire-injured arteries, as evident by pentachrome staining and morphometry (22.9 ± 2.1% vs. 15.6 ± 2.2% external elastic lamina (EEL) in controls, $n = 5$, $p < 0.05$) (Fig. 3A). No differences in the medial area were observed (data not shown). The decrease in neointimal area was associated with changes in cell composition. The relative content of macrophages was significantly diminished (5.8 ± 2.0% vs. 25.2 ± 1.5% in controls, $n = 5$, $p < 0.05$) (Fig. 3B), whereas smooth muscle cell content was increased in the neointima of PA508-treated mice (24.2 ± 2.6% vs. 14.5 ± 3.3% in controls, $n = 5$, $p < 0.05$) (Fig. 3C) and luminal $CD31^{+}$ lining as a measure of re-endothelialization was unaltered (data not shown). No differences in endogenous CCL2 serum levels were observed after the 22-day treatment with PA508 compared with vehicle (Online Fig. 1D). Neointimal CCL2 expression after 3 weeks was unaltered by PA508 (data not shown). Notably, PA508 failed to significantly reduce wire-induced neointima formation in CCR2-deficient $Apoe^{-/-}$ mice, confirming its specificity for CCR2 (Online Fig. 1E). Taken together, competition for GAG binding of CCL2 with the nonagonist PA508 markedly reduces neointima formation and generates a more stable plaque phenotype with reduced macrophage infiltration.

Effects of PA508 in a model of myocardial I/R. The major complication of coronary stenosis with unstable plaques is MI causing substantial tissue damage in part due to monocyte infiltration. Thus, we hypothesized that administration of PA508 might reduce myocardial damage

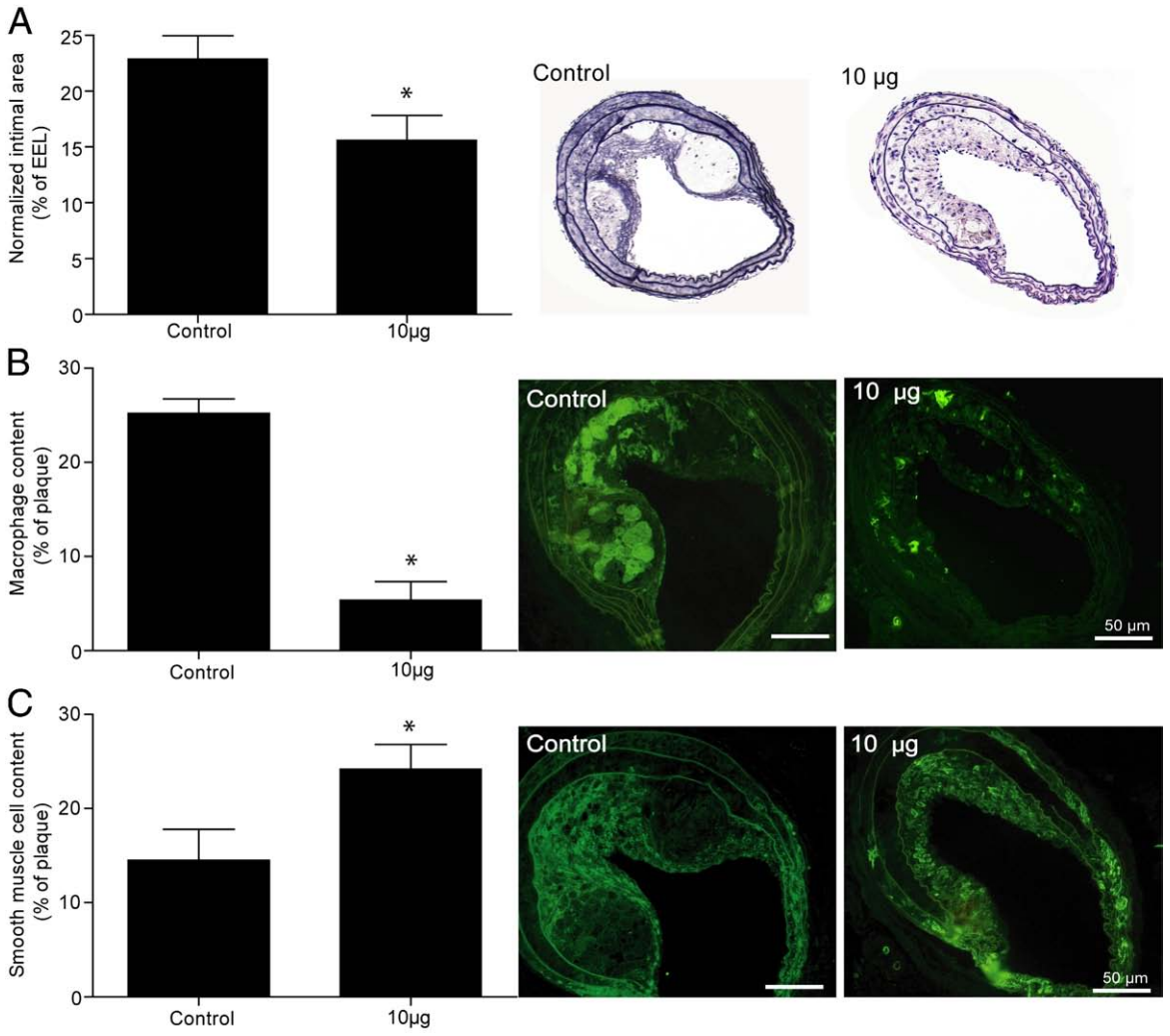

Figure 3 **Morphometry and Cell Content in Injured Arteries**

(A) Representative pentachrome-stained sections of carotid arteries 3 weeks after wire injury of *Apoe*$^{-/-}$ mice show a decrease in neointima formation in mice treated with PA508. Quantitative immunohistochemistry reveals that the relative content of macrophages **(B)** in the neointima was reduced, whereas the neointimal smooth muscle cell content **(C)** was increased in PA508-treated mice (**A** to **C**: all mean ± SEM, 5 per group, *$p < 0.05$ vs. control). EEL = external elastic lamina.

after MI. Mice underwent myocardial I/R and were treated with varying doses of PA508. After 7 days, isolated heart studies according to Langendorff were performed under constant perfusion pressure and electrical stimulation (600/min constant heart rate). We observed an impairment in LV function in wild-type mice, whereas LV function was significantly preserved in mice treated with 10-μg PA508 (54.2 ± 2.4 mm Hg vs. 33.7 ± 5.4 mm Hg in controls, n = 5 to 6, $p < 0.05$) (Fig. 4A). Positive (2,519 ± 238 mm Hg/s vs. 1,377 ± 215 mm Hg/s in controls, n = 5 to 6, $p < 0.05$) and negative pressures (−1,602 ± 172 mm Hg/s vs. −939 ± 122 mm Hg/s in controls, n = 5 to 6, $p < 0.05$) derived over time displayed similar patterns (Figs. 4B and 4C), indicating a marked improvement of contractility and relaxation in the hearts of PA508-treated mice. Moreover, mice treated with 5 or 10 μg of PA508 displayed a notable improvement in these parameters in response to adrenergic stimulation with dobutamine, unveiling a dose-dependent and even more apparent efficacy of the competitor under these conditions (Figs. 4B and 4C). Coronary flow analysis did not reveal differences between the groups. We verified these results by performing echocardiography and intraventricular measurements in control mice and mice treated with PA508 at 10 μg. Both groups compensated for the loss of cardiac mass and produced the same cardiac output; however, PA508-treated mice showed a better preserved cardiac function compared with control mice (ejection fraction 51.8 ± 1.2% vs. 40.4 ± 3.9%, n = 4 to 5, $p < 0.05$) (Table 3). In vivo measurements using the Millar Pressure Transducer Catheter confirmed a significantly preserved heart function (86 ± 6 mm Hg vs. 64 ± 5 mm Hg in controls, n = 4 to 5, $p < 0.05$) and improved contractility

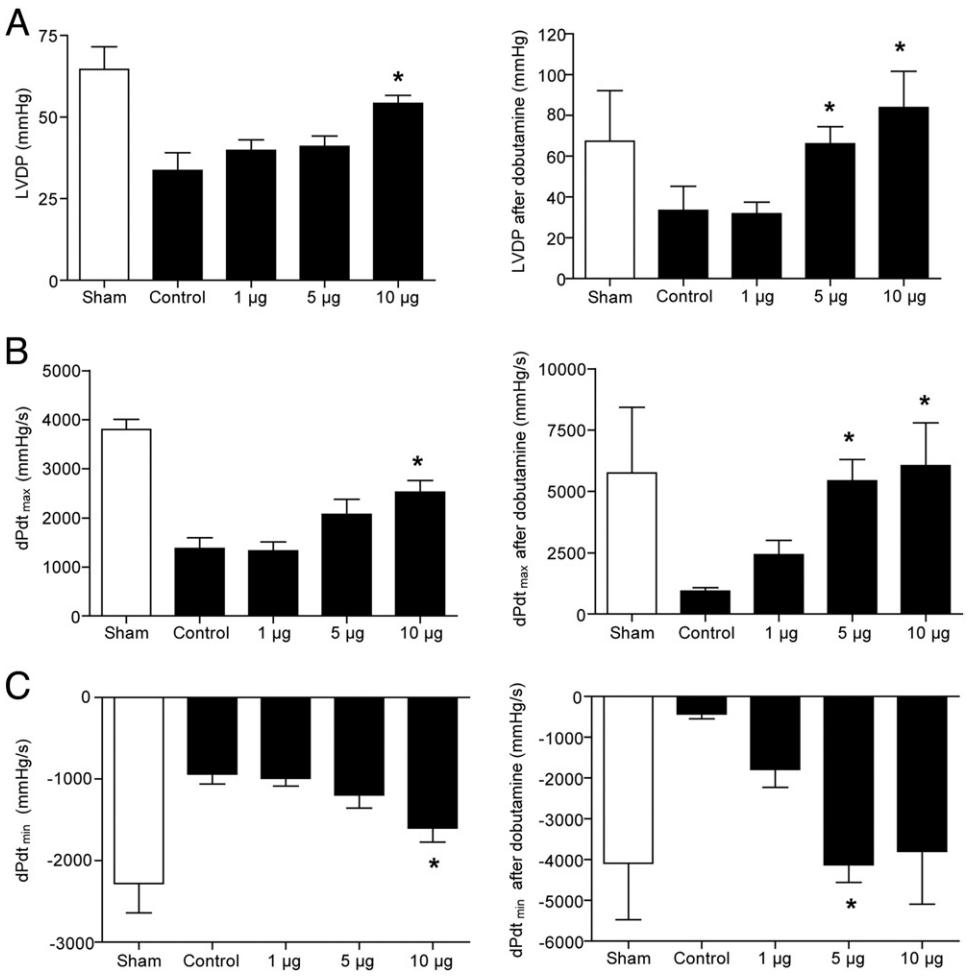

Figure 4 **Functional Assessment After Myocardial Ischemia/Reperfusion**

Hearts collected 1 week after ischemia/reperfusion were studied in a Langendorff perfusion system. Left ventricular developed pressure (LVDP) **(A)**, maximum time-derived pressure **(B)**, and minimum time-derived pressure **(C)** show a better preserved basal function of the PA508 (10 μg)–treated mice compared with control mice. Administration of dobutamine led to significant improvement of physiological parameters in PA508 (5 μg)–treated and PA508 (10 μg)–treated mice **(left panel)**. All data are reported as mean ± SEM (6 to 8 mice per group, *$p < 0.05$ vs. control). $dPdt_{max}$ = maximum pressure change over time.

(4,834 ± 393 mm Hg/s vs. 3,061 ± 404 mm Hg/s in controls, n = 4 to 5, $p < 0.05$) in PA508-treated mice. Due to anesthesia with ketamine/xylazine, the heart rate was decreased in this experiment but comparable between control and PA508-treated mice (Table 3). These data convincingly demonstrate a favorable effect of PA508 on heart function after I/R injury.

Histomorphometric analysis revealed a significant reduction of MI size in all PA508-treated groups compared with vehicle-treated controls (6.8 ± 0.5% at 10 μg, 7.5 ± 0.5% at 5 μg, and 11.3 ± 5.4% at 1 μg in the PA508 group vs. 16.5 ± 3.4% of LV area in controls, n = 5 to 6, $p < 0.05$) (Fig. 5A). Monocyte infiltration was markedly but dose-dependently reduced in all groups, indicating a high efficacy of PA508 (51 ± 17 cells/mm^2 at 1 μg, 25 ± 6 cells/mm^2 at 5 μg, and 36 ± 7 cells/mm^2 at 10 μg vs. 204 ± 68 cells/mm^2 in controls, n = 5 to 6, $p < 0.05$) (Fig. 5B). This suggests that attenuation of monocyte infiltration was a major mechanism contributing to preserved myocardial function. Myofibroblast content was found to be decreased only at the highest concentration of PA508 (935 ± 416 cells/mm^2 vs. 2,259 ± 173 cells/mm^2, n = 4 to 8, $p < 0.05$) (Fig. 5C). In parallel, we observed reduced collagen content in the infarcted area (Fig. 5D), corresponding to earlier findings in CCR2$^{-/-}$ mice (19,20). In contrast, neutrophil/T-cell content, CCL2 expression, angiogenesis, apoptosis, and proliferation did not differ between the groups (data not shown). Unlike CCL2, PA508 did not induce differentiation of cultured fibroblasts into myofibroblasts (Online Fig. 1F). These findings imply that, in addition to reduced monocyte infiltration, an alteration in scar myofibroblast and collagen content may account for improved heart function, in particular at higher concentrations of PA508.

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Table 3 In Vivo Assessment of Cardiac Function 7 Days After Myocardial Ischemia/Reperfusion

	Sham (n = 6)	Control (n = 4)	PA508 (10 μg) (n = 4)	p Value PA508 vs. Control
Echocardiography				
Heart weight, mg	115 ± 28	69 ± 7	76 ± 8	NS
Heart rate, beats/min	476 ± 66	385 ± 17	357 ± 27	NS
Cardiac output, ml/min	11.6 ± 5.1	10.2 ± 1.0	10.4 ± 2.0	NS
Ejection fraction, %	61.9 ± 6.4	40.4 ± 3.9	51.8 ± 1.2	<0.05
Invasive measurement of cardiac function				
Heart rate, beats/min	276 ± 7	242 ± 14	279 ± 15	NS
LVPD, mm Hg	107 ± 5	64 ± 5	86 ± 6	<0.05
$dPdt_{max}$, mm Hg/s	7,546 ± 969	3,061 ± 404	4,834 ± 393	<0.05

Value are mean ± SEM.
$dPdt_{max}$ = maximum pressure change over time; LVDP = left ventricular developed pressure; other abbreviation as in Table 2.

Discussion

Atherosclerotic cardiovascular disease with MI as its acute clinical manifestation is one of the leading causes of death worldwide. Atherosclerosis is a chronic inflammation of the arterial wall characterized by an influx of mononuclear cells releasing cytokines and chemokines, which in turn enhance their recruitment and activation (5,6). Acute MI also triggers a complex inflammatory reaction orchestrated by cytokines and chemokines, which cause monocyte recruitment at sites of ischemic damage (3,4,32). Studies in animal models featuring an impaired monocyte infiltration consistently yielded a reduction of neointima formation (13,17) and preserved heart function after experimental induction of MI (18–20). Therefore, interfering with monocyte recruitment represents a valuable therapeutic strategy for future clinical use. The chemokine CCL2, one of the main factors that mediate recruitment of mononuclear cells, namely, monocytes and T-cell subsets (12), modulates their phenotype and regulates fibrous tissue deposition and angiogenesis (13). In a hyperlipidemic mouse model of acute arterial injury, CCL2 rapidly up-regulated in the artery wall and presented by surface-adherent platelets mediates early monocyte arrest to denuded areas and subsequent neointima formation (17).

Here, we introduce the CCL2 competitor PA508 as a therapeutic candidate for treating monocyte-driven inflammatory conditions, with a particular focus on cardiovascular disease. Based on the concept that the interaction of chemokines with GAGs and their presentation on the surface of vascular endothelial cells or platelets are critical for their function (5,17,33), the modified CCL2 variant PA508 displayed exceptionally high retention to GAGs yet was functionally inactive, as shown by receptor binding, chemotaxis, and calcium influx assays. In vitro, PA508 inhibited monocyte chemotaxis and transendothelial migration toward a CCL2 gradient, implying that presentation of CCL2 on both monocytes and endothelial cells is necessary for efficient activation of CCR2. During the past decade, it has become clear that GAGs are important cofactors for chemokine receptor activation. For example, it has been shown that CCR1 and CCR5 ligand binding and functional activity were strongly reduced in the absence of GAGs on chemokine receptor–expressing cells (34). Thus, a mechanism can be envisioned where binding of a chemokine to GAGs takes place first and the chemokine receptor is activated subsequently. As PA508 reduces the availability of GAGs for CCL2 through occupying its binding sites, the net effect on CCR2 will be similar to an absence of GAGs.

When injected intraperitoneally into mice, PA508 rapidly entered the circulation, yet it did not influence circulating levels of endogenous CCL2 nor cause any abnormalities in serum liver and kidney parameters or organ histology. Thus, PA508 appears to be devoid of notable side effects. Treatment with PA508 effectively reduced leukocyte recruitment to air pouches in vivo and to injured atherosclerotic arteries ex vivo, suggesting that PA508 can actively cross-compete with endogenous CCL2 for binding sites on the vessel surface despite its lower affinity for CCR2. Indeed, evidence was recently provided that PA508 exhibits a 4-fold higher affinity toward the natural CCL2 GAG ligand heparan sulfate and thereby displaces wild-type CCL2 from heparan sulfate (23). Moreover, the absence of antagonistic activity against, for example, CCL5 in vitro and in $CCR2^{-/-}$ mice in vivo indicates that PA508 specifically attenuates the CCL2-CCR2 axis, while leaving the functions of other chemokines unaffected.

In the model of wire-induced neointimal hyperplasia, the effects of PA508 treatment on plaque formation were comparable to those seen with CCR2-deficiency in $Apoe^{-/-}$ mice (17), diminishing neointima formation and monocyte infiltration but increasing smooth muscle cell content. This demonstrates the potential of the competitor to limit neointimal growth and restenosis and to achieve a more stable, less inflammatory plaque phenotype after vascular injury. Similarly, interference with chemokine oligomerization and chemokine/heparin interactions using the chemokine mutant [^{44}AANA^{47}]-CCL5 was recently reported as a powerful approach to inhibit progression of established atherosclerosis in mice, and through inhibiting leukocyte

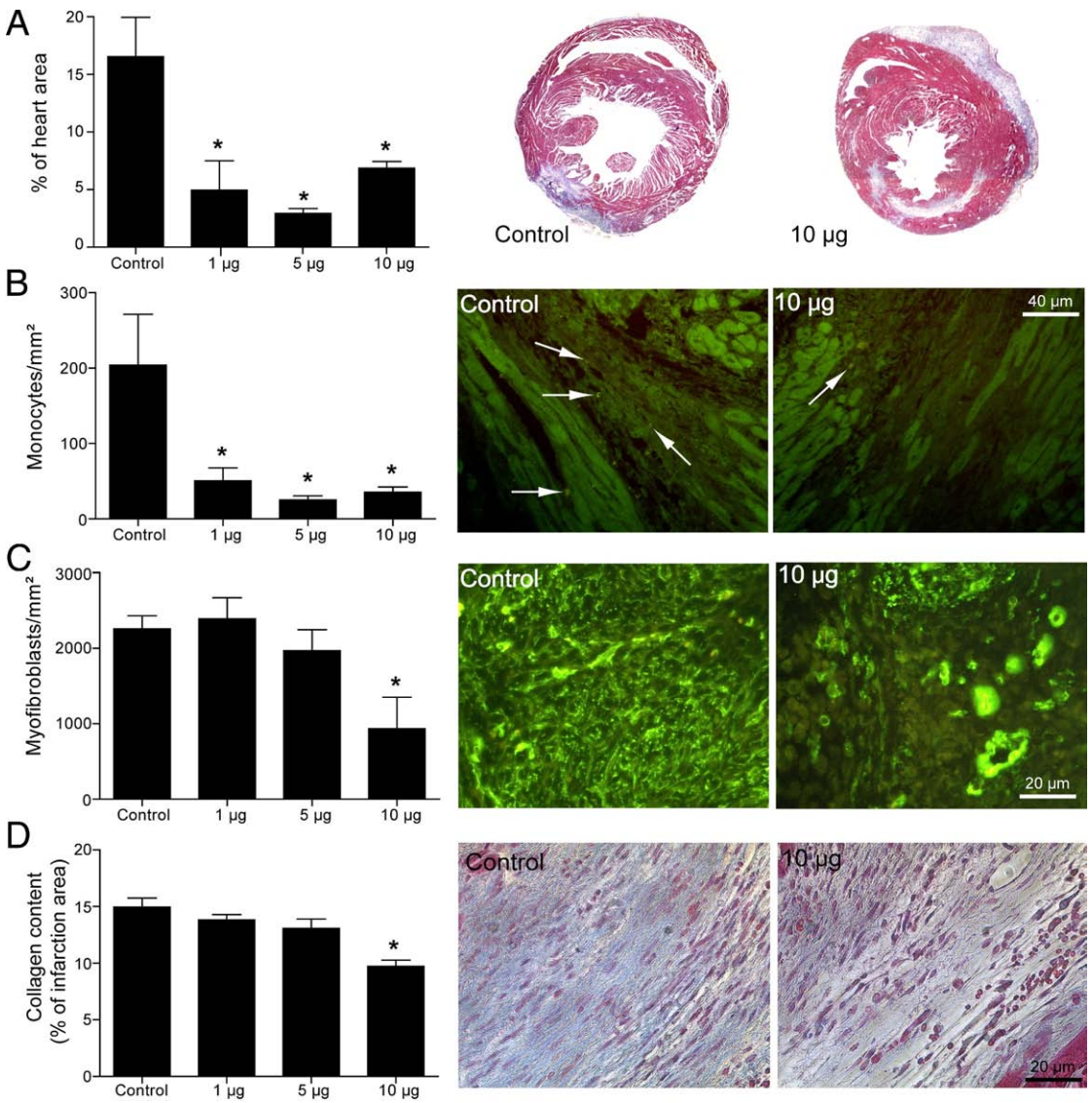

Figure 5 **Histomorphometry and Scar Remodeling After Myocardial Ischemia/Reperfusion**

Paraffin-embedded hearts were cut in serial sections and stained with Gomori 1-step, and heart wall and infarcted area were measured by planimetry **(A)**. Compared with vehicle-treated controls, PA508-treated mice (1, 5, or 10 μg) show significantly smaller infarction size. Monocyte infiltration was drastically reduced in all treated groups **(B, arrows)**, whereas myofibroblast **(C)** and collagen **(D)** content was significantly reduced only in PA508 (10 μg)–treated mice. All data are reported as mean ± SEM (6 to 8 mice per group, *$p < 0.05$ vs. control).

recruitment into plaques, to confer a less inflammatory plaque phenotype (35).

Previous work showed that CCL2 was markedly induced after MI and represented a key mediator of LV remodeling during the early phase of MI (20,36). CCL2-deficient mice exhibited decreased monocyte recruitment to the infarcted heart, attenuated fibroblast accumulation, and LV remodeling (19,20). Likewise, targeted deletion of CCR2 protected from development of adverse remodeling after MI (15,18). Here, we show that treatment of mice undergoing myocardial I/R with the CCL2 competitor PA508 preserved heart function and reduced MI size by inhibiting myocardial macrophage–related inflammation, myofibroblast infiltration, and subsequent collagen synthesis. It is noteworthy that monocyte recruitment into the infarcted area of PA508-treated mice was markedly reduced, already at the lowest dose administered. However, reduced monocyte infiltration alone does not appear to be sufficient to improve LV function. Therefore, additional effects of CCL2 unrelated to monocyte trafficking should be taken into account. We were able to show that, unlike CCL2, PA508 is unable to induce differentiation of fibroblasts into myofibroblasts. By analogy to results in CCR2$^{-/-}$ mice (19,20), this, combined with inhibition of CCL2, may lead

to altered collagen synthesis and a more compliant scar, which appears to be essential for improved heart function at higher concentrations of PA508. On the other hand, because PA508 limits the overall presentation and availability of CCL2, in particular at higher doses, it is conceivable that the competitor may decrease exposure of cardiac myocytes to CCL2, thereby reducing its potential apoptotic effects and contributing to cardiomyocyte survival and cardiac viability (21).

Developing specific therapies against CCL2-mediated inflammation is not limited to vascular diseases because CCL2 and its receptor play a critical role in many other acute and chronic inflammatory diseases. Treatment of CCL2-mediated diseases has, however, not yet resulted in a clinically applicable product or therapeutically efficient drug to date. Initially, promising biopharmaceutical approaches like the anti-CCL2 monoclonal antibody ABN912 (37) or the P8A CCL2 mutant (11) showed no or insufficient efficacy to reach final stages of drug development. On the other hand, small molecule CCR2 inhibitors remain in active but still early development and, like the JNJ-27141491 compound (38), may have significant advantages over biopharmaceuticals due to oral bioavailability. In general, strategies that encompass the direct blockade of chemokine receptors by small molecule antagonists have returned rather disappointing results (39). The therapeutic approach presented here makes use of a novel way to interfere with CCL2 pathological activity by targeting the GAG coreceptor(s) of the chemokine. Engineering chemokines to become protein-based GAG antagonists was outlined recently for CXCL8-related inflammatory diseases (40) and proved to work also in CCL5-related settings (41). The CCL2-based PA508 mutant therefore represents a novel biologic for the treatment of pathological situations in which binding of CCL2 to GAGs is crucial for disease onset or progression. On the other hand, potential side effects of PA508 on the immune system (e.g., during CCL2-mediated host defenses against infection with viruses or parasites) should be carefully profiled and scrutinized (42–44).

Conclusions

In summary, this study introduces an entirely new concept by demonstrating favorable effects of the nonagonist CCL2 competitor PA508 in 2 mouse models for the most common vascular diseases. Further pharmacological and clinical studies in larger animal models and in humans are mandatory to evaluate and validate the therapeutic potential of PA508 as an innovative drug for the treatment of vascular diseases.

Acknowledgments

The authors thank M. Garbe, S. Wilbertz, S. Winkler, S. Roubrocks, J. Tupiec, and R. Chehab for excellent technical assistance.

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Key Words: chemokines ■ inflammation ■ leukocyte ■ myocardial infarction ■ neointima formation.

APPENDIX

For additional Methods sections and figures, please see the online version of this article.