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Deficiency of MMP1a Collagenase Suppresses Development of Atherosclerosis in Mice: Translational Implications for Human Coronary Artery Disease

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Disclosure

All authors report that they have no other relationships relevant to the contents of the paper to disclose.

Supplemental Material

Figure I, Figure II, Figure III, Figure IV, Table I, Table II

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Abstract

OBJECTIVE: Destruction of arterial collagen allows monocyte and macrophage infiltration leading to atherosclerotic plaque formation, but it is not clear what role the MMP1 collagenase plays in this process in vivo. To define the specific contribution of MMP1 to atherosclerotic plaque burden and pathogenesis, we generated ApoE$^{-/-}$ mice deficient in the human MMP1 ortholog, MMP1a.

APPROACH AND RESULTS: After 12–16 weeks of western diet, genetic loss of MMP1a resulted in a significant 50% reduction in total aortic plaque burden compared to control ApoE$^{-/-}$ mice. MMP1a deficiency led to significant reductions in plaque monocytes/macrophages, SMCs, and necrosis, with increases in collagen content. Collagen invasion of oxidized-LDL activated PBMCs from MMP1a-deficient mice was markedly attenuated and was similar to suppressive effects with pharmacologic inhibitors of MMP1 and its receptor, PAR1. CAD and ACS patients undergoing cardiac catheterization in the TRIP-PCI trial were evaluated for circulating levels of all 3 major secreted collagenases, MMP1, MMP8 and MMP13 and total number of coronary lesions with ≥50% stenosis (CAD burden). MMP1 was significantly ($P<0.001$) higher by 19-fold and 5.7-fold relative to MMP13 and MMP8, respectively. MMP1 correlated with stenotic CAD burden, TNFα levels, and was co-expressed with PAR1 on monocytes. Treatment of patients with the PAR1 inhibitor, PZ-128, prevented a drop in monocytes following coronary catheterization, an acute protective effect that was reproduced in mice undergoing cardiac ischemia reperfusion.

CONCLUSIONS: These data provide evidence for an important role for the MMP1a collagenase in atherosclerotic lesion development and leukocyte behavior and validate MMP1 as a compelling target in CAD/ACS patients.

Graphical Abstract

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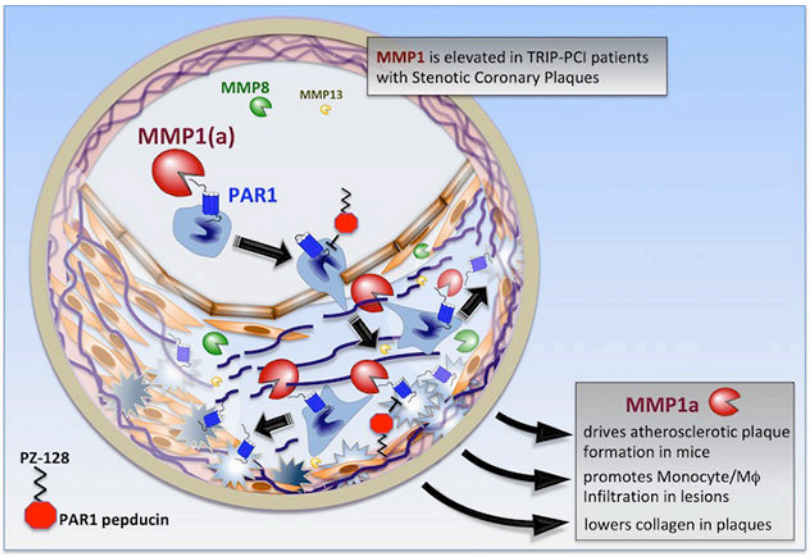

Keywords

Atherosclerosis; Coronary Artery Disease; MMP1; PAR1; Animal Models of Human Disease; Coronary Circulation; Inflammation; Translational Studies

Introduction

Matrix metalloproteases (MMPs) play an important role in atherosclerotic plaque formation by degrading the fibrillar collagens and matrix proteins that provide both vascular tensile strength and a sub-endothelial barrier which slows migration and subsequent expansion of leukocytes and smooth muscle cells. Pericellular matrix degradation by inflammatory cells is mediated by a number of secreted and membrane-bound MMPs which are constitutively expressed or upregulated by cytokines and chemokines.[1] Other cells present in the atherosclerotic plaque including smooth muscle cells, also express MMPs and other proteases that are engaged in remodeling and repair processes that help resolve inflammatory injury and enhance plaque stability.[2, 3]

There has been an increasing appreciation of the potential therapeutic and diagnostic value of targeting specific MMPs in patients with ACS, high-risk CAD,[4] and other chronic inflammatory conditions.[5] Macrophages are major suppliers of MMPs in plaques including MMP1, MMP3, MMP8, MMP12, MMP13 and MMP14,[1] which have been proposed to serve redundant, different, or opposing functions in foamy macrophages, inflammatory macrophages, and monocytes.[6, 7] This complexity makes it difficult to establish reliable MMP-based biomarkers that accurately predict lesion severity and prognosis of a particular patient.

Only a select subset of the secreted MMPs, namely the 3 interstitial collagenases MMP1 (collagenase-1), MMP8 (collagenase-2) and MMP13 (collagenase-3), are able to appreciably cleave fibrillar Type I and III collagens which comprise the majority of the matrix fabric of

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the arterial wall.[8] Studies using ApoE-deficient mice have proven to be quite useful in delineating the specific roles of the MMP8 and MMP13 collagenases in vivo. Individual or dual deletion of the MMP8 and MMP13 genes resulted in enhanced collagen accumulation in plaques, but unexpectedly had no effect on the size or development of the atherosclerotic plaques, nor on macrophage content.[9, 10] This would indicate that MMP8 and MMP13 may not be directly involved in plaque inflammation, but function primarily as collagen/matrix-degrading enzymes that regulate plaque structure and stability.

Evaluation of the role of the remaining major fibrillar collagenase in atherosclerotic plaque development, namely MMP1, has lagged over the past two decades as an early prevailing view was that there was no functional homolog of MMP1 in mice, and that transgenic overexpression of human MMP1 in mouse macrophages led to paradoxical reductions in plaque size.[11] Two genes homologous to MMP1 have been identified in mice: murine collagenase-like A (Mcol-A/MMP1a) and murine collagenase-like B (McolB/MMP1b) located in the MMP-rich gene locus of chromosome 9.[12] Unlike MMP1b, MMP1a demonstrates collagenase activity, generating the classic ¾ and ¼ length degradation fragments from fibrillar collagens. MMP1a has an unstable prodomain and is more rapidly converted to the active form than the other two collagenases MMP8 and MMP13.[13] As occurs in humans with MMP1, MMP1a is upregulated in response to inflammatory and proliferative stimuli and is expressed in mouse leukocytes, endothelium and smooth muscle cells.[14, 15] Using biochemical and pharmacologic approaches, we recently showed that both MMP1 and MMP1a could activate the protease-activated receptor 1 (PAR1) on endothelium through a non-canonical signaling pathway that greatly amplified TNFα induction of ICAM and VCAM to trigger monocyte transmigration through endothelium.[16] Unlike blockade of the canonical PAR1 agonist thrombin with bivalirudin, inhibition of either MMP1 with FN-439, or PAR1 with the PZ-128 pepducin[17, 18] gave similar results indicating that chronic inflammatory signaling through MMP1(a)-PAR1 may promote endothelial dysfunction and plaque inflammation.

Here, we use *ApoE*$^{-/-}$ mice that are genetically deficient in MMP1a in order to provide the first direct evidence for the involvement of collagenase-1 in atherosclerotic plaque development, monocyte/macrophage infiltration, and collagen content. Translational studies using patients from the TRIP-PCI study determined the relative amounts of the 3 secreted collagenases in CAD/ACS patients and correlations with angiographically defined stenotic coronary artery disease burden and systemic TNFα levels. Together, these data provide evidence for an important role for the MMP1(a) collagenase in regulating atherosclerotic plaque development and collagen content, and monocyte/macrophage infiltration in atherosclerosis.

Materials and Methods

The data and materials that support the findings of this study are available from the corresponding author upon reasonable request.

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TRIP-PCI Clinical Trial and Coronary Artery Disease Burden

The TRIP-PCI study[19] was a prospective, randomized, double-blinded, placebo-controlled, phase 2 study conducted at three academic medical centers in the US with the goal of testing the safety and efficacy of PZ-128 in CAD and ACS patients which targets protease-PAR1 signaling in vascular cells. Institutional review board approval was obtained before study initiation which was conducted in accordance with FDA regulations. All adult patients who met the inclusion criteria for the study provided written informed consent prior to enrollment/randomization. Full clinical trial inclusion and exclusion criteria are detailed at ClinicalTrials.gov NCT02561000 and are described previously.[19] Eligible patients underwent cardiac catheterization ± percutaneous coronary intervention (PCI) for either stable CAD or non-ST-segment elevation acute coronary syndrome (NSTE-ACS). Patients received the PAR1 pepducin inhibitor, PZ-128 (0.3 mg/kg or 0.5 mg/kg) or placebo (1:1:1) as a 2 hour intravenous infusion which started just prior to initiation of the procedure and typically finished 1 h after completion of cardiac catheterization/PCI. Whole blood and plasma samples from all patients were collected at baseline and various time-points during and after the coronary catheterization and intervention, and a subset of patients had the option to participate in additional blood sampling for the collagenase array and biomarker analysis. Cardiovascular angiography and interventions were performed according to professional practice guidelines (AHA, SCAI, ACCF). Patients for whom PCI was not indicated or performed that day based upon the coronary angiography findings were recommended for CABG and/or medical management. Coronary artery disease (CAD) burden was quantified as the number of coronary lesions with ≥50% stenosis based on 29-segment Coronary Artery Surgery Study (CASS) scoring.

ELISAs, Fireplex, and Flow Cytometry Assays of Patient Blood

Whole blood samples were collected from patients in sodium heparin vacutainer tubes to prepare plasma for storage at −80 °C and then shipped to Tufts Medical Center for analysis. Plasma levels of active and pro-MMP1 (R&D Systems-Fluorokine E Active MMP1, MDL-0.052 ng/mL) and TNFα (Thermo) were determined by ELISA. Plasma MMP8 and MMP13 (active and pro-forms) were quantified using a bead-based flow cytometry Fireplex assay (ABCAM) according to manufacturer's instructions. For flow cytometry, PBMCs were isolated from fresh whole patient blood using Ficoll-Paque Premium (GE Healthcare) gradients. PBMCs were washed with RBC lysis buffer (Biolegend), followed by a wash with phosphate-buffered saline (PBS, pH 7.4). PBMCs were stained for 1 h at 4 °C with MMP1 polyclonal antibody (raised against human MMP1 linker region) made by previously described methods[14, 20] and FITC-goat anti-rabbit as secondary antibody (Invitrogen), along with CD11b (APC-efluor780 Invitrogen), CD3 (PerCp-eFluor710 eBioscience) monoclonal antibodies. PAR1 was stained with ATAP2-PE (Santa Cruz, SC-13503) and PBMCs were analyzed with a BD CANTO II flow cytometer and FloJo (Version 8.8.7).

Atherosclerosis Mouse Model

We adhered to the guidelines for experimental atherosclerosis studies described in the AHA Statement.[21] *MMP1a*$^{-/-}$ mice were generated by targeted replacement of *MMP1a* exon 5 with PGK-neomycin as previously described.[13] Exon 5 encodes the zinc-binding portion of

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the catalytic domain and is essential for metalloprotease activity. *MMP1a*-deficient mice were back-crossed 10 generations into the C57BL/6 background and further crossed into the B6.129P2-ApoE^{tm1Unc}/J (Charles River). Starting at 8 weeks of age, male and female *MMP1a*$^{-/-}$*ApoE*$^{-/-}$ (KO) and *MMP1a*$^{+/+}$*ApoE*$^{-/-}$ (WT) mice were maintained on a high fat/high cholesterol 'Western Diet' (0.15% cholesterol, 40% kcal fat, 43% kcal carbohydrate, 17% kcal protein, Research Diets) for 12–16 weeks in climate controlled 12 h light/dark cycle rooms and maintained using IACUC-approved protocols. Mice received either a 100 μL daily subcutaneous injection of vehicle (5% DMSO, Sigma Aldrich) or the PAR1 inhibitor PZ-128 (10 mg/kg) for 6/7 days per week. At the conclusion of the 12 or 16 week endpoint, mice were perfused in the left ventricle with PBS for 1 min and then for 10 min with 10% NB-formalin at 120 mm Hg. Heart and thoracic/abdominal aortas were collected. Adventitial fat was removed and the splayed aortas pinned on black silicone for en face staining with oil-red-O. Aortic roots were either placed in 4% formalin for 2 h and then 100% ethanol until embedding in paraffin, or alternatively tissues were immediately frozen in OTC (Sakura) for sectioning. Aortic roots were sectioned to feature the three valve leaflets. Sections were cut at 6 μm. Digital photomicrographs were taken on a Nikon T2E at 10x and the area of staining measured as a percentage of total area using ImageJ.

To determine immune cell composition of aortic arch plaques, the aortic arch was dissected from additional cohorts of *MMP1a* WT and KO *ApoE*$^{-/-}$ mice maintained for 16 weeks on western diet. Tied-off arches were immediately filled with digestion buffer (12.5 U/mL collagenase type XI, 6 U/mL hyaluronidase type I, 45 U/mL collagenase type 1 (Sigma-Aldrich), with 6 U/mL DNAase (NEB). Tissue was then minced to 1 mm^3 sections using scissors and allowed to digest while shaking at 150 rpm for 45 min at 37 °C. Digests were then clarified with a 70 μm mesh cell strainer (FisherBrand) and washed 2x with PBS, before suspension in RBC lysis buffer (Biolegend). Following another PBS wash, cells were stained with CD45.2 APC (BD Pharmingen Clone 104), CD11b PerCpCy5.5 (BD Pharmingen clone M1/70), CD3 FITC (Biolegend clone 17A2), and Ly6c PE-Cy7 (Biolegend clone HK1.4), and FACS performed.

Myocardial Infarction/Ischemia Reperfusion Model

10 week old male C57BL6N mice (Charles River Laboratories) were administered a 100 μL sc dose of vehicle (5% DMSO) or PZ-128 10 mg/kg 1 h prior to cardiac surgery following procedures modified from published methods[22] as follows. Briefly, mice were shaved, and under isoflurane the thoracic cavity was opened between the 3rd and 4th ribs and the left descending coronary artery occluded with 7–0 silk suture (Ethicon) over PE-10 tubing for 30 mins. Tubing and ligature were then removed, the lungs re-inflated and the thoracic cavity closed. The mice were left to recover for 2 h, and then the thoracic cavity was again opened, the occluding suture re-tied. Blood anticoagulated with heparin was collected from the vena cava for isolation and quantification of monocytes from PMCBs by FACS staining and analysis as detailed above.

Immunohistochemistry

Masson's trichrome staining for collagen and matrix proteins used the Polysciences Kit according to their instructions. Following antigen retrieval (microwaved for 8 min in citrate

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buffer), plaque macrophages were detected using the F4/80 antibody (Biolegend clone BM8) developed by DAB with HRP secondary Ab (Sigma-Aldrich), and α-smooth muscle actin Ab-FITC (Santa Cruz #53015) by immunofluorescence with DAPI co-stain.

Collagen Invasion Assays

PMBCs were isolated from blood collected via cardiac puncture from C57BL6/MMP1a$^{+/+}$, ApoE$^{-/-}$/MMP1a$^{+/+}$, and ApoE$^{-/-}$/MMP1a$^{-/-}$ mice. Following Ficoll-Paque separation, PBMCs (composition was 90% lymphocytes, 9% monocytes, 1% basophils/eosinophils) were activated with 50 ng/mL ox-LDL (Kalon Scientific) for 16 h at 37 °C in polypropylene tubes. The following day, the ox-LDL activated PBMCs were resuspended in DMEM-F12 media/0.2% BSA and pre-treated for 45 mins with PZ-128 (10 μM), MMP1 inhibitor (FN-439, 3 μM), MMP8 inhibitor (25 nM), MMP9/13 inhibitor (2.5 μM) from Calbiochem, or 0.1% DMSO vehicle or media, before being placed (7000 PBMCs per well) in a micro Boyden chamber equipped with rat tail Collagen Type 1 (30 μg per membrane; BD Biosciences) or fibronectin (6 μg per membrane; BD Biosciences)-coated cellulose nitrate filters (Neuroprobe Inc.). Migration was towards TNFα (5 ng/mL, Sigma), MCP-1 (5 ng/mL, Sigma) or IL-8 (10 nM, Sigma) in RPMI in the lower chamber over 5 h at 37 °C. The distance invaded through Type I collagen or migration through fibronectin was measured in microns and normalized to 1.0-fold for vehicle.

Statistical analyses

Statistical analysis of experimental data was performed with GraphPad Prism 9.0. Results are expressed as median (25–75%), or mean ± SE. The data were analysed for equal variance by the Bartelett's test and for normality by the Shapiro-Wilk Test. If the data were homogeneous, a one-way or two-way ANOVA was performed to assess statistical significance with Tukey's multiple comparisons as post-test. If the data did not pass normality test, the Kruskal-Wallis non-parametric one-way ANOVA on Ranks was used with Dunn's post-hoc test.

Results

Effect of MMP1a-Deficiency in ApoE$^{-/-}$ Mice

Deficiency of MMP1a Results in Decreased Atherosclerotic Plaque Burden— We examined the effect of deletion of the mouse homolog of MMP1 on atherosclerosis. Mice deficient in *MMP1a* were bred into the *ApoE*$^{-/-}$ background to create the *MMP1a*$^{-/-}$*ApoE*$^{-/-}$ (KO) and *MMP1a*$^{+/+}$*ApoE*$^{-/-}$ (WT) controls (Figure I in the Data Supplement). In addition, the effect of blockade of the MMP1 receptor PAR1 was examined by treating cohorts of WT and KO mice with daily PZ-128 or vehicle. After 12 weeks of western diet, the aortas in vehicle-treated male and female WT *MMP1a*$^{+/+}$*ApoE*$^{-/-}$ mice showed large amounts of atherosclerotic plaque formation in aortic arches and descending aorta (Figure 1A–C). MMP1a-deficiency and/or 12 week treatment with PZ-128 had no effect on plasma cholesterol levels relative to WT or vehicle-treatment (Table I in the Data Supplement). In contrast, male *ApoE*$^{-/-}$ mice deficient in *MMP1a* showed significant 50% drops in lesion area in both descending aorta and aortic arches (Figure 1A–C). Female KO mice had similar significant drops in atherosclerotic plaque lesion area as compared to WT mice. Male and

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female WT mice treated with the PAR1 pepducin PZ-128 exhibited significant decreases in plaque formation in the whole aorta, at levels similar to that seen in the *MMP1a* deficient mice (Figure 1A–B), but with no significant decreases in the aortic arch (Figure 1C). Treatment of *MMP1a*$^{-/-}$*ApoE*$^{-/-}$ mice with PZ-128 had no additional significant protective effects on plaque area relative to MMP1a-KO alone (Figure 1B–C).

Deficiency of MMP1a Causes Reduction in Monocyte and Macrophages in Atherosclerotic Plaques—In order to determine why deficiency of *MMP1a* gave significant reductions in plaque formation, a second cohort of ApoE$^{-/-}$ mice were placed on western diet for 16 weeks and the immune cell content of the aortic arch plaques was examined. As shown in Figure 2A, the total number of CD45^{+} leukocytes was significantly reduced by more than 2-fold in the MMP1a-KO versus WT plaques. Monocyte/macrophage (CD45^{+}CD11b^{+}CD3^{-}) content was also significantly lower in the KO plaques relative to WT (Figure 2B), whereas T cell content was unaffected (Figure 2C). PZ-128 treatment also resulted in a significant decrease in monocyte/macrophage content of the plaques in WT mice, with no further significant decrease in the MMP1a-KO mice (Figure 2B). However, there was a significant decrease in T cell content of the plaques caused by PZ-128 in the KO mice (Figure 2C). Both MMP1a-deficiency or PZ-128 treatment of WT ApoE$^{-/-}$ mice caused a significant drop in circulating monocytes but no effect on circulating T cells (% of CD45^{+}) in the peripheral blood of the 16 week ApoE$^{-/-}$ mice (Figure 2D–F).

We then tested whether MMP1a deficiency had an effect on invasion of activated PBMCs through collagen matrices. PBMCs isolated from whole blood of WT and MMP1a-deficient mice were activated overnight with oxidized (ox)-LDL. The activated PBMCs were then allowed to invade through Type-I collagen. As shown in Figure 2G, ox-LDL activated PBMCs from MMP1a-deficient mice in both C57BL6 and ApoE$^{-/-}$ backgrounds had significant decreases in basal ability to invade through Type-I collagen as compared to WT mice.

Oxidized-LDL-activated PBMCs from WT and MMP1a-KO mice were then allowed to migrate through fibronectin-coated filters towards media containing MMP1a versus media alone. As shown in Figure 2H, inclusion of MMP1a in the media gave a highly significant 75% increase ($P<0.0001$) in PBMC migration over media alone. PBMCs from MMP1a-KO mice had a 40% loss in migration to media alone which could be fully recovered to baseline by addition of MMP1a to the media in the lower well. Addition of either the PAR1 inhibitor, PZ-128, or the MMP1 inhibitor, FN-439, gave significant reductions in migration of WT PBMCs, similar to MMP1a-KO, effects which were not reversed by supplementation with MMP1a in the media in the lower wells (Figure 2H). This is consistent with migration of the activated mouse PBMCs as being dependent on both MMP1a and its receptor, PAR1.

MMP1a Deficiency Causes an Increase in Intact Fibrillar Collagen in Media and Intima of Aortic Root Plaques—The media and intima of aortic root plaques of MMP1a-KO and WT ApoE$^{-/-}$ mice after 16 weeks of high fat/high cholesterol diet were examined for fibrillar collagen content using Sirius red staining under polarized light. As shown in Figure 3A, WT ApoE$^{-/-}$ mice had major loss of intact fibrillar collagen in the media underlying the aortic root plaques as compared to MMP1a-deficient ApoE$^{-/-}$ mice.

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Quantification of these data showed a highly significant increase in both the intensity and area of fibrillar collagen staining within the aortic root plaque media and intima in the MMP1a-KO mice (Figure 3B–C, Figure II in Data Supplement). Treatment with the PAR1 inhibitor, PZ-128, had no significant effect compared to vehicle on the intensity or area of the intact fibrillar collagen in either WT or MMP1a-KO ApoE$^{-/-}$ aortic root media or intima.

Loss of MMP1a Results in More Compact Collagen and Reduction of SMCs in the Media Underlying Aortic Root Plaques—Trichrome staining revealed more compact collagen fibers with less cellularity of the media underlying the plaque in the *MMP1a*-deficient mice as compared to WT mice, but no effect with PZ-128 on collagen (Figure 4A). Accordingly, there were lower numbers of αSMA-positive cells in the MMP1a-KO aortic root media as compared to the corresponding WT mice indicating less SMC expansion in the plaque media of the MMP1a-deficient mice (Figure 4A–B). There was relatively little αSMA staining of the plaque intima (Figure 4C) and a negative control stain with FITC-conjugated mouse anti-rabbit IgG showed no detectable background fluorescence (Figure III in the Data Supplement). Macrophage content by F4/80 stain, necrotic core area, and total plaque area were also significantly decreased in the aortic root plaques of the *MMP1a*-deficient mice as compared to WT mice (Figure 4D–F). PZ-128 caused significant reductions in F4/80, necrotic core and plaque area of aortic roots in WT mice but no significant extra inhibitory effects in the MMP1a-deficient mice consistent with PAR1 acting downstream of MMP1a (Figure 4D–F).

Effects of Collagenase and PAR1 Inhibitors in Activated PBMC Chemo-invasion through Collagen—In addition to expressing PAR1 and chemokine receptors such as CCR2 (MCP-1) and CXCR1/2 (IL-8), monocytes and macrophages can produce all 3 soluble collagenases MMP1(a), MMP8 and MMP13 that could potentially be involved in chemotactic invasion through collagen matrices.[1] We tested the effects of inhibitors against MMP1 (FN-439), MMP8, MMP9/13, PAR1 (PZ-128) versus MMP1a-deficiency on the ability of ox-LDL activated PBMCs from WT mice to invade through Type I collagen towards gradients of MCP-1, IL-8 and TNFα. FN-439 was previously shown to inhibit >94% of MMP-1 collagenase activity, 0–7% of MMP-2, MMP-3, and MMP-8, 21% of MMP-9 and 36% of MMP-13 activity, and has no inhibitory effects against the PAR1 agonist thrombin.[23] Deficiency of MMP1a gave reductions of 30–50% in collagen invasion of activated PBMCs towards MCP-1, IL-8 or TNFα as compared to WT PBMCs (Figure 5A–C). Blockade of PAR1 with PZ-128 gave similar reductions as both MMP1a-deficiency or the MMP1 inhibitor, consistent with MMP1a-PAR1 significantly contributing to monocyte collagen invasion as described for MMP1a-PAR1 in cancer cells.[13, 24] In comparison, the MMP8 inhibitor did not have any significant effect of WT PBMC invasion through collagen towards MCP-1, IL-8 or TNFα (Figure 5A–C). Similarly, the MMP9/13 inhibitor had no effect on invasion of WT PBMCs towards MCP-1 or IL-8 (Figure 5A–B), but did significantly inhibit invasion towards TNFα (Figure 5C).

PMBCs were then purified from whole blood from human subjects and activated with ox-LDL overnight. The activated human PBMCs were allow to invade through Type-I collagen

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towards gradients of IL-8, TNFα and MCP-1. Addition of the PAR1 inhibitor, PZ-128 or the MMP1 inhibitor, FN-439, gave highly significant reductions in collagen chemo-invasion towards both IL-8 and TNFα (Figure 5D–E), consistent with the mice data, but no effect with MCP-1 (Figure IV in the Data Supplement). Conversely, inhibition of MMP8 or MMP9/13 had no effect on human PBMC collagen invasion towards TNFα, and MMP8 inhibition gave a slight effect on invasion towards IL-8, indicating that MMP1 appears to be the dominant secreted collagenase in invasion through collagen for human PBMCs.

Translational Results

Angiographic Determination of Coronary Artery Disease Burden in TRIP-PCI Patients

Next, we examined whether human MMP1 versus MMP8 and MMP13, might be correlated with atherosclerotic plaque burden in CAD and ACS patients undergoing cardiac catheterization. To accomplish this, we used patients from TRIP-PCI, a prospective, randomized, double-blinded, multi-center, phase 2 trial that tested the safety and efficacy of the PAR1 pepducin, PZ-128, versus placebo in 100 patients undergoing cardiac catheterization with intent to perform percutaneous coronary intervention (PCI).[19] Nearly 50% of the patients had acute coronary syndromes (ACS), 39% had stable coronary artery disease (CAD) and 11% had positive stress/imaging tests (other), with a preponderance of CAD risk factors such as male sex (84%), dyslipidemia (89%), hypertension (80%), smoker (63%), diabetes (30%), prior MI (23%) or prior PCI/CABG (35%) (Figure 6A). Following cardiac catheterization, PCI was performed in 39%, CABG in 11% and the remainder underwent medical management. Angiographic CAD burden was quantified for each patient using the 29-segment CASS scoring system of the number and location of coronary arteries with ≥50% stenosis. Two-thirds of the patients had left coronary artery lesions and 45% had right coronary artery lesions (Figure 6B) with a mean of 2.9 stenotic lesions per patient (range 0–12).

Circulating MMP1 is Significantly More Abundant as Compared to MMP8 and MMP13 Collagenases in TRIP-PCI Patients

Baseline plasma collected from the TRIP-PCI patients was quantified for the 3 collagenases MMP1, MMP8, MMP13, along with TNFα. Median MMP1 levels were significantly ($P<0.001$) higher by 5.7-fold relative to MMP8 and 19-fold higher than MMP13 (Figure 6C). MMP1 was also highly significantly correlated with angiographically-determined CAD burden ($P<0.0013$) and TNFα levels ($P<0.0012$) in the TRIP-PCI patients (Figure 6D–E). MMP8 was correlated with CAD burden but not TNFα levels, whereas MMP13 exhibited no correlation with CAD burden or TNFα (Figure 6D–E).

PZ-128 Protects Against Transient Monocyte Depletion Following Cardiac Catheterization

As MMP1 may activate PAR1 on monocytes in the setting of atherothrombotic disease and acute coronary interventions, we determined whether circulating monocytes may co-express both agonist and receptor. Monocytes from patients with diagnosed CAD were analyzed by flow cytometry for surface MMP1 and PAR1 expression (Figure 7A). There was a significant correlation ($R=0.78$, $P=0.005$) between MMP1 and PAR1 expression on the monocytes of the CAD patients (Figure 7B). The mechanical process and stress of cardiac

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catheterization and PCI causes damage to the vasculature and may induce acute activation/adhesion and transient depletion of circulating monocytes. We assessed whether 2 h intravenous infusion of the PAR1 inhibitor, PZ-128, may have a protective effect against acute loss of circulating monocytes in the periprocedural period in the TRIP-PCI patients. As shown in Figure 7C, patients treated with PZ-128 had a significant protection against the drop in circulating monocytes observed at the 2 h time point (approximately 1 h after completing cardiac catheterization/PCI) in the placebo arm. By comparison, there were no significant changes in platelet counts, RBCs, neutrophils or lymphocytes at 2 h or 6 h relative to baseline in either the placebo or PZ-128 arms in the CAD/ACS patients (Table II in the Data Supplement). To model the acute monocyte effects experimentally, mice were subjected to 30 min of total left coronary artery occlusion followed by 2 h reperfusion or sham procedure. Treatment with PZ-128 also protected against a similar loss in monocytes at the 2 h 30 min time point as compared to placebo in the model of ischemia reperfusion injury (Figure 7D). Examination of the monocyte LyC6Hi and LyC6Lo subpopulations showed a preferential loss of the proinflammatory Ly6cHi monocytes in the placebo mice as compared to PZ-128 treated mice following cardiac ischemia reperfusion injury (Figure 7E). Together, these data would indicate that blockade of PAR1 suppresses acute loss of circulating monocytes following myocardial ischemia in both mice and humans.

Discussion

This study demonstrated that the collagenase MMP1a plays an important role in atherosclerotic plaque formation. Genetic deletion of MMP1a resulted in marked reduction of monocyte and macrophage content in plaques and 50% less overall plaque burden in the aortas of *ApoE*$^{-/-}$ mice. Inhibition of MMP1a or deletion suppressed invasion of activated PBMCs through type I collagen, consistent with the lower levels of infiltrated monocytes/macrophages observed in the aortic plaques. This is in contrast to the lack of effects on plaque size and inflammatory infiltrates previously observed with either single or dual deletion of the other two major secreted collagenases, MMP8 and MMP13, in the *ApoE*$^{-/-}$ model[10] or with a selective inhibitor of MMP13.[25] Likewise, we found that inhibition of either MMP8 or MMP9/13 had no significant effect on oxLDL-activated PBMC invasion though collagen matrices towards MCP-1 or IL-8 chemokines. These data therefore indicate that MMP1a is the dominant secreted collagenase compared to MMP8 and MMP13, acting to promote pro-inflammatory processes that drive atherosclerotic plaque formation in mice. In humans, we found that circulating levels of MMP1 were significantly more abundant relative to MMP8 (6-fold lower) and MMP13 (19-fold lower) in CAD/ACS patients from the TRIP-PCI study. MMP1 was significantly correlated with angiographically-determined stenotic coronary artery disease burden in these patients, thus providing consistent results for atherosclerotic human and mouse plaque burden.

Deletion of MMP1a also enhanced intact fibrillar collagen content and density in the aortic roots of *ApoE*$^{-/-}$ mice with correspondingly less smooth muscle cell expansion in the collagen matrix located in the media underlying the plaques. MMP1(a) was previously shown to activate non-canonical signaling through PAR1[26] including amplification of TNFα production via NF-κB/AP-1[16] and may therefore play a more complex role beyond collagen remodeling by regulating PAR1 dependent cellular activities on plaque SMCs[23] and other

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vascular cells.[16, 27, 28] We previously found that MMP1 cleavage and activation of PAR1 on primary SMCs caused significant loss of myocardin expression, the major transcriptional co-activator responsible for expression of the SMC gene box, along with suppression of SM-22α, calponin, and contractility signaling.[23] Consistent with those earlier findings, loss of MMP1a caused a more collagen-dense media with less SMC cellularity and compressed cell size in the medial regions underlying the aortic root plaques.

Suppression of collagenolysis in atherosclerotic plaques was also noted previously in both the MMP8 and MMP13 deficient mice in the *ApoE*$^{-/-}$ background.[10, 29] Therefore, unlike the effects on plaque size and inflammatory cell burden seen only with deletion of MMP1a, all 3 secreted collagenases appear to provide an overlapping ability to degrade fibrillar collagen in the arterial plaques and could potentially contribute to overall plaque stability or vulnerability in an additive manner, though this could not be directly assessed in the mouse model. Plasma levels of MMP1, MMP8 and/or MMP13 could therefore serve as biomarkers of plaque collagen indices in unstable angina or ACS, whereas plasma MMP1 may be an additional non-invasive marker of total plaque burden and vascular inflammation. In this regard, MMP1 was the only collagenase in the TRIP-PCI patients that was correlated with systemic levels of the inflammatory cytokine, TNFα. Previous studies showed that both MMP1 and MMP13 collagenases are expressed in human atheroma macrophages,[30] however unstable plaques demonstrated 8-fold higher MMP1 expression[31] and plasma MMP1 was significantly elevated in patients with high intimal/media thickness.[32] Similarly, in a large prospective study in ACS patients referred for coronary angiography, patients presenting with high amounts of plasma MMP1 had a nearly 50% increase in 5-year mortality (HR, 1.49; 95% CI, 1.23–1.80; P<0.0001).[33]

Despite circulating at 6-fold lower median levels than MMP1, we found that MMP8 also correlated with stenotic coronary artery disease burden in the TRIP-PCI patients. MMP8 is highly expressed in neutrophils with much lower levels in macrophages.[34] Unlike MMP1, MMP8 expression is down-regulated upon activation of macrophages,[34] and MMP8 plays opposing functions to MMP1 in systemic inflammation[14, 35] and in cancer.[36] Although likely to be a small minority of the plaque leukocytes, the neutrophils present in mature atherosclerotic lesions have recently been shown to express alarmins and cytokines such as S100A8/9 and CXCL2[6] which are processed by MMPs such as MMP8.[35, 37] Therefore, neutrophil-expressed MMP8 could potentially regulate the recruitment and retention of adjacent monocytes and macrophages and affect long-term plaque regression in humans. Similarly, MMP1 can activate and release pro-TNFα into its soluble form[38] which may perpetuate local pro-inflammatory pathways in atherosclerosis.

We found that circulating monocytes from CAD patients co-expressed both MMP1 and PAR1 and that the PAR1 inhibitor, PZ-128, significantly protected against transient loss of monocytes following cardiac catheterization. Akin to these protective effects on circulating monocytes in the periprocedural period in the TRIP-PCI patients, PZ-128 also prevented the acute transient loss of circulating monocytes in mice following total left coronary artery occlusion and reperfusion. Although the long-term significance, if any, of these acute protective effects on monocytes is not known, it is possible that monocytes activated by the cardiac procedure may attach to damaged endothelium and intravasate into the blood vessel

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wall in an MMP1-PAR1 dependent mechanism. In this regard, a previous study showed transient loss of flowing and rolling monocytes in ear blood vessels 15–30 min after acute MI in mice with simultaneous enrichment into infarcted hearts suggesting acute depletion of the vascular pool of monocytes prior to eventual replenishment by the splenic pool.[39] Circulating monocytes may also play a role in neointimal growth in stents after PCI and have been shown to become significantly elevated at 2-day time points,[40] with MI and ischemic injury accelerating subsequent plaque development in mice through a monocyte-dependent splenic release mechanism.[41] Interestingly, we found that either MMP1a deficiency or chronic treatment of ApoE$^{-/-}$ mice with the PAR1 inhibitor, PZ-128, for 16 weeks resulted in a significant 50% relative drop in circulating monocytes, indicating a potential systemic role of the MMP1-PAR1 system in regulating monocyte homeostasis under conditions of chronic inflammation.

Unlike the effects on monocytes and macrophages, MMP1a deficiency gave no effect on T cell content in plaques or systemically, however, there was a significant inhibition of plaque T cell content due to PZ-128 treatment in the MMP1a-KO mouse, pointing to a PAR1 effect on T cells that is independent of MMP1a. This could indicate that a different protease agonist could activate PAR1 on T cells or that PAR1 constitutive signaling might be suppressed by PZ-128 in the plaque microenvironment. The finding that MMP1a deficiency had no effect on plaque T cell content is consistent with previous work that showed that unlike monocytes, T lymphocytes crawl through fibrillar collagen independent of matrix remodeling by MMPs.[42] A potential limitation is that we did not include a non-DMSO control group that would have helped assess the pro-inflammatory effect, if any, of the DMSO vehicle (5% used to dissolve PZ-128) on the atherosclerosis parameters, monocyte numbers and expression of collagenolytic proteases in the mouse models. However, if the DMSO vehicle was acting as a proinflammatory (or anti-inflammatory) stimulus, it was still used at the same concentration, frequency, and route of administration for every mouse. Moreover, addition of 0.1% DMSO had no significant effects of chemotactic invasion of mouse and human PBMCs through type-I collagen (collagenolytic-enzyme requiring) relative to media in the absence of DMSO.

In conclusion, we provide long-awaited evidence that genetic loss of MMP1a has a major impact on both inflammatory infiltrates and collagen content in atherosclerotic lesion development in mice, and that the analogous secreted collagenase in humans, MMP1, is much higher in the blood of CAD and ACS patients with stenotic coronary lesions as compared to MMP8 and MMP13. As genetic deletion or pharmacologic blockade of MMP1a or its receptor PAR1 with PZ-128 suppressed atherosclerotic lesion development and inflammatory cell infiltrates, the work presented here indicates a new therapeutic strategy for potentially impacting both leukocyte infiltration and collagen invasion in lesions in the chronic time frame of atherosclerosis.

Supplementary Material

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Nonstandard Abbreviations and Acronyms

ACS	acute coronary syndromes
CASS	29-segment Coronary Artery Surgery Study Scoring
MMP1a	Matrix metalloprotease-1a
Ox-LDL	oxidized low density lipoprotein
PAR1	protease-activated receptor-1

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Highlights

- Humans have only 3 secreted collagenases, MMP1, MMP8 and MMP13, that are able to cleave triple-stranded fibrillar collagen located in the vessel wall in atherosclerotic plaques, however mice lacking MMP8 and/or MMP13 surprisingly had no reduction of atherosclerotic lesion burden nor inflammatory cells.
- As destruction of arterial collagen allows monocyte and macrophage infiltration leading to atherosclerotic plaque formation, it is not clear what role the remaining MMP1 collagenase plays in this process in vivo.
- We demonstrate that genetic loss of the MMP1a collagenase in ApoE$^{-/-}$ mice has major impact on plaque development, inflammatory infiltrates, and collagen content in lesions.
- In patients undergoing cardiac catheterization, plasma levels of MMP1 were found to greatly exceed those of MMP8 and MMP13, and that MMP1 was significantly higher in patients with more stenotic coronary lesions and systemic levels of TNFα.
- These data provide evidence for an important role for the MMP1(a) collagenase in atherosclerotic lesion development and leukocyte behavior and validate MMP1 as a potential target in atherosclerosis.

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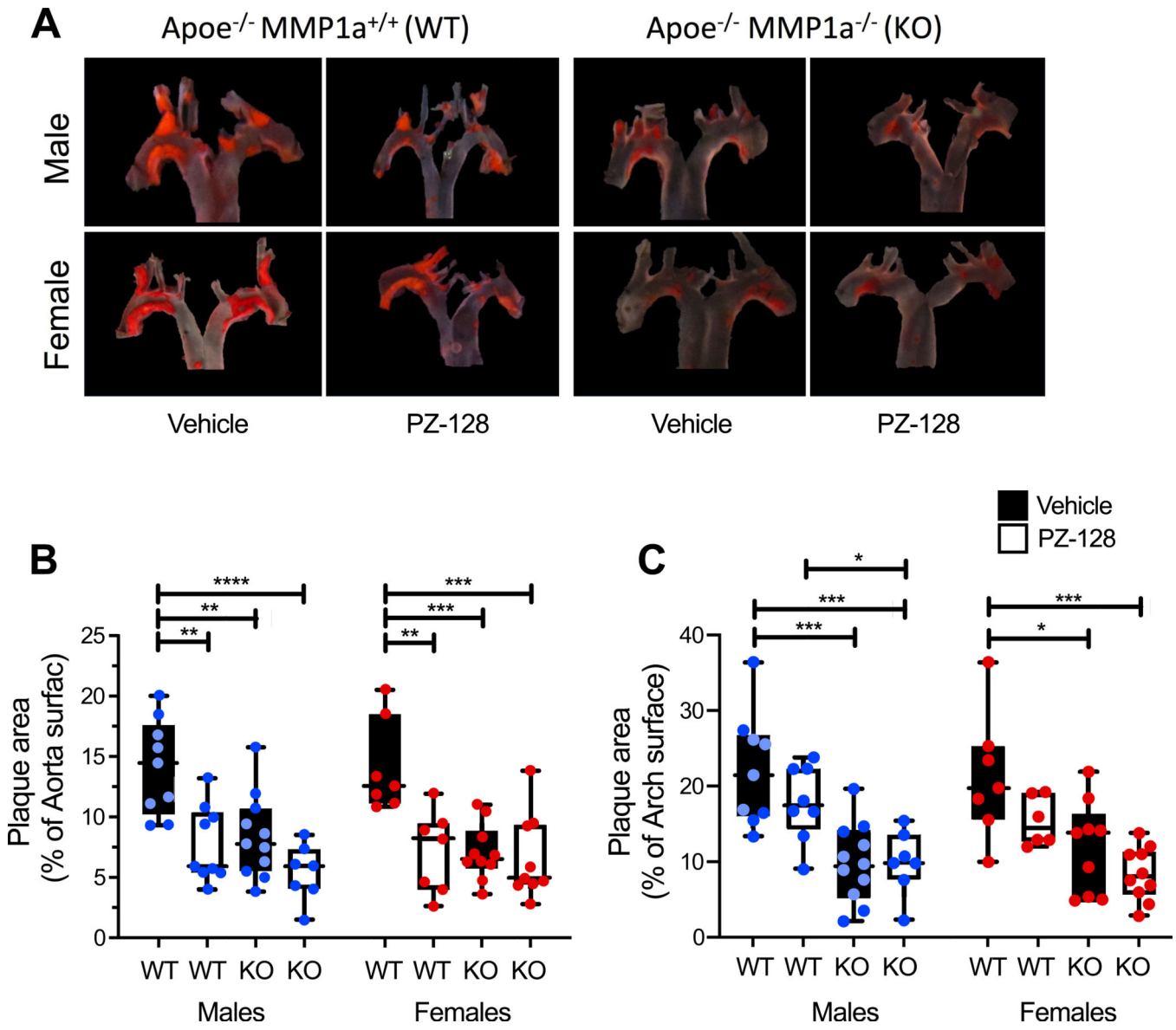

Figure 1. Genetic deficiency of MMP1a gives significant protection against atherosclerotic lesion development in the aortas of male and female mice.

A. Male and female MMP1a$^{+/+}$ApoE$^{-/-}$ (WT) and MMP1a$^{-/-}$ApoE$^{-/-}$ (KO) mice (n=7–10) were fed a western type diet (WD) for 12 weeks and treated daily with sc vehicle or PAR1 pepducin PZ-128 (10 mg/kg). After 12 weeks, mice were sacrificed and aortas stained with oil-red O. **B.** Quantification of plaque area as % of total Aortic surface or **C**, as % of Aortic Arch surface from mice in A. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 by 2-way ANOVA followed by Tukey's multiple comparisons.

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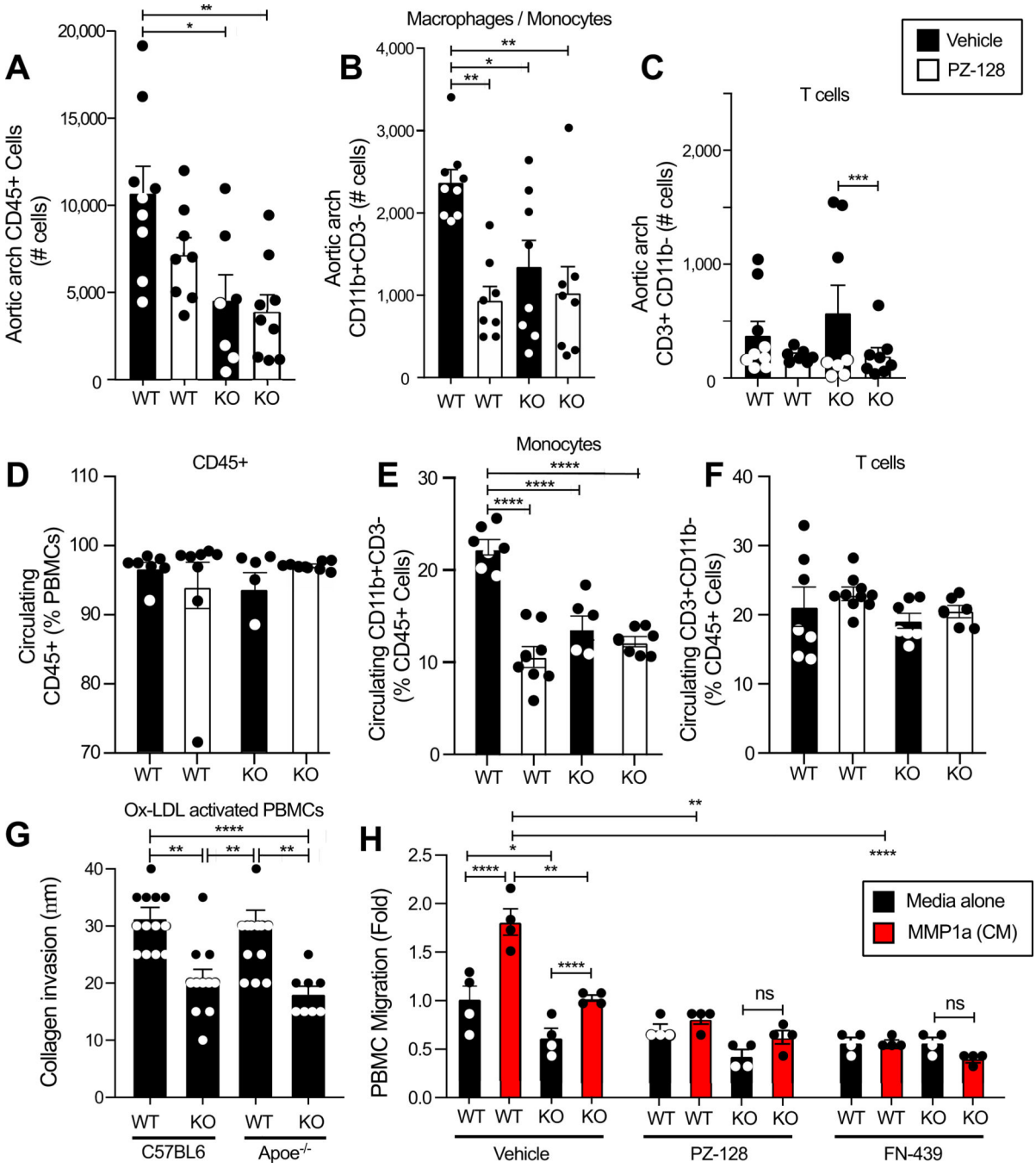

Figure 2. Effect of genetic deficiency of MMP1a on leukocytes in atherosclerotic plaques.

A-C. Atherosclerotic plaques were removed from the aortic arches of male MMP1a$^{+/+}$ApoE$^{-/-}$ (WT) and MMP1a$^{-/-}$ApoE$^{-/-}$ (KO) mice (n=7–9) on WD treated for 16 weeks with either daily sc vehicle or PZ-128. Plaques were digested and cells sorted by FACS using CD45.2, CD11b, CD3 antibodies to determine the amount of (A) CD45.2^{+} leukocytes, (B) macrophages/ monocytes, or (C) T cells. Peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood of mice in A-C and cells sorted by FACS using CD45.2, CD11b, and CD3 antibodies to determine the % of (**D**) CD45.2^{+} cells, (**E**) monocytes, and

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(**F**) T cells. **G.** PBMCs were isolated from the whole blood of 8 week old C57BL6/MMP1a$^{+/+}$, C57BL6/MMP1a$^{-/-}$, ApoE$^{-/-}$/MMP1a$^{+/+}$, and ApoE$^{-/-}$/MMP1a$^{-/-}$ mice and activated overnight with 50 ng/mL ox-LDL. The ox-LDL activated monocytes were then allowed to invade for 5 h through Type I collagen-coated nitrocellulose membranes in a micro-Boyden chamber (n=8–13). **H.** Isolated PMBCs from 8 week old C57BL6/MMP1a$^{+/+}$ (WT) and C57BL6/MMP1a$^{-/-}$ (KO) mice were allowed to migrate for 4 h through fibronectin-coated nitrocellulose membranes in a micro-Boyden chamber (n=4) towards either media alone or MMP1a-containing media in the presence of vehicle, PZ-128 (10 μM), MMP1 inhibitor (FN-439, 3 μM). *P<0.05, **P<0.01, ****P<0.0001 by 2-way ANOVA followed by Tukey's multiple comparisons.

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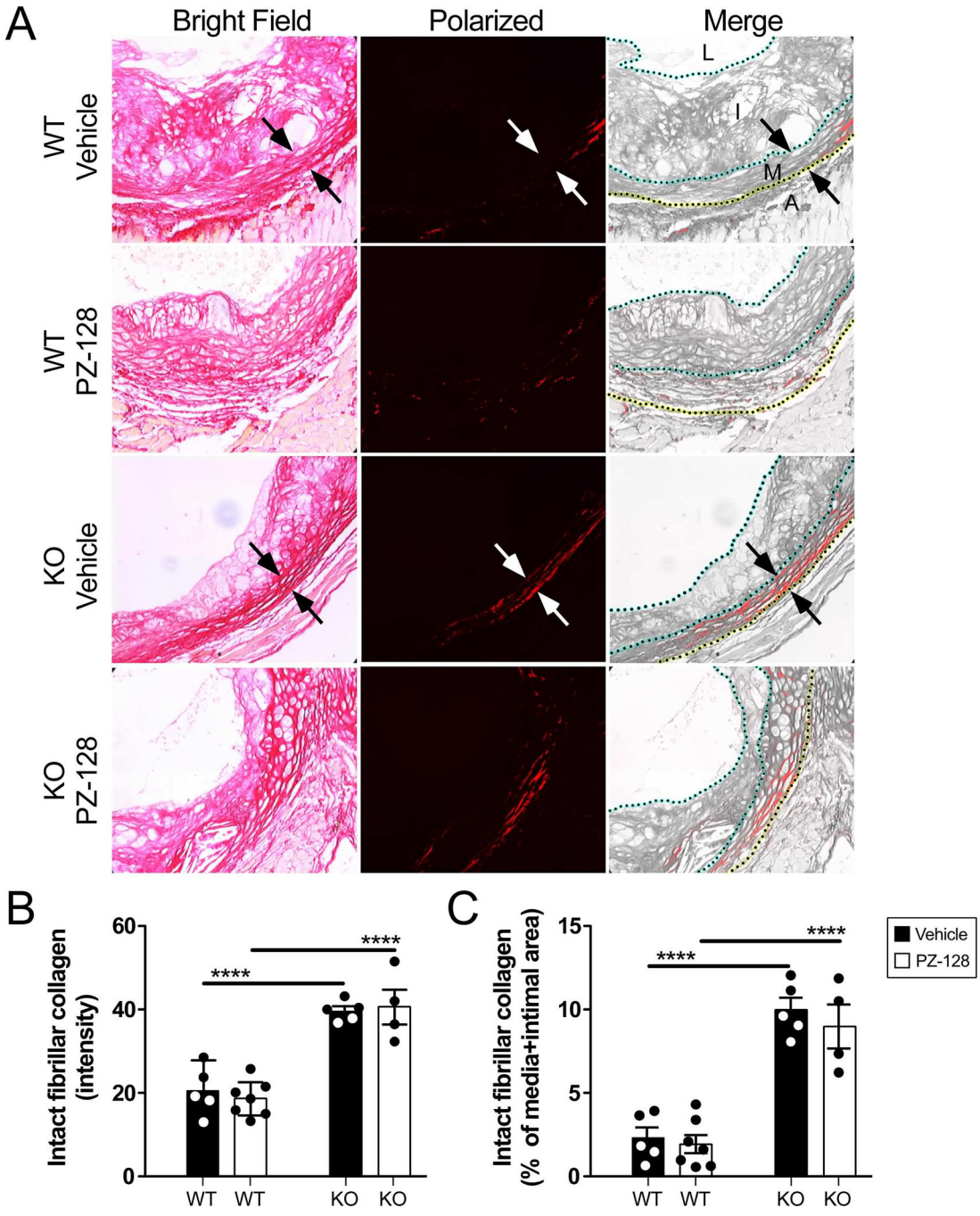

Figure 3. MMP1a deficiency causes an increase in intact fibrillar collagen in aortic root plaque media plus intima of ApoE$^{-/-}$ mice after 16 weeks western diet.

A. Aortic root sections were isolated from MMP1a$^{+/+}$ApoE$^{-/-}$ (WT) and MMP1a$^{-/-}$ApoE$^{-/-}$ (KO) mice fed a western diet with 16 weeks of daily treatment with vehicle or PZ-128 as in Figure 2. Sections were stained for collagen with sirius red and visualized under bright field or with polarized light (n=4–7) and (**B)** intact fibrillar collagen quantified as intensity per total field, or (**C)** as percent of plaque media+intimal area. ****P<0.0001 by 2-way Anova with Tukey's post-test. L=lumen, I=intima, M=media, A=adventia.

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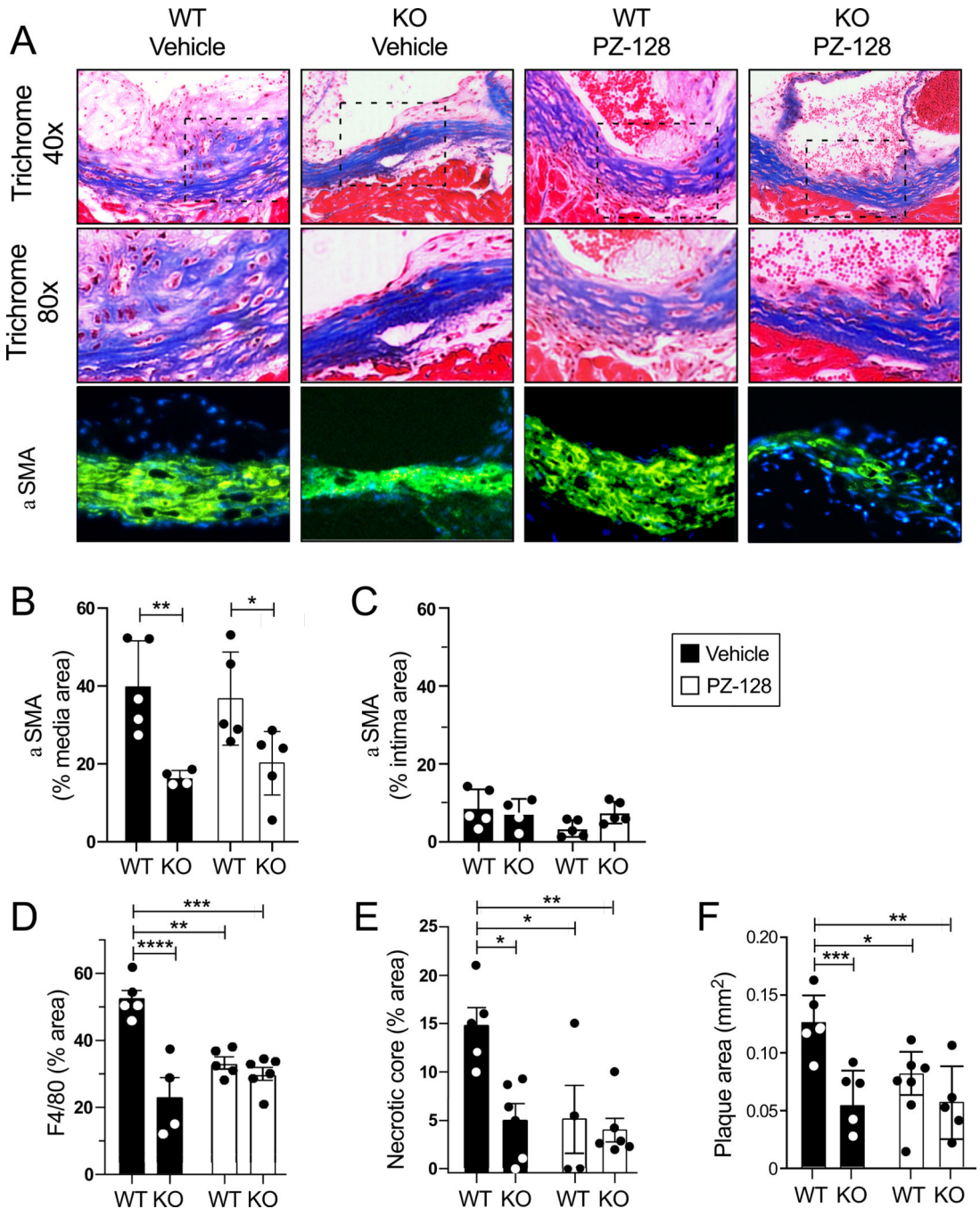

Figure 4. Deficiency of MMP1a results in more compact collagen and a reduction in SMC expansion in plaque media.

A. Aortic root sections were isolated from MMP1a$^{+/+}$ApoE$^{-/-}$ (WT) and MMP1a$^{-/-}$ApoE$^{-/-}$ (KO) mice fed western diet with 16 weeks of daily treatment with Vehicle or PZ-128 as in Figure 2. Sections were stained for collagen with trichrome (paraffin) or αSMA-antibody for smooth muscle actin (frozen sections). αSMA content (green fluorescence shown in bottom row of A) of the media and intima were quantified as % area of (**B**) media and (**C**) intima of the aortic root plaque, respectively. **D,** macrophage F4/80 staining and **E,** necrotic

core (assessed from oil-red O staining) were quantified as percent of arterial area. **F.** Mean aortic root plaque area. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 by 2-way Anova with Tukey's post-test.

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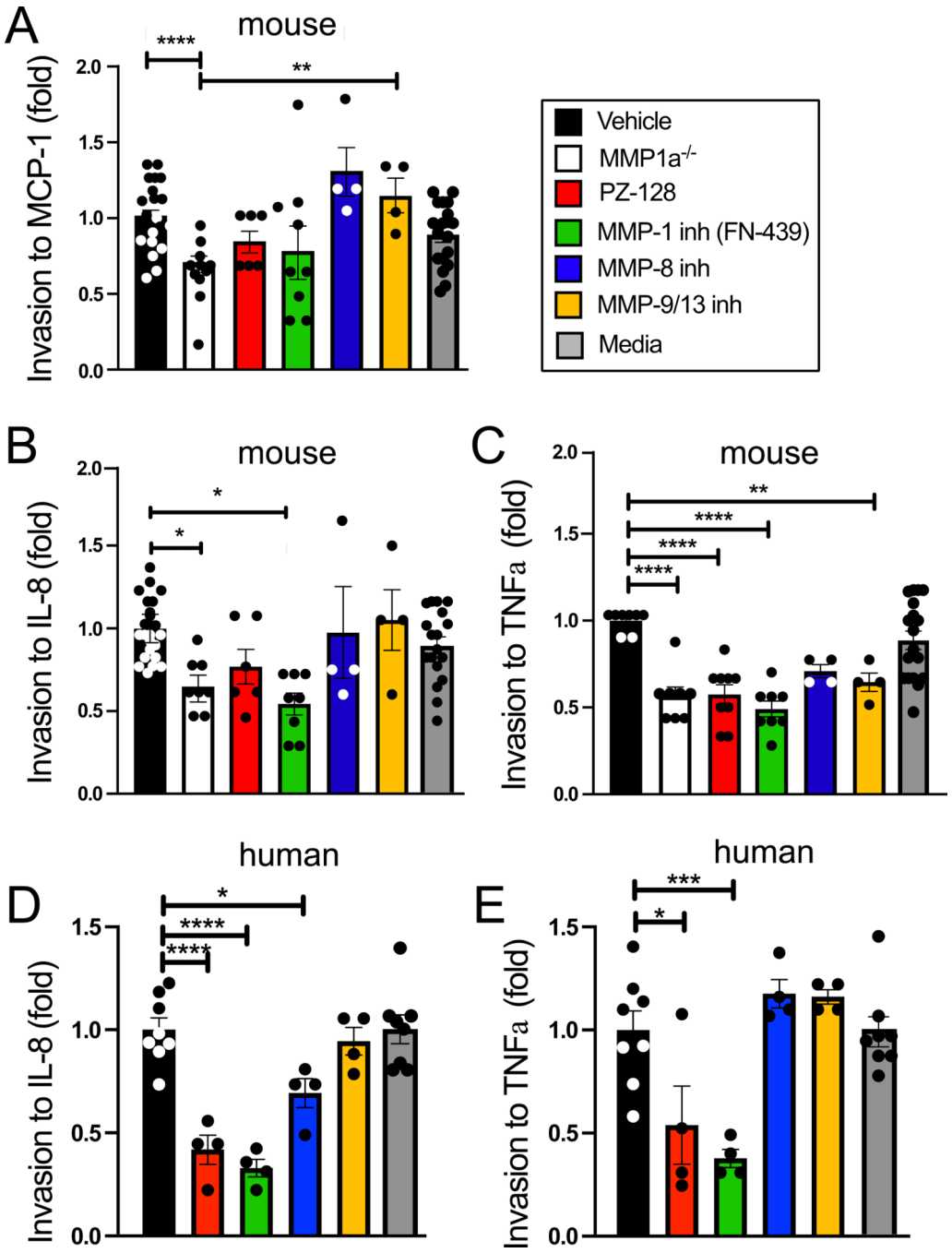

Figure 5. Effect of genetic deficiency of MMP1a or inhibitors of MMP1, MMP8, MMP13 or PAR1 on collagen invasion of ox-LDL-activated PBMCs.

PBMCs were isolated from the whole blood of WT (black/colored bars), MMP1a$^{-/-}$ (white bars) mice, or from healthy human volunteers and activated overnight with 50 ng/mL ox-LDL (n=4–20). The ox-LDL activated PBMCs were allowed to invade for 5 h through Type I collagen-coated nitrocellulose membranes in a micro-Boyden chamber in triplicate towards gradients of (**A**) 10 ng/mL MCP-1, (**B, D**) 10 nM IL-8, or (**C, E**) 5 ng/mL TNFα in the lower well, in the presence of inhibitor vehicle (WT-black bar, MMP1a$^{-/-}$-white bar),

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PZ-128 (10 μM), MMP1 inhibitor (FN-439, 3 μM), MMP8 inhibitor (25 nM), MMP9/13 inhibitor (2.5 μM), or media (omit 0.1% DSMSO) as indicated. *P<0.05, **P<0.01, ****P<0.0001 by ANOVA followed by Tukey's post-test.

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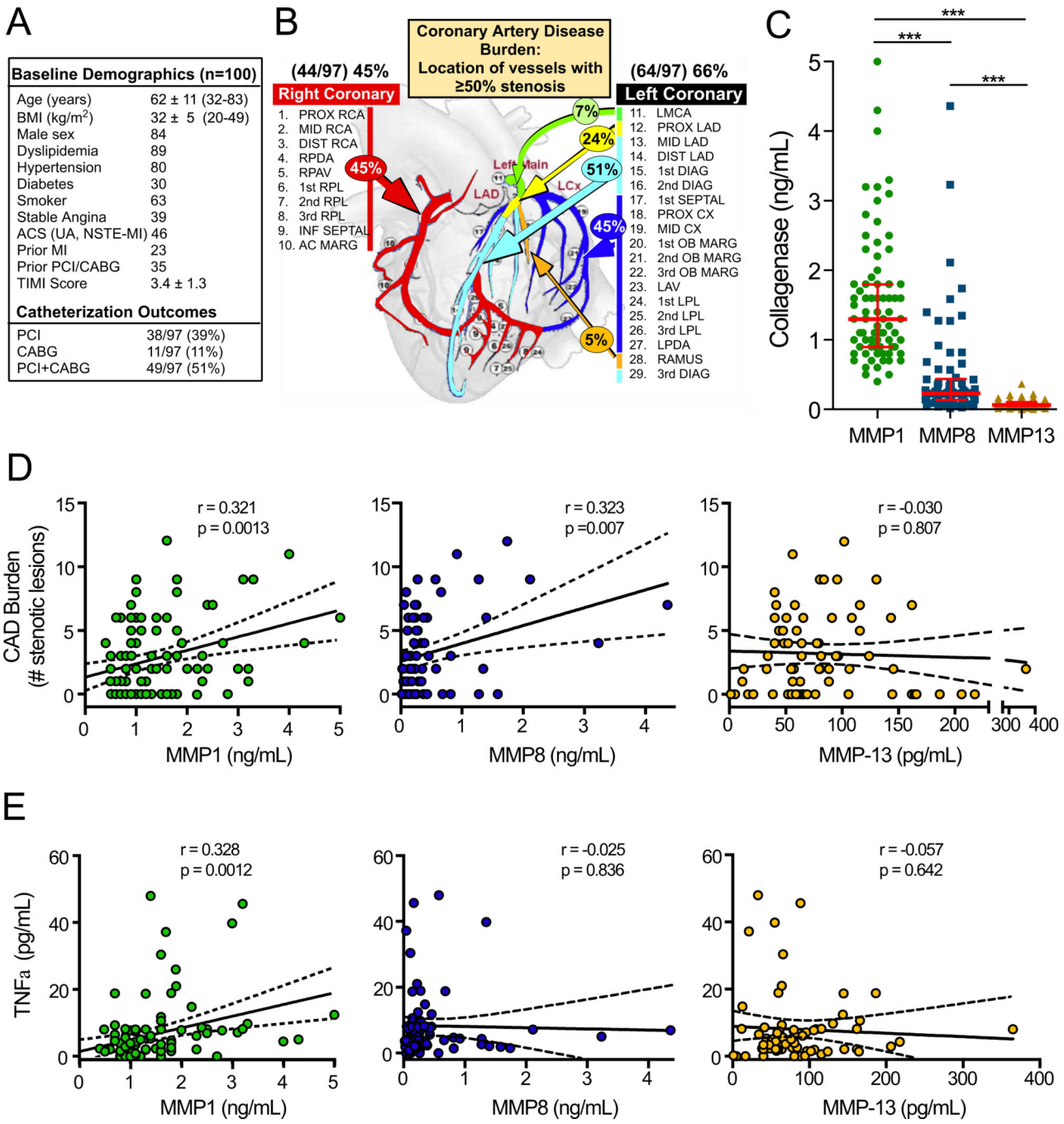

Figure 6. Relative levels of the 3 collagenases MMP1, MMP8 and MMP13 versus stenotic coronary artery disease burden in patients with CAD and ACS.

A. Baseline demographics and cardiac catheterization outcomes (PCI, percutaneous coronary intervention; CABG, coronary artery bypass surgery) of patients enrolled in the TRIP-PCI phase 2 trial (n=100). **B.** Location and percentage of patients with coronary lesions with ≥50% stenosis based on the 29-segment CASS score. **C.** Levels of the 3 circulating collagenases MMP1, MMP8 and MMP13 measured in the plasma of TRIP-PCI patients. Median (25%−75%) shown with Kruskal-Wallis non-parametric one-way ANOVA

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on Ranks and Dunn's post-hoc test ***P<0.001. **D.** Systemic MMP1, MMP8 and MMP13 collagenase levels were plotted versus CAD burden or (**E**) TNFα in plasma for each patient. Linear regression analyses were performed and least-squares lines (±95% CIs), R and P values for the slopes are shown.

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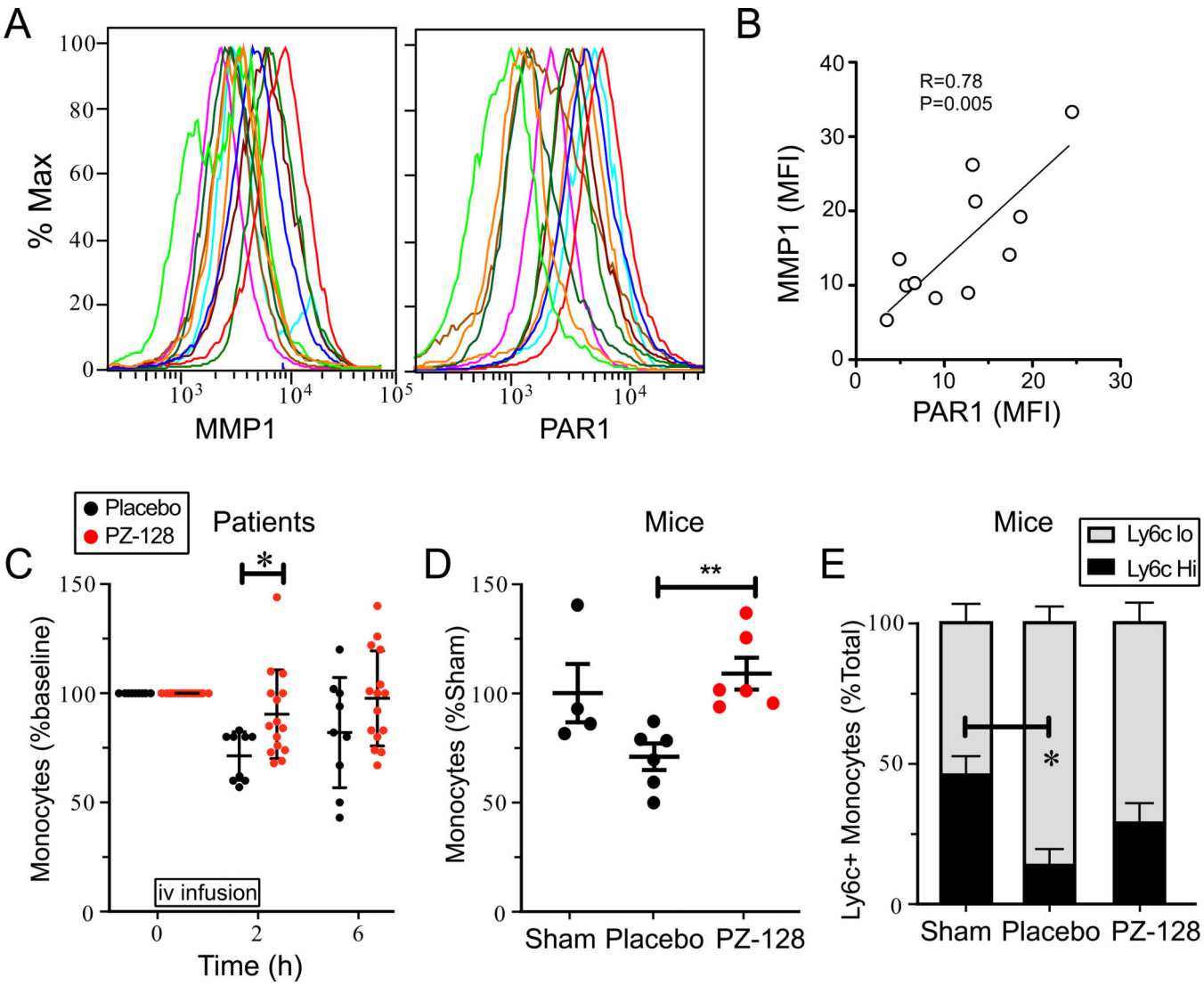

Figure 7. Co-expression of MMP1 and PAR1 in monocytes of CAD patients and effects of PZ-128 on circulating monocytes following cardiac catheterization.

A. Surface expression of MMP1 and PAR1 on circulating monocytes in CAD patients (n=11) at baseline using flow cytometry. PBMCs were isolated from whole blood by ficoll gradient and stained with antibodies to CD11b, CD3, MMP1 and PAR1. **B.** Correlation of MMP1 and PAR1 expression on CD11b+/CD3- monocytes from A using linear regression of mean fluorescent intensities of MMP1 and PAR1 relative to secondary antibody alone. R and P values for the slope of the line are shown. **C.** Circulating monocyte levels in patients from the TRIP-PCI study receiving either a 2 h infusion of 0.3–0.5 mg/kg PZ-128 (n=14) or 5% dextrose placebo (n=9). **D.** Circulating monocyte (CD11b+/CD3-) levels (at 2 h 30 min) in mice subjected to 30 min of total left coronary artery occlusion followed by 2 h of reperfusion or sham procedure. Mice were injected sc with either placebo (vehicle) or 10 mg/kg PZ-128, 1 h prior to cardiac surgery. **E.** Circulating monocytes from mice treated with either placebo or PZ-128 after 2 h cardiac reperfusion injury in D, were also quantified for high (Hi) or low (Lo) Ly6c surface expression as % of the total CD11b+/CD3- monocyte

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population. 2-way Anova followed by Tukey's post-test was performed in C, and 1-way Anova followed by Tukey's post-test in D and E, *P<0.05, **P<0.01.