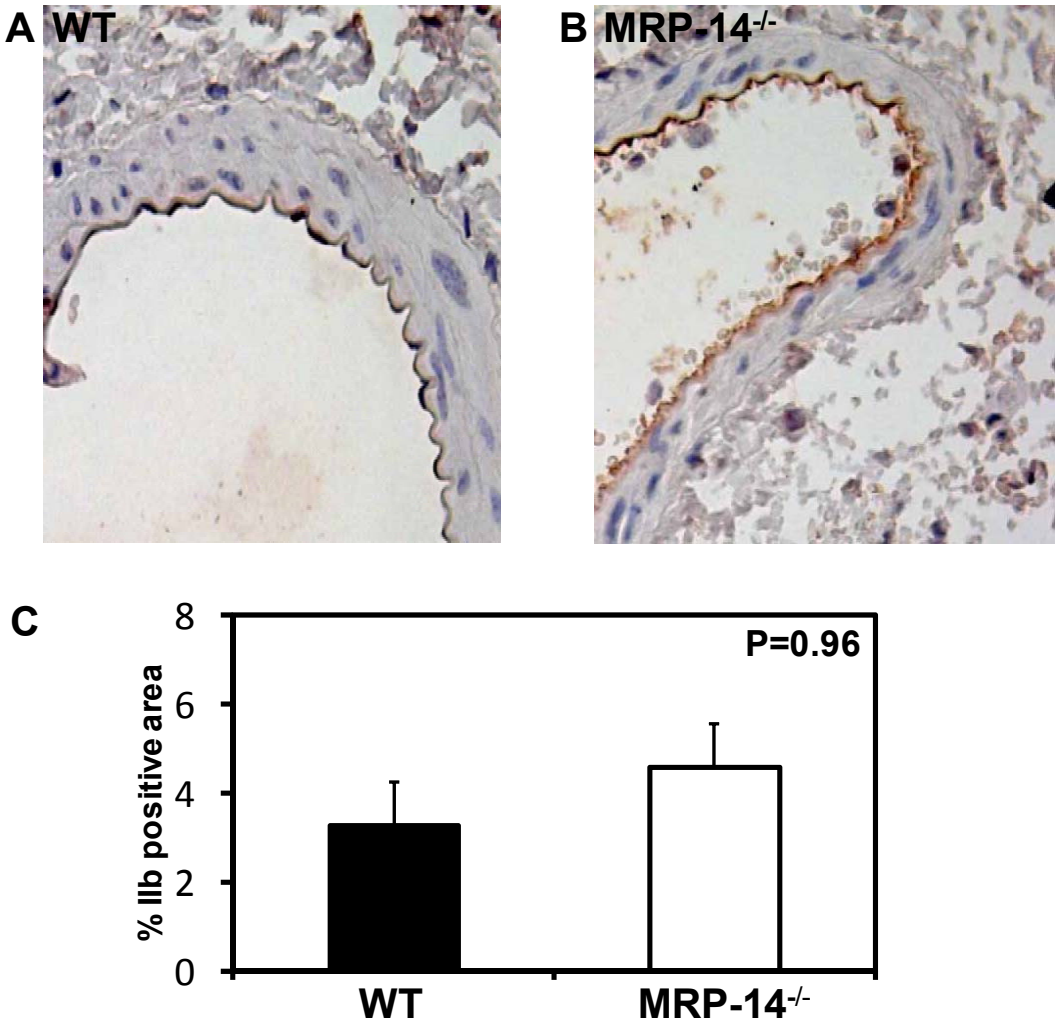
Supplemental Data and Methods:

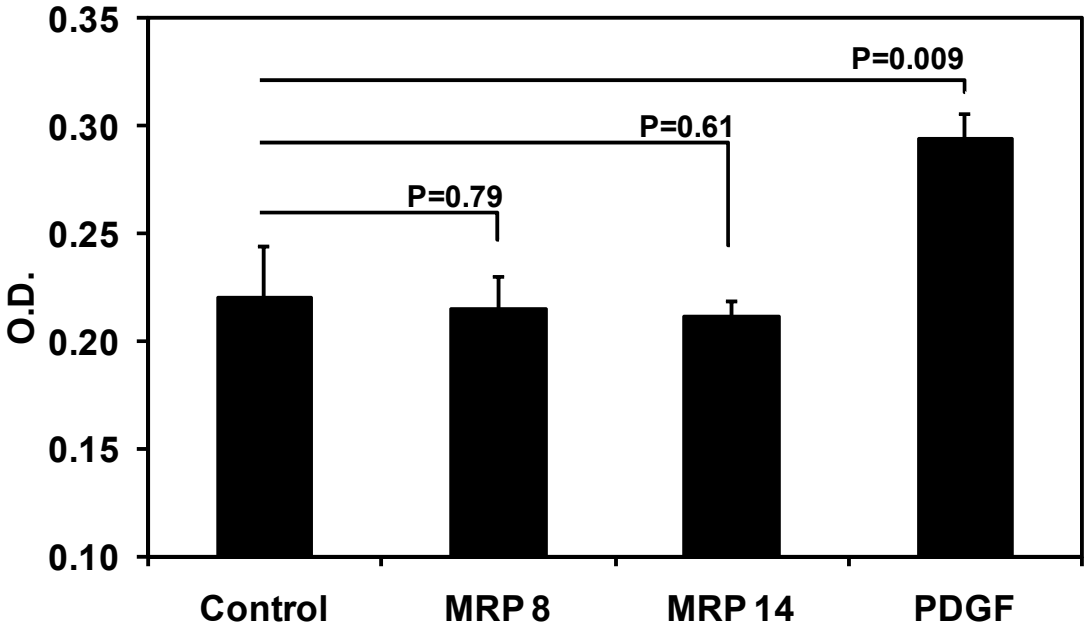
Supplemental Data:

Supplemental Figure 1



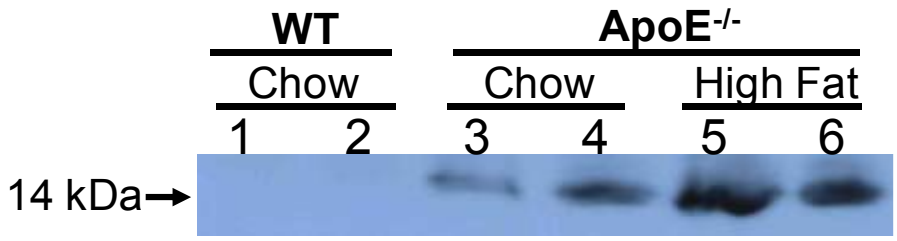
Supplemental Figure 1: Photomicrographs femoral arteries from WT and MRP-14$^{-/-}$ mice 6 hours after wire injury. A-B, IIb immunostaining (original magnification 40X). **C,** Quantification of platelet deposition (% IIb-positive area lumen). Mean ± standard error, n=6 mice per group.

Supplemental Figure 2



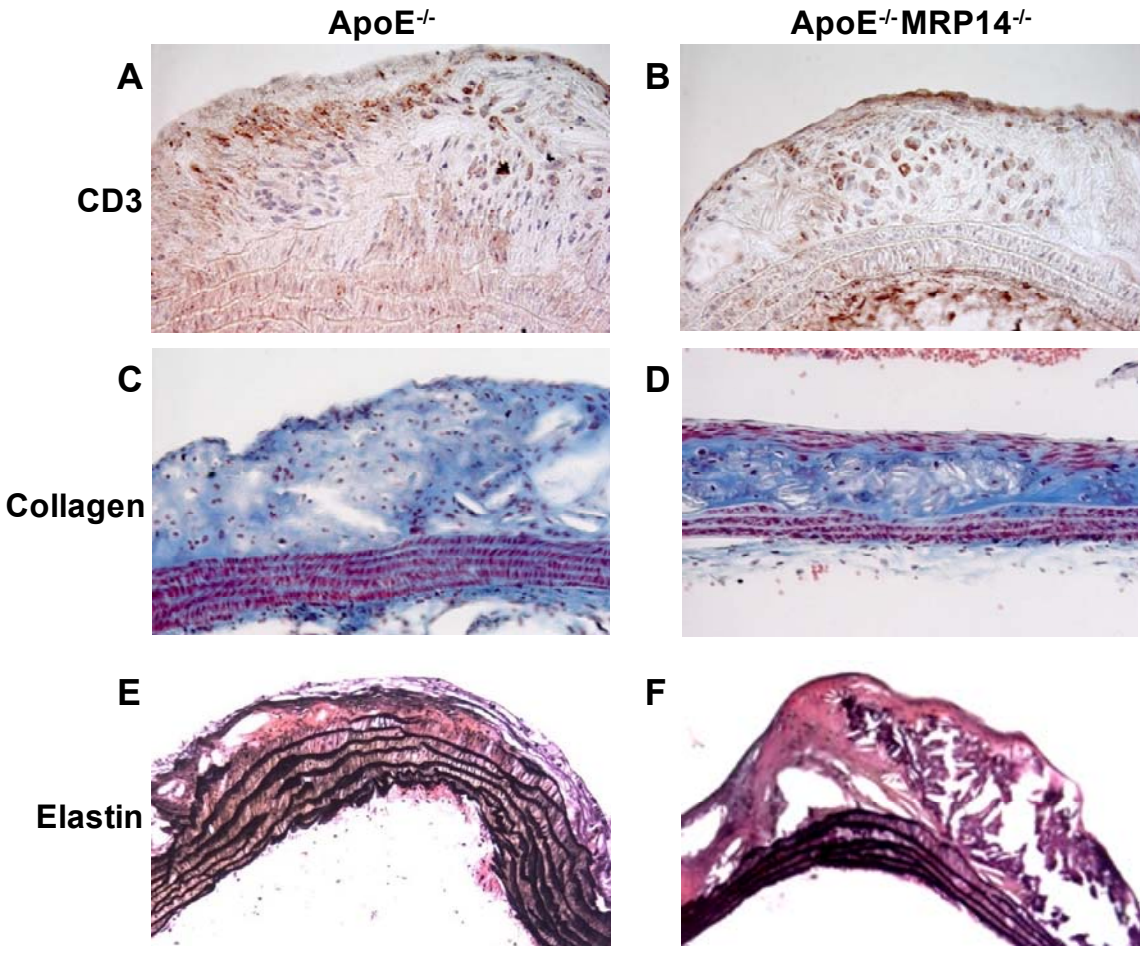
Supplemental Figure 2: Human aortic smooth muscle cell proliferation. Proliferative activity of cultured human aortic smooth muscle cells determined by MTT assay after 24 hour incubation (no addition, MRP-8, 2μg/ml, MRP-14, 2μg/ml, PDGF, 10ng/ml). Representative experiment, mean ± standard deviation, n= 3.

Supplemental Figure 3



Supplemental Figure 3: Plasma MRP-14 increases during high-fat diet-induced atherosclerosis. WT or ApoE$^{-/-}$ mice were maintained on regular chow or high-fat diet for 12 weeks, and plasma MRP-14 content was assessed by immunoblotting. Representative blot is shown in which equal amounts of plasma from individual mice (2 per treatment group) were added to each well.

Supplemental Figure 4



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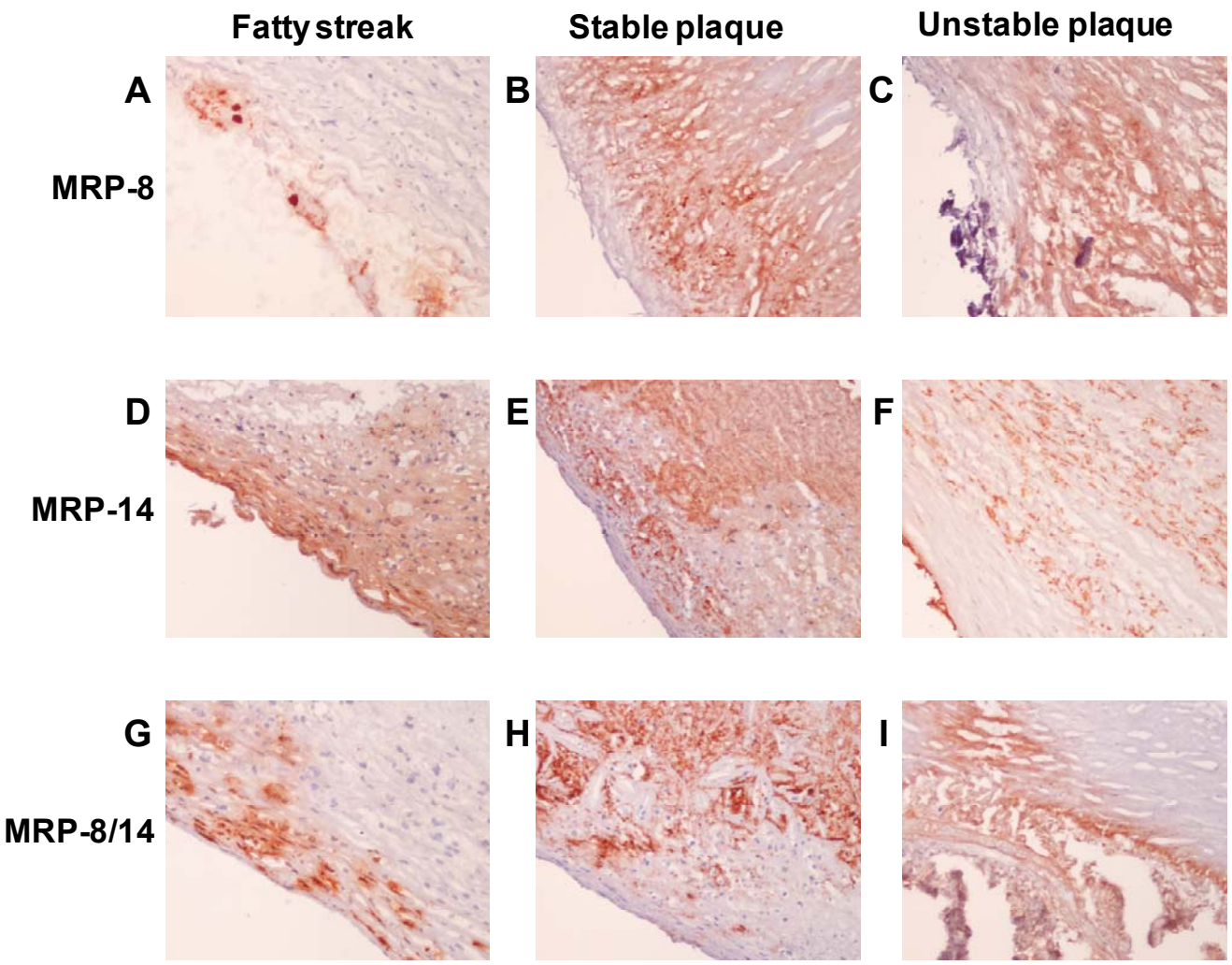
Quantitative Histology of Atheroma

	ApoE$^{-/-}$	ApoE$^{-/-}$MRP-14$^{-/-}$	P-value
% CD3-positive area	9.1±3.3	7.7±2.8	0.14
% Collagen-positive area	32.4±11.5	25.9±11.5	0.10
% Elastin-positive area	24.7±11.9	26.8±9.1	0.56

Supplemental Figure 4: T cell, collagen, and elastin staining of atherosclerotic plaques. Analysis of atherosclerotic plaques from the aortas of ApoE$^{-/-}$ and ApoE$^{-/-}$MRP-14$^{-/-}$ mice after 20 weeks of high-fat feeding. **A,B,** T cell staining for CD3. **C,D,** Collagen staining. **E,F,** Elastin staining. **G,** Table of % CD3-positive area, % collagen-positive area, % elastin-positive area from ApoE$^{-/-}$ and ApoE$^{-/-}$MRP-14$^{-/-}$ mice. Analysis of n=9-14 sections per data point. Data are presented as mean ± standard deviation.

Supplemental Figure 5



Supplemental Figure 5: MRP-8/14 expression in human carotid fatty streaks, stable, and unstable atherosclerotic plaques. A,B,C, Representative MRP-8 staining of carotid fatty streaks (n=6), stable plaques (n=6), and unstable plaques (n=4). **D,E,F,** Representative MRP-14 staining of carotid fatty streaks (n=6), stable plaques (n=6), and unstable plaques (n=4). **G,H,I,** Representative MRP-8/14 staining of carotid fatty streaks (n=6), stable plaques (n=6), and unstable plaques (n=4).

Supplemental Methods:

Femoral Artery Wire Injury

Bilateral wire injury (0.010 in) of the femoral artery was performed as described previously [13, 14]. Seven or 28 days after vascular injury, anesthesia was administered, and a 22-gauge butterfly catheter was inserted into the left ventricle for in situ pressure perfusion at 100 mm Hg with heparinized (20U/mL) 0.9% saline for 1 minute followed by fixation with 4% paraformaldehyde in 0.1 mol/L phosphate buffer, for 10 minutes. All animals received 5-bromo-2-deoxyuridine (BrdU) (50 mg/kg IP) 18 hours and 1 hour before euthanasia. The right and left femoral arteries were excised, paraffin embedded, and 2 cross sections were stained with hematoxylin and eosin (H&E) and Verhoeff tissue elastin stain. A histologist blinded to genotype measured the luminal, intimal, and medial areas of each cross-sectional plane using a microscope equipped with a CCD camera (Zeiss AxioCam MRc5) interfaced to a computer running NIH Image.

Morphometry results for each mouse were averaged (WT: n=9 mice; MRP-$14^{-/-}$: n=7 mice). For immunohistochemistry, standard avidin-biotin procedures were used for staining with antibodies against MRP-8 and MRP-14 (R&D Systems, Minneapolis, MN), CD45 (BD Biosciences, San Diego, Calif), mouse macrophage-specific marker Mac-3 (mAb M3/84, BD Biosciences), anti-neutrophil mAb 7/4 (AbD Serotec, North Carolina), mouse platelet-specific marker glycoprotein IIb (GPIIb)(BD Biosciences), and BrdU (DAKO, Carpinteria, Calif). Immunostained sections were developed with the Cytomation ARK Animal Research Kit (Dako) or with 3-amino-9-ethylcarbazole substrate, and sections were counterstained with Harris

hematoxylin solution. For CD45, Mac-3 7/4, and GPIIb, staining was quantified as the percent positive staining area. For BrdU staining was quantified as the number of immunostained positive cells per total number of nuclei. Images were captured using a microscope equipped with a CCD camera (Zeiss AxioCam MRc5). Computer-assisted imaging analysis (Zeiss Axiovision software Rel 4.5) was done by a histologist blinded to genotype. Percent-positive area was defined as the fraction of immunopositive staining to total area measured.

Human aortic SMC culture, mouse aortic SMC culture, and SMC MTT proliferation assay:

Human aorta specimens were obtained from autopsy using protocols approved by the Human Investigation Review Committee at the Brigham and Women's Hospital. Primary human aortic SMCs (huAoSMCs) were isolated and cultured in DMEM supplemented with 10% fetal bovine serum (FBS)(Hyclone). Cells were split into 96 well plates and rendered quiescent by overnight culture in serum-free medium prior to stimulation. HuAoSMCs were incubated with PDGF (10ng/ml)(Cell Signaling) or purified MRP-8- or MRP-14-gst fusion proteins (2µg/ml) (Novus Biologicals) for 24 hours. Cell proliferative activity was measured by MTT assay (ATCC).

Primary mouse aortic smooth muscle cells (muAoSMC) were isolated from WT or MRP-$14^{-/-}$ mice as described previously [42, 43]. After the adventitia was carefully removed, aortas were chopped and digested with type I collagenase (1 mg/mL, Worthington) and elastase III (0.125 mg/mL, Sigma). Culture and passage was done using DMEM with 20% FBS. MuAoSMCs were split into 96 well plates and rendered quiescent by overnight culture in serum-free medium

prior to stimulation with PDGF for 14 hours, and measurement of proliferative activity by MTT assay (ATCC).

Vasculitis Experiments: Local Shwartzman Reaction

Induction of vasculitis (the local Shwartzman reaction) was performed as described previously [15]. Briefly, male mice aged 6-10 week-old were used. On day 1, the hair on the back of the mice was removed with electric clippers and 18 hours later, 200 μg lipopolysaccharide (LPS) was injected subcutaneously into the shaved site. Twenty four hours after the LPS injection, 300ng TNF-α was injected in the same site to enhance vasculitic lesion formation. On day 4, mice were euthanized and skin tissue was harvested for immunohistochemical analysis.

Assessment of lesions. Semi-quantitative macroscopic grading of skin lesions was performed by an investigator blinded to genotype prior to harvesting tissue to generate a lesion hemorrhage score. Lesions were divided into 4 quadrants and were graded on the presence (+1) or absence of hemorrhage (0) in each quadrant (n=13 mice per group).

For microscopic analysis of lesions, the skin was excised and paraffin sections were made. MRP-8 and MRP-14 staining was done using standard avidin-biotin procedures and immunostained sections were developed with 3-amino-9-ethylcarbazole substrate followed by counterstaining with Harris hematoxylin solution. For quantification of % hemorrhage area, sections were H&E stained and four consecutive fields were captured at 40× magnification using a microscope equipped with a CCD camera (Zeiss AxioCam MRc5). The images were

analyzed using Zeiss Axiovision software (Rel 4.5). The extravascular red blood cell signal in H&E-stained sections indicated hemorrhage while the red signal inside arteries and veins was excluded. Percent hemorrhage area was defined as the fraction of extravascular red signal area to total area measured.

For neutrophil staining, immunohistochemistry was performed using the anti-neutrophil mAb 7/4 (AbD Serotec), and slides were developed with the Cytomation ARK Animal Research Kit (Dako). Four consecutive fields were captured at 20x magnification and neutrophil accumulation assessed by determining mAb 7/4-positive area using computer-assisted imaging analysis, as described above.

Western Blot Analysis of MRP-14 in the Plasma of Atherosclerotic Mice

WT or ApoE$^{-/-}$ mice were maintained on regular chow or high-fat diet for 12 weeks, and plasma MRP-14 content was assessed by immunoblotting by standard methods. Equal amounts of plasma from individual mice (2 per treatment group) were added to each well and the immunoblot was developed with goat anti-mouse MRP-14 antibody (R&D Systems).

Atherosclerosis Experiments: ApoE Model of High-Fat Diet-induced Atherosclerosis

To induce atherosclerosis, 8-week-old male ApoE$^{-/-}$ (n=22) or doubly deficient ApoE$^{-/-}$ MRP-14$^{-/-}$ (n=20) mice consumed a high-fat diet (Clinton/Cybulsky Rodent Diet D12108 with 1.25% cholesterol, Research Diets New Brunswick, NJ) for 20 weeks. Plasma lipid levels were

determined using commercial kits for total cholesterol, triglycerides, HDL, and LDL (Wako). At the time of harvest, mice were euthanized by CO_2 asphyxiation prior to in-situ perfusion fixation as described above.

The aortas were cleaned of excess adventitial tissue, and then stained in 0.5% Sudan IV (Sigma) for 15 minutes. After staining, aortas were differentiated in 80% ethanol prior to placement in buffered solution. Aortas were analyzed both en face (closed) and after they were opened longitudinally and pinned (opened). Images of Sudan IV-stained aortas were taken with a digital camera and lesion area quantified using computer-assisted imaging analysis with Zeiss Axiovision software (Rel 4.5). Macrophage (Mac-3-positive cells) or T cell (CD3-positive cells) accumulation in atherosclerotic lesions was assessed in the descending aorta and the aortic arch (lesser curvature) using 5µm serial sections from longitudinally embedded aortas. Percent-positive area was defined as the fraction of immunopositive staining to total area measured.

Isolation of Thioglycolate-elicited peritoneal macrophages and in vitro cytokine production:

Macrophages were obtained from the peritoneal cavity of WT or MRP-$14^{-/-}$ mice as described previously [44]. Briefly, peritonitis was induced in 12-week-old mice by intraperitoneal injection of 1 mL sterile 4% (wt/vol) thioglycolate broth. Forty eight hours later, peritoneal cavities were lavaged by injecting 10 mL sterile PBS buffer twice with gentle abdominal massage. Macrophages were enriched after brief adhesion to tissue culture plastic to remove nonadherent lymphocytes, resulting in more than 95% macrophages. Adherent macrophages

were rendered quiescent by overnight culture in low serum medium prior to stimulation with 5μg/mL E coli LPS for 18h at 37°C. TNF-α ,MCP-1, IL-1β,and IL-12 in cultured supernatants were quantified by immunoassay with Quantikine ELISA kits (R&D Systems, Minneapolis, MN).

Isolation of peripheral blood monocytes and transwell migration assay:

Mouse monocytes were isolated from peripheral blood (1.5 mL per mouse via direct inferior vena caval puncture) by Ficoll-Hypaque centrifugation followed by adhesion to tissue culture plastic to remove nonadherent lymphocytes. Cell migration was evaluated using 24-well transwell plates (Costar) with polycarbonate membrane containing 5-μm pores. Peripheral blood monocytes ($2x10^5$) were added to the upper chamber of each well. The lower chamber contained 10 nM MCP-1 (R&D Systems). Transwell plates were then incubated at 37°C for 90 minutes, polycarbonate membranes removed, and the under surface of the membrane rinsed with trypsin/EDTA for the determination of the number of migrated cells in the lower chamber.

Macrophage Uptake of Acetylated and Oxidized LDL

Macrophages were obtained from the peritoneal cavity of WT or MRP-$14^{-/-}$ mice as described above. To assess acetylated LDL uptake, adherent marcophages were incubated with 50μg/ml of fluorescent DiO-acetylated LDL for 4 hours (DiO-acLDL)(Biomedical Technologies Inc., Stoughton, MA). Following incubation, cells were washed with PBS, and DiO-acLDL uptake was measured using a fluorescent plate reader.[45] To assess oxidized LDL uptake, macrophages

were incubated with 50µg/ml of oxidized-LDL (oxLDL)(Biomedical Technologies Inc., Stoughton, MA) for 12 hours. Following incubation, cells were washed with PBS and then treated with 0.3% oil red O stain for 20 minutes. Cells were washed again with PBS followed by lipid extraction with isopropanol. Lipid content of isopropanol extracts was determined by measuring absorbance at 500nm.

Analysis of MRP-8/14 Expression in Human Carotid Atheroma

Surgical specimens of human carotid plaques from endarterectomies as well as nonatherosclerotic arteries (carotids from autopsies) were obtained by protocols approved by the Human Investigation Review Committee at the Brigham and Women's Hospital as described previously [46]. We used well-established criteria of plaque vulnerability[47]. Plaques with a minimal fibrous cap (FC) thickness ≥0.8 mm and an immunohistochemically determined positive area ≥10% for SMCs and ≤10% for macrophage and lipid content were designated as stable. In contrast, lesions with minimal FC thickness ≤0.3 mm, ≤10% positive area for SMC, and ≥20% positive area for macrophage and lipid content were designated as unstable. The tissue samples were embedded in OCT and serial fresh-frozen sections were fixed with 4% paraformaldehyde. Immunohistochemical staining used the avidin-biotin-peroxidase method. The reaction was visualized with 3-amino-9-ethylcarbazole substrate and sections were counterstained with Harris hematoxylin solution. Staining was done with antibodies to detect MRP-8, MRP-14 or MRP-8/14 (heterodimer complex specific antibody 27E10)(all from BMA Biomedical), and macrophages were identified with anti-CD68 antibody (Dako). Tissue sections

were viewed with a microscope (Nikon Eclipse 50i, Tokyo, Japan), and images were digitally captured with a CCD–SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, Mich).