Myeloid-Related Protein-8/14 Is Critical for the Biological Response to Vascular Injury

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Background—Myeloid-related protein (MRP)-8 (S100A8) and MRP-14 (S100A9) are members of the S100 family of calcium-modulated proteins that regulate myeloid cell function and control inflammation, in part, through activation of Toll-like receptor-4 and the receptor for advanced glycation end products. A transcriptional profiling approach in patients with acute coronary syndromes identified MRP-14 as a novel predictor of myocardial infarction. Further studies demonstrated that elevated plasma levels of MRP-8/14 heterodimer predict increased risk of first and recurrent cardiovascular events. Beyond its serving as a risk marker, whether MRP-8/14 participates directly in vascular inflammation and disease remains unclear.

Methods and Results—We evaluated vascular inflammation in wild-type and MRP-14−deficient (MRP-14−/−) mice that lack MRP-8/14 complexes with experimental arterial injury, vasculitis, or atherosclerosis. After femoral artery wire injury, MRP-14−/−mice had significant reductions in leukocyte accumulation, cellular proliferation, and neointimal formation compared with wild-type mice. In a cytokine-induced local Shwartzman-like reaction that produces thrombohemorrhagic vasculitis, MRP-14−/−mice had significant reductions in neutrophil accumulation, lesion severity, and hemorrhagic area. In response to high-fat feeding, mice doubly deficient in apolipoprotein E and MRP-8/14 complexes had attenuation in atherosclerotic lesion area and in macrophage accumulation in plaques compared with mice deficient in apolipoprotein E alone.

Conclusion—This study demonstrates that MRP-8/14 broadly regulates vascular inflammation and contributes to the biological response to vascular injury by promoting leukocyte recruitment. (Circulation. 2009;120:427-436.)

Key Words: atherosclerosis • inflammation • leukocytes • restenosis • vasculitis

A wealth of experimental and clinical research supports the premise that inflammation plays an important role in hyperplastic arterial diseases (eg, neointimal hyperplasia and restenosis after coronary intervention and cardiac allograft vasculopathy), in vasculitis, and in the initiation, progression, and complications of atherosclerosis.1 The pathobiology of arterial disease begins with an inciting injury that activates inflammatory responses from well-characterized cellular and molecular regulatory pathways. Vascular inflammation involves complex heterotypic interactions between endothelial cells, platelets, and inflammatory cells, including neutrophils, monocytes, lymphocytes, and mast cells.2 Under normal circumstances, the cellular and molecular processes that control vascular injury responses direct repair and vascular healing. In pathological conditions, however, dysregulation of inflammatory responses results in persistent vascular inflammation and adverse arterial remodeling and contributes to the development of clinical vascular diseases.

Clinical Perspective on p 436

Our previous study used a transcriptional profiling strategy to identify novel regulators of vascular inflammation and atherothrombosis by examining platelet mRNA transcripts that are differentially expressed in patients with ST-segment elevation myocardial infarction compared with stable coronary artery disease.3 Myeloid-related protein-14 (MRP-14, also referred to as S100A9) was one of the strongest candidate proteins that arose from the transcriptional profiling analysis. MRP-14 complexes with MRP-8 (S100A8), another member of the S100 family of calcium-modulated proteins; together, MRP-8 and MRP-14 regulate myeloid cell function by binding to Toll-like receptor-4 (TLR-4)4 and the receptor

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for advanced glycation end products and by modulating calcium signaling and cytoskeletal reorganization. In 2 prospective nested case-control studies, 1 in apparently healthy post-menopausal women and the other in patients presenting with acute coronary syndromes, elevated plasma levels of MRP-8/14 predicted the risk of future cardiovascular events independently of traditional cardiovascular risk factors and C-reactive protein. Elevated plasma levels of MRP-8/14 also serve as an early and sensitive marker of myocardial necrosis in the setting of chest pain.

Emerging evidence from studies using isolated cells from MRP-14-deficient (MRP-14−/−) mice strongly suggests that MRP-8 and MRP-14 regulate leukocyte migration. Despite the presence of MRP-8 mRNA transcripts, MRP-14−/− mice lack both MRP-8 and MRP-14 protein as a result of the instability of MRP-8 in the absence of MRP-14. In vitro studies with MRP-14−/− neutrophils show markedly diminished migration through endothelial monolayers and attenuated chemokinesis in a 3-dimensional collagen matrix. An essential role for MRP-14 in leukocyte recruitment in vivo is uncertain because MRP-14−/− mice have normal neutrophil migration during chemical peritonitis and interleukin-8 (IL-8)-induced skin inflammation but have diminished granulocyte recruitment during tissue wound healing and during acute pancreatitis. Recent studies using MRP-14−/− mice have demonstrated that MRP-8 and MRP-14 play a regulatory role in endotoxin-induced phagocyte function. MRP-8 and MRP-14 are endogenous regulators of TLR-4, affecting myeloid MyD88-dependent activation of nuclear factor-κB and expression of tumor necrosis factor-α (TNF-α).

Although MRP-8 and MRP-14 serve as risk biomarkers and can modulate inflammatory cell function, whether these proteins participate directly in vascular inflammation and in cardiovascular disease remains uncertain. The present study used MRP-14−/− mice to test the hypotheses that MRP-8/14 complexes contribute to neutrophil-dependent and monocyte/macrophage-dependent vascular inflammatory responses in experimental arterial injury, vasculitis, and atherosclerosis.

Methods

MRP-14−/− mice were generated in the laboratory of Dr Nancy Hogg. For the atherosclerosis experiments, MRP-14−/− mice were crossed with apolipoprotein E−deficient (ApoE−/−) mice (Jackson Laboratories, Bar Harbor, Me) to generate compound-mutant mice doubly deficient in MRP-14 and ApoE (MRP-14−/−ApoE−/−). Single-mutant ApoE−/− mice were used as controls for the atherogenesis experiments. All mice had a congenic C57BL/6 background and were maintained in animal facilities at Harvard Medical School and Case Western Reserve University School of Medicine. Animal care and procedures were reviewed and approved by the Institutional Animal Care and Use Committees and performed in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care and the National Institutes of Health.

Femoral Artery Wire Injury

Bilateral wire injury (0.010 in) of the femoral artery was performed as described previously and as detailed in the Material section of the online-only Data Supplement. Six hours, 7 days, or 28 days after vascular injury, the right and left femoral arteries were excised, embedded in paraffin, and stained with hematoxylin and eosin 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 charge-coupled device camera (Zeiss AxioCam MRc5, Oberkochen, Germany) interfaced to a computer running NIH Image. For immunohistochemistry, standard avidin-biotin procedures were used. Percent Mac-3–positive area, percent 7/4–positive area, and percent glycoprotein lb–positive area were defined as the fraction of immunopositive staining to total area measured. Methods for human aortic smooth muscle cell (SMC) culture, mouse aortic SMC culture, and SMC MTT proliferation assays are detailed in the Material section of the online-only Data Supplement.

Vasculitis Experiments: Local Shwartzman Reaction

Induction of vasculitis (the local Shwartzman reaction) was performed as described previously and as detailed in Material section of the online-only Data Supplement. Briefly, 200 μg lipopolysaccharide was injected subcutaneously into the skin, and 24 hours after the lipopolysaccharide injection, 300 ng TNF-α was injected into the same site to enhance vasculitic lesion formation. On day 4, mice were euthanized and skin tissue was harvested for semiquantitative macroscopic grading of skin lesions and histological analysis. For immunohistochemistry, standard avidin-biotin procedures were used. The extravascular red blood cell signal in hematoxylin and eosin–stained sections indicated hemorrhages, whereas 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. Neutrophil accumulation was assessed by determining mononclonal antibody 7/4–positive area.

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/Cybulsyk Rodent Diet D12108 with 1.25% cholesterol, Research Diets, New Brunswick, NJ) for 20 weeks. As detailed in the Material section of the online-only Data Supplement, Sudan IV staining was used to quantify atherosclerotic lesion area. Macrophage (Mac-3–positive cells) accumulation in atherosclerotic lesions was assessed in the descending aorta and aortic arch (lesser curvature), and percent macrophage area was calculated by determining the Mac-3–positive area using computer-assisted imaging analysis. Methods for in vitro cytokine production and transwell assays are detailed in the Material section of the online-only Data Supplement.

Statistical Analysis

Unless otherwise noted, data are presented as the mean±SD, and statistical analysis was done with a Student t test. Values of P<0.05 were considered significant. Correlation analyses for the atherosclerosis experiments (open versus closed lesion area, macrophage staining of the descending aorta versus aortic arch) were done using the correlation function in Microsoft Excel.

Results

MRP-8/14 Complexes Regulate Neutrophil and Monocyte Recruitment and Neointimal Thickening After Femoral Artery Injury

To determine whether MRP-8/14 complexes influence arterial injury responses and neointimal formation, we performed femoral artery wire injury in wild-type (WT) and MRP-14−/− mice. Wire injury causes endothelial denudation, medial injury, and platelet and fibrin deposition and yields prominent inflammation of all arterial layers. Inflammation after wire injury involves both neutrophils and monocytes. We and others have demonstrated the critical participation of inflammatory cell recruitment in neointimal formation in wire-injured mouse arteries.
Wire injury resulted in a significant increase in MRP-8 and MRP-14 expression in the vessel wall (no injury, Figure 1A and 1B; 7 days after injury, Figure 1C and 1D). As expected, neither MRP-8 nor MRP-14 expression was detected in the injured arteries of MRP-14⁻/⁻/⁻ mice (Figure 1E and 1F). We next examined leukocyte recruitment 7 days after wire injury, the time corresponding to peak inflammatory cell infiltration in this model.¹³ Accumulation of leukocytes in the developing neointima (CD45 staining) was reduced significantly by 45% (percent CD45-positive area: WT, 13.1±7.2%; MRP-14⁻/⁻/⁻, 7.2±5.4%; P=0.028) in MRP-14⁻/⁻/⁻ compared with WT mice (Figure 2A and 2B and Table 1). We expanded the CD45 analysis by immunostaining for the macrophage-specific marker Mac-3 and the neutrophil-specific marker 7/4 (Figure 2C through 2F and Table 1). In the absence of MRP-14, macrophages accumulating in the intima (percent Mac-3-positive area: WT, 27.8±7.5%; MRP-14⁻/⁻/⁻, 16.1±9.6%; P=0.013) and invading the media (WT, 6.9±5.2%; MRP-14⁻/⁻/⁻, 1.6±1.3%; P=0.023) at 7 days fell significantly by 42% and 76%, respectively, whereas neutrophils invading the intima fell significantly by 58% (percent 7/4-positive area: WT, 20.4±9.4%; MRP-14⁻/⁻/⁻, 8.4±5.5%; P=0.046).

Because increasing evidence suggests that leukocytes play an important role in regulating cellular proliferation, we assessed cellular proliferation by quantifying incorporation of BrdU. Substantial proliferation was observed 7 days after injury in WT vessels (15.2% of intimal cells, 5.3% of medial cells). Cellular proliferation at 7 days fell significantly by 72% in the intima (WT, 15.2±5.3%; MRP-14⁻/⁻/⁻, 4.2±3.5%; P=0.001) and 80% in the media (percent BrdU-positive cells: WT, 5.3±3.6%; MRP-14⁻/⁻/⁻, 1.1±1.2%; P=0.019) in MRP-14⁻/⁻/⁻ mice compared with WT mice (Figure 2G and 2H and Table 1). MRP-14⁻/⁻/⁻ deficient mice had less neointimal formation assessed 28 days after femoral artery injury compared with WT mice (neointimal area: WT, 17 561±4984 μm²; MRP-14⁻/⁻/⁻, 12 862±1743 μm²; P=0.031; Figure 2I and 2J and Table 1). Medial area increased slightly in the absence of MRP-14 (WT, 11 060±1573 μm²; MRP-14⁻/⁻/⁻, 12 862±1743 μm²; P=0.031). MRP-14⁻/⁻/⁻ mice had a 45% lower ratio of intima to media area at 28 days compared with WT mice (WT, 1.81±0.63; MRP-14⁻/⁻/⁻, 1.00±0.34; P=0.004; Table 1). Taken to-
Method (Table 2).

The awake and conscious states as measured by the tail cuff did not appear to influence inflammatory responses in the MRp-14 deficient mice. In addition, altered hemodynamics did not affect the proliferation of human SMCs derived from WT or MRp-14−/− mice (Figure 3). Aortic SMCs from MRp-14−/− mice had significantly reduced basal and platelet-derived growth factor–stimulated proliferation in vitro (P<0.001) by ANOVA). In contrast, the addition of purified human MRp-8 and MRp-14 had no effect on the proliferation of human aortic SMCs (Figure II of the online-only Data Supplement). Taken together, these data likely indicate a role for intracellular MRp-8/14 action (calcium transients, cytoskeletal reorganization, arachidonic acid metabolism) in the regulation of SMC proliferation.

### Table 1. Quantitative Morphometry and Immunohistochemistry

<table>
<thead>
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<th></th>
<th>WT</th>
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<tbody>
<tr>
<td>CD45-positive area</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>at 7 d, %</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Intima</td>
<td>13.1±7.2</td>
<td>7.2±5.4</td>
<td>0.028</td>
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<tr>
<td>Media</td>
<td>4.8±8.0</td>
<td>1.4±2.3</td>
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<tr>
<td>Mac-3–positive area</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>at 7 d, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intima</td>
<td>27.8±7.5</td>
<td>16.1±9.6</td>
<td>0.013</td>
</tr>
<tr>
<td>Media</td>
<td>6.9±5.2</td>
<td>1.6±1.3</td>
<td>0.023</td>
</tr>
<tr>
<td>7/4-Positive area at</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 d, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intima</td>
<td>20.4±9.4</td>
<td>8.4±5.5</td>
<td>0.046</td>
</tr>
<tr>
<td>Media</td>
<td>13.6±7.8</td>
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<tr>
<td>BrdU-positive cells</td>
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<tr>
<td>at 7 d, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intima</td>
<td>15.2±5.4</td>
<td>4.2±3.5</td>
<td>0.001</td>
</tr>
<tr>
<td>Media</td>
<td>5.3±3.6</td>
<td>1.1±1.2</td>
<td>0.019</td>
</tr>
<tr>
<td>Intimal area at 28 d, μm²</td>
<td>17 561±4084</td>
<td>12 252±4610</td>
<td>0.031</td>
</tr>
<tr>
<td>Media area at 28 d, μm²</td>
<td>11 060±1573</td>
<td>12 862±1743</td>
<td>0.031</td>
</tr>
<tr>
<td>Intima-to-media ratio</td>
<td>1.81±0.63</td>
<td>1.00±0.34</td>
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<tr>
<td>External elastic</td>
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<tr>
<td>lamina area at 28 d, μm²</td>
<td>47 585±6719</td>
<td>48 885±9752</td>
<td>0.73</td>
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</tbody>
</table>

Morphometry: WT, n=9 mice; MRp-14−/−, n=7 mice. Immunohistochemistry: analysis of n=9 to 14 sections per data point. Data are presented as mean±SD.

Together, these observations indicate that MRp-8/14 complexes modulate neutrophil- and monocyte-dependent vascular inflammation and neointimal thickening after experimental mechanical arterial injury and suggest that MRp-8/14 complexes modulate cellular proliferation that is stimulated by platelets and/or leukocytes in this setting. Importantly, differences in neointimal formation between WT and MRp-14−/− mice did not appear to be related to altered platelet deposition; WT and MRp-14−/− mice showed similar staining for the platelet marker glycoprotein IIb 6 hours after injury (P=0.96) (Figure I of the online-only Data Supplement). In addition, altered hemodynamics did not appear to influence inflammatory responses in the MRp-14−/− mice because WT and MRp-14−/− mice had similar systolic, diastolic, and mean arterial pressures in the awake and conscious states as measured by the tail cuff method (Table 2).

Vascular SMCs are known to express MRp-14. To examine the role of MRp-8/14 in SMC proliferation relevant to vascular injury and restenosis, we isolated SMCs from the aortas of WT and MRp-14−/− mice and examined basal and platelet-derived growth factor–stimulated proliferation in vitro by MTT assay (Figure 3). Aortic SMCs from MRp-14−/− mice had significantly reduced basal and platelet-derived growth factor–stimulated proliferation in vitro (P<0.0001 by ANOVA). In contrast, the addition of purified human MRp-8 and MRp-14 had no effect on the proliferation of human aortic SMCs (Figure II of the online-only Data Supplement). Taken together, these data likely indicate a role for intracellular MRp-8/14 action (calcium transients, cytoskeletal reorganization, arachidonic acid metabolism) in the regulation of SMC proliferation.

### Table 2. Blood Pressure Measurements

<table>
<thead>
<tr>
<th>Pressure</th>
<th>WT (n=8)</th>
<th>MRp-14−/− (n=8)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic, mm Hg</td>
<td>99.6±2.3</td>
<td>98.5±4.7</td>
<td>0.53</td>
</tr>
<tr>
<td>Diastolic, mm Hg</td>
<td>71.9±7.1</td>
<td>72.5±8.1</td>
<td>0.87</td>
</tr>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>81.1±4.8</td>
<td>81.2±6.1</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD. Measurements were made on age- and sex-matched mice that were 12 weeks of age.

MRp-8/14 Complexes Regulate Neutrophil-Dependent Thromboendothelial Vasculopathy

Next, we examined the role of MRp-8/14 complexes in cytokine- and neutrophil-dependent inflammation. MRp-14 regulates neutrophil functions in vitro, and neutrophil-derived cytotoxic products play an important role in cytokine-induced inflammation of the vessel wall. The local Shwartzman-like reaction (LSR), induced by successive lipopolysaccharide and cytokine injections into the skin, produces a thromboendothelial vasculitis. Consistent with prior reports, flow cytometry demonstrated equivalent expression of the lipopolysaccharide receptor TLR-4 on bone marrow–derived and thioglycolate-elicited peritoneal macrophages harvested from WT and MRp-14 mice (data not shown). The LSR depends on neutrophils and their interaction with activated platelets and endothelial cells. Neutrophil-derived microparticles promote the accumulation of fibrin, generation of occlusive thrombi, and eventual hemorrhage from inflamed blood vessels. The LSR produces hemorrhage in the intact skin 24 hours after TNF-α injection. MRp-14−/− mice subjected to LSR exhibited a significant reduction in the development of hemorrhagic lesions (n=13 mice per group; Figure 4A). In analysis blinded to genotype, MRp-14−/− mice had a 59% reduction in lesion severity (lesion hemorrhage score: WT, 3.1±1.52; MRp-14−/−, 1.24±0.91; P<0.0001; Figure 4A and 4B). Histopathology of vasculitic lesions demonstrated that the
As expected, neither MRP-8 nor MRP-14 was detected in the expression compared with normal skin (Figure 5A through 5D). We examined whether plasma levels of MRP-14 in events related to atherosclerotic plaque rupture and thrombosis, we quantified by computer-assisted imaging analysis (WT, 22.5 ± 5.4% of normal skin from a WT mouse. C and D, MRP-8 or MRP-14 immunostaining of vasculitic lesion from a WT mouse. E and F, MRP-8 or MRP-14 immunostaining of vasculitis from an MRP-14−/− mouse. G and H, Hematoxylin and eosin staining of representative vasculitic lesions demonstrating hemorrhage (original magnification ×40). I, Quantification of percent hemorrhage area. J and K, Immunohistochemical staining for neutrophils in vasculitic lesions (monoclonal antibody 7/4–positive cells) (×20). L, Quantification of percent neutrophil-positive area. Data represent mean ± SD; n = 13 mice per group. LPS indicates lipopolysaccharide.

Figure 5. Photomicrographs of skin lesions from WT and MRP-14−/− mice during cytokine-induced thrombohemorrhagic vasculitis. A and B, MRP-8 or MRP-14 immunostaining of normal skin of a WT mouse. C and D, MRP-8 or MRP-14 immunostaining of vasculitic lesion from a WT mouse. E and F, MRP-8 or MRP-14 immunostaining of vasculitic lesion from an MRP-14−/− mouse. G and H, Hematoxylin and eosin staining of representative vasculitic lesions demonstrating hemorrhage (original magnification ×40). I, Quantification of percent hemorrhage area. J and K, Immunohistochemical staining for neutrophils in vasculitic lesions (monoclonal antibody 7/4–positive cells) (×20). L, Quantification of percent neutrophil-positive area. Data represent mean ± SD; n = 13 mice per group. LPS indicates lipopolysaccharide.

Figure 4. Deficiency of MRP-14 attenuates lesion severity during cytokine-induced thrombohemorrhagic vasculitis. A, Necropsy images showing representative vasculitis lesions in WT and MRP-14−/− mice. B, Scoring of vasculitic lesions from WT and MRP-14−/− mice. C, Quantification of skin thickness (dermis plus epidermis) from histological section of vasculitic lesions. Data represent mean ± SD; n = 13 mice per group.

Histological analysis of skin tissue affected by the LSR revealed a significant increase in MRP-8 and MRP-14 expression compared with normal skin (Figure 5A through 5D). As expected, neither MRP-8 nor MRP-14 was detected in vasculitic lesions from MRP-14−/− mice (Figure 5E and 5F). The vasculitic lesions had substantial erythrocyte extravasation, edema, neutrophil accumulation, and occlusive thrombi containing neutrophils (Figure 5G through 5L). MRP-14−/− mice had an 82% reduction in hemorrhagic area, as assessed by computer-assisted imaging analysis (WT, 22.5 ± 4.5%; MRP-14−/−, 4.1 ± 5.9%; P < 0.015; Figure 5G through 5L). Neutrophil (monoclonal antibody 7/4–positive cells) accumulation in the vascular wall and skin also fell by 45% in the MRP-14−/− mice (percent neutrophil area: WT, 14.4 ± 2.0%; MRP-14−/−, 7.8 ± 1.5%; P = 0.013; Figure 5J through 5L).

These data indicate that in the LSR, MRP-8/14 complexes participate in neutrophil-dependent tissue injury, leading to the development of thrombohemorrhagic vasculitis.

MRP-8/14 Complexes Modulate Monocyte/Macrophage-Dependent Atherosclerotic Lesion Formation

Mice with experimental atherosclerosis, a process driven by monocyte recruitment and function, were used in examinations of the role of MRP-8/14 in monocyte/macrophage-dependent vascular inflammation.19–21 Because we have shown that plasma levels of MRP-8/14 predict cardiovascular events related to atherosclerotic plaque rupture and thrombosis,3,8 we examined whether plasma levels of MRP-14 increase during the development of atherosclerosis in mice. WT and atherosclerosis-prone ApoE−/− mice were started on an atherogenic diet at 8 weeks of age, and 12 weeks later, plasma levels of MRP-14 were assessed by immunoblotting. After 12 weeks of consumption of the atherogenic diet, ApoE−/− mice developed atherosclerotic lesions in the aorta (data not shown). Immunoblot analysis of MRP-14 expression demonstrated more MRP-14 in the plasma of ApoE−/− mice fed a normal chow diet compared with WT controls (Figure III of the online-only Data Supplement). In addition, ApoE−/− mice fed the atherogenic diet had further increases in MRP-14 levels compared with ApoE−/− mice that were maintained on normal chow (Figure III of the online-only Data Supplement).

We generated compound-mutant mice deficient in both ApoE and MRP-14 (ApoE−/− MRP-14−/−) and examined the development of atherosclerotic lesions in doubly deficient ApoE−/− MRP-14−/− compared with singly deficient ApoE−/− mice. Mice consumed an atherogenic diet from 8 weeks of age, and the thoracic and abdominal aortas were harvested at 20 weeks for atherosclerotic lesion analysis. Importantly, plasma lipid profiles did not differ significantly between ApoE−/− and ApoE−/− MRP-14−/− mice on an atherogenic diet (Table 3). ApoE−/− MRP-14−/− mice had a 34% reduction in lesion area in en face (closed) analysis of Sudan IV–stained thoracoabdominal aortas at 20 weeks (ApoE−/−, 44.0 ± 11.0%; ApoE−/− MRP-14−/−, 29.0 ± 13.2%; n = 9 per group; P = 0.023) and a 25% reduction in lesion area when the aortas were opened longitudinally, pined, and analyzed (ApoE−/−, 28.1 ± 9.8%; ApoE−/− MRP-14−/−, 21.2 ± 9.0%; n = 22 per group; P = 0.021; Figure 6A and 6B).
We examined plaque inflammation by staining atherosclerotic lesions for the macrophage-specific marker Mac-3. Plaque inflammation fell significantly in ApoE-/−MRP-14-/− mice in both the descending aorta atheroma (percent macrophage area: ApoE-/−, 21.6±13.3%; ApoE-/−MRP-14-/−, 8.7±2.9%; n=9 per group; P=0.021; Figure 6C, 6D and 6E) and the aortic arch atheroma (ApoE-/−, 17.1±6.6%; ApoE-/−MRP-14-/−, 7.3±2.8%; n=9 per group; P=0.0025; Figure 6C, 6F and 6G). Additional analyses demonstrated no significant differences in T-cell accumulation (CD3-positive cells), collagen content, or elastin content in atherosclerotic lesions from ApoE-/− and ApoE-/−MRP-14-/− mice (Figure IV of the online-only Data Supplement). These observations indicate that MRP-8/14 complexes promote monocyte/macrophage recruitment, plaque inflammation, and the development of atherosclerotic lesions in ApoE-/− mice.

To specifically examine the role of MRP-8/14 in the regulation of macrophage cytokine production relevant to atherosclerosis, we evaluated the production of atherogenic cytokines by cultured macrophages derived from WT and MRP-14-/− mice. Twelve-hour stimulation; representative experiment of n=3 per cytokine. A, TNF-α, MCP-1, IL-1β, and IL-12 production. B, Transwell assay of MCP-1-stimulated peripheral blood mononuclear cell migration. HPF indicates high-power field.

Table 3. Plasma Lipid Levels

<table>
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<tr>
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<th>ApoE-/−</th>
<th>ApoE-/−MRP-14-/−</th>
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<tbody>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>1168±200</td>
<td>1325±183</td>
<td>0.12</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>506±80.0</td>
<td>527±101</td>
<td>0.46</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>24.2±7.9</td>
<td>18.5±7.9</td>
<td>0.07</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>145±54</td>
<td>117±37</td>
<td>0.12</td>
</tr>
</tbody>
</table>

LDL indicates low-density lipoprotein; HDL, high-density lipoprotein. Data are presented as mean±SD. Measurements were made on mice that were fed a high-fat diet for 9 to 12 weeks (n=10 to 15 mice).

Figure 6. Deficiency of MRP-14 attenuates atherosclerotic lesion formation and plaque inflammation. A, Sudan IV staining of intact closed and longitudinally opened and pinned thoracoabdominal aortas harvested from ApoE-/− and ApoE-/−MRP-14-/− mice after 20 weeks of high-fat feeding. B, Quantification of percent lesion area as assessed by Sudan IV staining and computer-assisted imaging analysis. C, Quantification of plaque macrophage content (percent Mac-3-positive area) in longitudinal sections from the descending aorta and the lesser curvature of the aortic arch. D through G, Representative photomicrographs staining for macrophages (Mac-3-positive cells) within atherosclerotic lesions in the descending aorta (D, E) and aortic arch (F, G) (original magnification ×20). For paired samples in the atherosclerotic lesion area analysis, comparison of the percent lesion area in closed versus open analysis resulted in a correlation value of 0.63. For macrophage staining, comparison of the percent macrophage area in the descending aorta versus the aortic arch resulted in a correlation value of 0.41.

Figure 7. MRP-8/14 regulates macrophage cytokine production and migration. ELISA analysis of lipopolysaccharide-induced (5 μg/mL) cytokine production by cultured macrophages derived from WT and MRP-14-/− mice. Twelve-hour stimulation; representative experiment of n=3 per cytokine. A, TNF-α, MCP-1, IL-1β, and IL-12 production. B, Transwell assay of MCP-1-stimulated peripheral blood mononuclear cell migration. HPF indicates high-power field.
genic cytokine production, macrophages derived from MRP-14<sup>-/-</sup> mice had a 37% reduction in MCP-1–stimulated migration in transwell margination assays (WT, 61.2±14.9 cells per high-power field; MRP-14<sup>-/-</sup>, 38.7±14.0 cells per high-power field; P<0.0001; Figure 7B). Analysis of macrophage lipid uptake and foam cell formation in vitro by thioiglycolate-elicited macrophages from WT and MRP-14<sup>-/-</sup> mice demonstrated equivalent uptake of fluorescently labeled acetyl low-density lipoprotein (WT, 4460±248 relative fluorescence units; MRP-14<sup>-/-</sup>, 4587±728 relative fluorescence units at 48 hours; P=0.87) and similar uptake of oxidized low-density lipoprotein measured by Oil Red O (WT, 0.317±0.041 optical density; MRP-14<sup>-/-</sup>, 0.294±0.009 optical density at 48 hours; P=0.44). Taken together, these data demonstrate that MRP-8/14 influences the production of key cytokines involved in the pathobiology of restenosis, atherosclerosis, and atherosclerosis and that MRP-8/14 regulates cell migratory function in response to MCP-1, which is a critical mediator of vascular inflammation.

**Human Carotid Atheroma Contain MRP-8/14**

To begin to investigate the relevance of MRP-8/14 in human disease, we examined the expression of MRP-8, MRP-14, and MRP-8/14 in normal human carotid arteries and in carotid atheroma. Carotid atheroma, but not normal arteries (n=5), contain abundant MRP-8, MRP-14, and heterodimeric MRP-8/14 (identified through the use of the heterodimer complex–specific antibody 27E10) (Figure 8A through 8L). Analysis of adjacent tissue sections demonstrated that MRP-8, MRP-14, and MRP-8/14 staining is largely associated with lesional CD68-positive macrophages (×40 serial sections in Figure 8B, 8E, 8H, and 8K). MRP-8, MRP-14, and MRP-8/14 were expressed robustly in all types of atherosclerotic lesions, including fatty streaks (n=6 samples), SMC-rich atheroma with stable features (n=6), and inflammatory cell–rich atheroma with thin caps and features of plaque instability (n=4; Figure V of the online-only Data Supplement).

**Discussion**

This study shows that deficiency of MRP-8 and MRP-14 reduces neutrophil- and monocyte-dependent vascular inflammation and attenuates the severity of diverse vascular injury responses in vivo. These observations establish that MRP-8 and MRP-14 participate importantly in the biological response to vascular injury.

Analyses of mice that lack MRP-8 and MRP-14 have provided important insights into the function of these proteins. Although the homozygous deletion of MRP-8 results in embryonic lethality, deletion of the MRP-14 gene does not affect viability and results in the additional loss of MRP-8 protein. Failure to produce mature MRP-8 protein in the presence of normal MRP-8 mRNA production likely results from the instability of MRP-8 in the absence of MRP-14. Thus, the MRP-14<sup>−/−</sup> mice used in this and previous studies lack MRP-8 and MRP-14 protein and MRP-8/14 complexes.

Recent observations in MRP-14<sup>-/-</sup> mice indicate that MRP-8/14 is essential for lipopolysaccharide-induced septic shock, neutrophil-dependent *Staphylococcus aureus* killing, and neutrophil infiltration in acute pancreatitis; however, a direct role for MRP-8 and MRP-14 in vascular inflammation and in the pathophysiology of vascular disease was heretofore unrecognized. Prior studies examining MRP-8/14 in vascular inflammation found high MRP-8/14 levels in neutrophils in large-vessel vasculitis and in macrophages and foam cells in human and mouse atherosclerotic lesions. The 3 experimental forms of vascular inflammation used in our study depend variably on neutrophils (vasculitis), monocyte/macrophages (atherosclerosis), or their combination (arterial wire injury). This study shows that MRP-8 and MRP-14 promote the recruitment of inflammatory cells and tissue injury, as assessed by neointimal formation (wire injury), hemorrhage area (vasculitis), and atherosclerotic plaque area (atherosclerosis).

Our data strongly suggest that MRP-8/14 influences vascular inflammation and the development of vascular disease through multiple mechanisms that include regulation of vascular SMC proliferation, regulation of MCP-1-stimulated monocyte migration, and regulation of the production of key cytokines (TNF-α, MCP-1, IL-1β, and IL-12). A particularly notable feature of the phenotype of MRP-14<sup>−/−</sup> mice in all 3 disease models (wire injury, vasculitis, atherosclerosis) is the significant reduction in the accumulation of neutrophils and macrophages in the inflamed tissue. This phenotype of reduced leukocyte accumulation is supported by several studies demonstrating MRP-8/14 regulation of myeloid adhesion and transmigration across the blood vessel wall. In vitro studies with MRP-14<sup>−/−</sup> neutrophils show markedly diminished migration through endothelial monolayers and...
attenuated chemokinesis in collagen gels,\textsuperscript{11} and although \textit{MRP-14}\textsuperscript{−/−} mice have normal neutrophil migration during chemical peritonitis, \textit{MRP-14} promotes the trafficking of neutrophils into granulation tissue during wound healing in vivo,\textsuperscript{7} into pancreatic tissue during acute pancreatitis,\textsuperscript{12} and into the skin during IL-8–stimulated inflammation.\textsuperscript{6,11} Recently, \textit{MRP-8/14} was linked to leukocyte activation in lethal endotoxin-mediated shock, but that report did not identify the in vivo source of \textit{MRP-8/14} and did not study the effects of \textit{MRP-8/14} deficiency on leukocyte recruitment during sepsis.\textsuperscript{4}

It is possible that \textit{MRP-8/14} regulates leukocyte adhesion during vascular inflammation through control of the expression and function of the leukocyte integrin adhesion molecule Mac-1 (\textit{CD11b/CD18, αMβ2}).\textsuperscript{11,30,31} \textit{MRP-14}\textsuperscript{−/−} neutrophils have reduced Mac-1 expression at baseline and in response to cell activation,\textsuperscript{11} and \textit{MRP-14} binds to a distinct neutrophil receptor, resulting in inside-out signaling events that promote Mac-1 activation and increase cell adhesiveness.\textsuperscript{31} \textit{MRP-8/14} induces Mac-1 expression on monocytes and enhances endothelial transmigration via intracellular adhesion molecule-1–dependent pathways.\textsuperscript{32} We have shown previously that Mac-1 directs leukocyte recruitment and is critical for the biological response to vascular injury and neointimal formation.\textsuperscript{14,33} These studies linking \textit{MRP-8/14} function to leukocyte adhesion and transmigration are consistent with our data that \textit{MRP-14} deficiency reduces MCP-1–stimulated monocyte transmigration. It is also possible that extracellular \textit{MRP-8/14} regulates leukocyte recruitment during the development of vascular disease by functioning as a chemoattractant or by binding to cell receptors that activate leukocytes. \textit{MRP-8/14} interacts with the receptor for advanced glycation end products\textsuperscript{9} and TLR-4\textsuperscript{4} receptors, which both modulate vascular inflammation and have important roles in the pathobiology of atherosclerosis.\textsuperscript{34,35} Other putative receptors for \textit{MRP-8/14} on target cells include CD36,\textsuperscript{36} special carboxylated N-glycans,\textsuperscript{37} and heparin-like glycoaminoglycans.\textsuperscript{38}

In the vascular injury model, \textit{MRP-8/14} appears to control the inflammatory response at several levels, including regulation of macrophage and neutrophil accumulation and regulation of vascular SMC proliferation. Our finding that \textit{MRP-14} deficiency attenuates mouse vascular SMC proliferation is consistent with a recent report linking \textit{MRP-8/14} function to bacteria-induced proliferative responses in human vascular SMCs.\textsuperscript{16} Despite equivalent TLR-4 receptor expression in WT and \textit{MRP-14}\textsuperscript{−/−} mice, mechanistic interpretation of the LSR response in \textit{MRP-14}\textsuperscript{−/−} mice is complicated by the fact that lipopolysaccharide is used to stimulate the LSR. Because \textit{MRP-8/14} itself modulates lipopolysaccharide signaling via TLR-4,\textsuperscript{4} attenuated LSR response could be secondary to dampened TLR-4 signaling in \textit{MRP-14}\textsuperscript{−/−} mice. However, the LSR requires subcutaneous injection of TNF-α 24 hours after lipopolysaccharide, making it unlikely that reduced TLR-4 signaling (ie, production of TNF-α) could solely account for the effect in \textit{MRP-14}\textsuperscript{−/−} mice. Rather, we believe that a combination of the intracellular actions of \textit{MRP-14} (regulation of calcium-dependent signaling,\textsuperscript{5,6} integration of mitogen-activated protein kinase activity,\textsuperscript{7} regulation of microtubule reorganization,\textsuperscript{7} regulation of arachidonic acid metabolism, and trafficking\textsuperscript{27,39}) and extracellular actions of \textit{MRP-14} (receptor-dependent signaling through TLR-4,\textsuperscript{4} receptor for advanced glycation end products,\textsuperscript{5} CD36,\textsuperscript{36} and possibly other receptors\textsuperscript{30}) accounts for the attenuated thrombohemorrhagic vasculitic response in vivo. Furthermore, reduced leukocyte Mac-1 expression and ligand binding are well documented in \textit{MRP-14}\textsuperscript{−/−} mice, and reduced \textit{MRP-8/14}–dependent Mac-1 adhesion/activation\textsuperscript{11,30,31} could account for attenuation of the LSR response because this model is dependent on Mac-1–mediated neutrophil function.\textsuperscript{15}

With regard to the role of \textit{MRP-8/14} in atherosclerosis, recent studies have demonstrated that free cholesterol accumulation in macrophage membranes activates TLRs and p38 mitogen–activated protein kinase and induces production and secretion of \textit{MRP-8}.\textsuperscript{40} It is possible that hyperlipidemia-induced macrophage expression of \textit{MRP-8/14} could promote leukocyte recruitment and atherogenic cytokine production, a hypothesis that is supported by our demonstration of \textit{MRP-8/14} expression in fatty streaks and advanced atheroma. Furthermore, the demonstration of reduced cytokine production by \textit{MRP-14}\textsuperscript{−/−} macrophages and the demonstration of reduced atherosclerotic lesion size and plaque macrophage accumulation in \textit{ApoE}\textsuperscript{−/−/MRP-14}\textsuperscript{−/−} mice suggest a regulatory role for \textit{MRP-8/14} in leukocyte recruitment and cytokine production during atherogenesis. A regulatory role for \textit{MRP-8/14} in atherogenesis is further supported by the recent discovery that \textit{MRP-8/14} expression levels are increased in microarray analysis of gene expression in normal compared with atherosclerotic human arteries in which statistical analysis of gene expression patterns suggested a role for \textit{MRP-8/14} in the development of atherosclerotic vascular disease.\textsuperscript{41}

Limitations

The present study does not clarify whether neutrophil-, monocyte-, or platelet-derived \textit{MRP-8} and/or \textit{MRP-14} account for the action of these proteins in the pathophysiology of inflammatory vascular disease. Although platelet deposition after wire injury was similar in WT and \textit{MRP-14}\textsuperscript{−/−} mice, we have not excluded a regulatory role for \textit{MRP-14} in platelet functions that influence vascular injury responses. Tissue-specific knockdown of \textit{MRP-14} is required to establish the relative contributions of \textit{MRP-8} and \textit{MRP-14} action in specific hematopoietic cell types, and investigations into the role of \textit{MRP-14} in platelet function will be the focus of future research. The relevance of these observations to human disease awaits expanded studies with human tissues, although the present observations document high levels of \textit{MRP-8/14} in human carotid artery atheroma (Figure 8 and Figure V of the online-only Data Supplement), and elevated plasma levels of \textit{MRP-8/14} are associated with adverse cardiovascular outcomes in patients.\textsuperscript{3,8}

Conclusions

This study demonstrates that \textit{MRP-8/14} broadly regulates vascular inflammation and contributes to biological responses to various vascular insults by controlling neutrophil and macrophage accumulation, macrophage cytokine production,
and SMC proliferation. These findings implicate MRP-8/14 in the development of diverse forms of vascular disease, including hyperplastic responses to arterial intervention, vasculitis, and atherosclerosis.

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**Disclosures**

None.

**References**

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**CLINICAL PERSPECTIVE**

We previously identified myeloid related protein-14 (MRP-14; S100A9) as a differentially expressed gene in ST-segment elevation myocardial infarction and validated that elevated plasma levels of MRP-8/14 heterodimer predict increased risk of future cardiovascular events in apparently healthy postmenopausal women and recurrent events in patients presenting with acute coronary syndromes. Despite its utility as a biomarker, it was previously unknown whether MRP-14 itself participates in the pathogenesis of coronary artery disease and its clinical complications. Using MRP-14–deficient (MRP-14−/−) mice that lack MRP-8/14 complexes, we evaluated the biological response to vascular injury in 3 models of vascular disease. Vascular inflammation and lesion formation in femoral artery wire injury, vasculitis, and atherosclerosis were attenuated significantly in MRP-14−/− mice compared with wild-type controls. Mechanistic studies demonstrated that MRP-8/14 modulates neutrophil and macrophage accumulation and cellular proliferation. Because MRP-8/14 broadly regulates vascular inflammation, targeting MRP-8/14 may provide a novel therapeutic approach to modulate diverse forms of vascular injury, including restenosis, vasculitis, and atherosclerosis.
Myeloid-Related Protein-8/14 Is Critical for the Biological Response to Vascular Injury
Kevin Croce, Huiyun Gao, Yunmei Wang, Toshifumi Mooroka, Masashi Sakuma, Can Shi, Galina K. Sukhova, René R.S. Packard, Nancy Hogg, Peter Libby and Daniel I. Simon

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Supplemental Methods

Femoral Artery Wire Injury:

Bilateral wire injury (0.010 in) of the femoral artery was performed as described previously. 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 3,4. 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).

4  **Vasculitis Experiments: Local Shwartzman Reaction:**

Induction of vasculitis (the local Shwartzman reaction) was performed as described previously. 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 CO₂ 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. 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 5\(\mu\)g/mL E coli LPS for 18h at 37°C. TNF-\(\alpha\), MCP-1, IL-1\(\beta\), 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-\(\mu\)m 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 macrophages were incubated with 50\(\mu\)g/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. 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. We used well-established criteria of plaque vulnerability. 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).
Supplemental Figures and Figure Legends

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: 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, 2\(\mu\)g/ml, MRP-14, 2\(\mu\)g/ml, PDGF, 10ng/ml). Representative experiment, mean ± standard deviation, n= 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: T cell, collagen, and elastin staining of atherosclerotic plaques. Analysis of atherosclerotic plaques from the aortas of ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>MRP<sup>−/−</sup> 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<sup>−/−</sup> and ApoE<sup>−/−</sup>MRP<sup>−/−</sup> mice. Analysis of n=9-14 sections per data point. Data are presented as mean ± standard deviation.
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 References


