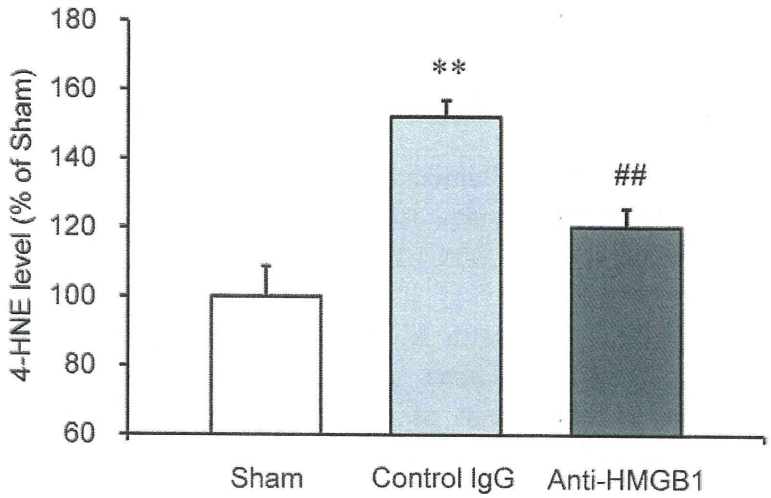

Supplemental Figure 4

Plasma 4-HNE adducts levels in control IgG-treated and anti-HMGB1 mAb-treated rats. Plasma samples were collected from ischemic rats at 6 h after reperfusion. 4-HNE adducts in the samples were detected by ELISA using 4-HNE-BSA as the standard. The results are the means ± SEM of 4 (sham), 5 (control IgG- and anti-HMGB1 mAb-treated group) rats. **P<0.01 compared with sham control, ##P<0.01 compared with control IgG group.

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Arteriosclerosis, Thrombosis, and Vascular Biology

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High-Mobility Group Box Protein 1 Neutralization Reduces Development of Diet-Induced Atherosclerosis in Apolipoprotein E –Deficient Mice

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Objective—High-mobility group box protein 1 (HMGB1) is a DNA-binding protein and cytokine highly expressed in atherosclerotic lesions, but its pathophysiological role in atherosclerosis is unknown. We investigated its role in the development of atherosclerosis in ApoE−/− mice.

Methods and Results—Apolipoprotein E–deficient (ApoE−/−) mice fed a high-fat diet were administered a monoclonal anti-HMGB1 neutralizing antibody, and the effects on lesion size, immune cell accumulation, and proinflammatory mediators were assessed using Oil Red O, immunohistochemistry, and real-time polymerase chain reaction. As with human atherosclerotic lesions, lesions in ApoE−/− mice expressed HMGB1. Treatment with the neutralizing antibody attenuated atherosclerosis by 55%. Macrophage accumulation was reduced by 43%, and vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1 expression was attenuated by 48% and 72%, respectively. CD11c+ dendritic cells were reduced by 65%, and the mature (CD83+) population was reduced by 60%. Treatment also reduced CD4+ cells by nearly 50%. mRNAs in lesions encoding tumor necrosis factor-α and interleukin-1β tended to be reduced. Mechanistically, HMGB1 stimulated macrophage migration in vitro and in vivo; in vivo, it markedly augmented the accumulation of F4/80+Gr-1(Ly-6C)+ macrophages and also increased F4/80+CD11b+ macrophage numbers.

Conclusion—HMGB1 exerts proatherogenic effects augmenting lesion development by stimulating macrophage migration, modulating proinflammatory mediators, and encouraging the accumulation of immune and smooth muscle cells. (***Arterioscler Thromb Vasc Biol.* 2011;31:313-319.**)

Key Words: atherosclerosis ■ macrophages ■ HMGB1

Atherosclerosis is a chronic inflammatory disease characterized by intimal accumulation of atherogenic lipoproteins, extracellular matrix, smooth muscle cells, and inflammatory cells. Cytokines within atherosclerotic lesions play a key role in both the development and progression of atherosclerosis.[1,2] Recently, we and others have identified a novel cytokine in human atherosclerotic lesions, high-mobility group box 1 (HMGB1), that could be important for regulating development of atherosclerosis.[3–5] HMGB1 has 2 main functions. As a nuclear protein, it stabilizes nucleosomes and bending of DNA, which facilitates gene transcription. It can also be released from necrotic cells[6] or secreted by inflammatory cells, such as macrophages,[3] and natural killer cells,[7] triggering inflammation.[8] HMGB1-nucleosome complexes released by necrotic cells activate macrophages and dendritic cells to produce cytokines, such as tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), IL-6, and IL-10[9]; also, native secreted HMGB1 stimulates monocytes to secrete proinflammatory cytokines.[10] With respect to the vasculature, HMGB1 stimulates the migration and proliferation of vascular smooth muscle cells[4,11] and activates endothelial cells.[12] It induces the migration of macrophages, activates dendritic cells,[13,14] and is required for dendritic cells maturation.[14,15] Notably, dendritic cells control T-cell activation by secreting HMGB1.[16]

Structurally, HMGB1 has a tripartite domain organization. It contains 2 similar DNA-binding domains, HMG Box A and Box B, and a unique C-terminal domain consisting of an acidic tail of 30 amino acids all connected by short amino acid sequences.[17] The proinflammatory activity of HMGB1 has been localized to Box B, and antibodies raised against Box B prevent HMGB1 actions[17,18]; a receptor for advanced glycation end products (RAGE)-binding domain is localized within the C-terminal component of Box B and the segment connecting to the acidic tail.[19] In contrast, Box A attenuates HMGB1-induced release of proinflammatory cytokines.[20,21] The acidic tail is thought to be unstructured and interacts with specific basic residues in both boxes, possibly regulating their conformation.[22]

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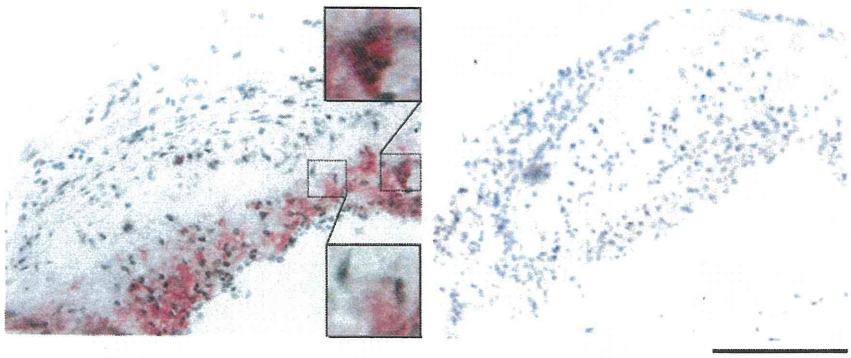

Figure 1. Immunohistochemical identification of HMGB1 expression in aortic sinus atherosclerotic lesions in ApoE−/− mice. Left, HMGB1 (red staining) was localized within intimal cells (top insert) and also diffusely distributed within lesions (bottom inset). Cells within the media did not express HMGB1. Right, Region similar to that shown in A incubated with nonspecific rabbit IgG instead of the primary rabbit HMGB1 IgG. Section was counterstained with hematoxylin. Scale bar represents 100 μm.

The proinflammatory effects of HMGB1 have been attributed to its interaction with a number of receptor types, which appears dependent on factors bound to HMGB1. RAGE, toll-like receptor (TLR)2, and TLR4 appear to mediate proinflammatory effects in HMGB1-stimulated macrophages.[23,24] HMGB1 in DNA immune complexes and nucleosome complexes appears to exert proinflammatory effects via TLR9 and TLR2, respectively.[12,25] More recently, HMGB1 has also been shown to interact with CD24 and Siglec-10 to attenuate tissue damage induced immune responses[26]; a balance between these and the detrimental effects of HMGB1 has been suggested to determine the overall magnitude of its detrimental effects.[26]

HMGB1 has been implicated in a number of immune-driven diseases, including systemic lupus erythematosus, autoimmune diabetes, and arthritis.[9,27,28] In arthritis, it triggers inflammation by activating macrophages and inducing IL-1.[28] Because HMGB1 is overexpressed in atherosclerotic lesions[3–5] and activates immune processes that can augment atherosclerosis,[4,11,13–15] we examined its role in the development of atherosclerosis in apolipoprotein E–deficient mice (ApoE−/− mice). We used an anti-HMGB1neutralizing monoclonal antibody, which interacts specifically with the C-terminal sequence of HMGB1 within the acidic tail, to determine its role in atherosclerosis. This antibody reacts with HMGB1 and not HMGB2 and has been shown to inhibit HMGB1 responses in macrophages and ameliorates brain infarction induced by transient ischemia.[29]

Materials and Methods

Six-week-old ApoE−/− mice were fed a high-fat diet for 8 weeks and administered either an anti-HMGB1 monoclonal antibody or IgG2a control (400 μg IV twice weekly). At the end of the study, mice were killed with an overdose of pentobarbitone, and blood, aortic sinus and arch, spleen, and lymph nodes were collected for histological and molecular studies. Monocyte proliferation in vivo was assessed after administering bromodeoxyuridine (1 mg IP) for 3 days before the mice were killed. The chemotactic effects of HMGB1 on monocytes was assessed in mice 5 hours after administering HMGB1 (20 μg) into the peritoneal cavity.

A detailed Supplemental Methods section is available online at http://atvb.ahajournals.org.

Results

HMGB1 Expression in Atherosclerotic Lesions

HMGB1 is highly expressed in human atherosclerotic lesions, mostly by macrophages in which nearly 50% of the cells contained HMGB1 in their cytoplasm.[3] Therefore, we investigated whether atherosclerotic lesions in ApoE−/− mice also expressed HMGB1. HMGB1 was expressed within the aortic sinus of ApoE−/− mice and restricted to cells within the atherosclerotic intima (Figure 1). HMGB1 appeared to be cell associated, frequently within the cytoplasm and also diffusely distributed within lesions; the latter most probably reflected secreted HMGB1 (Figure 1).

Effect of HMGB1 Neutralization on Atherosclerotic Lesion Size

At 14 weeks of age (8 of weeks antibody treatment) total plasma cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglyceride levels were not different between mice treated with control or anti-HMGB1 neutralizing antibody (Supplemental Table I). However, analysis of Oil Red O–stained aortic sinus sections revealed significant differences in lipid deposition and atherosclerotic plaque size (Figure 2). Morphometric analysis showed that the anti-HMGB1 neutralizing antibody reduced lesion size compared with treatment with control antibody, 89 253±13 098 μm^2 versus 183 903±11 784 μm ($P<0.05$; Figure 2). Similarly, macrophage accumulation was reduced in lesions of mice treated with the neutralizing antibody. Cross-sectional area of the aortic sinus that stained with anti-CD68 antibody averaged 83 400±15 451 μm^2 in mice treated with the anti-HMGB1 neutralizing antibody compared with 141 364±12 608 μm^2 with control antibody (Figure 2). To determine whether the effects of the HMGB1 antibody were due to neutralizing circulating HMGB1, we measured plasma HMGB1 levels in control and anti-HMGB1 antibody treated mice using a specific HMGB1 ELISA. Plasma HMGB1 levels were undetectable (<1 ng/mL) in both instances, similar to earlier reports in nonatherosclerotic mice,[30] suggesting that effects of the neutralizing antibody were local at the site of developing lesions.

Effect of Neutralizing HMGB1 on Atherosclerotic Lesion Composition

Analysis of atherosclerotic lesion composition also revealed differences between the 2 groups in terms of cellular composition. Because HMGB1 can stimulate the migration of dendritic cells,[14,15] we investigated whether neutralizing HMGB1 activity attenuated their accumulation in developing lesions. Immunohistochemical studies and quantitative analysis indicated a marked reduction in dendritic cell accumu-

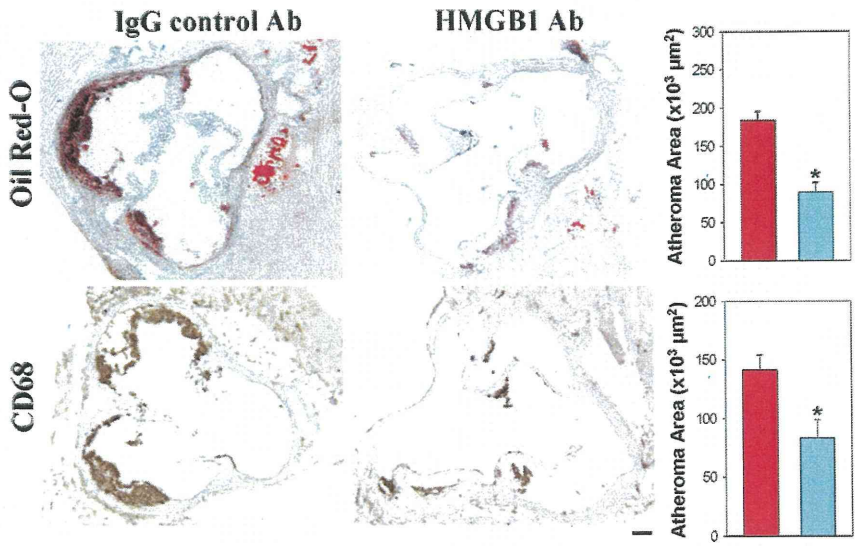

Figure 2. Photomicrographs of aortic sinus atherosclerotic lesions from ApoE−/− mice fed a high-fat diet and treated with control antibody (Ab; left) and anti-HMGB1 neutralizing antibody (right). Top sections show sections stained with Oil Red O and mean areas of staining (bar graph) in the 2 groups of mice. Bottom sections, Immunohistochemical staining using anti-CD68 (macrophage) antibody and mean areas of staining (bar graphs) in the 2 groups of mice. Red indicates control antibody; blue, anti-HMGB1 neutralizing antibody. *$P<0.05$ compared with control. Scale bar represents 100 µm.

lation, with CD11c staining averaging 8±1% of lesion area in aortic sinus of mice treated with anti-HMGB1 neutralizing antibody compared with 24±3% with control antibody ($P<0.05$; Figure 3). Because HMGB1 can also promote maturation of dendritic cells,[15] we investigated whether expression of CD83, a marker of dendritic cell maturation, was affected. CD83 expression in the lesions represented only a small fraction of the dendritic cell population, indicating that the dendritic cell population was largely immature (Figure 3). Treatment with the anti-HMGB1 antibody reduced the CD83 cell population by nearly 60% ($P<0.05$; Figure 3). Mature dendritic cells in atherosclerotic lesions are known to produce T-cell–attracting chemokines CCL19 and CCL21.[31] Consequently, we examined whether CD4+ T-cell accumulation was affected in mice treated with the anti-HMGB1 neutralizing antibody. Treatment with anti-HMGB1 neutralizing antibody reduced CD4+ T-cell accumulation in lesions by ≈50% ($P<0.05$; Figure 4). To determine the different T-cell subtypes that might be affected, we analyzed subset-specific mRNA expression. In lesions of anti-HMGB1 antibody–treated mice, we observed no change in expression of Tim-3, a known marker and negative regulator of Th1 lymphocytes ($P>0.05$; Figure 4)[32]; Tim-1, a marker of Th2 lymphocytes, was undetectable (Figure 4).[33]

Because HMGB1 stimulates the in vitro migration and proliferation of vascular smooth muscle cells,[4,11] and endothelial cells,[34] we also investigated whether it affected smooth muscle cell and endothelial cell numbers in developing lesions. Treatment with the anti-HMGB1 neutralizing antibody reduced smooth muscle cell accumulation within developing lesions by nearly 50% ($P<0.05$; Supplemental Figure I) but did not affect lesion-associated endothelial cells (Supplemental Figure I). Treatment with the neutralizing antibody was also associated with a 35% reduction in the number of proliferating cells within the lesions ($P<0.05$; Supplemental Figure I).

To further confirm that these cellular effects were largely restricted to developing atherosclerotic lesions, we also assessed lymphocyte populations in blood, spleen, and paraaortic lymph nodes. In these tissues anti-HMGB1 antibody treatment

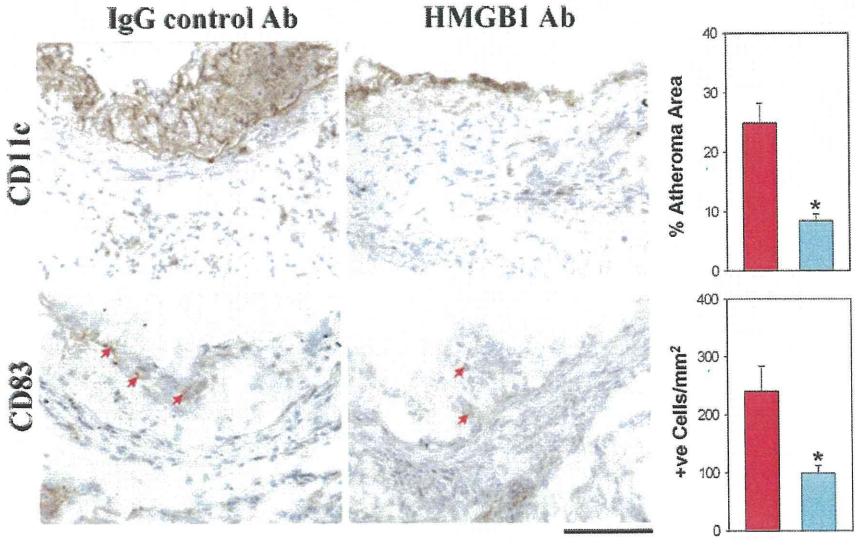

Figure 3. Immunohistochemistry of aortic sinus atherosclerotic lesions from control antibody (Ab; left) and anti-HMGB1 antibody (right)–treated ApoE−/− mice fed a high-fat diet. Cross-sections were stained with anti-CD11c antibody to detect dendritic cells (top) and anti-CD83, a marker of mature dendritic cells (bottom). Bar graphs represent the extent of immunostaining in the 2 groups. Red indicates control antibody; blue, anti-HMGB1 neutralizing antibody. *$P<0.05$ compared with control. Scale bar represents 100 µm.

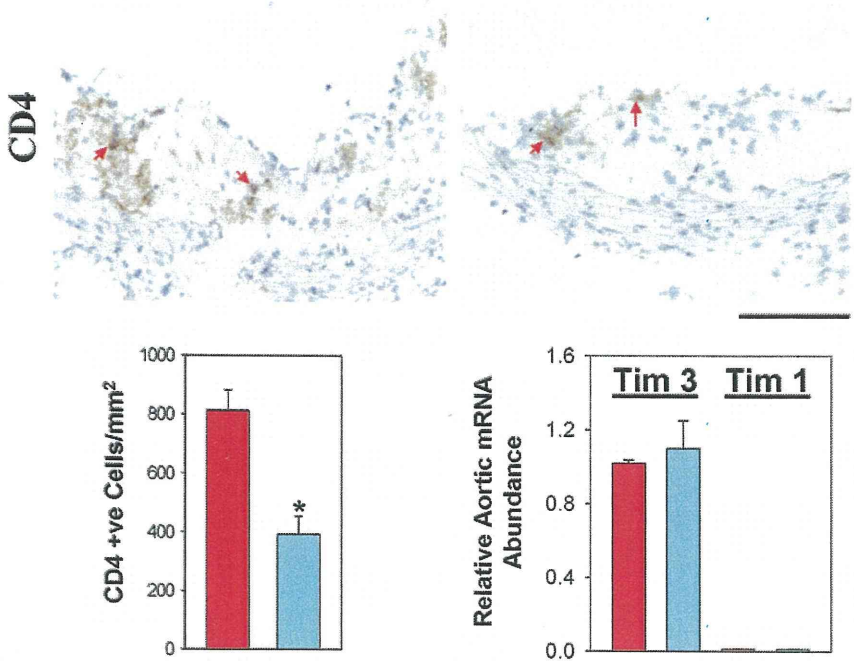

Figure 4. Distribution of CD4+ T cells in aortic sinus atherosclerotic lesions and their polarization following treatment of ApoE−/− mice with either control antibody or anti-HMGB1 neutralizing antibody. Top, cross-sections were stained with anti-CD4 antibodies to detect CD4+ lymphocytes in lesions of control (left) and anti-HMGB1 antibody (right)–treated mice. Bottom left, Bar graph indicating CD4+ lymphocyte density in the lesions. Bottom right, Bar graph indicating the relative abundance of mRNAs encoding Tim3 and Tim1 in lesions. Red indicates control; blue, anti-HMGB1 treatment. *$P<0.05$ compared with control. Scale bar represents 100 μm.

did not affect CD4+ T cells, B cells, natural killer (NK) or natural killer T cell or CD4+Foxp3+ regulatory T-cell numbers (all $P>0.05$; Supplemental Figure II). In addition, we assessed monoblast/promonocyte proliferation, as well as monocyte and dendritic cell numbers in blood of control and anti-HMGB1–treated mice and dendritic cell numbers in lymph nodes. BrdU+CD11b^{hi}Ly-6C^{hi}CD90^{lo}CD49b^{lo}NK1.1^{lo}Ly-6G^{lo}CD22^{lo}, CD11b^{hi}Ly-6C^{hi}CD90^{lo}CD49b^{lo}NK1.1^{lo}Ly-6G^{lo}CD22^{lo}, and CD11b^{hi}CD90^{lo}Cd49b^{lo}NK1.1^{lo}Ly-6G^{lo}CD22^{lo} monocytes in blood were unaffected by anti-HMGB1 antibody treatment ($P>0.05$; Supplemental Table II). Similarly, BrdU+CD11c^{hi}CD11b^{hi}CD90^{lo}CD49b^{lo}NK1.1^{lo}Ly-6G^{lo}CD22^{lo}, CD11c^{hi}CD11b^{hi}CD90^{lo}CD49b^{lo}NK1.1^{lo}Ly-6G^{lo}CD22^{lo}, and CD11c^{hi}CD11b^{hi}I-A$^{b(hi)}$CD115^{hi}CD90^{lo}CD49b^{lo}NK1.1^{lo}Ly-6G^{lo}CD22^{lo} monocyte-derived dendritic cells in blood were unaffected ($P>0.05$; Supplemental Table II). Also, CD11c^{hi}CD11b^{hi}I-A$^{b(hi)}$CD115^{hi}CD90^{lo}CD49b^{lo}NK1.1^{lo}Ly-6G^{lo}CD22^{lo} monocyte-derived dendritic cells in inguinal and mediastinal lymph nodes were unaltered by anti-HMGB1 antibody treatment; CD11c+I-A^{b}+CD115+ are thought to emigrate from lesions to lymph nodes during regression of lesions.[35] Together, these results indicate that monocytes/dendritic cell numbers in blood do not account for the reduction in macrophages and dendritic cells in lesions of anti-HMGB1 treated mice. Similarly, emigration of dendritic cells from lesions does not appear to be affected by anti-HMGB1 antibody treatment.

HMGB1 Neutralization and Proinflammatory Mediators in Lesions

HMGB1 can increase the expression of a number of proinflammatory mediators including MCP-1, vascular cell adhesion molecule-1 (VCAM-1) and a variety of cytokines including IL-1β, IL-6, and tumor necrosis-α (TNF-α).[12,36,37] We investigated whether treatment with anti-HMGB1 neutralizing antibodies might affect expression of such proinflammatory mediators in developing lesions. Treatment with anti-HMGB1 neutralizing antibodies reduced expression of VCAM-1 in lesions by 50% and MCP-1 expression by nearly 70% (both $P<0.05$; Supplemental Figure III). There also tended to be reductions in the expression of TNF-α, IL-1β, and interferon-α, whereas IL-6 was unaffected (Supplemental Figure III).

HMGB1 and Cell Migration

Because anti-HMGB1 antibody treatment affected immune cell accumulation in lesions, in particular macrophages, to a much greater extent than proinflammatory cytokines, we examined the possibility that HMGB1 might be influencing macrophage migration to developing lesions. In vitro, HMGB1 stimulates macrophage migration.[13] To confirm the chemoattractant actions of HMGB1 on mouse macrophages, we first assessed its ability to stimulate mouse RAW264.7 macrophage migration using 24-well chemotaxis chambers. HMGB1 stimulated their migration, more than doubling the number of migrated macrophages ($P<0.05$; Figure 5). To determine whether HMGB1 also stimulated macrophage cell migration in vivo, we injected HMGB1 into the peritoneal cavity of mice and 5 hours later assessed its effects on peritoneal macrophage. HMGB1 markedly increased the number of F4/80+Gr1(Ly-6C)+ macrophages ($P<0.05$; Figure 5) and also increased F4/80+CD11b+ macrophages ($P<0.05$; Figure 5).

Discussion

We have previously reported that HMGB1 is expressed by macrophages in human aortic fatty streaks and fibrofatty lesions.[3] In advanced atherosclerotic plaques, HMGB1 is also expressed by vascular smooth muscle cells.[5] Also, serum HMGB1 levels have been associated with coronary artery

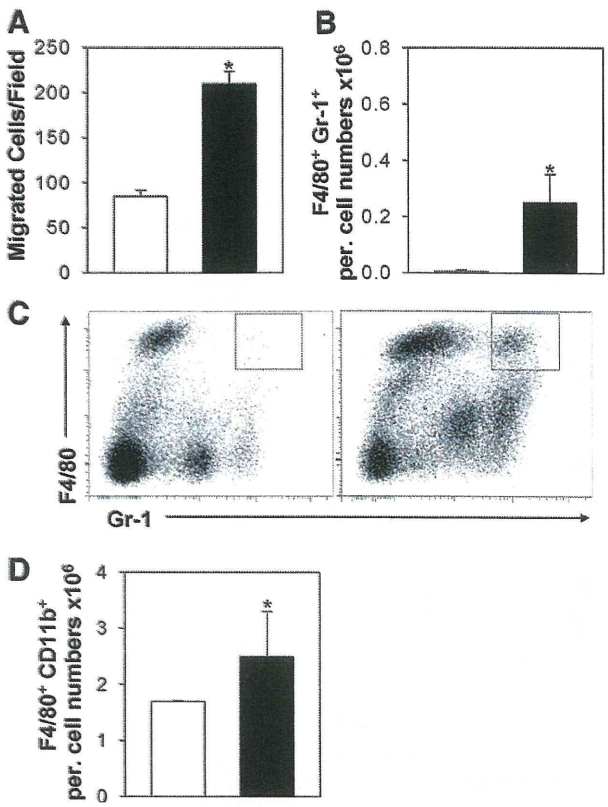

Figure 5. HMGB1-stimulated macrophage chemotaxis in vitro and intraperitoneal macrophage recruitment in vivo. A, Migration of mouse RAW264.7 macrophages in vitro in response to vehicle or HMGB1 (4 μg/mL). B, Number of F4/80+Gr-1(Ly-6C)+ macrophages recruited to the peritoneal (per.) cavity 5 hours after IP injection of vehicle or HMGB1 (20 μg/mL). C, Flow cytometric analysis of peritoneal lavage effluent demonstrating F4/80 and Gr-1 populations 5 hours after injection of vehicle or HMGB1. Boxed areas show the F4/80+Gr-1(Ly-6C)+ population. D, Number of F4/80+CD11b+ macrophages recruited to the peritoneal cavity 5 hours after IP injection of vehicle or HMGB1. *P<0.05 compared with vehicle control.

disease.[38,39] In this study, we demonstrated that atherosclerotic lesions within the aortic sinus of ApoE−/− mice also express HMGB1, which contributes to lesion development. Neutralizing HMGB1 using a monoclonal antibody targeting amino acids within the acidic tail,[29] attenuated development of atherosclerosis. Our study suggests that HMGB1 in lesions rather than circulating HMGB1 contributes to atherosclerosis, stimulating macrophage migration and increasing the accumulation of other immune cell types, as well as proinflammatory mediators.

HMGB1 exerts multiple effects on monocyte/macrophages in vitro. It stimulates macrophage migration,[13] monocyte adherence and spreading, monocyte-matrix interactions,[40] and dendritic cell migration.[14] Our observations extend these findings and indicate that HMGB1 is also chemoattractive in vivo for macrophages; HMGB1 stimulated the migration of F4/80+Gr-1(Ly-6C)+ macrophages, a subtype known to accumulate in atherosclerotic lesions.[41] It does not appear to influence monoblast/promonocyte proliferation, monocyte numbers in blood, or efflux of dendritic cells from lesions.

Our studies suggest that CD4+ T cells in lesions are also affected by HMGB1; CD4+ T-cell numbers were reduced in lesions of mice treated with the HMGB1 neutralizing antibody but unaffected in blood, spleen, or lymph nodes. In vitro HMGB1 acts as a costimulatory factor together with T-cell receptor stimulation to augment proliferation.[42] Also, dendritic cells produce and respond to HMGB1 elevating IL-12 secretion, which in turn polarizes CD4+ T cells to the Th1 phenotype.[37] Our finding that Tim-3 expression is unaffected by anti-HMGB1 antibody treatment, despite reductions in CD4+ T cells, suggests that the function of these cells may be impaired; Tim-3 is a negative regulator of Th1 cell function.[32,43] Because HMGB1 appears to regulate immune cell numbers in lesions we also investigated whether it affected cytokine levels. In vitro, HMGB1 potently stimulates macrophages and endothelial cells to secrete proinflammatory cytokines, TNF-α, IL-1β, and IL-6.[12,37] Surprisingly, we found that neutralizing HMGB1 only had small effects on the expression of proinflammatory cytokines, suggesting that HMGB1 in lesions was affecting immune cell function rather than expression of proinflammatory cytokines in lesions. However, HMGB1 augmented the expression of proinflammatory mediators VCAM-1 and MCP-1.

In addition to affecting immune cells, HMGB1 can affect vascular smooth muscle cells, stimulating their migration[11] and proliferation.[4] Treatment with anti-HMGB1 neutralizing antibodies reduced smooth muscle cell accumulation and the number of proliferating cells within developing lesions. The intima is considered to be the "soil" in which atherosclerosis develops,[44] and attenuating its development/growth by neutralizing HMGB1 activity and reducing the intimal smooth muscle population could contribute to the reductions in lesion size. Although HMGB1 has also been shown to stimulate endothelial cell proliferation and angiogenesis, endothelial cells did not appear to be affected by treatment with the anti-HMGB1 neutralizing antibody.

Although our studies indicate that HMGB1 contributes to lesion development by stimulating macrophage migration and activating dendritic cells, the receptors through which these effects are mediated remain to be identified. It is interesting to note that the effects we observed on macrophages, dendritic cells, and T cells, as well as vascular smooth muscle cells, have been largely attributed to interactions with RAGE.[11,13,15,16] Inhibition of RAGE attenuates lesion development and is associated with reductions in macrophage and smooth muscle cell accumulation, as well as reductions in VCAM-1 expression,[45] effects that we observed following neutralization of HMGB1. However, we cannot exclude the possibility that HMGB1 also interacts with other receptors during development of atherosclerosis, including TLR2, TLR4, or the CD24/Siglec-10 system. Our findings provide an encouraging basis for the development of a novel therapeutic approach for atherosclerosis, possibly using newer formats of recombinant antibodies.

In conclusion, our data extend earlier findings on the expression of HMGB1 in human atherosclerotic lesions and demonstrate that locally, I expressed HMGB contributes to lesion development by stimulating macrophage migration and modulating proinflammatory mediators such as MCP-1 and VCAM-1. HMGB1 also indirectly contributes to accumula-

tion of dendritic cells and CD4+ T cells. The findings define a new potential therapeutic target for atherosclerosis.

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Disclosures

None.

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Supplemental Material

Peter Kanellakis et al. HMGB1 Neutralization Reduces Development of Diet-Induced Atherosclerosis in Apolipoprotein E-Deficient Mice

Supplemental Materials and Methods

Animal Experiments

Twenty four male ApoE-/- mice on the C57BL/6 background were obtained from the Precinct Animal Facility, AMREP, Melbourne, Australia and fed a high-fat diet containing 0.15% cholesterol and 21% fat (Speciality Feeds) from 6 weeks of age for 8 weeks. Mice (12 per group) also received either a control IgG2a against *Keyhole Limpet* hemocyanin (400μg iv twice weekly) or anti-HMGB1 monoclonal antibody (400μg iv twice weekly) for the duration of the dietary feeding. This dose regime was based on our earlier findings that 400μg anti-HMGB1 i.v. is highly effective in reducing cerebral infarct volumes,[1] and the long plasma half-life of IgG2a, approximately 5 days.[2,3] In vitro, 1μg of the anti-HMGB1 neutralizing antibody attenuates the stimulatory effect of 10μg HMGB1 on macrophages, assessed as an increase in ICAM-1 expression, by 67% whilst 100μg of antibody attenuates this response by 93%.[1] At the end of the study mice were killed with an overdose of pentobarbitone (120mg/kg i.p.), blood collected by cardiac puncture and aortic sinus and arch collected for histology and molecular studies. To assess the effects of the anti-HMGB1 antibody treatment on monocytes and dendritic cells and proliferation of their precursors an additional 4 mice in each group were treated with bromodeoxyuridine (1mg, i.p. daily) for three consecutive days prior to culling. All experiments were approved by the Alfred Medical Research Education Precinct (AMREP) Animal Ethics Committee.

Monoclonal Antibodies

Monoclonal antibodies (anti-HMGB1 and control) were purified from culture media of growing hybridomas producing the anti-HMGB1 and anti-*Keyhole Limpet* hemocyanin

(control) antibodies using Mep HyperCel (Pall Life Sciences, Cerg, FGrance) and DEAE cellulose chromatographies. HMGB1 neutralizing activity was confirmed by determining its ability to attenuate HMGB1 bioactivity on cultured macrophages as previously described.[1]

HMGB1

Full-length recombinant HMGB1 was purchased from HMGBiotech (Milano, Italy); it was purified as previously described and endotoxins were removed by passage through Detoxy-Gel columns (Pierce Chemical Co).[4,5] HMGB1 was rigorously tested to be LPS-free.[6]

Plasma Cholesterol and Triglycerides

Plasma LDL- and HDL-cholesterol were determined enzymatically using a Cobas Mira Plus Autoanalyzer and a HDL and LDL/VLDL cholesterol quantification kit (BioVision, Mountain View, CA).Plasma triglycerides were determined using a triglyceride quantification kit (BioVision, Mountain View, CA).

Quantification of Atherosclerotic Lesions

The heart and proximal aorta were dissected from mice, embedded in OCT compound (Tissue-teck) and frozen at -80°C. Frozen sections (6μm) were cut from the aortic sinus, from where the valves or valve cusps first become visible to where the left and right coronary arteries branch off, a distance of approximately 250μm.[7] A total of 4 sections taken from identical aortic sinus locations in each mouse at 60μm intervals were stained with Oil Red O to delineate lipid deposits and counter stained with haematoxylin.[8] The aortic sinus was evaluated because this region of the aorta is particularly susceptible to the development of atherosclerotic lesions in mice fed a high fat diet.[7] Sections were examined using light microscopy and the cross-sectional area of lipid depositions quantified using image analysis software (Optimus 6.2 VideoPro-32). For each mouse, the

lesion size was measured in 4 cross-sections and lesion size per cross-section averaged to provide the mean lesion size per mouse.

Immunohistochemistry

Twenty seven 6μm cryo-sections from similar parts of the aortic sinus of ApoE-/- mice treated with control or anti-HMGB1 neutralizing antibody were used for immunohistochemistry to assess macrophage accumulation (CD68), CD4+ T-lymphocytes, dendritic cells (CD11c), vascular smooth muscle cells (alpha SM actin) and endothelial cells (CD31), VCAM-1, MCP-1, CD83, HMGB1 and PCNA. Briefly, sections were fixed in cold (-20°C) acetone for 20 min. The sections were then incubated in 3% hydrogen peroxide in PBS, 10% normal serum and biotin/avidin blocking reagents (Vector Laboratories). Then the sections were incubated (1hr) with primary antibodies in serum, rat anti-mouse CD68 (1-100; Serotec: cat#MCA1957), Armenian hamster anti-mouse CD11b (1-50; eBioscience: cat#14-0114), rat anti-mouse CD4 (1-20; BD Pharmingen: Cat#550280), rabbit anti-alpha smooth muscle actin (1-100; Abcam: #ab5694), rat anti-mouse CD31 (1-100, BD Pharmingen: cat# 550274), rat anti-mouse VCAM-1 (1-50; BD Pharmingen: cat#550547), rabbit anti rat MCP-1 (1-50; Abcam: cat#ab7202), rat anti-mouse CD83 (1-50; eBioscience: cat#14-0831),rabbit anti-HMGB1 antibody (0.125μg/ml; BD Pharmingen: cat#556528) and rabbit anti-human PCNA (1-50; Abcam: cat#ab2426). Subsequently the sections were washed and incubated with the appropriate secondary antibody [biotinylated mouse anti-rat (1-200; BD Pharmingen: cat#550325), biotinylated mouse anti-Armenian hamster (1-200; eBioscience: cat#13-4113-85) or biotinylated anti-rabbit (1-200; Vector Labs: cat#BA-1000)] for 40 minutes, followed by incubation with streptavidin horseradish peroxidase complex (Vector Laboratories). Antigens were visualized using 3,3-diaminobenzidine.[9] Sections were counterstained with hematoxylin. Expression of antigens was quantified either by cell counting or measuring stained areas using Optimus 6.2 VideoPro-32 and results expressed either as stained cross-sectional

area (CD68), cells per unit area (CD4, PCNA) or percent of total plaque area (CD31, alpha-SM actin, CD11c, CD83, VCAM-1, MCP-1).[10]

mAbs and Flow Cytometry

The following antibodies were used: anti-CD4-pacific blue (BD Pharmingen), anti-CD8-PerCP (BD Pharmingen), anti-TCRβ-APC (Pharmingen), anti-NK1.1-PE Cy7 (Pharmingen), anti-CD25-APC Cy7 (Pharmingen), Foxp3-PE (BD Pharmingen), anti-CD11c-PE (eBioScience), anti-F4/80-Alexfluor (647) eBioScience), anti-CD11b-PerCP-Cy5.5 (eBioScience) and anti-Gr1 (Ly6G and Ly6C)-AlexaFluor-488 (eBioScience).[11] Spleens and lymph nodes were gently dissociated between the frosted ends of glass slides to obtain single cell suspensions which were passed through a 70μM nylon strainer. Single cell suspensions were pre-incubated with anti-FcRγ (2.4G2) to prevent non-specific staining followed by incubation with fluorochrome-conjugated primary antibodies for 30 minutes at 4°C. For Foxp3 staining the cells were permeabilized using the BD Pharmingen Mouse Foxp3 buffer set according to the manufacture's directions. For analyses of monocytes and dendritic cells in blood and lymph nodes the following antibodies were used: NK1.1-PE, CD22-PE, CD90-PE, CD49b-PE, Ly-6G-PE, CD11b-APC-Cy7, Ly-6C-pacific blue, CD11c-APC and BrdU-PerCP (all from BD Pharmingen), I-A^b-PE-Cy7 (BioLegend) and CD115-Alexa-488 (eBiosciences). Monocytes in blood were identified as $CD11b^{hi}CD90^{lo}CD22^{lo}CD49b^{lo}NK1.1^{lo}Ly\text{-}6G^{lo}$ and monocyte-derived dendritic cells as $CD11c^{hi}CD11b^{hi}CD90^{lo}CD22^{lo}CD49b^{lo}NK1.1^{lo}Ly\text{-}6G^{lo}$.[12] Sample data was acquired using a BD FACS Canto II (BD Biosciences).

HMGB1 Elisa

HMGB1 in plasma was measured using an ELISA HMGB1 detection kit (Apotech Corp, Enzo Life Sciences GMBH, Germany) according to the manufacture's instructions. The sensitivity of the assay is approximately 1ng/ml.

Macrophage Chemotaxis in Vitro and in Vivo

Macrophage chemotaxis in vitro was measured using fibronectin-coated-8μm porous transwells (BD Biosciences). Briefly, RAW264.7 mouse macrophages (200,000) were cultured in DMEM containing 1% fetal calf serum prior to being loaded onto the inserts which were placed in 24-well plates containing DMEM in the presence or absence of 4μg/ml HMGB1 and incubated at 37°C for 15 hours. Non-migrated cells in the top chamber were removed using cotton swabs before fixing the migrated cells in methanol-glacial acetic acid (3:1) and staining with crystal violet. Chemotaxis was quantified by counting the number of migrated in five random high-power microscopy fields per well.[13] To determine whether HMGB1 induced macrophage chemotaxis in vivo mice were injected i.p. with 1ml of vehicle (0.9% NaCl) or HMGB1 (20μg/ml in 0.9% NaCl). After 5 hours the mice were sacrificed and the cellular contents of their intraperitoneal cavities were collected by sequential lavages in PBS. After centrifugation, red blood cells were lysed with ammonium chloride-potassium carbonate-EDTA buffer (0.15M NH_4Cl, 1mM $KHCO_3$ and 0.1mM Na_2EDTA in PBS) and total cell numbers determined. Then the cells were pre-incubated with anti-FcRγ (2.4G2) to prevent non-specific staining followed by incubation with fluorochrome-conjugated primary antibodies-anti-F4/80, anti-CD11b, anti-CD11c and anti-Gr-1 (see mAbs and Flow Cytometry) for 30 minutes at 4°C. Sample data was acquired using a BD FACS Canto II (BD Biosciences).

Analysis of Gene Expression

Total RNA was extracted from tissues as previously described[14] and resuspended in sterile water; any contaminating DNA was removed by incubating the RNA extracts with 2 U DNase (Stratagene), for 15 minutes at 37°C. Then 2 μL of 2 mol/L sodium acetate followed by an equal volume of isopropanol was added and the precipitated RNA was sedimented by centrifugation. The RNA pellet was washed by resuspension in 70% aqueous ethanol followed by centrifugation, and then air dried for 30 minutes. This purified RNA was dissolved in sterile water and quantitated by spectrophotometry at 260 nm. The

extracted total RNA was reverse transcribed using TaqMan methodology (Applied Biosystems) as described by the manufacturer. Then 40ng of cDNA was used for real-time PCR to determine the expression of each gene using Applied Biosystems SYBR Green PCR Mix and the ABI Prism 7500 system. Each amplification was performed in duplicate and included internal controls for CD68 (macrophages) to take into account any alterations in lesion size. Relative amounts of each mRNA for each of the genes in lesions from control and anti-HMGB1 treated ApoE-/- mice were calculated using comparative C_T values.[4] The sequences of oligonucleotides used were, TIM-1: sense,5'-AGTGACCTTTTCATTGCAAGTTAAAC-3' and antisense, 5'-GCTGTGG GCCTTGTAGTTGTG-3'; for TIM-3: sense, 5'-CAGCTTCTCCAAGAACCCTAACC-3' and antisense, 5'-TTATTATGGAGGGTCACCAGTGTCT-3'; for IL-6: sense, 5'-GAAATGAT GGATGCTACCAAACTG-3' and antisense, 5'-CCAGAAGACCAGAGGAAATTTTCA-3'; for TNF-α: sense, 5'-CTATGGCCCAGACCCTCACA-3' and antisense, 5'-TCCTCCACTTG GTGGTTTGC-3'; for IFN-α: 5'-TCCTCAGACTCATAACCTCAGGAA-3' and antisense, 5'-GGGAGAGTCTCCTCATTTGTACCA-3'; for IL-1β: sense, 5'-CCACCTCAATGGACAGAA TATCAA-3' and antisense, 5'-GTCGTTGCTTGGTTCTCCTTGT-3'; for 18s: sense, 5'-CGGCTACCACATCCAAGGAAGGCA-3' and antisense, 5'-GCTGGAATTACCGCGGCTGCTGGC-3' ; for CD68: sense, 5'-TGACCTGCTCTCTCTAAGGCTACA-3 and antisense, 5'-TGGTCACGGTTGCAAGAGAA-3.

Statistical Analyses

Statistical analyses were performed using Student's t-test when data followed a normal distribution or Mann-Whitney U test when data did not follow a normal distribution, using the software GraphPad Prism v4.01. $P < 0.05$ was considered statistically significant.

References for Supplemental Materials and Methods

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Legends for Supplementary Tables and Figures

Table I: Plasma Lipid Levels in ApoE-/- mice treated with control or anti-HMGB1 antibody (Ab) and fed a high fat diet for 8 weeks. Results are means ± SEM.

Table II. Monocyte subtypes and dendritic cells in blood (cells/ml) and lymph nodes (cell numbers) of ApoE-/- mice treated with control or anti-HMGB1 antibody (Ab) and injected with bromodeoxyuridine (1mg, i.p.) for 3 consecutive days before culling. Results are means ± SEM of 4 mice in each group.

Figure I. Immunohistochemistry of aortic sinus atherosclerotic lesions from control (left) and anti-HMGB1 neutralizing antibody (right) treated ApoE-/- mice fed a high fat diet. Cross sections were stained with anti-alpha-SM actin antibody to detect smooth muscle cells associated with lesions (top), anti-CD31 antibody to detect endothelial cells associated with lesions (middle) and anti-proliferating nuclear antigen (PCNA) antibody to detect cell proliferation (bottom). Bar graphs represent differences between the two groups. Red: control and blue: anti-HMGB1 antibody treatment. *P < 0.05 from control. Size bars on photomicrogrpahs represent 100μm.

Figure II. Lymphocyte populations (CD4+ T cells, CD19+ B cells, NK1.1+ NK cells, NK1.1+TCR+ NKT cells and CD4+Foxp3+ regulatory T cells [Tregs]) in blood, spleen and para-aortic lymph nodes following treatment with control (open boxes) or anti-HMGB1 neutralising antibodies (shaded boxes). Bar graphs represent means ± SEM of three mice in each group.

Figure III. Expression of proinflammatory mediators in atherosclerotic lesions following treatment with control (left) or anti-HMGB1 neutralizing antibodies (right). Top, photomicrographs of aortic sinus lesions stained with anti-VCAM-1 antibodies; middle:

aortic lesions stained with anti-MCP-1 antibodies and bottom real time PCR analyses of TNF-α, IFN-α, IL-6 and IL-1β expression in aortic atherosclerotic lesions. Bar graphs represent differences between the two groups. Red: control and blue: anti-HMGB1 antibody treatment. *P < 0.05 from control. Size bars on photomicrographs represent 100μm