Mesenchymal Stem Cells Attenuate NADPH Oxidase-Dependent High Mobility Group Box 1 Production and Inhibit Abdominal Aortic Aneurysms


Objective—Abdominal aortic aneurysm (AAA) formation is characterized by inflammation, smooth muscle activation, and matrix degradation. This study tests the hypothesis that macrophage-produced high mobility group box 1 (HMGB1) production is dependent on nicotinamide adenine dinucleotide phosphate oxidase (Nox2), which leads to increase in interleukin (IL)-17 production resulting in AAA formation and that treatment with human mesenchymal stem cells (MSCs) can attenuate this process thereby inhibiting AAA formation.

Approach and Results—Human aortic tissue demonstrated a significant increase in HMGB1 expression in AAA patients when compared with controls. An elastase-perfusion model of AAA demonstrated a significant increase in HMGB1 production in C57BL/6 (wild-type [WT]) mice, which was attenuated by MSC treatment. Furthermore, anti-HMGB1 antibody treatment of WT mice attenuated AAA formation, IL-17 production, and immune cell infiltration when compared with elastase-perfused WT mice on day 14. Elastase-perfused Nox2−/− mice demonstrated a significant attenuation of HMGB1 and IL-17 production, cellular infiltration, matrix metalloproteinase activity, and AAA formation when compared with WT mice on day 14. In vitro studies showed that elastase-treated macrophages from WT mice, but not from Nox2−/− mice, produced HMGB1, which was attenuated by MSC treatment. The production of macrophage-dependent HMGB1 involved Nox2 activation and superoxide anion production, which was mitigated by MSC treatment.

Conclusions—These results demonstrate that macrophage-produced HMGB1 leads to aortic inflammation and acts as a trigger for CD4+ T-cell–produced IL-17 during AAA formation. HMGB1 release is dependent on Nox2 activation, which can be inhibited by MSCs leading to attenuation of proinflammatory cytokines, especially IL-17, and protection against AAA formation. (Arterioscler Thromb Vasc Biol. 2016;36:908-918. DOI: 10.1161/ATVBAHA.116.307373.)

Key Words: aortic aneurysms, abdominal ◼ HMGB1 protein ◼ inflammation ◼ interleukins ◼ stem cells

Abdominal aortic aneurysm (AAA) is a significant medical problem with high mortality and a leading cause of death in elderly men.1 Currently, there are no directed therapies for aortic aneurysms which left untreated leads to an increased risk of aortic rupture. The pathobiology of AAAs remains poorly understood, but likely involves vascular inflammation characterized by leukocyte infiltration into the aortic wall associated with the production of many proinflammatory cytokines, as a part of the innate inflammatory response.2,3 It is during this stage that matrix-degrading enzymes are released leading to aortic wall elastin and collagen degradation, thereby causing AAA formation.4

Recent studies have shown that mesenchymal stem cells (MSCs) can effectively attenuate vascular inflammation and AAA formation.5,6 In a recent study using a murine elastase-perfused model of AAA, we demonstrated that CD4+ T-cell–produced interleukin (IL)-17 is a key proinflammatory cytokine regulating aortic inflammation, which can be attenuated by treatment with human MSCs.7 However, the exact signaling mechanism of MSC-mediated protection remains to be defined. Recent studies have demonstrated the ability of high mobility group box 1 (HMGB1), a damage-associated molecular pattern molecule, to be rapidly secreted by activated macrophages and to stimulate IL-17 production.4 In addition, blockade of HMGB1 has been shown to suppress the development of AAA formation in an animal model.7 Thus, in the present study, we investigated the role of the cross talk between macrophages and T cells, via HMGB1 and IL-17 secretion, respectively, as a pivotal axis important in vascular inflammation during AAA formation, which can be downregulated by MSCs.

Because reactive oxygen species in vascular tissue can regulate inflammation, we investigated the role of nicotinamide...
adenine dinucleotide phosphate (NADPH) oxidase in contributing to AAA formation via modulation of HMGB1 and IL-17 production. The NADPH oxidase (NOX) enzyme complex consists of transmembrane (eg, nicotinamide adenine dinucleotide phosphate oxidase 1-5 and p22phox) and cytosolic (p47phox, p67phox, and Rac) subunits, which assemble at the cell membrane and reduce molecular oxygen to superoxide anion by transferring electrons from NADPH. In particular, the nicotinamide adenine dinucleotide phosphate oxidase 2 isoform in phagocytic macrophages involves translocation and binding of the cytosolic subunits to the gp91phox/p22phox catalytic complex, which is facilitated and organized by p47phox. Previously, Miller et al10 have shown that NOX activity and its p47phox subunit are significantly upregulated in human atherosclerosis. Moreover, deletion of p47phox has been shown to attenuate oxidative stress and AAA formation in Ang II–infused apoE−/− mice.11

In the present study, we demonstrate that Nox2−/− mice have significantly decreased macrophage-dependent HMGB1 production and AAA formation. Furthermore, our findings demonstrate that human MSCs can attenuate NOX activity by decreasing superoxide anion production in macrophages leading to the mitigation of HMGB1 production. These results underline the importance of NOX-dependent release of HMGB1 by macrophages, which can upregulate IL-17 production and vascular inflammation, as well as loss of smooth muscle integrity leading to AAA formation. The mechanistic signaling pathway of MSC-mediated protection involves downregulation of Nox2 activation in macrophages to inhibit HMGB1 secretion, CD4+ T-cell–produced IL-17 leading to decreased inflammation and vascular remodeling, thereby offering protection against AAA.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
HMGB1 Expression is Increased in Human AAA
Protein levels of HMGB1 were significantly elevated in aortic tissue from AAA patients when compared with controls (50.7±4.07 versus 26.87±2.52 ng/mL; P<0.05; Figure 1A). Human aortic explants from male AAA patients were cultured in vitro with or without MSCs and transiently exposed to elastase. After 24 hours, a significant increase in HMGB1 expression was observed in cell culture supernatants after elastase treatment when compared with controls (57.22±4.24 versus 15.06±2.63 ng/mL; Figure 1B), which was significantly attenuated by MSC treatment (23.22±2.35 ng/mL). Human aortic explants treated with elastase also demonstrated a significant increase in matrix metalloproteinase (MMP) 2 and MMP9 activity that was attenuated by MSC treatment (Figure 1C–1E). These results indicate that HMGB1 may play an important proinflammatory role in human AAA and that MSCs have the ability to mitigate HMGB1 production in human aortic tissue.

MSCs Inhibit HMGB1 and AAA Formation in Murine Elastase-Perfusion Model
Using the elastase-perfusion model, aortic diameter was measured in wild-type (WT) mice treated with or without MSC treatment. Human umbilical cord MSCs were isolated and characterized as described in the Methods in the online-only Data Supplement (Figure I in the online-only Data Supplement). Elastase-perfused WT mice had a significant increase in aortic diameter when compared with heat-inactivated elastase controls (134.9±10.14% versus 50.98±3%; Figure 2A). There was no significant difference in aortic diameter in WT mice controls treated with or without MSCs. There was a significant decrease in aortic diameter on day 14 in elastase-perfused mice treated with MSCs when compared with elastase-perfused mice alone (99.39±8.44% versus 134.9±10.14%; P=0.02).

A significant increase in HMGB1 expression was observed in aortic tissue from elastase-perfused WT mice when compared with controls on day 14 (79.6±3.67 versus 10.58±1.64 ng/mL; P<0.05; Figure 2B). Treatment of WT mice with MSCs significantly attenuated HMGB1 expression in the elastase-perfused murine aortic tissue when compared with elastase-perfused mice alone (33.49±3.67 versus 79.6±3.67 ng/mL; P=0.001). Moreover, aortic tissue from elastase-perfused WT mice treated with MSCs showed a significant attenuation in MMP2 and MMP9 activity when compared with elastase-perfusion alone (Figure 2C–2E). These results suggest an important role of MSCs in the mitigation of aortic inflammation, matrix degradation, and AAA formation.

Macrophages Play a Key Role in AAA Formation via HMGB1 Production
Treatment of WT mice with anti-HMGB1 antibody significantly attenuated the elastase-perfused increase of aortic diameter at day 14 in WT mice when compared with elastase-perfused mice alone (70.43±6.34% versus 93.63±2.85%, respectively; P<0.05; Figure 3A). There was no significant difference in aortic diameter in WT mice controls treated with or without anti-HMGB1 antibody. A significant attenuation of IL-17 was observed in aortic tissue of anti-HMGB1 antibody-treated elastase-perfused WT mice when compared with elastase-perfused mice alone (47.64±5.28 versus 118.5±10.15 pg/mL, respectively; P<0.05; Figure 3B). Comparative histology and immunostaining of aortic tissue on day 14 showed a significant decrease in T lymphocytes (CD3+), macrophages, and neutrophils, as well as decrease in...
elastic fiber disruption in elastase-perfused WT mice treated with anti-HMGB1 antibody when compared with elastase-perfused mice (Figure 3C). Immunostaining with another macrophage-specific marker (ie, CD68) and HMGB1 in aortic tissue from elastase-perfused WT mice on day 14 also demonstrated colocalization of HMGB1 with macrophages (Figure II in the online-only Data Supplement). The respective IgG control antibodies showed negligible immunostaining for macrophages and HMGB1 (Figure III in the online-only Data Supplement).

Nox2 Mediates HMGB1 Production and AAA Formation

One feature of AAA pathogenesis is inflammation and vascular remodeling, which can be regulated by reactive oxygen species and oxidative stress. To determine whether oxidative stress via NOX can independently modulate the activation of HMGB1, Nox2−/− mice were exposed to elastase-perfusion AAA model. A significant attenuation of aortic diameter was observed in the elastase-perfused Nox2−/− mice when compared with elastase-perfused WT mice (83.14±9.33% versus 162.6±17.91% respectively; \( P < 0.05 \); Figure 4A). There was no significant difference in aortic diameter in Nox2−/− mice controls when compared with WT mice controls. Aortic tissue from elastase-perfused Nox2−/− mice demonstrated a significant attenuation in HMGB1 expression (Figure 4B) as well as MMP2 and MMP9 activity (Figure 4C–4E) when compared with elastase-perfused WT mice.

Furthermore, aortic tissue from Nox2−/− mice displayed a significant attenuation of proinflammatory cytokines, including IL-17, when compared with elastase-perfused WT mice (Figure 4F). Comparative histology and immunohistological analysis of aortic tissue also revealed a significant attenuation of inflammatory cell infiltration (CD3+ T cells, macrophages, and neutrophils) and a decrease in elastic fiber disruption in elastase-perfused Nox2−/− mice when compared with elastase-perfused WT mice (Figure 4G). HMGB1 expression was also markedly attenuated in the aortic tissue of elastase-perfused Nox2−/− mice when compared with WT mice on days 3, 7, and 14 by immunofluorescence via confocal microscopy (Figure 5). These results demonstrate that aortic diameter is significantly reduced in elastase-perfused Nox2−/− mice, and this is associated with concomitant mitigation of HMGB1 and IL-17 production, matrix degradation, and inflammatory cell infiltration.

Figure 1. Increased high mobility group box 1 (HMGB1) protein expression in human abdominal aortic aneurysm (AAA). A, Human aortic tissue demonstrated an increased expression of HMGB1 in AAA patients (n=16) compared with controls (n=8). B, Human aortic explants in culture treated with transient elastase treatment for 5 min and analyzed after 24 h showed a significant increase in HMGB1 production, which was significantly mitigated by mesenchymal stem cell (MSC) treatment (n=8 per group). C–E, Gelatin zymography of human aortic explant tissue and subsequent quantification of optical density (O.D.) demonstrates a significantly increased level of matrix metalloproteinase (MMP) 2 and MMP9 activity compared with controls and was attenuated by cocultures with MSCs (n=3–5 per group). Mean±SE; \(^*P<0.05\) vs other groups.
MSCs Suppress Macrophage and CD4+ T-Cell Activation to Attenuate HMGB1 and IL-17 Production

To determine the signaling events in Nox2-dependent mechanism of macrophage-produced HMGB1 and to decipher the cross talk between macrophages and CD4+ T cells via HMGB1/IL-17 axis, in vitro experiments were conducted. Primary CD11b+ macrophages (1×10⁵ cells) were grown in culture with or without MSCs and transiently (5 minutes) exposed to elastase (0.4 U/mL). Cells were washed with PBS, and fresh media was added. HMGB1 measurement in culture supernatants was performed after 24 hours. A significant increase in HMGB1 production was observed in elastase-treated WT macrophages, which were attenuated by coculture with MSCs (29.6±3.4 versus 12.8±2.9 ng/mL, respectively; P<0.05; Figure 6A). Moreover, elastase-treated macrophages from Nox2−/− mice showed a significantly decreased expression of HMGB1 when compared with elastase-treated WT macrophages (11.4±2.4 versus 29.6±3.4 ng/mL, respectively). There was no additional attenuation of HMGB1 levels by MSC treatment in elastase-perfused Nox2−/− macrophages when compared with elastase treatment alone (7.3±2.3 versus 11.4±2.4 ng/mL, respectively). These data suggest a prominent role of Nox2 in macrophage-dependent HMGB1 production.

Furthermore, NOX activity was assessed by measuring superoxide anion production in primary CD11b+ macrophages from WT mice. Elastase-treated WT macrophages had a significant increase in superoxide anion production, which was attenuated by coculture of macrophages with MSCs (Figure 6B). Elastase-exposed WT macrophages treated with apocynin and elastase-treated Nox2−/− macrophages displayed a significant decrease in superoxide anion production when compared with elastase-treated WT macrophages. These results demonstrate that MSCs can downregulate Nox2 activation, which then leads to decreased HMGB1 production.

CD4+ T cells were also separately treated with recombinant HMGB1 (10 ng/mL) for 24 hours and IL-17 production

Figure 2. Mesenchymal stem cells (MSCs) attenuate aortic diameter and high mobility group box 1 (HMGB1) expression in murine abdominal aortic aneurysm (AAA) model. A, Infrarenal mouse aortas were perfused with elastase (0.4 U/mL) or heat-inactivated elastase (control), and aortic diameter was measured on day 14. A multifold increase in aortic diameter observed in elastase-perfused WT mice compared with controls was significantly attenuated by MSC treatment (n=8 per group). B, A significant, multifold increase in HMGB1 expression was observed in aortic tissue of elastase-perfused wild-type (WT) mice compared with controls. Treatment of elastase-perfused WT mice with MSC significantly attenuated HMGB1 expression compared with elastase-perfused mice alone (n=8 per group). C–E, Gelatin zymography of murine aortic tissue and subsequent quantification of optical density (O.D.) demonstrates a significantly increased level of matrix metalloproteinase (MMP) 2 and MMP9 activity compared with controls and was attenuated by treatment with MSCs (n=3–5 per group). Mean±SE; *P<0.05 vs control; #P<0.05 vs elastase.
in the supernatants was measured. Elastase or HMGB1 treatment of CD4+ T cells significantly increased the production of IL-17, which was significantly attenuated by coculture of CD4+ T cells with MSCs (Figure 6C). There was no production of either IL-17 by macrophages or HMGB1 production by CD4+ T cells after elastase treatment (Figure IV in the online-only Data Supplement). Also, recombinant IL-17 treatment of CD11b+ macrophages did not induce HMGB1 production (Figure IV in the online-only Data Supplement), whereas recombinant HMGB1 treatment of CD4+ T cells induces IL-17 production (Figure 6C).

Conditioned media transfer experiments were performed to evaluate the potential cross talk between macrophages and CD4+ T cells via HMGB1-mediated signaling (Figure 6D). Conditioned media transfer from elastase-exposed WT macrophages to WT CD4+ T cells resulted in a significant enhancement of IL-17 production when compared with controls. Coclurures of MSCs with elastase-exposed macrophages and subsequent conditioned media transfer to WT CD4+ T cells significantly attenuated the CD4+ T-cell–produced IL-17 production. Similarly, treatment of elastase-exposed macrophages with anti-HMGB1 antibody and subsequent conditioned media transfer to WT CD4+ T cells blocked the CD4+ T-cell–dependent IL-17 production (Figure 6D). These results signify the importance of the cross talk between macrophages and CD4+ T cells via HMGB1 and IL-17 to induce aortic

**Figure 3.** High mobility group box 1 (HMGB1) inhibition leads to decreased abdominal aortic aneurysm (AAA) formation. A, Treatment of elastase-perfused wild-type (WT) mice with anti-HMGB1 antibody significantly attenuates the increase in aortic diameter observed in elastase-perfused WT mice on day 14. B, Interleukin (IL)-17 production in the aortic tissue on day 14 is attenuated in elastase-perfused WT mice treated with anti-HMGB1 antibody compared with elastase-perfused WT mice alone. C, Comparative histology and immunohistochemistry performed on day 14 indicates that WT mice treated with anti-HMGB1 antibody have a marked decrease in CD3+ T-cell, neutrophil (PMN) and macrophage (Mac-2) infiltration, as well as decrease in elastic fiber disruption (Verhoeff-Van Gieson staining for elastin) compared with elastase-perfused WT mice. Arrows indicate areas of immunostaining. Scale bars, 50 μm. n=5 mice per group; *P<0.05 vs control, #P<0.05 vs elastase+IgG.
Figure 4. Abdominal aortic aneurysm (AAA) formation is attenuated in nicotinamide adenine dinucleotide phosphate oxidase (Continued)
inflammation, which is markedly downregulated by MSCs via their actions on both macrophages and CD4+ T cells (Figure 6E).

**Discussion**

The present study demonstrates that Nox2 is a key regulator of HMGB1 production and subsequent inflammation in an animal model of AAA, and this can be mitigated by the treatment with MSCs. An increased expression of HMGB1 was observed in aortic tissue from human male AAA patients, and further investigation into the importance of HMGB1 and its induction of other proinflammatory cytokines, ie, IL-17, was then conducted using in vivo and in vitro studies. Aortic diameter and proinflammatory cytokine production were significantly attenuated in aortic tissue of elastase-perfused WT mice treated with anti-HMGB1 antibody. Furthermore, elastase-perfused Nox2−/y mice demonstrated a significant attenuation of aortic diameter, HMGB1 expression, proinflammatory cytokine production, matrix degradation, as well as decreased immune cell infiltration and preservation of aortic morphology. In vitro studies confirmed that elastase treatment modulates activation of macrophages to produce HMGB1 in an Nox2-dependent manner. Moreover, MSCs downregulated superoxide anion production by mitigation of Nox2 activation to inhibit HMGB1 production in elastase-treated macrophages. The ability of macrophage-produced HMGB1 to amplify IL-17 generation by CD4+ T cells demonstrates the critical signaling cross talk between these immune cells, which can be markedly reduced by immunomodulation of the Nox2 signaling pathway by MSCs.

Recent studies demonstrate an important role for macrophage-produced HMGB1 in vascular inflammation and its ability to rapidly cause cellular inflammation.12,13 HMGB1 has been shown to act as a danger signal that mediates cross talk between immune cells resulting in initiation of inflammation and injury.14,15 Immune cells, such as macrophages and monocytes, actively release HMGB1 in response to exogenous (ie, bacterial endotoxin) or endogenous host stimuli (tumor necrosis factor-α, IFN-γ, or H2O2).16–18 On release, it can bind to several receptors including receptor for advanced glycation end products, toll-like receptor-2, and toll-like receptor-4, thereby triggering cell signaling pathways involving mitogen-activated protein kinases, nuclear factor-κB, or PI3K/Akt to mediate cell migration, activation, proliferation, and differentiation.8,19,20 Recent studies have elucidated an increased expression of HMGB1 in aortic atherosclerosis in human and animal studies underlining the importance of HMGB1 and its receptors in regulating aortic inflammation.21,22 In the present study, we defined a crucial aspect of macrophage-mediated aortic inflammation via HMGB1, which upregulates CD4+
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T-cell–dependent IL-17 to initiate a signaling cascade resulting in AAA formation. HMGB1/toll-like receptor-4 signaling has also been shown to activate NOX activation in circulating neutrophils to mediate hemorrhagic shock/resuscitation. However, in our in vivo and in vitro studies, we demonstrated a critical link between Nox2 activation and HMGB1 release in macrophages. Thus, ROS generation via Nox2 can lead to increased HMGB1 production in activated, viable immune cells, and upon release, HMGB1 retains its proinflammatory cytokine activity to further induce inflammation.

ROS production is a major mediator of the inflammatory cascade, which is responsible for the pathogenesis of vascular inflammation and AAA formation. One of the key enzymes for ROS production is the NOX complex, which is a highly regulated membrane-bound enzyme complex that catalyzes the production of superoxide by the single electron reduction of oxygen using NADPH as the electron donor. In particular, a major contributor to redox imbalance is H$_2$O$_2$, which is converted from superoxide and can induce both active and passive release of HMGB1 from macrophages. Previous findings have established a key role for H$_2$O$_2$-dependent oxidative stress in inducing active HMGB1 release in macrophage/monocyte cultures via a mitogen-activated protein kinase- and chromosome region maintenance 1–dependent mechanism. Our present study suggests that Nox2 activation regulates HMGB1 release in macrophages, which is likely mediated by the p47phox subunit of the NOX complex. In fact, Aoki et al demonstrated that p47phox was mainly expressed in macrophages and upregulated during cerebral aneurysm formation. On the contrary, a recent study by Kigawa et al showed that NOX deficiency can exacerbate angiotensin-II–induced AAA formation in mice. This unexpected occurrence of hyperinflammation by Nox2 deficiency is contrary to our findings where elastase-perfused Nox2−/− mice were significantly protected.
Figure 6. Mesenchymal stem cells (MSCs) inhibit nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation in macrophages to attenuate high mobility group box 1 (HMGB1) production. A, CD11b+ macrophages from wild-type (WT) and nicotinamide adenine dinucleotide phosphate oxidase (Nox) 2−/y mice were exposed to transient exposure with elastase (0.4 U/mL for 5 min) and HMGB1 secretion was measured in cell culture supernatants after 24 h. A multifold increase in HMGB1 production was observed in WT macrophages compared with controls and was significantly attenuated by cocultures with MSCs. Also, elastase-treated Nox2−/y macrophages had a significant decrease in HMGB1 expression compared with elastase-treated WT macrophages. n=8 per group; *P<0.05 vs control; #P<0.05 vs WT elastase.

B, NADPH oxidase activity was measured by quantification of superoxide anion production in cell culture supernatants of WT and Nox2−/y-derived macrophages. Elastase-treated WT macrophages displayed a significant increase in superoxide anion production compared with controls, and this was attenuated by MSCs. However, there was no significant induction of NADPH oxidase activation in WT macrophages treated with apocynin or in elastase-treated Nox2−/y-derived macrophages. n=8 per group; *P<0.05 vs control; #P<0.05 vs elastase.

C, CD4+ T cells purified from WT mice were grown in culture with or without MSCs and treated with either recombinant (r)HMGB1 (10 ng/mL), transient elastase treatment or both. Cell culture supernatants were analyzed for interleukin (IL)-17 expression. rHMGB1 or elastase treatment stimulate CD4+ T cells to produce IL-17, which is significantly attenuated by MSCs. n=8 per group; *P<0.05 vs control; #P<0.05 vs elastase alone; ##P<0.05 vs rHMGB1; *P<0.05 vs elastase and δP<0.05 vs elastase+rHMGB1.

D, IL-17 production was significantly enhanced on conditioned media transfer (CMT) from elastase-exposed WT macrophages (MΦElastase) to WT CD4+ T cells compared with controls. Co-culture of MSCs with elastase-exposed macrophages blocked the enhancement of IL-17 production after CMT to CD4+ T cells. Pretreatment of elastase-exposed macrophages with anti-HMGB1 (1 μg/mL) significantly attenuated IL-17 production after CMT to CD4+ T cells. *P<0.05 vs control; #P<0.05 vs MΦElastase→CD4+ T; n=8 per group.

E, (Continued)
from AAA. The apparent contrast of Nox2 signaling in aortic aneurysms between the 2 studies could be attributed to differences in the mouse models as Kigawa et al. induced neutrophil-mediated inflammation in atherogenic mice that were fed a high cholesterol diet when compared with the elastase-perfusion model used in our study. The complex heterogeneity between different Nox isoforms and the deleterious role of free radicals in T cells and macrophages may account for the phenotypic differences in Nox2−/− mice between the 2 models of AAA. However, our findings are in conjunction with previous studies wherein the deletion of p47phox subunit of Nox results in protection from cerebral and aortic aneurysm formation as shown in animal models using p47phox−/− mice. Our study demonstrates the ability of HMGB1 to induce proinflammatory cytokines during the pathogenesis of AAA formation and specifically decipher the cross talk between macrophages and CD4+ T cells, as Nox2-dependent HMGB1 augmented CD4+ T-cell–produced IL-17, which leads to vascular remodeling and AAA formation. Importantly, we present evidence that human MSCs have the unique ability to immunomodulate Nox2 activation and downregulate HMGB1 and subsequent CD4+ T-cell–produced IL-17, thereby mitigating inflammation and AAA formation. MSCs can also inhibit CD4+ T-cell–produced IL-17 via its direct actions on T cells, which may involve Nox2-dependent mechanisms.

In summary, our results highlight the mechanistic aspect of MSC therapy for AAA treatment via its direct effects on macrophage-dependent inflammation. The initiation of aortic inflammation by key regulatory cytokines, i.e., macrophage-produced HMGB1 and CD4+ T-cell–produced IL-17, can lead to immune cell infiltration, upregulation of MMPs, aortic smooth cell remodeling, thereby causing AAA formation. The ability of MSCs to modulate the pivotal initial inflammatory triggers is essential to attenuate the molecular pathogenesis of AAA formation. The multiple effects of MSCs on targeting the Nox2 pathway in macrophages and inhibition of the HMGB1/IL-17 axis demonstrate the multifactorial capacities of MSCs as a nonsurgical therapeutic strategy for the treatment of aortic aneurysms. Additional studies are needed to decipher the impact of MSC-mediated upregulation of specific paracrine factors in the modulation of Nox subtypes on AAA formation.

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Disclosures
None.

References
Significance

Abdominal aortic aneurysm (AAA) remains a significant clinical problem in the elderly population. Although most patients are asymptomatic, the risk of mortality increases caused by aortic rupture on expansion of the aneurysm. Currently, the only definitive treatment is surgical intervention, and a therapeutic approach to treat AAs remains to be defined. In this study, we determined that high mobility group box 1, a proinflammatory cytokine secreted by macrophages, regulates AAA formation via upregulation of CD4+ T-cell–dependent interleukin-17 production. Furthermore, we observed that high mobility group box 1 production is dependent on nicotinamide adenine dinucleotide phosphate oxidase complex as Nox2−/− mice demonstrate a significant reduction in high mobility group box 1 production and AAA formation. Finally, our results further define a key role for human mesenchymal stem cells in decreasing high mobility group box 1 production via inhibition of Nox2 activation, thereby leading to inhibition of AAA growth.
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Ashish K. Sharma, Morgan D. Salmon, Guanyi Lu, Gang Su, Nicolas H. Pope, Joseph R. Smith, Mark L. Weiss and Gilbert R. Upchurch, Jr

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**Materials and Methods**

**Human Aortic Tissue Analysis**
Collection of human aortic tissue was approved by the University of Virginia’s Institutional Review Board (#13178). Preoperative consent was obtained from all patients. AAA tissue from male patients was resected during open surgical AAA repair, and abdominal aortic tissue was obtained from transplant donor patients to serve as controls. Tissue was washed in phosphate-buffer saline and homogenized using gentleMACS Dissociator (Miltenyi Biotec, San Diego, CA) in a 1X cell lysis buffer with protease inhibitor cocktail (BioVision, Inc., Milpitas, CA). The homogenates were centrifuged for 10 minutes at 10,000rpm at 4°C and quantified for protein concentration using BCA protein assay kit (Life Technologies, Grand Island, NY). Also, aortic explants from human AAA male patients were cultured on 25-mm plates using basement membrane matrigel (BD Biosciences). Aortic explants were transiently treated with elastase (0.4U/ml for 5 minutes) with or without co-cultures with MSCs (1x10^5 cells) and culture supernatants were collected after 24 hrs for further analysis.

**Animals**
All animal experimentation was approved by the University of Virginia’s Institutional Animal Care and Use Committee (Protocol # 3848). Male C57BL/6 and Nox2-/- mice (8-12 weeks of age) were obtained from Jackson Laboratories (Bar Harbor, ME).

**Elastase Perfusion Model of Aneurysm Formation**
A murine elastase perfusion model of AAA formation was used as previously described. Briefly, the infrarenal abdominal aorta was isolated *in situ* and perfused with porcine pancreatic elastase (Sigma, 0.4 U/mL) for 5 minutes at a pressure of 100 mm Hg. Control animals were perfused with heat-inactivated elastase for 5 minutes. WT mice were also treated with either MSCs (1x10^6 cells intravenously), IgG isotype control or anti-HMGB1 antibody (20 µg intravenously; Sigma-Aldrich, St. Louis, MO) on day 1 after perfusion. Video micrometric measurements of aortic diameters were made *in situ* before perfusion, after perfusion, and before harvesting the aorta on day 14.

**Enzyme-Linked Immunosorbent Spot Assay**
Primary CD4+ T cells and CD11b+ F4/80+ macrophages were purified from mouse spleens using a magnetic bead-based cell isolation kit following the manufacturer’s protocol (Miltenyi Biotec, San Diego, CA). Briefly, a single cell suspension from mouse spleens was prepared using the gentleMACS dissociator and CD4+ T cells or CD11b+ macrophages were isolated from the single cell suspensions using the respective isolation kits. Cells were fluorescently stained with CD4+ or CD11b+ cell cocktail and analyzed by flow cytometry after cell debris and dead cell exclusion from analysis based on scatter signals and propidium iodide fluorescence. We routinely achieved a relative enrichment of CD4+ T cells and CD11b+ macrophages of >90% using this selection process. 1x10^5 CD4+ T cells or CD11b+ macrophages were plated in 48-well culture dishes with or without co-cultures with MSCs. Cells were exposed to transient elastase treatment for 5 minutes followed by washing the cells with PBS and replacing the media. After 24hrs, cell culture supernatants were collected and analyzed. Conditioned
media transfer (CMT) experiments were performed by exposing CD11b+ macrophages to transient elastase treatment for 5 minutes followed by washing the cells with PBS and replacing the media with or without 10 μg/ml anti-HMGB1 antibody treatment or co-cultures with MSCs. After 24 hrs the conditioned culture media was transferred to CD4+ T cells and was analyzed for IL-17A (R&D Systems, Minneapolis, MN) production by ELISA as instructed by the manufacturer’s protocol.

**Cytokine Measurements**

Cytokine content in murine aortic tissue homogenates was quantified using the Bioplex Bead Array technique using a multiplex cytokine panel assay (Bio-Rad Laboratories, Hercules, CA) per the manufacturer’s instructions.

**HMGB1 Measurement**

HMGB1 was measured in cell culture supernatants using an ELISA kit per the manufacturer’s instructions (IBL International, Hamburg, Germany).

**Human Mesenchymal Stem Cell Isolation and Characterization**

Human umbilical cord-derived mesenchymal stem cells (MSCs) were isolated from Wharton’s jelly. Umbilical cord derived MSCs were differentiated with StemPro differentiation kits for chondrogenesis, adipogenesis and osteogenesis following the protocols included with the kits (Life Technologies, Grand Island, NY). Further characterization of MSCs done by flow cytometry confirms a pattern consistent with MSC population showing an expression of CD90, CD73, CD105 and CD44. MSCs lacked expression of CD45, CD34, CD11b, CD19, and HLA-DR. 1x10^6 MSCs were injected intravenously into WT mice via tail vein injection on day 1 in the *in vivo* experiments. After isolation and subsequent culture, early-passage MSCs were plated in a 48-well plate at a density of 1x10^6 cells per designated well for the *in vitro* assays.

**Histology and Immunohistochemistry**

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**Zymography for Matrix Metalloproteinases**

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**NADPH oxidase activity**

NADPH oxidase activity was measured by a chemiluminescence-based assay in WT and Nox2^−/−^ -derived primary CD11b+ macrophage cell culture supernatants using a Lumimax Superoxide Anion Detection Kit (Agilent Technologies, Santa Clara, CA) as per the manufacturer’s instructions. Apocynin (600µM) treatment of WT-derived macrophages was also used as a control to inhibit NADPH oxidase-dependent superoxide anion production.

**Statistical Analysis**

Values are presented as the mean ± standard error of the mean (SEM) and statistical evaluation was performed using GraphPad Prism 6 software. One-way analysis of variance after post hoc Tukey’s test was used to determine the differences among multiple comparative groups. The Mann-Whitney U test was applied on small-size comparisons with non-normal distributions between groups. The t-test was applied only on experiments with normal distributions between the comparison groups. A P value less than 0.05 was considered statistically significant.

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Materials and Methods

Human Aortic Tissue Analysis
Collection of human aortic tissue was approved by the University of Virginia’s Institutional Review Board (#13178). Preoperative consent was obtained from all patients. AAA tissue from male patients was resected during open surgical AAA repair, and abdominal aortic tissue was obtained from transplant donor patients to serve as controls. Tissue was washed in phosphate-buffer saline and homogenized using gentleMACS Dissociator (Miltenyi Biotec, San Diego, CA) in a 1X cell lysis buffer with protease inhibitor cocktail (BioVision, Inc., Milpitas, CA). The homogenates were centrifuged for 10 minutes at 10,000rpm at 4ºC and quantified for protein concentration using BCA protein assay kit (Life Technologies, Grand Island, NY). Also, aortic explants from human AAA male patients were cultured on 25-mm plates using basement membrane matrigel (BD Biosciences). Aortic explants were transiently treated with elastase (0.4U/ml for 5 minutes) with or without co-cultures with MSCs (1x10⁵ cells) and culture supernatants were collected after 24 hrs for further analysis.

Animals
All animal experimentation was approved by the University of Virginia’s Institutional Animal Care and Use Committee (Protocol # 3848). Male C57BL/6 and Nox2⁻/⁻ mice (8-12 weeks of age) were obtained from Jackson Laboratories (Bar Harbor, ME).

Elastase Perfusion Model of Aneurysm Formation
A murine elastase perfusion model of AAA formation was used as previously described¹. Briefly, the infrarenal abdominal aorta was isolated in situ and perfused with porcine pancreatic elastase (Sigma, 0.4 U/mL) for 5 minutes at a pressure of 100 mm Hg. Control animals were perfused with heat-inactivated elastase for 5 minutes. WT mice were also treated with either MSCs (1x10⁶ cells intravenously), IgG isotype control or anti-HMGB1 antibody (20 µg intravenously; Sigma-Aldrich, St. Louis, MO) on day 1 after perfusion. Video micrometric measurements of aortic diameters were made in situ before perfusion, after perfusion, and before harvesting the aorta on day 14.

Enzyme-Linked Immunosorbent Spot Assay
Primary CD4⁺ T cells and CD11b⁺ F4/80⁺ macrophages were purified from mouse spleens using a magnetic bead-based cell isolation kit following the manufacturer’s protocol (Miltenyi Biotec, San Diego, CA). Briefly, a single cell suspension from mouse spleens was prepared using the gentleMACS dissociator and CD4⁺ T cells or CD11b⁺ macrophages were isolated from the single cell suspensions using the respective isolation kits. Cells were fluorescently stained with CD4⁺ or CD11b⁺ cell cocktail and analyzed by flow cytometry after cell debris and dead cell exclusion from analysis based on scatter signals and propidium iodide fluorescence. We routinely achieved a relative enrichment of CD4⁺ T cells and CD11b⁺ macrophages of >90% using this selection process. 1x10⁵ CD4⁺ T cells or CD11b⁺ macrophages were plated in 48-well culture dishes with or without co-cultures with MSCs. Cells were exposed to transient elastase treatment for 5 minutes followed by washing the cells with PBS and replacing the media. After 24hrs, cell culture supernatants were collected and analyzed. Conditioned
media transfer (CMT) experiments were performed by exposing CD11b+ macrophages to transient elastase treatment for 5 minutes followed by washing the cells with PBS and replacing the media with or without 10 μg/ml anti-HMGB1 antibody treatment or co-cultures with MSCs. After 24 hrs the conditioned culture media was transferred to CD4+ T cells and was analyzed for IL-17A (R&D Systems, Minneapolis, MN) production by ELISA as instructed by the manufacturer’s protocol.

**Cytokine Measurements**
Cytokine content in murine aortic tissue homogenates was quantified using the Bioplex Bead Array technique using a multiplex cytokine panel assay (Bio-Rad Laboratories, Hercules, CA) per the manufacturer’s instructions.

**HMGB1 Measurement**
HMGB1 was measured in cell culture supernatants using an ELISA kit per the manufacturer’s instructions (IBL International, Hamburg, Germany).

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