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ApoE^{-/-} Mice Lacking Hemopexin Develop Increased Atherosclerosis *via* Mechanisms That Include Oxidative Stress and Altered Macrophage Function

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Abstract

Objective—We previously reported that Hemopexin (Hx), a heme scavenger, is significantly increased and associated with proinflammatory HDL under atherogenic conditions. Although it is established that Hx together with macrophages plays a role in mitigating oxidative damage, the role of Hx in the development of atherosclerosis is unknown.

Approach and Results—We used Hx and apoE double knockout mice (HxE^{-/-}) to determine the role of Hx in the development of atherosclerosis. HxE^{-/-} mice had significantly more free heme, ROS, and proinflammatory HDL in their circulation, when compared to control apoE^{-/-} mice. Atherosclerotic plaque area (apoE^{-/-} = $9.72 \pm 2.5 \times 10^4 \mu\text{m}^2$ and HxE^{-/-} = $27.23 \pm 3.6 \times 10^4 \mu\text{m}^2$) and macrophage infiltration (apoE^{-/-} = $38.8 \pm 5.8 \times 10^3 \mu\text{m}^2$ and HxE^{-/-} = $103.4 \pm 17.8 \times 10^3 \mu\text{m}^2$) in the aortic sinus were significantly higher in the HxE^{-/-} mice. Atherosclerotic lesions in the aortas were significantly higher in the HxE^{-/-} mice compared to apoE^{-/-} mice. Analysis of polarization revealed that macrophages from HxE^{-/-} mice were more M1-like. *Ex vivo* studies demonstrated that HxE^{-/-} macrophage cholesterol efflux capacity was significantly reduced when compared to apoE^{-/-} mice. Injection of human Hx (hHx) into HxE^{-/-} mice reduced circulating heme levels and hHx pretreatment of naïve bone marrow cells *ex vivo* resulted in a shift from M1-like to M2-like macrophages.

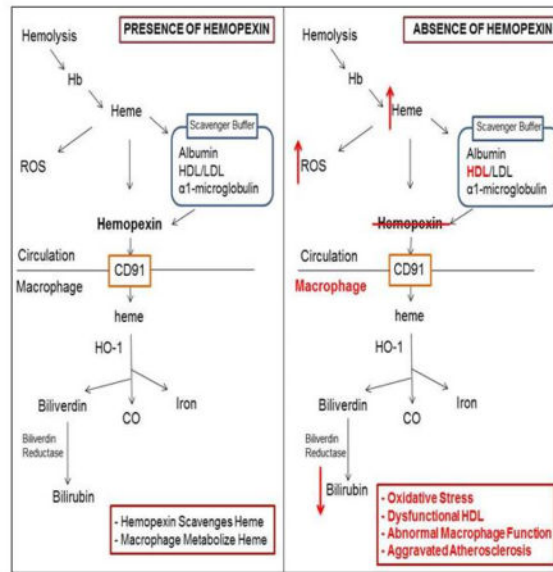
Conclusion—We conclude that Hx plays a novel protective role in alleviating heme induced oxidative stress, improving inflammatory properties of HDL, macrophage phenotype and function, and inhibiting the development of atherosclerosis in apoE^{-/-} mice.

Graphical abstract

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DISCLOSURES

MN, AMF and STR are principals in Bruin Pharma and AMF is an officer in Bruin Pharma.



Keywords

Heme; ROS; Inflammation; Macrophages/monocytes; ABCA1; Cholesterol/Efflux

INTRODUCTION

Atherosclerosis is a chronic and systemic vascular inflammatory process that forms the pathological basis of coronary artery disease (CAD) (1). The etiology of atherosclerosis is multi-faceted. Accumulation of reactive oxygen species (ROS) resulting in increased systemic oxidative stress and altered inflammatory properties of low-density lipoproteins (LDL) and high-density lipoproteins (HDL), are key mediators of the development of atherosclerosis.

Watanabe *et al.* (2) reported that hemoglobin (Hb) is associated with HDL in mouse models of atherosclerosis and demonstrated that Hb when associated with HDL is predominantly in the oxyHb form and can effectively consume nitric oxide and contract arterial vessels *ex vivo* (2). Hb is the most abundant and functionally important protein in erythrocytes (3). Under normal conditions, the reactivity of heme is controlled by its insertion into the “heme pockets” of Hb. Under oxidative stress however, Hb can release the heme. Haptoglobin (Hp) and Hemopexin (Hx) are acute phase proteins, produced by the liver, with the highest binding affinity for Hb ($K_d \sim 1\text{pM}$) and heme ($K_d < 1\text{pM}$), respectively (5, 6). Watanabe *et al.* (4) reported that Hb scavenger proteins, Hp and Hx are significantly increased in apoA-I-containing particles of HDL, both in mouse models of atherosclerosis and in CAD patients.

Andrew Levy’s group pioneered studies on Hp. Hp has been studied extensively both at the genetic and biochemical level in the context of diabetes and its macrovascular complications (7, 8). Andrew Levy and colleagues demonstrated that among the three Hp genotypes; Hp 1-1, Hp 2-1, and Hp 2-2, the Hp 2-2 genotype is associated with increased cardiovascular

disease in diabetes (7, 8). In humans, the potential for increased oxidative damage caused by the Hp 2-2-hemoglobin complex is attributed to elevated levels of Hp 2-2 in the circulation (9). Transgenic mice with diabetes and the Hp 2-2 genotype have higher levels of oxidative stress, greater macrophage infiltration and more atherosclerotic plaques (10). However, relatively very little is known on Hp's heme-scavenging counterpart Hx, which plays an important role in heme elimination.

Hx is a 60-KDa plasma glycoprotein and is considered to be a major transport vehicle of heme into macrophages, thereby inhibiting heme-mediated ROS production and preventing both heme-catalyzed and ROS mediated oxidative damage (11, 12). Cytotoxic properties of free heme are thought to be involved in many complex cellular mechanisms: release of redox-active iron, production of superoxide and hydroxyl radicals; and peroxidation of membrane lipids (13). A number of diseases are associated with free heme and lead to a state of endothelial dysfunction. This leads to enhanced expression of adhesion molecules on the endothelium, high levels of circulating proinflammatory cytokines and activated leukocytes, and, monocyte recruitment (14–16). Heme is a major source for the generation of ROS. High levels of ROS lead to lipid, protein, and DNA damage and eventually to cell death, and they too favor endothelial activation (17, 18) and leukocyte recruitment, thus promoting a chronic inflammatory state. The primary defense for cells against heme toxicity is currently thought to be provided by complexing of heme with Hx (19).

The heme-Hx complex is taken up by cells through receptor-mediated endocytosis. LRP/CD91 receptor on macrophages mediates the internalization of the heme-Hx complex, which induces the expression of anti-oxidant hemeoxygenase enzymes (HO-1 and HO-2). Heme is then catabolized by the heme oxygenase enzymes (20) to generate biliverdin, free iron and carbon monoxide as reaction products (21, 22). Biliverdin is processed further to bilirubin, a direct antioxidant (22). Another important recently discovered effect of heme internalization is the co-induction of Liver X Receptor (LXR) genes along with HO-1 via a common transcription factor in order to maintain the lipid-iron homeostasis in macrophages (23). Vinchi *et al.* have shown that Hx therapy can improve cardiovascular function by preventing heme-induced endothelial toxicity in mouse models of hemolytic diseases (24). Thus Hx serves to regulate the balance between free heme and bound heme, and/or to regulate heme degradation.

The role of Hx in the development of atherosclerosis has not been studied to date. We generated Hx^{-/-} mice on an apoE^{-/-} background (HxE^{-/-}) in order to determine the role of Hx in the development of atherosclerosis. Our results show that Hx protects against the development of atherosclerosis in apoE^{-/-} mice. We show that Hx deficiency results in increased oxidative stress and proinflammatory HDL in HxE^{-/-} mice. Furthermore, during our investigations to examine the effect of Hx deficiency on macrophage function, we discovered a novel role for Hx in determining macrophage phenotype.

MATERIALS AND METHODS

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

RESULTS

Hepatic heme detoxification is dysregulated in apoE^{-/-} mice lacking hemopexin

We observed significantly higher levels of heme in the serum (Figure 1A) and HDL fractions (Figure 1B) of HxE^{-/-} mice compared to apoE^{-/-} mice (n = 8–10). Levels of bilirubin, a heme breakdown product (Figure 1C) and albumin (Figure 1D) were significantly lower in the serum of HxE^{-/-} mice compared to apoE^{-/-} mice. The serum samples were collected with extreme care to avoid hemolysis during retro-orbital bleeding of the mice, which could potentially account for higher heme levels. The levels of Hb are slightly although not significantly elevated (Supplemental Figure II A) compared to their matched control apoE^{-/-} serum samples. The mean Corpuscular Hb, which is the average mass of hemoglobin per RBC, is significantly elevated in the HxE^{-/-} mice (Supplemental Figure II B) although the RBC levels were the same (Supplemental Figure II C) in both the groups of mice, indicating endogenous hemolysis. Furthermore, intraperitoneal administration of human Hx (hHx) to HxE^{-/-} mice for 24 hours significantly reduced the levels of heme in the serum of the mice (Figure 1E) suggesting that Hx regulates circulating heme levels.

Hepatic gene expression analysis was performed to determine the levels of the scavenger receptor CD91 and heme catabolizing enzymes HO-1 and HO-2. We recorded significantly lower gene expression levels of CD91, HO-1 and HO-2 in the livers of HxE^{-/-} mice compared to apoE^{-/-} mice (Figure 1F). Significantly lower gene expression of albumin in the liver of HxE^{-/-} mice (Figure 1F) supported the lower levels of albumin in the serum of HxE^{-/-} mice (despite the lower levels of albumin in the HxE^{-/-} mice compared to the apoE^{-/-} mice, the levels were within the normal range reported for C57BL/6J mice as shown in Materials and Methods). Together, these data indicated that lack of Hx in serum significantly affects the heme detoxifying potential of the liver. These results showed that Hx deficiency causes excess heme accumulation and reduced levels of anti-oxidants (bilirubin) in the circulation.

ApoE^{-/-} mice lacking hemopexin have higher oxidative stress and impaired HDL function

Cell-free heme promotes the generation of ROS, which is involved in the pathophysiology of several disorders (25, 26). We estimated levels of ROS by a cell free assay using DCFH₂. The relative fluorescence units (RFUs), which are reflective of the levels of ROS, were significantly higher in the serum and HDL of HxE^{-/-} mice compared to apoE^{-/-} mice. Individual serum samples were used to estimate ROS levels in circulation (Figure 2A). Additionally, serum was pooled from the apoE^{-/-} and HxE^{-/-} mice (n=6–8) and lipoproteins were separated using FPLC. ROS levels were estimated on all the FPLC fractions. HDL fractions from HxE^{-/-} mice exhibited significantly higher levels of ROS compared to the HDL fractions from apoE^{-/-} mice (Figure 2B). We estimated LCAT activity on the same HDL fractions and observed significantly lower LCAT activity in HDL from HxE^{-/-} mice compared to apoE^{-/-} mice (Figure 2C). Increased heme content and ROS levels associated with HDL and reduced LCAT activity further led us to determine HDL functionality by measuring anti- vs. proinflammatory activity of HDL. This was measured *ex vivo* by a monocyte migration assay as described in Materials and Methods. We observed a significantly higher degree of monocyte migration from the trans-wells treated with the

conditioned media collected from human aortic endothelial cells treated with HDL from HxE^{-/-} mice compared to HDL from apoE^{-/-} mice (Figure 2D). This demonstrated that HDL from HxE^{-/-} mice was more proinflammatory compared to the HDL from apoE^{-/-} mice. In addition we demonstrated that apoE^{-/-} mice lacking hemopexin had higher oxidative stress in the livers as measured by significantly higher malondialdehyde levels (Figure 2E) compared to the control apoE^{-/-} mice (n=7–8). However, levels of alanine transaminase (ALT) and aspartate transaminase (AST) enzymes (Supplemental Figure III A and B) were similar between the two groups of mice. Our results show that Hx deficiency in apoE^{-/-} mice leads to accumulation of proinflammatory HDL and higher systemic oxidative stress.

Hemopexin ablation in apoE^{-/-} mice causes increased atherosclerosis

We investigated the effect of Hx ablation on the extent of atherosclerosis in apoE^{-/-} mice. Six-month-old female mice (n = 13 apoE^{-/-}, n = 12 HxE^{-/-} mice) maintained on a chow diet were euthanized after overnight fasting. The HxE^{-/-} mice showed a higher Oil-Red-O staining of the sinus, indicative of an increase in the plaque area in the heart, compared to the control apoE^{-/-} mice (Figure 3A, representative images). Quantitative analysis showed a significant increase in plaque area in the HxE^{-/-} mice compared to the apoE^{-/-} mice. On average, a three-fold increase in the area of plaque was observed in the double knockout HxE^{-/-} mice compared to its respective control (Figure 3B). To examine the extent of atherosclerotic lesions, *en face* analysis was performed on paraformaldehyde fixed aortas from the two groups of mice (n = 24 apoE^{-/-}, n = 25 HxE^{-/-} mice). Figure 3C shows representative images of the whole aorta stained for lesions using Sudan IV. There was a significant increase in aortic atherosclerosis in the HxE^{-/-} mice compared to the apoE^{-/-} mice (Figure 3D). These results demonstrated that Hx deficiency in apoE^{-/-} mice causes increased atherosclerosis. Macrophage infiltration is a hallmark for the onset of atherosclerosis. Immunohistochemistry using macrophage marker CD68 was performed on sections of aortic sinus to examine the extent of macrophage content in the lesions (n = 11 apoE^{-/-}, n = 15 HxE^{-/-} mice). Morphometric analysis for antibody stained area revealed an augmentation of macrophage accumulation in the double knockout mice compared to control mice (Figure 3E, representative images). A two-fold increase in macrophage content in the plaque area was observed in the HxE^{-/-} mice compared to apoE^{-/-} mice (Figure 3F). Taken together, these results demonstrated that HxE^{-/-} mice develop augmented atherosclerosis compared to apoE^{-/-} mice.

BMDMs from HxE^{-/-} mice have impaired/reduced cholesterol efflux capacity through the ABCA1 receptor

Hx along with macrophages play an important role in the clearance of cell-free heme from the circulation, thereby mitigating oxidative damage caused by heme. Boyle *et al.* recently demonstrated that heme can transcriptionally co-activate HO-1 and LXR genes in macrophages (23). We used naïve and differentiated bone marrow cells (BMDMs) from 8–12 week old apoE^{-/-} and HxE^{-/-} mice (n=3–4) without and with hHx, to determine the expression of LXR genes using real time-quantitative PCR (RT-qPCR) gene expression analysis. Treatment of the naïve cells with MCSF dramatically increased the expression of LXR α and ABCA1, and the expression of these genes was significantly less on exposure to

LPS (data not shown). Interestingly, in contrast to LXR- α upon exposure to LPS, levels of LXR- β were not significantly reduced in MCSF-1 treated BMDMs from HxE^{-/-} mice compared to apoE^{-/-} mice (Figure 4A). ATP-binding cassette, sub-family A, member 1 (ABCA1), a downstream target of LXR- α was also significantly less expressed in HxE^{-/-} MCSF-1 treated BMDMs treated with LPS, however, there was no change in the expression of ABCG1 (Figure 4A) between the two groups of mice. ABCA1 protein expression was significantly lower (Figure 4B) in MCSF-1 treated BMDMs from HxE^{-/-} mice compared to apoE^{-/-} mice. ABCA1 is an important cell surface cholesterol efflux transporter. We determined levels of ABCA1 on the cell surface of the BMDMs by flow cytometry analysis as described in Materials and Methods. We observed a significant reduction in expression of cell surface ABCA1 in HxE^{-/-} MCSF-1 treated BMDMs compared to apoE^{-/-} BMDMs after exposure to LPS (Figure 4C).

Since ABCA1 is a key cholesterol efflux transporter we performed a cholesterol efflux assay using MCSF-1 treated BMDMs loaded with Ac-LDL (50 μ g/mL) and measured apoA-I (10 μ g/mL) mediated cholesterol efflux. Net percent cholesterol efflux was measured as a ratio of counts in media divided by counts in media plus cells. MCSF-1 treated BMDMs from HxE^{-/-} mice showed a significant reduction in cholesterol efflux capacity compared to apoE^{-/-} MCSF-1 treated BMDMs (Figure 4D). Since the efflux was apoA-I mediated we can propose that the efflux was via the ABCA1 transporter. Ac-LDL cholesterol uptake was similar in MCSF-1 treated BMDMs from both groups of mice (Figure 4D, lower panel) and serum apoA-I levels estimated by ELISA were not different between apoE^{-/-} and HxE^{-/-} mice (Supplemental Figure IIIC). These results led us to hypothesize that absence of Hx may inhibit the macrophage-heme driven LXR and ABCA1 pathway. In order to confirm this, we added hHx to the differentiated BMDMs and determined the expression of the genes using RT-qPCR. On adding hHx to the differentiated BMDMs, the expression of LXR- α , LXR- β and ABCA1 was significantly increased in differentiated BMDMs from HxE^{-/-} mice (Figure 4A) when compared to HxE^{-/-} differentiated BMDMs without hHX, (although significantly lower in HxE^{-/-} differentiated BMDMs compared to apoE^{-/-} differentiated BMDMs). The expression of LXR- α and ABCA1 increased in the hHx treated differentiated BMDMs from apoE^{-/-} mice, but the increase did not reach statistical significance (Figure 4A).

Bone marrow-derived macrophages from the HxE^{-/-} mice are proinflammatory

Oikawa *et al.* showed that oxidative stress and abnormal redox balance induce changes in the bone marrow microenvironment (27). Since there is a heightened level of heme and ROS in the circulation of HxE^{-/-} mice, we investigated whether the phenotype of the BMDMs from HxE^{-/-} were different when compared to those from apoE^{-/-} mice. We used bone marrow from 8–12 week old apoE^{-/-} and HxE^{-/-} mice (n=3–4) to determine the macrophage polarization of M1 (classically activated) vs. M2 (alternatively activated) cells. To evaluate the macrophage population in our mice, we used two treatment methods; naive bone marrow cells from apoE^{-/-} and HxE^{-/-} mice were differentiated in the presence of MCSF-1 (10 ng/ml) + IL-4 (10 ng/ml) (Figure 5A) or MCSF-1 (10 ng/ml) + LPS (10 ng/ml) (MCSF-1 + LPS in Figure 5B) as described in Materials and Methods. RNA was isolated and analyzed for gene expression analysis using RT-qPCR.

Macrophages differentiated in the presence of MCSF-1 + IL-4 from HxE^{-/-} mice showed a significantly lower expression of FIZZ1 and Ym1 genes compared to the control apoE^{-/-} mice (Figure 5A). The expression of the downstream target of LPS stimulation, TNF- α was significantly upregulated in naïve HxE^{-/-} macrophages compared to apoE^{-/-} naïve macrophages as well as in cells treated with MCSF-1 + LPS (Figure 5B). Chemokine Receptor-2 (CCR-2), a well-established M1 macrophage marker was significantly increased in the MCSF-1 + LPS cells from the HxE^{-/-} mice compared to the cells from the apoE^{-/-} mice (Figure 5B). The MCSF-1 + LPS HxE^{-/-} macrophages had significantly lower expression of F4/80, Arg-1 and anti-inflammatory IL-10 (Figure 5B) compared to their apoE^{-/-} controls. Together with the data in Figure 4A these results suggest that the macrophages from HxE^{-/-} mice are more M1-like (more proinflammatory). These results led us to hypothesize that absence of Hx in HxE^{-/-} mice may favor more M1-like macrophages. In order to test this hypothesis, we added hHx to differentiated BMDMs from apoE^{-/-} and HxE^{-/-} mice and determined the expression of two important M1 and M2 genes using RT-qPCR. On adding hHx to the MCSF-1 + LPS BMDMs, the expression of CCR-2, a well-established M1 macrophage marker was significantly reduced in HxE^{-/-} cells, but not in apoE^{-/-} cells (Figure 5C). In addition, the expression of Arg-1, an important M2 macrophage marker was significantly increased in HxE^{-/-} MCSF-1 + LPS treated BMDMs that were also treated with hHx (Figure 5C). We also performed, immunohistochemistry analysis using a macrophage M1 marker, MHCII in aortic root sections (n = 6 apoE^{-/-}, n = 6 HxE^{-/-} mice). We observed a significant increase in % MHCII positive staining in the macrophage stained area (CD68 positive) in the double knockout mice compared to control mice (Figure 5D). These results confirm that the absence of Hx modifies macrophage polarization not only in the bone marrow, but also in the atherosclerotic lesions.

DISCUSSION

The results presented here show that lack of Hx in the apoE^{-/-} mouse model on a chow diet causes increased atherosclerosis. Hx is an acute phase protein that binds to heme with high affinity (28). To limit free heme availability and prevent free radical formation, mammals use Hx as the major heme scavenger protein. Hx binds to free heme, and the resultant heme-Hx complex is taken up by macrophages and hepatocytes through the CD91 receptor via receptor-mediated endocytosis (29). Heme scavenging is a very important process to avoid oxidative stress and inflammation that occurs in the presence of increased levels of free heme. The results in Figure 1 show that the absence of Hx resulted in increased heme in the serum and HDL of HxE^{-/-} mice and supplementing the mice with hHx significantly reduced serum heme levels. HxE^{-/-} mice also showed decreased expression of the CD91 receptor and the anti-oxidant enzymes HO-1 and HO-2 in the liver. HO-1 is a vital enzyme for iron homeostasis and protection from oxidant stress. HO-1 catabolizes the pro-oxidant heme and generates biliverdin, free iron and carbon monoxide as reaction products (21, 22). Biliverdin is further processed to bilirubin, a direct antioxidant (22), which increases the anti-inflammatory efficacy of the CD91-HO-1 pathway.

Epidemiological studies have revealed that moderately increased plasma levels of bilirubin can decrease the risk of developing cardiovascular diseases (30). Interestingly, HxE^{-/-} mice showed reduced serum bilirubin levels compared to control apoE^{-/-} mice suggesting that

reduced heme breakdown by the CD91-HO-1 pathway may in part contribute to increased atherosclerosis. Albumin is known to also bind to heme and form a heme-albumin complex that can be taken up by endothelial cells (24). The results in Figure 1 show that HxE^{-/-} mice had reduced levels of albumin. The cause of the decreased albumin levels in the HxE^{-/-} mice is not evident from our studies. However, the decreased albumin levels may also contribute to the higher levels of free heme in the serum of HxE^{-/-} mice compared to apoE^{-/-} mice. Moreover, free heme can transiently bind to lipoproteins such as HDL and LDL (31), before being transferred to Hx for scavenging. We observed a significantly higher level of heme in HDL fractions from HxE^{-/-} mice compared to apoE^{-/-} mice (Figure 2) indicating that in the absence Hx free heme accumulates on transient carrier proteins such as HDL.

Free heme activates NADPH oxidase, a major source of ROS, and ROS has been shown to induce several pathological conditions. With augmented heme in the circulation, we observed significantly increased ROS accumulation in serum, HDL and post-HDL fractions of HxE^{-/-} mice compared to the apoE^{-/-} controls (Figure 2). Free heme toxicity is exacerbated by its ability to intercalate into lipid membranes. The extreme hydrophobicity of free heme allows it to enter the phospholipid bilayer. This catalyzes the oxidation of the cell membrane and promotes lipid peroxidation, increasing membrane permeability and ultimately leading to cell death (17, 18). Levels of malondialdehyde can be used as a biomarker to estimate oxidative stress *in vivo*. Malondialdehyde levels are a measure of hepatic lipid peroxidation. Our data showed that HxE^{-/-} mice had increased levels of malondialdehyde in their livers compared to apoE^{-/-} mice consistent with increased heme and ROS leading to increased lipid peroxidation. Watanabe *et al.* showed that in patients with cardiovascular disease, Hb and its scavenger proteins: Hp and Hx can associate with HDL and influence the inflammatory properties of HDL (4). This study also reported that absence of Hx could convert HDL from anti- to a proinflammatory HDL in C57BL/6J mice. Our results confirm these findings in apoE^{-/-} mice lacking Hx. In a monocyte chemotactic assay, the HDL from HxE^{-/-} mice stimulated cultured endothelial cells to produce more monocyte chemoattractant-1 (MCP-1) as determined by a bioassay. In addition, HDL from HxE^{-/-} mice had significantly lower LCAT activity compared to HDL from apoE^{-/-} mice. Our results suggest that Hx confers protection against heme and ROS driven HDL dysfunction and oxidative stress in apoE^{-/-} mice.

Excess heme and ROS are implicated in various aspects of cardiovascular pathology. Increased free radicals in the circulation cause endothelial dysfunction, platelet activation and induce smooth muscle proliferation (32–36), which are features of atherosclerosis and hypertension (37–39). HxE^{-/-} mice on a chow diet developed increased atherosclerotic lesions in the aortic sinus and the aorta compared to apoE^{-/-} mice. Macrophage content in the aortic sinus was also significantly higher in HxE^{-/-} compared to apoE^{-/-} mice as seen in Figure 3 of this study. In addition, total and LDL-cholesterol levels tended to be elevated and HDL-cholesterol levels tended to be lower in the HxE^{-/-} mice compared to apoE^{-/-} mice, but these changes did not reach statistical significance (Supplemental Table I). Collectively, these results demonstrated that Hx ablation in apoE^{-/-} mice causes aggravated plaque formation and macrophage infiltration leading to increased atherosclerosis.

Monocytes originate from bone marrow-derived progenitor cells, and at early stages of monocyte development may be regulated by cellular cholesterol content in a manner that can affect atherosclerosis. Mice, with monocyte progenitor cells genetically engineered to have defective cholesterol efflux due to deficiency of ABCA1 and ABCG1 transporters, showed an increase in atherosclerosis (40). We found a significant reduction in ABCA1 gene and protein expression in the HxE^{-/-} BMDMs compared to apoE^{-/-} BMDMs (Figure 4). PPAR is known to induce the expression of ABCA1 in macrophages through a transcriptional cascade mediated by the nuclear receptor LXR that hetero dimerizes with retinoic-X-receptor (41–45). LXR-alpha, a well-established transcriptional regulator for ABCA1, was also significantly down-regulated in the HxE^{-/-} BMDMs. The functional consequences of the reduced gene and protein expression of ABCA1 was demonstrated by a cholesterol efflux assay. BMDM isolated from HxE^{-/-} mice were found to have reduced apoA-I mediated cholesterol efflux (Figure 4). Interestingly, in an independent study it was shown that after heme internalization in macrophages HO-1 and LXR expression was increased, foam cell formation was prevented and the export of cholesterol to HDL was promoted (23, 46). This coordination of iron and lipid metabolism provides protection from oxidant stress and lipid overload. These findings are also consistent with our finding of reduced expression of LXR- α and ABCA1 in BMDMs from HxE^{-/-} mice. Interestingly, when the BMDMs from HxE^{-/-} mice were supplemented with hHx, we observed a significant increase in the expression of LXR- α and ABCA1 genes (Figure 4). This reduction in LXR genes in HxE^{-/-} BMDMs may contribute to increased foam cell formation due to reduced cholesterol efflux capacity, which may play a role in the increased atherosclerosis observed in our studies.

In pathological conditions such as aging, atherosclerosis, and diabetes, excess amounts of ROS in the bone marrow microenvironment may impair stem and progenitor cell function, and cause hematopoietic dysfunction (47,48). Thus, both dysregulation of ROS and a more oxidative environment may have deleterious effects on the bone marrow microenvironment. We observed that BMDMs from HxE^{-/-} mice were more classically activated M1-like macrophages compared to the apoE^{-/-} macrophages, which were more M2 in phenotype. Classically activated macrophages or M1 are important components of the host defense in the fight against various pathogens; M1 macrophages are thought to be more proinflammatory (49,50). In addition, proinflammatory M1 macrophages have been shown to exhibit reduced expression of ABCA1 (51). Cytokines such as IL-4 can antagonize classical macrophage activation and induce the development of alternatively activated or anti-inflammatory M2 macrophages (52). It is well established that the expression of FIZZ1 and Ym1 is induced in alternatively activated macrophages as compared with classically activated macrophages while TNF- α and CCR2 are highly expressed in M1 macrophages (53–56). BMDMs from HxE^{-/-} mice showed significantly reduced expression of Ym1 and FIZZ1 and significantly increased expression of TNF- α and CCR2, when compared to apoE^{-/-} mice (Figure 5). Myeloid-derived suppressor cells (MDSCs) are considered to be an immature population of myeloid cells associated with infections or tumors that are cable of suppressing proinflammatory responses. Mouse MDSCs have been found to express F4/80, IL-4R α , Arg1, IL-10, and TGF- β amongst many other markers (56). BMDMs from HxE^{-/-} mice showed a significant down regulation of F4/80, Arg1 and IL-10 compared to apoE^{-/-} BMDMs (Figure 5). Interestingly, when hHx was added to the BMDMs from HxE^{-/-} mice,

there was a significant reduction in expression of CCR2, and a significant increase in expression of Arg1. Our data suggests that BMDMs from HxE^{-/-} mice promote proinflammatory responses compared to BMDMs from apoE^{-/-} mice. Additionally, immunohistochemistry results for MHCII, a well-established M1 macrophage marker, confirm that absence of Hx results in more M1-like macrophages in atherosclerotic lesions. It has been shown that M1 macrophages have reduced expression of ABCA1, which has important implications in the development of atherosclerosis. We have performed immunohistochemistry using antibodies against both CD68 and ABCA1 on serial atherosclerotic lesion sections and quantified the data as ABCA1 expressed specifically in the macrophage area (Supplemental Figure IV). It should be pointed out that i) the lesions were elevated in HxE^{-/-} mice, and ii) CD68 positive staining is significantly elevated in HxE^{-/-} lesions. Therefore, the significant increase in ABCA1 per lesional macrophage area was not very informative of naive macrophages. The results using BMDM from the two groups of mice in Figures 4 and 5 demonstrate that lack of Hx alters ABCA1 expression and function. Taking into account the different gene expression profiles of BMDMs from HxE^{-/-} and apoE^{-/-} mice with and without hHx treatment and the MHCII immunohistochemistry results, we suggest that the lack of Hx from birth in apoE^{-/-} mice influences the bone marrow microenvironment resulting in macrophages in the lesions that are more proinflammatory or M1-like. There has been extensive evidence from in vitro, animal, and human studies relating Hp to cardiovascular disease and diabetes (7–10). We have previously demonstrated that Hp can bind directly to HDL and appears to be responsible for the increased amount of the Hb complex and reactive oxygen species associated with the HDL in mice. The amount of total lipid peroxides associated with HDL was also found to be increased in individuals with diabetes and the Hp 2-2 compared to the Hp 1-1 genotype (57). It was suggested that Hp's role in oxidative stress and HDL function might provide proof of concept for the hypothesis of dysfunctional HDL in diabetes (58). Since Hp and Hx are part of the Hb/heme scavenging complex and in light of the results from our current studies, it is very likely that Hx will have a significant role in diabetes and related cardiovascular complications.

In conclusion, our results demonstrate, for the first time, that mice lacking Hx are more susceptible to atherosclerosis, and suggest that targeting free heme in the circulation may be a novel approach in preventing and treating oxidative stress-mediated atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Hx	Hemopexin
ApoE^{-/-}	ApoE null
HxE^{-/-}	Hx-null/ApoE-null
HDL	High Density Lipoprotein
ROS	Reactive Oxygen Species
Hb	Hemoglobin
BMDM	Bone Marrow Derived Macrophages
ABCA1	ATP-binding cassette, sub-family A, member 1

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HIGHLIGHTS

1. Hemopexin (Hx) deficiency aggravates the development of atherosclerosis in apoE null mice.
2. Heme scavenging and regulation of macrophage phenotype/function appear to be the primary mechanisms by which Hx protects against increased atherosclerosis.
3. Hx could be a novel treatment for alleviating heme mediated oxidative damage and atherosclerosis.

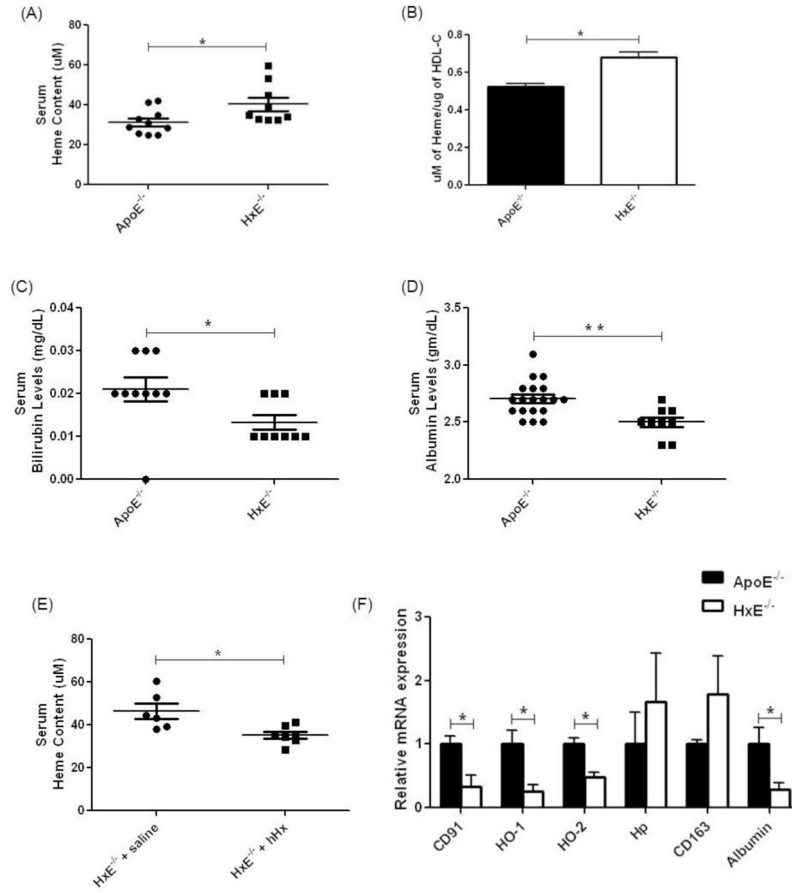


Figure 1. Hepatic heme detoxification is dysregulated in apoE^{-/-} mice lacking hemopexin
 (A and E) Heme content was measured colorimetrically in the serum and (B) in pooled HDL fractions of apoE^{-/-} and Hx/E^{-/-} mice (n = 8 - 10). (C) Bilirubin (n = 8 - 10) and (D) albumin levels (n = 20, apoE^{-/-}; n = 9, Hx/E^{-/-}) were measured in the serum of apoE^{-/-} and Hx/E^{-/-} mice as described in Materials and Methods. (E) Hx/E^{-/-} mice at 4 weeks of age were given a single injection of human Hx (hHx) or saline intraperitoneally. 24 hours later, retro-orbital bleeding was performed to obtain serum and the levels of serum heme were determined. (F) Hepatic gene expression was determined by RT- qPCR for apoE^{-/-} and Hx/E^{-/-} mice (n = 3-4). All genes were normalized to GAPDH. The mean value for each group of mice is indicated by the horizontal bars. The data shown are mean ± SEM. ** Significance: p<0.01; * Significance: p<0.05.

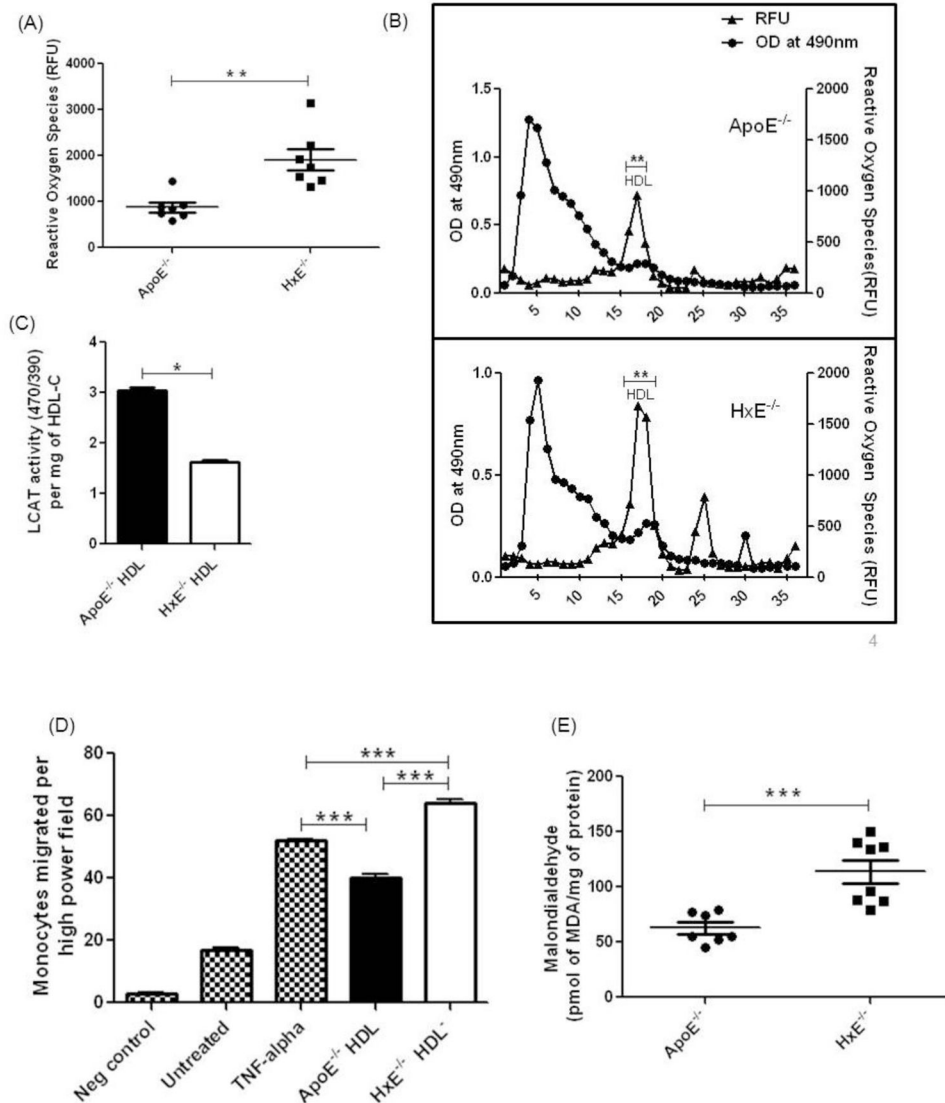
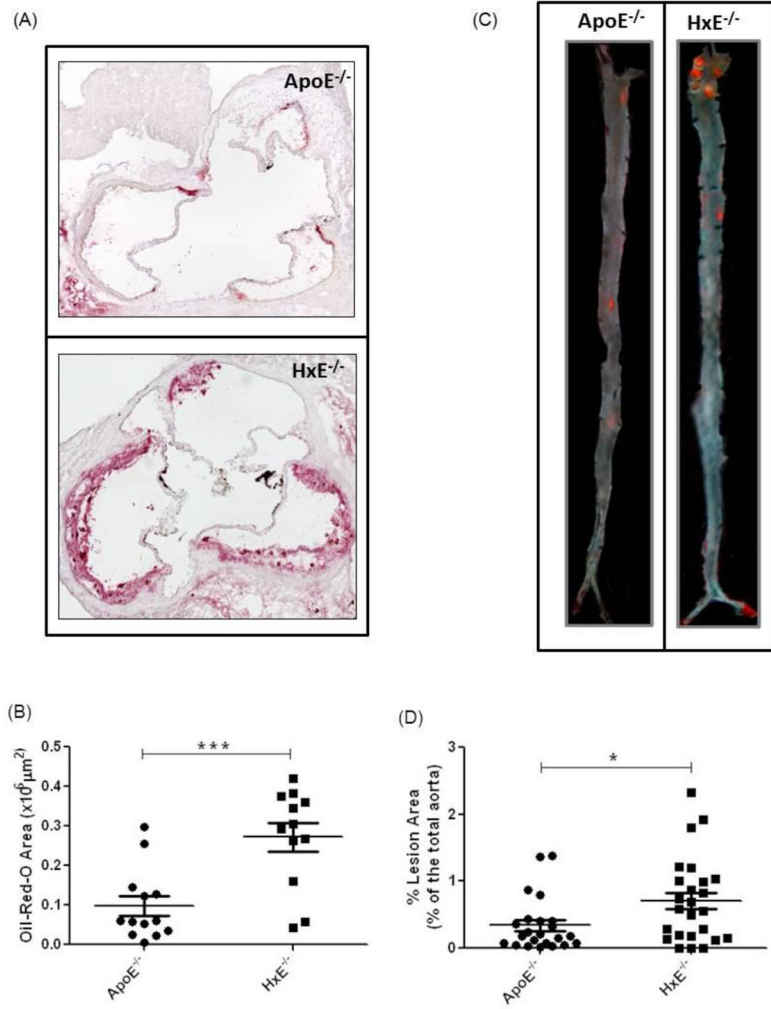


Figure 2. Absence of hemopexin significantly increases oxidative stress in apoE^{-/-} mice
 (A) ROS levels were determined and represented as relative fluorescence units (RFUs) in a cell free assay using DCFH₂ in serum samples of HxE^{-/-} and apoE^{-/-} mice (n = 7) as described in Materials and Methods. (B) Lipoproteins were separated using FPLC from pooled serum samples obtained from apoE^{-/-} and HxE^{-/-} mice (n = 6–8) and, cholesterol levels on the different lipoprotein fractions were obtained using a colorimetric assay. ROS levels were determined on all the FPLC fractions using a cell free assay. (C) LCAT activity was measured fluorometrically on concentrated pooled HDL fractions. (D) 50µg/dL of HDL cholesterol from apoE^{-/-} and HxE^{-/-} mice (n = 3) was used to perform a monocyte migration assay as described in Materials and Methods. (E) Malondialdehyde levels were determined using a colorimetric assay in livers of HxE^{-/-} and apoE^{-/-} mice (n = 7–8). The mean value for each group of mice is indicated by the horizontal bars. The data shown are mean ± SEM. *** Significance: *p*<0.001; ** Significance: *p*<0.01; *Significance: *p*<0.05.



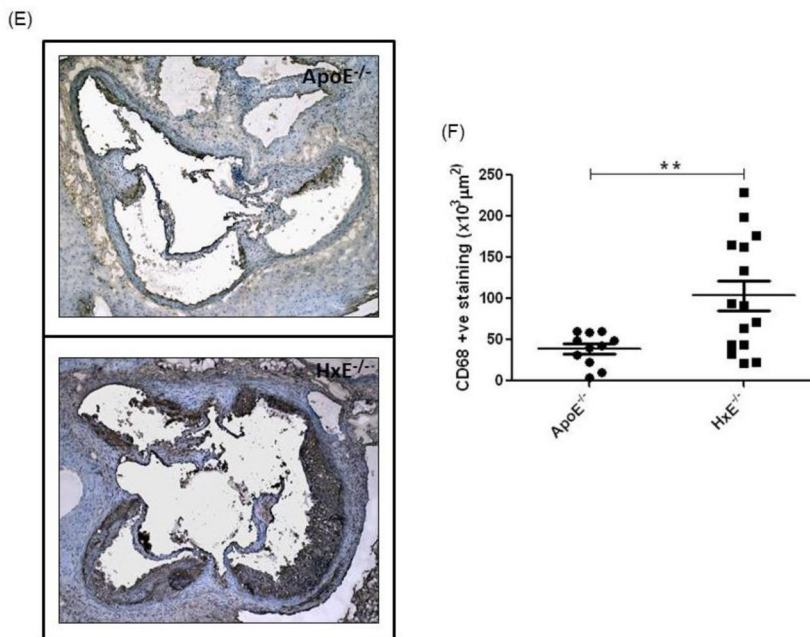


Figure 3. ApoE^{-/-} mice lacking hemopexin develop increased atherosclerosis

(A) Atherosclerotic plaque area was determined in the aortic sinus of the heart via Oil-Red-O staining of the lesions (Scale bar = 500 μ m). (B) Quantitative analysis was performed of the plaque area in Hx E^{-/-} (n = 12) and apoE^{-/-} (n = 13) mice. Each point represents the mean (8 - 10 sections per mouse) atherosclerotic plaque area per mouse. (C) Whole aorta lesions were determined using Sudan IV staining as described in Materials and Methods (Scale bar = 0.5cm). (D) Quantitative analysis of the extent of atherosclerosis was determined by calculating the percent of aortic surface area covered by atherosclerotic lesions in Hx E^{-/-} (n = 25) and apoE^{-/-} mice (n = 24). Each point represents the mean lesion area per mouse. (E) CD68 positive staining was performed in the aortic sinus for macrophage content (Scale bar = 500 μ m); the section for the Hx E^{-/-} mouse is from a mouse with values above the Mean in the data shown in panel F. (F) Quantitative analysis of the macrophage content in the Hx E^{-/-} (n = 15) and apoE^{-/-} (n = 11) mice was performed. Each point represents the mean macrophage area per mouse (3 sections per mouse). The mean area for each group of mice is indicated by the horizontal bars. *** Significance: $p < 0.001$; ** Significance: $p < 0.01$; * Significance: $p < 0.05$.

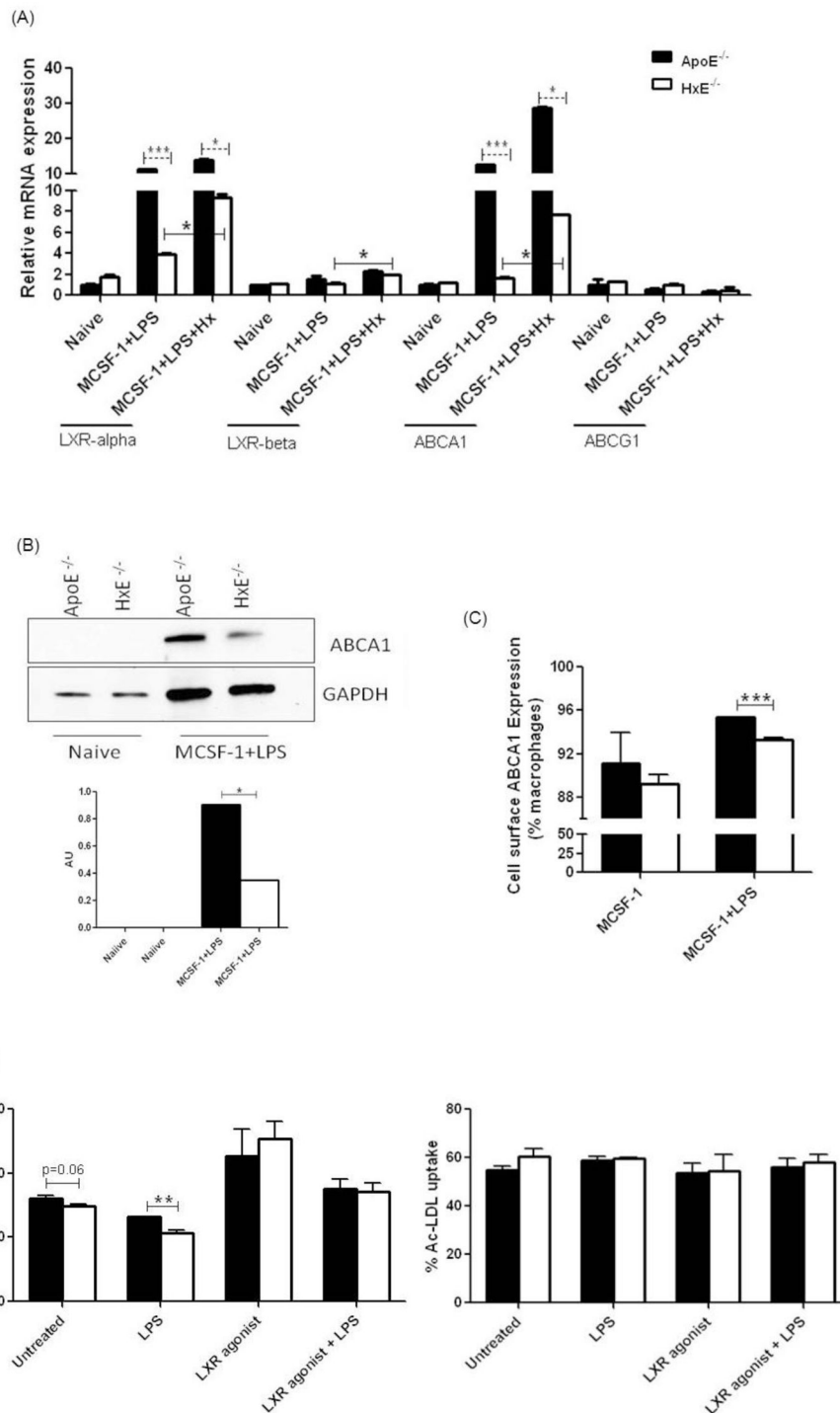


Figure 4. HxE^{-/-} macrophages have reduced cholesterol efflux capacity through the ABCA1 transporter

(A) MCSF-1 (10ng/ml) was used to differentiate the bone marrow derived cells isolated from apoE^{-/-} and HxE^{-/-} mice (n = 3–4) as described in Materials and Methods. RT-qPCR analysis was performed as described in Materials and Methods for untreated (Naive), and MCSF-1 treated BMDMs that were exposed to LPS (10 ng/ml) without or with human Hx

(hHx) added at 45 $\mu\text{g/ml}$. (B) Western blot analysis for ABCA1 was performed using 60 μg of whole cell lysates from the cells as described in Materials and Methods. Densitometric analysis of ABCA1 protein expression was performed as described in Materials and Methods. (C) Cell surface expression analysis of ABCA1 was performed using flow cytometry. (D) ApoA-I (10 $\mu\text{g/ml}$) mediated cholesterol efflux assay was performed using tritiated Ac-LDL (50 $\mu\text{g/ml}$) loaded MCSF-1 treated BMDMs from apoE^{-/-} and HxE^{-/-} mice (upper panel, n = 3–4) as described in Materials and Methods. All treatments in panel D were done in the presence of MCSF-1 (10ng/ml). Ac-LDL uptake assay was performed using tritiated Ac-LDL (50 $\mu\text{g/ml}$) loaded BMDMs from apoE^{-/-} and HxE^{-/-} mice (lower panel, n = 3–4) as described in Materials and Methods. The data shown are mean \pm SEM. *** Significance: $p < 0.001$. ** Significance: $p < 0.01$; * Significance: $p < 0.05$.

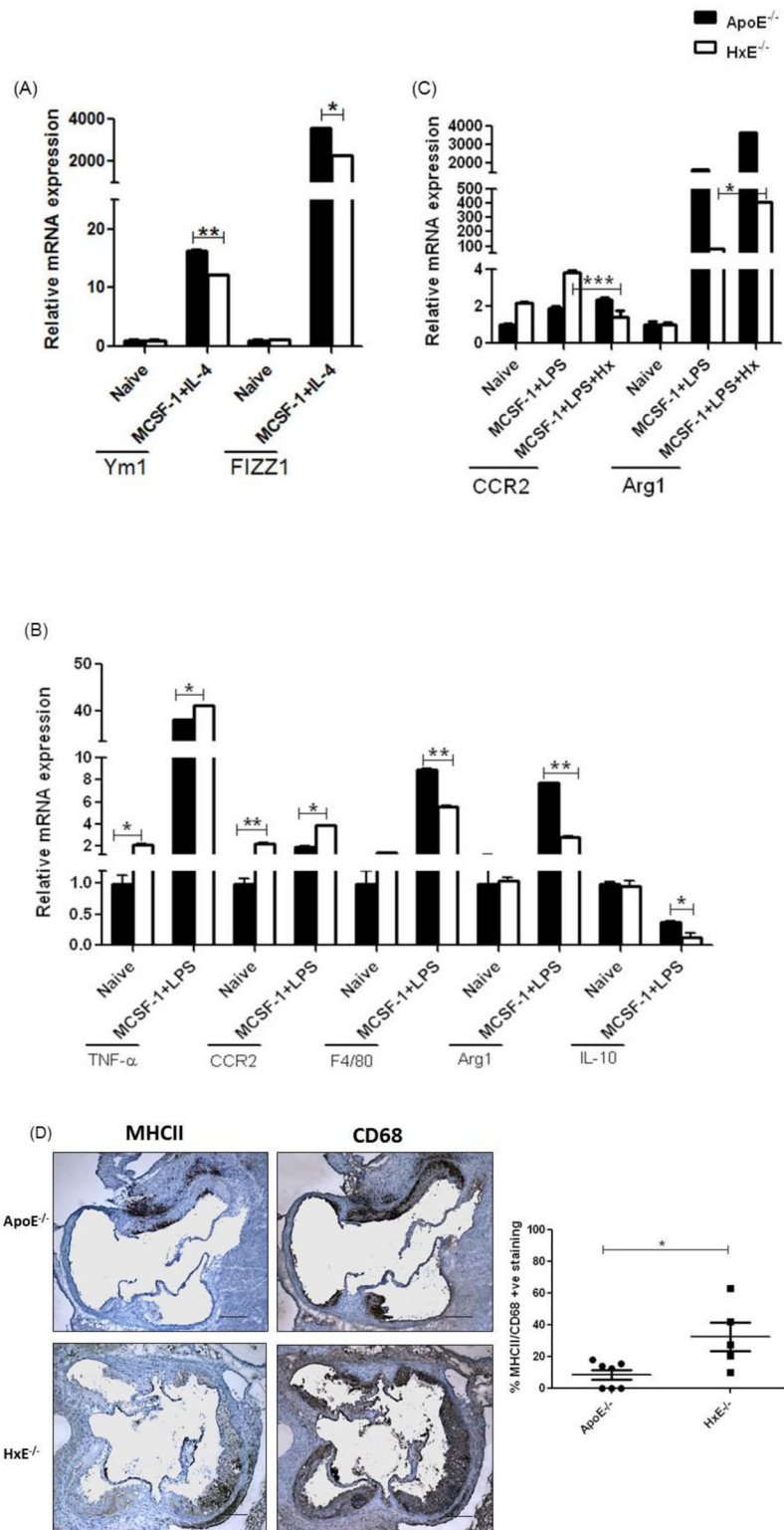


Figure 5. Bone marrow-derived macrophages from the HxEx^{-/-} mice are proinflammatory

(A) RT-qPCR analysis was performed using (A) naïve and MCSF-1 (10 ng/ml) + IL-4 (10ng/ml) treated bone marrow cells or (B) naïve and MCSF-1 (10 ng/ml) + LPS (10ng/ml) treated bone marrow cells isolated from HxE^{-/-} and apoE^{-/-} mice (n = 3 – 4) as described under Materials and Methods. (C) RT-qPCR analysis was performed using naïve, or MCSF-1 (10 ng/ml) + LPS (10ng/ml) without or with hHx (45 µg/ml) treated bone marrow cells isolated from HxE^{-/-} and apoE^{-/-} mice (n = 3 – 4) as described in Materials and Methods. (D) MHCII and CD68 positive staining was performed in aortic sinus sections to determine macrophage polarization as described in Materials and Methods. The data in panel D are shown as the %MHCII/CD68 positive staining in HxE^{-/-} (n = 15) and apoE^{-/-} (n = 11) mice. Each point represents the mean positive area per mouse (3 sections per mouse; Scale bar = 500µm). The mean area for each group of mice is indicated by the horizontal bars. The data shown are mean ± SEM. ***Significance: $p < 0.001$; **Significance: $p < 0.01$; * Significance: $p < 0.05$.