Paradoxical Suppression of Atherosclerosis in the Absence of microRNA-146a

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Rationale: Inflammation is a key contributor to atherosclerosis. MicroRNA-146a (miR-146a) has been identified as a critical brake on proinflammatory nuclear factor κ light chain enhancer of activated B cells signaling in several cell types, including endothelial cells and bone marrow (BM)–derived cells. Importantly, miR-146a expression is elevated in human atherosclerotic plaques, and polymorphisms in the miR-146a precursor have been associated with risk of coronary artery disease.

Objective: To define the role of endogenous miR-146a during atherogenesis.

Methods and Results: Paradoxically, Ldlr−/− (low-density lipoprotein receptor null) mice deficient in miR-146a develop less atherosclerosis, despite having highly elevated levels of circulating proinflammatory cytokines. In contrast, cytokine levels are normalized in Ldlr−/−;miR-146a−/− mice receiving wild-type BM transplantation, and these mice have enhanced endothelial cell activation and elevated atherosclerotic plaque burden compared with Ldlr−/− mice receiving wild-type BM, demonstrating the atheroprotective role of miR-146a in the endothelium. We find that deficiency of miR-146a in BM-derived cells precipitates defects in hematopoietic stem cell function, contributing to extramedullary hematopoiesis, splenomegaly, BM failure, and decreased levels of circulating proatherogenic cells in mice fed an atherogenic diet. These hematopoietic phenotypes seem to be driven by unrestrained inflammatory signaling that leads to the expansion and eventual exhaustion of hematopoietic cells, and this occurs in the face of lower levels of circulating low-density lipoprotein cholesterol in mice lacking miR-146a in BM-derived cells. Furthermore, we identify sortilin-1 (Sort1), a known regulator of circulating low-density lipoprotein levels in humans, as a novel target of miR-146a.

Conclusions: Our study reveals that miR-146a regulates cholesterol metabolism and tempers chronic inflammatory responses to atherogenic diet by restraining proinflammatory signaling in endothelial cells and BM-derived cells. (Circ Res. 2017;121:354-367. DOI: 10.1161/CIRCRESAHA.116.310529.)

Key Words: atherosclerosis ■ endothelial cells ■ hematopoiesis ■ inflammation ■ microRNAs

Atherosclerosis is a chronic inflammatory vascular disease characterized by the narrowing of blood vessels caused by the growth of lipid-rich plaques.1 The initiation of atherogenesis relies on the recruitment of circulating leukocytes by activated endothelial cells (ECs) to regions of deposited oxidized low-density lipoprotein (LDL).2 Activated ECs and leukocytes use the nuclear factor κ light chain enhancer of activated B cells (NF-κB) signaling pathway to propagate inflammatory gene expression, including induction of adhesion molecules, chemotaxicactants, and cytokines to drive inflammation in the vessel wall.3,4 NF-κB signaling is tightly controlled, and this includes regulation by a network of microRNAs, which titrate the expression of signaling components post-transcriptionally.5 In particular, microRNA-146a (miR-146a) has been well characterized...
**Novelty and Significance**

**What Is Known?**
- MicroRNA-146a (miR-146a) suppresses inflammatory responses in endothelial cells and bone marrow (BM)-derived cells by targeting adaptor proteins in the nuclear factor κ light chain enhancer of activated B cells signaling pathway.
- Increased levels of miR-146a have been detected in human atherosclerotic plaques, and polymorphisms in the miR-146a precursor are associated with risk of coronary artery disease.
- Injection of exogenous miR-146a reduces atherogenesis in mouse models.

**What New Information Does This Article Contribute?**
- Deletion of miR-146a in BM-derived cells enhances the production of proinflammatory cytokines, but paradoxically reduces circulating proatherogenic cells, ultimately resulting in decreased atherosclerosis.
- miR-146a in BM-derived cells protects against high cholesterol diet-induced hematopoietic progenitor cell exhaustion in the BM and prevents extramedullary hematopoiesis and splenomegaly.
- Circulating very-low-density lipoprotein levels are progressively decreased in mice lacking miR-146a in the BM, and this is accompanied by enhanced inflammation in the liver and dysregulation of a newly identified miR-146a target gene, sortilin 1 (Sort1).

**Nonstandard Abbreviations and Acronyms**

| Abbreviation | Description                  |
|--------------|------------------------------|
| BM           | bone marrow                  |
| BMT          | bone marrow transplant       |
| DKO          | double knockout              |
| EC           | endothelial cell             |
| HCD          | high cholesterol diet        |
| HSC          | hematopoietic stem cell      |
| HuR          | human antigen R              |
| ICAM-1       | intercellular adhesion molecule-1 |
| IL           | interleukin                  |
| IRAK1        | interleukin receptor–associated kinase 1 |
| KO           | knockout                     |
| LDL          | low-density lipoprotein      |
| LDLR         | low-density lipoprotein receptor |
| LPS          | lipopolysaccharide           |
| miR-146a     | microRNA-146a                |
| NCD          | normal chow diet             |
| NF-κB        | nuclear factor κ light chain enhancer of activated B cells |
| SELE         | E-selectin                   |
| siICAM-1     | soluble intercellular adhesion molecule-1 |
| SORT1        | sortilin-1                   |
| TNF-α        | tumor necrosis factor-α      |
| TRAF6        | TNF receptor–associated factor 6 |
| VCAM-1       | vascular cell adhesion molecule-1 |
| VLDL         | very-low-density lipoprotein |
| WT           | wild type                    |

**What New Information Does This Article Contribute?**
- Deletion of miR-146a in BM-derived cells enhances the production of proinflammatory cytokines, but paradoxically reduces circulating proatherogenic cells, ultimately resulting in decreased atherosclerosis. The study reveals a critical function for a single microRNA in the control of the intensity of inflammatory responses to hypercholesterolemia and highlights the detrimental effects of unrestrained inflammatory signaling in multiple organs: BM (hematopoietic stem cell exhaustion), spleen (extramedullary hematopoiesis and splenomegaly), liver (cholesterol homeostasis defects), and the vasculature (enhanced endothelial cell activation and monocyte recruitment). Importantly, these findings provide a further impetus to therapeutically augment miR-146a expression/function in atherosclerosis.

Elevation of miR-146a expression in atherosclerotic plaques in humans and polymorphisms in the miR-146a precursor that are associated with coronary artery disease are suggestive of a role for this microRNA in atherogenesis. Although numerous studies have placed miR-146a among the echelon of anti-inflammatory microRNAs, the role of endogenous miR-146a in atherosclerosis remains unknown. Surprisingly, despite the ability of this microRNA to restrain cytokine production in BM-derived cells, loss of this microRNA resulted in reduced atherosclerosis. This was accompanied by hematopoietic stem cell exhaustion and a corresponding reduction in levels of circulating proatherogenic cells. Enhanced inflammatory signaling occurred even though circulating levels of very-low-density lipoprotein cholesterol were diminished in these mice. Within the vasculature, miR-146a restrained endothelial activation, and loss of miR-146a in the vasculature enhanced atherosclerosis. This study reveals a critical function for a single microRNA in the control of the intensity of inflammatory responses to hypercholesterolemia and highlights the detrimental effects of unrestrained inflammatory signaling in multiple organs: BM (hematopoietic stem cell exhaustion), spleen (extramedullary hematopoiesis and splenomegaly), liver (cholesterol homeostasis defects), and the vasculature (enhanced endothelial cell activation and monocyte recruitment). Importantly, these findings provide a further impetus to therapeutically augment miR-146a expression/function in atherosclerosis.

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Characterization of miR-146a−deficient mice has revealed defects in multiple aspects of immune cell biology. Older (>1 year) miR-146a−/− mice develop multiorgan inflammation, bone marrow (BM) failure, splenomegaly, and lymphoadenopathy. When challenged by proinflammatory stimuli (eg, lipopolysaccharide [LPS] or IL-1β [interleukin-1β]), these mice have exacerbated NF-κB–dependent inflammatory responses and demonstrate expansion of proinflammatory Ly6C+ monocyt. Interestingly, the hyperactivation of NF-κB caused by low-grade inflammation during normal aging or through repeated LPS challenge drives the proliferation and eventual exhaustion of hematopoietic and progenitor stem cells in these mice, resulting in eventual loss of circulating leukocytes and lymphocytes.

NF-κB pathway is activated in ECs, macrophages, and smooth muscle cells within human atherosclerotic lesions. However, defining the role of NF-κB signaling in atherogenesis has been complicated, as ablation of NF-κB activity in ECs reduces atherogenesis, whereas inhibition within macrophages enhances atherogenesis. Of interest, recent studies have shown that injection of miR-146a mimic into athereprone mice reduces atherogenesis, and it has been suggested that this is because of suppression of macrophage NF-κB signaling. The role of endogenous miR-146a in atherogenesis remains undefined. Here, we show that genetic ablation of miR-146a in BM-derived cells reduces atherogenesis and that this is paradoxically accompanied by enhanced circulating levels of proinflammatory cytokines despite reduced levels of circulating LDL cholesterol. Lack of miR-146a in BM-derived cells leads to monocytosis in both ECs and leukocytes as a negative regulator of NF-κB activity through its ability to target upstream adaptor proteins, including TRAF6 (TNF receptor–associated factor 6) and IRAK1 (interleukin receptor–associated kinase 1).
in response to high cholesterol diet (HCD), followed by BM exhaustion, depleting circulating levels of proatherogenic cells. Conversely, deletion of miR-146a in the vasculature promotes atherogenesis by increasing endothelial activation. Thus, unrestrained inflammatory signaling in miR-146a-deficient tissues has diverse consequences during atherogenesis, and our studies emphasize the importance of tight control of inflammatory pathways in the setting of hypercholesterolemia.

Methods
A complete description of Methods is included in the Online Data Supplement.

Results
miR-146a Expression Is Increased in ECs and Intimal Cells During Murine Atherogenesis
Ldlr−/− (low-density lipoprotein receptor null) mice were placed on a HCD for 18 weeks to visualize the expression of miR-146a in atherosclerotic plaque (Figure 1A). In situ polymerase chain reaction on aortic root cross-sections revealed that miR-146a was expressed in intimal cells, including Mac-2+ macrophages, and was robustly expressed in CD31+ ECs. The in situ signal was specific for miR-146a, as staining was not detected in miR-146a−/− mice (Figure 1A). Expression of miR-146a in the aortic root seemed to progressively increase in the intima during the progression of atherosclerosis (Online Figure I). The absence of signal in the media implies that contractile smooth muscle cells in the aortic root do not express miR-146a at sufficient levels to be detected by this technique. In addition, using quantitative reverse transcriptase-polymerase chain reaction at an early stage of atherogenesis (ie, Ldlr−/− mice, 4-week HCD), we found a significant elevation of miR-146a expression in the lesser curvature of the aortic arch, a region of the aorta where atherosclerotic plaque forms, compared with regions that are protected from atherosclerosis, namely the greater curvature of the aortic arch and the descending thoracic aorta (Figure 1B and 1C). However, miR-146a expression was at appreciable levels in all regions examined (not shown), which may reflect the known expression of miR-146a in the vascular endothelium.

Global Deletion of miR-146a Activates Proinflammatory Pathways Yet Suppresses Atherogenesis and Is Accompanied by Reduced Circulating LDL Cholesterol Levels in Mice on HCD
To elucidate the role of miR-146a during atherogenesis, we generated global double knockout (DKO; miR-146a−/−; Ldlr−/−) mice by crossing miR-146a−/− mice with Ldlr−/− mice. Two time points (12 and 18 weeks of HCD) were assessed to determine the effect of miR-146a on the progression of atherosclerotic phenotypes (Figure 2A). Analyses of male and female mice were grouped together as we found no significant differences between sexes for the parameters measured, except

![Figure 1. MicroRNA-146a (miR-146a) is expressed in murine atherosclerotic plaques. A. Cross-sections of Ldlr−/− or Ldlr−/−;miR-146a−/− mouse aortic roots after 18 wk of high cholesterol diet (HCD). Expression of miR-146a, assessed by in situ polymerase chain reaction (red) overlaps with Mac-2–positive macrophages (purple) and CD31–positive endothelial cells (ECs; green) in the intima, and signal is absent in miR-146a−/− mice. miR-146a expression during the progression of atherosclerosis is shown in Online Figure I. B. Schematic of the aorta, indicating the aortic root (examined in A), the greater curvature (GC, atheroprotective) and lesser curvature (LC, atherosusceptible) of the aortic arch and the descending thoracic aorta (DTA, atheroprotective). C. Expression of miR-146a (normalized to U6 levels) in the specified regions of the aorta in Ldlr−/− mice after 4 wk of HCD (n=5). Ldlr indicates low-density lipoprotein receptor.](https://circres.ahajournals.org/content/10.1161/CIRCRESAHA.117.309255.f01a)
Figure 2. Reduced atherosclerosis in mice with global deletion of microRNA-146a (miR-146a). A, Schematic of high cholesterol diet (HCD) regimen for Ldlr−/− and Ldlr−/−; miR-146a−/− (double knockout [DKO]) mice. B, Weights of male mice after 12- or 18-wk HCD (n=3–5). Weights of female mice were also unchanged between genotypes (not shown). C, Food consumption in mice (n=4 mice per cage). T, is 18 wk of HCD. D, Percentage of Oil Red-0 (ORO) regions quantified from aortic arches of Ldlr−/− and DKO mice after HCD for 12 or 18 wk. Representative images are shown to the right. The descending side of the aorta is to the right. Aortic root and descending thoracic aorta analyses are shown in Online Figure II. n=18 to 22 for 12-wk time point and n=4 for 18-wk time point. E, Circulating levels of proinflammatory markers, IL-6 (interleukin-6) and soluble intercellular adhesion molecule-1 (sICAM-1) in wild-type and DKO mice (n=5–8). F, Time course of plasma cholesterol measurements (n=3–5; 1 group of mice were used for weeks 1, 6, and 9, and a separate group was used for weeks 12 and 18). Mice were fasted overnight before sample collection. G, FPLC (fast protein liquid chromatography) trace of cholesterol content in lipoprotein fractions in plasma after 18 wk of HCD (pooled analysis of 5 samples). H, Intrahepatic cholesterol and triglyceride levels in mice after 12-wk HCD (n=13–14). Eighteen-week HCD is shown in Online Figure IID. Bile cholesterol (n=3) and fecal cholesterol (n=4) in mice after 18-wk HCD. I, Assessment of very-low-density lipoprotein (VLDL) secretion by measurement of triglycerides and cholesterol in plasma after injection of Poloxamer 407 (12-wk HCD; n=4, 2). Ldlr indicates low-density lipoprotein receptor.
Next, we performed BM transplantation (BMT) experiments to elucidate the role of miR-146a in BM-derived cells during atherosogenesis. Ldlr−/− mice were lethally irradiated and reconstituted with either miR-146a+/+ (wild-type [WT]) or miR-146a−/− (knockout [KO]) BM (Figure 3A). Reconstitution of hematopoiesis after transplantation of WT or KO BM cells seemed to be normal, as circulating levels of leukocytes and lymphocytes were similar 8 weeks after BMT, before the administration of HCD (Online Figure IIIA). Body weight was similar between the 2 groups after 12 weeks of HCD (Figure 3B), as was food intake (Figure 3C). Although lipid plaque burden was not significantly altered at early stages (ie, 4-week HCD), mice receiving KO BM developed less lipid plaque in the aorta after 12 weeks of HCD (Figure 3D; Online Figure IIIB), and markers of macrophage content in the aortic arch were reduced (Online Figure IIIC). Plaque burden in the descending thoracic aorta (Online Figure IIIB) and aortic root (Online Figure IIID) appeared to be unchanged. The decrease in plaque burden in the aortic arch was paradoxically accompanied by signs of systemic inflammatory signaling, with higher levels of circulating sICAM-1, IL-6, and TNF-α (tumor necrosis factor-α) detected in the plasma of mice receiving KO BM after 12 weeks of HCD (Figure 3E), with a trend toward elevated IL-6 levels being observed after 4 weeks of HCD (Figure 3E). These findings suggest that loss of miR-146a expression in BM-derived cells surprisingly results in reduced atherosclerosis, despite the ability of miR-146a to restrain inflammatory signaling. The similarity in phenotypes observed in DKO mice and mice receiving KO BM suggests that loss of miR-146a function in BM-derived cells is the predominant contributor to the observed phenotypes.

Interestingly, we found a progressive decrease in total cholesterol, LDL, triglycerides, and high-density lipoprotein levels in the plasma of mice receiving KO BM (Figure 3F). FPLC revealed a marked reduction in cholesterol content in VLDL fractions (Figure 3G). However, levels of total and free cholesterol and triglycerides in the liver were not significantly different (Figure 3H), neither were cholesterol esters (not shown), and fecal cholesterol levels were also unchanged (Figure 3H). To determine potential mechanisms for the altered lipid metabolism, we assessed gene expression in livers of Ldlr−/− and DKO mice (18-week HCD), and Ldlr−/− mice receiving WT or KO BM (12-week HCD). We observed an elevation of a macrophage marker (F4/80), as well as several proinflammatory cytokines, such as IL-1β and IL-6, and an increase in IL-10, in DKO livers and in the livers of Ldlr−/− mice receiving KO BM, compared with their respective controls (Figure 3I). Importantly, dysregulation of IL-6 and IL-10 has previously been implicated in altered lipid metabolism. Indeed, we found that exposing primary hepatocytes to IL-6 decreased triglyceride secretion (Figure 3J). Acute phase response genes were elevated in DKO livers, but not in recipients of KO BM (not shown). We also assessed the expression of a panel of 84 lipid signaling and cholesterol metabolism genes by quantitative reverse transcriptase-polymerase chain reaction arrays. A small number of genes were significantly dysregulated in either experimental group (9 genes in DKO mice compared with Ldlr−/− mice, and 19 genes in KO BMT recipients compared with WT BMT recipients; Online Figure IV; Online Table I). However, the only genes that were significantly decreased in both models were ApoB (apolipoprotein B; 1.25-fold decrease in DKO mice), Ldlr−/− and 1.61-fold decrease in KO BMT versus WT BMT recipients) and Cebp (CCHC-type zinc finger nucleic acid binding protein); 1.43-fold decrease in DKO mice, Ldlr−/− and 1.69-fold decrease in KO BMT versus WT BMT recipients), but these changes were modest. Although not on the quantitative reverse transcriptase-polymerase chain reaction array, we also assessed the expression of sortilin-1 (Sort1) because it is a known regulator of circulating LDL levels that was identified by genome-wide association studies in humans, and it has been shown to promote IL-6 signaling and secretion in macrophages in mouse models. Interestingly, Sort1 is also predicted to be an miR-146a target gene (Figure 3K). We found that Sort1 expression in the liver was elevated in both models (2.21-fold increase in DKO mice versus Ldlr−/− and 1.68-fold increase in KO BMT versus WT BMT recipients; Figure 3I). Furthermore, we confirmed that Sort1 is a bona fide miR-146a target gene by luciferase assay (Figure 3K).

Thus, loss of miR-146a from BM-derived cells perturbs...
MicroRNA-146a (miR-146a) in bone marrow (BM)–derived cells contributes to atherogenesis. A, Ldlr−/− mice lethally irradiated and given BM transplantation (BMT) from wild-type (WT BM) or miR-146a−/− (knockout [KO] BM) donors followed by high cholesterol diet (HCD) for 4 or 12 wk. B, Body weights of female mice after 12-wk HCD (n=5–7). Weights of male mice were also unchanged (not shown). C, Food consumption in mice (n=4 mice per cage). Tt is 12 wk of HCD. D, Percentage of Oil Red-O (ORO) staining in aortas of Ldlr−/− mice with WT BM (top) and KO BM (bottom) after 12-wk HCD are shown to the right. Aortic root and descending thoracic aorta analyses are shown in Online Figure IIIB and IIIID. E, Circulating proinflammatory markers, soluble intercellular adhesion molecule-1 (sICAM-1), IL-6 (interleukin-6), and TNF-α (tumor necrosis factor-α), measured by ELISA of plasma samples (n=4–7). Values are relative to the controls for each group, as indicated. *Significant difference in expression.

Diet- and Age-Dependent Hematopoiesis Defects in miR-146a−/− Mice

Strikingly, the spleens of DKO mice fed HCD for 18 weeks (>8 months of age) have previously been shown to spontaneously develop splenomegaly, which is accompanied by BM hematopoiesis defects.32 Because we observed a splenomegaly phenotype in young mice on HCD, this suggests that atherogenic diet may accelerate the development of splenomegaly. Similar to global KOs, mice receiving KO BMT and fed HCD developed larger spleens and had pale femurs, suggestive of BM dysfunction (Figure 4B and 4C). We previously

miR-146a Regulates Atherogenesis

cholera metabolism, potentially through dysregulated NF-kB–dependent inflammatory pathways in the liver, including macrophage accumulation and IL-6 secretion, and perhaps through regulation of Sort1. Of note, despite the lower levels of VLDL/LDL cholesterol, miR-146a−/− mice display an exaggerated inflammatory response to HCD.
showed that prolonged hypercholesterolemia results in the outsourcing of hematopoiesis from the BM to the spleen. It seems that this phenotype may be accelerated and exaggerated in miR-146a−/− mice, even in the face of lower circulating VLDL/LDL cholesterol levels.

To further investigate the effects of aging on splenomegaly and atherogenesis, DKO mice were fed a 12-week HCD regime starting at 20 weeks of age (rather than the typical 10 weeks of age; Figure 4D). In contrast to younger DKO mice, which had unaltered plaque burden in the aorta after 12 weeks of HCD (Figure 2D), older mice had reduced atherosclerosis in the aortic arch after the same duration of diet (Figure 4E). No differences in plaque formation were observed in the descending thoracic aorta (not shown). This reduction in aortic arch atherosclerosis was accompanied by splenomegaly (Figure 4F and 4G). Importantly, the splenomegaly phenotype at this age was dependent on exposure to HCD, as this was not observed in DKO mice on a regular chow diet (Figure 4F and 4G). The pale femur phenotype in older DKO mice also seemed to be dependent on exposure to HCD (Figure 4F). The increased spleen size in older DKO mice on HCD corresponded with an increase in splenic CD45+ leukocytes (Figure 4H). Intriguingly, these findings highlight a potential relationship between the reduced atherogenesis observed in DKO mice on HCD and development of splenomegaly and pale femurs, suggesting that defective hematopoiesis may contribute to the phenotype. Although previous studies have linked splenomegaly with reduced circulating cholesterol,21
the contribution of splenomegaly to reduced LDL cholesterol in miR-146a−/− mice remains unclear. Although leukocyte content in the spleen at 12 weeks of HCD was not significantly different in DKO s (Online Figure VA) and spleens were not significantly larger (Figure 4A) — despite reduced levels of plasma cholesterol at this stage (Figure 2F) — mice receiving miR-146a−/− BMT had greatly enlarged spleens at 12 weeks of HCD (Figure 4C), which coincided with reduced circulating LDL (Figure 3F). Furthermore, oxidized LDL uptake and cholesterol efflux were similar in WT and miR-146a−/− macrophages (Online Figure VB and VC), suggesting that miR-146α−/− deficient macrophages seem not to be more avid at sequestering cholesterol. However, expansion of macrophages in the liver and spleen may contribute to the sequestering of cholesterol from circulation. The contribution of splenomegaly to cholesterol lowering in miR-146a−/− mice will require further exploration.

Loss of miR-146α Leads to Reduced BM Hematopoiesis While Promoting Extramedullary Hematopoiesis in the Spleen in Mice Fed a HCD

The enlarged spleens in DKO mice on 18 weeks of HCD contained more CD45+ leukocytes and lymphocytes (Figure 5A; Figure 5).

**Figure 5.** Global loss of microRNA-146a (miR-146a) inhibits bone marrow (BM) hematopoiesis and promotes extramedullary hematopoiesis in the spleen. Increase of splenic (A) and decrease in BM (B) CD45+ leukocytes and Ly6G−/CD115− lymphocytes in DKO mice on diet for 18 wk, determined by fluorescence-activated cell sorting (FACS) analysis (n=5). C, Decrease of multipotent progenitor cells (MPPs) and downstream progenitor cells (eg, Sca-1−stem cells antigen-1−)negative progenitor [LS−K], megakaryocyte–erythroid progenitor [MEP], common myeloid progenitor [CMP], and granulocyte–macrophage progenitor [GMP]) in BM of double knockout (DKO) mice after 18 wk of high cholesterol diet (HCD; n=5). D, Increase of splenic hematopoietic and multipotent stem cells in DKO mice after 18 wk of HCD (n=5; Online Figure VIII). HPC indicates hematopoietic progenitors cell; HSC, hematopoietic stem cell; Ldlr, low-density lipoprotein receptor; and LSK, lineage Sca-1+ Kit+. 
miR-146a in BM-Derived Cells Regulates BM and Extramedullary Hematopoiesis and Levels of Circulating Leukocytes and Lymphocytes

Similar to the nontransplanted DKO mice on 18-week HCD, mice receiving KO BM accumulated more CD45+ leukocytes and lymphocytes in their spleens; however, this occurred after just 12-week HCD (Figure 6A). The BM in these mice was depleted of these cells by 12-week HCD (Figure 6B). This was accompanied by elevated NF-κB signaling in the BM of KO mice, as well as enhanced expression of TRAF6, an miR-146a target gene (Figure 6C). Progenitors downstream of HSCs, namely multipotent progenitor cells, Sca-1–negative progenitors, common myeloid progenitors, granulocyte–macrophage progenitors, and megakaryocyte–erythroid progenitors and megakaryocyte–erythroid progenitors, were diminished in the BM (Figure 6D), whereas extramedullary hematopoiesis was evident in mice receiving KO BM (Figure 6E). Correspondingly, mice receiving KO BM had decreased levels of circulating atherogenic leukocytes, including neutrophils, B cells, and Ly6C+ monocytes, but levels of atheroprotective Ly6C+ monocytes were increased after 12 weeks of HCD (Figure 6F). Assessing circulating levels of leukocytes and lymphocytes at earlier stages (ie, 4 weeks of HCD) revealed monocytosis in mice receiving KO BM (Figure 6F), suggesting that the reduction of hematopoiesis at later stages of atherosclerosis is preceded by enhanced hematopoiesis at earlier stages, similar to previous studies that revealed HSC exhaustion in KO mice in the context of repeated LPS stimulation.10

We next assessed the functionality of WT (CD45.1) and KO (CD45.2) BM-derived cells in a competitive 1:1 BMT. After reconstitution of the BM compartment of lethally irradiated Ldlr−/− mice for 8 weeks, mice were placed on either normal chow diet (NCD) or HCD diet. Assessing circulating levels of leukocytes in mice fed an NCD revealed that KO BM cells preferentially contributed to neutrophil and Ly6C+ monocyte populations compared with WT BM cells (Figure 7A). A short duration on HCD (4 weeks) expanded the leukocyte populations examined, and KO cells were predominant compared with WT cells (Figure 7A). This was especially the case for neutrophils and Ly6C+ monocytes. However, in mice that received HCD for 12 or 32 weeks, the abundance of KO BM-derived cells was decreased. WT BM-derived cells were less affected (Figure 7A). This suggests that long-term HCD impairs the ability of KO BM-derived cells to contribute to circulating leukocyte populations. Assessing the abundance of WT versus KO leukocytes in the aorta at advanced stages of atherosclerosis revealed that neutrophils, macrophages, and monocytes (Ly6C+ and Ly6C+) seemed to be primarily WT BM derived, whereas B- and T-cell populations had similar contributions from WT and KO cells (Figure 7B). This was in contrast to the aorta in mice fed an NCD, where the majority of the cells seemed to be derived from KO cells (Figure 7B). Consistent with the reduced abundance of KO BM-derived cells in the circulation and atherosclerotic plaques in mice fed HCD, hematopoietic cells in the BM seemed to be primarily of WT origin under conditions of HCD feeding (Figure 7C). However, the opposite was observed in mice fed an NCD (Figure 7C).

miR-146a in the Vasculature Restrains EC Activation and Atherosclerosis

Deletion of miR-146a has a major effect on BM-derived cell function, promoting systemic inflammatory signaling, extramedullary hematopoiesis, BM failure, and lipid dysregulation. To further distinguish the role of miR-146a in BM-derived cells versus the rest of the body, we transplanted lethally irradiated Ldlr−/− and DKO mice with miR-146a+/− (WT) BM (Figure 8A). Transplanted mice were placed on HCD for 12 weeks. Interestingly, we found no differences in circulating IL-6, sICAM-1, or TNF-α levels (Figure 8B) or circulating cholesterol or lipoproteins (Figure 8C). This suggests that the dysregulation of inflammation and circulating lipoprotein levels are dependent on deletion of miR-146a from BM-derived cells, rather than in other cell types, such as hepatocytes. In addition, no changes were observed in spleen size (Figure 8D).

Levels of leukocytes in the spleen and in the circulation were also normalized, and only a modest decrease in leukocyte levels in the BM was seen (Figure 8E). Interestingly, NF-κB–dependent cytokines known to accelerate HSC proliferation (ie, IL-6, TNF-α, and IL-10)10,22,23 were highly expressed in the BM of Ldlr−/− mice reconstituted with KO BM, but this was not observed in DKO mice reconstituted with WT BM (Figure 8F). Finally, with the normalization of these parameters after transplantation of WT BM in DKO mice, lipid plaque burden in the aorta was elevated compared with Ldlr−/− mice receiving WT BMT (Figure 8G).

To determine whether miR-146a in the vasculature affects EC activation in the aorta, we stimulated WT or KO mice with the proinflammatory cytokine, IL-1β. We found that miR-146a target genes (eg, HuR [human antigen R] and TRAF6) were elevated in the aortic arch of KO mice, and that levels of VCAM-1 (vascular cell adhesion molecule-1), E-Selectin (SELE), and ICAM-1 (intercellular adhesion molecule-1) were induced to a greater extent in KO compared with WT mice (Online Figure VIII A and B).
VIIIB). In the setting of atherosclerosis, we found that expression of adhesion and chemokine genes seemed to be elevated in intimal cells of the aorta from DKO mice receiving WT BM compared with Ldlr−/− mice receiving WT BM (Online Figure VIIIC). These observations are consistent with our previous study that demonstrated that miR-146a restrains EC activation.6

Discussion

miR-146a has been identified as a vital brake in inflammatory signaling pathways,6,8,10,24 and levels are elevated in human atherosclerotic plaques.25 Recent studies have also uncovered a single-nucleotide polymorphism in the miR-146a gene that influences miR-146a expression and
susceptibility to coronary artery disease. However, no studies have directly assessed the function of endogenous miR-146a during atherogenesis. Here, we report that deletion of miR-146a within BM-derived cells surprisingly reduces atherosclerotic plaque formation, whereas deletion of miR-146a in the vasculature enhances endothelial activation and atherogenesis. These diverse phenotypes arise from a common defect in distinct cellular compartments, namely unrestrained NF-κB–dependent inflammatory signaling.

To our surprise, ablation of miR-146a from BM-derived cells reduced atherosclerosis, while paradoxically elevating indices of systemic inflammatory signaling (ie, proinflammatory cytokines and sICAM-1; an overview of miR-146a–deficient phenotypes is given in Online Figure IX). This increase in circulating cytokines would typically be accompanied by abundant inflammatory immune cells in circulation. However, we observed a decrease in proatherogenic cells, including Ly6C<sup>hi</sup> monocytes, T cells and neutrophils, and an increase in atheroprotective Ly6C<sup>lo</sup> monocytes. This implies that...
miR-146a–deficient leukocytes present in circulation are likely to be especially proinflammatory, demonstrating that miR-146a is important in quelling their activation. The paucity of circulating immune cells is the consequence of defective BM hematopoiesis, which likely arises because of hematopoietic cell exhaustion. Hypercholesterolemia stimulates hematopoiesis in the BM and spleen to produce proinflammatory cells, such as Ly6Chi monocytes that contribute to plaque growth.20,30–33 We find evidence of precocious monocytosis at early stages of atherogenesis in mice that received miR-146a−/− BM. In addition, transplanted miR-146a–deficient cells outcompete transplanted WT cells in the BM and in circulation during early atherogenesis. However, prolonged exposure to hypercholesterolemia seems to lead to a defect in the contribution of miR-146a−/− cells to hematopoietic cell populations in the BM, circulation, and in atherosclerotic plaques, implying that the activation of BM hematopoiesis by HCD cannot be sustained in the absence of miR-146a. Consequently, these mice initiate
extramedullary hematopoiesis in the spleen. Although spleen-derived Ly6C<sup>+</sup> monocytes can contribute to atherogenesis, we identify Sort1 as a novel miR-146a target gene. SORT1 has been shown to play an important, though controversial role in VLDL secretion. Genome-wide association studies in humans identified SORT1 as a causative gene in the regulation of circulating LDL levels and risk of atherosclerotic disease. Overexpression of SORT1 in the liver of mice enhances VLDL destruction in the liver and inhibits secretion. SORT1 can also bind extracellular LDL and direct its catabolism. However, other studies have found that deletion of Sort1 in mice can also result in reduced LDL levels, suggesting that the contribution of SORT1 to cholesterol metabolism remains to be fully resolved. Finally, additional studies found that lack of SORT1 in macrophages can inhibit secretion and signaling of cytokines, such as IL-6, whereas overexpression can enhance LDL uptake. This is of interest considering that Sort1 is likely dysregulated in BM-derived cells in our miR-146a loss-of-function models. Further studies will be required to delineate the contribution of miR-146a–dependent Sort1 regulation within BM-derived cells to the VLDL/LDL phenotype. Furthermore, additional miR-146a target genes in BM-derived cells may contribute.

Our study emphasizes the important role that miR-146a plays in controlling the output of inflammatory signaling pathways in ECs and hematopoietic cells in the setting of an atherogenic diet and further confirms the critical role of hypercholesterolemia in hematopoietic cell stress. Our findings are intriguing in light of polymorphisms in miR-146a in human patients that alters susceptibility to coronary artery disease. Furthermore, our studies suggest that elevating the expression of miR-146a in BM-derived cells or ECs is likely to suppress human atherogenesis by restraining NF-κB signaling, which is in agreement with recent studies in mouse models.

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Disclosures
None.

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Online Methods:

Mouse models of atherosclerosis: All animal protocols were approved by the Animal Care Committee at the University Health Network (Toronto). All mice used were age and sex matched unless stated otherwise and were all on the C57BL/6 background. Ldlr−/− and miR-146a−/−;Ldlr−/− mice were placed on high cholesterol diet (1.25% cholesterol, D12108C from Research Diets Inc.) at the age of 10 weeks. For bone marrow (BM) transplant models, 10 week-old recipient mice were subjected to whole-body irradiation (10 Gys) followed by injection of bone marrow (BM) donor cells (1 x 10^6 cells) by tail vein injection, followed by recovery for 8 weeks. Plasma was collected by retro-orbital bleeds or cardiac puncture followed by centrifugation at 13,000g for 10 minutes. Mice were perfused with PBS before tissue extraction. Gene expression analysis from lesser curvature intimal cells of the aorta was performed as before^1. Whole tissue RNA or protein extraction was performed by homogenization in Trizol or Laemmli sample buffer, respectively. Aortic roots were embedded in OCT (optimal cutting temperature compound) and sectioned by the MIRC Core Histology Facility (McMaster University).

Cells: Blood was collected by retro-orbital bleeds using heparin-coated capillary tubes (Fisherbrand K41B22365566). Erythrocytes were lysed using RBC Lysis Buffer (BioLegend). Total white blood cell count was determined by preparing a 1:20 dilution of (undiluted) peripheral blood in RBC Lysis Buffer, followed by counting using a hemocytometer. For solid organs, single-cell suspensions were obtained as follows: for bone marrow, the femur of one leg was crushed with mortar and pestle and homogenized through a 40-µm-nylon mesh. Spleens were homogenized through a 40-µm-nylon mesh,
after which RBC lysis was performed using RBC Lysis Buffer (BioLegend) for 10 minutes. For aortic tissue, the aorta was perfused with 10 ml PBS before digestion. The entire aorta (from the aortic sinus to the iliac bifurcation) was cut in small pieces and subjected to enzymatic digestion with 450 U ml⁻¹ collagenase I, 125 U ml⁻¹ collagenase XI, 60 U ml⁻¹ DNase I and 60 U ml⁻¹ hyaluronidase (Sigma-Aldrich) for 30 minutes at 37°C while shaking. Single-cell suspensions of digested tissues were obtained by homogenizing digested tissue though 40-μm-nylon mesh.

**Flow cytometry:** Antibodies used for flow cytometric analyses are provided in Online Table II. Data was acquired on an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo v8.8.6 (Tree Star, Inc.). Aortic single cell suspensions were treated with FcBlock (BD Biosciences) for 15 minutes before incubation with antibody cocktail for an additional 30 minutes. Single cell suspensions of peripheral blood, spleen and bone marrow were stained with antibody cocktails for 30 minutes. Samples were fixed before flow analysis (BD Cytofix).

**Gene expression analysis:** RNA was isolated using Trizol (Invitrogen), reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) and quantitative reverse-transcriptase PCR (qRT-PCR) was performed as described previously¹. MiR-146a and U6 were reverse-transcribed using the Taqman® MicroRNA Reverse Transcription kit (Applied Biosystems) and analyzed using Taqman Primer sets (Applied Biosystems). Real-time PCR was conducted in triplicate using a Roche Lightcycler 480® with Roche 480 Probes Master Mix or LC 480 SYBR Green I Master (Roche) for Taqman® and Sybr green chemistries, respectively. Data was normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) or U6 using the Delta-Delta Cₜ method. The primers used are indicated in Online Table III. Lipoprotein Signaling and Cholesterol Metabolism genes were assessed using an RT² Profiler PCR Array (Qiagen), which contains 84 genes in these pathways, according to the manufacturer’s suggestions.
**Luciferase assays:** Luciferase constructs (pGL3) containing approximately 250 bp of wild-type mouse *Sort1* 3'UTR (*Sort1*WT/pGL3) or a mutant version with the miR-146a binding site mutated (*Sort1*MUT/pGL3) were constructed. 3’ UTR sequences were generated as gBlocks gene fragments (Integrated DNA technologies) with XbaI linkers (red):

5’-AATTCTAGAAACTGTATAGTGACATGTTAATGATTATCAGATGCCCGAATTCCCTAGTGCAAGTTCTCATTTCTCCGCATGCCCCTCAGCTGTGGTGACTTCCTGTCCTGGCAGCTCTGAGCTCCTGCCCTGGCAGCTCTGCTGAGTCCCTGTGTTTGAGCCTCCAGGGAGAAGGGTTTGGGCTGCATTCTTTATCCCATGCCACAGAAACGCTCAAGGTTCCCACTGCCTGTGGTTCCCTCCCTCTTCTAGAATAT-3’
The underlined region contains the miR-146a binding site, which was mutated (GTTCTCA → GTTAGAA) to generate the *Sort1*MUT/pGL3 construct. After digesting with XbaI, the gBlock fragment was cloned into the XbaI site in the 3’ UTR of pGL3. The directionality and sequence of the insert was confirmed by sequencing.

BAEC (bovine aortic endothelial cells) grown in 12-well dishes were transfected with 1 μg of luciferase construct and 100 ng of pRL *Renilla* luciferase construct (Promega) (for normalization of transfection efficiency), using Lipofectamine 2000. Cellular lysates were isolated 24 h post-transfection using Passive Lysis Buffer and luciferase activity was monitored using the Dual Luciferase Reporter Assay System (Promega) using a GloMax 20/20 Luminometer (Promega).

**Lipid measurements in plasma:** Plasma measurements of total cholesterol, low- and high-density lipoprotein, triglyceride and glucose were performed by the Clinical Chemistry department at The Centre for Phenogenomics (Toronto). Animals were fasted overnight prior to acquisition of plasma samples. Analytes and LIH (lipemia, icterus, hemolysis) were scored on a Beckman AU480 Biochemistry Analyzer.

**Liver lipid analysis:** Liver lipid content was determined based upon the method described by Carr *et al*². A piece of frozen liver was thawed and minced with a razor blade. Following transfer to a tared 16x100mm glass tube, the wet weight of the liver piece was measured using an analytical balance. To extract the lipids from the tissue, 3
ml 2:1 chloroform:methanol (CHCl₃:MeOH) was added to the tube which was then incubated at 60°C for 3 hours and subsequently overnight at room temperature. After centrifuging the tube at 1,500xg for 10 min, the 2:1 CHCl₃:MeOH lipid extract was transferred to a new 16x100 mm glass screw top tube. The tube containing the extracted liver was washed with 2 ml 2:1 CHCl₃:MeOH and centrifuged as described above. The 2:1 CHCl₃:MeOH lipid extract and wash were combined and the solvent was evaporated under nitrogen at 55°C. The dried lipid extract was dissolved in 6 ml of 2:1 CHCl₃:MeOH. After the addition of 1.2 ml dilute H₂SO₄ (0.05%, v/v), the tube was vortexed for 20 seconds and the phases separated by centrifugation as described above. The upper aqueous phase was removed and an aliquot (typically 1 ml) of the lower phase lipid-containing organic phase was transferred to a new 16x100 mm glass screw top tube using a volumetric glass pipet. After adding 2 ml 1% Triton-X100 dissolved in CHCl₃, the organic solvent was evaporated under nitrogen at 55°C. The dried sample was dissolved in 1 ml water while being heated at 60°C for 10 min. After vortexing and centrifuging as above, samples were analyzed for lipids using commercially available enzymatic kits for total cholesterol (Pointe Scientific, cholesterol reagent), free cholesterol (Wako, Free Cholesterol E), and triglyceride (Wako, L-Type Triglycerides M). To determine protein content of the liver piece, the CHCl₃:MeOH was removed from the extracted liver by placing the uncapped tubes in a 100°C vacuum oven for 20 minutes. 4 ml 1N NaOH was added to the tube and the capped tube was incubated in a 100°C vacuum oven vortexed every 30 minutes until the tissue was dissolved. A modified Lowry assay using BSA as a standard was used to determine the protein concentration of the tissue lysate. Note: Organic-solvent resistant, Teflon lined caps were used to seal the tubes throughout the protocol.

**Gas Chromatography (GC) analysis of neutral sterol in feces:** Feces were transferred to a 20 mL glass scintillation vial and desiccated overnight in a vacuum oven set at 80°C. The dried feces were weighed and crushed into a fine powder using a mortar and pestle. A portion of fecal powder (~25 mg) was weighed in a 16x100 mm glass screw top tube containing 100 µg 5-alpha cholestane (Steraloids, C3300-000). To saponify the fecal lipid, 2 ml 95% EtOH and 200 µL 50% KOH were added to the tube, which was then
sealed with a Teflon-lined cap and incubated at 60°C for 3 hours with periodic vortexing. The neutral sterol was extracted from the sample by adding 2 mL hexane followed by 2 mL water with vortexing (20 seconds) between each addition. The tube was centrifuged at 1500xg for 10 min at room temperature. A 400 µl aliquot of the upper hexane phase was diluted 4-fold with hexane and transferred to a GC vial for analysis of sterol mass. The extracted sterol was analyzed by injecting 1 µL of sample onto a ZB50 (0.53-mm inner diameter × 15 m × 1 µm) gas-liquid chromatography column (Phenomenex) at 250°C and installed in a Agilent Technologies 7890B gas chromatograph equipped with a Agilent Technologies 7693 autosampler using on-column injection and a flame ionization detector (FID).

**GC analysis of biliary cholesterol:** Gallbladder bile (2 µl) was transferred into a 16x100mm glass screw top tube containing 10 µg 5α-cholestane and 0.75 ml water. To the tube was added sequentially, 2.25 ml 2:1 MeOH:CHCH₃, 1.5 ml CHCH₃, and 0.75 ml water. After each addition, the tube was capped and vortexed for 20 seconds. After centrifuging the tube at 1500xg for 10 minutes at room temperature, the organic, bottom phase of the bile extract was transferred to a new 16x100mm glass screw top. The organic solvent was evaporated under nitrogen at 55°C. To saponify the lipid, 1 ml 95% EtOH and 100 µL 50% KOH was added to the tube which was then sealed with a Teflon-lined cap and incubated at 60°C for 3 hours with periodic vortexing. The cholesterol was extracted from the sample by adding 1 mL hexane followed by 1 mL water with vortexing (20 seconds) between each addition. The tube was centrifuged at 1500xg for 10 min at room temperature. The upper hexane phase was transferred to a glass 12x75 mm tube. After evaporating the hexane as described above, the dried cholesterol was dissolved in 50 µl hexane and transferred to a tear drop GC vial insert. The sample was then analyzed by GC-FID as described above.

**FPLC analysis of lipoproteins:** 100 µL of pooled plasma was separated on a Superose column (Amersham) at a flow rate of 0.4 mL/min as described previously.³ Cholesterol in each fraction was measured using the Total Cholesterol E kit (Wako, 439-17501).
**VLDL secretion assay in vivo:** Mice were fasted for 4 hours prior to intraperitoneal injection with 300 μL of poloxamer 407 in PBS (1000 mg/kg, Sigma). Retro-orbital bleeds using heparinized capillary tubes were performed prior to poloxamer 407 injection (0 h), and then 30 minutes, 1 h, 2 h and 3 h after. Triglycerides from isolated plasma samples were quantified using L-Type Triglyceride M Enzyme kit (Wako Diagnostics), as specified by the manufacturer, and total cholesterol was measured by the Clinical Chemistry department at The Centre for Phenogenomics (Toronto).

**Triglyceride secretion in IL-6 treated primary mouse hepatocytes:** Primary mouse hepatocytes were isolated as described before \(^4,^5\) and cultured in DMEM/F-12 (Gibco, Waltham) with 2 mM L-Glutamine (Sigma-Aldrich Canada Co., Oakville), 10% fetal calf serum (Sigma-Aldrich Canada Co.), 1% ITS-A (Gibco), 1% Penicillin/Streptomycin (Wisent Inc. Saint-Bruno), and 0.04 μg/mL EGF (Sigma-Aldrich Canada Co.). 0.5-1.0 x 10\(^6\) cells were plated onto one well of a collagen-coated 12-well plate. Non-adherent/dead cells were removed after 4 h and cells were then cultured over-night. The following day, media was changed followed by the addition of recombinant mouse IL-6 (Peprotech, Cat. #216-16) for 6 h. Media was collected (1 mL total volume) and 100 μL was used to measure triglycerides using the L-Type Triglyceride M Enzyme kit (Wako Diagnostics), as specified by the manufacturer.

**Cholesterol efflux assays and oxLDL uptake assays:** Bone marrow cells were harvested from femurs of wild-type and miR-146a\(^{-/-}\) mice and differentiated into macrophages for 7 days in differentiation media (DMEM with 10% (v/v) FBS, 20% (v/v) L929 conditioned media and 1% (v/v) penicillin and streptomycin) to generate bone marrow-derived macrophages (BMDMs). Cholesterol efflux experiments were performed essentially as previously described\(^3,^6\). BMDMs were cholesterol-loaded with 37.5 μg/mL acetylated LDL (Alfa Aesar) and labeled with 1 μCi/mL [\(^3\)H] cholesterol (Perkin Elmer) for 24 h. BMDMs were washed extensively with PBS and equilibrated in 2% fatty acid-free BSA in DMEM media for 4 h prior to being treated with 50 μg/mL apoA1 (Alfa Aesar) (or BSA alone, where indicated) for 6 or 24 h. Medium and cellular
[3H] were counted and expressed as a percentage of total cellular [3H] cholesterol content.

Peritoneal macrophages were isolated from mice injected with 1 mL of thioglycolate 4 days prior. Peritoneal macrophages were grown overnight in DMEM with 10% FBS and Penicillin/Strep. Prior to flow analysis, macrophages were treated with 4 uL of Dil-MOX-LDL (5mg/mL) (Kalen Biomedical, LLC) for 2 hours.

**Enzyme-linked immunosorbent assay (ELISA):** IL-6, TNF-α and soluble ICAM-1 protein was quantified from plasma isolated from mice that were fasted overnight using Quantikine ELISA kits from R&D Systems, according to the manufacturer’s recommendations.

**Plaque measurement from aorta:** Mice were perfused with PBS followed by 2% paraformaldehyde before extraction of the aorta. Adipose tissues were removed from the aorta before staining with Oil Red-O (ORO) (Sigma-Aldrich). Stock stain was 0.3 g/10 mL isopropanol. Working solution stain was comprised of a 3:2 ratio of stock ORO to water. Aortas were stained for 30 minutes followed by 2 washes of 60% isopropanol. The aortic arch and descending thoracic aorta were pinned for en face plaque area measurement and pictures were captured using a stereo microscope (Leica M165FC). Plaque percentage was calculated using ImageJ.

**Combined in situ PCR and immunostaining:** Sections (4 µm thick) from aortic root were fixed with Paxgene (Qiagen) and incubated with recombinant DNase I (Roche) overnight in SecureSeal™ hybridization chambers (Applied Biosystems) at 37°C. In situ PCR was performed with a miR-146a-5p ultramer extension primer (GACCCCTTAATGCGTCTAAAGACCCCTTAATGCGTCTAAAGACCCCTTAATG CGTCTAAAAACCCATGGAATTCACTTCTCA) in digoxigenin-labeled PCR system at 50°C for 30 min in a Thermoblock (Eppendorf). After stringent washing with SSC buffer and blockade of nonspecific binding sites using TNB (Perkin Elmer) and biotin/avidin binding sites using a blocking kit (Vector Lab), sections were incubated with a peroxidase-conjugated anti-digoxigenin antibody (Fab fragments from sheep,
1:100 dilution; Roche) for 1 h at 37°C. A tyramide-based amplification system (TSA Plus Biotin; Perkin Elmer) and Dylight 549–conjugated streptavidin (KPL) were used to visualize the probe. Sections were subsequently incubated with anti-CD31 (PECAM-1) antibody (Santa Cruz, sc-1506, goat polyclonal, 200μg/ml) followed by anti-goat FITC secondary antibody (Jackson ImmunoResearch, 705-165-147; diluted 1:100 in PBS). For Mac-2 staining, supernatant of cultured M3/38.1.2.8 HL2 cells (ATCC TIB-166) were used as Primary Mac-2 antibody (one drop approximately 50μl) followed by either anti-rat Cy5 (diluted 1:200 in PBS, purple) or anti-rat FITC (diluted 1:100 in PBS, green) secondary antibody.

**Western blotting:** Western blotting was performed as described. The following antibodies were used: TRAF6 (Santa Cruz, D-10), HuR (Santa Cruz, 3A2), and GAPDH (Santa Cruz, 0411). HRP-conjugated secondary antibodies were from Cell Signaling or Santa Cruz, and blots were developed using SuperSignal West Pico Chemiluminescence Substrate (Pierce). For phospho-p65 western blots, 25 μL of Dynabeads® M-280 sheep anti-mouse IgG were mixed with 500 μL of ChIP dilution buffer and rotated for 10 min at 4 °C. Then, 5 μL of the anti-p65 rabbit polyclonal antibody (Santa Cruz Biotechnology, sc-372) was added to the mix and incubated at 4°C overnight with rotation. Afterwards, 150 μg of cell lysate was added to the antibody-Dynabeads mix and rotated overnight at 4°C. Beads were collected using a magnetic separator. The supernatant was removed and beads were washed four times with RIPA buffer. After washing, 30 μL of 4x loading buffer was added to the magnetic beads. Beads were heated at 95°C for 10 min and the supernatant was loaded to a 12% SDS-PAGE gel and Western blotting was performed using anti-p65 (the same as used for IP) and anti-phospho-p65 (Cell Signaling, Cat. #3033) antibodies. Anti-Actin antibody (Sigma, Cat. #A2066) was used as a loading control.

**Statistical analyses:** Unless otherwise indicated, data represent the mean of at least three independent experiments and error bars represent the standard error of the mean. Pair-wise comparisons were made using a Student’s t-test. Comparison of three or more groups was performed using a 1-way analysis of variance (ANOVA) with Newman–
Keuls post hoc test. A p-value of 0.05 or less was considered to be statistically significant. In all figures *, ** and *** represent a p-value of 0.05, 0.01 and 0.001, respectively.

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Online Table 1: Summary of qPCR array of lipoprotein signaling and cholesterol metabolism genes (differentially regulated genes are highlighted in red).

| Gene   | Relative change: DKO vs cdf-/- (18 w) (n = 4) | t-test p-value | Relative change: milt-1Hsa-/ BMT vs WT BMT (12 w) (n = 4) | t-test p-value |
|--------|-----------------------------------------------|----------------|------------------------------------------------------------|----------------|
| Abca1  | 0.822                                         | 0.604          | 0.018                                                      |                |
| Abca2  | 1.025                                         | 0.677          | 0.093                                                      |                |
| Abcg1  | 1.020                                         | 1.208          | 0.477                                                      | 0.005          |
| Acoa2  | 0.665                                         | 0.456          | 0.115                                                      |                |
| Akr1d2 | 0.931                                         | 0.535          | 0.036                                                      |                |
| Angpt3 | 0.930                                         | 0.656          | 0.227                                                      | 0.004          |
| Ankr24 | 0.922                                         | 0.791          | 0.335                                                      |                |
| Apoa1  | 0.791                                         | 0.540          | 0.046                                                      |                |
| Apoa2  | 0.861                                         | 0.572          | 0.084                                                      |                |
| Apoa4  | 1.304                                         | 0.868          | 0.670                                                      |                |
| Apob   | 0.805                                         | 0.627          | 0.048                                                      |                |
| Apoc3  | 0.686                                         | 0.454          | 0.002                                                      |                |
| Apod   | 0.793                                         | 0.716          | 0.665                                                      |                |
| Apoe   | 0.487                                         | 0.415          | 0.214                                                      |                |
| Apof   | 0.965                                         | 0.548          | 0.067                                                      |                |
| Apol8  | 2.378                                         | 0.684          | 0.353                                                      |                |
| Cehh3  | 0.941                                         | 0.784          | 0.124                                                      | 0.044          |
| Cel    | 0.818                                         | 0.767          | 0.601                                                      |                |
| Cela1b | 3.161                                         | 1054.995       | 0.423                                                      |                |
| Cebp   | 0.700                                         | 0.559          | 0.044                                                      |                |
| Colc12 | 0.834                                         | 0.558          | 0.006                                                      |                |
| Cyp    | 0.871                                         | 0.578          | 0.081                                                      |                |
| Cxcl16 | 0.869                                         | 0.823          | 0.371                                                      |                |
| Cyp6c3 | 1.695                                         | 0.513          | 0.229                                                      |                |
| Cyp11a1| 1.801                                         | 0.828          | 0.678                                                      |                |
| Cyp38a1| 0.853                                         | 0.898          | 0.767                                                      |                |
| Cyp40a1| 1.488                                         | 0.388          | 0.146                                                      |                |
| Cyp51  | 1.823                                         | 0.702          | 0.326                                                      |                |
| Cyp7a1 | 0.449                                         | 0.808          | 0.600                                                      |                |
| Cyp7b2 | 0.640                                         | 0.418          | 0.057                                                      |                |
| Dhcx24 | 1.812                                         | 0.750          | 0.192                                                      |                |
| Dhcx7 | 1.481                                         | 0.575          | 0.136                                                      |                |
| Elap   | 0.812                                         | 0.668          | 0.002                                                      |                |
| Fdh1  | 0.796                                         | 0.568          | 0.014                                                      |                |
| Fdps   | 2.644                                         | 0.457          | 0.150                                                      |                |
| Hdlap | 0.803                                         | 0.794          | 0.294                                                      |                |
| Hmgcr | 1.223                                         | 0.675          | 0.261                                                      |                |
| Hmgcs1 | 0.950                                         | 0.527          | 0.043                                                      |                |
| Hmgcs2 | 0.543                                         | 0.680          | 0.187                                                      |                |
| Id1    | 1.787                                         | 0.920          | 0.793                                                      |                |
| Id2    | 0.004                                         | 1.214          | 0.693                                                      |                |
| Id4    | 0.919                                         | 0.519          | 0.103                                                      |                |
| Insg1  | 1.604                                         | 0.462          | 0.085                                                      |                |
| Insg2  | 0.638                                         | 0.465          | 0.147                                                      |                |
| Lcat   | 0.702                                         | 0.803          | 0.208                                                      |                |
| Ldhnap1| 0.849                                         | 0.835          | 0.214                                                      |                |
| Lpl    | 7.050                                         | 0.347          | 0.450                                                      |                |
| Lpxe   | 1.055                                         | 0.780          | 0.210                                                      |                |
| Lppl10 | 0.910                                         | 0.915          | 0.663                                                      |                |
| Lppl12 | 0.643                                         | 0.568          | 0.076                                                      |                |
| Lppl18 | 0.004                                         | 0.000          | 0.391                                                      |                |
| Lppl6 | 0.866                                         | 0.695          | 0.023                                                      |                |
| Lrappl | 0.945                                         | 0.669          | 0.001                                                      |                |
| Mftpl1 | 0.895                                         | 0.692          | 0.023                                                      |                |
| Mfvd1  | 1.457                                         | 0.758          | 0.551                                                      |                |
| Mfkd1  | 1.341                                         | 0.652          | 0.305                                                      |                |
| Npc1l3 | 0.001                                         | 0.000          | 0.422                                                      |                |
| Nrdc2  | 0.865                                         | 0.341          | 0.052                                                      |                |
| Nthk4  | 0.794                                         | 0.861          | 0.685                                                      |                |
| Nvah1  | 1.716                                         | 0.599          | 0.219                                                      |                |
| Nvch1 | 0.857                                         | 0.574          | 0.177                                                      |                |
| Olr3l6 | 0.848                                         | 0.721          | 0.025                                                      |                |
| Olr3l7 | 0.973                                         | 0.943          | 0.697                                                      |                |
| Pck1e | 1.938                                         | 0.478          | 0.100                                                      |                |
| Pmk1  | 1.113                                         | 0.503          | 0.078                                                      |                |
| Ppadr | 1.013                                         | 0.829          | 0.585                                                      |                |
| Prkca1 | 0.853                                         | 0.994          | 0.971                                                      |                |
| Prkca2 | 0.817                                         | 0.683          | 0.023                                                      |                |
| Prkcg2 | 0.972                                         | 0.679          | 0.124                                                      |                |
| Sapos | 0.910                                         | 0.602          | 0.183                                                      |                |
| Saposf1 | 0.797                                       | 0.789          | 0.212                                                      |                |
| Saposf2 | 0.807                                       | 0.798          | 0.037                                                      |                |
| Saposf3 | 1.010                                       | 0.880          | 0.422                                                      |                |
| Saposf4 | 0.785                                       | 0.688          | 0.160                                                      |                |
| Saposf5 | 1.163                                       | 1.481          | 0.237                                                      |                |
| Saposf6 | 1.040                                       | 0.398          | 0.004                                                      |                |
| Saposf7 | 1.118                                       | 0.728          | 0.089                                                      |                |
| Stab2 | 0.988                                         | 0.942          | 0.705                                                      |                |
| Stab2 | 0.852                                         | 0.628          | 0.102                                                      |                |
| Stard6 | 1.626                                         | 0.477          | 0.006                                                      |                |
| Tm7sd2 | 1.231                                         | 1.055          | 0.839                                                      |                |
| Vldh | 0.565                                         | 0.322          | 0.056                                                      |                |

# of genes significantly dysregulated: 9 19
# Online Table II: List of antibodies used for FACS analysis

| ANTIGEN | COMPANY | CLONE |
|---------|---------|-------|
| Ly6C    | BD      | AL-21 |
| CD11c   | BD      | HL3   |
| CD11b   | BD      | M1/70 |
| CD115   | eBiosciences | AF598 |
| MHC II  | eBiosciences | M5/114.15.2 |
| CD45.1  | Biolegend | A20   |
| CD45.2  | eBiosciences | 104   |
| F4/80   | eBiosciences | BM8   |
| CD4     | BD      | L374  |
| CD8a    | BD      | 53-6.7|
| B220    | BD      | RA3-6B2|
| CD3e    | BD      | 17A2  |
| Ter119  | BD      | TER-119|
| CD127   | eBiosciences | A7R34 |
| NK1.1   | Biolegend | PK136 |
| Ly6G    | BD      | 1A8   |
| CD34    | eBiosciences | RAM34 |
| CD16/32 | BD      | 2.4G2 |
| CD117   | BD      | 2B8   |
| CD150   | eBiosciences | mShad150 |
| CD48    | Biolegend | HM48-1 |
| Sca1    | eBiosciences | D7   |
### Online Table III: Primer sequences for qRT-PCR analysis (mouse)

| Gene      | Forward Primer (5' -> 3') | Reverse Primer (5' -> 3') |
|-----------|---------------------------|---------------------------|
| IL-1β     | GTCCCTGTCATGCTTTCTGG      | ACCAGCAAGATGATCCCAAT      |
| Tnf-α     | GTAGCCCACGTGCTAGCAACAC    | GCACCACAGTTGGTGTCTTTGA    |
| Ccl12 (Mcp-1) | GTCCCTGTCATGCTTTCTGG  | ATGGGATCATCTTGCTTGGT      |
| IL-6      | TGGATGCTACCAAAACTGGAT     | CAAAGCCAGAGTCCTTCAGA      |
| IL-10     | GCCCAGAAATCAAGGAGCATT     | GCTCCACTGCTTGGCTTTATT     |
| Elav1 (HuR) | GTACACCACAGGCACAGAG     | CCAAGGGTGTAGATGAAGATGC    |
| Traf6     | TATGATCTGGACTGCTCCCAAC   | AGTCTCATGTGCAACTGGGTGA    |
| Irak1     | TTTATGCGCTGCTGCCCCAAAT   | TTTACATCAGGATAGCCCCCA     |
| Vcam1     | GCAACAAAGAAGGCTTTGAAGCA  | GATTTTGAGCAATCGTTTTTGTATTCAAG |
| Sele      | GAAACAAAGAAGTCTGGGATGT   | ATGACCACAGGATGAGCATCATT   |
| Icam1     | CTGCCTTGTTAGACTGACTGA    | AGGACAGGAGCTGAAAAGTTGTA   |
| Nos3      | CCAAGGTGATGAGCTCGTG      | GAAGATATCTCGGGGACAGCAG    |
| Sort1     | AATTGGAAGGATGGCTTTTGT    | GTGCAAACAGATCTCCCCCCCT    |
| Cd68      | AGCTGGCGAAGGGAGCAGCT     | AGGAGACAGGGCAGCCTGATG     |
| F4/80     | GGATGTACAGATGGGGGATG     | GGAAGCGCTGTTTACAGGGTG     |
| Hprt      | CAAGCTTGCTGGTGAAGAGA     | TGAAGTACATTAGTGCAAGCCATATC |
ONLINE FIGURE LEGENDS:

**Online Figure I:** Cross-sections of mouse aortic roots during the progression of atherosclerosis (Ldlr^{-/-} mice; 4, 12 and 24 weeks of HCD). Expression of miR-146a, assessed by in situ PCR (red) overlaps with Mac-2 positive leukocytes found in the intima. Regions of miR-146a expression adjacent to the lumen are suggestive of endothelial cell (EC) expression.

**Online Figure II:** (A) Representative images of en face Oil Red-O staining in the aorta of Ldlr^{-/-} and Ldlr^{-/-};miR-146a^{-/-} (DKO) mice after 18 weeks of HCD reveals changes in plaque formation in the aortic arch, but not in the descending thoracic aorta. (B) Representative images of sections through the aortic root of Ldlr^{-/-} and DKO mice after 18 weeks of HCD. (C) Quantification of plaque area per valve (n = 4). No major changes in plaque formation in the root were noted. (D) Intrahepatic content of total cholesterol (TC), free cholesterol (FC), cholesterol esters (CE) and triglycerides (TG) after 18 weeks of HCD (n = 4).

**Online Figure III:** Lethally-irradiated Ldlr^{-/-} mice received bone marrow (BM) transplantation from wild-type (WT BM) or miR-146a^{-/-} (KO BM) donors followed by 8 weeks recovery. (A) FACS analysis of CD45^{+} leukocytes and lymphocytes in peripheral blood (PB) prior to start of high cholesterol diet (HCD) regimen, revealing similar levels of circulating cells (n = 6-8). (B) Representative images of en face Oil Red-O staining in the aorta of Ldlr^{-/-} mice receiving WT or KO BM after 12 weeks of HCD. (C) Macrophage content in the aortic arch as assessed by mRNA levels of macrophage markers (CD68, F4/80). Data is normalized to HPRT (n = 3-4). (D) Representative images of sections through the aortic root of Ldlr^{-/-} mice receiving WT or KO BM after 12 weeks of HCD.

**Online Figure IV:** The expression of 84 lipoprotein signaling and cholesterol metabolism genes was assessed by qRT-PCR array of RNA isolated from livers of Ldlr^{-/-} or DKO mice (18 weeks of HCD) or Ldlr^{-/-} mice receiving WT or KO BMT (12 weeks HCD) (n = 4 per group). The relative change in expression in DKO mice was compared with Ldlr^{-/-} mice and relative change in expression in KO bone marrow transplant (BMT) mice was compared to WT BMT. Genes that were significantly dysregulated in either comparison are depicted in a heat map, with * indicating significantly dysregulated genes. Genes that were significantly dysregulated in both comparisons are highlighted in red. See Online Table I for the complete dataset.

**Online Figure V:** (A) FACS analysis of CD45^{+} leukocytes from spleen, BM and peripheral blood of Ldlr^{-/-} and DKO mice after 12 weeks HCD (n = 5-8). (B) Dil-labeled ox-LDL uptake analysis in peritoneal macrophages isolated from WT of KO mice (n = 4). (C) Cholesterol efflux assay in bone marrow-derived macrophages (BMDMs) isolated from WT or KO mice (n = 6).

**Online Figure VI:** Gating schematic for FACS analysis of (A) myeloid and lymphoid cells, and (B) hematopoietic stem cells.
**Online Figure VII:** (A) Representative FACS plots for HPC-1, HPC-2, HSC, and MPP populations from the BM. MPPs are decreased, while no changes to the long-term HSCs were observed in DKO mice after 18 weeks HCD. Quantification is in Figure 5C. (B) FACS analysis of PB neutrophils, B-cells, Ly6C\textsuperscript{hi} and Ly6C\textsuperscript{lo} monocytes in *Ldlr\textsuperscript{-/-}* and DKO mice (18 weeks of HCD) (n = 5).

**Online Figure VIII:** (A) RNA expression by qRT-PCR of aortic arches harvested from WT and *miR-146a\textsuperscript{-/-}* mice injected intravenously with IL-1β for 2 hours. n = 8. (B) Western blot of miR-146a target genes (HuR and TRAF6) in WT and *miR-146a\textsuperscript{-/-}* aortas. A representative blot of 4 is shown. (C) RNA expression of lesser curvature aortic cells (EC and intimal cells) from aged *Ldlr\textsuperscript{-/-}* and DKO mice (10 months) on HCD for 2 weeks. n = 2.

**Online Figure IX:** Schematic overview of the atherosclerotic phenotypes observed in mice with a deficiency of *miR-146a* in bone marrow-derived cells. Note that phenotypes are similar when *miR-146a* is deleted globally, but the phenotypes take longer to manifest. While total and LDL cholesterol levels are normal at the early stages of cholesterol, there is a progressive decrease in LDL cholesterol that appears to be due to defects in VLDL secretion, which is accompanied by inflammation (e.g. IL-6 expression) and *Sort1* expression in the liver. Despite the lower levels of LDL cholesterol, circulating inflammatory cytokines are increased, NF-κB activity is elevated and splenomegaly occurs. In the bone marrow, an initial increase in hematopoiesis in response to hypercholesterolemia is followed by a progressive decrease in hematopoiesis. Levels of circulating pro-atherogenic cells (such as Ly6C\textsuperscript{hi} monocytes, neutrophils and T-cells) are reduced as atherosclerosis progresses, and extramedullary hematopoiesis in the spleen occurs, but is unable to compensate for reduced hematopoiesis in the bone marrow.
Online Figure I

*Ldlr*^−/−^ mice

4 weeks HCD

12 weeks HCD

24 weeks HCD
A. Post-BM transplant, Pre-diet

PB CD45⁺

| Cell Number (x10⁷) | Ldlr⁺/- WT BM | Ldlr⁺/- KO BM |
|-------------------|---------------|---------------|
| 0                 | ns            | ns            |

PB Lymphocytes

| Cell Number (x10⁷) | Ldlr⁺/- WT BM | Ldlr⁺/- KO BM |
|-------------------|---------------|---------------|
| 0                 | ns            | ns            |

B. 12 weeks HCD

C. 12 weeks HCD

Aortic Arch

| Relative Expression | Ldlr⁺/- WT BM | Ldlr⁺/- KO BM |
|---------------------|---------------|---------------|
| Cd68                | 1.00          | 0.50          |
| F4/80               | 1.25          | 1.00          |

D. 12 weeks HCD

Online Figure III
Online Figure IV
Online Figure VI
Online Figure VII

A. Bone Marrow Hematopoietic Stem Cells

| CD48 | CD150 |
|------|-------|
| HPC-1 | HPC-2 |
| MPP   | HSC   |

CD48 vs CD150 plot for Ldlr^-/- and DKO strains.

B. 18 weeks HCD

- **PB Neutrophils**
  - Ldlr^-/-: $p=0.067$
  - DKO:

- **PB B Cells**

- **PB Ly6C^hi Monocytes**

- **PB Ly6C^lo Monocytes**

Ldlr^-/- (blue dots) and DKO (orange squares) represented.
Online Figure VIII

A

Relative Expression

0.0
0.5
1.0
1.5
2.0
2.5

HuR
Irk1
Traf6

B

wild-type
miR-146a

HuR
TRAF6
GAPDH

C

Relative Expression

30
20
10
10
0

HuR
Traf6
Irk1
Vcam1
Sele
Icam1
Mcp1
Nos3

LDLR
DKO
Online Figure IX

| Initiation | Early Athero | Late Athero |
|------------|-------------|-------------|
| LDL Cholesterol |  |  |
| Cytokines/Splenomegaly/NF-κB Activity |  |  |
| Bone Marrow/Hematopoiesis/Circulating Leukocytes |  |  |
| Atherogenesis |  |  |

- **Ldlr^-/-** or wild-type BMT
- **Ldlr^-/-;miR-146a^-/-** or **miR-146a^-/-** BMT