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

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ABSTRACT

**Rationale:** Inflammation is a key contributor to atherosclerosis. MicroRNA-146a (miR-146a) has been identified as a critical brake on pro-inflammatory NF-κB signalling in several cell types, including endothelial cells and bone marrow-derived cells. Importantly, miR-146a expression is elevated in human atherosclerotic plaques, and polymorphisms in the *miR-146a* pre-cursor have been associated with risk of coronary artery disease.

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

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

**Keywords:** MicroRNA, inflammation, atherosclerosis, endothelial cell, NF-κB, hematopoiesis.
Nonstandard Abbreviations and Acronyms:

acLDL    Acetylated LDL
BAEC    bovine aortic endothelial cells
Bm    bone marrow
BMDM    bone marrow-derived macrophage
BMT    bone marrow transplant
CMP    common myeloid progenitor
DKO    double knockout
DTA    descending thoracic aorta
EC    endothelial cell
ELISA    enzyme-linked immunosorbent assay
FACS    fluorescence activated cell sorting
GAPDH    glyceraldehyde-3-phosphate dehydrogenase
GC    greater curvature
GMP    granulocyte-macrophage progenitor
HCD    high cholesterol diet
HDL    high-density lipoprotein
HPRT    hypoxanthine-guanine phosphoribotransferase
HPC    hematopoietic progenitor cell
HSC    hematopoietic stem cell
HuR    human antigen R
ICAM-1    intercellular adhesion molecule-1
IL-1β    interleukin-1β
IL-6    interleukin-6
IL-10    interleukin-10
IRAK1    interleukin receptor associated kinase 1
KO    knockout
LC    lesser curvature
LDL    low-density lipoprotein
LDLR    LDL receptor
LPS    lipopolysaccharide
LS-K    Lin‘ Sca-1‘ c-Kit
MCP-1    monocyte chemoattractant protein-1
MEP    megakaryocyte-erythroid progenitor
MPP    multipotent progenitor cell
NCD    normal cholesterol diet
NF-κB    nuclear factor κ light chain enhancer of activated B cells
NOS3    nitric oxide synthase 3
ORO    oil red-O
oxLDL    oxidized LDL
PB    peripheral blood
qRT-PCR    quantitative reverse transcriptase-polymerase chain reaction
SELE    E-selectin
sICAM-1    soluble intercellular adhesion molecule-1
SMC    smooth muscle cell
SORT1    sortilin-1
TC    total cholesterol
TG    triglyceride
TNF-α    tumor necrosis factor α
TRAF6    TNF receptor associated factor 6
INTRODUCTION

Atherosclerosis is a chronic inflammatory vascular disease characterized by the narrowing of blood vessels due to the growth of lipid-rich plaques. The initiation of atherogenesis relies on the recruitment of circulating leukocytes by activated endothelial cells (ECs) to regions of deposited oxidized LDL. Activated ECs and leukocytes utilize the nuclear factor κ light chain enhancer of activated B cells (NF-κB) signalling pathway to propagate inflammatory gene expression, including induction of adhesion molecules, chemoattractants and cytokines to drive inflammation in the vessel wall. NF-κB signalling is tightly controlled, and this includes regulation by a network of microRNAs, which titrate the expression of signalling components post-transcriptionally. In particular, microRNA-146a (miR-146a) has been well characterized in both ECs and leukocytes as a negative regulator of NF-κB activity through its ability to target upstream adaptor proteins, including TRAF6 and IRAK.

Characterization of miR-146a deficient mice has revealed defects in multiple aspects of immune cell biology. Older (>1 year) miR-146a−/− mice develop multi-organ inflammation, bone marrow (BM) failure, splenomegaly and lymphoadenopathy. When challenged by pro-inflammatory stimuli (e.g. lipopolysaccharide [LPS] or interleukin-1β [IL-1β]) these mice have exacerbated NF-κB-dependent inflammatory responses, and demonstrate expansion of pro-inflammatory Ly6C hi monocytes. Interestingly, the hyperactivation of NF-κB caused by low-grade inflammation during normal ageing 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.

The NF-κB pathway is activated in ECs, macrophages and smooth muscle cells (SMCs) within human atherosclerotic lesions. However, defining the role of NF-κB signalling 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 atheroprone mice reduces atherogenesis, and it has been suggested that this is due to suppression of macrophage NF-κB signalling. 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 pro-inflammatory cytokines despite reduced levels of circulating LDL cholesterol. Lack of miR-146a in BM-derived cells leads to monocytosis in response to high cholesterol diet, followed by BM exhaustion; depleting circulating levels of pro-atherogenic cells. Conversely, deletion of miR-146a in the vasculature promotes atherogenesis by increasing endothelial activation. Thus, unrestrained inflammatory signalling 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.
RESULTS

MiR-146a expression is increased in endothelial cells and intimal cells during murine atherogenesis.

Ldlr<sup>-/-</sup> mice were placed on a high cholesterol diet (HCD) for 18 weeks to visualize the expression of miR-146a in atherosclerotic plaque (Fig. 1A). In situ PCR on aortic root cross-sections revealed that miR-146a was expressed in intimal cells including Mac-2<sup>+</sup> macrophages and was robustly expressed in CD31<sup>+</sup> ECs. The in situ signal was specific for miR-146a, as staining was not detected in miR-146a<sup>-/-</sup> mice (Fig. 1A). Expression of miR-146a in the aortic root appeared to progressively increase in the intima during the progression of atherosclerosis (Online Figure I). The absence of signal in the media implies that contractile SMCs in the aortic root do not express miR-146a at sufficient levels to be detected by this technique. Additionally, using quantitative RT-PCR (qRT-PCR) at an early stage of atherogenesis (i.e. Ldlr<sup>-/-</sup> mice, 4 weeks HCD) we found a significant elevation of miR-146a expression in the lesser curvature (LC) of the aortic arch, a region of the aorta where atherosclerotic plaque forms, compared to regions that are protected from atherosclerosis, namely the greater curvature (GC) of the aortic arch and the descending thoracic aorta (DTA) (Fig. 1B,C). 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<sup>6</sup>.

Global deletion of miR-146a activates pro-inflammatory 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<sup>-/-</sup>; Ldlr<sup>-/-</sup>) mice by crossing miR-146a<sup>-/-</sup> mice with Ldlr<sup>-/-</sup> 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 (Fig. 2A). Analyses of male and female mice were grouped together as we found no significant differences between sexes for the parameters measured, except for body weight (not shown). At the 12-week time-point, no differences in body weight (Fig. 2B) or aortic arch plaque burden (Fig. 2D) were observed between Ldlr<sup>-/-</sup> and DKO mice. However, the circulating inflammatory marker, IL-6, was elevated in the majority of DKO mice at this stage, although the difference did not reach statistical significance (Fig. 2E). By 18 weeks, despite no differences in body weight (Fig. 2B) or food intake (Fig. 2C), DKO mice surprisingly had less lipid plaque in the aortic arch (Fig. 2D). This decrease in atherosclerosis occurred despite signs of elevated systemic inflammatory signalling, including enhanced circulating levels of sICAM-1 and IL-6 (Fig. 2E). Atherosclerotic plaque formation was unaltered in the descending thoracic aorta of DKO mice: this region is typically protected from atherosclerosis (Online Figure II-A). Plaque burden in the aortic root was also comparable between groups (Online Figure II-B,C).

Unexpectedly, DKO mice displayed progressive lipid metabolism defects, resulting in lower circulating total cholesterol and LDL cholesterol. High-density lipoprotein (HDL) levels and triglyceride levels were modestly affected (Fig. 2F). Assessment of lipoprotein profiles by FPLC revealed a striking decrease in cholesterol content in very low-density lipoprotein (VLDL) fractions (Fig. 2G). Measurement of total cholesterol and triglyceride levels in the liver revealed no significant differences at 12 weeks (Fig. 2H) or 18 weeks of HCD (Online Figure II-D). Likewise, cholesterol levels in bile and feces were unchanged at 12 weeks of HCD (Fig. 2H). Assessment of VLDL secretion from the liver suggested a decrease in cholesterol and triglyceride secretion in DKO mice (Fig. 2I). Taken together these data demonstrate that lack of miR-146a decreases circulating VLDL/LDL cholesterol yet paradoxically enhances inflammatory signalling in mice on a pro-atherogenic diet.
Deletion of miR-146a in BM-derived cells enhances inflammatory signalling, yet paradoxically suppresses atherogenesis and alters cholesterol metabolism.

Next we performed BM transplantation experiments to elucidate the role of miR-146a in BM-derived cells during atherogenesis. Ldlr<sup>-/-</sup> mice were lethally irradiated and reconstituted with either miR-146a<sup>+/+</sup> (WT) or miR-146a<sup>-/-</sup> (KO) BM (Fig. 3A). Reconstitution of hematopoiesis following transplantation of WT or KO BM cells appeared to be normal, as circulating levels of leukocytes and lymphocytes were similar 8 weeks after BM transplantation, prior to the administration of HCD (Online Figure III-A). Body weight was similar between the two groups after 12 weeks of HCD (Fig. 3B), as was food intake (Fig. 3C). While lipid plaque burden was not significantly altered at early stages (i.e. 4 weeks HCD), mice receiving KO BM developed less lipid plaque in the aorta following 12 weeks of HCD (Fig. 3D; Online Figure III-B), and markers of macrophage content in the aortic arch were reduced (Online Figure III-C). Plaque burden in the descending thoracic aorta (Online Figure III-B) and aortic root (Online Figure III-D) appeared to be unchanged. The decrease in plaque burden in the aortic arch was paradoxically accompanied by signs of systemic inflammatory signalling, with higher levels of circulating soluble intercellular adhesion molecule (sICAM-1), interleukin-6 (IL-6), and tumour necrosis factor alpha (TNF-α) detected in the plasma of mice receiving KO BM after 12 weeks of HCD (Fig. 3E), with a trend towards elevated IL-6 levels being observed after 4 weeks of HCD (Fig. 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 signalling. 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 (TG) and HDL levels in the plasma of mice receiving KO BM (Fig. 3F). FPLC revealed a marked reduction in cholesterol content in VLDL fractions (Fig. 3G). However, levels of total and free cholesterol and triglycerides in the liver were not significantly different (Fig. 3H), neither were cholesterol esters (not shown), and fecal cholesterol levels were also unchanged (Fig. 3H). To determine potential mechanisms for the altered lipid metabolism we assessed gene expression in livers of Ldlr<sup>-/-</sup> and DKO mice (18 weeks HCD), and Ldlr<sup>-/-</sup> mice receiving WT or KO BM (12 weeks HCD). We observed an elevation of a macrophage marker (F4/80), as well as several pro-inflammatory cytokines such as IL-1β and IL-6, and an increase in IL-10, in DKO livers and in the livers of Ldlr<sup>-/-</sup> mice receiving KO BM, compared to their respective controls (Fig. 3I). Importantly, dysregulation of IL-6 and IL-10 have previously been implicated in altered lipid metabolism<sup>15-17</sup>. Indeed, we found that exposing primary hepatocytes to IL-6 decreased triglyceride secretion (Fig. 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 signalling and cholesterol metabolism genes by qRT-PCR arrays. A small number of genes were significantly dysregulated in either experimental group (9 genes in DKO mice compared to Ldlr<sup>-/-</sup> mice, and 19 genes in KO BMT recipients compared to WT BMT recipients) (Online Figure IV, Online Table I). However, the only genes that were significantly decreased in both models were ApoB (1.25-fold decrease in DKO mice vs. Ldlr<sup>-/-</sup> mice and 1.61-fold decrease in KO BMT vs. WT BMT recipients) and Cnbp (1.43-fold decrease in DKO mice vs. Ldlr<sup>-/-</sup> mice and 1.69-fold decrease in KO BMT vs. WT BMT recipients), but these changes were modest. Although not on the qRT-PCR array, we also assessed the expression of Sort1, since it is a known regulator of circulating LDL levels that was identified by GWAS in humans<sup>18</sup>, and it has been shown to promote IL-6 signalling and secretion in macrophages in mouse models<sup>19</sup>. Interestingly, Sort1 is also predicted to be a miR-146a target gene (Fig. 3K). We found that Sort1 expression in the liver was elevated in both models (2.21-fold increase in DKO mice vs. Ldlr<sup>-/-</sup> mice and 1.68-fold increase in KO BMT mice vs. WT BMT recipients) (Fig. 3I). Furthermore, we confirmed that Sort1 is a bone fide miR-146a target gene by luciferase assay (Fig. 3K). Thus, loss of miR-146a from BM-derived cells perturbs cholesterol metabolism, potentially through dysregulated NF-κB-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.

**Diet- and age-dependent hematopoiesis defects in miR-146a−/− mice.**

Strikingly, the spleens of DKO mice fed HCD for 18 weeks (6-7 months of age) were ~2.5 times larger than *Ldlr−/−* mice on the same atherogenic diet (Fig. 4A). At earlier stages (i.e. 12 weeks of HCD; 5-6 months of age) spleen weight was not significantly changed (Fig. 4A). Of note, aged *miR-146a−/−* mice (>8 months of age) have previously been shown to spontaneously develop splenomegaly, which is accompanied by BM hematopoiesis defects10. Since we observed a splenomegaly phenotype in young mice on HCD, this suggests that atherogenic diet may accelerate the development of splenomegaly. Similar to global knock-outs, mice receiving KO BM transplants and fed HCD developed larger spleens and had pale femurs, suggestive of BM dysfunction (Fig. 4B, C). We previously showed that prolonged hypercholesterolemia results in the outsourcing of hematopoiesis from the BM to the spleen20. It appears 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 ageing 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) (Fig. 4D). In contrast to younger DKO mice, which had unaltered plaque burden in the aorta following 12 weeks of HCD (Fig. 2D), older mice had reduced atherosclerosis in the aortic arch following the same duration of diet (Fig. 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 (Fig. 4F,G). 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 (Fig. 4F,G). The pale femur phenotype in older DKO mice also appeared to be dependent on exposure to HCD (Fig. 4F). The increased spleen size in older DKO mice on HCD corresponded with an increase in splenic CD45+ leukocytes (Fig. 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. While prior studies have linked splenomegaly with reduced circulating cholesterol21, the contribution of splenomegaly to reduced LDL cholesterol in *miR-146a−/−* mice remains unclear. While leukocyte content in the spleen at 12 weeks of HCD was not significantly different in DKOs (Online Figure V-A) and spleens were not significantly larger (Fig. 4A) – despite reduced levels of plasma cholesterol at this stage (Fig. 2F) – mice receiving *miR-146a−/−* BMT had greatly enlarged spleens at 12 weeks of HCD (Fig. 4C), which coincided with reduced circulating LDL (Fig. 3F). Furthermore, oxLDL uptake and cholesterol efflux were similar in wild-type and *miR-146a−/−* macrophages (Online Figure V-B,C), suggesting that *miR-146a* deficient macrophages appear to not 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-146a 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 (Fig. 5A; See Online Figure VI for flow cytometry gating strategies). This was in contrast to the depletion of these cells from the BM of DKO mice (Fig. 5B). Prior studies have observed defects in BM hematopoietic stem cell (HSC) longevity in aged (>8 months) *miR-146a−/−* mice, or in younger mice following repeated challenge with LPS10. Assessing the spectrum of hematopoietic cells in the BM revealed normal levels of hematopoietic progenitors cells (HPC-1, -2) and HSCs, but levels of multipotent progenitor cells (MPPs) were significantly decreased in DKO (Fig. 5C; Online Figure VII-A). The consequences of
decreased MPP levels were further evident in the decreased numbers of downstream progenitor cells (e.g. Sca-1 negative progenitors [LS-K], common myeloid progenitors [CMPs], granulocyte-macrophage progenitors [GMPs], megakaryocyte-erythroid progenitors [MEPs]) (Fig. 5C). Taken together, these data are suggestive of an HSC functional defect in the BM. Interestingly, despite this defect in HSC function in the BM, HSCs and downstream progenitor cells appeared in the spleens of DKO mice on HCD for 18 weeks but not in Ldlr-/– mice (Fig. 5D), demonstrating that loss of miR-146a accelerates extramedullary hematopoiesis. The dysregulation of hematopoiesis in the spleen and BM was accompanied by modest effects on circulating leukocytes such as neutrophils and B-cells, while anti-inflammatory Ly6Clo monocytes were significantly increased (Online Figure VII-B).

**miR-146a in BM-derived cells regulates BM and extramedullary hematopoiesis, and levels of circulating leukocytes and lymphocytes.**

Similar to the non-transplanted DKO mice on 18 weeks HCD, mice receiving KO BM accumulated more CD45+ leukocytes and lymphocytes in their spleens; however, this occurred following just 12 weeks HCD (Fig. 6A). The BM in these mice was depleted of these cells by 12 weeks HCD (Fig. 6B). This was accompanied by elevated NF-κB signalling in the BM of KO mice, as well as enhanced expression of TRAF6, a miR-146a target gene (Fig. 6C). Progenitors downstream of HSCs, namely MPPs, LS-K, CMPs, GMPs and MEPs were diminished in the BM (Fig. 6D), while extramedullary hematopoiesis was evident in mice receiving KO BM (Fig. 6E). Correspondingly, mice receiving KO BM had decreased levels of circulating atherogenic leukocytes including neutrophils, B-cells, and Ly6Cahi monocytes, but levels of atheroprotective Ly6Clo monocytes were increased after 12 weeks of HCD (Fig. 6F). Assessing circulating levels of leukocytes and lymphocytes at earlier stages (i.e. 4 weeks of HCD) revealed monocytosis in mice receiving KO BM (Fig. 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 stimulation10.

We next assessed the functionality of WT (CD45.1) and KO (CD45.2) BM-derived cells in a competitive 1:1 BM transplant. Following 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 a NCD revealed that KO BM cells preferentially contributed to neutrophil and Ly6Cahi monocyte populations compared to WT BM cells (Fig. 7A). A short duration on HCD (4 weeks) expanded the leukocyte populations examined, and KO cells were predominant compared to WT cells (Fig. 7A). This was especially the case for neutrophils and Ly6Cahi monocytes. However, in mice that received HCD for 12 or 32 weeks, the abundance of KO BM-derived cells were decreased. WT BM-derived cells were less affected (Fig. 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 vs. KO leukocytes in the aorta at advanced stages of atherosclerosis revealed that neutrophils, macrophages and monocytes (Ly6Cahi and Ly6Clo) appeared to be primarily WT BM-derived, while B and T cell populations had similar contributions from WT and KO cells (Fig. 7B). This was in contrast to the aorta in mice fed a NCD, where the majority of the cells appeared to be derived from KO cells (Fig. 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 appeared to be primarily of WT origin under conditions of HCD feeding (Fig. 7C). However, the opposite was observed in mice fed a NCD (Fig. 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 signalling, 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 (Fig. 8A). Transplanted mice were placed on HCD for 12 weeks. Interestingly we found no differences in circulating IL-6, sICAM-1, or TNF-\(\alpha\) levels (Fig. 8B) or circulating cholesterol or lipoproteins (Fig. 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 (Fig. 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 (Fig. 8E). Interestingly, NF-\(\kappa\)B-dependent cytokines known to accelerate HSC proliferation (i.e. IL-6, TNF-\(\alpha\) 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 (Fig. 8F). Finally, with the normalization of these parameters following transplantation of wild-type BM in DKO mice, lipid plaque burden in the aorta was elevated compared to Ldlr\(^{-/-}\) mice receiving wild-type BM transplant (Fig. 8G).

To determine whether miR-146a in the vasculature affects EC activation in the aorta, we stimulated WT or KO mice with the pro-inflammatory cytokine, IL-1\(\beta\). We found that miR-146a target genes (e.g. HuR and TRAF6) were elevated in the aortic arch of KO mice, and that levels of VCAM-1, E-Selectin (SELE) and ICAM-1 were induced to a greater extent in KO compared to WT mice (Online Figure VIII-A,B). In the setting of atherosclerosis, we found that expression of adhesion and chemokine genes appeared to be elevated in intimal cells of the aorta from DKO mice receiving WT BM compared to Ldlr\(^{-/-}\) mice receiving WT BM (Online Figure VIII-C). 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 signalling 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\(^{26-29}\). 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, while 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-\(\kappa\)B-dependent inflammatory signalling.

To our surprise, ablation of miR-146a from BM-derived cells reduced atherosclerosis, while paradoxically elevating indices of systemic inflammatory signalling (i.e. pro-inflammatory cytokines and sICAM-1) (see Online Figure IX for an overview of miR-146a deficient phenotypes). This increase in circulating cytokines would typically be accompanied by abundant inflammatory immune cells in circulation. However, we observed a decrease in pro-atherogenic cells including Ly6C\(^{hi}\) monocytes, T-cells and neutrophils, and an increase in athero-protective Ly6C\(^{lo}\) monocytes. This implies that miR-146a deficient leukocytes present in circulation are likely to be especially pro-inflammatory, 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 due to hematopoietic cell exhaustion. Hypercholesterolemia stimulates hematopoiesis in the BM and spleen to produce pro-inflammatory cells, such as Ly6C\(^{hi}\) 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 out-compete transplanted wild-type cells in the BM and in circulation during early atherogenesis. However, prolonged exposure to hypercholesterolemia appears 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 activation of BM hematopoiesis by HCD can not be sustained in the
absence of miR-146a. Consequently, these mice initiate extramedullary hematopoiesis in the spleen. Although spleen-derived Ly6C^hi monocytes can contribute to atherogenesis, the circulating cells generated in the spleen of mice receiving miR-146a deficient BM appear to be insufficient to compensate for the reduction in leukocyte output from the BM.

Defects in hematopoiesis have previously been observed in aged miR-146a^−/− mice. In this case, older mice (>8 months of age) developed a progressive loss of hematopoietic stem and progenitor cells (HSPCs) as a result of increased NF-κB-dependent IL-6 production in the BM. Enhanced IL-6 production in miR-146a^−/− mice promoted hematopoietic cell proliferation leading to eventual exhaustion. This phenotype could be accelerated by repeated challenge with LPS, which drives IL-6 production. Similarly, in our model of atherosclerosis we observed an increase in TRAF6 expression, NF-κB activity, and IL-6 expression (an NF-κB-regulated cytokine) in the BM, as well as IL-6 protein levels in circulation. Since hypercholesterolemia drives stress-induced hematopoiesis and premature HSC ageing and senescence, it is likely that increased cycling of hematopoietic cells leads to stem cell exhaustion in the absence of miR-146a.

A notable phenotype observed in both the global and BM-restricted miR-146a loss-of-function models, is the reduction in VLDL/LDL cholesterol in circulation. Defects in cholesterol homeostasis in miR-146a global knock-outs could be rescued by transplantation of wild-type BM, demonstrating that miR-146a in BM-derived cells, rather than hepatocytes, regulates lipid metabolism. It is important to note that the defects that we observe in miR-146a^−/− mice (i.e. enhanced levels of circulating cytokines, monocytosis, outsourcing of hematopoiesis to extramedullary sites, splenomegaly and eventual BM failure) are known to be driven by hypercholesterolemia. That we observe these phenotypes even in the face of lower VLDL/LDL cholesterol suggests that it is not lower cholesterol per se that is solely responsible for the decrease in atherosclerosis in these mice, although it likely contributes to altered initiation and progression of atherosclerosis. MiR-146a^−/− mice are especially sensitive to the inflammatory effects of hypercholesterolemia (even though VLDL/LDL cholesterol levels are decreased), and the hematopoietic phenotypes that we observe are not apparent in mice fed a normal cholesterol diet. The reduced atherosclerosis appears to be at least partly attributable to an unchecked chronic inflammatory response to hypercholesterolemia that drives BM hematopoiesis defects and a decrease in circulating pro-atherogenic inflammatory cells. The role of miR-146a in monocyte recruitment and macrophage biology in the plaque has not been explored here, but should be assessed in future studies.

We demonstrate that a lack of miR-146a appears to impair VLDL secretion from the liver. This is accompanied by macrophage accumulation, inflammatory gene expression (including IL-6) as well as an increase in Sort1 expression in the liver; each of which may contribute to the phenotype. Although miR-146a deficient bone marrow-derived macrophages have unaltered oxLDL uptake and cholesterol efflux, the expansion of macrophages in the liver or spleen may influence circulating cholesterol levels through cholesterol sequestration. Altered VLDL secretion also occurs in response to pro-inflammatory cytokines such as IL-6, and we confirm that exposure to IL-6 reduces triglyceride secretion from cultured primary mouse hepatocytes. Finally, 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. Over-expression 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 signalling of cytokines, such as IL-6, while over-expression can enhance LDL uptake. This is of interest considering that Sort1 is likely dysregulated in bone marrow-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 bone marrow-
derived cells to the VLDL/LDL phenotype. Furthermore, additional miR-146a target genes in bone marrow-
derived cells may contribute.

Our study emphasizes the important role that miR-146a plays in controlling the output of
inflammatory signalling 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 signalling, which is in
agreement with recent studies in mouse models.14,44

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None.

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**FIGURE LEGENDS**

**Figure 1.** MiR-146a is expressed in murine atherosclerotic plaques. (A) Cross-sections of Ldlr<sup>−/−</sup> or Ldlr<sup>−/−;miR-146a<sup>−/−</sup></sup> mouse aortic roots after 18 weeks of high cholesterol diet (HCD). Expression of miR-146a, assessed by in situ PCR (red) overlaps with Mac-2 positive macrophages (purple) and CD31 positive endothelial cells (EC) (green) in the intima, and signal is absent in miR-146a<sup>−/−</sup> mice. See Online Figure I for miR-146a expression during the progression of atherosclerosis. (B) Schematic of the aorta, indicating the aortic root (examined in panel 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<sup>−/−</sup> mice after 4 weeks of HCD (n = 5).

**Figure 2.** Reduced atherosclerosis in mice with global deletion of miR-146a. (A) Schematic of HCD regimen for Ldlr<sup>−/−</sup> and Ldlr<sup>−/−;miR-146a<sup>−/−</sup></sup> (DKO) mice. (B) Weights of male mice after 12 or 18 weeks 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<sub>0</sub> is 18 weeks of HCD. (D) Percentage of Oil Red-O (ORO) regions quantified from aortic arches of Ldlr<sup>−/−</sup> and DKO mice after HCD for 12 or 18 weeks. Representative images are shown to the right. The descending side of the aorta is to the right. See Online Figure II for aortic root and descending thoracic aorta analysis. n = 18-22 for 12 week time-point and n = 4 for 18 week time-point. (E) Circulating levels of pro-inflammatory markers, IL-6 and sICAM-1 in wild-type and DKO mice (n = 5-8). (F) Time-course of plasma cholesterol measurements (n = 3-5; one 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 prior to sample collection. (G) FPLC trace of cholesterol content in lipoprotein fractions in plasma after 18 weeks of HCD (pooled analysis of 5 samples). (H) Intrahepatic cholesterol and triglyceride levels in mice after 12 weeks HCD (n = 13-14). See Online Figure II-D for 18 weeks HCD. Bile cholesterol (n = 3) and fecal cholesterol (n = 4) in mice after 18 weeks HCD. (I) Assessment of VLDL secretion by measurement of triglycerides and cholesterol in plasma following injection of Poloxamer 407 (12 weeks HCD; n = 4, 2).

**Figure 3.** MiR-146a in bone marrow (BM)-derived cells contributes to atherogenesis. (A) Ldlr<sup>−/−</sup> mice lethally irradiated and given bone marrow (BM) transplantation from wild-type (WT BM) or miR-146a<sup>−/−</sup> (KO BM) donors followed by HCD for 4 or 12 weeks. (B) Body weights of female mice after 12 weeks HCD (n = 5-7). Weights of male mice were also unchanged (not shown). (C) Food consumption in mice (n = 4 mice per cage). T<sub>0</sub> is 12 weeks of HCD. (D) Percentage of Oil Red-O (ORO) regions quantified from aortic arches of Ldlr<sup>−/−</sup> mice with WT BM (top) and KO BM (bottom) after 12 weeks HCD are shown to the right. See Online Figure III-B,D for aortic root and descending thoracic aorta analyses. (E) Circulating pro-inflammatory markers, sICAM-1, IL-6 and TNF-α, measured by ELISA of plasma samples (n = 4-7). (F) Time-course of plasma cholesterol measurements in Ldlr<sup>−/−</sup> mice receiving WT or KO BM (n = 4-7; one group of mice was used for weeks 0 and 4, and a separate group was used for week 12). (G) FPLC trace of cholesterol content in lipoprotein fractions from plasma after 12 weeks of HCD (pooled analysis of 4 samples). (H) Intrahepatic total cholesterol (TC), free cholesterol (FC) and triglycerides (TG) after 12 weeks HCD (n = 4). Fecal cholesterol levels after 12 weeks of HCD (n = 8). (I) Expression of inflammatory genes, Sort1 and a macrophage marker (F4/80) from liver tissues. Shown is a heat map of qRT-PCR data (n = 4-8). Values are relative to the controls for each group, as indicated. * indicates a significant difference in expression. (J) Triglyceride measurements in the media of cultured primary mouse hepatocytes treated with recombinant mouse IL-6 for 6 h (n = 4). (K) The predicted miR-146a binding in the human and mouse SORT1 3' UTR (above) and luciferase analyses in BAECs (n = 5).

**Figure 4.** Diet- and age-dependent splenomegal in DKO mice. (A) Spleen weight in Ldlr<sup>−/−</sup> or DKO mice after 12 or 18 weeks of HCD (n = 12-16 for 12 weeks HCD; n = 5 for 18 weeks HCD). (B) Representative images of spleens and femurs in WT and KO BMT mice on HCD for 12 weeks. (C)
Quantification of spleen weight in WT and KO BMT mice on HCD for 12 weeks (n = 5-7). (D) Mice were placed on HCD or normal cholesterol diet (NCD) at 20 weeks of age for 12 weeks. (E) Percentage of ORO region per aortic arch measured en face (n = 3-4). Ldlr−/− mice on NCD were from a separate experiment and are included for comparison purposes (n = 5). (F) Representative images of spleens and femurs. (G) Quantification of spleen weights (n = 3-4). Ldlr−/− mice on NCD were from a separate experiment and are included for comparison purposes (n = 5). (H) Quantification of total CD45+ cells in spleens by FACS analysis (n = 3-4).

**Figure 5.** Global loss of miR-146a inhibits BM hematopoiesis and promotes extramedullary hematopoiesis in the spleen. Increase of splenic (A) and decrease in bone marrow (B) CD45+ leukocytes and Ly6G/CD115+ lymphocytes in DKO mice on diet for 18 weeks. Determined by FACS analysis (n = 5). (C) Decrease of MPPs and downstream progenitor cells (e.g. LS K, MEP, CMP, GMP) in BM of DKO mice after 18 weeks of HCD (n = 5). (D) Increase of splenic hematopoietic and multipotent stem cells in DKO mice after 18 weeks of HCD (n = 5). See also Online Figure VII.

**Figure 6.** MiR-146a in BM-derived cells regulates BM and extramedullary hematopoiesis and levels of circulating leukocytes and lymphocytes. Lethally-irradiated Ldlr−/− mice (mix of males and females) were reconstituted with bone marrow from wild-type (WT BM) or miR-146a−/− (KO BM) donors, followed by HCD for 12 weeks. Increase of splenic (A) and a decrease of bone marrow (B) leukocytes and lymphocytes, as assessed by FACS analysis (n = 5-7). (C) Western blot of TRAF6 and phospho-p65 (normalized to β-actin and total p65, respectively), in mice receiving WT or KO BM after 12 weeks of HCD. Phospho-p65/p65 blots from WT/KO animals are from the same membrane with identical imaging parameters. (D) Decrease of a subset of multipotent stem cells, but no changes to the long-term HSCs in the BM of mice receiving KO BM transplants (n = 5-7). (E) Increase of splenic hematopoietic and multipotent stem cells in mice receiving KO BM (n = 5-7). (F) Early monocytopoiesis (4 weeks HCD), followed by a decrease in peripheral blood (PB) pro-atherogenic cells (neutrophils, B-Cells, and Ly6CHi monocytes), and an increase of anti-atherogenic Ly6Clow monocytes after 12 weeks of HCD (n = 3 for 4 weeks HCD; n = 9-11 for 12 weeks HCD).

**Figure 7.** miR-146a deficient cells appear to be out-competed by wild-type cells in the bone marrow, circulation, and in atherosclerotic plaques in HCD-treated animals, but not in NCD-treated animals. Competitive bone marrow transplantation was performed into Ldlr−/− recipients. A 1:1 mix of BM from WT (CD45.1) and KO (CD45.2) was used. (A) Peripheral blood was analyzed by FACS following NCD or HCD for 4, 12 or 32 weeks (n = 8, 5, 4, 2, respectively). Comparison was made between WT and KO within each time-point. (B) Cells in the aorta (B) and the bone marrow (C) were analyzed by FACS in animals receiving NCD or HCD for 32 w (n = 2 per group).

**Figure 8.** MiR-146a in the vasculature restrains EC activation and atherosclerosis. (A) Schematic of lethally-irradiated Ldlr−/− or DKO mice given bone marrow transplantation from wild-type (WT BM) donors followed by HCD for 12 weeks. (B) Circulating pro-inflammatory markers, sICAM-1, IL-6 and TNF-α, measured by ELISA (n = 5-8). (C) Circulating cholesterol, HDL, TG, LDL and glucose levels after 12 weeks of HCD (n = 5). (D) Quantification of spleen weight after 12 weeks of HCD (n = 12-15). (E) FACS analysis of myeloid cells from spleen, BM, and peripheral blood (n = 12-15). (F) Gene expression in BM cells from BM transplanted animals (n = 3-7). (G) Percentage of ORO region per aortic arch measured by en face staining (n = 11-14). Representative images are shown to the right. See also Online Figure VIII for data on endothelial cell activation in the aorta.

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NOVELTY AND SIGNIFICANCE:

What Is Known?

- MicroRNA-146a (miR-146a) suppresses inflammatory responses in endothelial cells and bone marrow-derived cells by targeting adaptor proteins in the NF-κB signalling 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 bone marrow-derived cells enhances the production of pro-inflammatory cytokines, but paradoxically reduces circulating pro-atherogenic leukocytes, ultimately resulting in decreased atherosclerosis.

- MiR-146a in bone marrow-derived cells protects against high cholesterol diet-induced hematopoietic progenitor cell exhaustion in the bone marrow and prevents extramedullary hematopoiesis and splenomegaly.

- Circulating VLDL levels are progressively decreased in mice lacking miR-146a in the bone marrow, and this is accompanied by enhanced inflammation in the liver and dysregulation of a newly identified miR-146a target gene, sortilin 1 (Sort1).

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. While numerous studies have placed miR-146a amongst the echelon of anti-inflammatory microRNAs, the role of endogenous miR-146a in atherosclerosis remains unknown. Surprising, despite the ability of this microRNA to restrain cytokine production in bone marrow-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 pro-atherogenic cells. Enhanced inflammatory signalling occurred even though circulating levels of VLDL 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 signalling in multiple organs: bone marrow (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.
Figure 1
Figure 2
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Paradoxical Suppression of Atherosclerosis in the Absence of microRNA-146a

Henry S Cheng, Rickvinder Besla, Angela Li, Zhiqi Chen, Eric A Shikatani, Maliheh Nazari-Jahantigh, Adel Hammoutène, My-Anh Nguyen, Michele Geoffrion, Lei Cai, Nadiya Khyzha, Tong Li, Sonya A MacParland, Mansoor Husain, Myron I Cybulsky, Chantal M Boulanger, Ryan E Temel, Andreas Schober, Katey J Rayner, Clinton Robbins and Jason E Fish

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**Supplemental Material:**

**Paradoxical suppression of atherosclerosis in the absence of microRNA-146a**

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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<sup>-/-</sup>* and *miR-146a<sup>-/-</sup>;Ldlr<sup>-/-</sup>* 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<sup>6</sup> 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<sup>1</sup>. 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 through 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\textsuperscript{WT}/pGL3) or a mutant version with the miR-146a binding site mutated (Sort1\textsuperscript{MUT}/pGL3) were constructed. 3’ UTR sequences were generated as gBlocks gene fragments (Integrated DNA technologies) with XbaI linkers (red):

```
5’-AATTCTAGAAGCTATAGTGACATGTTAATGATTTATCAGATGCCGGGA
ATTCTAGTGAGTTTCTCAATTCGTGCTCTGGCATGCAGCTAGCTGTGGTCTCTGCAGTTCTCATTCTCCGCATGCCCTCAGCTGTGCTCTGGGGTATAGAATAT-3'
```

The underlined region contains the miR-146a binding site, which was mutated (GTTCTCA → GTTAGAAG) to generate the Sort1\textsuperscript{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 \textit{et al}\textsuperscript{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 previously³. 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 before4, 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 described3, 6. BMDMs were cholesterol-loaded with 37.5 μg/mL acetylated LDL (Alfa Aesar) and labeled with 1 μCi/mL [3H] 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

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 DiI-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 ulramer extension primer (GACCCCTTAATGCCTCTAAAGACCCCCTTAATGCGTCAAAGACCACCCCTTAAATGCGTCTAAAACCCATGGAAATTCAGTCTCA) 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\(^1\). 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. Pairwise 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.

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

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# of genes significantly dysregulated: 9

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Online Table III: Primer sequences for qRT-PCR analysis (mouse)

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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<sup>hi</sup> and Ly6C<sup>lo</sup> monocytes in Ldlr<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> aortas. A representative blot of 4 is shown. (C) RNA expression of lesser curvature aortic cells (EC and intimal cells) from aged Ldlr<sup>−/−</sup> 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<sup>hi</sup> 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
A 18 weeks HCD

B 18 weeks HCD

C

Plaque area per valve (mm²)

D 18 weeks HCD

Online Figure II
Online Figure III

A Post-BM transplant, Pre-diet

PB CD45+  |  PB Lymphocytes
---|---

Cell Number (x10^7)

- **Ldlr**^{-/-} WT BM
- **Ldlr**^{-/-} KO BM

ns

---

B 12 weeks HCD

---

C 12 weeks HCD

Aortic Arch

Relative Expression

- **Cd68**
- **F4/80**

macrophage markers

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D

---
Online Figure IV
Online Figure VI

A

- Side Scatter
- CD45
- CD115
- Ly6G
- Ly6C
- CD3
- B220
- CD8
- CD4

B

- Side Scatter
- CD45
- CD117
- Sca-1
- CD150
- CD16/32
- CD117
- CD150
Online Figure VII

A  

Bone Marrow Hematopoietic Stem Cells

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Ldlr^/-  

DKO

CD48 vs CD150 scatter plots showing cell distribution.

B 18 weeks HCD

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p=0.067

Ldlr^/-  

DKO

Graphs depicting cell counts for each category.

Online Figure VII