Rationale: Hemizygous deficiency of the transcription factor Krüppel-like factor 2 (KLF2) has been shown previously to augment atherosclerosis in hypercholesterolemic mice. However, the cell type responsible for the increased atherosclerosis due to KLF2 deficiency has not been identified. This study examined the consequence of myeloid cell-specific KLF2 inactivation in atherosclerosis.

Methods and Results: Cell-specific knockout mice were generated by Cre/loxP recombination. Macrophages isolated from myeloid-specific Klf2 knockout (myeKlf2<sup>−/−</sup>) mice were similar to myeKlf2<sup>+/+</sup> macrophages in response to activation, polarization, and lipid accumulation. However, in comparison to myeKlf2<sup>+/+</sup> macrophages, myeKlf2<sup>−/−</sup> macrophages adhered more robustly to endothelial cells. Neutrophils from myeKlf2<sup>−/−</sup> mice also adhered more robustly to endothelial cells, and fewer myeKlf2<sup>−/−</sup> neutrophils survived in culture over a 24-hour period in comparison with myeKlf2<sup>+/+</sup> neutrophils. When myeKlf2<sup>−/−</sup> mice were mated to Ldlr<sup>−/−</sup> mice and then fed a high fat and high cholesterol diet, significant increase in atherosclerosis was observed in the myeKlf2<sup>−/−</sup>Ldlr<sup>−/−</sup> mice compared with myeKlf2<sup>+/+</sup>Ldlr<sup>−/−</sup> littermates. The increased atherosclerosis in myeKlf2<sup>−/−</sup>Ldlr<sup>−/−</sup> mice was associated with elevated presence of neutrophils and macrophages, with corresponding increase of myeloperoxidase as well as chlorinated and nitrosylated tyrosine epitopes in their lesion areas compared with myeKlf2<sup>+/+</sup>Ldlr<sup>−/−</sup> mice.

Conclusions: This study documents a role for myeloid KLF2 expression in modulating atherosclerosis. The increased neutrophil accumulation and atherosclerosis progression with myeloid-specific KLF2 deficiency also underscores the importance of neutrophils in promoting vascular oxidative stress and atherosclerosis. Collectively, these results suggest that elevating KLF2 expression may be a novel strategy for prevention and treatment of atherosclerosis. (Circ Res. 2012;110:1294-1302.)

Key Words: Krüppel-like factor ■ neutrophils ■ oxidative stress ■ atherosclerosis

The importance of KLF2 in vascular development has led to the hypothesis that KLF2 may also have a protective role in the vascular system under pathological conditions. This hypothesis is supported by the finding that mice lacking 1 copy of the KLF2 gene exhibit increased atherosclerosis when mated with apoE-deficient mice and fed a high fat and cholesterol diet.4 However, as these mice have reduced KLF2 levels in all cells, and the pathogenesis of atherosclerotic lesions includes vascular endothelial and smooth muscle cell abnormalities as well as infiltration and proinflammatory
activation of circulating leukocytes, the importance of KLF2 expression in each of these cell types toward atheroprotection has not been completely elucidated.

In the vascular endothelium, KLF2 is expressed in areas of high laminar flow but at low levels where little shear stress occurs such as at bifurcations and bends. The shear stress element of the KLF2 gene has been identified and shown to bind several transcription factors, with MEF2 acting as one of the factors that links shear stress with KLF2 expression. Under laminar flow conditions, KLF2 expression is induced, leading to the downregulation of inflammatory genes such as vascular cell adhesion molecule and E-selectin and the upregulation of vascular protective genes such as endothelial nitric oxide synthase. Several antithrombotic genes are also upregulated including thrombomodulin and tissue plasminogen activator, whereas plasminogen activator inhibitor-1, protease activated receptor 1, and tissue factor are downregulated. Two of these genes, endothelial nitric oxide synthase and thrombomodulin, have been shown to be KLF2 regulated and the promoter regions that bind KLF2 have been identified. These results indicate that KLF2 expression in endothelial cells protects against endothelial activation by modulating the expression of these genes.

In the peripheral circulation, KLF2 is expressed in mature thymocytes, naive T cells, and memory T cells as well as in cells from the myeloid lineage. In the hematopoietic T cells, KLF2 binds to and transactivates the promoter for sphingosine-1-phosphate receptor-1 to promote thymocyte emigration and recirculation through peripheral lymphoid organs. KLF2 has also been shown to be involved in monocyte differentiation, with diminished expression on monocyte differentiation into macrophages. The expression of KLF2 is required to maintain immune cell quiescence, but its expression in both T cells and monocytes is suppressed during cell activation and inflammation. Overexpression of KLF2 inhibits immune cell activation and the expression of inflammatory genes is repressed. Interestingly, KLF2 expression can be induced by statin treatment, suggesting that the lipid-independent atheroprotective effects of statin may be due to induction of KLF2 gene expression.

Another related transcription factor, KLF4, has also been shown to play a role in the proinflammatory response of macrophages. KLF4-deficient progenitor cells of the myeloid lineage differentiate preferentially into granulocytes at the expense of monocytes, whereas overexpression of KLF4 results in almost exclusive differentiation of these progenitor cells into monocytes. Because neutrophil infiltration is an early event of atherogenesis and the depletion of neutrophils reduces atherosclerotic lesion size, expression of these Krüppel-like factors in myeloid cells may significantly impact on atherosclerosis development and progression. To explore the role of myeloid cell KLF2 in lesion formation, mice were developed in which KLF2 was deleted in myeloid cells. The myeloid KLF2-deficient mice were then placed on an Ldlr<sup>−/−</sup> background and fed a high fat diet. The resulting animals exhibited an increase in vascular lesion formation, emphasizing the importance in myeloid KLF2 in this process.

**Methods**

An expanded Methods section is available in the Online Data Supplement. The Klf2<sup>−/−</sup> mice in C57BL/6 background were generated by homologous recombination using the strategy described in the Online Supplemental Methods and shown schematically in Figure 1A. The Klf2<sup>−/−</sup> mice were mated with Ldlr<sup>−/−</sup> mice (Jackson Laboratories) and then crossed with transgenic mice expressing Cre recombinase under the control of the LysM promoter (LysM-Cre, Jackson Laboratories) to generate Ldlr<sup>−/−</sup> mice with or without KLF2 expression in myeloid cells. The Klf2<sup>−/−</sup> mice without the Cre recombinase transgene were used as controls throughout the study. Detailed experimental protocols, including animal maintenance and dietary conditions, blood cell count, macrophage and neutrophil functional studies, immunoblot analysis, and atherosclerosis lesion analysis and characterization, are also described in the Online Supplemental Methods. All polymerase chain reaction primers used are in Online Table I. All animal care and experimental procedures were reviewed and approved by the University of Cincinnati Institutional Animal Care and Use Committee.

**Results**

**Development of Myeloid-Specific KLF2-Deficient Mice**

The approach for determining whether KLF2 in myeloid cells alters the course of vascular lesion formation and atherosclerosis was to develop mice that lack this transcription factor in the myeloid cell lineage. This was accomplished by mating mice carrying conditional Klf2<sup>−/−</sup> allele in which a portion of the Klf2 gene was flanked by LoxP sites with transgenic mice with LysM promoter driven Cre recombinase expression (Figure 1A and 1B). Analysis of genomic DNA from bone marrow cells of the progeny mice by PCR amplification showed the replacement of a DNA fragment in the Klf2<sup>−/−</sup> genome with a smaller DNA fragment expected from cre-mediated recombination indicative of successful Klf2 gene knockout in bone marrow cells (Figure 1C).

Real-time PCR quantification of RNA from bone marrow–derived and peritoneal–elicited macrophages and CD11b<sup>Ly6G<sup>−Ly6C<sup>+</sup></sup></sup> neutrophils confirmed the absence of Klf2–specific reaction products in myeloid cells of the LysM-Cre, Klf2<sup>−/−;</sup>LysM-Cre mice (Figure 1D and 1E). Therefore, these mice can be referred to as myeloid-specific Klf2 knockout (myeKlf2<sup>−/−</sup>) mice. The Klf2<sup>−/−</sup> mice without cre recombinase expression are referred to as myeKlf2<sup>−/−</sup> mice. The inactivation of KLF2 in myeloid cells did not influence KLF4 gene expression in macrophages of myeKlf2<sup>−/−</sup> and myeKlf2<sup>−/−</sup> mice (Online Figure 1).

Additionally, KLF4 expression was not detectable in neutrophils of either myeKlf2<sup>−/−</sup> or myeKlf2<sup>−/−</sup> mice.

**Myeloid KLF2 Inactivation Reduces Lymphocyte Cell Count and Alters Neutrophil-Lymphocyte Ratio**

The influence of myeloid KLF2 expression on leucopoesis and cell differentiation was examined by comparing total
leukocyte cell counts between myeKlf2/H11001/H11001 and myeKlf2/H11002/H11002 mice. Interestingly, total number of monocytes and granulocytes, including neutrophils, eosinophils, and basophils, was not affected by KLF2 expression. In contrast, total lymphocyte cell count was significantly reduced in myeKlf2/H11002/H11002 mice compared with myeKlf2/H11001/H11001 mice (Online Figure II, A). The reduced lymphocyte cell count resulted in alteration in leukocyte cell population with a trend toward higher monocyte:lymphocyte and neutrophil:lymphocyte ratios in myeKlf2/H11002/H11002 mice that did not reach statistical significance (Online Figure II, B and C).

Myeloid KLF2 Inactivation Increases Atherosclerosis in Hypercholesterolemic Mice

Elevation of neutrophil-lymphocyte ratio is an indicator of inflammation and is associated with poor cardiovascular outcome. Although this relationship is generally attributed to inflammation associated with atherosclerosis and inflammation-induced granulopoiesis at the expense of lymphopoiesis, potential direct contribution of elevated neutrophil-lymphocyte ratio toward atherosclerosis has not been explored. Therefore, myeKlf2−/− mice were mated to Ldlr−/− mice to generate myeKlf2−/−Ldlr−/− mice. The Klf2floxfloxLdlr−/− mice without the LysM-cre transgene (myeKlf2+/−Ldlr−/−) retained normal myeloid Klf2 expression in Ldlr−/− background and therefore were used as control. The LysM-cre transgenic mice with normal Klf2 gene expression were not included as controls because numerous studies from several laboratories have reported that myeloid cre recombinase expression in this mouse line has no effect on atherosclerosis lesion area in the aortic roots. The mice were fed a high fat and high cholesterol Western-type diet for 8 weeks beginning at 6 weeks of age. Plasma cholesterol and triglyceride levels were similar between myeKlf2+/−Ldlr−/− and myeKlf2−/−Ldlr−/− mice under both basal and Western diet feeding conditions (Figure 2A). However, significant increase in atherosclerosis was observed in the aortic roots in mice with myeloid KLF2 deficiency.
KLF2 Deficiency Increases Macrophage Adhesion to Endothelial Cells

The increase in atherosclerosis observed in myeloid KLF2-deficient hypercholesterolemic mice may be due to elevated macrophage inflammation and/or propensity for lipid accumulation in response to atherogenic lipoprotein stimulation. To test these possibilities, bone marrow–derived macrophages from myeloid 

\[ \text{myeKlf2}^{+/+} \] and \[ \text{myeKlf2}^{-/-} \] mice were incubated with or without lipopolysaccharide (LPS) and interleukin (IL)-4. Both \[ \text{myeKlf2}^{+/+} \] and \[ \text{myeKlf2}^{-/-} \] macrophages responded to LPS-induced M1 polarization with elevated expression of IL-6, tumor necrosis factor-\( \alpha \), and inducible nitric oxide synthase (iNOS) (Nos2) (Figure 3A). Macrophages from \[ \text{myeKlf2}^{+/+} \] and \[ \text{myeKlf2}^{-/-} \] mice also responded to IL-4–induced M2 polarization with elevated expression of Arg-1, Ym1, and Fizz1 (Figure 3B). Interestingly, both LPS and IL-4 also increased KLF2 expression in \[ \text{myeKlf2}^{+/+} \] macrophages, which may explain the lower LPS-induction of iNOS and IL-6 and IL-4-induction of Fizz1 observed in \[ \text{myeKlf2}^{-/-} \] cells. When the thioglycolate-elicited peritoneal macrophages were added to endothelial monolayer, more \[ \text{myeKlf2}^{-/-} \] macrophages were found to adhere to endothelial cells in comparison with that observed with \[ \text{myeKlf2}^{+/+} \] macrophages (Figure 4A). Additional experiments revealed no statistically significant difference in neutral lipid accumulation between peritoneal macrophages isolated from \[ \text{myeKlf2}^{+/+} \] and \[ \text{myeKlf2}^{-/-} \] mice (Figure 4B). Taken together, these data indicate that KLF2 deficiency does not alter lipid accumulation in macrophages but may promote...
atherosclerotic lesion progression via increased adhesion to endothelial cells.

**KLF2 Deficiency Increases Neutrophil Adhesion and Cell Death**

Neutrophils from myeKlf2<sup>−/−</sup> mice were also defective in KLF2 expression compared with neutrophils from myeKlf2<sup>+/+</sup> mice (Figure 1E). Neutrophils from myeKlf2<sup>−/−</sup> mice showed elevated CD11b expression during fasting as well as after feeding a lipid-rich meal (Figure 5A). Additionally, more myeKlf2<sup>−/−</sup> neutrophils than myeKlf2<sup>+/+</sup> neutrophils were found to adhere to activated endothelial cells (Figure 5B), which probably is due to the elevated expression of CD11b in myeKlf2<sup>−/−</sup> neutrophils. Expression of CD11b in polymorphonuclear leukocytes has also been shown to promote apoptosis. Indeed, fewer myeKlf2<sup>−/−</sup> neutrophils were found to survive after 24 hours in culture compared with myeKlf2<sup>+/+</sup> neutrophils (Figure 5C).

**Myeloid KLF2 Deficiency Promotes Macrophage and Neutrophil Accumulation and Activity in Atherosclerotic Lesions of Hypercholesterolemic Mice**

The increased propensity of myeKlf2<sup>−/−</sup> macrophage and neutrophil adherence to endothelial cells observed in vitro suggested that increased atherosclerosis observed in myeKlf2<sup>−/−</sup>Ldlr<sup>−/−</sup> mice may be due to elevated infiltration of these inflammatory cells to the lesion area. Analysis of atherosclerotic lesions in female myeKlf2<sup>+/+</sup>Ldlr<sup>−/−</sup> and myeKlf2<sup>−/−</sup>Ldlr<sup>−/−</sup> mice did reveal statistically significant increase in both macrophage (Figure 6A) and neutrophil (Figure 6B) numbers in the lesions of myeKlf2<sup>−/−</sup>Ldlr<sup>−/−</sup> mice. The higher number of neutrophils present in the lesion areas of myeKlf2<sup>−/−</sup>Ldlr<sup>−/−</sup> mice coincided with increased accumulation of myeloperoxidase (Figure 6C), an enzyme that directly contributes to the oxidative stress and endothelial dysfunction characteristics of atherosclerosis. Indeed, increased chlorinated tyrosine (Figure 6D) and nitrosylated tyrosine (Figure 6E) immunoreactivities, indicative of myeloperoxidase-induced and oxidative stress–induced reactive oxygen and reactive nitrogen species, respectively, were observed in the lesion areas of myeKlf2<sup>−/−</sup>Ldlr<sup>−/−</sup> mice.

**Discussion**

The importance of KLF2 in cell quiescence, trafficking, and differentiation has been demonstrated in several cell types, including T lymphocytes, adipocytes, monocytes, and erythrocyt cells. The role of KLF2 in the vascular system was documented by observations that mice lacking the KLF2 gene die in utero due to vascular abnormalities. Endothelial specific KLF2 knockout mice are also embryonically lethal, thus establishing the importance of endothelial KLF2 expression in vascular functions. Mice with hemizygous deficiency of KLF2 have been shown to augment atherosclerosis in apoE-deficient mice. The latter study showed that increased atherosclerosis due to heterozygous KLF2 deficiency was due to elevated macrophage infiltration into the atherosclerotic lesion areas as well as increased oxidized LDL accumulation.
in the macrophages of the Klf2−/− mice. Because KLF2 expression in the hemizygous mice is reduced in all tissues, including endothelial cells of the vessel wall and leukocytes in circulation, the cell type responsible for the accelerated atherosclerosis in the Klf2−/− mice has not been identified. The current study used conditional gene knockout approach to establish the importance of KLF2 expression in myeloid cell lineage in atheroprotection. The data revealed myeloid-specific KLF2 inactivation increases atherosclerosis in hypercholesterolemic Ldlr−/−/− mice. The mechanism appears to be related to increased myeloid cell adhesion to endothelial cells, with the consequence of elevated macrophage and neutrophil accumulation in atherosclerotic lesion areas to promote oxidative stress.

Although our results of increased atherosclerosis with myeloid-specific KLF2 inactivation are consistent with results reported earlier with hemizygous Klf2−/− mice, the two studies differed with respect to macrophage lipid accumulation in vitro. Atkins et al showed that peritoneal macrophages isolated from hemizygous Klf2−/− mice accumulated more neutral lipids accompanied by elevated fatty acid binding protein 4 expression in response to oxidized LDL. In contrast, our results showed that peritoneal macrophages with total KLF2 deficiency were similar to control macrophage in lipid accumulation in response to acetylated LDL stimulation. Differences between these two studies are not immediately evident but may be related to KLF2 expression levels in the macrophages or due to different forms of modified lipoproteins used in the in vitro experiments. Additionally, although both our study with conditional myeloid-specific KLF2 knockout mice and the Atkins study with global hemizygous Klf2−/− mice showed increased atherosclerosis with elevated macrophage infiltration into the lesion areas, our data additionally indicated that KLF2 expression in neutrophils also playing prominent role in accounting for the difference in atherosclerosis between control and KLF2-deficient mice.

It is interesting to note that in comparison to control macrophages, KLF2-deficient macrophages expressed less IL-6 and iNOS in response to LPS-induced M1 polarization, yet more atherosclerosis was observed in myeKlf2−/−/−Ldlr−/−/− mice compared with myeKlf2+/−/−Ldlr−/−/− mice. Although lower levels of inflammatory M1 markers are typically associated with less atherosclerosis, the robust atheroscle-
rosis observed in myeKlf2−/−Ldlr−/− mice may be due to more myeKlf2−/− macrophages recruited to the atherosclerotic lesion areas as a consequence of increased CD11b expression and their adhesion to endothelial cells. Alternatively, the myeKlf2−/− macrophages were also less polarized to M2 macrophages with reduced Fizz1 expression in response to IL-4, suggesting their reduced ability to resolve inflammation at the lesion site.48 Our results showing persistent presence of significantly more neutrophils in the lesion areas of myeKlf2−/− mice compared with myeKlf2+/+ mice is consistent with the latter possibility. Typically, neutrophils are prominent in atherosclerotic lesions only during the early phase of atherogenesis preceding the recruitment of macrophages and the establishment of mature plaques.49 However, significant presence of neutrophils was observed in hypercholesterolemic myeKlf2−/− mice even after 8 weeks of feeding the high fat and high cholesterol diet when mature lesions were well established. The high levels of neutrophils in the atherosclerotic plaques of myeKlf2−/− mice may also reflect increased CD11b expression in myeKlf2−/− neutrophils, leading to their sustained infiltration into atherosclerotic lesion areas throughout the atherosclerosis process.

Results of the current study also showed that myeKlf2−/− mice have reduced lymphocyte cell count in the blood. Because the LysM promoter sequence used in the current study to drive cre expression and conditional Klf2 knockout is specific for myeloid cells without direct influence on gene expression in lymphoid cells,50 the reduced number of lymphocytes in myeKlf2−/− mice is probably an indirect effect reflecting myeloid cell regulation of lymphopoiesis.27,51 However, despite the contributing role of lymphocytes in vascular inflammation and atherosclerosis,52 the reduction in the number of lymphocytes in circulation of myeKlf2−/− mice did not reduce atherosclerosis in hypercholesterolemic Ldlr−/− mice. In contrast, atherosclerosis was significantly increased in myeKlf2−/−Ldlr−/− mice compared with myeKlf2+/+Ldlr−/− mice. The difference is likely due to elevated macrophage and neutrophil adhesion to activated endothelial cells and their infiltration into the atherosclerotic lesion areas. The contributory role of neutrophils toward atherosclerosis progression is recently highlighted by data showing neutrophilia promotes atherosclerosis, whereas neutropenia reduces atherosclerosis.53 Neutrophil accumulation in the vessel wall may increase atherosclerosis through its abundant expression of myeloperoxidase, which promotes endothelial dysfunction and elevates oxidative stress.54,55 Increases in myeloperoxidase as well as reactive oxygen and nitrogen species were indeed observed in the lesion areas of myeKlf2−/−Ldlr−/− mice compared with myeKlf2+/+Ldlr−/− mice. In view of reports showing that statin induces KLF2 expression in several cell types,56−58 including its reduction of CD11b expression59,60 and transendothelial migration of leukocytes in the vessel wall,61−63 the cholesterol-independent properties of statin in atheroprotection may in part be related to induction of KLF2 expression in macrophages and neutrophils, leading to their reduced adherence to activated endothelial cells.

Acknowledgments
We thank Dr Marie-Dominique Filippi for her expert advice in neutrophil biology and Dr Junqi Yang for assistance with cell sorting.

Sources of Funding
This study was supported by National Institutes of Health grants HL78806 (to J.B.L.) and DK74932 (to D.Y.H.).

Disclosures
None.

References


---

**Novelty and Significance**

**What Is Known?**

- The transcription factor Krüppel-like factor 2 (KLF2) plays a protective role in the vascular system and suppresses atherosclerotic lesion formation in hypercholesterolemic mice.
- KLF2 expression in vascular endothelial cells protects against endothelial activation by downregulating endothelial adhesion molecules and increasing the expression of vascular protective genes.
- KLF2 is also expressed in myeloid cells and participates in inflammatory responses.

**What New Information Does This Article Contribute?**

- Myeloid cell-specific inactivation of KLF2 increases atherosclerosis in hypercholesterolemic mice.
- KLF2 inactivation elevated cell surface expression of CD11b in neutrophils and macrophages, leading to their increased adhesion to endothelial cells and recruitment to atherosclerotic lesions.
- The increased presence of neutrophils and macrophages in atherosclerotic lesion areas of myeloid-specific KLF2-deficient mice exacerbates vascular oxidative stress to further promote atherosclerosis.

The transcription factor KLF2 plays a protective role in endothelial cells by repressing proinflammatory genes and inducing those that are anti-inflammatory. Nevertheless, the role of KLF2 in myeloid cells in atherosclerosis is currently unknown. Therefore, we examined the effects of deleting KLF2 in neutrophils and macrophages on atherosclerotic lesion formation in LDL receptor–deficient mice fed with a high fat, high cholesterol diet. We found that mice lacking KLF2 in myeloid cells (myeKlf2−/−) displayed increased atherosclerosis, with elevated neutrophil and macrophage infiltration due to their increased adhesion to endothelial cells. Increased presence of neutrophils and macrophages in the lesions of myeloid cell KLF2-deficient mice was associated with increased oxidative stress, myeloperoxidase, and chlorinated and nitrosylated tyrosine residues. These findings suggest that myeloid cell KLF2 plays a critical role in protecting against atherosclerotic lesion formation by limiting neutrophil and macrophage recruitment in to the lesions and reducing oxidative stress in the vasculature in response to hypercholesterolemia. An important implication of this study is that induction of KLF2 expression in myeloid cells may confer atheroprotection. The atheroprotective effects of statins, independent of cholesterol lowering, may also be related in part to KLF2 induction in myeloid cells.
Myeloid-Specific Krüppel-Like Factor 2 Inactivation Increases Macrophage and Neutrophil Adhesion and Promotes Atherosclerosis

Jerry B Lingrel, Robyn Pilcher-Roberts, Joshua E. Basford, Palanikumar Manoharan, Jon Neumann, Eddy S. Konaniah, Ramprasad Srinivasan, Vladimir Y. Bogdanov and David Y. Hui

_Circ Res._ 2012;110:1294-1302; originally published online April 3, 2012; doi: 10.1161/CIRCRESAHA.112.267310

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/110/10/1294

Data Supplement (unedited) at: http://circres.ahajournals.org/content/suppl/2012/04/03/CIRCRESAHA.112.267310.DC1

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the _Permissions and Rights Question and Answer_ document.

**Reprints:** Information about reprints can be found online at: http://www.lww.com/reprints

**Subscriptions:** Information about subscribing to _Circulation Research_ is online at: http://circres.ahajournals.org/subscriptions/
Supplemental Methods

Strategy to create $Klf2^{\text{flox.flox}}$ mice

The $Klf2^{\text{flox.flox}}$ mice in C57BL/6 background were generated by homologous recombination in embryonic stem cells. Two LoxP sites were inserted into the mouse $Klf2$ gene flanking exon 2. The 5’ LoxP site was introduced into the BglIII site in intron 1 and the 3’ LoxP site replaced a 120-bp EcoRI fragment in intron 2, as shown schematically in Figure 1A of the main text. Exon 2 encodes 271 amino acid residues of the total 354-residue KLF2 protein. Upon cre recombinase-mediated excision at the LoxP sites, the flanked region of exon 2 of the $Klf2$ genomic locus is deleted with introduction of a frameshift mutation in any truncated mRNA. The floxed $Klf2$ allele was introduced into SV/129 embryonic stem cells to generate $Klf2^{\text{fl/fl}}$ mice using standard techniques. The $Klf2^{\text{fl/fl}}$ mice were identified by PCR amplification of genomic DNA using allele-specific primers: 5’-GGAGGTAGACTTCAGGCTGTG-3’ and 5’-GTTGTTAGGTCCTCATCCGTG-3’, which detect the presence or absence of the 5’-LoxP site resulting in a 260-bp or 230-bp amplicon, respectively.

Animals and Diets

The $Klf2^{\text{fl/fl}}$ mice backcrossed to C57BL/6 background were mated with $Ldlr^{-/-}$ mice (Jackson Laboratories) and then crossed with transgenic mice expressing Cre recombinase under the control of the LysM promoter (LysM-cre, Jackson Laboratories) to generate $Ldlr^{-/-}$ mice with or without KLF2 expression in myeloid cells. The $Klf2^{\text{fl/fl}}$ mice without the Cre recombinase transgene were used as controls throughout the study. The animals were fed normal rodent chow diet containing 4.5% fat without added cholesterol (Harlan NIH-07) and then placed on a high fat (21.5% by weight, 40% kcal) and high cholesterol (1.25% by weight) Western type diet (Research Diets D12108C) beginning at 6 weeks of age. Blood was obtained from anesthetized mice after an overnight fast for lipid determinations. Tissues were obtained from euthanized mice after 8 weeks of experimental diet feeding. Meal induced hypertriglyceridermia was accomplished by oral gavage of olive oil (8 μl/gm body weight). Blood was collected after 2 hr for analysis. All procedures and animal care were reviewed and approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Blood Leukocyte Cell Counts

Leukocyte cell composition was determined in blood samples by differential cell count using a Hemavet 950 FS (Drew Scientific) automated hematology system. CD11b surface expression on blood neutrophils was quantified using a Guava Easycyte 8HT system (Millipore) flow cytometer. Heparinized blood samples were cooled to 4°C and Fc receptors were blocked using purified anti-mouse CD16/CD32 antibody (eBioscience) in flow cytometry buffer containing Hank’s balanced salt solution with 1% bovine serum albumin and 0.1% sodium azide. Cells were labeled with fluorochrome-conjugated antibodies CD11b:APC-Cy7, Ly6G:PerCP-Cy5.5, Ly6:APC or rat IgG2b κ isotype control APC (eBioscience). CD11b median fluorescence intensity was determined from Ly6G+ gated neutrophils with fluorescence greater than 99% of isotype control and appropriate side scatter profile.
Macrophage Polarization

Bone marrow cells were collected from the femurs and tibias of 3-5 control or myeKlf2−/− mice, pooled, and differentiated in vitro by incubating for 7 days at 37°C in RPMI 1640 medium containing 20% fetal bovine serum (FBS) and 10% L929 conditioned media. Greater than 98% of the adhering cells were differentiated to macrophages based on F4/80 expression as determined by flow cytometry. The bone marrow-derived macrophages were activated by incubation with RPMI 1640 medium plus 20% FBS containing either 0.01 µg/ml lipopolysaccharide (LPS) plus 0.02 µg/ml interferon-γ (IFN-γ) or 0.05 µg/ml interleukin-4 (IL-4) for 24 hr. The cells were washed extensively and RNA was isolated for real-time PCR amplification to assess M1 and M2 polarization, using sequence specific primers as indicated in the supplement (Online Supplement Table I).

Macrophage Function

Macrophage adhesion to endothelial monolayers was determined using peritoneal macrophages collected three days after intraperitoneal injection of 3% thioglycolate solution. Peritoneal exudates were seeded onto plastic culture dishes for 2 hr, non-adherent cells were removed and adherent macrophages were cultured in RPMI 1640 containing 10% FBS overnight at 37°C and 5% CO₂. Macrophages were labeled with 1µM calcein AM and added onto bEND3 mouse endothelial monolayers. Non adherent cells were removed after 1 hr and adherent cells were quantified by measuring the fold increase of fluorescence over control at 485 nm excitation and 520 nm emission.

To measure neutral lipid accumulation, thioglycolate-elicited peritoneal macrophages were plated in 12-well dishes at a density of 5 x 10⁵ cells/ml then incubated overnight with 50 µg/ml of acetylated LDL (acLDL) as described previously. Neutral lipid accumulation in macrophages was stained with Oil Red O, and quantified by optical density measurement at 510 nm after lipid extraction. The values were normalized to total cellular protein content in each sample.

Immunoblot Analysis

Peritoneal macrophages were solubilized in RIPA buffer and cellular protein concentration was measured at A280nm. Equal amounts of total protein samples (150 µg) were resolved using 10% SDS PAGE and immobilized to Immobilon–P membranes (Millipore). These membranes were probed with anti-KLF2 (Millipore) and anti-β-Actin (Abcam) antibodies at 1:1000 and 1:5000 dilutions, respectively. Secondary detection using goat anti-rabbit Alexa680 (Life Technologies) and goat anti-mouse IRDye 800CW (LICOR Biosciences) were performed at 1:10,000 dilution. Membranes were scanned using Odyssey Infrared Imaging System (LICOR Biosciences).

Neutrophil Isolation and Function

Bone marrow neutrophils were isolated from the femurs and tibias and purified by discontinuous Percoll gradient centrifugation. Any red cell contamination was further removed using Histopaque 1119 (Sigma). Purity of the resulting neutrophil preparation was determined by cytoospin and Diff-Quik (Siemens) staining. Peritoneal neutrophils were elicited by 1 ml intraperitoneal injection of a 9% casein solution with a subsequent injection the following morning. Peritoneal exudates were harvested 3 hr after the final injection and then purified via magnetic cell sorting using Anti-Ly-6G MicroBead Kit and autoMACS Separator (Miltenyi
Biotec) according to manufacturer’s protocol. Peritoneal neutrophils were labeled with 5 µM calcein AM and then added to endothelial monolayers that have previously been activated by incubation for 4 hr with 0.05 µg/ml TNF-α. Non-adhering cells were removed after 1 hr. The number of adherent neutrophils was determined by measuring the fold increase of fluorescence over control at 485 nm excitation and 530 nm emission.

Peritoneal neutrophils were cultured at 5.0x10^5 cells per well in 12-well dishes in IMDM media containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cell viability was assessed after 0, 6 and 22 hr using Annexin V:APC and propidium iodide (Ebioscience, San Diego, CA). Viable cells were counted in the Annexin V, propidium iodide double negative quadrant and expressed as a percentage of viable cell number at time zero.

**Characterization of Atherosclerotic Lesions**

Atherosclerosis was assessed in mice after 8 weeks of high fat and high cholesterol feeding. The heart and aorta were perfusion fixed with 4% neutral formalin solution. The upper heart section was embedded in OCT medium, frozen, and sectioned with a cryostat at 10-µm starting at the valve nubs and the appearance of the coronary artery branch throughout the aortic sinus until the valve separates at the base. The sections were stained with oil red O and counterstained with hematoxylin. Tissue macrophages were detected by immunostaining with anti-CD68 (1:100 dilution, Abcam, Cambridge, MA), HRP-conjugated secondary and Vectastain ABC detection reagents (Vector Labs, Burlingame, CA). Additional serial sections were immunostained with antibodies against myeloperoxidase (MPO) (1:200, Abcam), chlorinated tyrosines (1:100, Hycult Biotech, Plymouth meeting, PA), or nitrosylated tyrosines (1:100, Life Technologies, Grand Island, NY), followed by detection using anti-rabbit secondary antibodies conjugated to Alexa594 (1:5000, Life Technologies) and counterstained with 4,6-diamidino-2-phenylindole (DAPI). Neutrophil-specific Leder staining was performed on citrate-acetone-formaldehyde fixed frozen sections by incubation in Napthol AS-D chloroacetate solution and counterstained with hematoxylin. Percent CD68-positive nuclei or positive Leder stained nuclei were counted from four high power fields obtained from five sections per mouse. Mean lesion area and lesion area with positive immunostaining were calculated from analysis of digitalized images obtained from 5 sections per mouse with 10 mice in each group.

**Statistical Analysis**

Statistical analysis was performed with SigmaPlot Version 11. Values were expressed as mean ± SD. Multiple comparisons were tested by Student’s t test or ANOVA, with Student Newman-Keuls post-hoc analysis. A difference of $P < 0.05$ was considered statistically significant.

**Reference**

**Supplemental Data**

**Supplemental Table I.** RT-qPCR primer sequences for experimental and reference genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Primer Sequence</th>
<th>Amplicon</th>
</tr>
</thead>
</table>
| Cyclophilin A (Ppia) | NM_008907     | sense 5’ tcatgtgccagggtgtgcac (20 bp)  
                        |               | antisense 5’ ccattcagtctctgcagtgc (20 bp)  | 184 bp |
| IL-6         | NM_031168     | sense 5’ tccaccccagtgtgcac (21 bp)  
                        |               | antisense 5’ gggagtggtcctctgtgaagtc (24 bp)  | 100 bp |
| Nos2         | NM_010927     | sense 5’ ttcaccccagtgtgcac (24 bp)  
                        |               | antisense 5’ tccatgtgccacccccacaacga (24 bp)  | 141 bp |
| Tnf-α        | NM_013693     | sense 5’ aatacgagatcgactgac (18 bp)  
                        |               | antisense 5’ accgcctgaggctgaga (19 bp)  | 71 bp |
| Arg-1        | NM_007482     | sense 5’ ggaatctgcatggcac (24 bp)  
                        |               | antisense 5’ agggtctacgtgcaccaac (22 bp)  | 141 bp |
| Ym1 (Chi3l3) | NM_009892     | sense 5’ tccacgccatgtgcac (23 bp)  
                        |               | antisense 5’ tttgtctcttaggctgctcct (23 bp)  | 438 bp |
| Fizz1 (Retlna) | NM_020509    | sense 5’ ggtcccaqtgcatatggatgagca (26 bp)  
                        |               | antisense 5’ cacctctttcactgagcag (26 bp)  | 297 bp |
| Klf2         | NM_008452     | sense 5’ tctgctgtcactacacacaggtgaa (24 bp)  
                        |               | antisense 5’ acatgtgcctttcatgtgcag (24 bp)  | 192 bp |

**Supplemental Figure I.** KLF4 expression in (A) peritoneal macrophages and (B) bone marrow derived macrophages of myeKlf2+/+ (control) and myeKlf2−/− (KO) mice.
**Supplemental Figure II.** Blood cell analysis of myelogenic KLF2-deficient mice. (A) Total number of leukocytes in blood samples from superficial facial veins of 10 myeKlf2<sup>+/+</sup> (Control, filled bars) and 11 myeKlf2<sup>-/-</sup> (KO, open bars) mice. (B) Monocyte to lymphocyte and (C) neutrophil to lymphocyte ratios increased in the KO but compared to control mice were not statistically different. *P < 0.01, Student’s t test.
Supplemental Figure III. Negative controls and magnified image of aortic root atherosclerotic lesions. (A) Negative control of IgG isotype control staining of atherosclerotic lesions in myeKlf2<sup>−/−</sup>Ldlr<sup>−/−</sup> aortas. (B) Negative control of IgG isotype immunofluorescence, with DAPI nuclear counter. The white dash lines marked the luminal margin of the atherosclerotic lesion. (C) Amplified image of Figure 6B showing Leder staining of neutrophil esterases (arrow heads) in atherosclerotic lesions in myeKlf2<sup>−/−</sup>Ldlr<sup>−/−</sup> mice. Scale bar = 100 μm.