Regulation of Csf1r and Bcl6 in Macrophages Mediates the Stage-Specific Effects of MicroRNA-155 on Atherosclerosis

Yuanyuan Wei, Mengyu Zhu, Judit Corbalán-Campos, Kathrin Heyll, Christian Weber, Andreas Schober

Objective—The function of microRNAs is highly context and cell type dependent because of their highly dynamic expression pattern and the regulation of multiple mRNA targets. MicroRNA-155 (miR-155) plays an important role in the innate immune response by regulating macrophage function; however, the effects of miR-155 in macrophages on atherosclerosis are controversial. We hypothesized that the stage-dependent target selection of miR-155 in macrophages determines its effects on atherosclerosis.

Approach and Results—The expression of miR-155 increased in lesional macrophages of apolipoprotein E–deficient mice between 12 and 24 weeks of a high-cholesterol diet. Mir155 knockout in apolipoprotein E–deficient mice enhanced lesion formation, increased the lesional macrophage content, and promoted macrophage proliferation after 12 weeks of the high-cholesterol diet. In vitro, miR-155 inhibited macrophage proliferation by suppressing colony-stimulating factor-1 receptor, which was upregulated in lesional macrophages of Mir155+/− apolipoprotein E–deficient mice. By contrast, Mir155 deficiency reduced necrotic core formation and the deposition of apoptotic cell debris, thereby preventing the progression of atherosclerosis between 12 and 24 weeks of the high-cholesterol diet. miR-155 inhibited efferocytosis in vitro by targeting B-cell leukemia/lymphoma 6 and thus activating RhoA (ras homolog gene family, member A). Accordingly, B-cell leukemia/lymphoma 6 was upregulated in lesional macrophages of Mir155+/− apolipoprotein E–deficient mice after 24 weeks, but not after 12 weeks of the high-cholesterol diet.

Conclusions—Our findings demonstrate a stage-specific role of miR-155 in lesion formation. miR-155 suppressed macrophage proliferation by targeting colony-stimulating factor-1 receptor in early and impaired efferocytosis by downregulating B-cell leukemia/lymphoma 6 in advanced atherosclerosis. Therefore, targeting the interaction between miR-155 and B-cell leukemia/lymphoma 6 may be a promising approach to inhibit the progression of atherosclerosis.

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Key Words: atherosclerosis ■ macrophages ■ microRNAs

Atherosclerosis is a leading, but potentially preventable, cause of death and disability worldwide and results in devastating diseases, such as myocardial infarction because of coronary artery disease or stroke.1 Modified forms of low-density lipoprotein (LDL), for example, resulting from oxidation, in the vessel wall induce the expression of adhesion molecules and chemokines in the endothelium, and this supports the adhesion and transmigration of circulating monocytes.2 In the vessel wall, monocytes differentiate into macrophages, which transform into foam cells by intracellular cholesterol accumulation. In addition to monocyte recruitment, the proliferation of macrophages within plaques contributes to the lesional macrophage burden.3 Colony-stimulating factor-1 (Csf1) secreted from endothelial cells promotes atherosclerosis by regulating the survival, proliferation, and differentiation of macrophages via the Csf1 receptor (Csf1r).4–7 The removal of apoptotic macrophages by efferocytosis induces an anti-inflammatory response, thereby promoting inflammation resolution and limiting the progression of atherosclerosis.8,9 However, a continuous supply of LDL to the intima and the limited reverse cholesterol transport capacity results in intracellular accumulation of cholesterol-ester in lipid droplets. The accumulation of free cholesterol in endoplasmic reticulum membranes increases macrophage apoptosis via activation of the unfolded protein response.10 In addition to increased macrophage apoptosis, efferocytosis is defective in advanced atherosclerosis, partly owing to the deregulation of efferocytosis receptors.11 Insufficient removal of apoptotic macrophages leads to secondary necrosis, which impairs inflammation resolution and promotes the formation of a highly thrombogenic necrotic core, the hallmark of vulnerable plaques.8

MicroRNAs (miRNAs) are small, noncoding RNAs that regulate gene expression at the post-transcriptional level.12

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The expression and targets of miRNAs are context and cell type dependent, which determines the biological function of miRNAs.\textsuperscript{13} MiRNAs play important roles in the development of atherosclerosis.\textsuperscript{14–17} Previously, we found that a hematopoietic microRNA-155 (\textit{Mir155}) deficiency limits atherosclerosis in partially ligated carotid arteries of apolipoprotein E–deficient (\textit{Apoe–/–}) mice by increasing the expression of B-cell leukemia/lymphoma 6 (\textit{Bcl6}).\textsuperscript{18} \textit{Mir155} knockout and inhibition by antagomirs in \textit{Apoe–/–} mice reduce lesion formation after consuming a high-cholesterol diet (HCD).\textsuperscript{19,20} By contrast, hematopoietic \textit{Mir155} suppresses atherosclerosis in LDL receptor–deficient mice.\textsuperscript{21} However, the cause of these contradictory findings is unclear.

Here, we demonstrate that miR-155 has opposite roles during early and advanced atherosclerosis. During the early stage of atherosclerosis, miR-155 suppressed lesion formation via reducing the lesional macrophage content by inhibiting \textit{Csf1r}-mediated macrophage proliferation, whereas miR-155 promoted atherosclerosis by diminishing \textit{Bcl6}-mediated phagocytosis of apoptotic cells during the advanced stage.

Materials and Methods

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

Results

Role of miR-155 in Atherosclerosis at Different Stages

The expression level of miR-155 was upregulated in the aortas of \textit{Apoe–/–} mice after 24 weeks as compared with 4 and 12 weeks of the HCD and tended to increase from 4 to 12 weeks (Figure 1A). Moreover, miR-155 expression was mainly detectable in Mac2\textsuperscript{+} mononuclear phagocytic cells after 12 and 24 weeks of the HCD as determined by combined in situ PCR and Mac2 immunostaining (Figure 1A). In vitro, treatment of bone marrow–derived macrophages (BMDMs) with oxidized LDL for 72 hours increased the expression level of miR-155 and cholesterol loading compared with 24 hours (Figure I in the online-only Data Supplement). To study the function of miR-155 at different stages of atherosclerosis, \textit{Mir155\textsuperscript{+/+}Apoe–/–} and \textit{Mir155\textsuperscript{–/–}Apoe–/–} mice were fed the HCD for 12 or 24 weeks. After 12 weeks, lesion formation and lipid deposition in the aortic roots and aortas, respectively, were increased in \textit{Mir155\textsuperscript{–/–}Apoe–/–} compared with \textit{Mir155\textsuperscript{+/+}Apoe–/–} mice (Figure 1B and 1C). By contrast, a significant reduction in lesion size was found in the aortic roots but not in the aortas of \textit{Mir155\textsuperscript{–/–}Apoe–/–} mice after the 24-week HCD feeding program (Figure 1B and 1C). Notably, a lack of \textit{Mir155} prevented

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** Effects of microRNA-155 (miR-155) on atherosclerosis at different stages. \textit{Mir155\textsuperscript{+/+}Apoe–/–} and \textit{Mir155\textsuperscript{–/–}Apoe–/–} mice were fed the high-cholesterol diet for 4, 12, or 24 weeks. A, miR-155 expression in atherosclerotic aortas and aortic root lesions from \textit{Mir155\textsuperscript{+/+}Apoe–/–} and \textit{Mir155\textsuperscript{–/–}Apoe–/–} mice as determined by quantitative real-time polymerase chain reaction (PCR; left) and in situ PCR combined with Mac2 immunostaining (right), respectively. The lesion areas are outlined. B, Lesion formation in aortic roots quantified in sections stained with elastic van Gieson (EVG) stain. C, Lesion formation in Oil red O–stained, en face prepared aortas. D, Necrotic core area in aortic roots quantified in sections stained with EVG stain. Scale bars, 20 \textmu m (A) and 500 \textmu m (B). *\textit{P}<0.05, **\textit{P}<0.01, and ***\textit{P}<0.001. \textit{n}=8 to 15. DAPI indicates 4′,6-diamidino-2-phenylindole.
the progression of atherosclerosis in aortic roots between 12 and 24 weeks of the HCD (Figure 1B). The decreased lesion progression in Mir155 −/−Apoe −/− mice was associated with a reduced necrotic core area compared with that of Mir155 +/+Apoe −/− mice (Figure 1D). Moreover, a larger necrotic core area formed in Mir155 +/+Apoe −/− mice fed the HCD for 24 weeks than for 12 weeks (Figure 1D); thus, we confirmed this as the advanced stage of atherosclerosis. The lesional content of Mac2+ macrophages and smooth muscle cells was higher in Mir155−/−Apoe−/− mice than in Mir155+/+Apoe−/− mice after 12 weeks of the HCD (Figure 2A and 2B; Figure IIA in the online-only Data Supplement). After 24 weeks, the smooth muscle cell content was decreased in Mir155−/−Apoe−/− mice, whereas the macrophage content did not differ between the groups (Figure 2A and 2B; Figure IIA in the online-only Data Supplement). The lesional myeloperoxidase+ neutrophil content and the serum cholesterol level were not substantially affected by Mir155 deficiency (Figure 2C; Figures IIB and III in the online-only Data Supplement).

Role of Hematopoietic mir-155 in Atherosclerosis at the Early Stage

To study whether miR-155 derived from macrophages mediates the atheroprotective effects in early atherosclerosis, BM chimeric mice were generated. Increased lesion formation (Figure 3A and 3B) and lesional macrophage accumulation (Figure 3C) was found in Mir155+/+Apoe−/− mice harboring Mir155−/−Apoe−/− BM, whereas atherosclerosis was not altered in Mir155−/−Apoe−/− mice harboring Mir155+/+Apoe−/− BM compared with Mir155+/+Apoe−/− mice repopulated with Mir155−/−Apoe−/− BM after the 12-week HCD feeding program. Similar to the results of the ubiquitous Mir155 knockout, the necrotic core area was not different between the 3 groups after 12 weeks of the HCD (Figure 3A).

Mechanism of the Opposite Roles of miR-155 During Atherogenesis

The accumulation of macrophages in early atherosclerotic lesions is dominated by their local proliferation and the formation of the necrotic core during lesion progression occurs because of an imbalance between macrophage apoptosis and

Figure 2. Effects of microRNA-155 (miR-155) on plaque composition in apolipoprotein E–deficient (Apoe−/) mice at different stages. Mir155+/+Apoe−/− and Mir155−/−Apoe−/− mice were fed the high-cholesterol diet for 12 or 24 weeks. A, Quantification of lesional macrophages in aortic roots determined by Mac2 immunostaining. Representative images are shown and the lesion areas are outlined. Scale bars, 50 μm. B, Accumulation of lesional smooth muscle cells in aortic roots determined by smooth muscle actin (SMA) immunostaining. C, Accumulation of lesional neutrophils in aortic roots determined by myeloperoxidase (MPO) immunostaining. *P<0.05 and **P<0.001. n=8 to 15. DAPI indicates 4′,6-diamidino-2-phenylindole.

Figure 3. Effects of hematopoietic microRNA-155 (miR-155) expression on early atherosclerosis. Mir155+/+Apoe−/− mice transplanted with Mir155+/+Apoe−/− or Mir155−/−Apoe−/− bone marrow (BM) cells and Mir155−/−Apoe−/− mice transplanted with Mir155+/+Apoe−/− BM cells were fed the high-cholesterol diet for 12 weeks. A, Lesion formation quantified in elastic van Gieson–stained aortic root sections. B, Lesion formation quantified in Oil red O–stained, en face prepared aortas. C, Accumulation of lesional macrophages determined by Mac2 immunostaining. Scale bars, 500 μm. *P<0.05. n=7 to 8.
**miR-155 Regulates Macrophage Efferocytosis by Targeting Csf1r**

We previously showed that increased expression of Bcl6 in Mir155+/− macrophages reduces inflammatory responses and limits atherosclerosis. However, the mechanism of the protective effect of Bcl6 is not completely understood. Notably, the Bcl6 mRNA expression level was increased in lesions of Mir155+/− mice compared with the control after 24 weeks, but not after 12 weeks of the HCD (Figure 6A). The stage-dependent effect of Mir155 deletion on the expression of its targets in the lesions was only observed for Bcl6, and not for other targets, such as Socs1, PU.1, Rhoa, Ship1, and Hpp1 (Figure IV in the online-only Data Supplement). Accordingly, the number of macrophages expressing Bcl6 protein was higher in lesions of Mir155+/− mice after 24 weeks (Figure 6B).

In vitro, the Bcl6 expression level was increased in Mir155+/− BMDMs (Figure 6C) and downregulated by treatment with a miR-155 mimic in a dose-dependent manner (Figure V in the online-only Data Supplement). Interestingly, 20 nmol/L was sufficient for the miR-155 mimic to reduce Csf1r; however, reduction of Bcl6 expression was only observed for 30 nmol/L (Figure V in the online-only Data Supplement), suggesting an effect of miR-155 availability on the regulation of Csf1r and Bcl6. To study whether miR-155 regulates efferocytosis by targeting Bcl6, Calcein AM–labeled and BAY11-7085–induced wild-type apoptotic macrophages were incubated with lipopolysaccharide (LPS)/IFN-γ-stimulated Mir155+/− or Mir155+/− BMDMs. Apoptotic cells were more effectively phagocytized by Mir155+/− BMDMs than by Mir155+/− BMDMs (Figure 6D). Mir155 deficiency had no effect on the apoptosis of macrophages treated with BAY11-7085 (Figure VI in the online-only Data Supplement). The increased efferocytosis capability of Mir155+/− BMDMs was impaired after treatment...
with an inhibitor of Bcl6 (Figure 6D), suggesting that the effect of miR-155 on macrophage efferocytosis is mediated by the targeting of Bcl6. Moreover, activation of the efferocytosis suppressor ras homolog gene family, member A (RhoA) was reduced in Mir155−/− BMDMs, and this reduction was rescued by treatment with a Bcl6 inhibitor, indicating that the role of miR-155 in efferocytosis is mediated by Bcl6–RhoA axis (Figure 6E). However, the RhoA expression levels were not affected by Mir155 deficiency in either LPS/IFN-γ–stimulated macrophages or aortic roots, although the Rhoa mRNA level was increased in unstimulated Mir155−/− BMDMs (Figures IV and VII in the online-only Data Supplement).

Furthermore, pro- and anti-inflammatory markers were measured in lesions from Mir155+/+Apoe−/− and Mir155−/−Apoe−/− mice at various stages of atherosclerosis. The pro-inflammatory marker Nos2 was reduced, whereas the anti-inflammatory markers Fizz1 and Ym1 were increased after 24 weeks in Mir155−/−Apoe−/− mice (Figure VIII in the online-only Data Supplement). In addition, Fizz1 was increased by Mir155 deficiency after 12 weeks (Figure VIII in the online-only Data Supplement).

**Discussion**

Our data demonstrate that miR-155 has a protective effect against early atherosclerosis but promotes advanced atherosclerosis. These opposite roles of miR-155 are attributable to the inhibition of macrophage proliferation by suppressing Csf1r in early and decreased efferocytosis by targeting Bcl6 in advanced atherosclerosis (Figure 6F).

The role of miR-155 in atherosclerosis has been studied previously by others and us; however, the results are controversial. Knockout of Mir155 in BM cells reduces atherosclerotic lesion size, lipid deposition, and lesional macrophage content in aortic roots after a 16-week HCD feeding program in 16-week-old Apoe−/− mice. These data indicate a proatherogenic role of macrophage-derived miR-155 at the advanced stage of atherosclerosis. Accordingly, we found that knockout of Mir155 reduces advanced lesion formation and increases Bcl6 expression after 24 weeks of the HCD. By contrast, LDL receptor–deficient mice repopulated with Mir155−/− BM cells develop more atherosclerosis after 10 weeks of a HCD, indicating antatherogenic effects of miR-155 expression in macrophages during early atherosclerosis. In line with this result, miR-155 limited lesion formation in Apoe−/− mice after 12 weeks of the HCD via reduced macrophage proliferation. Thus, miR-155 has opposite effects in early and advanced atherosclerosis and plays a crucial role in the progression of atherosclerosis.

Macrophage proliferation plays an important role in the development of atherosclerosis. The growth-promoting effect of Csf1 maintains tissue macrophages and contributes to macrophage proliferation under inflammatory conditions. Genetic deletion of Csf1 or its receptor Csf1r limits atherosclerosis, and blocking Csf1r with an antibody reduces early lesion formation, but has no effect on lesion progression. Thus, the effect of Csf1 on macrophage proliferation may be essential in the initiation of atherosclerosis. We found that miR-155 suppresses its target Csf1r in lesional macrophages from early and advanced atherosclerosis and inhibits macrophage proliferation by suppressing Csf1r in vitro. However, the expression of Csf1 was greatly diminished during the progression of atherosclerosis. Therefore, the effect of miR-155 on Csf1r expression may only affect macrophage proliferation in early atherosclerosis because of the reduced availability of Csf1 in advanced atherosclerosis (Figure 6F). Csf1 upregulates the expression level of scavenger receptor class A, which mediates the proliferation...
of lesional macrophages.3,11 Our finding that the expression level of scavenger receptor class A is increased in Mir155+/+ macrophages suggests that the miR-155-mediated suppression of Csf1r reduces macrophage proliferation in early lesions by downregulating scavenger receptor class A expression. In advanced atherosclerosis, increased cell apoptosis is accompanied by defective efferocytosis, thereby promoting the formation of a necrotic core and accelerating atherosclerosis.8,10,11 We found that knockout of the miR-155 gene results in a smaller necrotic core size and reduced lesion formation during advanced atherosclerosis. Although miR-155 did not affect the apoptosis of lesional macrophages, the extracellular deposition of DNA from apoptotic cells in the necrotic core was substantially reduced in Mir155−/− mice. These results indicate that miR-155 plays an important role in the progression of atherosclerosis by impairing efferocytosis. Unlike various other miR-155 targets, the expression of Bcl6 was selectively upregulated by Mir155 deficiency in advanced atherosclerosis. Notably, Bcl6 can inhibit the activity of RhoA, which negatively regulates efferocytosis by activating ROCK1.32–34 Moreover, Bcl6 deficiency in macrophages increases lesion formation and the necrotic core area in Apoe−/− mice.35 Hence, miR-155 may impair efferocytosis by targeting Bcl6. Accordingly, we found that suppression of Bcl6 by miR-155 increases RhoA activity and inhibits efferocytosis. This effect of miR-155 on RhoA activity is in contrast to previous findings that RhoA is a target of miR-155.26,27 Our findings indicate that miR-155 targets Rhoa mRNA in resting macrophages, but not in inflammatory macrophages and atherosclerotic lesions, suggesting a switch of the miR-155 effect on RhoA activity during macrophage activation. Taken together, miR-155–mediated suppression of Bcl6 inhibited efferocytosis via

Figure 6. Role of microRNA-155 (miR-155) in efferocytosis. Mir155+/+Apoe−/− and Mir155−/−Apoe−/− mice were fed the high-cholesterol diet (HCD) for 12 or 24 weeks (A and B). A, Lesional expression of Bcl6 mRNA determined by quantitative real-time polymerase chain reaction (qRT-PCR). n=3 to 5. B, Double immunostaining for Mac2 and Bcl6 in lesions after 24 weeks of the HCD. Arrows indicate the Bcl6-positive macrophages. n=6 to 8. Scale bars, 25 μm. C, Bcl6 mRNA expression in lipopolysaccharide (LPS)/interferon (IFN)−γ-stimulated Mir155+/+ or Mir155−/− BMDMs determined by qRT-PCR. n=3 to 4. D, Apoptosis of wild-type BMDMs labeled with Calcein AM was induced by treatment with BAY11-7085. The apoptotic cells were then incubated with LPS/IFN−γ-stimulated Mir155+/+ or Mir155−/− BMDMs in the presence or absence of a Bcl6 inhibitor (INH). The percentage of phagocytes engulfing apoptotic cells was quantified by flow cytometry. n=3 to 4. E, Measurement of the activation of ras homolog gene family, member A (RhoA) in LPS/IFN−γ-stimulated Mir155+/+ or Mir155−/− BMDMs in the presence or absence of a Bcl6 INH (relative to Mir155−/− BMDMs without Bcl6 inhibitor treatment). The activity of RhoA was normalized to the total RhoA protein. n=4 to 6. F, Model for the stage-specific effects of miR-155 in atherosclerosis. In the early stage, miR-155 inhibits lesion formation by suppressing Csf1r-mediated macrophage proliferation. Although miR-155 targets Csf1r at the advanced stage as well, its effect on macrophage proliferation is limited by low expression of colony stimulating factor 1 (CSF1). The expression of miR-155 is upregulated by modified low-density lipoprotein (LDL) during the progression of atherosclerosis, resulting in the suppression of both Bcl6 and Csf1r by miR-155 in advanced lesions. Targeting Bcl6 by miR-155 diminishes efferocytosis by macrophages, and thus enhances atherosclerosis. *P<0.05, **P<0.05, and ***P<0.001. DAPI indicates 4′,6-diamidino-2-phenylindole; oxLDL, oxidized LDL; and SR-A, scavenger receptor class A.
increased RhoA activity and thereby promoted necrotic core formation and the progression of atherosclerosis.

Our results indicate that miR-155 targets Bcl6 in lesional macrophages from advanced but not early atherosclerotic lesions. miR-155 was substantially upregulated in macrophages during the progression of atherosclerosis and a lower expression level was required to target Csf1r than Bcl6 in vitro. Thus, upregulation of miR-155 expression levels in advanced lesions may be required for the targeting of Bcl6 and the inhibition of efferocytosis (Figure 6F). Short-term treatment with modified or native LDL can increase the expression of miR-155 in macrophages.18,20 In addition, we found that long-term treatment of macrophages with oxidized LDL, resulting in foam cell formation and overloading with free cholesterol,16 which can trigger TLR4 activation,17,21 strongly upregulates miR-155 expression levels. Of note, TLR4 signaling induces miR-155 expression via activation of inflammatory transcription factors, such as NF-κB (nuclear factor kappa light polypeptide gene enhancer in B cells 1), AP-1 (activator protein 1), and ETS-2 (E26 avian leukemia oncogene 2).19,42–45 Therefore, accumulation of free cholesterol in foam cells impairs phagocytosis presumably by cholesterol-induced upregulation of miR-155 expression and the suppression of Bcl6.46,47

In conclusion, the data in this study demonstrate that miR-155 protects from early, but promotes advanced atherosclerosis. These opposite roles of miR-155 are attributable to the inhibition of macrophage proliferation by suppressing Csf1r in early stages and decreased efferocytosis by targeting Bcl6 in advanced atherosclerosis (Figure 6F), highlighting the context-dependent roles of this miRNA. These findings indicate that targeting the interaction between miR-155 and Bcl6 may be a promising approach to prevent the progression of atherosclerosis.

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Disclosures
None.

References


## Significance

Although the roles of microRNA-155 during atherogenesis have been demonstrated, it has both pro- and antitherogenic effects. In this study, we provide evidence for stage-specific effects of microRNA-155 on atherosclerosis by regulating macrophage proliferation and effec-tocytosis via different targets, highlighting the context-dependent roles of miRNAs in diseases. MicroRNA-155 suppresses early lesion formation by inhibiting colony-stimulating factor-1 receptor–mediated macrophage proliferation and promotes the development of advanced lesions by limiting Bcl6-mediated phagocytosis of apoptotic cells. This finding points toward the development of miRNA-based therapies against atherosclerosis by targeting specific microRNA–mRNA interactions rather than the miRNA, generally.
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Supplemental Figure I. Expression of miR-155 during foam cell formation. A, miR-155 expression in macrophages treated with oxLDL for 24 h and 72 h. *$P < 0.05$, $n = 3–4$. B, Oil-red O staining of macrophages treated with oxLDL for 24 h and 72 h. Representative images are shown. Scale bars, 50 µm.
Supplemental Figure II. Accumulation of lesional SMCs and neutrophils in aortic roots. Mir155\(^{+/+}\) Apoe\(^{-/-}\) and Mir155\(^{-/-}\) Apoe\(^{-/-}\) mice were fed the HCD for 12 or 24 weeks. A, Accumulation of lesional SMCs in aortic roots determined by smooth muscle actin (SMA) immunostaining. Representative images are shown. Scale bars, 50 µm. B, Accumulation of lesional neutrophils in aortic roots determined by myeloperoxidase (MPO) immunostaining. Representative images are shown. Nuclei were stained with DAPI. Scale bars, 20 µm.
Supplemental Figure III. Effect of miR-155 on serum cholesterol levels. Serum cholesterol levels in Mir155+/+ Apoe−/− and Mir155−/− Apoe−/− mice fed an HCD for 12 or 24 weeks. n = 7–15 mice per group.
Supplemental Figure IV. Expression of miR-155 targets in atherosclerotic lesions. Expression levels of Socs1, PU.1, Rhoa, Ship1 and Hbp1 were quantified by qRT-PCR in aortic root lesions from Mir155+/+Apoe–/– and Mir155−/−Apoe−/− mice fed the HCD for 12 or 24 weeks. **P < 0.01. n = 4–5 mice per group.
Supplemental Figure V. Effect of miR-155 levels on the expression of Csf1r and Bcl6. Mir155−/− BMDMs were transfected with miR-155 mimics or scrambled controls at various concentrations. Csf1r and Bcl6 expression levels were quantified by qRT-PCR. *$P < 0.05$ compared to the control mimic. $n = 4–5$. 
Supplemental Figure VI. Effect of miR-155 on macrophage apoptosis. *Mir155^{+/+}* and *Mir155^{-/-}* BMDMs were treated with the NF-κB inhibitor BAY11-7085 and stained with Annexin V followed by flow cytometric analysis. Means ± SEM are shown. n = 3–4.
Supplemental Figure VII. Effect of miR-155 on RhoA expression. RhoA mRNA (A; n = 3–4) and protein (B; n = 5–6) levels in BMDMs stimulated with or without LPS/IFN-γ determined by qRT-PCR and ELISA, respectively. **P < 0.01.
Supplemental Figure VIII. Effect of miR-155 on the expression of macrophage phenotype markers in atherosclerotic lesions. *P < 0.05. n = 4–5 mice per group.
Supplemental Materials and Methods

**Animal models**

*Mir155^+/+Apoe^{−/−}* or *Mir155^{−/−}Apoe^{−/−}* mice (6–8 weeks; The Jackson Laboratory, Bar Harbor, ME, USA) were fed a high-cholesterol diet (HCD, Altromin, Germany) comprising 21% crude fat, 0.15% cholesterol, and 19.5% casein for 12 or 24 weeks. Early and advanced atherosclerosis was defined according to the duration of the HCD feeding. Hence, early atherosclerosis was studied after 12 weeks and advanced atherosclerosis after 24 weeks of the HCD. For bone marrow (BM) transplantation, BM cells from *Mir155^{+/+}Apoe^{−/−}* and *Mir155^{−/−}Apoe^{−/−}* mice (5–10 × 10^6 cells) were injected into the tail vein of *Mir155^{+/+}Apoe^{−/−}* or *Mir155^{−/−}Apoe^{−/−}* recipients treated with an ablative dose of whole-body irradiation (2 × 6.5 Gy). After a 3-week treatment with antibiotic water, the mice were fed the HCD for 12 weeks. *In situ* perfusion fixation was performed with paraformaldehyde. All animal experiments were approved by the local authorities (LANUV NRW) in accordance with German animal protection laws.

**Histology and immunostaining**

Serial sections (5 µm thick) of aortic roots (3-5 sections per mouse) were stained with Elastic van Gieson stain. Images were obtained with a bright-field microscope (DM6000B, Leica, Wetzlar, Germany) connected to a CCD camera. The lesion and the necrotic core area, which was defined as the lipid-rich, acellular region within the lesion, were quantified using planimetry (Diskus software, Bonn, Germany) or image analysis software (Image J). The quantitative immunostaining of α-SMA (Dako, Glostrup, Denmark), Mac2 (Cederlane, Ontario, Canada), Myeloperoxidase (Abcam, Cambridge, UK), TUNEL (In situ cell death detection kit, Roche, Basel, Switzerland), Ki67 (Abcam), Csf1r (Abcam) and Bcl6 (Santa Cruz Biotechnology, Dallas, TX, USA) were performed. Non-specific primary antibodies were used as negative controls. The primary antibodies were detected with a fluorescently labeled secondary antibody. Digital images were acquired with a fluorescence microscope (DM6000B, Leica) that was connected to a CCD camera and LAS software. The size of the positively stained area or the number of the positively stained cells per lesion area (2–3 sections per mouse) were determined with Image J, and the threshold was set according to the background of the negative control staining. To analyze TUNEL staining, the number of Mac2^+^ cells with TUNEL^+^ nuclei was counted, and the percentage of TUNEL^+^/Mac2^+^ cells in the total Mac2^+^ cells were calculated. In addition, the TUNEL-positive area regardless of Mac2 staining was measured.

**Cholesterol measurement**

Serum samples were analyzed by dry chemistry using a Vitros 250 Analyzer (Ortho Clinical Diagnostics GmbH, Neckargemünd, Germany) to determine the cholesterol levels.

**In situ PCR**

*In situ* PCR of miR-155 was performed according to previous methods, with small
modifications. In brief, sections (4 µm in thickness) from 4% paraformaldehyde-fixed and paraffin-embedded aortic roots were cooked in citrate buffer and treated with DNase I (Roche) after deparaffinization. After DNase digestion, the tissue sections were covered with the miRNA extension solution and then incubated for 30–60 min at 55°C. The extension solution consisted of mature miR-155 primer (5'-GACCCCTTAATGCGTCTAAAGACCCCTTAATGCGTCTAAAGACCCCTTAATGC GTCTAAACCCCTATCACAATTAGCATTAA-3'), digoxigenin dUTP (Roche), RNase inhibitor (Roche) and SuperScript One-Step RT-PCR System with Platinum Taq DNA Polymerase (Life Technologies, Carlsbad, CA, USA). The scrambled primer was used as a negative control. The digoxigenin-labeled miRNA cDNA was detected with digoxigenin antibody (Roche).

**Cell culture and transfection**
Bone marrow-derived cells from the femurs of Mir155+/+Apoe−/− or Mir155−/−Apoe−/− mice were harvested and cultured in DMEM/F12 supplemented with 10% FBS and 10% L929-conditioned medium. Native LDL (Merck, Kenilworth, NJ, USA) was incubated with CuSO4 (10 µM) at 37°C for 12–14 h to generate oxidized LDL (oxLDL). The macrophages were stimulated with either oxLDL (100 µg/ml) or LPS (100 ng/ml, 14 h) and IFN-γ (10 ng/ml, 6 h).

Lipofectamine 2000 (Life Technologies) was used to transfect Mir155−/− bone marrow-derived macrophages (BMDMs) with a miR-155 mimic (Life Technologies) or a scrambled control at doses of 10 nM, 20 nM and 30 nM. Total RNA was isolated after 24 h of the transfection with the NucleoSpin miRNA Kit (Macherey-Nagel, Düren, Germany).

**Oil-red O staining in vitro**
Wild-type bone marrow-derived macrophages were treated with oxLDL for 24 or 72 h and fixed in 4% paraformaldehyde. After rinse in 60% isopropanol, the cells were stained with Oil-red O solution for 10 mins followed with the rinse in 60% isopropanol and PBS. The nuclei were stained with Hematoxylin. The images were acquired under the microscope (IX50, Olympus).

**Proliferation assay in vitro**
Mir155+/+ and Mir155−/− BMDMs were cultured in DMEM/F12 supplemented with or without 10% L929 conditional medium (CM) and treated with or without Csf1r inhibitor3 (Ki20227, Tocris Bioscience, Bristol, UK) at the dose of 300 nM for 1h. Proliferation of these cells was assessed using flow cytometry by analyzing the cell cycle after fixation with ice-cold 70% ethanol before staining with the solution containing propidium iodide (20 µg/ml), ribonuclease A (10 µg/ml) and 0.1% Triton-X 100 (all Sigma-Aldrich, St. Louis, MO, USA). Sample acquisition and analysis was performed on the Attune Acoustic Focusing Cytometer (Life Technologies).

**Efferocytosis assay**
BM cells were isolated from Mir155+/+Apoe−/− or Mir155−/−Apoe−/− mice and cultured in
vitro. The wild-type cells were labeled with Calcein AM (Life Technologies) and induced apoptotic by incubation with BAY11-7085 (20 µmol/L, Calbiochem, Millipore, Billerica, MA, USA) for 2.5 hours. Apoptotic wild-type cells were then incubated for 30 min with fresh LPS (100 ng/ml, Sigma-Aldrich) and IFN-γ (10 ng/ml, PeproTech, Rocky Hill, NJ, USA)-stimulated Mir155+/+ and Mir155−/− BMDMs with or without pre-treatment with Bcl6 inhibitor4 (250 µM, Calbiochem, Millipore) for 24h. After vigorous washing with phosphate-buffered saline, the macrophages engulfing labeled apoptotic cells were analyzed with the Attune Acoustic Focusing Cytometer (Life Technologies).

**Determination of RhoA activity**

RhoA activity and total RhoA were determined in LPS/IFN-γ-stimulated Mir155+/+ or Mir155−/− BMDMs treated with or without Bcl6 inhibitor using the RhoA Activation Assay G-LISA Kit (Cytoskeleton, Denver, CO, USA) and the Total RhoA ELISA Kit (Cytoskeleton) according to the manufacturer’s instructions, respectively. RhoA activity was normalized to the total RhoA protein level.

**Apoptosis measurement by Annexin V staining**

Mir155+/+ and Mir155−/− BMDMs were treated with BAY11-7085 (20 µmol/L) for 2.5 h and stained with Annexin V (Life Technologies) in Annexin V binding buffer for 15 min. The Annexin V staining was analyzed with Attune Acoustic Focusing Cytometer (Life Technologies).

**Quantitative real-time PCR**

Total RNA was reverse-transcribed with a High Capacity cDNA Reverse Transcription Kit or TaqMan MicroRNA Reverse Transcription Kit (Life Technologies). The mRNA qRT-PCR assay was performed with gene-specific primers and a SYBR Green Master Mix (Life Technologies) or Taqman Master Mix II (Life Technologies). The miRNA qRT-PCR was performed with TaqMan MicroRNA Assays (Life Technologies). The data were normalized to single or multiple reference genes (b2m and actb for mRNA, U6 and sno135 for miRNA), scaled to the sample with the lowest expression (qBase software, Biogazelle, Gent, Belgium), and logarithmically transformed (log10).

**Statistical analysis**

The data represent the means ± SEM and were compared using unpaired t-tests (Prism, GraphPad Software, La Jolla, CA, USA). A P-value less than 0.05 was considered significant.

**References:**

