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Macrophages in atherosclerotic plaques drive inflammatory responses, degrade lipoproteins, and phagocytose dead cells. MicroRNAs (miRs) control the differentiation and activity of macrophages by regulating the signaling of key transcription factors. However, the functional role of macrophage-related miRs in the immune response during atherogenesis is unknown. Here, we report that miR-155 is specifically expressed in atherosclerotic plaques and proinflammatory macrophages, where it was induced by treatment with mildly oxidized LDL (moxLDL) and IFN-γ. Leukocyte-specific *Mir155* deficiency reduced plaque size and number of lesional macrophages after partial carotid ligation in atherosclerotic (*Apoe*–/–) mice. In macrophages stimulated with moxLDL/IFN-γ in vitro, and in lesional macrophages, loss of *Mir155* reduced the expression of the chemokine CCL2, which promotes the recruitment of monocytes to atherosclerotic plaques. Additionally, we found that miR-155 directly repressed expression of BCL6, a transcription factor that attenuates proinflammatory NF-κB signaling. Silencing of *Bcl6* in mice harboring *Mir155*–/– macrophages enhanced plaque formation and CCL2 expression. Taken together, these data demonstrated that miR-155 plays a key role in atherogenic programming of macrophages to sustain and enhance vascular inflammation.
MicroRNA-155 promotes atherosclerosis by repressing Bcl6 in macrophages

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Macrophages in atherosclerotic plaques drive inflammatory responses, degrade lipoproteins, and phagocytose dead cells. MicroRNAs (miRs) control the differentiation and activity of macrophages by regulating the signaling of key transcription factors. However, the functional role of macrophage-related miRs in the immune response during atherogenesis is unknown. Here, we report that miR-155 is specifically expressed in atherosclerotic plaques and proinflammatory macrophages, where it was induced by treatment with mildly oxidized LDL (moxLDL) and IFN-γ. Leukocyte-specific Mir155 deficiency reduced plaque size and number of lesional macrophages after partial carotid ligation in atherosclerotic (Apoe−/−) mice. In macrophages stimulated with moxLDL/IFN-γ in vitro, and in lesional macrophages, loss of Mir155 reduced the expression of the chemokine CCL2, which promotes the recruitment of monocytes to atherosclerotic plaques. Additionally, we found that miR-155 directly repressed expression of BCL6, a transcription factor that attenuates proinflammatory NF-κB signaling. Silencing of Bcl6 in mice harboring Mir155−/− macrophages enhanced plaque formation and CCL2 expression. Taken together, these data demonstrated that miR-155 plays a key role in atherogenic programming of macrophages to sustain and enhance vascular inflammation.

Introduction

Macrophages are the principal effector cells in atherosclerosis, in which they perpetuate the vascular inflammatory response to lipid overloading (1, 2). The subendothelial deposition of lipoproteins precedes infiltration by macrophages, which take up modified lipoproteins in an unrestricted manner via scavenger receptors (2). The lysosomal degradation of lipoproteins generates free cholesterol, which is then converted to, and stored as, cholesterol esters (1). Macrophages can dispose of cholesterol by reverse transport to the liver through several mechanisms of HDL-mediated cholesterol efflux (3, 4). In atherosclerosis, the influx of lipoproteins into macrophages exceeds cholesterol efflux, resulting in the intracellular accumulation of cholesterol esters within lipid droplets, a hallmark of macrophage-derived foam cells (5). Although lipid-handling macrophages show an antiinflammatory phenotype characterized by activation of PPARγ and NR1H3/NR1H2, excessive intracellular lipid storage can induce inflammatory activation by increasing free cholesterol or by inducing the formation of cholesterol crystals (6–10). Conversely, stimulation with inflammatory mediators (such as TNF-α, IL-1β, or the Th1 cytokine IFN-γ) and activation of TLR4 (e.g., by minimally modified lipoproteins) not only induces an inflammatory response, but also enhances intracellular lipid accumulation in macrophages (11, 12). NF-κB–dependent signaling pathways are of crucial importance in the proinflammatory activation of macrophages; however, in vivo studies on the role of the myeloid NF-κB module in atherosclerosis show inconsistent results, indicative of the complexity of the different NF-κB signaling pathways that control both the activation and the resolution phases of inflammation (13–15). Genetic deficiency of TLR4 or its downstream signaling molecule, MYD88, both of which are potent activators of classical NF-κB signaling, limits atherosclerosis by reducing macrophage recruitment and is also associated with reduced expression of chemokines, such as CCL2 (16, 17). In atherosclerosis, macrophage-derived CCL2 acts in concert with endothelial CXCL1 to induce additional macrophage accumulation through a positive feedback regulatory mechanism (18, 19).

BCL6 is primarily expressed in B cells during the germinal center phase of differentiation and acts as a proto-oncogene in diffuse larger B cell lymphomas by compromising the cells’ ability to sense DNA damage (20). The effect of BCL6, at least in part, on the germinal center reaction is mediated by counter-regulation of NF-κB activation (20). Several mechanisms of BCL6-mediated inhibition of NF-κB signaling have been described, including direct repression of p50, inhibitory binding of BCL6 to NF-κB proteins, and opposing regulation of NF-κB target genes (21–24). In macrophages, Bcl6 is transiently induced in a NF-κB–dependent manner and directly represses Ccl2 expression, which indicates that BCL6 inhibits the acute inflammatory response (23, 24).

MicroRNAs (miRs) play an important role in macrophage biology by regulating their differentiation from precursor cells and modifying their inflammatory capacity (25). The typical effect of an individual miR lowers the protein level by less than 2-fold, primarily by destabilizing the target mRNA (26). However, an miR can effectively control the function of its target, either by switch or by fine-tuning interactions (27). In macrophages, functional polarization is associated with the upregulation of a distinct set of miRs (28, 29). TLR4-mediated activation of NF-κB induces a negative feedback loop by upregulating miRs such as miR-21, miR-147, miR-210, miR-34a, and miR-146, which dampen TLR-induced signaling and cytokine expression (30–32). In contrast, miR-155
shows both anti- and proinflammatory effects by regulating TAB2 and SOCS1, respectively (29, 32, 33). Furthermore, upregulation of miR-29b and miR-125a-5p in inflammatory macrophages promotes the expression of proinflammatory cytokines, suggestive of feed-forward regulation (29). Interestingly, treatment of macrophages with oxidized LDL (oxLDL) appears to suppress several miRs induced after inflammatory stimulation, such as miR-146a, miR-155, and miR-21 (34, 35). oxLDL-mediated downregulation of miR-146a enhances both lipid uptake and TLR4 signaling (34). However, oxLDL can also upregulate miR-125a-5p and miR-155, which reduce the accumulation of lipids and cytokine secretion in macrophages (36, 37). miRs that are crucial for the inflammatory macrophage response, such as miR-21, miR-210, miR-146a, miR-34a, miR-147, and miR-125a-5p, are significantly upregulated in human atherosclerotic lesions, which indicates that macrophage-derived miRs contribute to atherogenesis (38, 39). In addition, miR-33 promotes the progression of atherosclerosis and impairs ABCA1-dependent cholesterol removal from lesional macrophages (40). Although these data suggest that miRs play a key role in regulating macrophage function at the intersection of lipid-handling and inflammatory activation, it is still unclear how miRs affect macrophages in atherosclerosis.

Here, we found that miR-155 was upregulated in inflammatory macrophages and in macrophages in atherosclerotic lesions. Increased expression of miR-155 induced CCL2 in macrophages stimulated with mildly oxLDL (moxLDL) and IFN-γ via direct suppression of Bcl6, a transcription factor that counter-regulates NF-κB activation. Furthermore, we demonstrated that deficiency of Mir155 in hematopoietic cells reduced advanced atherosclerotic plaque formation and decreased CCL2 expression in lesional atherosclerotic lesions.
macrophages by derepressing Bcl6. In summary, we identified what we believe to be a novel miR-155–dependent inflammatory pathway in macrophages that plays a role in atherosclerosis.

Results

**MiR expression profile in atherosclerosis and inflammatory macrophages.** To study the miR expression signature in atherosclerosis, flow-mediated plaque formation was induced in ApoE−/− mice by partial carotid ligation (41). When combined with high-fat diet (HFD) feeding, this process resulted in advanced stenotic plaques within 6 weeks, as detected by micro–computed tomography (42) and immunostaining (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI61716DS1). We found that 33 miRs, including miR-155 and miR-147, were increased, and 83 miRs were decreased, in carotid arteries with plaques versus those without (Figure 1A and Supplemental Tables 1 and 2). Notably, miR-155 and miR-147 were also among the 6 upregulated miRs identified in BM-derived macrophages (BMDMs) after stimulation with LPS and IFN-γ compared with unstimulated BMDMs (Figure 1B and Supplemental Table 3). Moreover, stimulation with LPS and IFN-γ suppressed the expression of 26 miRs in BMDMs (Figure 1B and Supplemental Table 4).

To further study the role of miR-155 and miR-147 expression during atherosclerosis, we quantified their expression in a mouse model of diet-induced atherosclerosis. Expression of miR-155 in the aortic wall of ApoE−/− mice was significantly higher after 10 months of HFD feeding than after 3 months of HFD or regular chow diet (P = 0.0111; Figure 1C). However, miR-155 expression in plaque samples of ApoE−/− mice was already increased after 3 months of HFD feeding compared with that in the normal arterial wall (Figure 1D). Expression of miR-155 was also higher in human carotid plaques than in the artery wall (Figure 1E). Similar to miR-155, expression of miR-147 in the murine aortic wall only increased after 10 months of HFD feeding (Figure 1F). Although miR-147 expression was not significantly increased in murine plaques (P = 0.6161 at 3 months and P = 0.3567 at 10 months; Figure 1G), the expression of miR-147b—the human homolog of murine miR-147—was increased in human carotid plaques compared with that in the vessel wall (Figure 1H). Thus, upregulation of miR-155 was consistently observed in different mouse models of atherosclerosis and in human plaques, whereas miR-147 expression was more variable.

Macrophages play a key role in the inflammatory response during atherosclerosis, and M1 polarized macrophages have been described in atherosclerotic lesions. Therefore, we performed in situ hybridization to identify the cellular source of miR-155 in atherosclerotic lesions. First, we verified the specificity of in situ hybridization for miR-155 using Mir155−/− mice as negative controls (Supplemental Figure 2). In atherosclerotic lesions, additional immunostaining for the macrophage marker MAC2 demonstrated that the majority of lesional macrophages expressed miR-155 (Figure 2A). Additionally, some SMCs also expressed miR-155, as detected by combined immunostaining for miR-155 and SMA (Figure 2B). Therefore, both hematopoietic and vessel wall–derived cells in atherosclerotic lesions expressed miR-155.

**Effect of miR-155 on atherosclerosis.** To study the function of miR-155 in atherogenesis, partial carotid ligation was performed in Mir155−/− ApoE−/− mice transplanted with Mir155+/+ ApoE−/− BM cells and in Mir155−/− ApoE−/− mice transplanted with Mir155−/− ApoE−/− BM. Mir155+/+ ApoE−/− mice repopulated with Mir155−/− ApoE−/− BM cells were used as controls. A significant reduction in plaque size and in the number of macrophages per plaque was found in Mir155−/− ApoE−/− mice harboring Mir155−/− ApoE−/− BM compared with the other 2 experimental groups (Figure 3, A and B). However, the medial area, external elastic lamina length, relative macrophage plaque content (determined as macrophage number or MAC2+ area normalized to the plaque area), and macrophage size were not different among the groups (Figure 3A and Supplemental Figures 3 and 4). Moreover, the relative SMC content tended to be decreased in Mir155−/− ApoE−/− mice harboring Mir155−/− ApoE−/− BM (Figure 3C), whereas the relative lesional CD3+ T cell and MPO+ neutrophil counts and the collagen type I content were not substantially affected by Mir155 deficiency (Figure 3, D–F). The number of apoptotic macrophages in the lesions was similar in all 3 groups (Figure 3G). No differences were observed in the serum lipoprotein profile among the groups (Supplemental Figure 5). A combination of immunostaining for MAC2 and Oil red O staining demonstrated that lipid accumulation in Mir155−/− macrophages in atherosclerotic lesions was significantly less than that in Mir155+/− macrophages (P = 0.0466; Supplemental Figure 6). These results indicate that the expression of miR-155 in lesional macrophages promotes atherosclerosis by increasing the number of lesional macrophages and critically regulating their function.

**Role of miR-155 in proinflammatory macrophage activation.** miR-155 is implicated in the regulation of proinflammatory M1 macrophage polarization (43); however, its functional role in the development of atherosclerotic plaque containing activated macrophages is unclear. Our analysis of macrophage-specific miR-155 expression, which was downregulated during macrophage differentiation (Figure 4A), showed that polarization of BMDMs into proinflammatory M1-type macrophages upon stimulation with LPS and IFN-γ (in contrast to antiinflammatory M2-type polarization induced by IL-4) induced miR-155 expression (Figure 4B). Native LDL and mox-LDL slightly increased miR-155 expression, whereas highly oxLDL reduced the expression of miR-155 (Figure 4B).
and induced expression of the M2 marker Mrc1 (Supplemental Figure 7). This indicates that the degree of LDL oxidation plays a role in the effect of modified LDL on miR-155 expression. Similar to LPS and IFN-γ, stimulation with moxLDL and IFN-γ induced miR-155 together with M1-type markers, such as Nos2 and Tnf (Figure 4B and Supplemental Figure 7). Thus, moxLDL-stimulated macrophages also required IFN-γ, which is highly expressed in atherosclerotic plaques (44), to develop a M1 phenotype and to substantially upregulate miR-155 expression. In contrast to moxLDL- and IFN-γ–stimulated Mir155+/+ BMDMs, Mir155−/− BMDMs mainly showed reduced expression of Ccl2 mRNA (and also some reduction in Il1b mRNA), whereas no substantial difference in Il1b and Nos2 expression was detectable between Mir155+/+ and Mir155−/− BMDMs (Figure 4C). The effect of miR-155 on CCL2 expression and secretion was confirmed at the protein level (Figure 4, D and E). In contrast, macrophage migration and apoptosis appeared to be unchanged in the absence of miR-155 in vitro (Figure 4, F and G). To study the effect of miR-155 on CCL2 expression in lesional macrophages, combined immunostaining for CCL2 and MAC2 (Figure 5) was performed (Figure 3). In line with the results obtained for BMDMs, the number of CCL2-expressing macrophages was substantially reduced in carotid lesions in Mir155+/+Apoe−/− mice harboring Mir155−/−Apoe−/− BM compared with that in Mir155+/+Apoe−/− and Mir155−/−Apoe−/− mice harboring Mir155+/+Apoe−/− BM (Figure 5), which may lead to impaired macrophage infiltration into the plaques in Apoe−/− mice with Mir155-deficient leukocytes.
Mechanism of miR-155–mediated cytokine expression. Several targets for miR-155 have been described in macrophages, including Socs1 and Sfpi1, which regulate inflammatory and developmental processes, respectively (33, 45). To identify the mechanism of miR-155–induced CCL2 expression, we studied 7 potential miR-155 targets that were increased in either unstimulated Mir155–/– BMDMs or in Mir155 –/– BMDMs stimulated with moxLDL and IFN-γ (Figure 6, A and B) using EIF2C2-specific IP. miR-155 was highly enriched in EIF2C2-containing miR-induced silencing complexes (mRISCs) in Mir155 +/+ , but not Mir155 –/– , BMDMs (Figure 6C). Of the 7 potential miR-155 targets in BMDMs, Rela, Tcf7l2, Socs1, Bcl6, and Sfpi1 were associated with EIF2C2 in Mir155 +/+ , but not Mir155 –/– , BMDMs (Figure 6D and Supplemental Figure 8), indicative of direct repression by miR-155, in moxLDL- and IFN-γ–stimulated macrophages. Although 2 binding sites for miR-155 are predicted to reside within the 3′ UTR of Bcl6, to our knowledge, targeting Bcl6 has not been described previously. Therefore, using luciferase reporter assays, we verified the direct targeting of the 3′ UTR of Bcl6 by miR-155 (Figure 6, E and F). Mutation of one of the predicted binding sites within the 3′ UTR of Bcl6 prevented suppression by miR-155 (Figure 6F).

To study which miR-155 target regulates expression of Ccl2 and Tnf, silencing of Socs1, Bcl6, and Sfpi1 was performed using RNA interference (Supplemental Figure 9). Silencing Bcl6, but not Socs1 or Sfpi1, increased Ccl2 and Tnf mRNA expression in Mir155 –/– BMDMs (Figure 7 and Supplemental Figure 10). Upregulation of CCL2 in Mir155 –/– BMDMs by Bcl6-specific siRNA was confirmed at the protein level, whereas TNF-α protein levels only showed a tendency toward an increase after Bcl6 siRNA treatment (Figure 7). Thus, derepression of the miR-155 target Bcl6 in Mir155 –/– macrophages reduced atherogenic cytokine expression.

Regulation of the antiinflammatory role of BCL6 in atherogenic macrophages. BCL6 acts as an NF-κB antagonist either by opposing the upregulation of many NF-κB–responsive target genes in stimulated macrophages or by directly inhibiting NF-κB activity (22, 23, 46). We found that blocking NF-κB activation using the NF-κB inhibi-
tor BAY11-7085 reduced Ccl2 and Tnf mRNA expression in Bcl6 siRNA–treated Mir155+/+ macrophages (Figure 8, A and B). To determine whether autocrine stimulation by TNF-α contributes to the effect of miR-155 on CCL2 expression, TNF-α in the cell culture medium was blocked using an anti–TNF-α antibody. Although the antibody inhibited CCL2 secretion by Mir155+/+ macrophages, blocking TNF-α did not abrogate the reduced CCL2 secretion in Mir155+/+ BMDMs compared with Mir155–/– macrophages (Figure 8, C and D), which indicates that miR-155 increases CCL2 secretion independently of elevated C and D), which indicates that miR-155 increases CCL2 secretion.

In contrast to Mir155–/– macrophages, Bcl6 mRNA in Mir155+/+ macrophages was upregulated 24 hours after stimulation with moxLDL and IFN-γ, reducing the difference in Bcl6 expression between unstimulated Mir155–/– and Mir155+/+ macrophages (Figure 8E). Similar results were obtained at the protein level (Figure 8, F and G); however, upregulation of the BCL6 protein in Mir155+/+ BMDMs was only observed 24 hours after stimulation, whereas BCL6 protein expression was still higher in Mir155–/– than in Mir155+/+ macrophages 6 and 10 hours after stimulation (Figure 8G). Blocking NF-kB activation in Mir155+/+ BMDMs reduced the expression of Bcl6 mRNA 24 hours after stimulation with moxLDL and IFN-γ (Figure 8H), which indicates that upregulation via NF-kB activation offsets the suppressive effect of miR-155 on Bcl6 expression at later stages of the inflammatory macrophage response.

Role of BCL6 in miR-155–mediated protection against atherogenesis. To investigate the role of BCL6 in protection against atherosclerosis in mice harboring Mir155–/– BM cells, we analyzed BCL6 expression in lesional macrophages using a combined immunostaining approach (Figure 9A). The number of BCL6-expressing macrophages was substantially increased in Mir155–/– Apo−/− mice harboring Mir155+/+ Ape−/− BM compared with Mir155–/– Ape−/− and Mir155–/– Ape−/− mice harboring Mir155+/+ Ape−/− BM (Figure 9B). Furthermore, Bcl6 mRNA expression was higher in carotid plaques from mice harboring Mir155+/+ BM than in those from mice with Mir155–/– BM (Figure 9C). Next, we studied the effect of increased Bcl6 expression in Ape−/− mice harboring Mir155+/+ BM on atherosclerosis by silencing Bcl6 in the carotid artery after partial ligation (Supplemental Figure 11). Treatment with Bcl6 siRNA led to a marked increase in plaque formation compared with nonspecific control siRNA, but did not affect the medial area in Ape−/− mice harboring Mir155+/+ BM (Figure 10A). Whereas the number of macrophages per lesion was substantially higher after treatment with Bcl6 siRNA, relative macrophage content and macrophage size were unchanged (Figure 10B and Supplemental Figure 12). Lesional SMC content tended to increase after Bcl6 siRNA treatment (Supplemental Figure 13). Of note, immunostaining of lesions showed more CCL2-expressing Mir155–/– macrophages in arteries treated with Bcl6 siRNA than with control siRNA (Figure 10C).

Discussion

Chronic, nonresolving inflammation driven by lipid-laden macrophages is crucial for atherosclerotic lesion formation. Here, we found that miR-155 promoted the inflammatory response of macrophages during atherosclerosis. Expression of miR-155 in unstimulated macrophages was essential for the upregulation of CCL2 after inflammatory stimulation by direct suppression of Bcl6 expression. Thus, miR-155 sensitized macrophages to inflammatory activation by abrogating BCL6-mediated inhibition of NF-kB signaling. The absence of miR-155 in macrophages limited atherosclerotic plaque formation by upregulating Bcl6, which decreased the expression of CCL2 in lesional macrophages. Therefore, priming of macrophages for inflammatory activation by miR-155 supported the development of advanced atherosclerotic lesions.

miRs play a crucial role in cell fate transitions and regulate the development and function of immune cells (47). Accordingly, macrophage polarization to inflammatory M1 — and alternatively activated M2 — subtypes is associated with the differential regulation of a distinct set of miRs (29). Upregulation of miR-155 and miR-147 was observed in macrophages upon stimulation of TLRs and after polarization into an M1-type (29, 30, 48); however, the functional roles of these 2 miRs in macrophages differ substantially. Whereas miR-155 primarily enhances proinflammatory cytokine expression, miR-147 is part of a negative feedback loop that constrains the inflammatory response (29, 30, 33). Our present results demonstrated that increased miR-155 and miR-147 expression was characteristic not only of M1-type
Figure 6

Identification of miR-155 targets in BMDMs. (A and B) Effect of Mir155 deficiency on expression of potential miR-155 targets Hif1a (n = 4) and Rela, Soc1, Tcf7l2, Bcl6, Pparg, and Sfp1 (n = 3 for each) in unstimulated (A) and moxLDL- and IFN-γ-stimulated (B) BMDMs. *P < 0.05 vs. Mir155+/+. (C) Quantitative RT-PCR of miR-155 in immunoprecipitates from moxLDL- and IFN-γ-stimulated Mir155+/+ and Mir155−/− BMDMs. Precipitates were obtained after incubation of cell lysates with an anti-EIF2C2 or nonspecific IgG control antibody (n = 4 independent experiments per group). *P < 0.05 vs. IgG. (D) Enrichment of potential miR-155 targets in EIF2C2-IP from moxLDL/IFN-γ-stimulated Mir155+/+ and Mir155−/− BMDMs by quantitative RT-PCR (n = 3–4 independent experiments per group). Results are expressed as target enrichment in Mir155−/− BMDMs normalized to that in Mir155+/+ BMDMs. *P < 0.05 vs. Mir155+/+. (E) Potential target sites for miR-155 in the 3′ UTR of murine Bcl6 mRNA, as predicted by the miRanda prediction algorithm (sites A and B; blue). Target site A (sequence highlighted in green) was mutated in the binding region. The same sites in the human Bcl6 3′ UTR are also predicted binding sites for human miR-155, differing from mouse miR-155 by 1 nucleotide. (F) Luciferase reporter assays in HEK293 cells treated with miR-155 mimics or nontargeting control mimics using the pEZX-MT01 vector containing the Bcl6 3′ UTR or the Bcl6 3′ UTR with mutations in predicted miR-155 binding site A (n = 3 independent experiments per group). *P < 0.05 vs. control. Data are mean ± SEM.
The results of the present study clearly showed that miR-155 derived dendritic cells and oxLDL-treated THP-1 macrophages and Ccl2 of miR-155 on Tnf expression were mediated by suppression of inflammatory cytokines (such as IL-1β) has previously been linked to the inhibition of miR-155 transcription, resulting in increased expression of IFN-α (50). Therefore, our results demonstrated that targeting of the transcriptional repressor Bcl6 by miR-155 is a key mechanism underlying the inflammatory activation of macrophages.

Accordingly, we found that targeting of Bcl6 by miR-155 in unstimulated macrophages promoted subsequent proinflammatory activation. However, once fully activated, the suppressive effect of miR-155 was offset by the NF-κB–mediated upregulation of Bcl6, which may be important for the resolution of the inflammatory response. Thus, our data suggest that miR-155–mediated suppression of Bcl6 is crucial for the acute inflammatory activation of macrophages, whereas feedback inhibition through NF-κB–mediated Bcl6 expression prevails during the postactivation state.

We also found that deficiency of miR-155 in hematopoietic cells, but not in vascular cells, attenuated advanced atherosclerosis induced by acute flow disturbance and hyperlipidemia via increased BCL6 expression by macrophages. The reduction in lesion size observed in mice harboring Mir155–/– BM was due to fewer macrophages in the plaques. Uptregulation of BCL6 in lesions of Mir155–/– macrophages repressed CCL2 expression, which is known to promote the recruitment of monocytes to atherosclerotic plaques (18). In addition to its immunoregulatory functions, miR-155 can enhance or prevent the apoptosis of infected macrophages (52, 53). However, we did not observe any effect of miR-155 on the apoptosis of lesional macrophages. In contrast to our findings, increased HFD-induced early atherosclerosis and neutrophil infiltration into lesions was described in Ldlr–/– mice harboring Mir155–/– BM (54). Neutrophils primarily play a role during early atherogenesis by enhancing the recruitment of monocytes, whereas advanced atherosclerosis appears to be less
In addition to upregulating miR-155 in inflammatory macrophages, we found that both native LDL and moxLDL increased the expression of miR-155, although to a lesser extent than did LPS and IFN-γ. In contrast, profoundly oxLDL suppressed the expression of miR-155 in macrophages. Previous results from studies of the effects of oxLDL on miR-155 expression are conflicting. For example, both up- and downregulation of miR-155 in response to oxLDL has been reported in THP-1 macrophages (34, 37). Moreover, treatment with oxLDL can induce the expression of miR-155 in human primary monocytes (36). According to our present results, regulation of miR-155 expression depended on the level of oxLDL modification, which may explain the controversial findings previously reported in the literature. Interestingly, higher levels of LDL oxidation inhibited both the expression of miR-155 and Bcl6 in macrophages, consistent with our findings that Bcl6 expression was increased in mice with high levels of oxLDL oxidation (29). In addition to the downregulation of miR-155, oxLDL treatment also increased the expression of CCL2, a key inflammatory cytokine involved in the recruitment of neutrophils to atherosclerotic lesions (19, 25). Our results suggest that oxLDL-induced expression of CCL2 is mediated by the activation of NF-κB and TNF-α signaling pathways.

Figure 8
Role of NF-κB and TNF-α in regulating CCL2 and BCL6 expression during macrophage stimulation. (A and B) Role of NF-κB signaling in increased Ccl2 (A) and Tnf (B) mRNA expression in Bcl6 siRNA-treated Mir155−/− BMDMs, as determined using the NF-κB inhibitor BAY11-7085. (C) BCL2 protein levels in the medium of Mir155−/− BMDMs after stimulation with moxLDL and IFN-γ, quantified by ELISA. A blocking antibody against TNF-α or an isotype control antibody was added to the medium. (D) CCL2 protein levels in the medium of Mir155−/− or Mir155+/− BMDMs after stimulation with moxLDL and IFN-γ, quantified by ELISA. A blocking antibody against TNF-α (5 μg/ml) was added to the medium in both groups. (E) Effect of moxLDL and IFN-γ stimulation on Bcl6 mRNA expression in Mir155−/− and Mir155+/− BMDMs. (F) BCL6 protein expression in unstimulated Mir155−/− and Mir155+/− BMDMs, determined by Western blot. (G) Time course of BCL6 protein expression after moxLDL and IFN-γ stimulation of Mir155+/− and Mir155−/− BMDMs, determined by Western blot. The intensity of the BCL6 bands relative to that of ACTB bands is expressed as a percentage of that in unstimulated BMDMs (F) or in Mir155−/− BMDMs 6 hours after stimulation (G). Lanes in G were run on the same gel but were noncontiguous (white lines). (H) Bcl6 mRNA expression in BMDMs stimulated with moxLDL and IFN-γ after treatment with BAY11-7085 or vehicle. *P < 0.05. n = 3 independent experiments per group. Data are mean ± SEM.

Influenced by neutrophils (55). Accordingly, we did not observe any effect of miR-155 on the neutrophil content of advanced carotid lesion that were induced by disturbed flow, which suggests that miR-155 affects atherosclerosis in a stage-dependent manner. In contrast to early atherosclerosis, in which Mir155 deficiency disturbs neutrophil homeostasis, the proinflammatory role of miR-155 in macrophage activation promoted advanced atherosclerosis. In line with our results, Bcl6 deficiency in BM cells from Ldlr−/− mice exacerbates atherosclerosis and increases the expression of genes involved in atherogenic inflammation, such as CCL2 (56). Taken together, our findings suggest that miR-155–mediated suppression of Bcl6 sensitizes macrophages to atherogenic activation and that this is a key inflammatory mechanism in advanced atherosclerosis.
inflammatory cytokines in macrophages and the activation of NF-κB, which is essential for the transcriptional upregulation of miR-155 (57–59). In addition to the regulation of miR-155 in macrophages by modified LDL, we found that lesional \( \text{Mir}155^{+/+} \) macrophages accumulated fewer lipids. This is in contrast to previous in vitro results, which report increased lipid accumulation in \( \text{Mir}155^{+/+} \)–/– macrophages (10). Although our methods do not indicate that miR-155 targets PPAR\( \gamma \), it plays a key role in the alternative activation and effective lipid handling in macrophages (10). Interestingly, the expression of PPAR\( \gamma \) in inflammatory macrophages promoted atherosclerotic lesions (60). Alternatively, PPAR\( \gamma \) levels might be reduced indirectly in miR-155–expressing macrophages via enhanced NF-κB activation (60, 61). Despite the reduced lipid accumulation in \( \text{Mir}155^{+/+} \)–/– macrophages, the size of the macrophages in atherosclerotic lesions was not altered by \( \text{Mir}155 \) deficiency. Therefore, we conclude that differences in lipid accumulation are not caused by reduced lesion formation in mice harboring \( \text{Mir}155^{+/+} \) macrophages.

In summary, we showed that increased miR-155 expression in proinflammatory macrophages promoted atherosclerosis by derepressing BCL6-mediated inhibition of CCL2 transcription. We therefore conclude that miR-155 plays a key role in the atherogenic programming of macrophages, which sustains and amplifies vascular inflammation.

**Methods**

See Supplemental Methods for details regarding human carotid plaque sample collection, micro–computed tomography, quantitative RT-PCR, moxLDL preparation, cell culture, migration and apoptosis assays, miR-155 target gene prediction, Western blot analysis, ELISA, and lipoprotein profiling.

**Animal models of atherosclerosis.** Female Apoe–/– mice (6–8 weeks old; Jackson Laboratory) were fed HFD (21% crude fat, 0.15% cholesterol, 19.5% casein; Altromin) for 3 or 10 months. The aorta was then harvested after in situ perfusion with RNAlater (Ambion) via the left ventricle. For the control group, arteries were collected from female Apoe–/– mice (8 weeks old) fed regular Chow diet.

\( \text{Mir}155^{+/+} \)–/– mice (Jackson Laboratory) were crossed with Apoe–/– mice to obtain double-deficient \( \text{Mir}155^{+/+} \)–/– BM. Cells from \( \text{Mir}155^{+/+} \)–/– and \( \text{Mir}155^{+/+} \)–/– mice (5 × 10^6 cells) were injected into the tail vein of \( \text{Mir}155^{+/+} \)–/– recipients treated with an ablative dose of whole-body irradiation (2 × 6.5 Gy). In HFD-fed mice, atherosclerotic plaques were induced 4 weeks after BM transplantation by partial carotid ligation (41). In brief, mice were anesthetized with ketamin and xylazine, and the left external carotid, internal carotid, and occipital arteries were ligated. This partial carotid ligation resulted in acutely reduced blood flow velocity in the common carotid artery due to restricted outflow through the superior thyroid artery. Perivascular treatment of the carotid artery with Bcl6 siRNA (Accell siRNA, 4 nmol/treatment; Dharmacon) was performed once weekly starting 2 weeks after partial ligation. In the control group, the partially ligated carotid arteries were treated with nontargeting siRNA (Dharmacon). The siRNA was mixed with transfection reagent (DharmaFECT 4; Dharmacon) and dissolved in pluronics gel (35%; Sigma-Aldrich) as described previously (19). The left and right carotid arteries were harvested 6 weeks after partial ligation following in situ perfusion with RNAlater (Ambion), paraformaldehyde, or PAXgene Tissue Fix (PAXgene Tissue Containers; Qiagen) through the left ventricle.

**Laser capture microdissection.** Serial sections (20 μm thick) of the carotid and innominate arteries and the aortic root were mounted on membrane-mounted metal frame slides (Molecular Machines and Industries), defparaffinized under RNase-free conditions, and completely dried. Laser capture microdissection was performed using a laser microdissection system (MMI cellcut plus; Molecular Machines and Industries) attached to an inverted microscope (IX71; Olympus). At least 40 sections of plaque tissue or morphologically normal vessel wall tissue were collected from each mouse. RNA was isolated using the RecoverAll total nucleic acid isolation kit (Life Technologies) according to the manufacturer’s instructions.

**miR real-time PCR array.** Total RNA was isolated from carotid arteries and cultured BMDMs using the mirVana kit (Invitrogen), and the quality of the RNA was determined using an Agilent 2100 Bioanalyzer. Reverse transcription and preamplification was performed using the Megaplex RT & Preamp Rodent Pool Set (Life Technologies) according to the manufacturer’s instructions. Samples were loaded onto preconfigured 384-well microfluidic cards (TaqMan Array MicroRNA Cards) for real-time analysis of 518 mouse miRs (Sanger miRBase v10) using a 7900HT RT-PCR System (Life Technologies). Data were analyzed using StatMiner software.
Images were obtained under a brightfield microscope (Leica DMLB) connected to a CCD camera (JVC). The plaque area was quantified by planimetry (Diskus software; Hilger, Bonn).

Quantitative immunostaining for TAGLN (rabbit polyclonal; Abcam); SMA, MAC2, and CD3E (rabbit polyclonal; Dako); MPO (rabbit polyclonal; Abcam); and collagen type I (rabbit polyclonal; Cedarlane) was performed on carotid artery sections. The primary antibody was detected with a fluoresceinlabeled secondary antibody. The percentage of positively stained area per total plaque area (2–3 sections/mouse; 20- to 100- μm distance between sections) was determined using image analysis software (ImageJ), with the threshold set according to the background of negative control staining. Combined immunostaining for CCL2/MAC2 or BCL6/MAC2 was performed by sequential incubation of the carotid sections with either an anti-CCL2 antibody (goat polyclonal; Santa Cruz Biotechnology) or an anti-BCL6 antibody (rabbit polyclonal; Abcam) followed by a FITC-labeled secondary antibody and an anti-MAC2 antibody, which was visualized using a Dylight549-conjugated anti-rat antibody (KPL).

Combined in situ hybridization and immunostaining. Sections (7 μm thick) from carotid arteries fixed with Paxgene (Qiagen) were hybridized with double digoxigenin-labeled probes (miR-155 and scrambled probes, 50 nM; U6 probe, 5 nM) in hybridization buffer (Exiqon) at 51°C for 1 hour 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 hour 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-MAC2 (rat, clone M3/38; Cederlane) or anti-SMA (mouse, clone1A4; Dako) antibodies followed by a FITC-conjugated secondary antibody.

**EIF2C2 IP.** BMDMs from Mir155+/+Apoe–/– and Mir155–/–Apoe–/– mice were washed in ice-cold PBS and incubated with lysis buffer (100 mM KCl, 5 mM MgCl2, 10 mM HEPES [pH 7.0], 0.5% NP40, 5 mM DTT, 250 U/ml RNase OUT [Invitrogen], 400 μM vanadyl ribonucleoside complexes [New England Biolabs], and protease inhibitors [Complete Protease Inhibitor Cocktail Tablets; Roche]) for 15 minutes on ice. After homogenization in a Dounce homogenizer, cell extracts were centrifuged. Input RNA was extracted from the supernatant using TRIzol (Invitrogen). Before IP, protein A/G conjugated to magnetic beads (Millipore) was incubated with a mouse monoclonal anti-EIF2C2 antibody (clone 2E12-1C9; Abnova) or mouse IgG (Millipore). The antibody-conjugated beads were then incubated with the cell extracts for 5 hours at 4°C before immobilization of the precipitate with (Integromics) according to the ΔΔCt method using multiple internal control genes. The most stable combination of internal controls was determined using the Genorm algorithm. A Ct value less than 40 was defined as the limit of detection of the individual assays. The fold change compared with the control group was calculated and logarithmically transformed (log10). Array data were deposited in GEO (http://www.ncbi.nlm.nih.gov/geo/; accession nos. GSE26555 and GSE33453).

**Immunostaining and histology.** Serial sections (5 μm thick) from the common carotid artery were collected within 1 mm of the area of bifurcation and stained (3–5 sections per mouse) with elastic van Gieson (EVG) stain. Figure 10. Role of BCL6 in miR-155–mediated atherosclerosis. Partially ligated carotid arteries from Apoe–/– mice harboring Mir155+/+ BM were perivascularly treated with Bcl6 siRNA or non-targeting control siRNA. (A) Lesion area and medial area were determined in carotid artery sections stained with EVG by planimetry. Representative images are shown. (B) Number of macrophages in lesions from the carotid artery, analyzed by immunostaining for MAC2 (green). Representative images are shown, and the number of MAC2+ cells per lesion was determined. (C) Combined immunostaining for CCL2 (green) and MAC2 (red) was performed in carotid artery sections, and the percentage of CCL2+ macrophages (yellow) was quantified. n = 4–5 per group. *P < 0.05. Scale bars: 100 μm. Data are mean ± SEM.
a magnetic separator (Millipore). RNA was isolated from the precipitate with TRIzol, reverse transcribed with random primers, and amplified using the SYBR Green PCR Master Mix (Fermentas). The fold enrichment (FE) of target genes in the EiF2C2-IP RNA compared with that in IgG-IP RNA was determined as follows: (a) $\Delta \Delta Ct_{\text{EiF2C2-IP}}$ was calculated as $Ct_{\text{input}} - Ct_{\text{EiF2C2-IP}}$; (b) $\Delta Ct_{\text{IgG-IP}}$ was calculated as $Ct_{\text{input}} - Ct_{\text{IgG-IP}}$; (c) $\Delta \Delta Ct$ was calculated as $\Delta Ct_{\text{EiF2C2-IP}} - \Delta Ct_{\text{IgG-IP}}$; and (d) FE was calculated as $2^{\Delta \Delta Ct}$.

Luciferase assay. Constructs of the pEZX-MT01 vector with or without the 3′ UTR of the murine Bcl6 gene were purchased from GeneCopoeia. Mutagenesis PCR was performed at the miR-155 target site of Bcl6. HEK293 cells were transfected with 100 ng luciferase reporter or empty vector and treated with miR-155 mimic (30 nM, Ambion Pre-miR miRNA Precursor; Life Technologies) or nontargeting control oligonucleotides (30 nM, Pre-miR miRNA Precursor Negative Control) using Lipofectamine 2000 (Invitrogen). Firefly and Renilla luciferase activity were assessed using the Dual-Glo Luciferase Assay System (Promega) and a microplate reader (Tecan) 72 hours after transfection.

Statistics. miR array data are presented as means and were compared using unpaired, moderated, 2-tailed t test (Statminer 4.2; Integromics). A value less than 0.05 was considered significant. More than 2 groups were compared using paired, 2-tailed t test; more than 2 groups were compared using 1-way ANOVA followed by Newman-Keuls post-test (Prism 5.0; GraphPad). A value less than 0.05 was considered significant. 2 groups were compared using paired, 2-tailed t test; more than 2 groups were compared using 1-way ANOVA followed by Newman-Keuls post-test (Prism 5.0; GraphPad). A P value less than 0.05 was considered significant.

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