MicroRNA-33–dependent regulation of macrophage metabolism directs immune cell polarization in atherosclerosis

Mireille Ouimet,1 Hasini N. Ediriweera,1 U. Mahesh Gundra,2 Frederick J. Sheedy,1 Bhama Ramkhelawon,1 Susan B. Hutchison,1 Kaitlyn Rinehold,1 Coen van Solingen,1 Morgan D. Fullerton,3 Katharine Cecchini,4 Katey J. Rayner,3 Gregory R. Steinberg,5 Phillip D. Zamore,4 Edward A. Fisher,16 P’ng Loke,2 and Kathryn J. Moore16

1Marc and Ruti Bell Vascular Biology and Disease Program and 2Department of Microbiology, New York University (NYU) School of Medicine, New York, USA. 3University of Ottawa, Ottawa, Ontario, Canada. 4RNA Therapeutics Institute, Howard Hughes Medical Institute, and Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts, USA. 5Division of Endocrinology and Metabolism, Department of Medicine, McMaster University, Hamilton, Ontario, Canada. 6Department of Cell Biology, NYU School of Medicine, New York, New York, USA.

Cellular metabolism is increasingly recognized as a controller of immune cell fate and function. MicroRNA-33 (miR-33) regulates cellular lipid metabolism and represses genes involved in cholesterol efflux, HDL biogenesis, and fatty acid oxidation. Here, we determined that miR-33–mediated disruption of the balance of aerobic glycolysis and mitochondrial oxidative phosphorylation instructs macrophage inflammatory polarization and shapes innate and adaptive immune responses. Macrophage-specific Mir33 deletion increased oxidative respiration, enhanced spare respiratory capacity, and induced an M2 macrophage polarization–associated gene profile. Furthermore, miR-33–mediated M2 polarization required miR-33 targeting of the energy sensor AMP-activated protein kinase (AMPK), but not cholesterol efflux. Notably, miR-33 inhibition increased macrophage expression of the retinoic acid–producing enzyme aldehyde dehydrogenase family 1, subfamily A2 (ALDH1A2) and retinal dehydrogenase activity both in vitro and in a mouse model. Consistent with the ability of retinoic acid to foster inducible Tregs, miR-33–depleted macrophages had an enhanced capacity to induce forkhead box P3 (FOXP3) expression in naïve CD4+ T cells. Finally, treatment of hypercholesterolemic mice with miR-33 inhibitors for 8 weeks resulted in accumulation of inflammation-suppressing M2 macrophages and FOXP3+ Tregs in plaques and reduced atherosclerosis progression. Collectively, these results reveal that miR-33 regulates macrophage inflammation and demonstrate that miR-33 antagonism is atheroprotective, in part, by reducing plaque inflammation by promoting M2 macrophage polarization and Treg induction.

Introduction

Macrophages are critical effectors of inflammation and innate immunity; they also regulate adaptive immunity by recruiting and/or activating other immune cells at inflammatory foci (1). In addition, macrophages play important roles in tissue homeostasis and resolution of inflammation (2). To fulfill these functions, macrophages can adopt a spectrum of activation programs depending on the context. M1, or classical activation, represents one end in response to products from bacterial infection such as LPS and IFN-γ, whereas M2, or alternative activation, occurs in response to the cytokines IL-4 and IL-13 (2). Although originally defined in the context of host defense against parasitic helminths (3), M2 macrophages are now appreciated as being important orchestrators of inflammation resolution and metabolic homeostasis through their secretion of antinflammatory cytokines (e.g., IL-10 and TGF-β), mediators of tissue repair (e.g. collagen), and catecholamines (4). Moreover, monocyte-derived M2 macrophages were recently shown to be an important source of retinoic acid (5), a hormone-like metabolite that promotes the differentiation of immunosuppressive Tregs (6, 7).

Recent evidence points to a pivotal role for cellular metabolism in macrophage activation. In particular, M1 and M2 macrophages use different metabolic programs to fuel their effector functions. M1 macrophages use aerobic glycolysis to rapidly provide energy needed for intense, short-lived bactericidal or proinflammatory responses, whereas M2 macrophages use a more efficient ATP-generating program of fatty acid oxidation (FAO) capable of being sustained for long periods of time (8, 9). Although these metabolic programs were originally thought to merely reflect the cell’s energy substrate utilization, recent findings indicate that disrupting cellular energy metabolism can directly alter macrophage M1/M2 fate and inflammatory functions. When the macrophage metabolism is shifted toward glycolysis, this drives a proinflammatory phenotype (10), whereas inhibition of glycolysis by 2-deoxyglucose (2-DG) decreases the
production of inflammatory cytokines such as IL-1β (11). Similarly, M2 activation is hindered by inhibiting FAO (12), whereas overexpression of the nuclear receptor PPARγ coactivator peroxisome proliferator–activated receptor γ coactivator 1β (PGC1β) to drive mitochondrial oxidative phosphorylation primes macrophages for alternative activation and inhibits proinflammatory cytokine production (9). These studies suggest a paradigm in which cellular metabolism and the M1/M2 inflammatory axis are intimately linked, each influencing the other. There is thus considerable interest in identifying the molecular regulation of metabolic pathways controlling M1/M2 macrophage polarization and harnessing these to favorably intervene in chronic inflammatory conditions.

One clinically important chronic inflammatory condition is atherosclerotic cardiovascular disease, in which persistent macrophage accumulation in the artery wall underlies the pathogenesis. In the setting of hypercholesterolemia, monocyte-derived macrophages infiltrate the arterial intima to clear retained apolipoprotein B–containing (apoB-containing) lipoproteins (e.g., LDL) and are transformed into lipid-laden macrophage foam cells (13). For reasons that are poorly understood, these macrophage foam cells persist in the artery wall, setting off a maladaptive immune response that promotes the formation of plaques. These macrophages are a source of inflammatory mediators, including cytokines and chemokines that mediate the recruitment and/or activation of other immune cells, thereby chronically sustaining the inflammation that fuels plaque progression. While both M1 and M2 macrophages have been documented in human and mouse atherosclerotic plaques (14), the preponderance of evidence suggests that M1 macrophages promote plaque progression. Studies in mouse models of atherosclerosis have shown that conditions that increase macrophage polarization toward an M1 phenotype (15–17) or attenuate polarization toward M2 (18–20) accelerate atherosclerotic plaque formation, whereas administration of the M2-polarizing factor IL-13 inhibits disease progression (21). Moreover, aggressive lipid lowering or raising of HDL levels in mouse models induces atherosclerosis regression that is characterized by a switch from M1 to M2 macrophages in plaques (22, 23), suggesting that M2 polarization is central to resolving atherosclerotic inflammation. Nonetheless, the factors in the plaque microenvironment that regulate the polarization of these cells in vivo remain incompletely defined.

An appealing possibility that the regulatory factors include microRNAs (miRNAs) is suggested by their roles as important posttranscriptional fine tuners of many biological and metabolic programs. Recent studies from our lab and others identified microRNA-33 (miR-33a) and miR-33b as intronic miRNAs that are coexpressed with their host genes, SREBF2 and SREBF1, which code for transcription factors that regulate cholesterol and fatty acid synthesis/uptake (24–26). miR-33a/b repress the expression of genes involved in pathways that these oppose SREBP-driven functions, including cholesterol efflux (ABCA1, ABCG1, NPC1) (24–26) and FAO (HADHB, CROT, CPT1A, PRKAA1) (27–29). Studies in mice and nonhuman primates show that inhibitors of miR-33 may hold promise for the treatment of atherosclerosis, as they increase the expression of the cholesterol transporter ABCA1 in the liver and macrophages, thereby raising plasma levels of HDL and macrophage cholesterol efflux (24–26, 30–32). This is supported by studies of miR-33 inhibition or targeted deletion in atherosclerotic mouse models, which showed increased plasma HDL cholesterol (HDL-C) and reduced atherosclerotic plaque size (31, 33). However, recent studies in hyperlipidemic LDL receptor–knockout (Ldlr−/−) mice fed a Western diet concurrent with anti–miR-33 treatment showed atheroprotection even in the absence of changes in plasma levels of HDL-C (34), indicating that miR-33 inhibition has antiatherosclerotic properties that are distinct from its HDL-raising abilities. Given that miR-33 is a metabolic regulator, we investigated whether miR-33 regulation of cellular metabolism can control macrophage inflammatory phenotype and function to alter atherosclerotic plaque inflammation and disease progression.

We show herein that, indeed, miR-33 directly controls macrophage polarization and that this is independent of its effects on cholesterol efflux. Furthermore, we show that the effects are a result of targeting the AMP-activated protein kinase (AMPK), a key integrator of cellular energy homeostasis, thereby reducing FAO and promoting a proglycolytic state that fuels M1 activation. Inhibitors of miR-33 reverse this shift in metabolism by upregulating cellular FAO and driving macrophage polarization to the M2 state in vitro and in vivo, as evidenced by the enrichment of markers that characterize alternatively activated macrophages. Furthermore, anti–miR-33 increased macrophage expression and activity of the retinoic acid–producing enzyme aldehyde dehydrogenase (ALDH) family 1, subfamily A2 (ALDH1A2), endowing these cells with the capacity to promote the differentiation of inducible forkhead box P3–positive (FOXP3+) Tregs. Consistent with this, anti–miR-33 treatment of Western diet–fed Ldlr−/− mice increased the accumulation of atheroprotective M2 macrophages and FOXP3+ Tregs in plaques, while reducing atherosclerotic plaque size (~35%) independently of changes in plasma HDL-C. Collectively, these data indicate that miR-33 promotes a proinflammatory M1 state by altering macrophage FAO and retinoid metabolism, consequently affecting both innate and adaptive immune responses.

Results

miR-33 controls the macrophage inflammatory phenotype by altering the balance of cellular FAO and glycolysis. To understand the relationship between miR-33 and macrophage inflammation, we measured levels of miR-33 in BM-derived macrophages (BMDMs) classically (M1) activated or alternatively activated (M2) by treatment with IFN-γ and LPS or IL-4 for 24 hours, respectively. Expression of miR-33 was higher in M1 macrophages as compared with M2, pointing to a potential role for miR-33 in macrophage polarization (Figure 1A). To investigate this, we next profiled the expression of key genes that mark commitment to M1 or M2 activation in mouse peritoneal macrophages transfected with miR-33 mimic or control oligonucleotides. Overexpression of miR-33 increased macrophage mRNA expression of M1 markers such as Il6, Nos2, and Il1b, and reduced expression of the M2 macrophages Mrc1 (mannose receptor CD206) and Fizz1/Retnla (Figure 1B). Conversely, macrophages transfected with inhibitors of miR-33 showed a marked increase in expression of M2 markers (Arg1, Mrc1, Fizz1) and reduced expression of M1 markers (Il6, Nos2, Il1b) compared with macrophages treated with control oligonucleotides (Figure
M1 with IFN-γ/LPS and reduced macrophage polarization to M2, as measured by the expression of M1 and M2 marker genes (Supplemental Figure 1). Notably, none of the altered genes contain potential miR-33–binding sites in their 3′ UTRs, with the exception of Arg1. However, overexpression of miR-33 failed to alter Arg1 3′ UTR luciferase activity, excluding Arg1 as a direct target of miR-33 (Figure 1D). Previous studies in macrophages lacking the cholesterol transporter ABCA1, a bona fide target of miR-33, suggested 1C), suggesting that miR-33 may alter macrophage inflammatory polarization. Furthermore, miR-33 inhibition or targeted deletion in BMDMs reduced the expression of M1 markers in macrophages classically activated with IFN-γ/LPS and increased expression of M2 markers in macrophages alternatively activated with IL-4 (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI81676DS1). Conversely, treatment with miR-33 mimics enhanced macrophage polarization to M1 with IFN-γ/LPS and reduced macrophage polarization to M2, as measured by the expression of M1 and M2 marker genes (Supplemental Figure 1). Notably, none of the altered genes contain potential miR-33–binding sites in their 3′ UTRs, with the exception of Arg1. However, overexpression of miR-33 failed to alter Arg1 3′ UTR luciferase activity, excluding Arg1 as a direct target of miR-33 (Figure 1D). Previous studies in macrophages lacking the cholesterol transporter ABCA1, a bona fide target of miR-33, suggested
Classical M1 activation of macrophages using IFN-γ and LPS treatment increased aerobic glycolysis and lactate production, which was reflected by a high extracellular acidification rate (ECAR). On the other hand, alternatively activated M2 macrophages induced by IL-4 preferentially rely on β-oxidation of fatty acids to fuel their functional activation, which was reflected by increased mitochondrial oxygen consumption rates (OCRs) in cells supplemented with carnitine and palmitate to support FAO (Figure 1G). Consistent with enhanced M1 activation, miR-33 overexpression shifted macrophage metabolism toward aerobic glycolysis and reduced FAO, as that enrichment of cellular membranes with free cholesterol can enhance macrophage proinflammatory responses (35, 36). However, the effects of miR-33 inhibition on macrophage M1/M2 gene programs were maintained in Abca1−/− macrophages (Figure 1E), indicating that miR-33 alters macrophage polarization by a mechanism independent of its regulation of ABCA1 expression.

As recent studies indicate that the activation of macrophages to M1 and M2 phenotypes is marked by differences in core metabolic programs (4), we next investigated whether miR-33–induced changes in macrophage polarization gene profiles were accompanied by changes in cellular energy metabolism. As shown in Figure 1F and consistent with published reports (12), classical M1 activation of macrophages using IFN-γ and LPS treatment increased aerobic glycolysis and lactate production, which was reflected by a high extracellular acidification rate (ECAR). On the other hand, alternatively activated M2 macrophages induced by IL-4 preferentially rely on β-oxidation of fatty acids to fuel their functional activation, which was reflected by increased mitochondrial oxygen consumption rates (OCRs) in cells supplemented with carnitine and palmitate to support FAO (Figure 1G). Consistent with enhanced M1 activation, miR-33 overexpression shifted macrophage metabolism toward aerobic glycolysis and reduced FAO, as

**Figure 2.** miR-33 regulates the macrophage metabolic and inflammatory phenotype via targeting of AMPK. (A) Ratio of OCR to ECAR in BMDM in the presence or absence of 2-DG to decrease glycolysis. Data represent mean ± SEM of 4 experiments. Un, untreated. (B) Real-time PCR analysis of markers of M2 activation in macrophages in A treated with vehicle or 2-DG for 24 hours. Data represent mean ± SEM of 3 experiments. (C and D) Real-time PCR analysis of (C) BMDMs transfected with control or miR-33 mimics and inhibitors for 48 hours and (D) BMDMs from WT or Mir33−/− mice. (E) ECAR of WT or Mir33−/− BMDMs sequentially treated (arrows) with oligomycin, FCCP, and rotenone plus antimycin. Data are representative of 3 independent experiments (C–E). (F) Maximal ECAR and OCR of WT or Ampk−/− BMDMs transfected with miR-33 or control mimics and (G) real-time PCR analysis of markers of classical M1 and alternative M2 activation in these cells. (H) Maximal ECAR and OCR in WT or Ampk−/− BMDMs treated with anti–miR-33 or control anti-miR and (I) real-time PCR analysis of markers of classical M1 and alternative M2 activation in those cells. Data are representative of 2 independent experiments (F–I). Statistical comparisons were made using 2-tailed Student’s t test (A, B, D, and F–H) or ANOVA (C, G, and I). *P ≤ 0.05; **P ≤ 0.005, compared with controls. All values are mean ± SEM.
evidenced by increased cellular ECAR and reduced OCR (Figure 1H). Furthermore, this pattern was reversed upon inhibition of endogenous miR-33 in macrophages, which markedly increased the mitochondrial oxidation rate in the presence of palmitate and decreased glycolytic activity (Figure 1I). Increased FAO in macrophages treated with anti-miR33 compared with control anti-miR was confirmed by measuring 14-C-oleic acid oxidation, and this increase was abolished in the presence of the CPT1 inhibitor etomoxir, which blocks FAO (Figure 1J). Thus, modulation of miR-33 expression can promote distinct activation programs in macrophages, measurable by changes in both M1/M2 markers and the metabolic programs that fuel their bioenergetic needs.

Recent studies suggest that manipulation of cellular metabolism can directly affect the inflammatory state of immune cells (e.g., refs. 9, 10). Consistent with these reports, we show that treatment of macrophages for 24 hours with 2-DG, a glucose analog that acts as a competitive inhibitor for glycolysis, increases the ratio of oxidative phosphorylation to aerobic glycolysis, and promotes the expression of genes that define M2 activation (Figure 2, A and B). Thus, we considered whether miR-33 targeting of genes involved in fatty acid or glucose metabolism could be responsible for reprogramming the macrophage inflammatory phenotype. We previously demonstrated that miR-33 targets genes involved in FAO in hepatocytes, including Cpt1a, Crot, Hadhb, and Prkaa1 (27), and show here that miR-33 overexpression and inhibition in macrophages reciprocally regulates the expression of Cpt1a, Crot, Hadhb, and Prkaa1 mRNA (Figure 2C). Consistent with the active repression of these genes in macrophages by miR-33, we show that BMDMs from miR-33−/− deficient mice have increased levels of Cpt1a, Crot, Hadhb, and Prkaa1 mRNA (Figure 2D) and this is associated with a marked upregulation of basal mitochondrial OCR and spare respiratory capacity (SRC) (the quantitative difference between maximal uncontrolled OCR and the initial basal OCR) (Figure 2E). Of these miR-33 target genes, Prkaa1 was the most highly derepressed in Mir33−/− macrophages and codes for AMPKα1, an evolutionary conserved kinase that acts as a cellular energy sensor. Upon activation, AMPK switches off ATP-consuming pathways and promotes ATP-producing pathways, including FAO (37). We show that inhibition of miR-33 in BMDMs increases both AMPK protein and activity, as evidenced by elevated levels of phosphorylated AMPK (Supplemental Figure 2). To investigate whether miR-33 regulates macrophage activation by targeting AMPK, we overexpressed and silenced miR-33 in WT and Prkaab1/− (also known as Ampkβ1−/−, herein referred to as Ampk−/−) macrophages, which have approximately 90% reduced AMPK activity (38). Whereas WT macrophages transfected with miR-33 showed increased ECAR and reduced OCR, these effects were abolished in macrophages lacking AMPK activity (Figure 2F). Furthermore, miR-33 overexpression failed to increase markers of M1 macrophage activation (Il6, Nos2, Il1b) in Ampk−/− macrophages consistent with a loss of metabolic reprogramming by miR-33 in the absence of AMPK (Figure 2G). Similarly, miR-33 inhibition failed to reduce ECAR or increase OCR in Ampk−/− macrophages, and this was paralleled by a loss of macrophage polarization to the M2 phenotype (Figure 2, H and I). Together, these results indicate that miR-33 targeting of AMPK to alter macrophage metabolism is central to its immunomodulatory function.
matory protein-1 (MIP-1β), and MIP-2 in mice treated with anti–miR-33 compared with control anti-miR (Figure 4E). Together, these data indicate that anti–miR-33 can reduce local and systemic inflammation by altering the plaque macrophage inflammatory phenotype in the face of continued hyperlipidemia.

Anti–miR-33 treatment restores impaired ALDH1A2 expression and activity in macrophages and macrophage foam cells. Among the genes induced by anti–miR-33 in plaque macrophages, we noted an increase in Aldh1a2 (Figure 4A), a gene recently reported to be expressed in alternatively activated macrophages (6). Aldh1a2 codes for the enzyme retin ALDH type 2 (RALDH2), which regulates the synthesis of retinoic acid, a metabolite with immunoregulatory function (7). Although not a predicted target of miR-33, we found that peritoneal macrophages elicited from mice treated with anti–miR-33 also had increased Aldh1a2 mRNA expression 3 days after injection, compared with macrophages from control anti-miR–treated mice (Figure 5A). Correspondingly, F4/80+ peritoneal macrophages from these mice treated with anti–miR-33 showed increased ALDH activity, as measured by flow cytometry, which could be blocked by the ALDH-specific enzyme inhibitor diethylaminobenzaldehyde (DEAB) (Figure 5B). Consistent with a key role for miR-33 targeting of AMPK in regulating macrophage polarization, we found that modulation of Aldh1a2 mRNA by miR-33 overexpression or inhibition was abolished in Ampk–/– macrophages (Figure 5, C and D). These data suggest that miR-33 modulation of Aldh1a2 expression is downstream of AMPK and metabolic pathways that dictate macrophage polarization. Indeed, acute inhibition of glycolysis in macrophages using 2-DG increased the expression of Aldh1a2, whereas inhibition of FAO using etomoxir reduced Aldh1a2 mRNA (Figure 5E), showing that expression of Aldh1a2 in macrophages is controlled by metabolic programs that determine M1/M2 polarization.

Recent studies indicate that in progressing atherosclerotic plaques, M1 macrophages predominate over M2 macrophages (40). Consistent with this idea, the expression of Aldh1a2 in plaque macrophages declined with plaque progression in West-
ern diet–fed \textit{Ldlr}\(^{-/-}\) mice (Figure 5F). Interestingly, we also observed reduced ALDH activity in peritoneal macrophages isolated from Western diet–fed \textit{Ldlr}\(^{-/-}\) mice compared with chow-fed WT mice (Figure 5G), suggesting that Aldh1a2 function may become impaired in macrophage foam cells. Notably, anti–miR-33 treatment enhanced ALDH activity in peritoneal macrophages isolated from Western diet–fed \textit{Ldlr}\(^{-/-}\) mice compared with those treated with control oligo (Figure 5H), suggesting that miR-33 inhibition can restore defective ALDH1A2 function in cholesterol-loaded macrophages.

\textbf{Inhibition of miR-33 in macrophages promotes Treg differentiation in vitro and in vivo.} The metabolic product of ALDH activity, retinoic acid, can promote the development of extrathymic inducible FOXP3\(^+\) Tregs (5), which exert a protective role in atherosclerosis (reviewed in ref. 41). To determine whether inhibition of miR-33 in macrophages could promote Treg differentiation, we cocultured naive T cells with macrophages that were transfected with anti–miR-33 or control oligo (Figure 5H), suggesting that miR-33 inhibition can restore defective ALDH1A2 function in cholesterol-loaded macrophages.

Importantly, in macrophage cocultures, the addition of retinoic acid to T cells cocultured with anti–miR-33–treated macrophages showed no further enhancement in the extent of Foxp3\(^+\) induction (Figure 6B). Consistent with these findings, we observed a significant increase in Treg expansion when naive CD4\(^+\) T cells were coincubated with \textit{Mir33}\(^{-/-}\) macrophages compared with WT macrophages, and this was inhibited when RA signaling was blocked using the retinoic acid receptor pan-antagonist LE540 (Figure 6C). Collectively, these results indicate that anti–miR-33–treated macrophages have an enhanced capacity to induce Treg differentiation from a naive T cell population in vitro via increased retinoic acid production. To assess whether miR-33 inhibition increased Treg populations in vivo, we measured the expression of T cell markers and their relevant cytokines in the aortic arches of mice treated with control or anti–miR-33 oligonucleotides. Notably, we observed significant increases in \textit{Aldh1a2}, \textit{Foxp3}, and \textit{Tgfb} mRNA in anti–miR-33–treated mice compared with control oligonucleotide–treated mice (Figure 6D). Consistent with this finding, immunofluorescence staining of the aortic root of atherosclerotic lesions revealed greater numbers of FOXP3\(^+\) cells in plaques and surrounding adventitia of anti–miR-33–treated mice compared with the control group (Figure 6, E and F). We observed no evidence for the expansion of FOXP3\(^+\) Tregs in secondary lymphoid
adaptive immune responses. We demonstrate that by reducing FAO and related SRC in macrophages, miR-33 promotes an inflammatory M1-like macrophage phenotype that is associated with metabolic diseases such as atherosclerosis. Notably, inhibition of miR-33 metabolically reprograms macrophages to the M2 phenotype by upregulating FAO (via the master FAO switch AMPK as well as multiple enzymes that execute the process), which fuels alternatively activated macrophage responses involved in resolving inflammation and tissue repair (Figure 6G).

### Discussion

Our study shows that miR-33 regulates macrophage cellular metabolism in a way that is intimately linked to the cell’s inflammatory phenotype, with important consequences on innate and adaptive immune responses. We demonstrate that by reducing FAO and related SRC in macrophages, miR-33 promotes an inflammatory M1-like macrophage phenotype that is associated with metabolic diseases such as atherosclerosis. Notably, inhibition of miR-33 metabolically reprograms macrophages to the M2 phenotype by upregulating FAO (via the master FAO switch AMPK as well as multiple enzymes that execute the process), which fuels alternatively activated macrophage responses involved in resolving inflammation and tissue repair (Figure 6G). In atherosclerosis, where macrophages are key integrators

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**Figure 5. miR-33 regulates Aldh1a2 expression and activity in vitro and in vivo.**

(A) Real-time PCR analysis of Aldh1a2 mRNA expression in thioglycollate-elicited peritoneal macrophages from C57BL/6 mice isolated 72 hours after i.p. injection of control or anti–miR-33 antisense oligonucleotides. Data are representative of 3 independent experiments. (B) Flow cytometric analysis of ALDH activity of macrophages prepared as indicated in A in the presence and absence of the ALDH inhibitor DEAB to show specificity. Data represent the mean ± SEM of n = 7–8 mice/group. (C and D) Real-time PCR analysis of Aldh1a2 mRNA expression in BMMDs from WT or Ampk–/– mice transfected with (C) control or miR-33 mimics or (D) control anti–miR or anti–miR-33 at 48 hours after transfection. Data are representative of 2 independent experiments. (E) Real-time PCR analysis of Aldh1a2 mRNA in BMMDs cultured for 24 hours with 2-DG to inhibit glycolysis or etomoxir to inhibit FAO. Data represent mean ± SEM of 3 experiments. (F) Nanostring measurement of Aldh1A2 mRNA expression in macrophages isolated from aortic root plaques of Ldlr–/– mice fed a Western diet for 8 or 14 weeks (n = 5 mice/group). (G and H) Flow cytometric analysis of ALDH activity in (G) thioglycollate-elicited peritoneal macrophages from Ldlr–/– mice fed a chow or Western diet (n = 6 mice/group) and (H) resident peritoneal macrophages isolated from Western diet–fed Ldlr–/– mice treated with control anti-miR or anti–miR-33 for 8 weeks. ALDH activity was measured in the presence and absence of DEAB to show specificity. Statistical comparisons were made using 2-tailed Student’s t test (A, B, and F–H) or ANOVA (C–E). *P ≤ 0.05; **P ≤ 0.005, compared with controls. All values are mean ± SEM.
Figure 6. Anti–miR-33–treated macrophages induce differentiation of Tregs. (A) Naive CD4+ T cells were cultured in the presence or absence of retinoic acid for 6 days with macrophages transfected with control anti-miR or anti-miR33 and (B) subsequently analyzed by flow cytometry for nuclear FoxP3 expression to determine Treg differentiation. Data are representative of 2 independent experiments. (C) Percentage of naive CD4+ T cells differentiated into Tregs in coculture with WT and Mir33–/– macrophages in the presence or absence of LE540 to block RA signaling. Data are representative of 2 independent experiments. (D) Real-time PCR analysis of mRNA from aortic arches of Western diet–fed Ldlr–/– mice treated with control anti-miR or anti–miR-33 (8 weeks) for markers of T cell subsets and associated cytokines. Data represent the mean ± SEM of n = 6 mice/group. (E) Immunofluorescent staining for Foxp3 in plaques of Western diet–fed Ldlr–/– mice treated with control anti-miR or anti–miR-33 (8 weeks). Dotted line indicates the outline of the intima. L, lumen. FoxP3+ cells accumulate in both the intima (arrowheads) and adventitia (arrows) of plaques of anti–miR-33–treated mice. Data represent the mean ± SEM of n = 6 mice/group. Scale bar: 100 μM. (F) Quantification of FoxP3 staining is shown in D. n = 5 mice/group. (B–D and F) All values are mean ± SEM. Statistical comparisons were made using 2-tailed Student’s t test. *P ≤ 0.05; **P ≤ 0.005, compared with controls. (G) Model of molecular mechanisms involved in miR-33 regulation of macrophage polarization and Treg differentiation. When miR-33 levels are high, AMPK, CPT1A, CROT, and HADHB are repressed, leading to reduced FAO and increased glycolysis, which polarizes macrophages to the inflammatory M1 phenotype. Conversely, when miR-33 levels are low, AMPK, CPT1A, CROT, and HADHB are no longer repressed, leading to increased FAO and polarization of macrophages to the M2 phenotype. M2 macrophages suppress inflammation through their production of antiinflammatory cytokines (e.g., IL-10) and retinoic acid, which can foster the differentiation of iTregs.
of inflammatory and metabolic signals that drive plaque progression, metabolic reprogramming by anti-miR-33 promoted the accumulation of M2 macrophages and FOXP3+ Tregs in plaques, decreased markers of systemic inflammation, and reduced plaque size. Importantly, we found that miR-33 exerts its effects on macrophage polarization and inflammation independently of its previously described roles in regulating macrophage cholesterol efflux and plasma HDL levels. Together, these data define what we believe to be a novel role for miR-33 in regulating the cellular pathways that underpin macrophage polarization and suggest that metabolic reprogramming of plaque macrophages to alternatively activated M2 cells by miR-33 inhibition promotes the resolution of atherosclerosis.

Recent studies indicate that metabolic changes in cells that participate in inflammation are critical to their function. Classically activated macrophages and dendritic cells as well as Th17 cells exhibit a shift toward aerobic glycolysis that accompanies their secretion of proinflammatory mediators and reactive oxygen species (8, 42, 43). Conversely, cells that limit inflammation, such as alternatively activated macrophages and Tregs, exhibit oxidative metabolism and the secretion of antiinflammatory cytokines (8, 43). Studies manipulating glycolytic and oxidative pathways have shown that these metabolic programs can control cellular inflammatory responses, revealing a close relationship between metabolism and immunity. Our data indicate that, while miR-33 is not required for macrophage polarization, it tempers macrophage M1/M2 polarization programs by altering the balance of cellular glycolysis/FAO. Consistent with this, we identify a major role for miR-33 targeting of AMPK, a master metabolic switch, in mediating its effects on macrophage inflammation. AMPK increases FAO via a 2-pronged mechanism: (a) inhibitory phosphorylation and inhibition of acetyl-CoA carboxylase, lowering malonyl-CoA-mediated inhibition of CPT1 and stimulating uptake of activated fatty acids into the mitochondria (44, 45), and (b) enhancement of expression of SIRT1 and PGC-1α, resulting in transcriptional activation of genes involved in mitochondrial FAO and mitochondrial biogenesis (46, 47). Mechanistically, we show that miR-33 overexpression reduces cellular oxygen consumption and increases extracellular acidification indicative of increased glycolysis, which is paralleled by an increase in markers of M1 macrophages (e.g., Il1b, Il6, Nos2) in WT, but not AMPK-deficient, macrophages. Furthermore, miR-33 silencing decreases ECARs and promotes FAO and the expression of M2 markers (e.g., Ym1, Cd206) only in AMPK-expressing macrophages, effects that could be reproduced by blocking glycolysis with 2-DG. Our findings are in agreement with studies showing that expression of a constitutively activated form of AMPK in macrophages can counterregulate inflammatory signaling, whereas siRNA targeting or genetic deletion of AMPK increases the expression of inflammatory cytokines in macrophages and obesity-induced insulin resistance (38, 48). However, it is possible that miR-33 targeting of other genes involved in fatty acid β-oxidation, including Crot, Cpt1a, and Hadh (27–29), may also contribute to its effects on macrophage polarization by reinforcing the effects of AMPK. It is interesting to note that AMPK-dependent mechanisms have also been shown to contribute to differentiation of CD4+ T lymphocytes into inducible Tregs (iTregs), where AMPK acts to simultaneously reduce levels of the glucose transporter Glut1 and increase FAO (49). Finally, although previous studies have shown that free cholesterol enrichment of cellular membranes can enhance inflammatory signaling from lipid rafts, particularly TLR signaling and activation of NF-κB (35, 50, 51), we did not observe a role for miR-33 targeting of the cholesterol transporter ABCA1 in macrophage inflammatory polarization.

Our findings that miR-33 inhibition can promote M2 polarization independently of effects on ABCA1 provide a framework for understanding the HDL-independent mechanisms by which miR-33 inhibitors protect from atherosclerosis. miR-33 was one of the earliest identified miRNAs to regulate cholesterol homeostasis, and much of the research involving this miRNA has focused on its role in repressing cholesterol efflux and plasma levels of HDL-C by targeting ABCA1 (52). Studies in mice and nonhuman primates showed that miR-33 inhibitors increase plasma levels of HDL (24–26, 30–32), a lipoprotein whose plasma level in observational studies is inversely associated with cardiovascular risk, suggesting that it may hold value as a therapeutic target for atherosclerosis. Indeed, a previous study of Ldlr−/− mice with established atherosclerotic plaques that were treated with anti–miR-33 in conjunction with chow diet showed increased plasma levels of HDL-C and regression of atherosclerosis (31). Notably, that study was designed to simulate a clinical scenario in which plasma LDL-C levels were lowered in conjunction with anti-miR-33 treatment. However, 2 studies subsequently reported that the HDL-raising effects of anti–miR-33 in mice are blunted during Western diet feeding, presumably due to decreased hepatic expression of SREBF2/miR-33 (34, 39). Despite this, in a study by Rottlan et al. there were significant reductions in atherosclerotic lesion area in the aortas of Western diet–fed (0.3% cholesterol) Ldlr−/− mice treated with anti–miR-33 for 8 weeks (34), suggesting that anti-miR-33’s atheroprotective effects might not be entirely explained by its ability to increase HDL. Yet this conclusion remained controversial, as Marquart et al. found no differences in either plasma HDL-C or atherosclerosis progression in anti–miR-33 LNA-treated Ldlr−/− mice fed a high cholesterol–containing diet (1.25%) for 12 weeks (39). In agreement with Rottlan et al, we observed a 37% reduction in atherosclerosis in the aorta en face in Ldlr−/− mice treated with anti–miR-33 for 8 weeks on a Western diet (0.3% cholesterol) in the absence of changes in plasma levels of total or HDL cholesterol. Furthermore, we also observed similar reductions (33%) in atherosclerotic plaque size in cross-sections of the aortic root of anti–miR-33–treated mice, and gene expression profiling of plaque macrophages in this site revealed increased expression of genes indicating M2 polarization (Arg1, Mrc1, Fizz1/Retnla, Ym1/Chi3l3). Immunofluorescent staining of plaques showed that macrophages that had taken up anti–miR-33, but not control oligonucleotides, had increased expression of the canonical M2 marker arginase 1, supporting our in vitro findings that anti–miR-33 directly polarizes macrophages to the M2 phenotype. In addition, anti–miR-33 may alter macrophage M2 polarization and decrease Western diet–induced inflammation in other tissues, such as the liver and adipose tissue, which may contribute to the observed reductions in plasma chemokine levels and systemic inflammation. That such an enrichment of M2 mac-
mRNA and FOXP3+ cells in plaques of anti–miR-33–treated mice

tially described (38).

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Methods

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Plasma analysis. Plasma was collected at baseline by tail vein bleeding and at sacrifice by cardiac puncture. Total cholesterol was assayed using the Cholester-E Kit (Wako Chemicals) as described (25). For HDL measurements, apoB-containing lipoproteins were precipitated by the phosphotungstate-magnesium method, and HDL-C
was measured using the HDL Cholesterol Kit (Wako) (25). Triglycerides were measured using the L-type enzymatic triglyceride assay (Wako). Analysis of plasma chemokines and apoA-I was performed by Myriad RBM using the rodent Multi-Analyte Profile.

**Atherosclerosis analysis.** Hearts were sectioned through the aortic root (6 μm) and stained with H&E for lesion quantification or used for immunohistochemical analysis as previously described (63). For morphometric analysis of lesions, 6 sections per mouse were imaged (Nikon Eclipse) with Image-Pro Plus, spanning the entire aortic root, and lesions and necrotic area were quantified using iVision software. For collagen analysis, 6 sections per mouse were stained with picrosirius red and imaged under polarized light using a Zeiss Axiosplan microscope. For detection of neutral lipid, oil red O staining was performed as previously described (63, 64). For macrophage analysis, 6 sections per mouse were incubated with an anti-CD68 antibody (AbD Serotec) and antibody reactivity was visualized using the Vectastain ABC Alkaline Phosphatase Kit (Vector Laboratories) and diaminobenzidine (DAB) substrate. For quantification of CD68+ area, images were analyzed using Fiji software (65). LCM was performed as previously described using the Leica DM-6000B instrument and Leica LMD CC7000 camera (Leica Microsystems) (65). A guide slide for LCM was created using the Zeiss Axiolab (Zeiss). The CD68+ areas in serial sections were collected by LCM. RNA was extracted from each animal as previously described using the RNeasy FFPE Tissue kit (QIAGEN). LCM was performed as previously described; alternatively, 2 ng of unamplified total RNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad) and 20 ng using the iScript cDNA Synthesis Kit (Bio-Rad). For relative quantification, miRNA levels were normalized to the corresponding firefly luciferase activity and plotted as fold-change relative to control. Experiments were performed in triplicate using KAPA SYBR FAST Universal 2× qPCR Master Mix (Kapa Biosystems) on the iCycler Real-Time Detection System (Bio-Rad). The mRNA levels were normalized to Gapdh as a housekeeping gene. The following primer sequences were used: Ahoal, 5′-AAAAACCGCACATCTTCCAG-3′ and 5′-CATACCGAATCTGCTTACCC-3′; Gapdh, 5′-TGGTAGGAGGATGCTCAGT-3′ and 5′-TGGTCCCTACCCCAAATGTGT-3′; Pkaa1, 5′-GTCAAGGCGACCAATGATA-3′ and 5′-CTGATCAACAGCAGCCCAATG-3′; Nos2, 5′-TGTCTAGGCCCCCAATTATCACAGA-3′ and 5′-GGTGCTCAGTTGCGTCCAC-3′; Il6, 5′-GGCAAGTTCTTCTGACTCAACT-3′ and 5′-ATCTTTGGGGTGCCTGCAACT-3′; Il6, 5′-TGTCTAGGCCCCCAATTATCACAGA-3′ and 5′-GGTGCTCAGTTGCGTCCAC-3′; Arg1, 5′-CTCCAAGGCAAAATTTAGGGTT-3′ and 5′-ATCTTTGGGGTGCCTGCAACT-3′; Retnla, 5′-CTCTGCTTCCACCTTCTTCA-3′ and 5′-TAGAAGGAGCTGCCAATGAC-3′; Mrc1, 5′-CTCTGCTTCCACCTTCTTCA-3′ and 5′-TAGAAGGAGCTGCCAATGAC-3′.

miRNA quantification. For mature miRNA quantification, the miScript II RT Kit (QIAGEN) was used for reverse-transcription reactions, using HiSpec Buffer, according to the manufacturer’s protocol. miR-33 and miR-33* miScript miRNA mimics were used to generate a standard curve of Ct values (y axis) against log copy number (x axis), according to the instructions in QIAGEN’s miScript PCR System Handbook, to ensure equivalent miR-33 and miR-33* primer efficiencies and for absolute quantification. miScript Primer Assays and miScript SYBR Green PCR were used on the iCycler Real-Time Detection System (Bio-Rad). For relative quantification, miRNA levels were normalized to RNU6-2 miScript PCR control (QIAGEN).

**3′ UTR luciferase reporter assays.** The mouse Argl (Mm:1026580) 3′ UTR target clone was purchased from GeneCopoeia. Point mutations in the predicted miR-33 site within the 3′ UTR of mouse Argl were generated using MultiSite-Quickchange (Stratagene) according to the manufacturer’s protocol. HEK293T cells (ATCC) were plated in antibiotic-free media in 96-well plates and cotransfected with 0.2 ng of reporter vector and the corresponding firefly luciferase vector and normalized to Renilla luciferase activity for transfection efficiency. Luciferase activity was measured using the Sequare-Plate Dual Luminescence Assay Kit (GeneCopoeia). Renilla luciferase activity was normalized to the corresponding firefly luciferase activity and plotted as fold-change relative to control. Experiments were performed in quadruplicate wells of a 96-well plate and repeated 3 times.
Seahorse extracellular flux analysis. BMDMs were seeded into XF24 cell culture microplates (Seahorse Bioscience) and transfected with anti-miR-33 or control anti-miR as described above, and 48 hours later, OCRs and ECARs were quantified using the XF24 instrument (Seahorse Bioscience) according to the manufacturer’s protocol. For glycolysis assessments, the XF Glycolysis Stress Test Kit was used according to the manufacturer’s protocol. For cellular FAO quantification, the OCR was measured following exposure to BSA-conjugated palmitate (200 μM) in Krebs-Henseleit buffer (KHB; 111 mM NaCl, 4.7 mM KCl, 2 mM MgSO4, 1.2 mM Na2HPO4, 2.5 mM glucose, 0.5 mM carnitine) using the XF24 instrument and the XF Cell Mito Stress Test kit.

FAO analysis. 1 × 10^6 BMDMs were cultured in 12-well plates and transfected with control or miR-33 miRNA inhibitors. Cells were incubated for 3 hours with 0.3% BSA/100 μM oleic acid/0.4 μCi/ml 1-14C-oleic acid (PerkinElmer) in DMEM, in the presence or absence of etomoxir (25 μM, Sigma-Aldrich), as previously described (67). Oxidation was stopped by the addition of 80 μl of 50% TCA per well, and 14CO2 was trapped on Whatman paper disks soaked with 20 μl of 1M NaOH for 1 hour in a fume hood, after which trapped 14CO2 was measured by liquid scintillation counting.

Flow cytometry. Cells were stained with LIVE/DEAD Blue (Invitrogen), blocked with 4 μg/ml aCD16/32 (2.4G2; Bioxcell), and stained with the cell surface markers CD11b eFluor450 (eBioscience), F4/80 PE-Cy7 (eBioscience), Siglec-F BV421 (BD Biosciences), CD4 BV650 (BioLegend), and CD25 PB (BioLegend). For intracellular nuclear staining of FOXP3, cells were washed with PhosFlow permeabilization buffer (BD Biosciences) and blocked with anti-mouse CD16/32 following surface staining with antibodies against CD4 and CD25, and stained with a FOXP3 antibody (PE-CF594, BD Biosciences) for 1 hour at 4°C in Foxp3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent (eBioscience) as previously described (5). ALDH activity was measured using the ALDEFLUOR staining kit (StemCell Technologies) in the presence of the ALDH inhibitor DEAB (15 μM), as previously described (5, 6). In this assay, BODIPY aminoacetaldehyde, the nontoxic substrate for ALDH that is able to diffuse freely into cells, is converted (5, 6). In this assay, BODIPY aminoacetaldehyde, the nontoxic sub-

Treg differentiation assay. Naive T cells were isolated from the spleens and lymph nodes of C57BL/6 mice using the Naive CD4+ T Cell Isolation Kit II (Miltenyi Biotec) and were cultured together with macrophages as previously described (6). 4 × 10^6 naïve T cells were cocultured with 4 × 10^6 peritoneal macrophages that were transfected with control anti-miR or anti–miR-33 at 48 hours prior to the coculture. Cells were cultured in complete RPMI containing soluble anti-CD3 (1 μg/ml), soluble anti-CD28 (1 μg/ml), and recombinant human IL-2 (5 ng/ml) for 6 days. The cocultures were supplemented with fresh medium and IL-2 on day 3. Retinoic acid (100 nM) was added to some culture wells. Cells were harvested on day 6 and stained for FOXP3 by intracellular nuclear staining as described above.

Western blots. Protein was extracted in RIPA buffer with protease and phosphatase inhibitors, and samples (60 μg/well) were electrophoresed on 8% SDS-polyacrylamide gels and transferred to nitrocel-

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Address correspondence to: Kathryn J. Moore, New York University School of Medicine Institutional Animal Care and Use Committee.

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