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ORIGINAL ARTICLE

miR-34a regulates macrophage-associated inflammation and angiogenesis in alcohol-induced liver injury

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Abstract

Background: Alcohol-associated liver disease (ALD) is a syndrome of progressive inflammatory liver injury and vascular remodeling associated with long-term heavy intake of ethanol. Elevated miR-34a expression, macrophage activation, and liver angiogenesis in ALD and their correlation with the degree of inflammation and fibrosis have been reported. The current study aims to characterize the functional role of miR-34a-regulated macrophage- associated angiogenesis during ALD.

Methods & Results: We identified that knockout of miR-34a in 5 weeks of ethanol-fed mice significantly decreased the total liver histopathology score

Abbreviations: ALD, Alcohol-associated liver disease; ALT, alanine transaminase; BMDMs, bone marrow-derived macrophages; CM, conditioned media; EtOH, ethanol; HIF1α, hypoxia inducible factor 1 α; HSCs, hepatic stellate cells; ICAM1, Intercellular Adhesion Molecule 1; IκBα, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α; KO, knockout; LCM, laser capture microdissection; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; miR-34a, microRNA-34a; miRNA, microRNA; MO, morpholino; NF-κB, Nuclear Factor Kappa B; OCR, oxygen consumption rate; RFU, relative fluorescence unit; TLR4, toll-like receptor 4; UTR, untranslated region; WT, wild-type.

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and miR-34a expression, along with the inhibited liver inflammation and angiogenesis by reduced macrophage infiltration and CD31/VEGF-A expression. Treatment of murine macrophages (RAW 264.7) with lipopolysaccharide (20 ng/mL) for 24 h significantly increased miR-34a expression, along with the enhanced M1/M2 phenotype changes and reduced Sirt1 expression. Silencing of miR-34a significantly increased oxygen consumption rate (OCR) in ethanol treated macrophages, and decreased lipopolysaccharide-induced activation of M1 phenotypes in cultured macrophages by upregulation of Sirt1. Furthermore, the expressions of miR-34a and its target Sirt1, macrophage polarization, and angiogenic phenotypes were significantly altered in isolated macrophages from ethanol-fed mouse liver specimens compared to controls. TLR4/miR-34a knockout mice and miR-34a Morpho/AS treated mice displayed less sensitivity to alcohol-associated injury, along with the enhanced Sirt1 and M2 markers in isolated macrophages, as well as reduced angiogenesis and hepatic expressions of inflammation markers MPO, LY6G, CXCL1, and CXCL2.

Conclusion: Our results show that miR-34a-mediated Sirt1 signaling in macrophages is essential for steatohepatitis and angiogenesis during alcohol-induced liver injury. These findings provide new insight into the function of microRNA-regulated liver inflammation and angiogenesis and the implications for reversing steatohepatitis with potential therapeutic benefits in human alcohol-associated liver diseases.

INTRODUCTION

Alcohol-associated liver disease (ALD) is a major health concern that encompasses a broad spectrum of liver injuries ranging from simple steatosis through alcohol-associated hepatitis to cirrhosis, vascular dysfunction, and HCC.[1,2] The pathophysiology of ALD is complex, but Lipopolysaccharide (LPS), macrophage activation, and angiogenesis are considered the key players, as alcohol metabolism and LPS increase the production of reactive oxygen species and lower cellular antioxidant levels, which cause macrophage activation and angiogenesis through several mechanisms.[3] Targeting hepatic macrophages and angiogenesis has high therapeutic potential for liver diseases. Alcohol and LPS-induced macrophage activation and angiogenesis are linked to the development of steatohepatitis, liver fibrosis, and cirrhosis.[4] However, the detailed understanding of the mechanisms of macrophage activation and angiogenesis involved in the pathogenesis of ALD and ethanol metabolism remains incomplete. Thus, the comprehensive investigation of the molecular mechanisms involved in hepatic macrophage activation and angiogenesis is important and may lead to more effective therapeutic approaches for human alcohol-associated liver disorders.

miRNAs are non-coding RNAs recently found to downregulate a large subset of human genes, and increasing evidence suggests that some miRNAs have powerful regulation effects on cellular functions.[5] miR-34a, the most upregulated miRNA in the ethanol-fed mice liver from our study, has been demonstrated to emerge as a specific miRNA modulated in liver disease. miR-34a suppresses SIRT1, which regulates the activity of transcriptional factor p53, a known regulator of cellular senescence.[6,7] Multiple miRNAs, including the miR-34 family, are transcriptionally activated by p53. In turn, miRNAs regulate the expression of many p53-induced genes.

Angiogenesis is another double-edged sword in health and disease, and it is the biggest ally of macrophages allowing its dissemination.[8,9] During chronic liver diseases, macrophages support angiogenesis not only by secreting pro-angiogenic growth factors and matrix-remodeling proteases but also by physically interacting with the sprouting vasculature to assist the formation of complex vascular networks. Recent studies have shown that miR-34a is an essential factor for macrophage polarization and

functions, especially for M2 subsets, and is dependent on Krüppel-like factor 4-Klf4.[10] We hypothesize that miR-34a plays a vital role in macrophage polarization, angiogenesis, and functions promoting the activation of other hepatic cells, such as hepatocytes and neutrophils, during alcohol-associated liver injury. In this study, we show the effects of miR-34a depletion in macrophage-associated angiogenesis and its roles in alcohol-associated liver injuries by using animal models.

METHODS

Animal models

All animal experiments were performed in accordance with protocols approved by Baylor Scott & White Hospital, University of Southern California, and Indiana University School of Medicine IACUC Committees. The mice (10 wk old, n = 20) were randomly divided into 4 groups: wild-type (WT) mice, ethanol-fed mice (Leiber-DeCarli liquid diet Ethanol (EtOH), ethanol feeding for 5 wk), miR-34a knockout mice, and ethanol-fed miR-34a knockout mice (for 5 wk). For chronic intragastric ethanol administration, mice were aseptically implanted with gastrostomy catheters as described.[11,12] A dose of liquid ethanol (5%) or control solution was infused for 5 wk.[11]

Human healthy control and steatohepatitis samples

Healthy human liver (n = 4) and liver samples of steatohepatitis patients with heavy alcohol consumption (n = 4) were purchased from Xenotech (Kansas, KS). The samples were used for RNA extraction, frozen section slides, and protein extraction. The characteristics of patients are as listed.[13]

Chemotaxis assay

Neutrophil chemotaxis in miR-34a deficient macrophages with or without LPS/Ethanol treatment was measured using Chemotaxis Assay Kit (96-well, 8 μm, ab235673) from Abcam (Waltham, MA) following the manufacturer's instructions.

Analysis of cytokine production or M1/M2 marker expression

Total mRNAs were harvested from frozen liver tissues, cultured cells, or LCM-isolated macrophages using RNeasy Mini Kit (QIAGEN, Valencia, CA) or PicoPure RNA isolation kit (Thermo Fisher Scientific). Two-step RT-PCR was performed using RT^2 qPCR Primer Assay (QIAGEN) and ViiA 7 Real-Time PCR System (Thermo Fisher Scientific).

Seahorse XFp metabolic assay

The seahorse XFp analyzer was used to perform metabolic analysis of ethanol (25 mM, 24 h) and LPS (20 ng/mL, 24 h) treated the RAW 264.7 murine macrophage after anti-miR-34a or control anti-miRNA transfections. The RAW 264.7 murine macrophage (1×10^5 cells/well) was plated on an XFp Cell Culture miniplate (Agilent Technologies) and treated as specified. Oxygen consumption rate (OCR) was assessed using the XFp Analyzer (Seahorse Bioscience) following manufacture's guidelines.

Statistical analysis

Data were expressed as mean ± SE. The statistical significance of differences between control and experimental groups was analyzed by unpaired Student *t*-test (2 groups) or 1-Way ANOVA (more than 2 groups) using GraphPad Prism 8 (GraphPad Software, La Jolla, CA). Values of $p < 0.05$ were considered statistically significant.

Please see Supplementary materials and methods, http://links.lww.com/HC9/A204, for more detailed information on this section.

RESULTS

Expression of miR-34a is upregulated along with enhanced inflammatory responses in the livers of patients with steatohepatitis induced by heavy alcohol consumption

miR-34a plays a critical role in liver physiology and the pathogenesis of ALD.[14,15] Northern blot analysis revealed that the expression of miR-34a increased in the livers of patients with steatohepatitis induced by heavy alcohol consumption compared with healthy controls (Figure 1A). Enhanced expressions of macrophage infiltration markers CD68 and CD43, as well as leukocyte adhesion marker ICAM1 and miR-34a mediator p53, were observed in liver sections from patients with steatohepatitis compared with healthy controls (Figure 1B&C), along with the increased expression of liver angiogenesis markers CD31, and VEGFR2 (Figure 1D). IPA was performed based on the data from the Inflammatory Response & Autoimmunity PCR Array from human ALD

liver tissues, with enhanced miR-34a expression (Figure 1E) to determine the cellular context of the differentially expressed signaling mechanisms related to miR-34a-mediated liver injury. IPA analysis indicated that NF-κB, LY6G, EMR1, CXCL1, CXCR2, and TNFα signaling molecules are involved in the inflammatory response in the liver of the patient with ALD that was verified by real-time PCR analysis (Figure 1F).

Ethanol feeding increases miR-34a expression, and miR-34a depletion decreases inflammatory response and angiogenesis in ethanol-fed mice

Typical pathological changes were observed in liver sections from ethanol-fed mice with steatohepatitis compared with WT controls by H&E staining (Figure 2A, left). Ethanol feeding significantly increased the hepatic expression of miR-34a when compared with control mice (Figure 2A, right). Furthermore, miR-34a expression was significantly decreased in the miR-34a knockout (miR-34a KO) mice fed with EtOH compared with WT EtOH-fed mice (Figure 2A, right). In the EtOH-fed miR-34a KO mice, there were decreased serum ALT levels compared with WT EtOH-fed mice (Figure 2B). Furthermore, in EtOH-fed mice, liver cell swelling, irregular nucleus size, shrinkage in varying degrees, and fatty degeneration of a large number of liver cells were all observed (Figure 2A. left). These pathologic changes were reduced in EtOH-fed miR-34a KO mice compared with WT EtOH-fed mice, along with significant reductions in the initiation of liver fibrosis detected by Sirius red staining (Figure 2C).

Immunohistochemistry was performed to analyze the macrophage infiltration and angiogenesis in the liver of WT and miR-34a KO mice. CD68 staining showed that the population of CD68+ macrophages was increased in WT EtOH-fed mice compared to WT control mice but significantly attenuated by the miR-34a knockout compared to WT EtOH-fed mice (Figure 2D, top). Meanwhile, adhesion marker ICAM1 was reduced in EtOH-fed miR-34a$^{-/-}$ mice compared with WT EtOH-fed mice (Figure 2D, bottom), along with the significant reductions of angiogenesis markers CD31 and VEGFR2 (Figure 2E&F). The expression of ICAM1 by neutrophils is also associated with phagocytosis and neutrophil-endothelial interaction. Additionally, the hepatic mRNA expressions of inflammation markers and the angiogenesis markers Ly6G, CXCL1, CXCR2, MCP-1, TNFα, and E-selectin, as well as the fibrosis markers TGFβ and Collagen 1A1, were significantly decreased after miR-34a knockout in ethanol-fed mice (Figure 2G).

miR-34a Morpholino treatment inhibits the macrophage-neutrophil interactions in ethanol-fed mice

As we had shown before, ethanol feeding significantly increased the hepatic expression of miR-34a compared with WT control mice. Furthermore, miR-34a expression was decreased in ethanol-fed mice treated with miR-34a Morpholino compared with ethanol-fed mice. Treatment with miR-34a Morpholino on ethanol-fed mice decreased alanine aminotransferase serum levels compared with ethanol-fed mice. Hepatic macrophages regulate the influx of neutrophils, which may play a direct role in alcohol-induced liver injury. Quantitative PCR assays were also performed for neutrophil marker MPO and inflammatory cytokines LY6G, CXCL1, CXCR2, TNFα, and MCP-1. The hepatic mRNA expression of MPO, LY6G, CXCL1, CXCR2, TNFα, and MCP-1 was significantly decreased after miR-34a Morpholino treatment in ethanol-fed mice (Figure 3A), along with the significant reductions of the adhesion markers ICAM1 and E-Selectin in isolated macrophages by laser capture microdissection (LCM) using F4/80 as the specific marker (Figure 3, B&C), suggesting that anti-miR-34a treatment also blocked macrophage activation, which may impact neutrophil recruitment into the liver by means of decreased chemokine production and also through reduced integrin expression during alcohol-associated liver injury.

LPS/TLR4 mediated the expression of miR-34a and inflammatory cytokines during alcohol-associated liver injury

It has been demonstrated that LPS upregulates miR-34a in human and mouse liver tissues and cells. By quantitative PCR, we showed that LPS increased inflammatory genes EMR1, CXCL1, and CXCR2 in cultured human hepatocytes plus LY6G in mouse RAW 264.7 macrophages (Figure 4, A&B). However, only the increase of CXCL1 in both hepatocytes and macrophages plus the increase of LY6G in macrophages was blocked by the inhibition of miR-34a (Figure 4, A&B and Supplementary Figure 1, http://links.lww.com/HC9/A204). Knockdown of miR-34a significantly inhibited CXCL1 up-regulations in cultured hepatocytes induced by the conditioned media from macrophage treated by LPS and EtOH (Figure 4C). To further test the effects of LPS/TLR4 mediated miR-34a signaling during ALD, alcohol-associated liver injury was induced by 5 weeks of ethanol feeding in wild-type (WT) and TLR4 knockout (KO) mouse, and significantly reduced expressions of miR-34a were observed by real-time PCR analysis in EtOH-treated TLR4 KO mouse livers (Figure 4D, left) and LCM-isolated

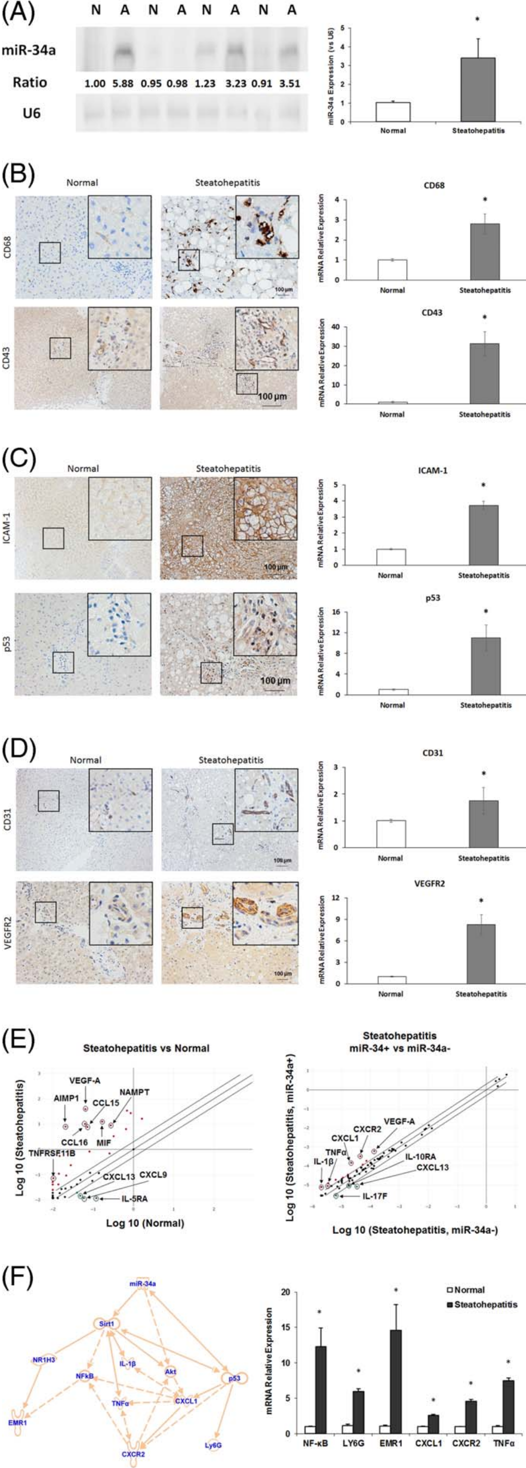

FIGURE 1 The expression of miR-34a and angiogenesis are increased in livers from heavy alcohol consumers with steatohepatitis. (A) Northern blot analysis has shown that expression of miR-34a was upregulated in the livers from steatohepatitis patients with heavy alcohol consumption compared with healthy controls. (B, C) Enhanced macrophage infiltration was detected in steatohepatitis patients with heavy alcohol consumption by CD68 and CD43 staining (Panel B) along with increased expressions of leukocyte adhesion marker ICAM1 and miR-34a mediator p53 (Panel C) relative to healthy controls. (D) Increased angiogenesis in heavy alcohol consumers with steatohepatitis was verified by CD31 and VEGFR2 staining compared with healthy controls. (E) miR-34a-regulated inflammatory cytokine releases in liver tissues from heavy alcohol consumers with steatohepatitis. (F) Ingenuity Pathway Analysis software showed that several genes implicated in liver inflammation are regulated by miR-34a, including NF-kB, LY6G, EMR1, CXCL1, CXCR2, and TNFα (left panel). The mRNA expressions of these inflammation mediators were verified in the liver from steatohepatitis patient versus normal controls by real-time PCR assays (right panel). *$p < 0.05$ versus healthy controls.

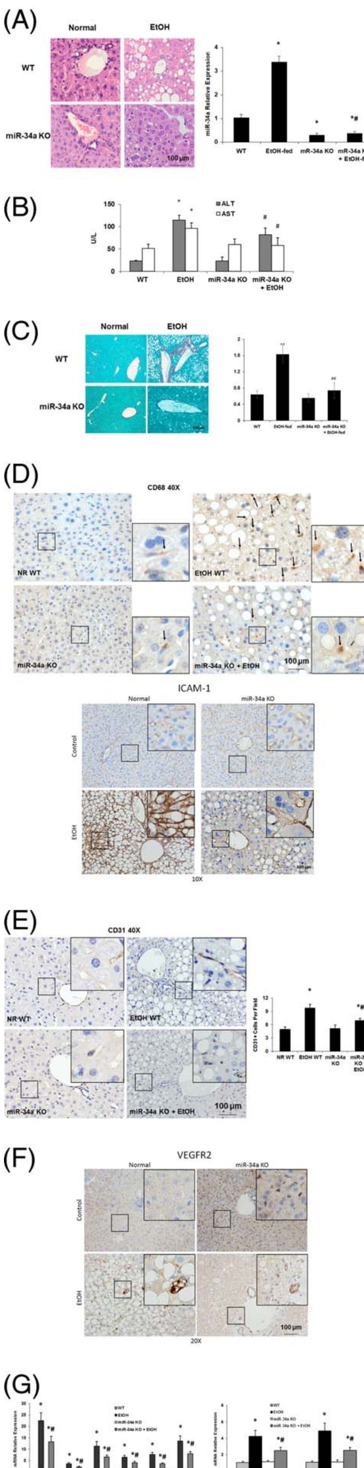

FIGURE 2 microRNA-34a (miR-34a) depletion attenuates alcohol-associated liver injury and angiogenesis in ethanol-fed mice. (A) Left: in EtOH-fed mice, liver cell swelling, irregular nucleus size, shrinkage in varying degrees, and fatty degeneration of a large number of liver cells were all observed by H&E staining. Right: miR-34a expression was assessed by Taqman real-time PCR assay in wild-type (WT) and miR-34a KO mice with ethanol treatment relative to controls (n = 5). (B) In EtOH-fed miR-34a KO mice there were decreased serum Alanine Transaminase (ALT) levels compared with WT EtOH-fed mice. (C) in EtOH-fed mice, the initiation of liver fibrosis was significantly increased by Sirius red staining, which was greatly reduced by miR-34a knockout. (D–F) macrophage infiltration markers CD68, adhesion marker ICAM1, angiogenesis markers CD31 and VEGFR2 were reduced in EtOH-fed miR-34a KO mice compared with WT EtOH-fed mice (n = 4). (G) The hepatic mRNA expressions of inflammation, angiogenesis and fibrosis markers were decreased after miR-34a knockout in ethanol-fed mice. *$p < 0.05$, relative to controls. #$p < 0.05$, relative to EtOH-fed mice. Abbreviations: ALT, alanine transaminase; KO, knockout; WT, wild-type.

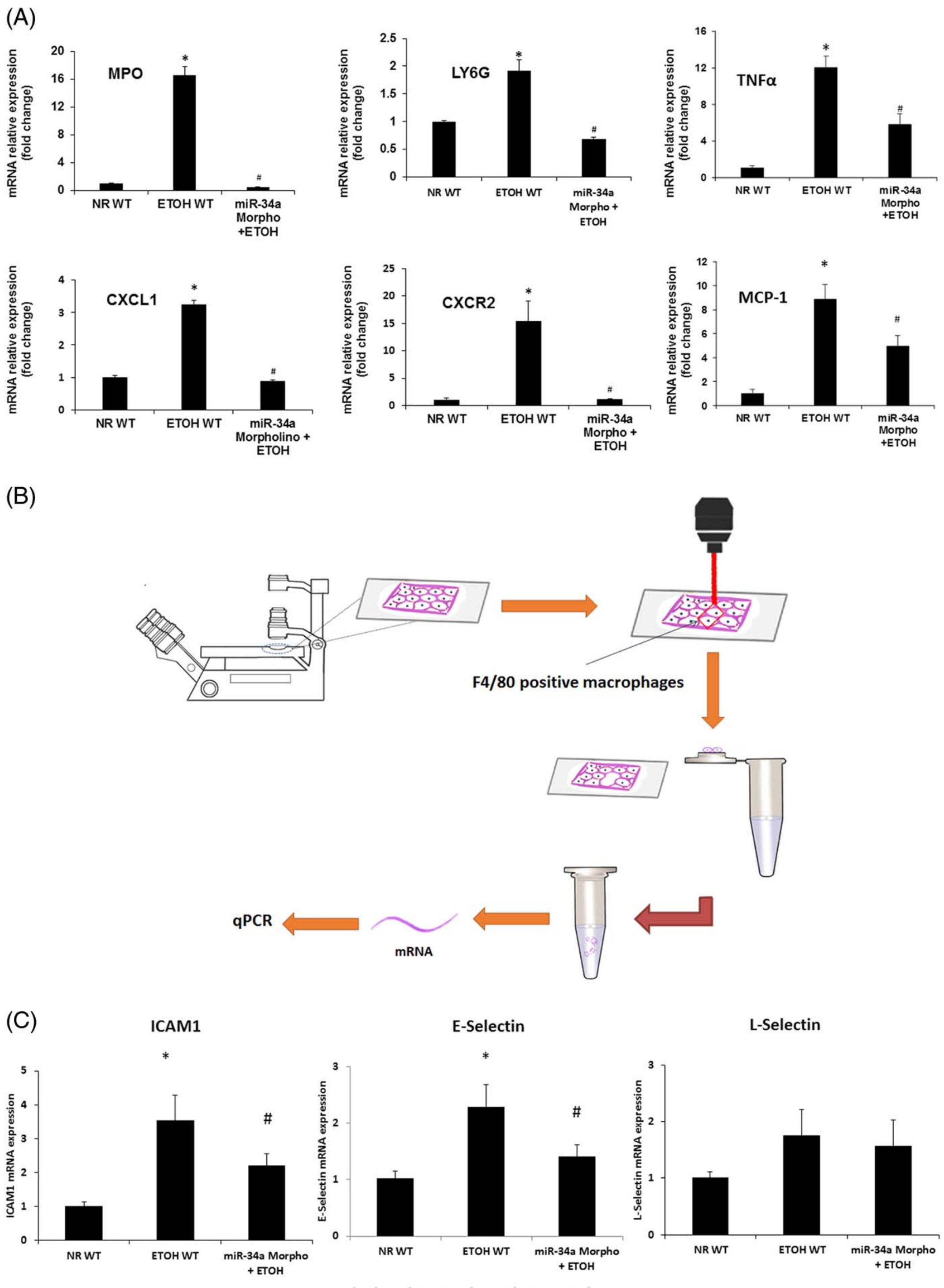

FIGURE 3 Anti-microRNA-34a Morpholino (anti-miR-34a Morpho) treatment inhibits the expressions of inflammatory cytokines during alcohol-associated liver injury. (A) Quantitative PCR assay for neutrophil marker MPO and inflammatory cytokines LY6G, CXCL1, CXCR2, MCP-1 and TNFα. (B) Schematic representation of laser capture microdissection (LCM) procedures was displayed. **(C)** The mRNA expressions of leukocyte adhesion molecules ICAM1 and E-Selectin were significantly upregulated in LCM-isolated hepatic macrophages from EtOH-fed WT mice and substantially reduced after anti-miR-34a Morpholino treatment. $n = 3$. $^{*}p < 0.05$, versus control mice; $^{\#}p < 0.05$, versus EtOH-fed mice. Abbreviations: WT, wild-type.

macrophages (Figure 4E, left) when compared with EtOH control. Meanwhile, significantly reduced expressions of inflammatory cytokines CXCL1 and LY6G were also observed after TLR4 knockout in ethanol-treated mouse liver (Figure 4D, right) and LCM-isolated macrophages (Figure 4E, right), which suggested

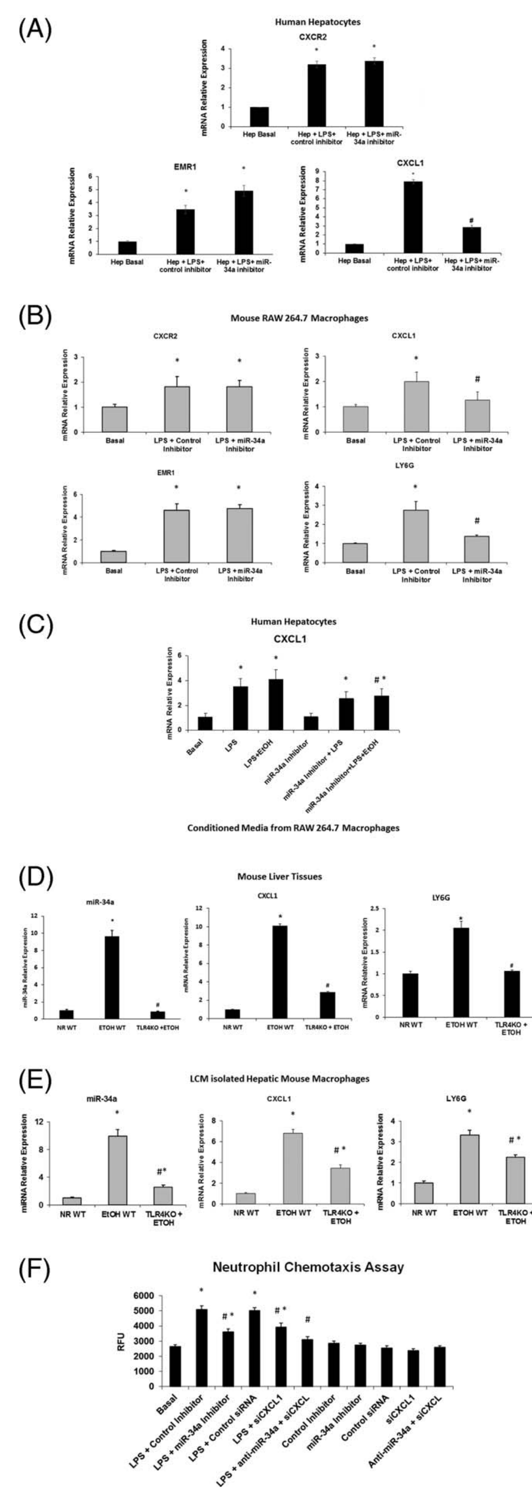

FIGURE 4 Regulation of the expression of miR-34a and inflammatory genes by LPS *in vitro* and *in vivo*. (A, B) Silencing of miR-34a decreases lipopolysaccharide (LPS)-induced CXCL1 gene expression in cultured human hepatocytes (A) and mouse RAW 264.7 macrophages (B). (C) RAW 264.7 mouse macrophages were transfected with control and anti-miR-34a inhibitors (100 nM) for 24 h and then treated with LPS (20 ng/mL) and/or ethanol (25 mM) for 24 hours. The conditioned media were collected and stimulated with normal human hepatocytes. (D, E) Knockout of TLR4 decreases the expression of miR-34a and inflammatory genes. (F) Mouse peripheral neutrophils were isolated from male C57BL/6J mice by Ficoll gradient centrifugation and real-time assay for neutrophil chemotaxis was performed in cell migration plates using a chemotaxis assay kit from Abcam Inc. *$p < 0.05$ relative to controls. #$p < 0.05$ relative to EtOH or LPS treated groups. Abbreviation: LPS, lipopolysaccharide

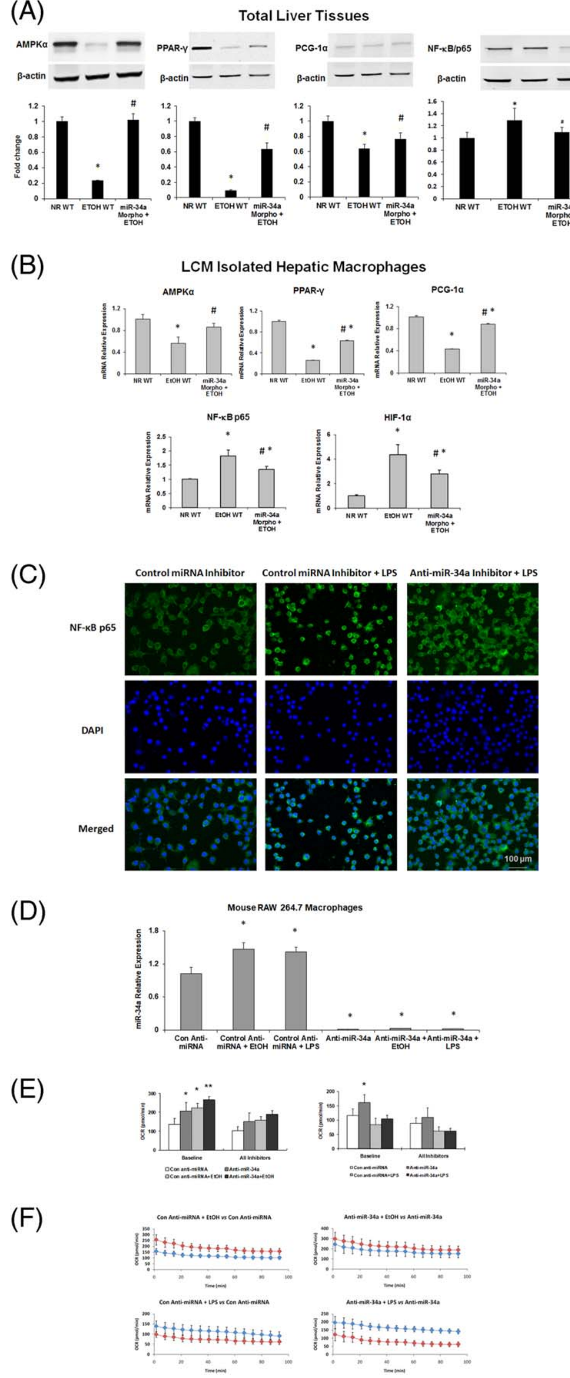

FIGURE 5 Inhibition of miR-34a results in reduced metabolic switching and inflammatory macrophage phenotypes via the inactivation of AMPK, PPARγ and PGC-1α. (A, B) Liver tissue homogenates (A) from control, EtOH and EtOH+miR-34a morpholino mice were either labeled with antibodies against AMPK, PPARγ, PGC-1α, NF-κB p65 and β-actin. β-actin was used as the internal control. Real-time PCR assays for AMPK, PPARγ, PGC-1α, NF-κB p65 and HIF-1α were also carried out in LCM-isolated mouse macrophages from the same set of mouse groups using F4/80 as the specific marker (B). (C) Inhibition of miR-34a by anti-miR-34a inhibitor transfection decreased NF-κB nuclear translocation in cultured mouse RAW 264.7 macrophages. Representative immunofluorescence pictures of NF-κB p65 in miR-34a inhibitor and control anti-miRNA inhibitor transfected RAW 264.7 cells are shown. (D–F): Seahorse extracellular flux analysis for metabolic potential and glucose dependency in the RAW 264.7 murine macrophages. Agilent Seahorse XFp Mito Fuel Flex Test kit was used with Agilent Seahorse XFp Analyzer to analyze miR-34a/Ethanol/LPS regulated the RAW 264.7 murine macrophage's metabolic potential and glucose dependency. RAW 264.7 mouse macrophages were transfected with control and anti-miR-34a inhibitors (100 nM) for 24 hours and then treated with LPS (20 ng/mL) or ethanol (25 mM) for 24 hours. Oxygen consumption rate (OCR) represents mitochondrial respiration under basal and all inhibitor conditions. (D) miR-34a expressions in LPS (20 ng/mL for 24 h) and EtOH (25 mM for 24 h) stimulated RAW 264.7 murine macrophages with anti-miR-34a inhibitor relative to control anti-miRNA inhibitor (100 nM for 24 h). (E) The metabolic differentiation and oxygen metabolism induced by miR-34a inhibition in EtOH and LPS treated mouse macrophages. (F)The RAW 264.7 murine macrophage's metabolic flux through glycolysis to mitochondrial OXPHOS. Statistical differences were calculated significant as $*p < 0.05$; $**p < 0.01$ versus Con-Anti-miRNA group. Abbreviation: LPS, lipopolysaccharide; OCR, Oxygen consumption rate.

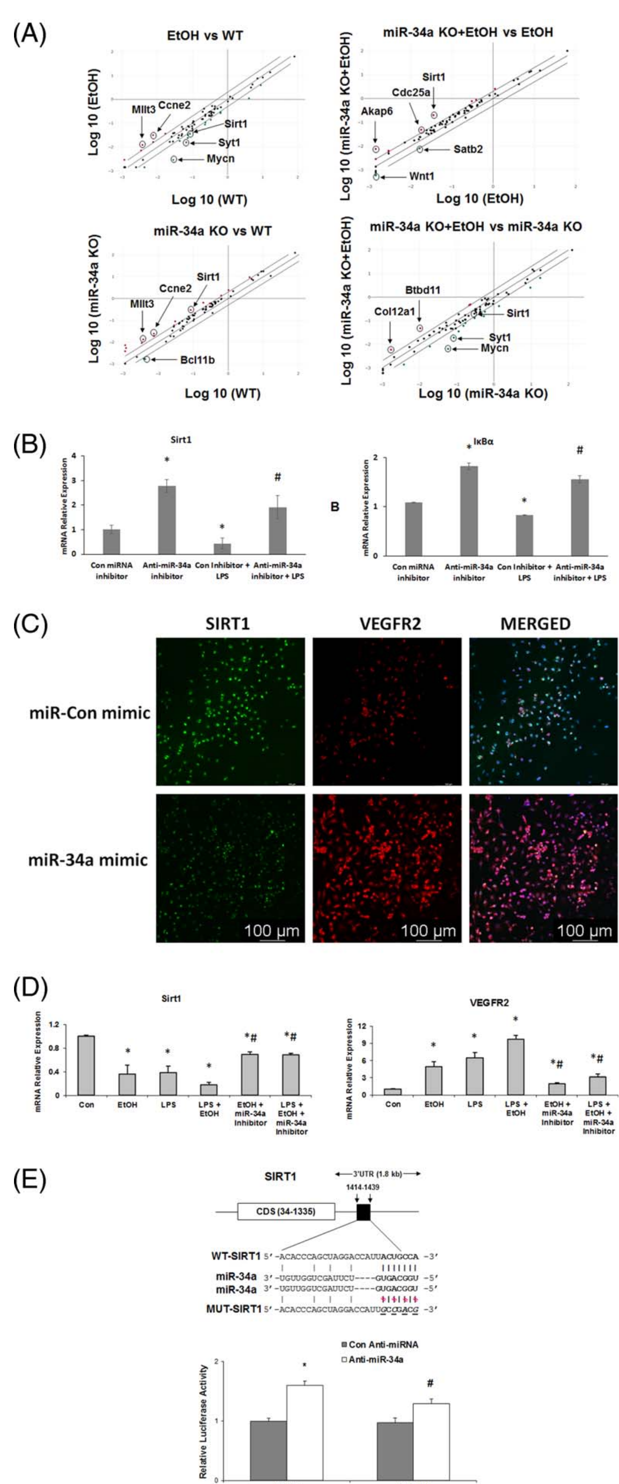

FIGURE 6 Sirt1 is the direct target gene of miR-34a during alcohol-induced liver injury. (A) The expression of a panel of diverse miR-34a target genes was evaluated by real-time PCR assay using The miR-34a Targets RT² Profiler PCR Array (PAMM-6006Z from Qiagen). SIRT1 is the only one that was downregulated in ethanol-treated mice liver and increased after miR-34a knockout with or without ethanol feeding. (B) LPS decreased mRNA expressions of SIRT1 and NF-κB inhibitor IκBα in cultured RAW 264.7 macrophages, which was reversed after anti-miR-34a inhibitor treatment. (C) Overexpression of miR-34a by miR-34a mimic transfection decreased Sirt1 expression and enhanced VEGFR2 staining in cultured RAW 264.7 macrophages. (D) Real-time PCR studies were carried out for SIRT1 and VEGFR2 in ethanol-treated mouse macrophages prior to LPS stimulation with or without miR-34a inhibition. (E) The increases in relative firefly luciferase activity in the presence of miR-34a inhibitor indicate the presence of a miR-34a modulated target sequence in the 3′-UTR of SIRT1 in macrophages ($n = 5$). *$p < 0.05$ relative to control anti-miRNA inhibitor group, # $p < 0.05$ relative to anti-miR-34a inhibitor group.

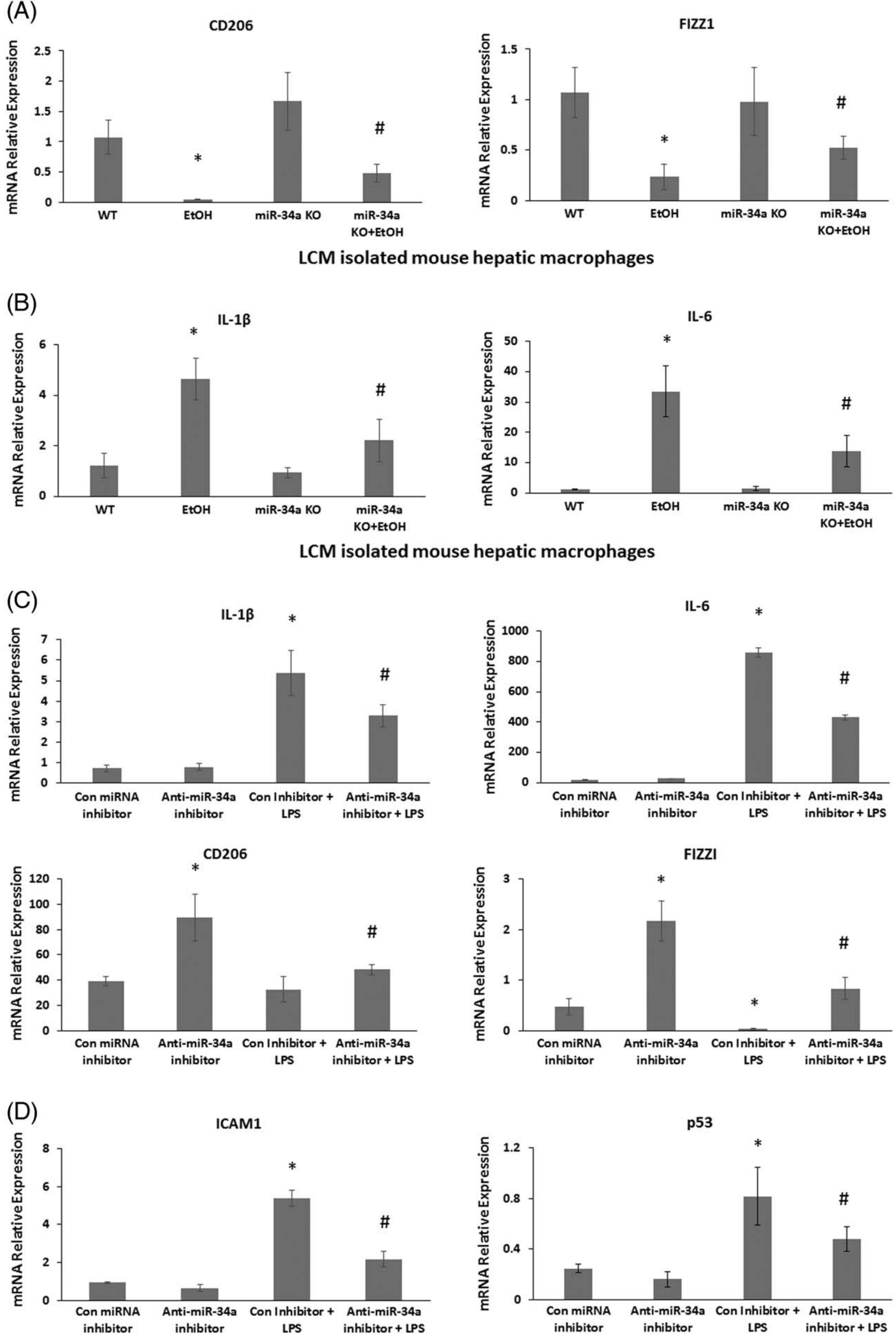

FIGURE 7 Macrophage polarization phenotype changes after miR-34a depletion during alcohol-induced liver injuries. (A, B) The LCM procedure was used to isolate F4/80-positive hepatic macrophages. The macrophage M2 markers CD206 and FIZZ1, as well as M1 markers IL-1β and IL-6 were evaluated with real-time PCR after LCM of F4/80-positive cells in liver sections. (C, D) Real-time PCR analysis confirmed reduced expressions of macrophage M1 markers IL-1β and IL-6 plus adhesion mediator ICAM1 and M2 polarization modulator p53, as well as the enhanced macrophage M2 markers CD206 and FIZZ1 in anti-miR-34a transfected murine macrophages after LPS treatment when compared with LPS controls. *$p < 0.05$, relative to WT control group; # $p < 0.05$, relative to EtOH group. Abbreviation: WT, wild-type.

inhibition of macrophage activation, which may impact neutrophil recruitment into the liver through decreased chemokine production after TLR4 depletion. Inhibition of miR-34a and silencing CXCL1 in mouse macrophages also significantly reduced their neutrophil chemotaxis activities associated with LPS stimulation (Figure 4F).

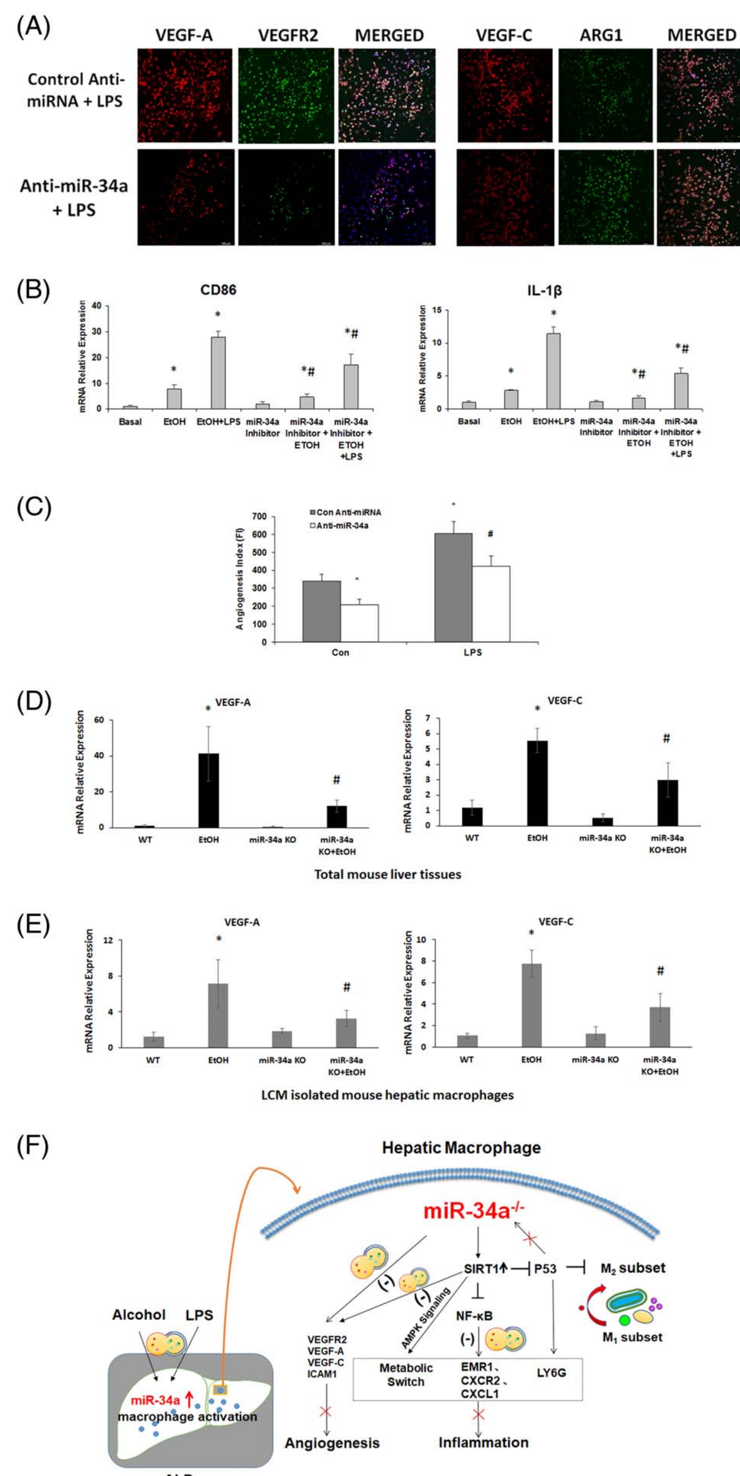

FIGURE 8 Functional miR-34a link between macrophage polarization and liver angiogenesis. (A) Immunofluorescence studies were carried out in control anti-miRNA and anti-miR-34a transfected RAW 264.7 macrophages with LPS treatment (20 ng/mL, 24 h). The expressions of angiogenesis factors VEGF-A, VEGFR2, VEGF-C, as well as the macrophage M2 markers Arginase 1 were detected by immunofluorescence staining. (B) Real-time PCR studies were carried out for prototypical proinflammatory markers CD86 and IL-1ß in ethanol-treated mouse macrophages prior to LPS stimulation with or without miR-34a inhibition. Ethanol treatment/sensitization prior to LPS significantly increased the mRNA expression of CD86 and IL-1ß which was recovered by miR-34a inhibition. *$p < 0.05$ relative to control anti-miRNA inhibitor group, #$p < 0.05$ relative to LPS and/or EtOH treatment group. (C) Anti-miR-34a inhibitor and con-anti-miRNA transfected RAW 264.7 macrophages were co-cultured with human sinusoidal endothelial cells for 24 hours. Anti-miR-34a inhibitor treated macrophages significantly reduced angiogenesis index when co-cultured with hepatic sinusoidal endothelial cells as detected by In Vitro Angiogenesis Assay from R&D Systems. (D, E) The mRNA expressions of VEGF-A and VEGF-C were significantly upregulated in ethanol-treated mice liver (C) and LCM-isolated hepatic macrophages (D), and substantially reduced after miR-34a depletion in both liver tissues and isolated hepatic macrophages. (F) Summary of miR-34a regulating macrophage-associated inflammation and angiogenesis in alcohol-induced liver injury. *$p < 0.05$ relative to WT or control anti-miRNA group; #$p < 0.05$ relative to EtOH or control anti-miRNA+LPS group. Abbreviation: Abbreviation: LPS, lipopolysaccharide; WT, wild-type.

Inhibition of miR-34a results in changes in macrophage-associated metabolic switching and inflammatory phenotypes

Macrophage phenotypes were also affected by metabolic switching through the inactivation of AMPK signaling. It has been demonstrated that AMPK acts as a central regulator of macrophage inflammatory function.[16] The expressions of key AMPK signaling mediators, AMPKα, PPARγ, and PGC-1α, were detected in miR-34a Vivo-Morpholino mice treated with ethanol *in vivo*. Liver tissue homogenates from control, EtOH, and EtOH+miR-34a morpholino mice were labeled with antibodies against AMPKα, PPARγ, PGC-1α, and β-actin. Inhibition of miR-34a results in reduced macrophage-associated metabolic switching and inflammatory phenotypes through the inactivation of AMPKα, PPARγ, and PGC-1α (Figure 5, A&B). The interactions between AMPK and its downstream target NF-κB as well as HIF-1α, the metabolic marker related to polarization, have also been investigated in anti-miR-34a morpholino-treated mice. Inhibition of miR-34a by anti-miR-34a inhibitor transfection significantly decreased NF-κB nuclear translocation in cultured mouse RAW 264.7 macrophages (Figure 5C). Expression of NF-κB/p65 and HIF-1α has also decreased in total liver and LCM-isolated macrophages from anti-miR-34a vivo morpholino-treated mice fed with ethanol, suggesting that anti-miR-34a induced AMPK activation attenuates inflammatory reaction and macrophage-associated metabolic switching through inhibiting NF-κB activation and HIF-1a transcription response (Figure 5, A&B). The metabolic assay using Agilent Seahorse XFp Mito Fuel Flex Test kit also showed the metabolic reprogramming and oxygen metabolism induced by miR-34a inhibition in ethanol-treated mouse macrophages. RAW 264.7 mouse macrophages were transfected with control and anti-miR-34a inhibitors (100 nM) for 24 hours and then treated with LPS (20 ng/mL) or ethanol (25 mM) for 24 hours. Either ethanol or LPS stimulation significantly increased miR-34a expression in cultured mouse macrophages (Figure 5D). Anti-miR-34a inhibition induces significantly increased oxygen consumption rates in RAW 264.7 murine macrophages under basal and EtOH-stimulated conditions (Figure 5, E&F). This strongly suggests that under pathological conditions, miR-34a is a driver that promotes metabolic reprogramming in macrophages by altering its target genes, resulting in either an increased proinflammatory phenotype or decreased anti-proinflammatory phenotype. Treating mouse macrophages with ethanol (25 mM for 24 h) also increased OCR (Figure 5F), suggesting altered metabolic reprogramming in macrophages with ethanol stimulation. This increase in OCR by ethanol may be due to the fact that ethanol is a nutrient whose metabolism increases metabolic fluxes to the mitochondria and remains to be elucidated. Consistent with previous studies,[17] the treatment of RAW 264.7 murine macrophages with LPS (20 ng/mL for 24 h) significantly decreased OCR (Figure 5F), suggesting that glycolysis is preferentially adopted by mouse macrophages upon activation with LPS.

miR-34a directly regulates SIRT1 expression

In general, miRNAs inhibit gene expression; therefore, we focused on genes upregulated by miR-34a knockout in ethanol-treated mouse liver. In particular, miR-34a Target PCR array analysis showed that miR-34a knockout upregulated SIRT1 mRNA expression in ethanol-treated mouse liver, and TargetScan Human database and our IPA analysis revealed that SIRT1 mRNA is a putative target of miR-34a. Additionally, among 84 miR-34a target genes, SIRT1 is the only one downregulated in ethanol-treated mice liver and increased after miR-34a knockout with or without ethanol feeding, suggesting that SIRT1 is the most important target gene of miR-34a during alcohol-induced liver injuries (Figure 6A).

To validate whether miR-34a regulates SIRT1 mRNA expression, we performed real-time PCR analysis of RAW 264.7 macrophages transfected with anti-miR-34a or anti-miR-Con with or without LPS stimulation. We confirmed the significant up-regulation of SIRT1 mRNA expression in the macrophages transfected with anti-miR-34a (Figure 6B).

To examine whether miR-34a inhibits SIRT1 expression at the protein level, we also performed immunofluorescence analyses of RAW 264.7 macrophages transfected with miR-34a mimic or miR-Con mimic. A significant downregulation of SIRT1 expression in the macrophages expressing miR-34a, along with the up-regulation of VEGFR2, was observed at the protein level (Figure 6C). Ethanol treatment/sensitization before LPS significantly altered the mRNA expression of Sirt1 and VEGFR2, which was recovered by miR-34a inhibition (Figure 6D).

To confirm that miR-34a directly regulates SIRT1 expression in cultured macrophages, we examined whether its overexpression inhibited the activity of a luciferase reporter construct containing SIRT1 3´-UTR (wild) or SIRT1 3´-UTR (mutant). Anti-miR-34a treatment significantly increased the luciferase activity of SIRT1 3´-UTR (wild) but not the SIRT1 3´-UTR (mutant), suggesting that the mutation in the seed sequence prevented the binding of miR-34a to 3´-UTR. Taken together, these data demonstrate that SIRT1 is a direct target of miR-34a in cultured macrophages (Figure 6E).

miR-34a drives M1 and inhibits M2 hepatic macrophage polarization

The role of miR-34a in the control of macrophage polarization remains to be determined. We further

determined the expression profile of M1 and M2 markers by real-time PCR in hepatic macrophages isolated from ethanol-treated miR-34a KO mice liver through LCM using F4/80 as the specific marker. Our data showed that the M1 markers IL-1β and IL-6 were significantly increased in hepatic macrophages from ethanol-treated mice related to WT controls (Figure 7A). We also found that the M2 markers studied (CD206 and FIZZ1) were detected at lower levels in macrophages from ethanol-treated mice compared to WT macrophages (Figure 7B). The opposite genotype-specific expression patterns were observed in ethanol-treated miR-34a KO mice liver relative to WT mice with ethanol treatment. Thus, further experiments were performed in cultured RAW 264.7 macrophages. We confirmed that anti-miR-34a treatment inhibited the expression of genes present in M1 macrophages and enhanced the expression of genes enriched in M2 macrophages with or without LPS stimulation (Figure 7C), along with the significantly lower level of adhesion marker ICAM1 and M2 polarization modulator p53 (Figure 7D). Our findings indicate that miR-34a is a homeostatic regulator of macrophage polarization and angiogenesis during alcohol-induced liver injuries.

miR-34a associated macrophage polarization functionally regulates the angiogenic potential of human sinusoidal endothelial cells

To demonstrate that the anti-miR-34a treatment diminishes the angiogenic potential of macrophage M1 polarization, we inhibited miR-34a in LPS-stimulated M1 macrophages. Blocking miR-34a in M1-polarized macrophages by anti-miR-34a construct, we analyzed the angiogenesis-inducing capacity of cells in comparison with control macrophages. The treatment of LPS-induced M1 macrophages with anti-miR-34a resulted in a substantial downregulation of angiogenesis mediator VEGF-A and VEGFR2 but no significant effects on the production of VEGF-C (Figure 8A). Ethanol treatment/sensitization before LPS also significantly increased the mRNA expression of prototypical proinflammatory markers CD86 and IL-1β in cultured mouse macrophages, which was recovered by miR-34a inhibition (Figure 8B). Additionally, anti-miR-34a inhibitor-treated macrophages significantly reduced angiogenesis index when co-cultured with hepatic sinusoidal endothelial cells as detected by In Vitro Angiogenesis Assay from R&D Systems (Figure 8C). Furthermore, the mRNA expressions of VEGF-A and VEGF-C were significantly upregulated in ethanol-treated mice liver and LCM-isolated hepatic macrophages but substantially reduced after miR-34a depletion in both liver tissues and isolated hepatic macrophages (Figure 8D&E). Together, these findings indicate that, within inflamed tissues in alcohol-induced liver injuries, the polarization of macrophages is miR-34a dependent, leading to pro-angiogenic events occurring in ALD.

DISCUSSION

ALD encompasses the liver manifestations of alcohol overconsumption, including fatty liver, alcoholic hepatitis, and chronic hepatitis with liver fibrosis or cirrhosis.[18,19] The current study, focusing on the functional roles of miR-34a in macrophage infiltration, activation, and angiogenesis, demonstrated that miR-34a deficiency had anti-inflammatory effects in alcohol-associated liver injury by activating SIRT1 signalling and macrophage-associated angiogenesis. Functions of miR-34a may differ depending on liver diseases, animal models, or the kinds of liver cells.[20] Similarly, acute attenuation of angiogenesis is good for ALD but may have dire consequences in the future. Since hepatic cells communicate with each other and orchestrate to maintain liver homeostasis during liver injury, miR-34a could play a vital role only in specific liver cells, such as hepatic macrophages and endothelial cells.

Macrophages are heterogeneous, and the balance of proinflammatory and anti-inflammatory macrophages may be critical for liver conditions.[21] Balb/c mice have dominant anti-inflammatory macrophage subsets and show better liver conditions in NASH feeding models compared to proinflammatory macrophage-dominant C57BL/6 mice.[22] Proinflammatory macrophages express genes involved in the initiation of angiogenesis,[23] including VEGF-A and FGF2, whereas the acquisition of high angiogenesis-inducing capacity by human and murine macrophages requires their polarization toward the anti-inflammatory macrophage phenotype.[24] Although our study demonstrated the therapeutic effects of inhibiting macrophage activation as proinflammatory macrophage subsets leading to attenuated cytokine production, the induction of macrophage polarization as anti-inflammatory macrophage subsets may be another strategy for novel treatments for liver injury. In addition, there are not only proinflammatory and anti-inflammatory macrophages but also other phenotypes for macrophage subsets. Anti-inflammatory macrophages have several subclasses.[25] Although we performed RT-PCR for F4/80$^+$ macrophages isolated by laser capture microdissection, F4/80$^+$ macrophages have subclasses; CD11b$^-$CD68$^+$, CD11b$^+$CD68$^-$, and CD11b$^+$CD68$^+$.[26] Hepatic F4/80$^+$ macrophages are Kupffer cells or BMDMs. Functions of macrophages may differ depending on their phenotypes or origins, and the effects of miR-34a inhibition could be varied in different subsets/subclasses/origins. Macrophages are important in all phases of the adult wound healing process, which includes inflammation, proliferation, and remodelling. Different subsets of macrophages have been suggested to exist related to anti-inflammatory versus pro-resolution during wound healing. During this process and as wounds heal, the local macrophage population transitions from predominantly

proinflammatory macrophage phenotypes to anti-inflammatory macrophage phenotypes.[27] Other markers, including IL-10, TGF-beta, and PGDF, could differentiate if miR-34a is driving which subtypes of macrophages exist in the liver during alcohol-induced liver injury. Further studies are required by cell sorting using multiple antibodies to elucidate the roles of miR-34a in different macrophage phenotypes.

Multiple cell types orchestrate the pathogenesis of ALD. The current study has demonstrated that miR-34a KO or morpholino inhibition in macrophages as well as hepatocytes show less cytokine production during ALD. LPS or cytokines such as IL-6 can activate hepatocytes and induce cell proliferation and angiogenesis.[28] Angiogenesis is a major contributor to liver complications during ALD, including portal hypertension, portal-systemic collaterals, and hyperdynamic splanchnic circulation. Our study has demonstrated decreased infiltration and cytokine production of neutrophils in miR-34a KO mice with alcohol-associated liver injury. It is known that macrophages and neutrophils cooperate to repair various tissue damages.[29,30] A previous study has shown that inhibition of miR-34a induces apoptosis and blocks the infiltration of neutrophils.[31] These previous studies and our findings suggest that hepatic cells orchestrate and contribute to liver inflammation and vascular remodelling through the expression of cytokines, and depletion of miR-34a inhibits these inflammatory responses and angiogenesis. Macrophages display various phenotypes, for example, proinflammatory activation versus pro-resolution, in different tissues under different pathophysiological states. To further validate the role of miR-34a in driving changes in macrophage phenotype, we performed in vitro assays to examine the direct effect of altering miR-34a on macrophage energy metabolism that is shown to critically regulate macrophage activation. As shown in Figure 5, knocking down miR-34a in cultured macrophages caused significant increases in oxygen consumption rates under both basal and EtOH-stimulated conditions. This strongly indicates that miR-34a is a driver, which functions by altering its target genes to promote metabolic reprograming in macrophages in a manner resulting in increased anti-inflammatory or decreased proinflammatory phenotype.

In conclusion, the current study demonstrates that miR-34a deficiency inhibits the activation of hepatic macrophages and cytokine production as well as macrophage and neutrophil infiltration, leading to attenuated angiogenesis and liver inflammation (Figure 8E). Inhibition of miR-34a could be a novel therapeutic approach to protect the liver from inflammation and the initiation of fibrosis.

AUTHOR CONTRIBUTIONS

Ying Wan, Elise Slevin, Chiung-Kuei Huang, Hidekazu Tsukamoto, and Fanyin Meng conceived and designed the research; Xuedong Li, Kelly Harrison, Sugeily Ramos Lorenzo, Tian Li, Yudian Zhang, and Wenjuan Xu performed the experiments; Ying Wan, Elise Slevin, Sachiko Koyama, Bingru Zhou, Tian Li, and Fanyin Meng analyzed the data; Chaodong Wu, Ashok K Shetty, Hidekazu Tsukamoto, and Fanyin Meng interpreted results of the experiments; Ying Wan, Elise Slevin, and Fanyin Meng prepared the figures; Ying Wan, Chiung-Kuei Huang, Hidekazu Tsukamoto, and Fanyin Meng drafted the manuscript; James E. Klaunig, Chiung-Kuei Huang, Sachiko Koyama, Chaodong Wu, James E. Klaunig, Hidekazu Tsukamoto, and Fanyin Meng edited the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to report.

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REFERENCES

1. Yang Y, Sangwung P, Kondo R, Jung Y, McConnell MJ, Jeong J, et al. Alcohol-induced Hsp90 acetylation is a novel driver of liver sinusoidal endothelial dysfunction and alcohol-related liver disease. J Hepatol. 2021;75:377–86.
2. Gao B, Bataller R. Alcoholic liver disease: pathogenesis and new therapeutic targets. Gastroenterology. 2011;141:1572–85.
3. Bhattacharyya A, Chattopadhyay R, Mitra S, Crowe SE. Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. Physiol Rev. 2014;94:329–54.
4. Park JK, Shao M, Kim MY, Baik SK, Cho MY, Utsumi T, et al. An endoplasmic reticulum protein, Nogo-B, facilitates alcoholic liver disease through regulation of kupffer cell polarization. Hepatology. 2017;65:1720–34.
5. Gomes AQ, Nolasco S, Soares H. Non-coding RNAs: multi-tasking molecules in the cell. Int J Mol Sci. 2013;14:16010–39.
6. Navarro F, Lieberman J. miR-34 and p53: New Insights into a Complex Functional Relationship. PLoS One. 2015;10:e0132767.
7. Song L, Chen TY, Zhao XJ, Xu Q, Jiao RQ, Li JM, et al. Pterostilbene prevents hepatocyte epithelial-mesenchymal transition in fructose-induced liver fibrosis through suppressing miR-34a/Sirt1/p53 and TGF-beta1/Smads signalling. Br J Pharmacol. 2019;176:1619–34.
8. Ramirez-Pedraza M, Fernandez M. Interplay Between Macrophages and Angiogenesis: A Double-Edged Sword in Liver Disease. Front Immunol. 2019;10:2882.
9. Freyer L, Lallemand Y, Dardenne P, Sommer A, Biton A, Gomez Perdiguero E. Erythro-myeloid progenitor origin of Hofbauer cells in the early mouse placenta. Development. 2022;149:dev200104.
10. Pan Y, Hui X, Hoo RLC, Ye D, Chan CYC, Feng T, et al. Adipocyte-secreted exosomal microRNA-34a inhibits M2 macrophage polarization to promote obesity-induced adipose inflammation. J Clin Invest. 2019;129:834–49.
11. Tsukamoto H, Mkrtchyan H, Dynnyk A. Intragastric ethanol infusion model in rodents. Methods Mol Biol. 2008;447:33–48.
12. Xiong S, She H, Zhang AS, Wang J, Mkrtchyan H, Dynnyk A, et al. Hepatic macrophage iron aggravates experimental alcoholic steatohepatitis. Am J Physiol Gastrointest Liver Physiol. 2008;295:G512–21.
13. Wu N, McDaniel K, Zhou T, Ramos-Lorenzo S, Wu C, Huang L, et al. Knockout of microRNA-21 attenuates alcoholic hepatitis through the VHL/NF-kappaB signaling pathway in hepatic stellate cells. Am J Physiol Gastrointest Liver Physiol. 2018;315:G385–98.
14. Wan Y, McDaniel K, Wu N, Ramos-Lorenzo S, Glaser T, Venter J, et al. Regulation of Cellular Senescence by miR-34a in Alcoholic Liver Injury. Am J Pathol. 2017;187:2788–98.
15. Meng F, Glaser SS, Francis H, Yang F, Han Y, Stokes A, et al. Epigenetic regulation of miR-34a expression in alcoholic liver injury. Am J Pathol. 2012;181:804–17.
16. Zhang M, Zhu H, Ding Y, Liu Z, Cai Z, Zou MH. AMP-activated protein kinase alpha1 promotes atherogenesis by increasing monocyte-to-macrophage differentiation. J Biol Chem. 2017;292:7888–903.
17. Vijayan V, Pradhan P, Braud L, Fuchs HR, Gueler F, Motterlini R, et al. Human and murine macrophages exhibit differential metabolic responses to lipopolysaccharide - A divergent role for glycolysis. Redox Biol. 2019;22:101147.
18. Mandrekar P, Ambade A, Lim A, Szabo G, Catalano D. An essential role for monocyte chemoattractant protein-1 in alcoholic liver injury: regulation of proinflammatory cytokines and hepatic steatosis in mice. Hepatology. 2011;54:2185–97.
19. King JA, Nephew BC, Choudhury A, Poirier GL, Lim A, Mandrekar P. Chronic alcohol-induced liver injury correlates with memory deficits: Role for neuroinflammation. Alcohol. 2020;83:75–81.
20. Escutia-Gutierrez R, Rodriguez-Sanabria JS, Monraz-Mendez CA, Garcia-Banuelos J, Santos-Garcia A, Sandoval-Rodriguez A, Armendariz-Borunda J. Pirfenidone modifies hepatic miRNAs expression in a model of MAFLD/NASH. Sci Rep. 2021;11:11709.
21. Fulham MA, Ratna A, Gerstein RM, Kurt-Jones EA, Mandrekar P. Alcohol-induced adipose tissue macrophage phenotypic switching is independent of myeloid Toll-like receptor 4 expression. Am J Physiol Cell Physiol. 2019;317:C687–700.
22. Maina V, Sutti S, Locatelli I, Vidali M, Mombello C, Bozzola C, et al. Bias in macrophage activation pattern influences non-alcoholic steatohepatitis (NASH) in mice. Clin Sci. 2012;122:545–53.
23. Spiller KL, Anfang RR, Spiller KJ, Ng J, Nakazawa KR, Daulton JW, et al. The role of macrophage phenotype in vascularization of tissue engineering scaffolds. Biomaterials. 2014;35:4477–88.
24. Zajac E, Schweighofer B, Kupriyanova TA, Juncker-Jensen A, Minder P, Quigley JP, et al. Angiogenic capacity of M1- and M2-polarized macrophages is determined by the levels of TIMP-1 complexed with their secreted proMMP-9. Blood. 2013;122:4054–67.
25. Orecchioni M, Ghosheh Y, Pramod AB, Ley K. Macrophage polarization: Different gene signatures in M1(LPS+) vs. classically and M2(LPS-) vs. alternatively activated macrophages. Front Immunol. 2019;10:1084.
26. Kinoshita M, Uchida T, Sato A, Nakashima M, Nakashima H, Shono S, et al. Characterization of two F4/80-positive Kupffer cell subsets by their function and phenotype in mice. J Hepatol. 2010;53:903–10.
27. Krzyszczyk P, Schloss R, Palmer A, Berthiaume F. The Role of Macrophages in Acute and Chronic Wound Healing and Interventions to Promote Pro-wound Healing Phenotypes. Front Physiol. 2018;9:419.
28. Sato K, Meng F, Venter J, Giang T, Glaser S, Alpini G. The role of the secretin/secretin receptor axis in inflammatory cholangiocyte communication via extracellular vesicles. Sci Rep. 2017;7:11183.
29. Butterfield TA, Best TM, Merrick MA. The dual roles of neutrophils and macrophages in inflammation: a critical balance between tissue damage and repair. J Athl Train. 2006;41:457–65.
30. Bian Z, Guo Y, Ha B, Zen K, Liu Y. Regulation of the inflammatory response: enhancing neutrophil infiltration under chronic inflammatory conditions. J Immunol. 2012;188:844–53.
31. Syed M, Das P, Pawar A, Aghai ZH, Kaskinen A, Zhuang ZW, et al. Hyperoxia causes miR-34a-mediated injury via angiopoietin-1 in neonatal lungs. Nat Commun. 2017;8:1173.

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