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Method to Regulate Monocyte Function by Silencing HIF-1α mRNA in a Model of Retinal Neovascularization

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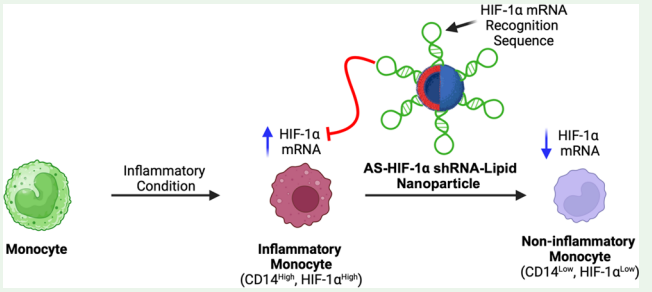
ABSTRACT: Circulating monocytes migrate into the retina in response to inflammation and neovascularization. Furthermore, under inflammatory conditions such as diabetes, healthy monocytes become activated in the circulation. However, the contribution of activated monocytes to neovascularization is largely unknown. HIF-1α has been shown to contribute to the pathogenesis of neovascularization. We describe here the synthesis of a hybrid nanomaterial for targeted delivery and gene silencing in activated monocytes that are associated with pathological neovascularization. To test the gene silencing ability of AS-shRNA-lipids in vitro, we used the probe to inhibit HIF-1α mRNA induced in mouse monocytes by exposing them to hypoxia. In addition, we tested AS-shRNA-lipids for inhibition of neovascularization in vivo using the mouse model of oxygen-induced retinopathy (OIR). Significant reduction of neovascularization was achieved in mouse OIR by targeting activated monocytes using intraperitoneal injections of AS-shRNA-lipids. Expression of HIF-1α and CD14 mRNA were both inhibited in circulating cells, suggesting normalization of the activated monocytes in P17 OIR animals treated with AS-shRNA-lipids. We hypothesized that inhibition of HIF-1α mRNA in activated monocytes may have a direct impact on VEGF expression in the retinal tissues in vivo. We observed that VEGF mRNA expression was inhibited in P17 retinal tissues after systemic treatment with HIF-1α-targeted AS-shRNA-lipids. These findings may provide a framework for a strategy to inhibit retinal neovascularization by targeting circulating activated monocytes.



KEYWORDS: *targeted delivery, activated monocytes, gene therapy, short hairpin RNA, neovascularization*

INTRODUCTION

Neovascularization is a hallmark characteristic of many types of retinopathies, including retinopathy of prematurity (ROP), age-related macular degeneration (AMD), and proliferative diabetic retinopathy (PDR).[1] Retinal neovascularization involves the pathological proliferation of endothelial cells from existing blood vessels in the retina. In contrast to existing healthy vasculature, abnormal neovascular structures lack essential tight junction proteins and are therefore prone to hemorrhaging into surrounding tissues, including the vitreous.[2,3] The resultant structural strain on the retina often leads to vision loss. Additionally, neovascularization is often accompanied by neuroinflammation, which triggers the migration of bone-marrow-derived activated monocytes and progenitor cells to the retina.[4,5] However, the contribution of these migratory cells to the pathological progression of neovascularization remains largely unknown.

Oxygen-induced retinopathy (OIR) is a reliable mouse model to study neovascularization in the retina. In this model, mouse pups are exposed to a hyperoxic environment consisting of 75% oxygen from postnatal day 7 (P7) until postnatal day 12 (P12).[6−8] This hyperoxic treatment causes the regression of capillary blood vessels at the central retina in the mouse pups. Upon return to normal room air oxygen (i.e., normoxia) at P12, the central avascular retina experiences hypoxia, leading to the proliferation of endothelial cells from existing blood vessels that may lead to neovascularization.[6,9,10] Studies have demonstrated that the hypoxia-inducible factor-1α (HIF-1α) plays a central role in the pathogenesis of neovascularization.[11−13] Thus, there has been a focus on developing therapies that stabilize HIF-1α as a means to protect capillary blood vessel from high-oxygen-induced vascular regression and thus protect from subsequent hypoxia-derived neovascularization.[12] However, the contribution of bone-marrow-derived activated monocytes to neovascularization remains largely unknown. We observed that HIF-1α mRNA is overexpressed in activated monocytes from OIR blood samples.[4] Thus, we hypothesized that targeted delivery and HIF-1α mRNA inhibition in these activated monocytes using a newly designed hybrid oligonu-

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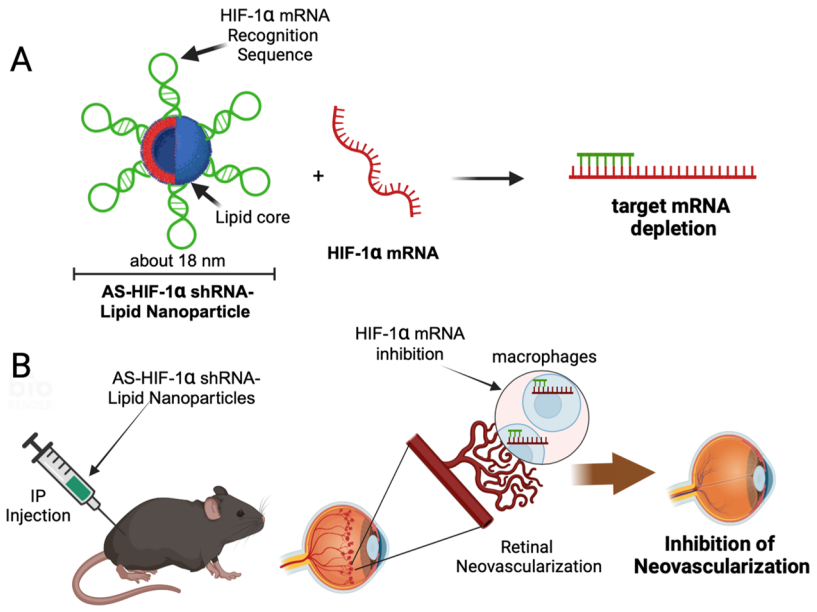

Figure 1. Schematic drawing and hybridization motif of shRNA-lipid conjugates. (A) Design and synthesis of HIF-1α mRNA-targeted antisense shRNA-lipid conjugates (AS-HIF-1α-shRNA-lipid) for targeted delivery and gene silencing in vivo. AS-shRNA-lipid conjugates are designed for transfection-free delivery of shRNA to activated monocytes and inhibition of HIF-1α mRNA. AS-shRNA-lipid conjugates are designed by incorporating an antisense sequence complementary to HIF-1α mRNA and are stabilized using 2′-MeO nucleotides. A nonsense conjugate (NS-shRNA-lipid) was also designed from scrambled sequence of the HIF-1α mRNA recognition sequence. Physical properties of the freshly conjugated shRNA-lipids were analyzed using transmission electron microscopy (TEM) and dynamic light scattering (DLS) measurements as shown in Figure S2. (B) After systemic injections (intraperitoneally), the AS-shRNA-lipid allows efficient internalization of the conjugates by activated monocytes. Upon hybridization to the target sequence, depletion of target mRNA leads to inhibition of retinal neovascularization.

cleotide conjugate could inhibit neovascularization in the OIR retina. These hybrid shRNA-lipid conjugates were synthesized by conjugating diacyl-lipids to antisense short hairpin RNA with an antisense sequence complementary to HIF-1α mRNA. The HIF-1α-targeted AS-shRNA-lipids are able to hybridize with HIF-1α mRNA in vivo.[4] In addition, AS-shRNA-lipids are highly sensitive to hybridize to the target sequence and are also capable of detecting single-mismatch in the target RNA.[4,5] We believe that our newly synthesized AS-shRNA-lipids will introduce a novel method for the targeted delivery and regulation of activated monocyte functions in retinal neovascularization. Current gene delivery methods, including AAV-viral vectors, require intraocular injections of the construct for gene delivery.[14] Though the AAV-viral vector-mediated gene delivery method provides genetic stability and high gene transduction, this method has several limitations including vector-induced immunogenicity, genotoxicity, narrow selectivity, and off-target mutations. We designed and synthesized AS-shRNA-lipid nanoparticles for transfection-free gene delivery to activated monocytes to overcome these limitations. In addition, the shRNA-lipid-derived gene delivery method is designed for systemic injections. Systemic injection offers less trauma to the patient, which translates into greater patient compliance without the risk of ophthalmitis associated with intravitreal injections. Herein, we report our results.

MATERIALS AND METHODS

Reagents. All reagents were used as received from the company unless noted otherwise. Antibodies used include the following: DyLight 649-conjugated GSL I Isolectin B4 (Catalog No. DL-1208, Vector Laboratories), IBA-1 (Catalog No. AB178847, Abcam), and Alexa Fluor 488 Donkey antirabbit IgG secondary antibody (Catalog No. A21206, Thermo Fisher Scientific). Chemical reagents used in shRNA modification include dibenzocyclooctyne-PEG4-*N*-hydroxy-succinimidyl ester (Catalog No. 764019, Sigma-Aldrich), simethyl sulfoxide or DMSO (Catalog No. D8418, Sigma-Aldrich), diisopropylethylamine or DIPEA (Catalog No. 496219, Sigma-Aldrich), PBS (Catalog No. 10010023, Life Technologies Corporation), and DSPE-PEG2000-azide (Catalog No. 880228, Avanti Polar Lipids). Primary mouse bone marrow monocytes (Catalog No. C57−6271F) and complete leukocyte medium (Catalog No. M3366) were purchased from CellBiologics (Chicago, IL).

Design and Synthesis of AS-ShRNA-Lipids. HIF-1α mRNA-targeted or nonsense short hairpin RNA (shRNA) oligonucleotides were designed computationally, and BLAST search was used to verify no significant overlap with any other mouse mRNA transcripts. The antisense and the nonsense sequences are located within the loop of the hairpin structure as shown in Figure 1. A self-complementary sequence was incorporated to form the stem of the shRNA hairpin. The sequences were synthesized and purified by Biosearch Technologies (Petaluma, CA). The antisense and nonsense shRNA were then conjugated to the diacyl-lipid according to our previously described method with slight modification.[4] Briefly, amine-functionalized shRNA was reacted with 10-fold molar excess of dibenzocyclooctyne-PEG4-*N*-hydroxy-succinimidyl ester (DBCO-PEG4-NHS) predissolved at 25 mM in DMSO. The reaction was carried out overnight at room temperature at a 1 mM oligonucleotide concentration in 30% DMSO and 70% PBS with 8 mM diisopropylethylamine (DIPEA). The product was then diluted 3-fold in water, and the excess reagents were removed by centrifugal filtration using a 3 kDa Amicon Ultra-0.5 Centrifugal Filter Unit (UFC5003Millipore), washed twice using PBS, and then reacted with 5-fold molar excess of DSPE-PEG2000-azide for 24 h at a 0.1 mM oligonucleotide concentration in 50% methanol and 50% water. The sample was diluted in water and purified using a 10 kDa Amicon Ultra-0.5 Centrifugal Filter Unit (Catalog No. UFC5010Millipore). The pure conjugate was collected and diluted in PBS for characterization and in vitro and in vivo applications. Molecular weight was confirmed using a Voyager STR-DE MALDI-TOF-MS (Applied Biosciences). The freshly conjugated lipid-shRNA was stored at 4 °C until used.

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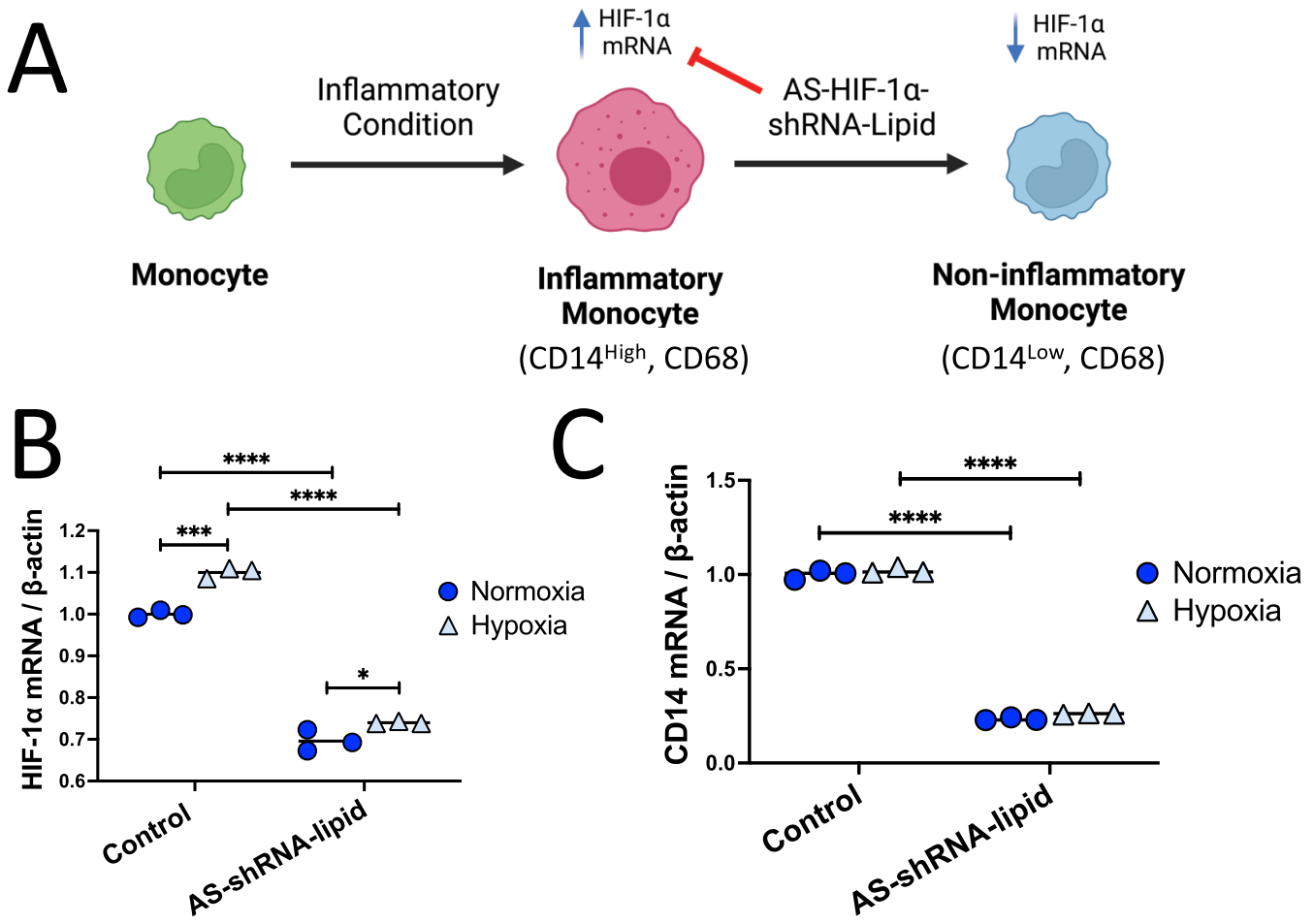

Figure 2. Monocyte activation and inhibition of HIF-1α mRNA. (A) Graphical representation of monocyte activation and HIF-1α mRNA inhibition using AS-shRNA-lipids. (B) HIF-1α mRNA was induced in monocytes by treating under hypoxic conditions. Expression of HIF-1α mRNA was inhibited significantly using AS-shRNA-lipids. (C) Under hypoxic conditions, monocytes get activated as monitored by CD14 expression and normalized using AS-shRNA-lipids. Monocyte cultures were treated with PMA for 78 h to induce activation. Next, AS-shRNA-lipid was added and cells were incubated for 4 h at 1% oxygen (hypoxia) and 21% oxygen (normoxia). qPCR data analysis showed both HIF-1α and CD14 expressions were significantly decreased in AS-shRNA-lipid-treated monocytes compared to PBS control. Hypoxia has little effect on CD14 expression in cultured monocytes. These data repetitive of three independent experiments with n = 3 for each samples size for statistical analysis.

Mouse OIR Model. All animal procedures performed in this study were approved by the Vanderbilt University Institutional Animal Care and Use Committee and conducted in line with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research. Multi-timed pregnant C57BL/6 mice were obtained from Charles River Laboratories. Dams and pups were placed in a 75% oxygen chamber from postnatal day 7 (P7) to postnatal day 12 (P12). On P12, animals were removed from the hyperoxic environment and kept under normal oxygen conditions and remained under normoxia until sacrificed on P17.

Treatment Groups. During hyperoxic treatment, mice pups were intraperitoneally injected with either 10 mg/kg antisense or nonsense shRNA-lipid conjugate in sterile PBS on days 12, 13, and 15 after hyperoxic treatment (P12, 13, and 15). Control group received PBS via intraperitoneal injections in the OIR mice. Tissues were harvested on P17.

Collection of Mice Retina and PBMCs. Tissues were harvested from all treatment groups on P17 and fixed overnight at 4 °C in 10% neutral buffered formalin (Catalog No. R04586−76, EMD Millipore). Retinal tissues were dissected, and mRNA was extracted using mini RNeasy kit (Catalog No. 74104, Qiagen). cDNA synthesis was performed using High Capacity cDNA Reverse Transcription Kit (Catalog No. 4368814, Thermo Fisher Scientific) according to the manufacturer's protocol, with the MiniAMP Thermal Cycler (Catalog No. A37834, Thermo Fisher Scientific). Cycling parameters are as follows: 50 μL sample, 105 °C cover, 25 °C for 10 min, 37 °C for 2 h, 85 °C for 5 min, and 4 °C until sample collection. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using QuantStudio 3 (Catalog No. #A28567, Thermo Fisher Scientific) with the following primers: β-actin control (Catalog No. 4351315, Life technologies), HIF-1α (Catalog No. Mm80468869, Thermo Fisher Scientific), and CD14 (Mm01158466, Thermo Fisher Scientific). In addition, blood samples were collected from the mice in each group. Blood samples were collected, added to BD Vacutainer Plastic Blood Collection Tubes with Sodium Citrate (Catalog No. 366560, BD), gently layered onto 3 mL of Ficoll-Paque Premium sterile solution (Catalog No. 17−5442−02, GE Healthcare), and centrifuged at 400g for 35 min at room temperature. The PBMC was isolated by density gradient centrifugation using Hank's Balanced Salt Solution (Catalog No., 14025, Gibco), centrifuged at 100g for 10 min at room temperature. Cells were resuspended again in Hank's Balanced salt solution and centrifuged at 500 × g for 10 min. The supernatant was removed, and cells were resuspended in 1 mL of Hank's Balanced salt solution. Cells were counted using trypan blue and centrifuged again. RNA was extracted, cDNA was synthesized, and qRT-PCR was performed as described above.

Immunostaining of Mice Retinal Tissues. At P17, mice from all groups were euthanized and enucleated, and eyeballs were fixed in 10% neutral buffered formalin (NBF). Retinas were dissected and

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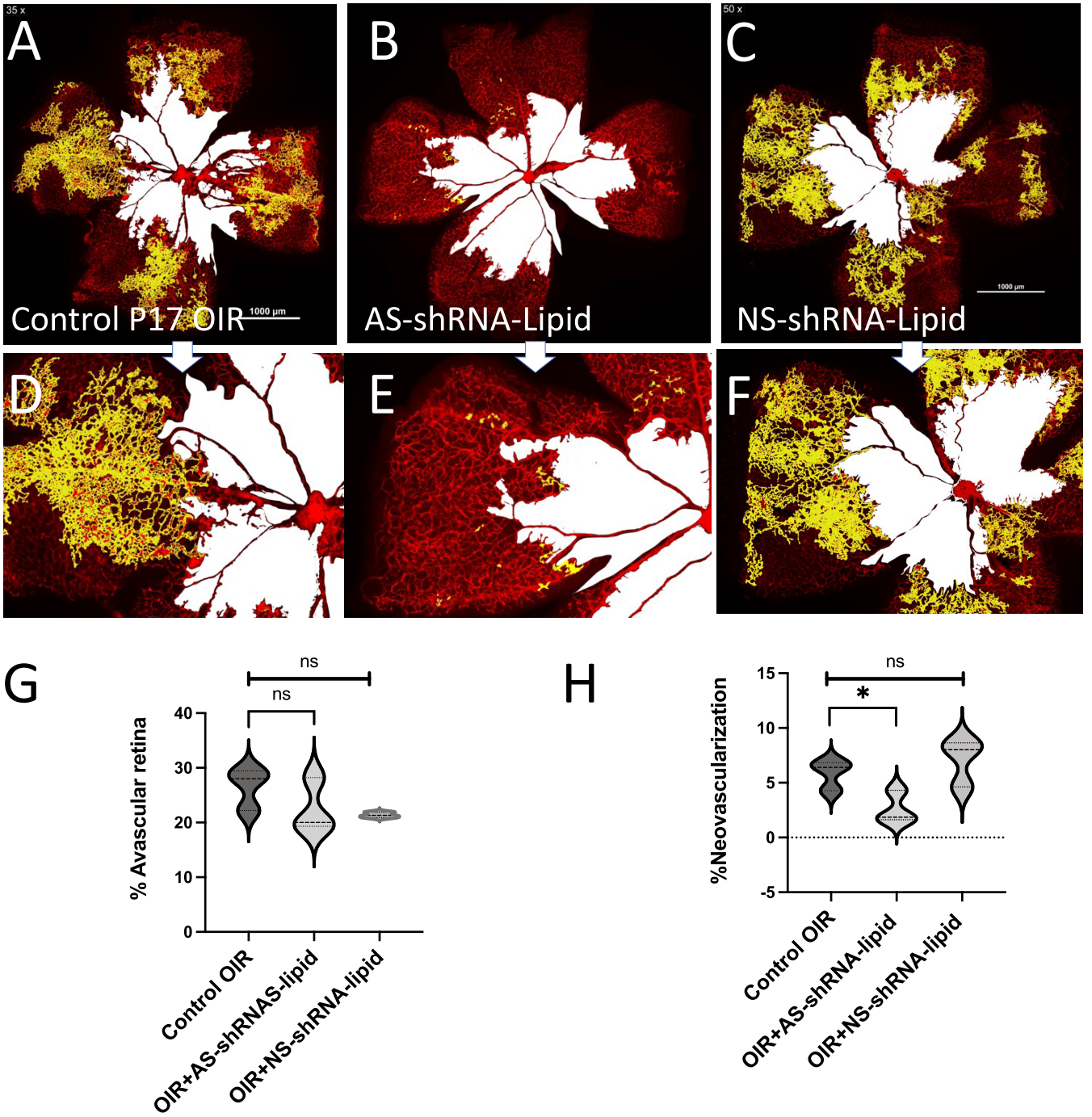

Figure 3. Quantification of avascular and neovascular areas in P17 mouse OIR retinas after inhibition of HIF-1α mRNA in activated monocytes via intraperitoneal injections of shRNA-lipids. The OIR pups were injected with AS(NS)-shRNA-lipid on P12, P13, and P15 posthyperoxic treatment, and tissues were collected at P17. (A−C) Retinal tissues were stained with IB4 to analyze % avascular areas (white) and % NV areas (yellow). Panels (D−F) are zoomed images of panels (A−C), respectively. (G, H) The AS-shRNA-lipid significantly inhibited (over 90%) NV areas compared to controls, though changes in avascular areas were minimal. For each group, a total of 12 pups were used.

blocked/permeabilized in a solution containing wash buffer (TBS, 0.05% sodium azide, 0.33% Tween 20, and 0.0033% Triton-X), 1% bovine serum albumin (BSA), 10% donkey serum, and 0.2% fish gelatin, for 2 h at room temperature. After blocking, the retinas were incubated with IBA-1 (dilution 1:100) overnight at 4 °C. Then, tissues were stained with DyLight 649-conjugated GSL I Isolectin B4 (dilution 1:100) and Donkey antirabbit IgG secondary antibody (dilution 1:100) for two h at room temperature. The tissues were then mounted with Prolong Gold Mounting medium with DAPI (Catalog No. P36931, Life Technologies Corp.). Images were taken using an epifluorescence Nikon Eclipse TiE inverted microscope.

Analysis of Retina Avascular and Neovascular Areas. Retinal flat mount images were analyzed using Adobe Photoshop, following the protocol outlined by Connor et al.[15] To determine the % avascular area, the total vascular area of the retina and avascular area were measured by tracing using the Polygonal Lasso tool. The pixel value for the avascular area was divided by the value for the total retinal area and multiplied by a hundred to calculate the percent avascular area. Neovascular areas were determined by using the magic wand tool to select neovascular tufts, identified by their protrusion from the superficial layer and fluorescent intensity.[15] The neovascular

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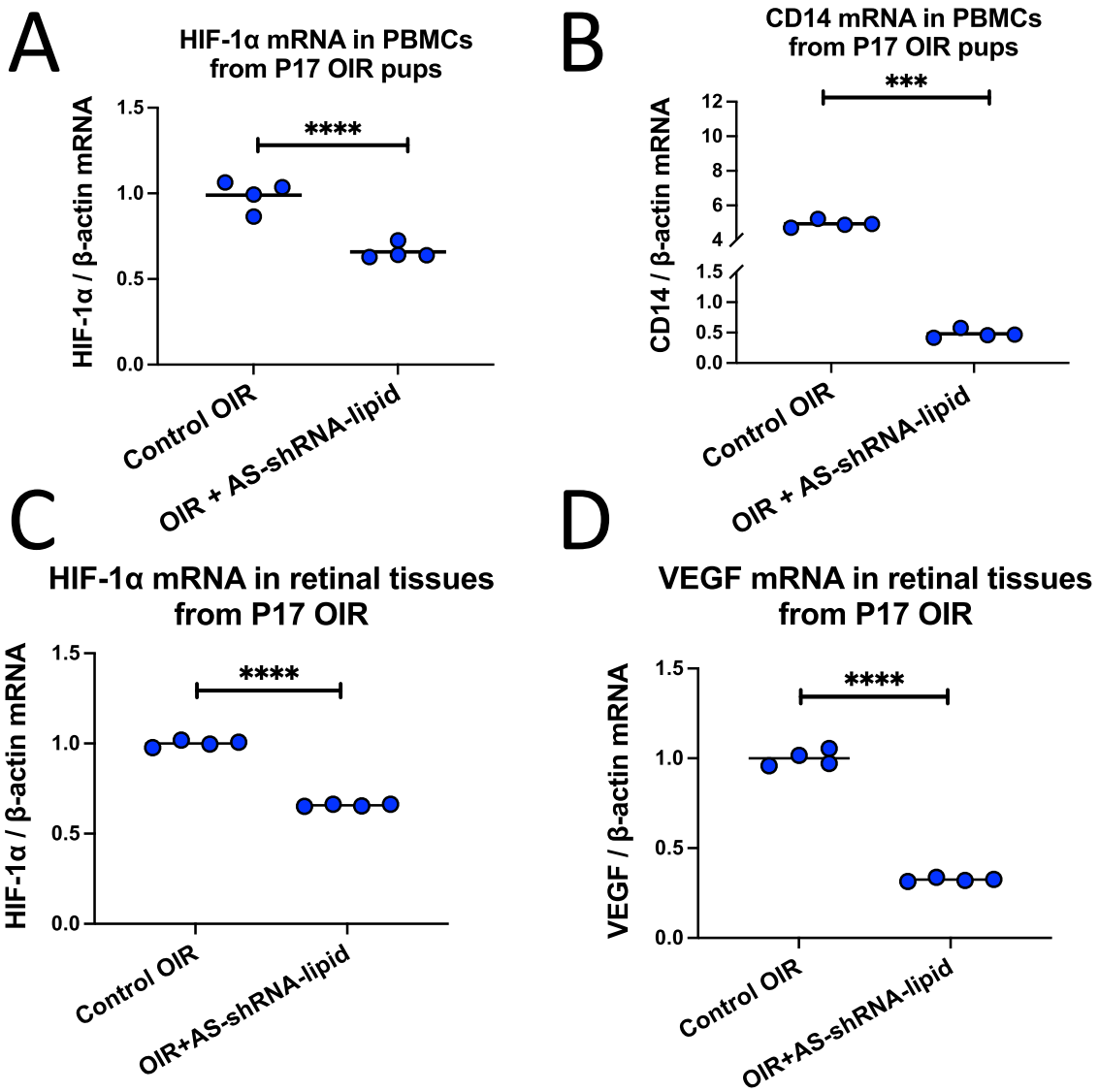

Figure 4. Expression of HIF-1α and CD14 mRNA in monocytes and HIF-1α and VEGF mRNA in retinal tissues from mouse OIR at P17 after treatment with PBS or AS-shRNA-lipids. (A, B) HIF-1α mRNA and CD14 mRNA expressions were significantly lower ($P < 0.0001$) in monocytes from OIR mice treated with the AS-shRNA-lipid compared to untreated OIR control mice. (C, D) Both HIF-1α mRNA and VEGF mRNA expressions were significantly decreased ($P < 0.0001$) in As-shRNA-lipid-treated mice compared to control mice. For each group, a total of 12 pups were used with pooled tissues from 3 pups for each experiment ($n = 4$).

area was divided by the total retina area and multiplied by a hundred to calculate percent neovascularization.

Monocyte Activation and Treatment with AS(or NS)-HIF-1α shRNA-Lipids. Primary mouse bone marrow monocytes were cultured in six well plates and treated with phorbol 12-myristate 13-acetate (PMA, from Sigma-Aldrich Catalog No. P1585) for 78 h. Cells were then treated with AS(or NS)-shRNA-lipids (50 nM concentration in complete leukocyte medium) and incubated for 4 h at 1% oxygen (hypoxia) or 21% oxygen (normoxia); then, cells were lysed to obtain mRNA samples using the Qiagen protocol. HIF-1α and CD14 mRNA expressions were analyzed in the AS(or NS)-shRNA-lipid-treated monocytes and control monocytes using qRT-PCR.

Statistical Analysis. Graphpad Prism 9 was used to perform statistical analysis and to make all graphs. Multiple comparison t tests were performed using the two-stage Benamini–Krüger–Yekutieli method with $Q = 1\%$. Data are expressed as the mean ± SEM.

RESULTS

AS-shRNA-Lipid Regulates HIF-1α and CD14 mRNA Expression. To quantify the effect of the newly synthesized hybrid-lipid conjugates on target gene expression, monocytes were treated with AS-shRNA-lipids under hypoxia and normoxia. The mRNA samples were analyzed using qRT-PCR. The group treated with AS-shRNA-lipids showed a significant reduction in the expression of HIF-1α under both hypoxic and normoxic conditions, compared to the control group (Figure 2B). CD14 mRNA expression was also significantly decreased in both monocytes treated with As-shRNA-lipids in the hypoxia and normoxia conditions (Figure 2C), suggesting that inhibition of HIF-1α mRNA could normalize the monocyte activation as shown in Figure 2A.

AS-shRNA-Lipid Inhibits Neovascularization in P17 OIR Mice by Regulating HIF-1α and CD14 mRNA Expression. The OIR mice were treated with AS(or NS)-shRNA-lipid to observe the effect of HIF-1α inhibition on

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retina neovascularization. We observed that the NS-shRNA-lipid-treated group did not show any significant change in both neovascular and avascular areas compared to the PBS-treated control group. However, there was a significant reduction in the percentage of neovascularization observed in the As-shRNA-lipid (Figure 3H), though there was minimal change in percent avascular area after HIF-1α inhibition in circulating activated monocytes.

Inhibition of HIF-1α mRNA in Activated Monocytes In Vivo. Blood samples were collected from control mouse and mouse OIR treated with the AS-shRNA-lipid to analyze HIF-1α and CD14 mRNA expressions. We have confirmed the expression of HIF-1α in CD14 positive activated monocytes using single-cell RNaseq data analysis (Figure S1).[4] Elevated levels of CD14 were detectable using qRT-PCR in PBMCs from OIR mice compared to room air control mice (Figure S1−C). However, changes in HIF-1α mRNA expressions were not detectable using qRT-PCR in blood samples from the OIR compared to mice kept in room air conditions, as shown in Figure S1D. This may be due to contributions from T- and B-cells that also express HIF-1α and also smaller number of activated monocytes in blood samples from OIR pups. However, decreased levels of both HIF-1α mRNA and CD14 mRNA expressions were detectable in PBMCs collected from the OIR mice treated with the AS-shRNA-lipid compared to the control OIR Mice (Figure 4A,B). Interestingly, significant decreased levels of both HIF-1α mRNA ($P < 0.0001$) and VEGF mRNA ($P < 0.0001$) expressions were observed in retinal tissues collected from OIR mice treated with the As-shRNA-lipid (Figure 4C,D). These observations suggest that the regulation of retinal VEGF levels could be achieved by targeting activated monocytes.

DISCUSSION

It is well-known that monocytes accumulate in the retina in response to inflammation and migrate in response to neovascularization in proliferative diseases such as PDR.[16,17] Yet, monocytes' exact contribution to disease progression is unknown. It has been observed that activated monocytes express HIF-1α mRNA which regulates angiogenesis.[18] Studies in the past have shown that in vitro and in vivo inhibition of HIF-1α through intravitreal and subconjunctival injections, using gene therapy such as RNAi, leads to decrease in neovascularization.[19,20] Neither of these studies specifically targeted activated monocytes. Therefore, we hypothesized that the inhibition of HIF-1α mRNA in activated monocytes could reduce neovascularization. Unlike past studies, this could be accomplished using HIF-1α-targeted As-shRNA-lipid conjugates developed in our laboratory. These conjugates are delivered systemically through intraperitoneal injection and evidence suggest that this novel gene therapy targets circulating monocytes that are recruited to the retina.[4,21] This can be done in vivo without using toxic transfection agents or more invasive routes of delivery that can cause inflammation themselves.[22]

In our in vitro model, HIF-1α mRNA-targeted AS-shRNA-lipid conjugates significantly decreased HIF-1α expression concurrently with the monocyte activation marker CD14 in both normoxic and hypoxic conditions (Figure 2), showing that we can regulate HIF-1α expression using our gene delivery method. With decreased HIF-1α and CD14 expressions, our gene therapy system could regulate monocyte function and phenotype.[23,24]

To test the efficacy of the As-shRNA-lipids in vivo, we used a mouse OIR model for the treatment of retinal neovascularization. In this model, mouse pups are treated under hyperoxic conditions from P7 to P12, where HIF-1α has a lower expression and then treated under normoxic condition from P12 to P17, where HIF-1α expression is elevated during this period.[25] During this relatively hypoxic stage (75% oxygen is hyperoxic compared to room air), abnormal neovascularization results in angiogenic tufts that are similarly seen in many ocular diseases.[26] By inhibiting HIF-1α specifically in monocytes during the hypoxic stage (p12 to p17), we showed that the percent neovascular tufts were significantly decreased, with a minimal change in the percent avascular area. This indicates that the HIF-1α As-shRNA-lipid protects against abnormal preretinal neovascularization without negatively affecting normal intraretinal revascularization in the retina.

Currently, in diseases such as in PDR, neovascularization is treated with anti-VEGF antibodies/VEGF traps.[27,28] HIF-1α regulates the expression of pro-angiogenic molecules including VEGF.[18,29] In the OIR mice treated with HIF-1α shRNA, retinal levels of VEGF mRNA were significantly decreased by regulating HIF-1α mRNA in activated monocytes. In the clinic, anti-VEGF treatments are highly effective for some patients but have two limitations. First, they require invasive intraocular injections, which can lead to decrease in patient compliance.[30] Second, many patients can develop resistance to the anti-VEGF treatments.[31] Therefore, developing therapies that are less invasive or can overcome the resistance is important. As-shRNA-lipid conjugates have the potential to overcome both of these limitations. First, this gene therapy is injected systemically and without the use of toxic transfection agents. Systemic injection is a better route because it offers less trauma to the patient, which translates into greater patient compliance and does not carry the risk of ophthalmitis associated with intravitreal injections. Second, the anti-VEGF resistance is believed to arise from increases in other pro-angiogenic factors.[31] Many of these factors are either regulated by HIF-1α or promote angiogenesis, partially by stabilizing HIF-1α.[32−36] Hence, HIF-1α regulation could overcome the limitation of anti-VEGF resistance with broader therapeutic advantages for the treatment of neovascularization.[19]

CONCLUSIONS

In summary, we showed that inhibition of neovascularization could be achieved by systemic injection of the HIF-1α-targeted As-shRNA-lipid that regulates HIF-1α mRNA expression in activated monocytes in vivo. Thus, HIF-1α mRNA could be a potential therapeutic target for the treatment of retinal neovascularization that is a component of both retinopathy of prematurity (ROP) and proliferative diabetic retinopathy (PDR).

ASSOCIATED CONTENT

Data Availability Statement

All data that support the results of this study are available within the article and its Supporting Information or upon request to the corresponding author (M.I.U.).

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsanm.3c04300.

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Additional images including HPLC chromatogram and HRMS spectrum of the AS(NS)-shRNA and lipid conjugates (PDF)

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Author Contributions

R.E.A. and B.W.D. performed the experiments, collected, and analyzed the data, and wrote the manuscript. J.S.P. helped revise the manuscript. M.I.U. conceived and supervised the project, designed and synthesized the compounds, designed and performed experiments, and revised the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

HIF-1α =hypoxia-inducible factor-1α
OIR =oxygen-induced retinopathy
shRNA =short hairpin RNA
NV =neovascularization
PBMCs =peripheral blood mononuclear cells
DR =diabetic retinopathy
PDR =proliferative diabetic retinopathy
AMD =age-related macular degeneration
ROP =retinopathy of prematurity

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