Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2026**; 31(10): 54786
https://doi.org/10.31083/FBL54786

Original Research

Utilizing Liposomes That Selectively Target Inflamed Endothelium to Upregulate Macrophage-Like Cell ABCA1 Through Endothelial Cell-Derived Exosome-Mediated Processes

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Academic Editor: Giordano Pula
Submitted: 11 June 2026 Revised: 7 August 2026 Accepted: 20 August 2026 Published: 10 October 2026

Abstract

Background: Atherosclerosis is caused by lipid accumulation in arterial endothelial cells and intimal cells. Atherosclerosis generally occurs in arteries that exhibit vascular inflammation, thereby making pro-inflammatory arterial endothelial cells a potential target for delivering atheroprotective material. Lipid-laden macrophages and vascular smooth muscle cells that have transdifferentiated into macrophage-like cells from cholesterol accumulation are the two primary intimal cell types that drive atherosclerosis. We have constructed a novel liposome-based therapeutic system (LP-pXMoA5p) that can specifically target inflamed arterial endothelial cells, resulting in endothelial cells that internalize LP-pXMoA5p to express and then package anti-miR-33a-5p into exosomes that are released basolaterally, which may lead to effective transfer of atheroprotective anti-miR-33a-5p to intimal cells. We have previously tested this delivery method with cultured macrophages being the recipient of endothelial-cell-derived exosomes filled with anti-miR-33a-5p, resulting in enhanced macrophage ABCA1-dependent cholesterol efflux. **Methods:** In this study, we wanted to test whether our LP-pXMoA5p delivery system is successful when utilizing cultured macrophage-like cells as the recipient cells for endothelial-cell-derived exosomes loaded with anti-miR-33a-5p. To test the atheroprotective efficacy of our LP-pXMoA5p delivery method, we measured miR-33a-5p expression, ABCA1 protein expression, and apoAI-mediated cholesterol efflux in recipient macrophage-like cells. **Results:** In our experiments, we observed that cultured macrophage-like cells incubated with anti-miR-33a-5p-filled exosomes exhibited high levels of cellular anti-miR-33a-5p, which resulted in decreased miR-33a-5p expression. Moreover, inhibition of miR-33a-5p within macrophage-like cells caused enhanced apoAI-mediated cholesterol efflux in these cells, which was likely the result of increased ABCA1 protein expression. **Conclusions:** Our findings reveal that LP-pXMoA5p appears capable of enhancing ABCA1-dependent cholesterol efflux within cultured macrophage-like cells, with this outcome potentially being due to macrophage-like cells internalizing anti-miR-33a-5p-loaded exosomes which are secreted by pro-inflammatory cultured immortalized mouse aortic endothelial cells (iMAECs), but we acknowledge this may not translate to similar effects *in vivo*.

Keywords: atherosclerosis; cardiovascular disease; extracellular vesicle; high-density lipoprotein; ischemic heart disease; low-density lipoprotein; nanoparticle; reverse cholesterol transport; vascular inflammation

1. Introduction

Despite advances in treatments, atherosclerotic cardiovascular disease (athCVD) remains the number one cause of death globally [1,2,3,4]. The two major consequences that lead to death from athCVD are myocardial infarction (i.e., heart attack) and cerebrovascular accident (i.e., ischemic stroke) [5,6,7,8]. The main arteries afflicted by cholesterol accumulation caused by atherosclerosis, resulting in heart attacks and strokes, are the coronary and carotid arteries, respectively [9]. However, the most common prescriptions and medications for atherosclerosis primarily function to lower serum/plasma cholesterol levels within the blood [10,11,12,13], instead of removing cholesterol precisely from atherosclerotic arteries. Furthermore, current treatments for athCVD are only partially effective at treating this disease [3]. Therefore, therapies aimed at removing excess cholesterol precisely from diseased lesions may be more effective at reducing mortality caused by athCVD.

The conventional view of atherosclerosis is that this condition is predominantly the result of intimal macrophages accumulating cholesterol, which results in the formation of lipid-laden foam cells that lead to plaque development within arteries [14,15,16,17,18,19]. How-

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ever, there is emerging evidence that medial vascular smooth muscle cells (VSMCs) may play a more prominent role in atherosclerosis development via migrating into the intima, internalizing cholesterol, then converting into a macrophage-like cell (MLC) [20,21,22,23,24,25,26,27]. Indeed, convincing data have suggested that ≥50% of cells detected within human atherosclerotic lesions may be MLC of VSMC origin [28]. We [29,30,31,32] and others [33,34] have shown that removing cholesterol from cultured MLC of VSMC-origin reverts these cells to their original VSMC phenotype. Thus, it is tempting to speculate that augmenting the removal of excess cholesterol from intimal MLC may effectively treat athCVD, as this may result in removing cholesterol from the cell type that may be most detrimental to atherosclerosis formation.

Cholesterol efflux is the process cells use for removing excess cholesterol [35,36]. Though there are four distinctly known pathways for cholesterol efflux [37], ABCA1-dependent cholesterol efflux has been deemed most anti-atherogenic [38,39]. Cell-surface ABCA1 participates in cholesterol efflux via interacting with lipid-poor apoAI particles in circulation, which leads to both intracellular cholesterol removal and nascent HDL biogenesis [40,41,42,43,44]. Increasing arterial ABCA1 expression has been proposed as a powerful treatment for atherosclerosis [45]. A potential strategy to increase ABCA1 in arterial cells is by inhibiting the microRNA miR-33a-5p because a main function of this microRNA is to silence expression of ABCA1 [46,47]. Indeed, miR-33a-5p has a prominent effect on suppressing expression of ABCA1 due to multiple miR-33a-5p binding sites located within the 3′UTR of the *ABCA1* gene, which leads to downregulation of ABCA1 [47,48,49]. Further, compelling evidence has shown that inhibition of miR-33a-5p causes ABCA1 de-repression, which results in upregulation of ABCA1 [50,51,52,53,54,55,56,57,58]. Regarding arterial cells, most of the published work pertaining to miR-33a-5p manipulation has focused on macrophages, and some data suggest inhibition of miR-33a-5p within this cell type leads to atheroprotective effects [59]. Our laboratory has also shown that ablating/inhibiting miR-33a-5p within cultured MLC of VSMC origin results in these cells exhibiting atheroprotective properties [30,31]. Therefore, precisely delivering anti-miR-33a-5p to intimal MLC may be anti-atherogenic, but there are challenges associated with delivering anti-miRs and other small RNAs to intimal cells due to the endothelium acting as a barrier that prevents delivery of nucleic acids and other molecules to the intima [60]. However, our laboratory has generated a delivery method that combines lipoparticle and endothelial cell-derived exosome-mediated approaches, which allows endothelial cells to release anti-miR-33a-5p-filled exosomes basolaterally so that these particles may reach subendothelial cells [61].

The purpose of our study is to test whether liposomes that specifically target pro-inflammatory endothelial cells (ECs) have the capacity to facilitate effective delivery of anti-miR-33a-5p to cultured MLC through exosome-mediated processes. This is an extension of our previous work that has shown this delivery system is able to increase macrophage ABCA1-dependent cholesterol efflux via EC-derived exosome-mediated transfer of anti-miR-33a-5p to cultured macrophages [61]. The liposomes used in our delivery method are decorated with a VHPK peptide that binds to the EC adhesion molecule VCAM-1 with high efficiency [61,62]. These liposomes also encapsulate a plasmid that expresses XMOTIF-tagged anti-miR-33a-5p, with this XMOTIF sequence allowing anti-miR-33a-5p to become preferentially packaged into EC-derived exosomes [61,63]. In our present study, we exposed cultured pro-inflammatory EC to these liposomes and then collected exosomes released basolaterally from these cells to treat cultured MLC. Therefore, a major objective in this study is to directly test whether incubating MLC with the anti-miR-33a-5p-filled EC-derived exosomes detailed above results in decreased MLC miR-33a-5p expression, leading to enhanced ABCA1-dependent cholesterol efflux in the treated MLC.

2. Materials and Methods

2.1 Maintenance and Treatment of Cultured Cells

The cell lines used in our experiments were immortalized mouse aortic endothelial cells (iMAECs) originally generated by Dr. Hanjoong Jo's laboratory [64] and the immortalized vascular smooth muscle cell line MOVAS cells [32] originally obtained from American Type Culture Collection (ATCC) (CRL-2797; Manassas, VA, USA). All cell lines were validated by STR profiling and tested negative for mycoplasma. The conditions to maintain cultured iMAEC and MOVAS cells included use of standard growth medium containing high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Corning, New York, NY, USA), 10% fetal bovine serum (VWR Life Science Seradigm, Radnor, PA, USA), 1% penicillin/streptomycin (Corning), and 500 µg/mL of geneticin (VWR Life Science Seradigm). Both types of cells were maintained in a CO_2 incubator (5%) set at 37 °C. The iMAEC line was initially maintained in culture as previously described [65] and then transferred to a 0.4 µm pore-size transwell (Corning, New York, NY, USA) containing sterile PBS within the basolateral compartment as detailed previously [61]. Briefly, iMAECs were either treated with vehicle (VEH) only or LPS (10 ng/mL; Sigma-Aldrich, St. Louis, MO, USA) for 24 hours. After the respective treatments, iMAECs were washed with sterile PBS and PBS from the basolateral compartment was aspirated; this compartment was washed with sterile PBS, then subsequently refilled with sterile PBS. We immediately incubated iMAECs with liposomes for one hour at a concentration of 1 liposome per iMAEC. After treatment,

we washed iMAECs with sterile PBS, and PBS from the basolateral compartments was aspirated, followed by washing these compartments with sterile PBS. We replenished iMAECs as well as filled the basolateral compartments with serum-free medium and then collected the serum-free medium from the basolateral compartments 24 hours later, which contained basolaterally secreted iMAEC-derived exosomes [61], to use in our downstream experiments.

To convert MOVAS cells into MLC, we used a cholesterol-loading strategy that induces MLC transdifferentiation in this vascular smooth muscle cell line [32]. Briefly, MOVAS cells maintained in culture were treated with 10 µg/mL of cholesterol–methyl-beta-cyclodextrin (MBCD:chol; Sigma-Aldrich) diluted in serum-free medium for 72 hours. After loading cells with MBCD:chol, the MOVAS MLC were treated with conditioned medium (CM) originating from the basolateral compartments of transwells that contained either LPS-challenged iMAECs or VEH-treated iMAECs exposed to liposomes. Detailed descriptions of downstream use for MOVAS MLC treated with VEH-CM versus LPS-CM are defined below.

2.2 Liposome Construction

We created liposomes used for treating iMAECs as described previously [61,62]. Briefly, 500 µL (1 µg/µL concentration) of an XMOTIF-tagged anti-miR-33a-5p expressing plasmid (pXMoA5p; System Biosciences, Palo Alto, CA, USA) [61] was used during liposome preparation via adding this plasmid to 0.78 mg of 1,2-dioleoyl-3-trimethylammonium propane (Avanti Polar Lipids, Alabaster, AL, USA) dissolved in 500 µL of chloroform and 1040 µL of methanol. After monophase formation, we incubated the solution at ambient temperature for 30 minutes, then added 500 µL of both deionized water and chloroform, which formed a biphasic solution that we mixed and subsequently centrifuged for 10 minutes at 800 ×g and 4 °C. Once spinning was complete, we collected the organic phase, and added it to 1.47 mg of cholesterol (Avanti Polar Lipids), 0.95 mg of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (Avanti Polar Lipids), and 4.55 mg of hydrogenated soy phosphatidylcholine (Avanti Polar Lipids). We proceeded to add 500 µL of diH2O to this solution, mixed via vortexing, performed emulsification with a sonicator, and then utilized a BUCHI Rotavapor® R-100 (New Castle, DE, USA) to evaporate the organic phase. We added another 500 µL of diH2O to this preparation, followed by a second round of evaporation using the Rotavapor. The liposome preparation was extruded for a total of 21 passages using a 100 nm polycarbonate membrane, with the temperature set at 55 °C. We purchased customized VHPK (Val-His-Pro-Lys-Gln-His-Arg-Gly-Gly-Ser-Lys(stearic_Lys(stearic)-PEG27)-Gly-Cys) from CPC Scientific (San Jose, CA, USA) to decorate the liposomes by directly adding 10 mg of this purified, lyophilized peptide to each liposome preparation and immediately mixing, inverting, and stirring this formulation using gentle agitation. This generated liposomes that contained pXMoA5p, with the surface of the liposomes also being decorated with VHPK peptides (LP-pXMoA5p). We utilized a Cytiva HiTrap column (Marlborough, MA, USA) for collecting LP-pXMoA5p fractions and characterized these preparations via nanoparticle tracking analysis, measuring zeta potential, and performing transmission electron microscopy, all as previously described [61,62,66]. To first validate that pXMoA5p can be effectively delivered to iMAEC, we assessed plasmid loading efficiencies for aliquots of LP-pXMoA5p to confirm ≥90% loading efficiencies [61] for plasmid DNA into LP that were later utilized in downstream experiments.

2.3 Gene Expression

After treating MOVAS MLC with either VEH-CM or LPS-CM for 24 hours, we harvested cells, and then isolated the total RNA from MOVAS MLC using Direct-Zol spin columns (Zymo Research, Irvine, CA, USA) specifically formulated to isolate both small RNA and mRNA transcripts [67]. We used this purified RNA as a template to synthesize cDNA via utilizing a Takara Mir-X miRNA First-Strand cDNA Synthesis Kit (San Jose, CA, USA). This synthesized cDNA was then used in qPCR reactions, along with a Takara TB Green Advantage qPCR kit, so that we could measure small RNA expression. The primer pairs used for PCR product amplification of small RNA were as follows: U6 housekeeping/reference gene: forward primer (5′-GGAACGATACAGAGAAGATTAGC-3′), reverse primer (5′-TGGAACGCTTCACGAATTTGCG-3′); miR-33a-5p: forward primer (5′-CGCGTGCATTGTAGTTGCATTGC-3′), Takara universal reverse primer (proprietary), anti-miR-33a-5p: forward primer (5′-TGCAATGCAACTACAATGCAC-3′), Takara universal reverse primer (proprietary).

Expression of mRNA was also measured in treated MOVAS MLC. After these cells were treated with VEH-CM or LPS-CM for 24 hours, we removed CM, washed cells with sterile PBS, and then treated MOVAS MLC with 100 µg/mL of apoAI (Academy Bio-Medical Company, Houston, TX, USA) diluted in serum-free medium supplemented with 2 mg/mL of BSA-FAFA (Sigma-Aldrich). After incubating cells with apoAI for 72 hours, we removed the medium, washed cells with sterile PBS, harvested MOVAS MLC, and isolated total RNA using Zymo Research Direct-Zol spin columns. We subsequently converted total RNA into cDNA with qScript cDNA Ultra SuperMix kits (Quantabio, Beverly, MA, USA) and used this cDNA as a template in PerfeCTa SYBR Green FastMix kits (Quantabio) for performing qPCR

reactions. Primer pairs to measure mRNA expression were as follows: GAPDH housekeeping/reference gene: forward primer (5′-CGTGCCGCCTGGAGAAAC-3′), reverse primer (5′-TGGGAGTTGCTGTTGAAGTCG-3′); SM-MHC: forward primer (5′-AAGCTGCGGCTAGAGGTCA-3′), reverse primer (5′-CCCTCCCTTTGATGGCTGAG-3′); F4/80: forward primer (5′-CTTTGGCTATGGGCTTCCAGTC-3′), reverse primer (5′-GCAAGGAGGACAGAGTTTATCGTG-3′). All small RNA and mRNA qPCR reactions were quantified via utilizing a qTOWER^3 G touch qPCR instrument (Analytik Jena US, Upland, CA, USA) and gene expression data were analyzed with the comparative C(T) method [68].

2.4 Protein Expression

We initially treated MOVAS MLC with VEH-CM versus LPS-CM for 48 hours, removed CM, washed cells with sterile PBS, and collected MOVAS MLC lysates using protease inhibitors diluted in RIPA lysis buffer (VWR Life Science, Radnor, PA, USA). We subsequently measured protein mass in cell lysates with a BCA assay kit (BioVision, Milpitas, CA, USA). We then loaded equal masses of protein from lysates onto SDS-PAGE gels for protein separation, transferred these separated proteins onto PVDF membranes (Merck Millipore Ltd., Burlington, MA, USA), and blocked membranes using milk protein-based blocking buffer. After blocking PVDF membranes, we incubated these membranes with the following antibodies: GAPDH primary antibody (1:3500 dilution; sc-365062; Santa Cruz Biotechnology); ABCA1 primary antibody (1:1250 dilution; sc-58219; Santa Cruz Biotechnology); HRP-conjugated IgG secondary antibody (1:8000 dilution; AP181P; Sigma-Aldrich). After antibody incubation, we submerged PVDF membranes in ECL substrate solution (Immobilon ECL Ultra Western HRP Substrate; Millipore-Sigma, Billerica, MA, USA) and detected proteins with a ChemiStudio imaging system (Analytik Jena US LLC, Waltham, MA, USA), followed by quantifying protein expression with NIH ImageJ software (version 1.53a, National Institutes of Health, Bethesda, MD, USA) [69].

2.5 ApoAI-Mediated Cholesterol Efflux

To measure cholesterol efflux within MOVAS MLC, we utilized an established protocol from our laboratory [29,30,31,32]. Briefly, we cholesterol-loaded MOVAS cells by treating these cells with 10 µg/mL of MBCD:chol (Sigma-Aldrich) and [^{3}H] cholesterol (1 µCi/mL; PerkinElmer, Waltham, MA, USA) for 72 hours, with the latter tritium-based reagent allowing us to measure cellular cholesterol efflux. During this 72-hour incubation period, cells were cultured in serum-free medium supplemented with 2 mg/mL of BSA-FAFA (Sigma-Aldrich). After cholesterol loading, MOVAS MLC were washed with sterile PBS, and incubated for 72 hours in cholesterol efflux medium, which was composed of serum-free medium, 2 mg/mL of BSA-FAFA (Sigma-Aldrich), and 100 µg/mL of apoAI (Academy Bio-Medical Company). After exposure to cholesterol efflux medium, we filtered this medium to remove non-adherent cells, washed the MOVAS MLC with sterile PBS, and collected cell extracts with sodium hydroxide. A PerkinElmer Tri-Carb 4910TR liquid scintillation counter was utilized to count the [^{3}H] in both the filtered medium and cell extracts. We calculated apoAI-mediated cholesterol efflux [70] by dividing [^{3}H] within the medium by [^{3}H] counted in both medium and cell extracts, after subtracting background cholesterol efflux that was analyzed in vehicle-treated (i.e., no apoAI) MOVAS MLC and the medium used to culture these cells.

2.6 Immunofluorescence Imaging of iMAEC

Cultured iMAECs were transferred to sterile 4-well chamber tissue slides (Corning) and maintained in standard growth medium until reaching optimal confluence. The iMAECs were subsequently treated with either VEH only or challenged with 10 ng/mL LPS (Sigma-Aldrich) for 24 hours, washed with sterile PBS, and then used for staining in non-permeabilized conditions. Briefly, cells were fixed in 4% paraformaldehyde. Samples were then blocked in 3% BSA/20 mM glycine in PBS, followed by incubation in PE-conjugated VCAM-1 (1:20 dilution; FAB6432P, R&D Systems, Minneapolis, MN, USA) for 1 hour at room temperature. Samples were washed, incubated with DAPI to counterstain the nuclei, washed, and mounted in ProLong Gold (Invitrogen, Carlsbad, CA, USA). Cells were imaged using a Leica Stellaris confocal microscope system (Leica Microsystems, Buffalo Grove, IL, USA), equipped with 405 nm and 561 nm lasers, HyD detectors, a 63× oil immersion lens (NA = 1.4), and Leica LAS X software (Version 4.7.0.28176). To capture images, we employed 1.5 zoom and collected emission wavelengths of 410 to 450 nm (DAPI, colorized in cyan) and 570 to 630 nm (PE-VCAM-1, colorized in magenta).

2.7 Statistical Analysis

All types of statistical analysis were carried out using SigmaPlot (Systat Software Inc. v14.0, Chicago, IL, USA) and we set $p < 0.05$ to determine statistical significance between the two tested groups. Before analyzing potential significant differences, we first conducted a Shapiro–Wilk test to assess the normal distribution of data. If the Shapiro–Wilk test failed, we subsequently performed a Mann–Whitney rank-sum test to analyze statistical differences between the two groups. If normality was not violated, then we conducted a Brown–Forsythe test to assess equal variances between groups. If equal variance was not assumed, then we performed a Welch's *t*-test to analyze statistical differences between the two groups. However, if equal variance was assumed, we instead performed a Student's *t*-test to analyze statistical differences between the two groups.

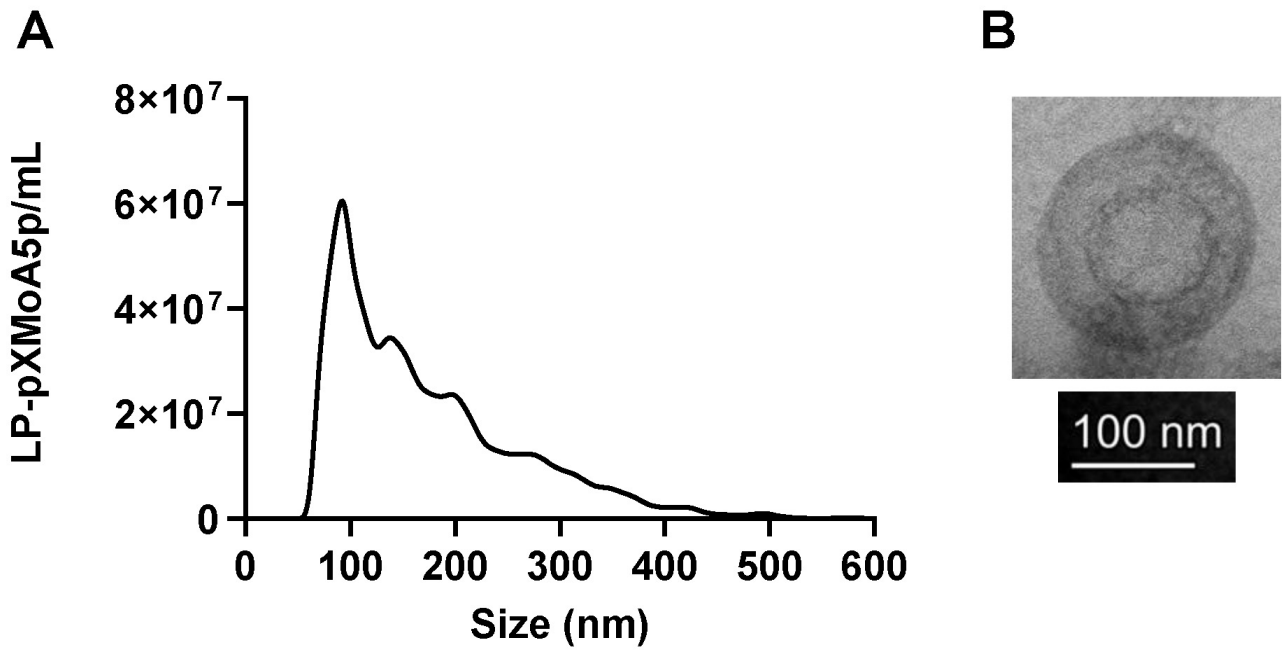

Fig. 1. Characterizing LP-pXMoA5p. (A) Aggregate data displaying size distribution and particle number of three separate, independent LP-pXMoA5p preparations via nanoparticle tracking analysis (NTA). (B) Representative transmission electron micrograph of LP-pXMoA5p derived from equal volumes of pooled aliquots originating from the three LP-pXMoA5p preparations initially utilized for NTA. Scale bar: 100 nm.

3. Results

3.1 Characterizing LP-pXMoA5p

In a previously published report [61], our laboratory characterized liposomes by conducting nanoparticle tracking analysis and transmission electron microscopy. In this current study, we utilized similar methods and strategies to characterize LP-pXMoA5p, with these particles demonstrating similar characterization profiles (Fig. 1A,B) as our previously generated liposomes [61]. We also measured the zeta potential of these newly generated liposomes to assess nanoparticle stability [71,72,73], which showed an average zeta potential of 8.43 mV, with these findings indicating that LP-pXMoA5p exhibits moderate colloidal stability [74].

3.2 Reduction of miR-33a-5p Expression Occurs in MOVAS MLC Incubated With LPS-CM

For cellular internalization of LP-pXMoA5p to be efficient, cell surface VCAM-1 should be present, since VHPK effectively binds to VCAM-1 [75,76,77,78]. While we have previously demonstrated that robust expression of VCAM-1 occurs within LPS-challenged iMAECs at both the mRNA and protein level [61,65,79,80], we have yet to examine cell surface VCAM-1 after iMAECs are challenged with LPS. By using immunofluorescent imaging, we do show that robust cell surface VCAM-1 expression is indeed present in LPS-challenged iMAECs (Fig. 2A), which implies higher numbers of LP-pXMoA5p can become internalized by these cells when compared to VEH-treated iMAECs, as we have demonstrated previously [61]. When we proceeded to incubate MOVAS MLC with LPS-CM or VEH-CM, we detected increased levels of anti-miR-33a-5p in the cells treated with LPS-CM when expression of this transgene was compared to VEH-CM-treated MOVAS MLC (Fig. 2B). Moreover, when we measured miR-33a-5p expression within these two groups of treated MOVAS MLC, we observed a significant decrease in miR-33a-5p levels within MOVAS MLC incubated with LPS-CM versus MOVAS MLC exposed to VEH-CM (Fig. 2C). From these findings, we conclude that treating MOVAS MLC with LPS-CM leads to decreased miR-33a-5p expression in these cells and that this downregulation of miR-33a-5p is likely due to high anti-miR-33a-5p uptake.

3.3 MOVAS MLC Exposed to LPS-CM Exhibit Enhanced ABCA1-Dependent Cholesterol Efflux

Since inhibition of miR-33a-5p has potential for upregulating ABCA1 [46,81], we next measured ABCA1 protein expression within MOVAS MLC incubated with either LPS-CM or VEH-CM. In these sets of experiments, we detected a significant increase in ABCA1 protein expression within MOVAS MLC treated with LPS-CM when compared to protein levels measured in MOVAS MLC treated with VEH-CM (Fig. 3A,B). ABCA1 controls apoAI-mediated cholesterol efflux [82,83], as apoAI is thought to exclusively participate in cholesterol efflux via interacting with ABCA1 [40]. Therefore, we next measured apoAI-mediated cholesterol efflux in MOVAS MLC incu-

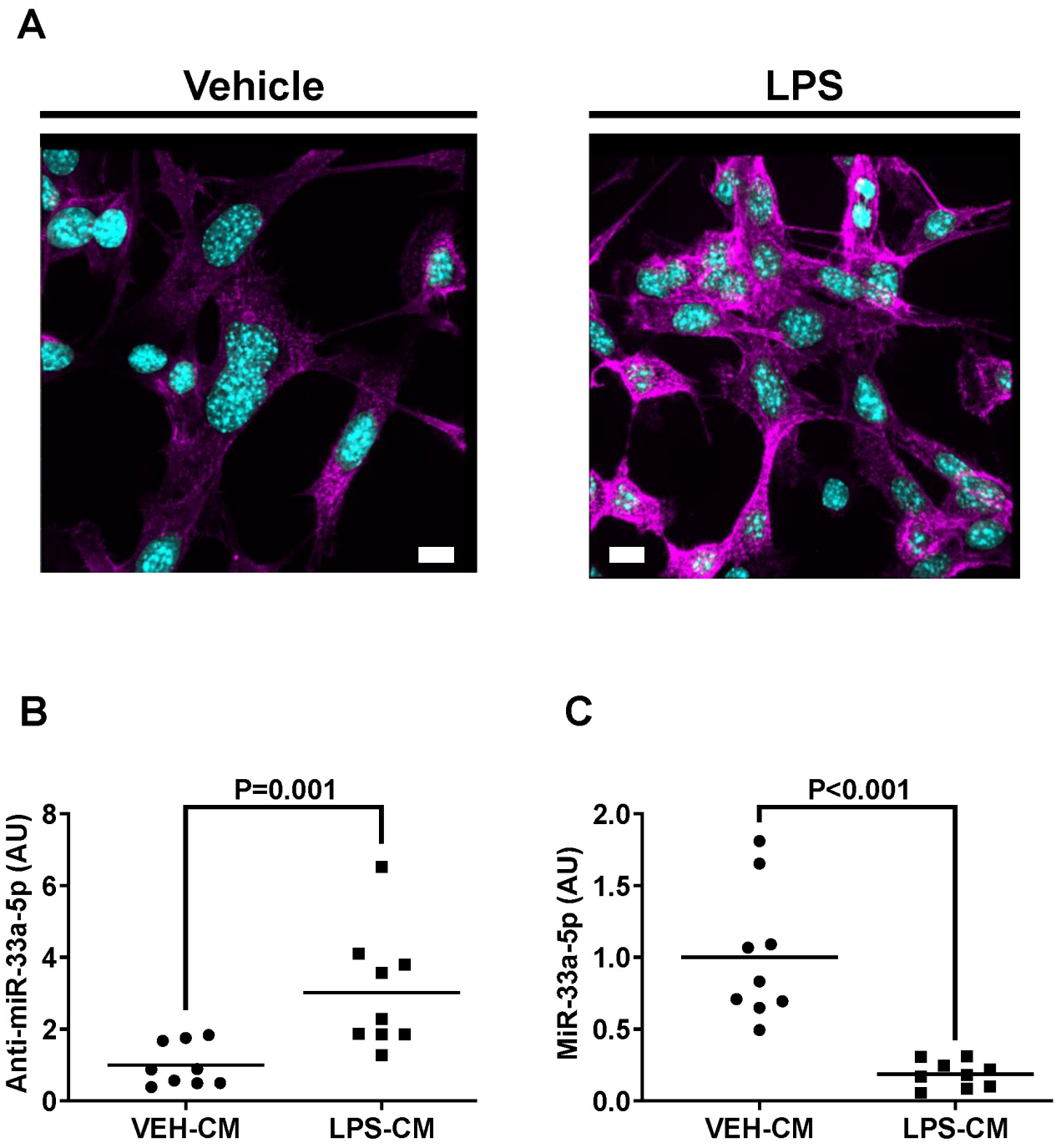

Fig. 2. LP-pXMoA5p facilitates effective anti-miR-33a-5p delivery to MOVAS macrophage-like cells (MLCs) through pro-inflammatory endothelial cell-derived exosome-mediated transfer. (A) Representative images illustrating VCAM-1 surface expression being assessed in vehicle-treated and LPS-challenged immortalized mouse aortic endothelial cells (iMAECs) using immunofluorescent staining. White scale bars = 10 µm. (B,C) RT-qPCR analyses of anti-miR-33a-5p (B) and miR-33a-5p (C) levels measured in MOVAS MLC incubated with either vehicle (VEH)-CM or LPS-CM. Data points show three independent experiments, including three biological replicates for each independent experiment. Bars are group means and AU are arbitrary units.

bated with LPS-CM versus VEH-CM. Results from these assays show apoAI-mediated cholesterol efflux was significantly increased in MOVAS MLC exposed to LPS-CM when percentage efflux was compared to MOVAS MLC exposed to VEH-CM (Fig. 3C). Hence, these results suggest that exosomes released basolaterally from pro-inflammatory iMAECs internalizing LP-pXMoA5p have the capacity to increase ABCA1-dependent cholesterol efflux in MOVAS MLC when taking up these exosomes.

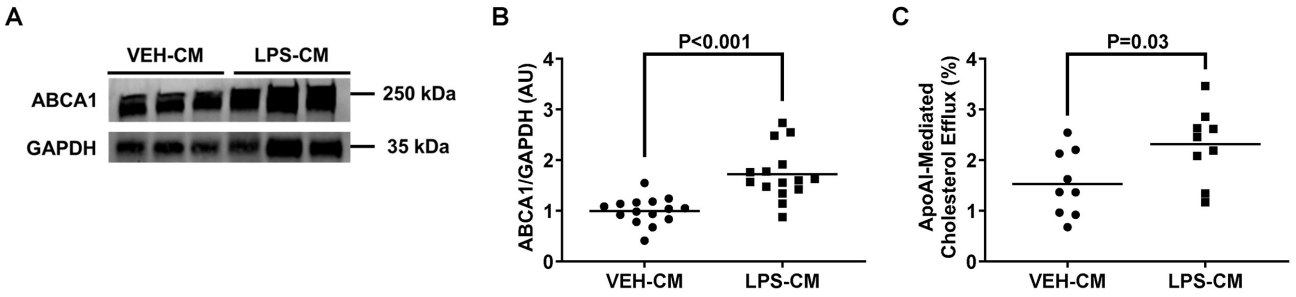

Fig. 3. ABCA1-dependent cholesterol efflux is enhanced in MOVAS MLC incubated with LPS-CM. (A,B) Representative immunoblot (A) for assessment of ABCA1 protein expression (B) measured in MOVAS MLC exposed to VEH-CM versus LPS-CM. (C) Cholesterol efflux assay measuring apoAI-mediated cholesterol efflux in MOVAS MLC incubated with either VEH-CM or LPS-CM. (B) Data points show five independent experiments, which include three biological replicates for each independent experiment, with bars being group means and AU being arbitrary units. (C) Data points show three independent experiments, including three biological replicates per independent experiment, with bars showing group means.

3.4 Vascular Smooth Muscle Cell Phenotype is Restored in Cholesterol-Loaded MOVAS Cells Pre-Incubated With LPS-CM and Then Treated With ApoAI

Cholesterol accumulation within vascular smooth muscle cells induces MLC transdifferentiation in this cell type [84], which is recognized as pro-atherogenic and thus removing excess cholesterol from these cells may result in atheroprotection [85]. MLCs lose expression of classical vascular smooth muscle cell markers while simultaneously beginning to express classical macrophage markers, due to the remarkable functional plasticity vascular smooth muscle cells/MLC exhibit [86]. Moreover, when MLCs deplete excess cholesterol, they revert to a vascular smooth muscle cell phenotype by suppressing macrophage marker expression and stimulating expression of vascular smooth muscle cell markers [34]. So, in our next experiments, we measured the expression of the classical vascular smooth muscle cell marker SM-MHC and classical macrophage marker F4/80 [87,88,89] in MOVAS MLC pretreated with either LPS-CM or VEH-CM, and then later exposed to the cholesterol acceptor, apoAI. In this study, we observed an increase in SM-MHC expression accompanied by a reduction in F4/80 expression in MOVAS cells treated with LPS-CM when compared to expression levels of MOVAS MLC treated with VEH-CM (Fig. 4A,B). We concluded from these findings that MLCs become at least partially restored to a VSMC-like phenotype when this cell type is exposed to basolaterally secreted exosomes released by pro-inflammatory endothelial cells that have internalized LP-pXMoA5p.

4. Discussion

In this report, we wanted to directly test whether MOVAS MLC exposed to LPS-CM may lead to atheroprotective effects driven by miR-33a-5p inhibition within these cells. The therapeutic angle of our approach hinges upon pro-inflammatory endothelial cells internalizing LP-pXMoA5p, resulting in these cells secreting anti-miR-33a-5p-filled exosomes basolaterally, which leads to uptake of these exosomes by lipid-laden intimal cells. In a previous study [61], we did show that LPS-challenged iMAECs effectively internalize LP-pXMoA5p, resulting in these cells predominantly releasing exosomes basolaterally that contained anti-miR-33a-5p, and we observed an increase in ABCA1-dependent cholesterol efflux within macrophages that internalized these exosomes. Thus, we wanted to extend testing of the possible atheroprotective properties of this delivery system by utilizing MOVAS MLC as the target for exosome delivery. In our current study, we did detect a significant decrease in miR-33a-5p within MOVAS MLC that were treated with LPS-CM and this outcome was likely due to robust transgene delivery of anti-miR-33a-5p to these cells. We also showed significant increases in both ABCA1 protein expression and apoAI-mediated cholesterol efflux within MOVAS MLC. Furthermore, we observed gene expression patterns in MOVAS MLC that are consistent with this cell type reverting to a vascular smooth muscle cell phenotype when these cells were pre-incubated with LPS-CM and then treated with the apoAI cholesterol acceptor. Hence, our report suggests LP-pXMoA5p is capable of successfully transferring anti-miR-33a-5p to intimal MLC through pro-inflammatory endothelial cell-derived exosome-mediated processes (Fig. 5, Ref. [61]) and this outcome leads to atheroprotective effects via enhancing ABCA1-dependent cholesterol efflux within intimal MLC.

Though the concept of nanotechnology has existed for the past 65 years [90], only recently have therapeutics utilizing nanoparticles been utilized globally. Indeed, the advent of lipid nanoparticle-based COVID-19 vaccines has now made nanotherapy use mainstream [91]. Historically, though, nanotherapy has mainly been used to treat certain forms of cancer [92,93,94] and little attention to this technology has focused on atherosclerotic cardiovascular dis-

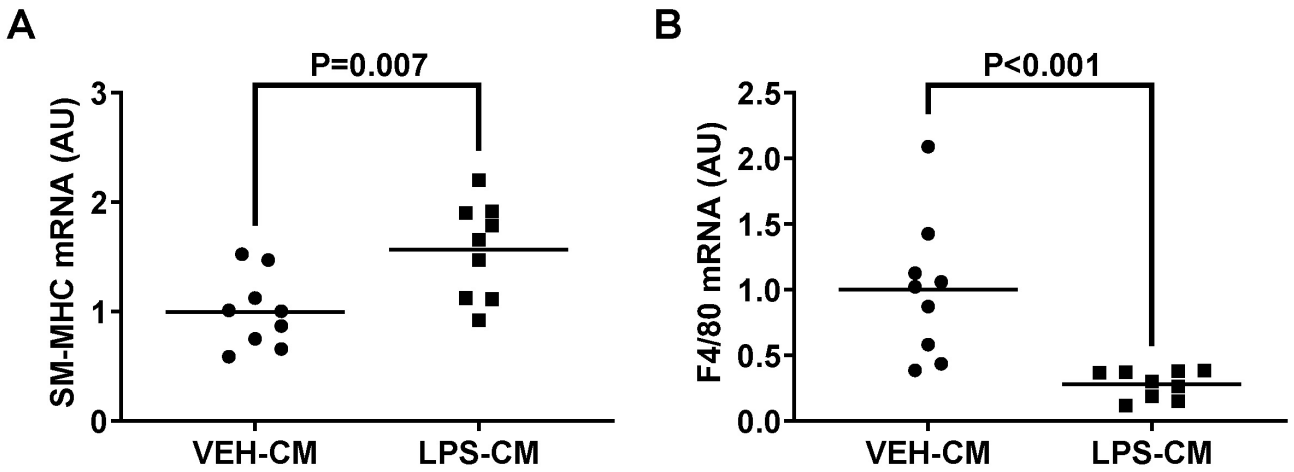

Fig. 4. Stimulation of SM-MHC expression compounded with suppressed F4/80 expression within cholesterol-loaded MOVAS cells initially treated with LPS-CM, then incubated with apoAI. (A,B) Gene expression of SM-MHC (A) and F4/80 (B) measured via RT-qPCR. Data points for the VEH-CM group and LPS-CM group indicate three independent experiments that include three biological replicates for each independent experiment. Bars show group means and AU indicates arbitrary units.

ease. One major reason for this is due to nanoparticles likely being incapable of reaching lipid-laden intimal cells, since these particles are too large to cross intact endothelium due to their diameter [95]. Therefore, endothelial cells serve as a gatekeeper to the intima and this cell type secretes large amounts of exosomes [96]. As we have shown previously [61], pro-inflammatory endothelial cells typically release exosomes basolaterally, and so our delivery method exploits this process to allow atheroprotective anti-miR-33a-5p to be effectively delivered to intimal cells, which we show here and in our previous publication [61] leads to increased ABCA1-dependent cholesterol efflux in both macrophages and MLC.

While still not entirely elucidated, there have been modern breakthroughs in better understanding atherosclerosis pathophysiology [97]. One of these major milestones is accepting vascular smooth muscle cells as a major cell type that drives atherosclerotic cardiovascular disease due to these cells converting into MLC during atherosclerosis progression [98]. While this has been speculated for decades, cell-lineage tracing techniques have confirmed that the majority of foam cells present within atherosclerotic plaques are vascular smooth muscle cell-derived [99,100]. What stimulates vascular smooth muscle cell transdifferentiation into MLC is when these cells become engorged with cholesterol and this process is generally acknowledged as encouraging atherogenesis [101]. MLCs are also able to revert to their original, healthy vascular smooth muscle cell phenotype via cholesterol removal [34]. Hence, promoting cholesterol efflux within intimal MLCs may be atheroprotective and our delivery system tested within this study shows promise in accomplishing that through delivering anti-miR-33a-5p to these cells using liposome/exosome-based strategies.

Atherosclerosis pathogenesis is intricately linked to vascular inflammation, with arterial endothelial cells largely contributing to this pathology [102]. Indeed, it is important to note that atherosclerosis is not considered a generalized disease of the arteries, but mainly occurs where vascular endothelium resides [103]. In particular, endothelial cells serve as a catalyst for this process via robustly expressing VCAM-1 and other pro-inflammatory adhesion molecules that aid in initiating atherosclerosis formation [104]. Our delivery method capitalizes on using endothelial cell surface VCAM-1 as a target to efficiently deliver atheroprotective anti-miR-33a-5p to pro-inflammatory endothelium. Interestingly, in our study, we did observe iMAEC surface VCAM-1 being present when these cells were cultured in basal (i.e., vehicle-only) conditions. Though we have also detected both mRNA (RT-qPCR) and protein expression (immunoblotting) for VCAM-1 in vehicle-treated iMAECs [61,65,79,80], it would be prudent to later analyze the number of VCAM-1-positive iMAECs not challenged with LPS via FACS, and experiments in our laboratory are currently underway to assess this. If we do observe appreciable levels of VCAM-1-positive iMAECs during basal states, then it may be possible that targeting another pro-inflammatory adhesion molecule besides VCAM-1 may lead to more precise delivery of atheroprotective material to vascular endothelium. However, in this scenario, we would first test basal levels of VCAM-1 within primary mouse arterial endothelial cells before deciding to change targets for the exploitation of delivering anti-miR-33a-5p to arteries, as this phenomenon may strictly occur in iMAECs but not primary arterial endothelial cells [105].

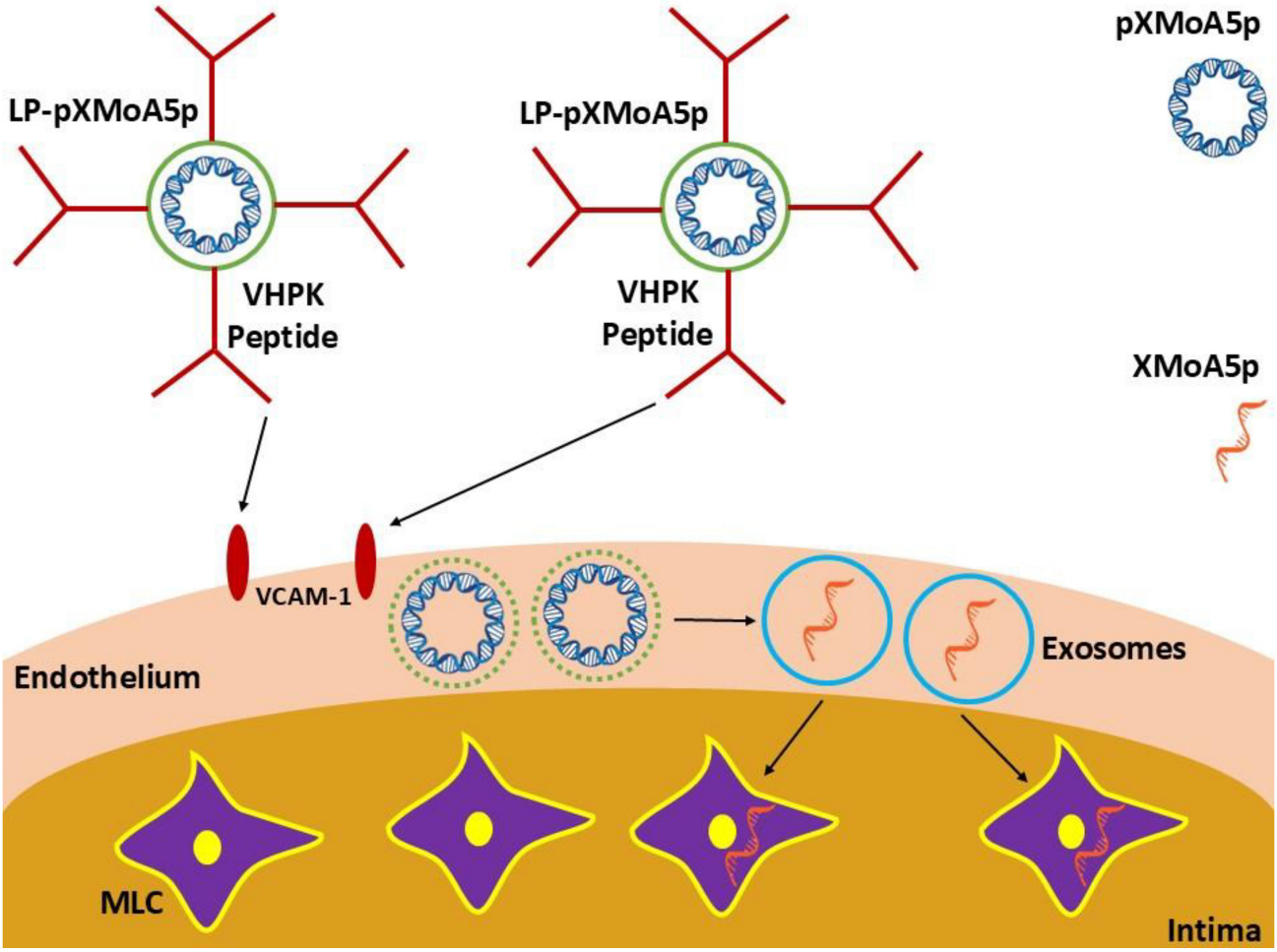

Fig. 5. Proposed working model. LP-pXMoA5p becomes internalized by pro-inflammatory arterial endothelium through VCAM-1 binding, resulting in endothelial cells that internalize LP-pXMoA5p to express and then package XMoA5p into exosomes that are released basolaterally, which allows intimal MLC to internalize these exosomes, leading to effective transfer of XMoA5p to these intimal cells. Figure adapted from Echesabal-Chen J, Constructing Lipoparticles Capable of Endothelial Cell-Derived Exosome-Mediated Delivery of Anti-miR-33a-5p to Cultured Macrophages; published by MDPI, 2023 [61].

5. Limitations

In our study, we want to address several limitations. One major concern is that while the overall goal for our laboratory is to utilize LP-pXMoA5p as an effective means to treat atherosclerosis safely, there are obvious obstacles that may impede our progress to using this type of therapy clinically [106,107,108]. One such hurdle includes verifying whether LP-pXMoA5p and subsequent exosome-mediated transfer of anti-miR-33a-5p are precisely delivered to pro-inflammatory endothelial cells and underlying intimal cells, respectively. While an exhaustive biodistribution panel has previously been performed in mice injected with VHPK-decorated LP [62], there have been no studies our laboratory is aware of that have assessed the biodistribution of XMOTIF-tagged anti-miRs administered to mice using VHPK-decorated LP. Indeed, potential consequences may arise with unintended exosome-mediated transfer of anti-miRs to off-target tissues [109,110,111], possibly leading to deleterious outcomes [112,113]. From a feasibility perspective, there are also potential challenges with LP-pXMoA5p-based therapy being a chronic, even lifelong treatment. This may deter clinical use of this type of therapy, particularly if utilized for atherosclerosis prevention [114], because patient adherence may be suboptimal, especially since treatments would involve injections [115,116]. These specific barriers may require refinement of our proposed atheroprotective nanotherapy over time.

Regarding limitations, another issue to address is the possibility that anti-miR-33a-5p may be delivered to MLC by methods that do not involve transfer within intact exosomes derived from pro-inflammatory iMAEC. For instance, it may be possible that free anti-miR-33a-5p within the CM used to treat MLC may lead to delivery of this anti-miR instead of exosome-mediated anti-miR-33a-5p delivery. Therefore, future studies that involve utilizing exosome-depleted CM, in addition to confirming high anti-

miR-33a-5p content within intact exosomes derived from pro-inflammatory iMAECs incubated with LP-pXMoA5p [63], should be conducted. Another limitation is the use of the immortalized vascular smooth muscle cell line MOVAS cells, as this cell line may behave differently when compared to primary vascular smooth muscle cells. While mouse primary aortic smooth muscle cell isolation, culture, and maintenance is considered a tedious and cumbersome process [117], these cells are commercially available [32], and so future studies should be devoted to attempting to repeat our laboratory's findings with primary aortic smooth muscle cells. Other limitations of our study involve how our *in vitro* models cannot mimic *in vivo* atherogenesis or atherosclerosis progression and thus our findings may not adequately recapitulate clinical atherosclerotic conditions. Therefore, before confirming whether our LP-pXMoA5p delivery system is effective at precisely targeting atherosclerotic plaque, restoring VSMC phenotype, and treating atherosclerosis, experiments that include physiologically relevant co-culture/flow-based models, primary cell *ex vivo* models, and *in vivo* testing in atherogenic models are needed. Lastly, failure to utilize miR-33a-5p mimic/inhibitor regulation experiments and ABCA1 manipulation assays is another limitation, as these studies could offer stronger evidence that LP-pXMoA5p provides atheroprotection from ABCA1 cellular upregulation through downregulation of miR-33a-5p.

6. Conclusions

Our study shows that LP-pXMoA5p has the capacity to enhance ABCA1-dependent cholesterol efflux within MLC *in vitro*, and this effect is likely from anti-miR-33a-5p transfer to these cells that is facilitated by exosomes secreted basolaterally by pro-inflammatory endothelial cells. This outcome, combined with our earlier report, which exhibited similar effects in cultured macrophages when utilizing LP-pXMoA5p [61], demonstrates proof-of-principle that our unique method of delivering anti-miR-33a-5p to intimal cells may result in atheroprotection. Therefore, future studies should eventually be conducted to directly test whether LP-pXMoA5p is capable of treating atherosclerosis in atherogenic animal models by removing excess cholesterol from arteries through intimal cell ABCA1 derepression.

Availability of Data and Materials

The data used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

AS; conceptualization. JEC, RRP, TB, LV, HJ, and AS; methodology. SD, KH, RRP, TB, LV, and AS; formal analysis. SD, JEC, AP, AB, KH, RRP, and LV; investigation. TB, LV, HJ, and AS; resources. SD, RRP, and AS; manuscript draft preparation. RRP and AS; manuscript review and editing. TB, LV, HJ, and AS; supervision. AS; funding acquisition. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

Funding

This research was funded in part by an NIH/NHLBI R15 Academic Research Enhancement Award (AREA); grant number 1R15HL152261-01A1. This work was also supported by a USDA/NIFA-Hatch Project; Project Number SC-1700690 (Accession Number: 7008395).

Conflicts of Interest

The authors declare no conflicts of interest.

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