Published in final edited form as:

J Control Release. 2018 December 10; 291: 106–115. doi:10.1016/j.jconrel.2018.10.015.

PECAM-1 directed re-targeting of exogenous mRNA providing two orders of magnitude enhancement of vascular delivery and expression in lungs independent of apolipoprotein E-mediated uptake

Hamideh Parhiz^{a,*}, Vladimir V. Shuvaev^a, Norbert Pardi^b, Makan Khoshnejad^a, Raisa Yu Kiseleva^a, Jacob S. Brenner^a, Thomas Uhler^a, Steven Tuyishime^b, Barbara L. Mui^c, Ying K. Tam^c, Thomas D. Madden^c, Michael J. Hope^c, Drew Weissman^{b,*}, and Vladimir R. Muzykantov^{a,*}

^aDepartment of Pharmacology, The Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

^bDepartment of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

^cAcuitas Therapeutics, Vancouver, BC V6T 1Z3, Canada

Abstract

Systemic administration of lipid nanoparticle (LNP)-encapsulated messenger RNA (mRNA) leads predominantly to hepatic uptake and expression. Here, we conjugated nucleoside-modified mRNA-LNPs with antibodies (Abs) specific to vascular cell adhesion molecule, PECAM-1. Systemic (intravenous) administration of Ab/LNP-mRNAs resulted in profound inhibition of hepatic uptake concomitantly with ~200-fold and 25-fold elevation of mRNA delivery and protein expression in the lungs compared to non-targeted counterparts. Unlike hepatic delivery of LNP-mRNA, Ab/LNP-mRNA is independent of apolipoprotein E. Vascular re-targeting of mRNA represents a promising, powerful, and unique approach for novel experimental and clinical interventions in organs of interest other than liver.

Keywords

Vascular targeting; mRNA delivery; Apolipoprotein E; Inflammation; Endothelial targeting

*Corresponding authors. parhizh@pennmedicine.upenn.edu (H. Parhiz), dreww@upenn.edu (D. Weissman), muzykant@mail.med.upenn.edu (V.R. Muzykantov).

Conflict of interest

H.P., V.V.S., D.W., and V.R.M. are inventors on a patent filed on some aspects of this work. Those interests have been fully disclosed to the University of Pennsylvania.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jconrel.2018.10.015>.

1. Introduction

Messenger RNA (mRNA)-based therapeutic approaches emerged as alternative treatment options in the fields of vaccination, protein replacement therapy, and cellular reprogramming [1,2]. One of the most promising delivery platforms is nucleoside-modified and purified mRNA encapsulated in lipid nanoparticles (LNPs) [3]. Nucleoside modification and HPLC purification of the mRNA are important to increase protein production *in vivo* and eliminate inflammatory responses after administration [4,5]. LNPs containing ionizable amino lipids are employed to pack mRNA and protect cargo en route to the site of action [6]. We and others have recently demonstrated that administration of antibody-encoding mRNA-LNPs resulted in high levels of functional antibodies that protected mice from infectious pathogens [7,8], and toxins [8], as well as increased tumor clearance in murine models [8,9]. In addition to potency, mRNA has several beneficial features over other therapeutic delivery platforms, such as the inability to integrate into the host genome, and transient and controllable translation in cells.

Organ and cell type-specific delivery of mRNA after systemic administration is a major challenge. Upon systemic delivery, mRNA-LNPs mainly target the liver due to their ability to bind apolipoprotein E (apoE) and target apoE receptors on the surface of hepatocytes [10]. Coupling to the surface of nanocarriers affinity ligands, for example, antibodies to specific target molecules provides an alternative approach for targeted delivery. Affinity targeting may provide more precise control of distribution in an organ and destination in the target cells [11].

Endothelial cells lining the vascular lumen represent targets for pharmacological interventions in many cardiovascular, neurological, and pulmonary conditions [11–14]. Endothelial targeting of diverse agents and carriers to the pulmonary, cerebrovascular, and other vascular areas has been achieved using antibodies and other affinity ligands that bind endothelial surface determinant CD31 (aka platelet-endothelial cell adhesion molecule-1 (PECAM-1), among others [15–20]. Here, we describe potent vascular targeting of nucleoside-modified and fast protein liquid chromatography (FPLC)-purified mRNA to the lungs using LNP-mRNA-coupled PECAM-1 antibodies in mice.

2. Materials and methods

2.1. Ethics statement

The investigators faithfully adhered to the “Guide for the Care and Use of Laboratory Animals” by the Committee on Care of Laboratory Animal Resources Commission on Life Sciences, National Research Council. The animal facilities at the University of Pennsylvania are fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC). All studies were conducted under protocols approved by the University of Pennsylvania IACUC.

2.2. Reagents

N-succinimidyl *S*-acetylthioacetate (SATA) was purchased from Pierce Biotechnology (Rockford, IL). Radioactive isotope ^{125}I was purchased from Perkin-Elmer (Wellesley, MA).

Whole molecule rat IgG was from ThermoFisher (Waltham, MA). Anti-mouse-PECAM-1/CD31 monoclonal antibody was obtained from BioLegend (San Diego, CA). Monoclonal antibodies to human PECAM-1 (anti-PECAM, Ab62) were kindly provided by Dr. Marian Nakada (Centocor) [21]. All chemical reagents were purchased from Sigma Aldrich, unless stated otherwise.

2.3. Cell culture

Human mesothelioma REN cells, either stably expressing human PECAM-1 (REN-PECAM) or not (REN wild type), have been previously described [22–25]. REN cells were maintained in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Life Technologies, Carlsbad, CA). Maintenance media for REN-PECAM cells also contained Geneticin (G418) at 200 µg/mL, as a selection antibiotic.

Human umbilical vein endothelial cells (HUVECs), purchased at passage 1 from Lonza (Walkersville, MD) were subcultured up to six passages in endothelial basal medium (EBM) supplemented with EGM-bulletkit (Lonza). Passages between 4 and 6 were used throughout the studies.

2.4. mRNA production and formulation into lipid nanoparticles

mRNAs were produced, as described previously [26], using T7 RNA polymerase (Megascript, Ambion) on linearized plasmids encoding codon-optimized firefly luciferase (pTEV-Luc2-A101) and eGFP (pTEV-eGFP-A101). To make modified nucleoside-containing mRNA, m¹ψ-5′-triphosphate (TriLink) was incorporated instead of UTP. mRNAs were transcribed to contain 101 nucleotide-long poly(A) tails. They were capped using the m7G capping kit with 2′-O-methyltransferase (ScriptCap, CellScript) to obtain cap1. mRNA was purified by Fast Protein Liquid Chromatography (FPLC) (Akta Purifier, GE Healthcare) [27]. All prepared RNAs were analyzed by electrophoresis using denaturing or native agarose gels and stored at −20 °C.

FPLC-purified m¹ψ-containing firefly luciferase and eGFP-encoding mRNAs were encapsulated in LNPs using a self-assembly process in which an aqueous solution of mRNA at pH = 4.0 is rapidly mixed with a solution of lipids dissolved in ethanol [28]. LNPs used in this study were similar in composition to those described previously [28,29], which contain an ionizable cationic lipid (proprietary to Acuitas)/ phosphatidylcholine/cholesterol/PEG-lipid (50:10:38.5:1.5 mol/mol) and were encapsulated at an RNA to total lipid ratio of ~0.05 (wt/wt). They had a diameter of ~80 nm as measured by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) instrument. mRNA-LNP formulations were stored at −80 °C at a concentration of mRNA of ~1 µg/µL.

2.5. Preparation and characterization of targeted lipid nanoparticles

LNPs were conjugated with mAb specific for PECAM-1. Targeting antibodies or control isotype-matched IgG were conjugated to LNP particles *via* SATA–maleimide conjugation chemistry [30]. The LNP construct was modified with maleimide functioning groups (DSPE-PEG-mal) by a post-insertion technique with minor modifications [31]. The antibody

was functionalized with SATA (N-succinimidyl *S*-acetylthioacetate) (Sigma-Aldrich) to introduce sulfhydryl groups allowing conjugation to maleimide. SATA was deprotected using 0.5 M hydroxylamine followed by removal of the unreacted components by G-25 Sephadex Quick Spin Protein columns (Roche Applied Science, Indianapolis, IN). The reactive sulfhydryl group on the antibody was then conjugated to maleimide moieties using thioether conjugation chemistry. Purification was carried out using Sepharose CL-4B gel filtration columns (Sigma-Aldrich). mRNA content was calculated by performing a modified Quant-iT RiboGreen RNA assay (Invitrogen).

Size and surface charge of the mRNA containing lipid nanoparticles was determined using dynamic light scattering (DLS) and laser doppler velocimetry (LDV), respectively on a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). For size measurements, LNPs were diluted in PBS pH 7.4 at 25 °C in disposable capillary cuvettes. A non-invasive back scatter system (NIBS) with a scattering angle of 173° was used. Diameters of unconjugated and antibody-modified particles were interpreted as normalized intensity size distribution as well as z-average values for particle preparations. Zeta potential measurements were also carried out in PBS buffer using disposable folded capillary cells. To assess the stability of antibody-modified LNPs, the particles were incubated in a range of 0–1000 mM NaCl solution and their hydrodynamic diameters were measured on DLS.

Morphologic characterization was carried out on a JEOL1010 transmission electron microscope (TEM), as described [19]. Briefly, carbon-coated 200-mesh copper grids were placed on a drop of the sample for 2 min and washed with Milli-Q water. Negative staining was done using 2% uranyl acetate. The stain was then wicked off with a filter paper and the grids were dried and imaged at an acceleration voltage of 120 K.

2.6. In vitro cell binding assay with radiolabeled particles

LNPs were first radiolabeled with Na¹²⁵I using Iodination Beads (Pierce). The reaction was performed for 15 min at room temperature. Unreacted materials were then removed by Quick Spin Protein Columns (G-25 Sephadex, Roche Applied Science, Indianapolis, IN) [19]. Anti PECAM-1 targeted LNP antibody conjugation was evaluated by incubation on REN-PECAM-1 cells, which stably express PECAM-1. Wildtype REN cells, a human mesothelial cell line that has no endogenous expression of PECAM-1, was tested in parallel to assess non-specific binding of particles. REN cells were incubated with increasing quantities of LNPs for one hour at room temperature. Incubation medium was then removed and cells were washed with PBS buffer three times to remove the unbound nanoparticles from the cell surface. The cells were lysed with 1% Triton X100 in 1 N NaOH and the cell-associated radioactivity was measured by a Wallac 1470 Wizard gamma counter (Gaithersburg, MD) and compared to total added activity.

2.7. In vitro cell transfection with reporter mRNA-loaded LNPs

REN cells were seeded in 48-well plates. After 18 h, LNPs carrying reporter firefly luciferase mRNA were added at increasing concentrations to the cells, and incubated for 1.5 h. Plates were then washed three times with PBS and complete medium was added to the cells. After culturing for 24 h in complete media, cells were washed with PBS, lysed in

luciferase cell culture lysis reagent (Promega, Madison, WI) and the firefly luciferase enzymatic activity as luminescence (Luciferase assay system, Promega) was measured. Transfections were performed in triplicate.

For fluorescence microscopy, REN cells or HUVECs were plated at 150,000 cells per well in 24-well plates. At ~70% cell confluence, LNPs carrying eGFP mRNA were added to the media as 6 µg mRNA per well and cells were incubated for 18 h. The level of eGFP production was then evaluated by imaging the cells under an EVOS-FL imaging system (ThermoFisher scientific, Waltham, MA).

2.8. Cell metabolic activity (MTS assay)

Cell metabolic activity (MTS assay) was performed on REN cells after incubating with LNPs. Cells were seeded at the same density as luciferase assay and were incubated with a relevant concentration range of particles for 4.5, 24, and 48 h. MTS reagent was added to the wells based on manufacturer's recommendation (MTS Assay Kit, abcam, Cambridge, MA), incubated for 2 h at 37 °C and the absorbance was read at 490 nm.

2.9. Detection of expression of inflammation marker, endothelial vascular cell adhesion molecule (VCAM)

Cell lysates were first run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 4–15% gradient gel (Mini- PROTEAN® TGXTM Gel, Bio-Rad, Hercules, CA). After gel transfer to PVDF membrane (Millipore, Billerica, MA), membrane was blocked with 3% nonfat dry milk in TBS-T (100 mM Tris, pH 7.5; 150 mM NaCl; 0.1% Tween 20) and was incubated with the corresponding antibodies. The blot was detected using ECL reagents (GE Healthcare, New York, NY, USA).

2.10. Pharmacokinetics/biodistribution studies upon intravenous injection of radiolabeled LNP-mRNAs in mice

Radiolabeled LNP-mRNAs were administered by retro-orbital injection in C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME). Animals were sacrificed at 5, 15, 30, and 60 min post-injection and their blood was collected from the inferior vena cava. Organs (liver, spleen, lung, kidney, and heart) were harvested, rinsed with saline, blotted dry, and weighed. Tissue radioactivity in organs and 100-µL samples of blood was measured in a gamma counter (Wallac 1470 Wizard gamma counter, Gaithersburg, MD). Radioactivity values and weight of the samples were then used to calculate targeting parameters of nanoparticles, including tissue uptake as percent of injected dose per gram tissue (%ID/g), and localization ratio (LR) as organ-to-blood ratio. Immunospecificity index (ISI) was also calculated as the ratio of the LR of targeted particles to that of non-targeted (IgG) control. These parameters were employed to discuss biodistribution and effectiveness of antibody-targeted formulation uptake in desired tissue.

2.11. Functional activity –firefly luciferase transfection in vivo

Mice (The Jackson Laboratory, Bar Harbor, ME) were intravenously injected with unconjugated or antibody-conjugated LNP-mRNA formulations. At desired time points,

animals were euthanized and all the vital organs were resected, washed with PBS, and stored at -80°C until analysis.

Organ samples were homogenized in 1 mL of cell lysis buffer (1 $\times$) (Promega Corp, Madison, WI) containing protease inhibitor cocktail (1 $\times$) and mixed gently at 4°C for one hour. The homogenates were then subjected to cycles of freeze/thaw in dry ice/ 37°C . The resulting cell lysate was centrifuged for 10 min at 16,000 g at 4°C . Luciferase activity was assayed in the supernatant using a Victor³ 1420 Multilabel Plate Counter (Perkin Elmer, Wellesley, MA).

2.12. Cytokine measurement

Mice (The Jackson Laboratory, Bar Harbor, ME) were intravenously injected with antibody-conjugated LNP-mRNA formulations as described above. At 4.5 h post-treatment, animals were euthanized and blood samples were collected in heparin and spun at 1500 $\times$ g for 10 min at 4°C .

Livers were also collected and homogenized in 1 mL of PBS containing protease inhibitor cocktail (1 $\times$). Lysis buffer was added to the homogenates and mixed at 4°C for one hour. The cell lysate was centrifuged for 10 min at 16,000 g at 4°C . The levels of macrophage inflammatory protein 2 (MIP-2) cytokine in liver and Interleukin 6 (IL-6) in plasma were assessed by ELISA according to manufacturer's protocol (DuoSet ELISA kits, R and D systems, Minneapolis, MN).

2.13. Bioluminescence imaging

Bioluminescence imaging was performed as described previously [3] using an IVIS Spectrum imaging system (Caliper Life Sciences, Waltham, MA). Mice were administered an intraperitoneal injection of D-luciferin at a dose of 150 mg/kg. After 5 min, the mice were euthanized; organs were quickly harvested, and placed on the imaging platform. Organ luminescence was measured on the IVIS imaging system using an exposure time of 5 s or longer to ensure that the signal obtained was within operative detection range. Bioluminescence values were also quantified by measuring photon flux (photons/s) in the region of interest using LivingImage software provided by Caliper.

2.14. Flow cytometry

LNPs containing DIO-tagged lipids were conjugated to antibodies, as described above. 30 min after IV administration of antibody-modified fluorescent LNPs into mice, the lungs were perfused and harvested. Briefly, the lungs were first digested and cell suspensions were passed through 100 μm nylon strainer. To lyse RBCs, ACK lysis buffer (Quality Biological, Gaithersburg, MD) was used. Anti-CD31, anti-CD45, and anti-F4/80 were used for staining endothelial cells, leukocytes, and macrophages/monocytes, respectively. Flow cytometry was performed on Accuri C6 instrument (Becton Dickinson, San Jose, CA).

2.15. Statistical analysis

Unless specified otherwise, the data have been calculated and presented as mean $\pm$ standard error of mean (SEM). When comparing two groups, a Student's *t*-test was used assuming a

Gaussian distribution with unequal variances. All probability values are two-sided, and values of $p < 0.05$ were deemed statistically significant.

3. Results and discussion

3.1. Characterization of targeted LNP-mRNAs

Ab/LNP-mRNA complexes assembled as illustrated (Fig. 1A) have physicochemical properties generally similar to those of unconjugated LNP-mRNAs. Dynamic light scattering revealed a hydrodynamic diameter of 82.5 ± 1.8 nm with narrow size distribution ($PDI = 0.062$) for unconjugated LNP-mRNA. Upon coupling antibody to LNPs, the mean z-average of particles increased up to ~ 100 nm (for example, 101.9 ± 0.7 nm and 103.3 ± 0.2 nm for IgG/LNP-mRNA and PECAM-1 Ab/LNP-mRNA, respectively, with $PDI \sim 0.2$) (Fig. 1B and C). Incubation of antibody-conjugated LNPs in a range of ionic strength solutions did not affect the particle size (Supplementary Fig. 1) demonstrating robust stability of antibody-conjugated LNPs. Moreover, conjugation of IgG and antibodies did not affect the zeta-potential and morphology of the nanoparticles (Fig. 1C, D, and E). Transmission electron microscopic analyses of unconjugated (Fig. 1D) and PECAM-1-conjugated LNP-mRNAs (Fig. 1E) demonstrated maintenance of morphology.

There are ~ 2 mRNAs per LNP. To evaluate antibody conjugation efficiency to LNPs, radiolabeled antibodies were monitored throughout conjugation steps. Quantified radioactivity of the conjugated antibody to LNPs, corresponded to ~ 80 antibody molecules per particle.

3.2. Targeting to endothelial cells in vitro

CD31 (PECAM-1) is mainly expressed by the endothelium and is mostly localized on the endothelial intercellular junctions [32]. PECAM-targeted Ab/LNP-mRNA bound to REN cells transfected with human PECAM-1 (REN-PECAM [21]), but not to wild type REN cells (Fig. 2A). This led to the PECAM-dependent expression of the reporter proteins firefly luciferase (Fig. 2B) and enhanced green fluorescent protein (eGFP) (Fig. 2C and Supplementary Fig. 2) encoded by the mRNA.

3.3. Targeting of mRNA-LNPs to vascular endothelium after systemic delivery in mice

Next, we studied tissue binding of LNP-mRNA formulations directly labeled with ^{125}I prior to antibody conjugation, by determining the percent of injected dose per gram of tissue ($\%ID/g$) in mice after intravenous administration. Consistent with previous reports [3,28], unconjugated LNP-mRNAs accumulated mainly in the liver (Fig. 3A) and to a lesser extent in the spleen. Comparing with pristine LNP-mRNA, IgG/LNP-mRNA showed higher level in blood and spleen, which squares well with concomitant reduction of IgG/LNP-mRNA level in liver (Fig. 3A). It is likely that elevation of blood level due to direct or/ and indirect inhibition of hepatic uptake by IgG coat allows IgG/LNPmRNA to accumulate in the organs and cells that otherwise are outcompeted by massive uptake in liver. On the other hand, since IgG/ LNP-mRNA has no affinity to the major target such as endothelium, there is no blood depletion unlike anti PECAM/LNP-mRNA that exerts prominent accumulation in the lungs.

In accordance with the literature, PECAM-1 provided targeting for vascular delivery to the lungs [19,33,34]. Pulmonary uptake of PECAM-1 targeted Ab/LNP-mRNA reached $105.03 \pm 3.5\%$ ID/g (Fig. 3A), providing a 16-fold increase compared to IgG/LNP-mRNA. To compare Ab/LNP-mRNA with IgG/LNP-mRNA, we calculated an immunospecificity index (ISI, the ratio of the %ID/g of these formulations in a given organ, normalized to blood level). According to this index, comparing these two nearly identical formulations, targeting affords ~200-fold enhancement of delivery to the pulmonary vasculature (Fig. 3B).

Kinetic studies revealed that ^{125}I -labeled unconjugated LNP-mRNA was quickly cleared from blood, reflecting uptake in liver and spleen (Fig. 3E). Blood clearance of PECAM-Ab/LNP-mRNA was even more expeditious, but reflected mostly pulmonary uptake (Fig. 3C). Specific lung uptake of PECAM-Ab/LNP-mRNA was rapid and sustained through the last time point studied (60 min post-injection) (Fig. 3C).

To assess the cellular distribution of PECAM-1 targeted LNPs *in vivo*, DIO-labeled LNPs were conjugated to anti-PECAM-1 and injected i.v. in mice. Flow cytometry was then performed on the cell suspension obtained from lung homogenates. Cells were co-stained with antibodies against CD31, an endothelial cell marker; CD45, a leukocyte marker; and F4/80 a monocyte/macrophage marker. Only a small percentage (4%) of total cells recovered from the lung were stained positive for CD31 (Fig. 4A). However, 100% of the recovered endothelial cell population were positive for LNPs, showing high uptake of targeted particles by endothelial cells.

3.4. In vitro and in vivo toxicity/inflammatory studies

To evaluate the effect of unconjugated and Ab/conjugated LNPs on cellular metabolic activity, MTS assays were performed on REN cells upon LNP treatment. As shown in Fig. 5A₁₋₃, incubation of cells with LNPs for up to 48 h did not lower the viability of cells below 90% for any of the constructs at tested concentrations.

Pro-inflammatory conditions can induce the expression of leukocyte adhesion molecules such as VCAM-1 and ICAM-1 [35,36]. To assess if LNP-mRNA treatment might induce pro-inflammatory markers, we analyzed VCAM expression on HUVECs after incubation with LNPs. Cell lysates from HUVECs were collected 4.5 h after LNP incubation. The expression of VCAM was analyzed by western blot (Fig. 5B). LNP-mRNA treatment did not induce VCAM expression, while the positive control (LPS, 0.5 $\mu\text{g/mL}$) showed strong VCAM expression.

To determine if LNP-mRNA treatment might affect pro-inflammatory cytokine profile *in vivo*, we collected plasma and liver tissues from mice 4.5 h after i.v. administration of LNP-mRNA (8 $\mu\text{g/ mouse}$). Plasma or liver tissue from mice challenged with intravenous LPS (2 mg/kg) was used as a positive control. IL-6 in plasma and MIP-2 in liver did not elevate upon LNP-mRNA treatment *in vivo* (Fig. 5C), when compared to untreated mice.

3.5. Tissue transfection pattern after i.v. administration of targeted LNP-mRNA

Protein expression with mRNA delivery is expected to peak around 4–5 h after i.v. injection [3]. Therefore, 4.5 h following injection, we used bioluminescence and luciferase assay to

investigate the biodistribution and levels of protein expression from the delivered mRNA. Retro-orbital injection of 8 μ g (0.32 mg/kg) of unconjugated LNP-mRNA led to firefly luciferase expression mainly in liver and at lower level in spleen (Fig. 6A and B). PECAM-targeted Ab/LNP-mRNA, but not IgG/LNP-mRNA showed profound and specific protein expression in the lung concomitant with reduced hepatic expression, providing an ~25 fold elevation of the reporter protein signal in the lungs compared to untargeted counterparts (Fig. 6A and C). The lung/liver ratio for Ab/ LNP-mRNA increased ~200 and 50-fold compared to unconjugated LNP-mRNA and IgG/LNP-mRNA, respectively (Fig. 6D). Dose escalation studies demonstrated that within the range of injected doses, expression of the signal does not reach saturation (Fig. 6E). This aligns well with the known high level of surface expression of PECAM in endothelial cells [32,37,38].

We further characterized the time course of mRNA translation using luciferase mRNA containing LNPs. Four time points, 1, 4.5, 24, and 96 h post-injection were chosen, based on previous studies [3]. It is notable that all three formulations, unconjugated-, IgG-, and anti-PECAM/LNP-mRNAs reached their maximal expression after 4.5 h and declined slowly in the next 24 h (Fig. 7).

3.6. Ab/LNP-mRNA targeting is independent of the apoE pathway(s) involved in hepatic delivery of untargeted LNP-mRNA

Interaction with various compounds in blood modulates the behavior of nanocarriers [39]. For example, apolipoprotein E (apoE) mediates hepatic uptake of LNP-mRNA [10]. Here, we demonstrated in apoE^{-/-} mice that affinity moieties like anti-PECAM could maintain targeting to desired tissues, lung, in the absence of apoE. Indeed, the hepatic luminescence signal measured after firefly luciferase mRNA-LNP delivery, was an order of magnitude lower in Apo-E^{-/-} mice compared to wild type animals (Fig. 8). Similarly, lower signal intensities were observed in other organs (Supplementary table 1). One potential explanation for the observation that apo E KO inhibits hepatic uptake without redistribution to other organs is that LNPs apparently have no or little affinity to other organs. For this reason, clearance mechanisms outcompete inefficient distribution of LNPs from blood to the organs in apoE KO mice. In sharp contrast, anti-PECAM/LNPs that have affinity to endothelium, do accumulate in the lungs of KO mice. No significantly reduced firefly luciferase activity in the lungs of apoE^{-/-} mice was observed after PECAM-1 targeted Ab/LNP-mRNA delivery compared to wild-type animals (Fig. 8). This further highlights the significance of targeting moieties in mitigating the influence of endogenous serum components like apoE from averting the transfection from desired tissue to liver.

4. Conclusion

Systemic mRNA delivery using antibody-coupled nucleoside-modified mRNA-LNPs provides highly effective vascular immunotargeting to target organs other than the liver in mice. Our targeted delivery platform was designed with one of the most efficient LNP-mRNA systems developed, thus far, that uses modified nucleosides to decrease innate immune activation and increase protein translation from mRNA *in vivo* [4,7,40]. Specific, rapid, and transient protein expression from firefly luciferase-encoding mRNA was

measurable in the lungs at a time window of 4–24 h, with limited off-target biodistribution. Flow cytometry analysis showed efficient uptake in endothelial cells. Notably, we demonstrated that antibody-targeted LNP-mRNAs is independent of the apo-E mediated uptake pathway(s). These studies provide the basis for development of targeted delivery systems for mRNA therapeutics in pulmonary conditions, including acute lung injury, pulmonary hypertension, and beyond. Taking into consideration the diversity and extension of endothelial phenotypes in the vascular system, and the pivotal roles played by these cells under physiologic and pathologic conditions, we believe that targeting strategies for vascular delivery of mRNA will find widespread medical utility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

H.P. acknowledges fellowship through NIH (NHLBI T32 HL007915). V.R.M. received funding from NIH (NHLBI R01 HL128392). D.W. was supported by the NIAID of the NIH under award numbers R01-AI124429 and R01-AI084860, and an amfAR ARCHE grant.

References

- [1]. Sahin U, Kariko K, Tureci O, mRNA-based therapeutics-developing a new class of drugs, *Nat. Rev. Drug Discov* 13 (2014) 759–780. [PubMed: 25233993]
- [2]. Pardi N, Hogan MJ, Porter FW, Weissman D, mRNA vaccines — a new era in vaccinology, *Nat. Rev. Drug Discov* (2018), 10.1038/nrd.2017.243.
- [3]. Pardi N, Tuyishime S, Muramatsu H, Karikó K, Mui BL, Tam YK, Madden TD, Hope MJ, Weissman D, Expression kinetics of nucleoside-modified mRNA delivered in lipid nanoparticles to mice by various routes, *J. Control. Release* 217 (2015) 345–351, 10.1177/1948550614552729.Behavior. [PubMed: 26264835]
- [4]. Karikó K, Muramatsu H, Welsh FA, Ludwig J, Kato H, Akira S, Weissman D, Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability, *Mol. Ther* 16 (2008) 1833–1840, 10.1038/mt.2008.200. [PubMed: 18797453]
- [5]. Karikó K, Muramatsu H, Ludwig J, Weissman D, Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA, *Nucleic Acids Res.* (2011), 10.1093/nar/gkr695.
- [6]. Kauffman KJ, Webber MJ, Anderson DG, Materials for non-viral intracellular delivery of messenger RNA therapeutics, *J. Control. Release* 240 (2016) 227–234, 10.1016/j.jconrel.2015.12.032. [PubMed: 26718856]
- [7]. Pardi N, Secreto AJ, Shan X, Debonera F, Glover J, Yi Y, Muramatsu H, Ni H, Mui BL, Tam YK, Shaheen F, Collman RG, Karikó K, Danet-Desnoyers GA, Madden TD, Hope MJ, Weissman D, Administration of nucleoside-modified mRNA encoding broadly neutralizing antibody protects humanized mice from HIV-1 challenge, *Nat. Commun* 8 (2017) 14630, 10.1038/ncomms14630. [PubMed: 28251988]
- [8]. Thran M, Mukherjee J, Pönisch M, Fiedler K, Thess A, Mui BL, Hope MJ, Tam YK, Horscroft N, Heidenreich R, Fotin-Mleczek M, Shoemaker CB, Schlake T, mRNA mediates passive vaccination against infectious agents, toxins, and tumors, *EMBO Mol. Med* 9 (2017) e201707678, 10.15252/emmm.201707678.
- [9]. Stadler CR, Bahr-Mahmud H, Celik L, Hebich B, Roth AS, Roth RP, Kariko K, Tureci O, Sahin U, Elimination of large tumors in mice by mRNA-encoded bispecific antibodies, *Nat. Med* 23 (2017) 815–817 10.1038/nm.4356. [PubMed: 28604701]

- [10]. Akinc A, Querbes W, De S, Qin J, Frank-Kamenetsky M, Jayaprakash KN, Jayaraman M, Rajeev KG, Cantley WL, Dorkin JR, Butler JS, Qin L, Racie T, Sprague A, Fava E, Zeigerer A, Hope MJ, Zerial M, Sah DWY, Fitzgerald K, Tracy MA, Manoharan M, Kotliansky V, De Fougères A, Maier MA, Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms, *Mol. Ther* 18 (2010) 1357–1364, 10.1038/mt.2010.85. [PubMed: 20461061]
- [11]. Shuvaev VV, Brenner JS, Muzykantov VR, Targeted endothelial nanomedicine for common acute pathological conditions, *J. Control. Release* 219 (2015) 576–595, 10.1016/j.jconrel.2015.09.055. [PubMed: 26435455]
- [12]. Aird W, The role of the endothelium in severe sepsis and multiple organ dysfunction syndrome, *Blood* 101 (2003) 3765–3777, 10.1182/blood2002-06-1887 (Supported). [PubMed: 12543869]
- [13]. Maniatis NA, Orfanos SE, The endothelium in acute lung injury/acute respiratory distress syndrome, *Curr. Opin. Crit. Care* 14 (2008) 22–30. [PubMed: 18195622]
- [14]. Thorpe PE, Vascular targeting agents as cancer therapeutics, *Clin. Cancer Res.* 10 (2004) 415–427. [PubMed: 14760060]
- [15]. Han J, Shuvaev VV, Muzykantov VR, Targeted interception of signaling reactive oxygen species in the vascular endothelium, *Ther. Deliv* 3 (2012) 263–276. [PubMed: 22834201]
- [16]. Howard M, Zern BJ, Anselmo AC, Shuvaev VV, Mitragotri S, Muzykantov V, Vascular targeting of nanocarriers: Perplexing aspects of the seemingly straight-forward paradigm, *ACS Nano* 8 (2014) 4100–4132, 10.1021/nn500136z. [PubMed: 24787360]
- [17]. Spragg DD, Alford DR, Greferath R, Larsen CE, Lee K-D, Gurtner GC, Cybulsky MI, Tosi P, Francois, Nicolau C, Gimbrone MA, Immunotargeting of liposomes to activated vascular endothelial cells: A strategy for site-selective delivery in the cardiovascular system, *Proc. Natl. Acad. Sci. U. S. A* 94 (1997) 8795–8800, 10.1073/pnas.94.16.8795. [PubMed: 9238057]
- [18]. Nowak K, Hanusch C, Nicksch K, Metzger RP, Beck G, Gebhard MM, Hohenberger P, Danilov SM, Pre-ischaemic conditioning of the pulmonary endothelium by immunotargeting of catalase via angiotensin-converting-enzyme antibodies, *Eur. J. Cardio-Thoracic Surg* 37 (2010) 859–863, 10.1016/j.ejcts.2009.10.029.
- [19]. Khoshnejad M, Shuvaev VV, Pulsipher KW, Dai C, Hood ED, Arguiri E, Christofidou-Solomidou M, Ivan J, Greineder CF, Muzykantov VR, Vascular accessibility of endothelial targeted ferritin nanoparticles, *Bioconjug. Chem.* 27 (2016) 628–637, 10.1021/acs.bioconjchem.5b00641. Vascular. [PubMed: 26718023]
- [20]. Albelda SM, Endothelial and epithelial cell adhesion molecules, *Am. J. Respir. Cell Mol. Biol* 4 (1991) 195–203. [PubMed: 2001288]
- [21]. Gurubhagavatula I, Amrani Y, Pratico D, Ruberg FL, Albelda SM, Panettieri RA, Engagement of human PECAM-1 (CD31) on human endothelial cells increases intracellular calcium ion concentration and stimulates prostacyclin release, *J. Clin. Invest* 101 (1998) 212–222, 10.1172/JCI269. [PubMed: 9421484]
- [22]. Sun J, Williams J, Yan H, Amin KM, Albelda SM, Delisser HM, Platelet endothelial cell adhesion molecule-1 (PECAM-1) homophilic adhesion is mediated by immunoglobulin-like domains 1 and 2 and depends on the cytoplasmic domain and the level of surface expression, *J. Biol. Chem* 271 (1996) 18561–18570. [PubMed: 8702505]
- [23]. Sun J, Paddock C, Shubert J, Zhang HB, Amin K, Newman PJ, Albelda SM, Contributions of the extracellular and cytoplasmic domains of platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31) in regulating cell-cell localization, *J. Cell Sci* 113 (2000) 1459–1469. [PubMed: 10725228]
- [24]. Delisser H, Chilkotowsky J, Yan H, Daisa M, Buck C, Sm A, Deletions in the cytoplasmic domain of platelet-endothelial cell adhesion molecule-1 (PECAM-1, CD31) result in changes in ligand binding properties, *J. Cell Biol.* 124 (1994) 195–203 <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2119897/>. [PubMed: 8294502]
- [25]. Jackson DE, Kupcho KR, Newman PJ, Characterization of phosphotyrosine binding motifs in the cytoplasmic domain of platelet / endothelial cell adhesion molecule-1 (PECAM-1) that are required for the protein-tyrosine phosphatase, SHP-2, *J. Biol. Chem* 272 (1997) 24868–24875. [PubMed: 9312087]

- [26]. Pardi N, Muramatsu H, Weissman D, Karikó K, In vitro transcription of long RNA containing modified nucleosides, in: Rabinovich PM (Ed.), *Synth. Messenger RNA Cell Metab. Modul. Methods Protoc*, Humana Press, Totowa, NJ, 2013, pp. 29–42., 10.1007/978-1-62703-260-5_2.
- [27]. Weissman D, Pardi N, Muramatsu H, Karikó K, HPLC purification of in vitro transcribed long RNA, in: Rabinovich PM (Ed.), *Synth. Messenger RNA Cell Metab. Modul. Methods Protoc*, Humana Press, Totowa, NJ, 2013, pp. 43–54., 10.1007/978-1-62703-260-5_3.
- [28]. Maier M. a, Jayaraman M, Matsuda S, Liu J, Barros S, Querbes W, Tam YK, Ansell SM, Kumar V, Qin J, Zhang X, Wang Q, Panesar S, Hutabarat R, Carioto M, Hettinger J, Kandasamy P, Butler D, Rajeev KG, Pang B, Charisse K, Fitzgerald K, Mui BL, Du X, Cullis P, Madden TD, Hope MJ, Manoharan M, Akinc A, Biodegradable lipids enabling rapidly eliminated lipid nanoparticles for systemic delivery of RNAi therapeutics, *Mol. Ther* 21 (2013) 1570–1578, 10.1038/mt.2013.124. [PubMed: 23799535]
- [29]. Jayaraman M, Ansell SM, Mui BL, Tam YK, Chen J, Du X, Butler D, Eltepu L, Matsuda S, Narayanannair JK, Rajeev KG, Hafez IM, Akinc A, Maier MA, Tracy MA, Cullis PR, Madden TD, Manoharan M, Hope MJ, Maximizing the potency of siRNA lipid nanoparticles for hepatic gene silencing in vivo, *Angew. Chem. Int. Ed. Engl* 51 (2012) 8529–8533, 10.1002/anie.201203263. [PubMed: 22782619]
- [30]. Howard MD, Hood ED, Greineder CF, Alferiev IS, Chorny M, Muzykantov V, Targeting to endothelial cells augments the protective effect of novel dual bioactive antioxidant/anti-inflammatory nanoparticles, *Mol. Pharm* 11 (2015) 2262–2270.
- [31]. Ishida T, Iden DL, Allen TM, A combinatorial approach to producing sterically stabilized (Stealth) immunoliposomal drugs, *FEBS Lett.* 460 (1999) 129–133, 10.1016/S0014-5793(99)01320-4. [PubMed: 10571074]
- [32]. Scherpereel A, Rome JJ, Wiewrodt R, Watkins SC, Harshaw DW, Alder S, Christofidou-Solomidou M, Haut E, Murciano JC, Nakada M, Albelda SM, Muzykantov VR, Platelet-endothelial cell adhesion molecule-1-directed immunotargeting to cardiopulmonary vasculature, *J. Pharmacol. Exp. Ther* 300 (2002) 777–786. [PubMed: 11861781]
- [33]. Muro S, Dziubla T, Qiu W, Leferovich J, Cui X, Berk E, Muzykantov VR, Endothelial targeting of high-affinity multivalent polymer nanocarriers directed to intercellular adhesion molecule 1, *J. Pharmacol. Exp. Ther* 317 (2006) 1161–1169, 10.1124/jpet.105.098970. [PubMed: 16505161]
- [34]. Bhowmick T, Berk E, Cui X, Muzykantov VR, Muro S, Effect of flow on endothelial endocytosis of nanocarriers targeted to ICAM-1, *J. Control. Release* 157 (2012) 485–492, 10.1016/j.jconrel.2011.09.067. [PubMed: 21951807]
- [35]. Shuvaev VV, Han J, Yu KJ, Huang S, Hawkins BJ, Madesh M, Nakada M, Muzykantov VR, PECAM-targeted delivery of SOD inhibits endothelial inflammatory response, *FASEB J.* (2011), 10.1096/fj.10-169789.
- [36]. Thomas SR, Witting PK, Drummond GR, Redox control of endothelial function and dysfunction: Molecular mechanisms and therapeutic opportunities, *Antioxid. Redox Signal.* (2008), 10.1089/ars.2008.2027.
- [37]. Ding B-S, Hong N, Christofidou-Solomidou M, Gottstein C, Albelda SM, Cines DB, Fisher AB, Muzykantov VR, Anchoring fusion thrombomodulin to the endothelial lumen protects against injury-induced lung thrombosis and inflammation, *Am. J. Respir. Crit. Care Med* 180 (2009) 247–256, 10.1164/rccm.200809-1433OC. [PubMed: 19342415]
- [38]. Scherpereel A, Wiewrodt R, Christofidou-Solomidou M, Gervais R, Murciano JC, Albelda SM, Muzykantov VR, Cell-selective intracellular delivery of a foreign enzyme to endothelium in vivo using vascular immunotargeting, *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol* (2001), 10.1096/fj.00-0022com.
- [39]. Semple SC, Chonn A, Cullis PR, Interactions of liposomes and lipid-based carrier systems with blood proteins: Relation to clearance behaviour in vivo, *Adv. Drug Deliv. Rev* 32 (1998) 3–17, 10.1016/S0169-409X(97)00128-2. [PubMed: 10837632]
- [40]. Karikó K, Buckstein M, Ni H, Weissman D, Suppression of RNA recognition by toll-like receptors: The impact of nucleoside modification and the evolutionary origin of RNA, *Immunity* 23 (2005) 165–175, 10.1016/j.immuni.2005.06.008. [PubMed: 16111635]

Fig. 1. Physicochemical characterization of targeted mRNA containing lipid nanoparticles. (A) Schematic illustration of the use of antibodies against endothelial cell surface markers for development of lung-targeted LNPs. Amino groups on antibodies were functionalized with heterobifunctional crosslinker (SATA) for introduction of thiol moieties on antibody surface followed by maleimide-thiol conjugation to maleimide-bearing LNP-mRNAs. (B) The average ($n = 3$) intensity size distribution curves for the unconjugated LNP-mRNA (gray trace) and antibody-conjugated LNP-mRNAs (black and red traces). (C) Particle size (z-average) and surface charge of particles measured using dynamic light scattering (DLS) and laser doppler velocimetry (LDV), respectively ($n = 3$). Images taken by transmission electron microscopy of (D) unconjugated LNP-mRNA, and (E) antibody-conjugated LNP-mRNA, scale bar: 100 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Binding and functional activity of targeted particles *in vitro*. (A) *In vitro* binding of targeted LNP-mRNAs to PECAM-1 positive and negative REN cells after 1 h incubation of 125 I-labeled anti-PECAM-1/LNP-mRNA with cells at RT (* $P < 0.05$). (B) mRNA encoded protein expression of anti-PECAM-1/LNP-mRNA in REN-PECAM-1 positive cells compared to control IgG/LNP-mRNA (# $P < 0.05$). The inset shows the luciferase activity for unconjugated LNP-mRNA. (C) *In vitro* eGFP expression of control IgG and anti-PECAM-1 conjugated eGFP-mRNA-LNPs in REN-PECAM-1 positive cells, 6 μ g mRNA per well.

Fig. 3.

Targeting of LNP-mRNA to PECAM-1 *in vivo*. (A) Biodistribution of ^{125}I -labeled anti-PECAM mAb- and control IgG-LNP-mRNAs in mice at 30 min. Tissue uptake is indicated as mean $\pm$ SEM ($n=3$). (* $P < 0.05$ and ** $P < 0.001$). (B) Immunospecificity index, calculated as the ratio of %ID/g of selected organs in mice treated with targeted (anti-PECAM-1) vs. non-targeted (control IgG)-LNP-mRNAs, normalized to blood levels. *In vivo* kinetics of LNP-binding as quantitative measurement of the percentage of PECAM-1-targeted (C), Control IgG- (D) and unconjugated (E) mRNA-loaded LNPs evaluated by radioactivity analysis in selected organs, after intravenous injection of nanoparticles.

Fig. 4. Flow cytometric analysis of cell populations receiving PECAM-1 targeted LNPs in lung tissue. Staining was performed against CD31 for endothelial cells, CD45 for leukocytes, and F4/80 for monocytes/macrophages. (A) Pie chart representative of total cell recovery from lung. (B) Percent of sub-cell populations positive for LNPs.

Fig. 5.

Cell toxicity/inflammatory profile of LNP-mRNA. Effect of anti-PECAM mAb-, control IgG-, and unconjugated LNP-mRNAs on REN cell viability measured by colorimetric MTS assay upon 4.5 h (A1), 24 h (A2), and 48 h (A3) incubation with LNPs. % Viability is indicated as mean $\pm$ SEM ($n = 3$). (B) Western blot showing HUVEC cell lysates (10 μ g total protein/lane) stained for human VCAM-1 and actin. An increase in VCAM-1 protein expression was induced by LPS, but not by LNP-mRNA treatment. Pro-inflammatory cytokines IL-6 in plasma (C) and MIP-2 in liver homogenate (D) upon treatment with LNP-mRNA (8 μ g/mouse) were compared to the untreated samples. LPS (2 mg/kg) was used as positive control here.

Fig. 6.

Organ distribution of firefly luciferase mRNA expression 4.5 h after intravenous administration of unconjugated, anti-PECAM-1 mAb- and control IgG/LNP-mRNAs demonstrated as (A) firefly luciferase activity and (B) luminescence imaging. (A) Quantitative expression as LU/mg protein values compared between non-targeted and targeted LNP. Data presented as mean $\pm$ SEM (n = 3), (*P < 0.05). (B) A representative sample set of mouse organs, which were analyzed 5 min after the administration of D-luciferin. (C) Transfection-specificity index, calculated as the ratio of luciferase activity in selected organs of mice treated with targeted (anti-PECAM-1) vs. non-targeted (control IgG)-LNP-mRNAs. (D) Lung to liver ratio, calculated as the ratio of transfection efficiency of lung to that of liver for each formulation. (E) Dose-response relationship of Luc mRNA containing anti-PECAM-1-LNPs. Mice received LNPs at doses of 1, 2, 4, and 8 μ g mRNA per mouse *via* intravenous administration. Selected organs were harvested at 4.5 h post-treatment and firefly luciferase activity was measured in tissue extracts.

Fig. 7.
In vivo kinetics of firefly luciferase expression following LNP-mRNA administration. Quantitative measurement of firefly luciferase activity in (A) liver and (B) lung upon intravenous injection of unconjugated- and anti-PECAM-1/LNP-mRNA; mRNA dose: 8 μ g/mouse.

Fig. 8.

Firefly luciferase mRNA expression in apoE knockout mice. Unconjugated, control IgG, and anti PECAM-1 Luc mRNA-LNPs were intravenously injected into mice. Mice were sacrificed 4.5 h after injection and firefly luciferase activity in livers and lungs of wild type mice was compared to apoE knockout mice. Data presented as mean $\pm$ SEM (n =3); (*P < 0.05).