Efficient Biodistribution and Gene Silencing in the Lung epithelium via Intravenous Liposomal Delivery of siRNA

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RNA interference (RNAi) may provide a therapeutic solution to many pulmonary epithelium diseases. However, the main barrier to the clinical use of RNAi remains the lack of efficient delivery vectors. Research has mainly concentrated on the intranasal route of delivery of short interfering RNA (siRNA) effector molecules for the treatment of respiratory diseases. However, this may be complicated in a disease state due to the increased fluid production and tissue remodeling. Therefore, we investigated our hydration of a freeze-dried matrix (HFDM) formulated liposomes for systemic delivery to the lung epithelium. Here, we show that 45 ± 2% of epithelial murine lung cells receive siRNA delivery upon intravenous (IV) liposomal administration. Furthermore, we demonstrate that liposomal siRNA delivery resulted in targeted gene and protein knockdown throughout the lung, including lung epithelium. Taken together, this is the first description of lung epithelial delivery via cationic liposomes, and provides a proof of concept for the use of IV liposomal RNAi delivery to specifically knockdown targeted genes in the respiratory system. This approach may provide an attractive alternate therapeutic delivery strategy for the treatment of lung epithelium diseases.

Introduction

The pulmonary system is the primary site of entry and establishment of many airborne viral and bacterial pathogens as well as the site for a number of diseases such as asthma, chronic obstructive pulmonary diseases, tumor metastasis, and cystic fibrosis. The discovery and development of RNA interference technologies has resulted in a promising new therapy for a variety of diseases of the respiratory tract.1

However, the main barrier to the use of RNAi targeting pulmonary diseases is the absence of efficient delivery vectors, particularly in the diseased state.2 The majority of studies have concentrated on the delivery of RNAi via inhalation as it has the benefits of being less invasive, avoids potential systemic toxicity and has increased specificity for the pulmonary system. However, intranasal delivery and inhalation techniques must overcome several biological barriers to enable sufficient delivery for target gene suppression. These barriers include the clearing sweeping motions of respiratory cilia as well as the mucous and pulmonary surfactant layer.3 In addition, tissue inflammation and extensive deposition of extracellular matrix might hamper delivery to the lower respiratory tract potentially limiting the therapeutic use of inhalation delivery systems. Indeed, the modest efficacy of inhaled siRNA in clinical trials for the treatment of respiratory syncytial virus has been suggested to result from inefficient delivery during acute infection.3,5

An alternative route for pulmonary delivery is the intravenous (IV) route. IV liposome siRNA delivery to the steady state pulmonary environment has successfully shown the delivery and knockdown of endothelial-specific genes.6,7 However, delivery to the lung epithelium via this method has historically been unsuccessful. Novel therapeutic strategies for the treatment of many diseases, such as viral infections or epithelial diseases would require efficient delivery to lung epithelial cells. Significant research has been conducted for the successful liposome delivery of RNAi to lung epithelial tumors.6,9 However, the disrupted vascular architecture in a tumor environment limits the comparisons that can be made to normal pulmonary architecture and thus delivery. Polyethyleneimines (PEI) have been used to protect siRNA molecules and achieve delivery to the lung epithelium via IV delivery.10 However, PEI has been associated with pro-inflammatory responses11–13 and toxicity upon multiple doses14 thus reducing its potential for clinical use. Therefore, a novel approach for IV delivery of liposomes to lung epithelium is warranted for the treatment of pulmonary diseases.

In this report, we investigated the potential of stealth liposomes to achieve RNAi delivery to the lungs following IV injection. Liposome uptake was noted in lung endothelial and epithelial cells as well as leukocytes. We subsequently investigated the ability of our lipid siRNA delivery to achieve targeted knockdown of gene and protein expression in the lung and analyzed silencing in epithelial cells. To our knowledge, this is the first report which describes the systemic delivery of siRNAs to the epithelial cells of the lungs via cationic liposomes.

Results

Characterization of lipid nanoparticles and evaluation of in vivo biodistribution

We previously described a novel method of liposome formulation, the hydration of a freeze-dried matrix (HFDM) method.15 We observed that IV injection of HFDM-formulated...
liposomes containing N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP), cholesterol, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), polyethylene glycol (PEG) 2000-C₁₆Ceramide (50:35:5:10 molar ratio), and a lipophilic dye, DiR, showed significant accumulation in the lung, liver and spleen (Figure 1a). Minimal delivery of HFDM-formulated liposomes was seen in other organs such as the kidney, heart and bladder (data not shown). Of delivery to the three main organs, we observed that 34 ± 4% of the HFDM-formulated PEGylated lipid particles localized to the lung with further distribution to the liver (55 ± 5%) and spleen (11 ± 1%) (Figure 1b). This is consistent with previous reports using PEG-containing delivery systems.\(^{16-20}\) It was unclear whether lung DiR accumulation resulted from liposome entrapment in the fine capillary beds, though further examination of delivery intensity throughout the lung (Figure 1a) suggested that the majority of delivery had uniform distribution. These observations lead us to investigate if these PEGylated liposomes could be used to deliver siRNA into the lung. We thus generated HFDM-prepared PEGylated siRNA-containing particles. Upon rehydration the size, polydispersity index and zeta potential of the resulting lipid particles, as well as the entrapment efficiency of siRNA were measured (Table 1). The results showed that the liposomes have desirable characteristics for \textit{in vivo} delivery including high entrapment efficiency and a size below 200 nm.\(^{15}\)

**Figure 1** Biodistribution of siRNA-loaded, DiR-labeled liposome nanoparticles after intravenous (IV) injection in mice. (a) Livers, spleens, and lungs were removed 48 hours after IV administration and analyzed on the fluorescent imager (\(\lambda_{\text{ex}} = 720\) nm; \(\lambda_{\text{em}} = 790\) nm). Scale shows intensity of DiR fluorescence. (b) Quantitative analysis of fluorescence intensity for each individual organ. Results are the mean ± SEM of \(n = 6\) from two independent experiments.

**Table 1** Characterization of siRNA-loaded PEGylated lipid particles

| Size (nm)\(^a\) | 190.25 ± 11.6 |
|-----------------|---------------|
| Polydispersity index | 0.326 ± 0.03 |
| Zeta potential (mV) | 52.1 ± 0.832 |

\(\text{a}\)Size represents \(Z_{\text{ave}}\pm \text{SD} \) as measured by Malvern Nano Zetasizer.

Each sample contained 40 µg siRNA in 300 µl isotonic sucrose solution. Three batches HFDM liposomes were analyzed (\(n = 3\)).

**Cellular distribution in the lung**

We examined which cell types in the lung were targeted and transfected by our HFDM liposomes. Mice were injected with DiR-labeled liposomes and at 48 hours lungs were removed and single cell suspensions were analyzed by flow cytometry.
Of total lung cells, 47 ± 3% were positive for the fluorescent dye, DiR (Figure 3a). We next defined the individual cell populations using cell-specific markers (Supplementary Figure S1). A high proportion (64 ± 10%) of CD146+ endothelial cells were DiR+ (Figure 3b). This observation was expected as the endothelial cells would be exposed to IV delivery of liposomes via the pulmonary vasculature. Surprisingly, we also observed that 45 ± 7% of CD326+ (epithelial cell adhesion molecule, EpCAM) cells were also positive for liposome delivery (Figure 3b). In humans, CD326 expression is restricted to epithelial cells, whereas in mice CD326 is expressed by epithelial cells and several leukocyte populations, including mononuclear phagocytes.29 To further define liposomal epithelial cell delivery, we used the hematopoietic marker CD45 to differentiate CD326+ CD45− epithelial cells from CD326+ CD45+ hematopoietic cells (Figure 3c,d). We observed that 23 ± 1% of CD326+ cells were CD45− indicating that they were part of the hematopoietic lineage. CD326−CD45− hematopoietic cells were 42 ± 6% positive for liposome delivery. The other 76 ± 2% of CD326− cells were CD45− indicating that they were lung epithelial cells. CD326−CD45− lung epithelial cells were 45 ± 1% positive for liposome delivery. Cationic liposomes have not previously been shown to transfect pulmonary epithelial cells, likely due to their tendency to aggregate, which we have shown does not occur in our particles due to PEGylation.15

Immune cells also take up HFDM liposomes
Given the propensity for macrophages and monocytes to take up liposomes, we subsequently investigated the ability of immune cells within the lung and lung vasculature to become transfected. We used CD11b/CD11c, B220, and CD3 markers to distinguish general populations of myeloid, B lymphocytes and T lymphocytes respectively. We observed that 35 ± 7% and 29 ± 4% of CD11b+ cells, 17 ± 3% of B220+ cells and 8 ± 1% of CD3+ cells were DiR+ (Figure 3c). In addition, CD45+ cells were 32 ± 1% positive for DiR liposome delivery (Figure 3e). As myeloid cells play a central role in the removal of foreign particles, we expected that the majority of systemically delivered particles would be taken up by these cells. In fact, cells of the myeloid lineage showed the highest liposomal delivery after IV injection in the spleen and liver (Supplementary Figure S2).

Ability to deliver to epithelial and endothelial cells of the lower respiratory tract
To further confirm that the liposomes were delivered to epithelial cells within the lung, we performed confocal immunofluorescent microscopy. By using this method, we could examine if delivery was achieved in the small alveoli, bronchi and connecting vasculature or if it was contained to the large airways and blood vessels of the pulmonary system. In addition, we were able to further examine epithelial...
specific delivery rather than immune cell delivery. Markers for endothelial (CD146) and epithelial (CD326) cells were used to costain frozen lung sections 48 hours after IV delivery of a single siRNA dose. Delivery to both epithelial and endothelial cells was confirmed with liposomes visualized in the small airways and blood vessels of the lower respiratory tract (Figure 4a). Upon higher magnification to further investigate CD326+ epithelial cell delivery, we observed colocalization of DiR-stained liposomes and epithelial cells with distribution of liposome throughout epithelial cell surface and cytoplasm (Figure 4d).

To exclude passive transfer of the DiR fluorescent lipophilic dye, unlabeled liposomes were loaded with AlexaFluor-750-labeled siRNAs to confirm direct delivery to target cells. Confocal microscopy confirmed the presence of AlexaFluor-750-siRNAs in both CD146+ endothelial and CD326+ epithelial cells of the lung and thus validated delivery to these cell populations in the lung (Figure 4b).

Figure 3 Liposomal delivery to lung cells. (a) Total lung cells were purified 48 hours after a single IV administration of unstained or DiR-stained liposomes. Cells were analyzed by flow cytometry. Delivery was quantified as a percentage of DiR+ cells/total cells. Data shown are mean ± SEM of n = 6 mice per group analyzed in two independent experiments. (b) Representative FACS histogram of unstained and DiR-stained liposomes are shown. (c) Individual cell populations were analyzed by flow cytometry for DiR fluorescence. Delivery was quantified as a percentage of DiR+ cells/total cells in the specific population. Control was mice treated with unstained liposomes. Data shown are mean ± SEM of n = 6 mice per group analyzed in two independent experiments. (d) Gating strategy for the analysis of CD45 and CD326+ cell populations by flow cytometry. (e) Analysis of the CD326+ cell population showing the level of DiR-stained liposome delivery to CD45− epithelial cells and CD45+ immune cells. Delivery was quantified as the percentage of total cells in the specific population. Data shown are mean ± SEM of n = 4 mice per group from two independent experiments.

Potent silencing of gene targets in the lung after IV injection
After demonstrating successful delivery of siRNA to the lung using the PEG-liposomes, we examined whether this delivery resulted in functional gene silencing. We evaluated reductions in mRNA and protein levels of the ubiquitously expressed Lamin A/C. Mice were injected IV with 2 mg/kg of siRNA (~40 µg per dose per mouse) contained in PEG-liposomes. mRNA and protein expression of Lamin A/C were determined 48 hours after injection. We compared the impact of a previously validated Lamin A/C specific siRNA versus animals injected with an identical formulation containing a control siRNA targeting either β-galactosidase or a scrambled control. 48 hours after single IV siRNA injection, we observed a significant reduction of Lamin A/C mRNA expression by 80 ± 0.1% in whole lung tissue (Figure 5a).

To examine the Lamin A/C protein, mice were injected with siRNA-containing PEG-liposomes on day 0 and 1 and protein expression of Lamin A/C in lungs was examined on day 4. This strategy was adopted as previous in vitro silencing of Lamin A/C indicated that significant reductions in protein levels were only seen after 48 hours. 48 hours after single IV siRNA injection, we observed a significant reduction of Lamin A/C mRNA expression at day 4.

Protein reductions in whole lung lysates from mice treated with the Lamin A/C specific siRNA indicated highly effective 55 ± 0.2% knockdown of Lamin A/C, thus corroborating the mRNA silencing results.
Gene silencing in epithelial and endothelial cells of the lung after IV injection

A key question for therapeutic gene silencing in the pulmonary system is whether silencing can be achieved in epithelial and endothelial cells that receive liposome delivery. Moreover, as epithelial cell delivery by cationic liposomes has not been previously reported, we sought to determine if siRNA was functional in these cells. Although the analysis of the entire pulmonary system showed high levels of gene silencing, it did not allow us to quantitate the level of silencing in each of these clinically relevant target cells. To address this, we injected a single 2 mg/kg dose of Lamin A/C siRNA IV in PEG-liposomes. At 48 hours, single cell suspensions of lungs were prepared and enriched for CD146+ endothelial and CD326+ epithelial cells via antibody mediated magnetic separation (Supplementary Figure S3). Lamin A/C mRNA expression was determined by quantitative real-time PCR (qRT-PCR). CD146+ endothelial cells exhibited 49 ± 0.2% reduction in Lamin A/C mRNA expression when compared to control siRNA (Figure 6a). Similarly, CD326+ cells achieved 54 ± 0.2% silencing of Lamin A/C mRNA (Figure 6b). The specific reduction of Lamin A/C mRNA in both CD326+ and CD146+ cells of the lungs indicated that delivery and functional silencing was achieved.

To provide direct evidence that the potent silencing of Lamin A/C was caused by the RNAi mRNA cleavage mechanism, we undertook a Taqman-based stem-loop RT-PCR assay to detect 5′ cleavage products predicted from Lamin A/C siRNA cleavage. This method uses a stem-loop primer that binds to the individual 5′ cleavage product at the 3′ cleavage site and primes reverse transcription (Supplementary Figure S4). To ensure that only the specific siRNA-generated cleavage was detected, we performed a Taqman PCR using Lamin A/C specific forward primer, a loop-specific reverse primer and a probe spanning the specific ligation site. Using this assay, we were able to detect the exclusive 5′ Lamin A/C cleavage product in the siLamin A/C liposome–treated CD146+ and CD326+ murine lung cells, and the positive control (Figure 6c). There was no amplification of the 5′ cleavage product was in either the control siRNA or untreated murine lung samples. Overall, the stem-loop Taqman qPCR analysis confirmed that the specific reduction of Lamin A/C mRNA was caused by the RNAi mechanism.

Discussion

Here, we show for the first time that cationic liposomes are highly effective at transecting not only endothelial cells but also epithelial cells of the lung following IV delivery. Moreover, this delivery was functional with 80% loss of mRNA and a 50% loss of target protein in lung tissue. For delivery of siRNA to the lung, many different routes using a diverse range of nanoparticles have been previously tested. These include intratracheal, intranasal, and IV delivery and each have shown varying degrees of success in mice and humans (reviewed in ref. 34). The most popular route is intranasal delivery where transfection of epithelial cells is achieved at high levels, although subsequent systemic circulation is poor and delivery of siRNAs into lung endothelial cells by this route has proven difficult. Furthermore, many obstructive barriers are present in intranasal delivery due to respiratory secretions, mucus, and cilia, which potentially limit access to target cells. This is particularly exacerbated in the diseased state. Indeed, this may be the reason for the diminished effectiveness of intranasal siRNA therapies targeting viruses.
in the lung. For example, post-exposure therapies of siRNA for respiratory syncytial virus, typically only 4–24 hours after infection, resulted in significantly reduced effectiveness of the treatment of viral burden in the lung in a mouse model.\(^{35}\) In lung transplant patients treated for respiratory syncytial virus, typically only 4–24 hours after infection, resulted in a 1,000-fold reduction in virus titers, but treatment 24 hours after infection only gave a twofold reduction, despite the high doses used in this study.\(^{36}\) This suggests that significant loss of efficacy occurred due to impaired delivery.

Successful IV delivery to pulmonary endothelial cells has been well established (reviewed in ref. 7). However, this delivery route has thus far only resulted in little or no epithelial cell penetration. Limited transfection of large type 2 pneumocytes within lung epithelia were reported using PEI-conjugated plasmid DNA injected intravenously.\(^{37}\) Cationic liposomes containing DODAC:DOPE (50:50) have previously been shown to efficiently transfect endothelial cells, macrophages and monocytes, but only rare delivery to epithelial cells was found.\(^{38}\)

Therefore, in this present study, we investigated cationic stealth liposomes that were generated using our published HFDM method.\(^{15}\) This is a simple method of formulating PEGylated siRNA-loaded lipid particles with characteristics favorable for \textit{in vivo} delivery. The polydispersity index was consistent with our previous findings and, as expected, was higher than that of extruded particles.\(^{15}\) We have previously shown that despite the positive zeta potential these particles do not induce liver toxicity\(^{39}\) or inflammatory cytokine responses.\(^{36,37}\) In this study, we have further shown that there was no morphologic evidence of inflammation, including immune cell infiltration, at 48 hours in liposome-treated
murine lungs, suggesting the treatment does not cause significant immune activation.

A potential reason that we observe siRNA transfection of epithelial cells with HFDM liposomes may be due to the combination of PEG stealth liposomes, together with the positive zeta potential. This would diminish uptake by professional phagocytes and encourage interaction with negatively charged endothelial cells. It is well known that intravenously delivered particles travel to the lungs and indeed 34 ± 4% of total liposomes were found to be lung associated at 48 hours. Liposomes distribute via the enhanced permeability and retention effect and thus the majority of particles accumulate in the liver due to the fenestrated architecture of the blood vessels. Cationic liposomes that use the enhanced permeability and retention effect for biodistribution must also use strategies that prevent aggregation of the delivery vector in the presence of serum. These include the use of a hydrophilic polymer, such as PEG, to shield the positive charge on the particle surface and inhibit interaction with cells of the reticuloendothelial system. Still, a significant proportion of leukocytes had taken up labeled PEG-liposomes. The PEG-ceramide is known to dissociate from the liposome surface so this may explain the delivery to myeloid cells. However, non-targeting liposomes have previously thought to be inefficient for transfecting cells at sites other than lung. Our study now shows that lung endothelial and epithelial cells are proficiently transfected by a single dose of liposomes delivered via the IV route.

Our data demonstrates that cationic liposome-mediated siRNA delivery leads to transfection of epithelial cells beyond the endothelial barrier. Although the detailed molecular mechanisms by which this occurs remain to be determined, passive mechanisms of sequestration were eliminated due to the lack of diffused equal value delivery throughout every cell population of the lung. Thus, possible mechanisms of siRNA positive sequestration may occur via direct cell contact with or exosomal transfer from transfected endothelial cells. Indeed, plants have been known to transmit dsRNA between cells and recently, endogenous microRNAs in endothelial cells. Further investigation is required to elucidate this mechanism.

Overall our data demonstrate that highly effective delivery of siRNA resulting in gene silencing in both endothelial and epithelial cells of the lung can be achieved using cationic liposomes via IV injection. We are presently investigating the significance of this in achieving gene knockdown in pathology conditions, including viral infections. The precise mechanism of how epithelial transfection occurs remains to be determined. However, one can envision that using this method may provide a more efficient means of treating diseased or infected lungs when compared to other routes of delivery.

Materials and methods

Antibodies and siRNAs. Primary antibodies used in western blot were rabbit anti-mouse Lamin A/C (Cell Signaling Technology, Beverly, MA) and rabbit anti-mouse β-actin (Cell Signaling Technology) polyclonal antibodies. Secondary reagent was a goat anti-rabbit IgG HRP-linked polyclonal antibody (Sigma-Aldrich, St Louis, MO).

Directly conjugated monoclonal primary antibodies were used for flow cytometry; CD146-FITC (clone ME-9F1; BioLegend, San Diego, CA), CD326-PE (clone G8.8; BioLegend), CD11b-PE (clone M1/70; BD Pharmingen, San Jose, CA), CD11c-PE (clone N418; eBiosciences, San Diego, CA), CD3-PE (clone 145-2C11; eBiosciences), B220-FITC (clone RA3-6B2; BD Pharmingen), and CD45.2-FITC (clone 104; eBiosciences).

siRNAs used in this study were siLamin A/C and siBgal24 obtained from Genesearch (Shanghai, China) and ScmR7 obtained from Integrated DNA Technologies (Coralville, IA).

Transfection of TC-1 cells. The murine epithelial cell line TC-1 (ATCC CRL-2785) was maintained in RPMI 1640 supplemented with 10% (v/v) heat inactivated fetal calf serum, 100 units/ml Penicillin G, 100 µg/ml Streptomycin sulphate and 2 mmol/l L-glutamine. TC-1 cells were cultured to 90% confluence in 12-well plates for 24 hours before transfection at 37 °C under a 5% CO2/95% air atmosphere. Cells were transfected with 40 nmol/l siLamin A/C or control siRNA for 4 hours in OptiMem serum free media (Invitrogen, Carlsbad, CA) using Oligofectamine (Invitrogen) according to the manufacturers’ guidelines. Cells were incubated in complete media for a further 24 hours before RNA purification.

Formulation of lipid nanoparticles. Liposomes were prepared via the HFD method as described previously. Briefly, DOTAP, DOPE, cholesterol, and PEG2000-Ceramide were dissolved in 1 ml of tert-butanol at a molar ratio of 50:5:35:10. Forty micrograms of siRNA was added to 1 ml of 55.5 mg/ml filtered sucrose solution before mixing with the lipid solution. The resultant formulation was then snap-frozen and freeze-dried overnight (ALPHA 1–2 LDPlus, Martin Christ, Germany) at −80 °C and <0.1 mbar. Freezedried matrix was then hydrated with sterile water before use to obtain a 300 µl isotonic sucrose solution.

Nanoparticle characterization. Size, polydispersity, and zeta potential of the resultant liposomes were measured using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) following appropriate dilution in distilled water. Measurements were carried out at room temperature. Two size measurements were performed with 10 runs per measurement undertaken.

In vivo delivery. 1,1-dioctadecyl-3,3,3,3-tetramethylindotri-carboxylic anhydride (DiR) (155 ng; Invitrogen) was dissolved in 10% ethanol. DiR of 155 ng in 1 µl was added to each nanoparticle formulation. Female C57BL/6 mice of 10–12 weeks old (Animal Resource Center, Perth, Australia) were injected intravenously via the tail vein with 300 µl of DiR-labeled PEGylated liposomes containing 40 µg of siRNA. At 24 hours after administration, each mouse was anaesthetized using isoflurane (Abbott, Kurnell, Australia). The biodistribution...
of DiR-labeled PEGylated liposomes was examined with the Kodak In Vivo Imager (Carestream Health, Rochester, NY) using excitation and emission wavelengths of 720 and 790 nm, respectively. Results were analyzed using the Kodak molecular imaging software. At 48 hours after administration, mice were euthanized and the lungs, liver, and spleen were removed, washed in phosphate-buffered saline (PBS) and examined via the Kodak In Vivo Imager. All experiments were approved by the University of Queensland Animal Ethics Committee.

Flow cytometry. Organs were removed and digested with collagenase D (1 mg/ml; Worthington Biochemical, Lakewood, NJ) and DNaseI (0.1 mg/ml; Roche Diagnostics, Indianapolis, IN) and passed through a 70 µm cell strainer (BD Biosciences, North Ryde, Australia) to obtain single cell suspensions. Cells were resuspended in PBS, 10% fetal calf serum, 10 mmol/l EDTA at a concentration of 0.5–1 × 10^7 cells/ml and 1 µg/ml anti-mouse Fc γ receptor II/IIIFc Block (clone 2.4G2; BD Pharmedics) for 30 minutes at 4 °C, washed and stained with Aqua amine-reactive viability dye (Invitrogen) and fluorescently labeled specific antibodies at 4 °C for 30 minutes. After staining, the cells were washed twice with PBS; 10% fetal calf serum, 10 mmol/l EDTA and fixed in 4% paraformaldehyde. Cells were analyzed using a Gallios Flow Cytometer (Beckman Coulter, Brea, CA) and Kaluza flow cytometry software (Beckman Coulter).

Histological analysis. Lungs were perfused and fixed overnight in 10% neutral buffered formalin, prior to embedding in paraffin wax, sectioning at 5 µm and staining with Harris hematoxylin and eosin by routine histological methods. Four serial sections from each mouse were evaluated by light microscopy by a board-certified veterinary pathologist (RA) with extensive experience in laboratory animal tissue analysis. Tissues observed in all mice included lung, heart, mediastinum, thymus, and oesophagus.

Immunofluorescence microscopy. Lungs were perfused with optimal cutting temperature (OCT) compound:PBS (1:1) solution, placed in Tissue-Tek OCT compound and snap frozen in a dry-ice ethanol bath. Sections (10 µm) were then cut using a cryostat (Leica Microsystems, Wetzlar, Germany) and were mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA).

Tissue sections were air dried for 1 hour and then fixed in ice-cold acetone for 5 minutes. Three PBS washes were used to remove the residual acetone and sections were placed in a humid chamber. Sections were blocked with 1% bovine serum albumin (Sigma-Aldrich)/5% normal rat serum in PBS for 15 minutes. Blocking solution was subsequently removed and primary antibodies (diluted in blocking solution) were incubated with the sections for 60 minutes. Sections were washed three times in PBS and a coverslip with Prolong gold anti-fade mountant (Invitrogen) and DAPI nuclear stain (1:1,000) was applied. Images were obtained with a Zeiss LSM 510 Meta Confocal microscope using Zen 2008 software (Carl Zeiss, Oberkochen, Germany).

In vivo knockdown. Liposomes were prepared via the HFD method containing cationic or neutrally charged siRNAs. Female C57BL/6 mice of 10–12 weeks old (Animal Resource Center) were injected IV via the tail vein with 300 µl of stealth liposomes containing 40 µg of siRNA. At 48 hours after administration, each mouse was euthanized and the lungs were removed, washed in PBS and processed for mRNA analysis. For protein analysis, mice had an additional stealth liposome IV injection at 24 hours and were processed at 72 hours after administration.

Magnetic-activated cell sorting. Cell populations from single cell suspensions were enriched by magnetic-activated cell sorting (MACS). Briefly, cells were stained with FITC and PE fluorescein isothiocyanate labeled specific antibodies for CD146 and CD326 cells respectively. The cells were subsequently labeled with Anti-FITC and Anti-PE microbeads (MiltenyiBiotec, Cologne, Germany) and loaded onto an autoMACS column. Purified CD146 and CD326 cells were obtained using positive selection and cellular composition was determined before and after enrichment by flow cytometry.

For gene expression studies, cells were lysed in Trizol (Invitrogen). For western blot, cells were lysed with a 25 gauge needle in RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich) and 1 mmol/l PMSF.

qRT-PCR, stem-loop PCR, and western blot. Total RNA was purified using standard protocols (Invitrogen). Total RNA of 0.5–2 µg was used for reverse transcription (RT) using an Omniscript RT kit (Qiagen, Valencia, CA) as per the manufacturer’s instructions. qPCR was performed in duplicate using the Rotor-gene system (Qiagen) using GoTaq qPCR SyBr green mix (Promega, Alexandria, New South Wales, Australia). ΔCt of the gene of interest was compared to the ΔCt of the reference gene to determine relative gene expression. Gene of interest primers were specific for murine Lamin A/C, 5′-GAGAAGGCTAAGAAGCAGC-3′ (sense) and 5′-ACGCAGTTCCTCGTGTA-3′ (antisense),3 murine TNFα, 5′-CATCTCTCTAAATTCGAGTGACA-3′ (sense) and 5′-TGAGTTAGCAGAACCC-3′ (antisense), murine IFNβ, 5′-AGCTCCAAGAAAGGACGACACT-3′ (sense) and 5′-GGCGTGATGGAGTTGATCT-3′ (antisense), whereas reference gene primers were specific for murine β-actin, 5′-GCTACAGCTTCCACACCACA-3′ (sense) and 5′-TTCAGGGAGGAAGAGGATT-3′ (antisense).

For stem-loop PCR analysis, a Taqman-based stem-loop RT-PCR assay for detection of 5′ cleavage product was adapted from the protocol described by Chen and colleagues.54 Total RNA of 500 ng was reverse transcribed with 1X RT buffer, 0.25 mmol/l dNTPs, 0.3 U/µl Omniscript RT, 0.25 U/µl RNase inhibitor, and 50 nmol/l Lamin A/C cleavage stem-loop RT primer 5′-CTGATATCCAGTCAGGTGTGGCCTGAGGATT-3′. The RT reaction was incubated initially for 30 minutes at 16 °C, then for 30 minutes at 42 °C followed by 5 minutes at 85 °C and finally held at 4 °C. The RT reaction was subjected to a standard Taqman PCR assay which comprised 1 µl of RT product, 1X Taqman universal PCR master mix, 0.2 µmol/l Taqman probe 5′-6-FAM-ATGCTGAGGCGAGTGTGATGCGAGGATT-3′, Black Hole Quencher 1 (Integrated DNA technologies), 1.5 µmol/l forward murine Lamin A/C primer 5′-CCTGAGGAGGAGTACCAGAGC-3′, and 0.7 µmol/l reverse loop–specific primer 5′-GGATACGGAGAAAGTCAAGC-3′. The qPCR was performed on an Applied Biosystems 7900HT real-time PCR system (Life Technologies, New York, NY) at
95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minutes.

For western blot analyzes, total cell lysates were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Polyvinyliden difluoride membranes (Sigma-Aldrich) using a Trans-Blot System (Bio-Rad, Hercules, CA). Membranes were blocked in Tris-buffered saline containing 0.5% Tween and 5% milk powder for 2 hours at room temperature. Primary antibody binding was achieved overnight at 4 °C, whereas HRP-linked secondary antibody binding was achieved in 1 hour at room temperature. Enhanced chemiluminescence was used for visualization.

**Statistical analysis.** Groups were compared using two-sided Student t-test. P values of <0.05 were considered to be statistically significant.

**Supplementary material**

**Figure S1.** Liposomal delivery to pulmonary epithelial and endothelial cells.

**Figure S2.** Intravenous liposomal delivery to the spleen and liver.

**Figure S3.** Puriﬁcation of epithelial and endothelial cells.

**Figure S4.** Schematic diagram depicting Taqman-based stem-loop RT-PCR strategy.

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