An impaired alveolar-capillary barrier *in vitro*: effect of proinflammatory cytokines and consequences on nanocarrier interaction

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The alveolar region of the lung is an important target for drug and gene delivery approaches. Treatment with drugs is often necessary under pathophysiological conditions, in which there is acute inflammation of the target organ. Therefore, *in vitro* models of the alveolar-capillary barrier, which mimic inflammatory conditions in the alveolar region, would be useful to analyse and predict effects of novel drugs on healthy or inflamed tissues. The epithelial cell line H441 was cultivated with primary isolated human pulmonary microvascular endothelial cells (HPMECs) or the endothelial cell line ISO-HAS-1 on opposite sides of a permeable filter support under physiological and inflammatory conditions. Both epithelial and endothelial cell types grew as polarized monolayers in bilayer coculture and were analysed in the presence and absence of the proinflammatory stimuli tumour necrosis factor- α (TNF- α) and interferon- γ (IFN- γ). In addition, the nanocarrier polyethyleneimine (PEI) was chosen as a model compound to study cell uptake (Oregon Green (OG)-labelled PEI) and gene transfer (PEI–pDNA complex). Upon treatment with TNF- α and IFN- γ , both cocultures exhibited comparable effects on the trans-bilayer electrical resistance, the transport of sodium fluorescein and the increase in secondary cytokine release. Basolateral (endothelial side) exposure to TNF- α or simultaneous exposure to TNF- α and IFN- γ generated an alveolar-capillary barrier with inflammation-like characteristics, impaired barrier function and a local disruption of the continuous apical labelling of the tight junction plaque protein zonula occludens-1 (ZO-1). Although transfection rates of 8 per cent were obtained for H441 cells in non-polarized monocultures, apical–basolateral-differentiated (polarized) H441 in coculture could not be transfected. After basolateral cytokine exposure, uptake of fluorescently labelled PEI in polarized H441 was predominantly detected in those areas with a local disruption of ZO-1 expression. Accordingly, transfected cells were only sparsely found in coculture after basolateral costimulation with TNF- α and IFN- γ . We designed a coculture model that mimics both the structural architecture of the alveolar-capillary barrier and inflammatory mechanisms with consequences on barrier characteristics, cytokine production and nanoparticle interaction. Our model will be suitable to systematically study adsorption, uptake and trafficking of newly synthesized nanosized carriers under different physiological conditions.

Keywords: alveolar-capillary barrier; lung injury; nanocarrier interaction; tight junctions; bilayer

1. INTRODUCTION

Biological diffusion barriers are indispensable for the physiological functioning of epithelial and endothelial cells, and are an essential prerequisite for life. These

barriers also represent a considerable obstacle for an efficient delivery of a given drug or gene from the site of administration to the intended side of action. Thus, the development of efficient nanoscale drug or gene carriers is an obvious target for new strategies in drug and gene delivery. Besides optimizing the technologies to prepare and to characterize appropriate nanocarriers,

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the cell biological and (patho)physiological properties of each barrier and delivery route must be adequately addressed. In addition, little is known about the effects of nanocarrier application to diseased tissue, especially when the barrier is impaired, for example as a consequence of inflammatory processes.

The alveolar region of the human lung is of considerable interest for drug and gene delivery via nanocarriers owing to its impressively large surface area of approximately 100–140 m² (Gehr *et al.* 1978) and a variety of pulmonary and systemic diseases that might be addressed by this approach. The alveolar epithelial barrier is maintained through interactions of domains between adjacent cells (e.g. adherens junctions (AJs), tight junctions (TJs)) via highly regulated events that establish cell differentiation (apical–basolateral membrane polarity). Inhaled nanocarriers may interact with the apical plasma membrane of the alveolar epithelial cells and enter or pass them via different passive or active uptake mechanisms, e.g. vesicle-mediated transport. In the healthy lung, the gate function of the TJs regulates the passage of ions and macromolecules through the paracellular pathway and is a limiting factor for the passage of nanocarriers. In contrast, in cases of impaired barrier function, permeation of larger molecules, e.g. albumin (65 000 kDa, atomic radius approx. 3.5 nm), can appear via the paracellular route (Nilsson & Wollmer 1992; Cavanaugh *et al.* 2006).

The alveolar-capillary barrier function and its relevance in lung inflammatory events, e.g. acute lung injury (ALI), has gained attention in recent years, as it may contribute to an increased nanoparticle translocation. In the course of ALI, increased capillary endothelial permeability (as reviewed by Maniatis *et al.* 2008), injury to the alveolar epithelium and a loss of the tight permeability barrier (Crandall & Matthay 2001) are observed. Among others, two cytokines, which contribute to the proinflammatory cascade, tumour necrosis factor- α (TNF- α) and interferon- γ (IFN- γ), are both elevated in ALI patients. We previously described a coculture model of the human distal lung epithelial cell line H441 and human pulmonary microvascular endothelial cells (HPMECs) as a potential experimental system to study mechanisms of ALI at the level of the alveolar-capillary barrier (Hermanns *et al.* 2004). This model mimics the alveolar-capillary barrier in terms of a structurally and functionally tight barrier established by cells with alveolar type II characteristics and a second diffusion barrier of microvascular endothelial cells.

In the present study, we investigated the barrier properties of H441 in coculture with either HPMECs or the microvascular endothelial cell line ISO-HAS-1 under physiological and proinflammatory conditions. To this aim, we have analysed both cell types grown as polarized monolayers in bilayer coculture in the presence and absence of proinflammatory stimuli such as TNF- α and IFN- γ . Furthermore, the established model of an inflamed alveolar-capillary barrier was used to identify its interaction with the nanocarrier polyethyleneimine (PEI) under physiological and pathological conditions (impaired barrier function).

2. MATERIAL AND METHODS

2.1. Materials

Branched PEI (MW 25 000 g mol⁻¹) was obtained from Aldrich (Bornem, Belgium) and OG 488-X succinimidyl-ester (OG) was from Molecular Probes (Leiden, The Netherlands) and was used as received. MCDB 131, RPMI, foetal calf serum (FCS), Glutamax, gelatin and penicillin/streptomycin (Pen/Strep) were purchased from Invitrogen (Karlsruhe, Germany). The following antibodies were used: mouse monoclonal anti-human E-cadherin (uvomorulin, L-CAM, Monosan CellSystems, St Katharinen, Germany), rabbit polyclonal anti-ZO-1 antibody (Zymed Laboratories Inc., San Francisco, CA, USA), mouse monoclonal anti-human cadherin-5 (VE-cadherin) (BD Transduction Laboratories, Heidelberg, Germany), anti-human PECAM-1 (Dako, Hamburg, Germany). The secondary antibodies, Alexa fluor 488-conjugated anti-mouse, anti-goat IgG and Alexa fluor 594-conjugated anti-rabbit IgG, were purchased from Molecular Probes (MoBiTec, Göttingen, Germany).

2.2. Cells and cell culture

HPMECs were obtained from macro- and microscopically normal portions of lung specimens surgically resected from patients who underwent lobectomies for early-stage lung cancer (Departments of Pathology and Surgery, Johannes Gutenberg University, Mainz, Germany). The present study was approved by the responsible state ethics committee and written consent of the patient. HPMECs were isolated as previously described (Hermanns *et al.* 2009). More than 99 per cent of the isolated cells were shown to be endothelial cells by staining for CD31 and von Willebrand factor. Cells were cultured in the HPMEC medium (MCDB 131 + 15% FCS + 2 mM Glutamax + Pen/Strep (100 U/100 μ g ml⁻¹) + 10 μ g ml⁻¹ heparin + 2.5 μ g ml⁻¹ basic fibroblast growth factor) in tissue culture flasks coated with gelatin (0.2%) for several days.

The human lung adenocarcinoma cell line, NCI H441 (H441, ATCC-HTB-174), was obtained from ATCC (Promochem, Wesel, Germany). The angiosarcoma cell line, ISO-HAS-1, derived from a single clone of the haemangiosarcoma cell line ISO-HAS, has been previously described (Unger *et al.* 2002). The H441 cells and the ISO-HAS-1 cells were propagated in RPMI 1640 medium with L-glutamine supplemented with 10 per cent FCS and Pen/Strep (100 U/100 μ g ml⁻¹) at 37°C, 5 per cent CO₂.

2.3. Coculture on HTS 24-Transwell filter plates

HPMECs or ISO-HAS-1 cells (5×10^4 cm⁻²) were seeded on the lower surface of inverted collagen type I-coated (12 μ g cm⁻², IBFB) HTS 24-Transwell filter membranes (polycarbonate; 0.4 μ m pore size; Costar) and incubated for 2 h at 37°C. The filter plates were then inverted and H441 (2×10^4 cm⁻²) cells were subsequently seeded on the top surface of the Transwell HTS filters and grown to confluence simultaneously

with the HPMECs on the lower surface over 10–14 days. To induce the differentiation of the H441 cell line, mono- and cocultures were treated with dexamethasone (1 μ M) beginning at day 3 of cultivation.

2.4. Immunocytochemical staining

Cells were grown in monoculture on micro-slides (μ -slide, Ibidi GmbH, Martinsried, Germany) or in coculture on collagen-coated (12 μ g cm⁻²) permeable filter supports. Forty-eight hours after incubation with PEI-OG or PEI-plasmid DNA (pDNA) the cells were fixed with paraformaldehyde (3.7%) in CS buffer (PIPES 0.1 M, EGTA 1 mM, 4% polyethylene glycol 800, NaOH 0.1 M) for 20 min, and washed twice with phosphate-buffered saline (PBS). The cell membranes were further permeabilized with 0.5 per cent Triton X-100 in PBS, and the respective primary antibodies (in PBS + 3% bovine serum albumin) were added and incubated overnight at 4°C. Cells were washed four times and incubated with a fluorochrome-coupled secondary antibody for 3 h. The nuclei were counterstained with a blue fluorescent Hoechst dye (HOE 33 342, Sigma). The filter was punched out from the plastic support and mounted on a slide in Gel/Mount (Biozol, Eching, Germany). The specimens on both sides of the permeable filter support were observed by fluorescence microscopy (personalDV, Applied Precision, Issaquah, USA).

2.5. Bioelectrical measurements

The trans-monolayer and trans-bilayer electrical resistance (TER) was measured using an EVOM voltohmmeter (World Precision Instruments, Berlin, Germany) equipped with a pair of STX-2 chopstick electrodes and the values expressed in Ω cm². Briefly, HTS 24-Transwell filter membranes without cells (as controls) and filter membranes with cells in the culture medium were examined over a cultivation period of 3–11 days. Resistance was shown as the mean resistance $\times$ area of three independent experiments. Calculations for Ω cm² were made by subtracting the resistance measurement of the blank filter coated with collagen type I (approx. 110 Ω) and multiplying by the area of the monolayer (0.33 cm²).

2.6. Exposure to tumour necrosis factor- α and interferon- γ

When average TER values of 400–550 Ω cm² were achieved both cocultures were exposed to the proinflammatory cytokines TNF- α (300 U ml⁻¹ $\approx$ 0.732 g ml⁻¹) and IFN- γ (200 U ml⁻¹ $\approx$ 10 ng ml⁻¹) or a combination of TNF- α (300 U ml⁻¹) + IFN- γ (200 U ml⁻¹). Basolateral exposure means exposure from the lower well (endothelial side) and apical exposure refers to upper well (epithelial side) treatment in the presence of 1 μ M dexamethasone. The corresponding opposite side of the coculture was exposed to the culture medium without cytokine. After 24 h, the medium was changed, whereby one fraction of the cocultures was continuously exposed to TNF- α or/and IFN- γ and the other fraction was exposed to fresh culture

medium (medium without (w/o) cytokine) to evaluate the recovery of TER. Thus, the cocultures were washed once with the culture medium, and the culture medium w/o cytokine was added to the upper and lower compartments. TER was measured after 12, 24, 36, and 48 h.

2.7. Transport study

Cocultures of H441 and HPMECs and cocultures of H441 and ISO-HAS-1 cells after 48 h of TNF- α or IFN- γ exposure were used in the transport study. The paracellular flux of sodium fluorescein (MW 376.3 Da; Acid Yellow 73) was measured across the bilayers in an apical-to-basolateral direction. Transport experiments were started by replacing the apical supernatant with sodium fluorescein at a concentration of 10 μ g ml⁻¹ in the culture medium. Samples from the acceptor fluid in the lower compartment (basolateral) were taken every 20 min over a time period of 3 h, and replaced by an equal volume of fresh culture medium. The samples were diluted 1 : 5 with 1 mM NaOH and assayed in a fluorescence plate reader (Genesis Plus, Tecan, Germany) at excitation and emission wavelengths of 485 and 530 nm, respectively. During the experiment, TER was measured to monitor the cell mono- and bilayer integrity. The transport studies after TNF- α or IFN- γ exposure were carried out in the culture medium (RPMI, 5% FCS). The cumulative amount of sodium fluorescein in the acceptor compartment was plotted as a function of time. Apparent permeability coefficients P_{app} were calculated using the equation: $P_{app} = (1/(A \times C_0)) \times (dQ/dt)$, where A is the surface area of the filter (0.33 cm²), C_0 the initial concentration of sodium fluorescein in the donor fluid and Q the amount of sodium fluorescein passing across the cell layer.

2.8. Detection of cytokines released into the apical and basolateral compartment

For quantification of the cytokines released, the culture supernatant from the apical and basolateral compartments was collected after 48 h. Multi-plex cytokine array analysis was performed using the Bio-plex protein multi-array system, which uses Luminex-based technology (Prabhakar *et al.* 2002). For current experiments, a human 3-plex assay was used according to the recommendations of the manufacturer (BioRad, Munich, Germany).

2.9. Preparation of polyethyleneimine nanocarriers and DLS measurements

OG 488-conjugated branched PEI nanocarriers (MW 25 kDa) were obtained as described previously (Dubruel *et al.* 2004). Briefly, PEI was dissolved at a concentration of 100 mg ml⁻¹ in NaHCO₃ (pH = 8.3). To this solution, an amount of OG 488-X succinimidylester was added, corresponding to a maximum conversion of 2 mol % of the PEI primary and/or secondary amine functions. After 1 h reaction, the reaction mixture was dialysed extensively against 1 M NaCl. Finally, the product was obtained as a powder after lyophilization. Unlabelled PEI nanocarriers were used as controls.

Table 1. Particle size (nm) and ζ potential (mV) of PEI–DNA complexes. (n.d., not determined.)

	water				cell culture medium		
charge ratio	0.5 : 1	1 : 1	2 : 1	4 : 1	1 : 1	2 : 1	4 : 1
size	n.d.	55 $\pm$ 5	54 $\pm$ 7	50 $\pm$ 3	350 $\pm$ 37	150 $\pm$ 19	67 $\pm$ 2
ζ potential	40 $\pm$ 7.4	41.8 $\pm$ 4.4	48.4 $\pm$ 4.4	55.1 $\pm$ 8.9	n.d.	n.d.	n.d.

Dynamic light scattering (DLS) measurements were performed for particle size detection using a Malvern 4800 photon correlation spectrometer equipped with an air-cooled Ar laser (488 nm). Hydrodynamic diameters (d_H , expressed in nm) were calculated from the diffusion coefficient (D) using the Stokes–Einstein equation: $d = (k_B T) / (3\pi \eta D)$, with k_B the Boltzmann constant, T the absolute temperature (K) and η the viscosity (mPas) of the solvent. Zeta potentials were measured on a Malvern Zetasizer 3000. Calibration was carried out with latex particles of known zeta potential. Sample preparation was carried out as described for DLS measurements. The values reported for DLS and zeta potential analysis are an average of three measurements.

PEI and pDNA formed stable polyplexes both in water and in the cell culture medium at 1 : 1 to 4 : 1 charge ratios. Polyplexes prepared in water possessed particles sizes in the range of 50–55 nm irrespective of the polyplex charge ratio (table 1). In the cell culture medium, polymer–DNA complexes possessed sizes in the range of 70–300 nm (table 1). The higher particle size of polyplexes prepared in the cell culture medium is related to the presence of various salts in the cell culture medium. The zeta potential of the PEI–DNA complexes increased with an increasing charge ratio from 40 to 55 mV. This increase is due to the larger amount of PEI positive charges with an increasing charge ratio.

2.10. Loading polyethyleneimine nanocarriers with plasmid DNA

Branched 25 kDa PEI was loaded with pDNA encoding enhanced green fluorescent protein (EGFP) gene (BD Clontech, Heidelberg, Germany). The PEI suspension was added to DNA EGFP-plasmid in a charge ratio of 2 : 1 in water, mixed and left at room temperature for at least 30 min before use. Loading efficiency of the PEI–pDNA complexes was above 99 per cent as calculated by measuring the difference between the amount of DNA added and the measured non-entrapped DNA remaining in the aqueous phase after complex formation. Therefore, the PEI–pDNA complexes were centrifuged for 15 min at 20 800 g and the supernatant was checked for the non-bound DNA concentration with the PicoGreen–dsDNA quantitation assay (Invitrogen) using a fluorescence plate reader (Genesis Plus, Tecan).

2.11. Toxicity studies and transfection

One day after cells were seeded into the 96-well plates, cells were washed and PEI, PEI–OG or PEI–pDNA formulations in a serum-free RPMI medium were applied

on cells at concentrations of 0.14–2.23 $\mu\text{g cm}^{-2}$ PEI, corresponding to 0.11–1.75 $\mu\text{g DNA cm}^{-2}$ in the case of PEI–pDNA complexes. The cell culture medium was used as a positive control for cell viability. After 2 h, cells were washed twice and the CellTiter Aqueous One Solution Cell Proliferation Assay (Promega, Mannheim, Germany) was performed according to the manufacturer’s instructions. Absorbance was measured at 492 nm. For uptake and transfection studies, the cells were cultivated for a further 48 h period, fixed with 3.7 per cent paraformaldehyde in CS buffer followed by fluorescence reading at excitation and emission wavelengths of 485 and 530 nm. Transfection efficiency was calculated by counting EGFP-expressing cells compared with the total cell number ($n = 500$ cells) using an image analysing system (ImageJ 1.40G).

2.12. Statistical analysis

From several independent measurements, means and standard deviations were calculated. Analyses are shown as mean $\pm$ s.d. from at least three separate experiments. Testing for significant differences between means was carried out using one-way ANOVA at a probability of error of 5, 1 and 0.1 per cent.

3. RESULTS

In the present study, we compared two *in vitro* models of the human alveolar-capillary unit in terms of their barrier properties under physiological and inflammatory conditions. Therefore, we analysed the epithelial cell line H441 in bilayer coculture with primary isolated HPMECs or with the angiosarcoma cell line ISO-HAS-1, respectively.

3.1. Morphological characterization of cocultures

The general assembly of an alveolar-capillary unit is presented in figure 1. Figure 1a depicts an abstracted view of a cross section through the alveolus surrounded by a capillary. Cross sections of the *in vitro* models were prepared on day 10 of cultivation by cutting the cocultures in the y – z axis. Sections were viewed by transmission electron microscopy to examine morphologically TJ formation. In both coculture systems with either HPMECs or ISO-HAS-1, H441 established a cuboidal morphology with apical microvilli and an apical–basolateral differentiation (figure 1b). Both endothelial cell types on the opposite side, that is, the primary isolated HPMECs and the endothelial cell line ISO-HAS-1, showed a typical fattened morphology

with a protruding nucleus and long cytoplasmic extensions (shown for ISO-HAS-1 in figure 1b).

H441 cells formed several lateral contacts between adjacent cells on day 10 of coculture. TJ formation was assessed by staining for the tight junctional cytoplasmic plaque protein, ZO-1, and was localized at the cell periphery in a sharp continuous band surrounding each cell at its apical border (figure 1d). HPMECs and ISO-HAS-1 on the opposite side of the filter membrane established close cell-to-cell contacts with well-defined AJs, as shown by a fine circumferential staining pattern of the AJ transmembrane protein with VE-cadherin in figure 1e,f, respectively. The two polarized monolayers in the bilayer coculture on 24-Transwell filters generated a compartmentalized system with two distinct compartments, the upper or apical compartment (apical) corresponding to the alveolar lumen and the lower or basolateral compartment (basolateral) corresponding to the pulmonary microvascular lumen (figure 1c).

3.2. Transmembrane electrical resistance after exposure to tumour necrosis factor- α and interferon- γ

The transmembrane electrical resistance across the cell bilayer is shown in figure 2 as a function of time in culture. To study the sensitivity of the bilayer cocultures on exposure to proinflammatory cytokines, the cocultures were exposed to TNF- α , a typical mediator of inflammation released by alveolar macrophages, and IFN- γ , produced by T-lymphocytes at the onset of ALI. Maximum TER values after 10–12 days of cocultivation averaged $400\text{--}550\ \Omega\text{ cm}^2$ for both cocultures. This time schedule was chosen for the exposure of the coculture to the proinflammatory cytokines.

Apical stimulation of both cocultures with TNF- α (300 U ml^{-1}) gave no significant effect on TER values (data not shown), whereas basolateral exposure to TNF- α caused a significant reduction of TER to $69 \pm 2\%$ of the control value after 24 h. This decrease in TER could be re-established to control levels after removal of the cytokine within 24 h for both cocultures (figure 2a,b). For H441 in coculture with HPMECs, apical and basolateral exposure for 24 h to IFN- γ (200 U ml^{-1}) caused a slight increase in TER values compared with control w/o cytokine (figure 2a). Both cocultures showed a reduction of TER to $72 \pm 6\%$ of control after 48 h basolateral exposure to IFN- γ . In the case of apical stimulation, only the combination of TNF- α + IFN- γ generated a significant reduction of TER after 48 h (data not shown). Exposure of TNF- α + IFN- γ from the lower compartment (basolateral, endothelial side) showed a costimulatory reduction of TER (57 ± 10 and $39 \pm 5\%$ of the control value after 24 and 48 h, respectively). Contrary to a basolateral exposure to TNF- α , basolateral exposure to IFN- γ and TNF- α + IFN- γ showed incomplete recovery (figure 2a,b). These findings correlate with an increased permeation of sodium fluorescein, shown for H441 in coculture with HPMECs (figure 3c) or with ISO-HAS-1 (figure 3d) by measuring the apparent permeation coefficient (P_{app}) after 48 h of basolateral

exposure. With apical exposure, only TNF- α + IFN- γ in combination significantly increased the P_{app} of sodium fluorescein (figure 3a,b).

3.3. Tumour necrosis factor- α and interferon- γ -induced secondary cytokine release in cocultures

To study the reactivity of both bilayer cocultures to proinflammatory cytokines, the cocultures were exposed to TNF- α and IFN- γ and the release of the secondary cytokines interleukin-6 (IL-6), interleukin-8 (IL-8, CXCL8) and monocyte chemoattractant protein-1 (MCP-1, CCL2) was measured within the apical and basolateral compartment. Thus, we exposed either the apical side (upper compartment, epithelial side) or the basolateral side (lower compartment, endothelial side) to 300 U ml^{-1} TNF- α , 200 U ml^{-1} IFN- γ or a combination of both cytokines for 24 h.

The release patterns of the secondary cytokines were comparable for H441 in the bilayer coculture with either HPMECs or ISO-HAS-1 (figure 4a–f). Compared with H441/HPMEC cocultures (figure 4c,e), H441 in coculture with ISO-HAS-1 showed a higher basal amount of IL-8 and MCP-1 released to the basolateral side (endothelial side, ISO-HAS-1) of the non-treated cocultures (figure 4d,f). This indicates a higher basal production of IL-8 and MCP-1 by the endothelial cell line ISO-HAS-1. For both cocultures, the release of the secondary cytokines at the side of exposure was clearly modified by TNF- α and a combination of TNF- α + IFN- γ . IFN- γ alone, apically or basolaterally exposed, did not stimulate the release of IL-6, IL-8 or MCP-1.

Substantially higher amounts of IL-6, IL-8 and MCP-1 were found in the basolateral compartment following basolateral exposure to TNF- α or to TNF- α + IFN- γ in combination. Basolateral exposure to TNF- α raised the release of IL-6 into the basal compartment about 8.8-fold for H441/HPMECs or fourfold for H441/ISO-HAS-1. TNF- α + IFN- γ had more than a costimulatory effect on IL-6 release (figure 4a,b). After exposure to TNF- α , the basal IL-8 release of the untreated control ($2069 \pm 282\text{ pg cm}^{-2}$) increased more than fourfold ($9632 \pm 2020\text{ pg cm}^{-2}$) and for H441/ISO-HAS-1 more than twofold. Additionally, MCP-1 release into the basolateral compartment was raised more than eightfold ($4209 \pm 499\text{ pg cm}^{-2}$) compared with the control without stimulation ($517 \pm 402\text{ pg cm}^{-2}$) for H441/HPMECs. Owing to the high basolateral MCP-1 production of the untreated H441/ISO-HAS-1 coculture, TNF- α exposure increased the basolateral release only 1.7-fold. However, the absolute amount of MCP-1 detected in the basolateral compartment of H441/ISO-HAS-1 ($4154 \pm 392\text{ pg cm}^{-2}$) after basolateral TNF- α exposure was comparable to that induced in H441/HPMECs ($4209 \pm 499\text{ pg cm}^{-2}$). For both cocultures, a combination of TNF- α + IFN- γ did not further increase the basolateral release of IL-8 and MCP-1 compared with TNF- α alone (figure 4c–f).

Additionally, after basolateral exposure to TNF- α and TNF- α + IFN- γ in combination, an increased

Figure 1. Assembly of the alveolar-capillary barrier model. (a) Expanded view of an alveolus surrounded by a capillary, depicting type I and type II alveolar epithelial cells that cover the alveolar lumen and the nearby endothelial cells that line the vessel wall (adapted from Ware & Matthay 2000). (b) Electron micrograph of a 10-day-old coculture in cross section. H441 grown as a polarized monolayer on the upper surface and endothelial cells (EC, ISO-HAS-1) on the lower surface of a polycarbonate filter membrane (fm). (c) Image of an HTS 24-Transwell filter unit showing the apical and the basolateral compartment. (d) Immunofluorescent labelling of the TJ cytoplasmic plaque protein ZO-1 of the H441 monolayer on day 10 of coculture. (e) AJs visualized by VE-cadherin staining of HPMECs. (f) VE-cadherin staining of ISO-HAS-1 on day 10 of coculture. Nuclear counterstain Hoechst 33342. Scale bar, 10 μm .

Figure 2. Transmembrane electrical resistance after basolateral (lower compartment, endothelial side) exposure to $\text{TNF-}\alpha$ (300 U ml^{-1}) and $\text{IFN-}\gamma$ (200 U ml^{-1}) as a function of time. (a) Coculture of H441 with HPMECs or (b) coculture of H441 with ISO-HAS-1. TER values are depicted as a percentage of the untreated control. Basolateral exposure to $\text{TNF-}\alpha$, $\text{IFN-}\gamma$ or $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$ in combination ($\text{TNF} + \text{IFN}$) for 24 h and a further 24 h without ($\leftrightarrow$) or with cytokine. Results are shown as means $\pm$ s.e. of three independent experiments. * $p < 0.05$ versus non-treated control. Black bars, 12 h; white bars, 24 h; light grey bars, 36 h; dark grey bars, 48 h.

Figure 3. P_{app} values for the transport of sodium fluorescein across cell bilayers of H441 in coculture with HPMECs or ISO-HAS-1 48 h after treatment with TNF- α , IFN- γ or TNF- α and IFN- γ in combination (TNF + IFN) for 24 h and a further 24 h without (light grey) or with cytokine (dark grey). (a) Apical (upper compartment, epithelial side) exposure to H441 with HPMECs or (b) H441 with ISO-HAS-1 in coculture. (c) Basolateral (lower compartment, endothelial side) exposure to H441 with HPMECs or (d) H441 with ISO-HAS-1 in coculture. Results are shown as means $\pm$ s.e. of three independent experiments. * p < 0.05 or ** p < 0.01 versus control without (w/o) cytokine.

release on the opposite side of the exposed compartment was measured for all cytokines. This release of IL-6, IL-8 and MCP-1 to the opposite side of the exposed compartment was more obvious for H441/ISO-HAS-1 cocultures, indicating that the permeability of the ISO-HAS-1 monolayer might be higher than that of HPMECs.

3.4. Immunofluorescent labelling of tight junction and adherens junction proteins after exposure to tumour necrosis factor- α and interferon- γ

The formation of AJ cell-to-cell contacts in the exposed cocultures was characterized by immunofluorescent labelling of the endothelial AJ-associated protein PECAM-1 and the epithelial AJ transmembrane protein E-cadherin. The integrity of the TJ complexes within the H441 cells was assessed by ZO-1 staining following immunofluorescence microscopy.

Apical as well as basolateral exposure to TNF- α , IFN- γ or a combination of both caused no obvious changes in the staining pattern of ZO-1. In a few cases, a local disruption of the continuous ZO-1 band was seen after basolateral exposure to TNF- α (figure 5a, asterisk) or TNF- α + IFN- γ (figure 5c, asterisk). Additionally, basolateral

exposure to TNF- α (figure 5d) or TNF- α + IFN- γ (figure 5f) caused a less distinctive and locally fragmented paracellular labelling of E-cadherin than the non-treated control or basolateral IFN- γ exposure (figure 5e).

Immunofluorescent staining of PECAM-1 revealed a gap formation between adjacent endothelial cells after basolateral exposure to TNF- α or TNF- α + IFN- γ (shown for HPMECs in figure 5g,i, respectively), whereas IFN- γ alone or apical stimulation with cytokines caused no such effect. Correlating with the recovery of TER values, samples following 24 h basolateral TNF- α stimulation and 24 h incubation in medium without cytokine showed a continuous circumferential labelling of PECAM-1 after 48 h (data not shown).

3.5. Toxicity studies and detection of uptake and transfection efficiency

The 25 kDa PEI formulations were evaluated for their biological activity, including their toxicity (MTS assay) and their adhesion/uptake (PEI-OG) or transfection efficiency (EGFP expression) (determination of green fluorescence) using the cell lines H441 and ISO-HAS-1 in monoculture on tissue culture plastic.

Figure 4. Secondary cytokine release by bilayer cocultures after exposure to TNF- α and IFN- γ . Supernatants were collected from the apical (values above zero) and the basolateral (values below zero) compartment of the two-chamber system after exposure from the apical (left four columns of each graph) or the basolateral (right four columns of each graph) compartment for 24 h. (a,b) IL-6, (c,d) IL-8, and (e,f) MCP-1 were quantified for H441 in coculture with HPMECs or ISO-HAS-1, respectively. Each bar represents the mean $\pm$ SD of three independent experiments. The asterisk signifies a significantly different ($p < 0.05$) change compared with the controls without (w/o) cytokine.

Figure 6a shows the MTS reduction seen in H441 cells 120 min after incubation with unlabelled PEI, PEI-OG and PEI-pDNA complexes prepared at a 2:1 charge ratio. The results obtained for ISO-HAS-1 are depicted in figure 6c.

After 120 min exposure, a concentration of $0.56 \mu\text{g cm}^{-2}$ unlabelled PEI already reduced the mitochondrial activity (MTS assay) of the endothelial cells.

For OG-conjugated and pDNA-complexed PEI, this effect on ISO-HAS-1 was not seen until exposed to a concentration of $2.23 \mu\text{g cm}^{-2}$ (figure 6c). For H441 cells, $2.23 \mu\text{g cm}^{-2}$ unlabelled PEI significantly reduced mitochondrial activity (figure 6a). The cellular adhesion/uptake of PEI-OG depended on the concentration of the nanocarrier in the medium. For the analysed concentrations of 0.14 – $2.23 \mu\text{g cm}^{-2}$,

Figure 5. Immunofluorescent labelling of intercellular junctions performed 48 h after exposure to TNF- α and IFN- γ . (a–c) ZO-1 labelling of H441, (d–f) E-cadherin labelling of H441, and (g–i) staining of HPMECs for PECAM-1. The different treatments are arranged as separate columns: (a,d,g) TNF- α , (b,e,h) IFN- γ and (c,f,i) TNF- α + IFN- γ in combination. A local disruption of the continuous ZO-1 band is denoted by asterisks. Gap junction formation in HPMECs is denoted by arrows. The nuclei out of focus are the nuclei of cells on the opposite side of the filter membrane and are stained with Hoechst dye (Hoechst 33342). Scale bar, 10 μ m.

adhesion/uptake of OG-conjugated PEI continuously increased in both cell types, shown as increased fluorescence signal for PEI-OG in figure 6b,d, respectively.

To assess transfection efficiency, the fluorescence of the EGFP-expressing cells was detected 48 h after transfection with pDNA-complexed PEI. ISO-HAS-1 cells showed the highest fluorescence signal at concentrations of $0.56 \mu\text{g cm}^{-2}$ PEI-pDNA complex. For increasing concentrations of PEI-pDNA, the fluorescence signal decreased, indicating an influence of PEI toxicity. EGFP-expressing H441 cells were detected after exposure to concentrations higher than $1.12 \mu\text{g cm}^{-2}$ PEI-pDNA complex ($0.88 \mu\text{g cm}^{-2}$ pDNA). A higher concentration of PEI-pDNA ($2.24 \mu\text{g cm}^{-2}$) did not increase transfection efficiency in H441 any further (figure 6b). The maximum fluorescence signal of the epithelial cells, detected at a concentration of $1.12 \mu\text{g cm}^{-2}$ PEI-pDNA complex ($0.88 \mu\text{g cm}^{-2}$ pDNA), showed less than one-half of the fluorescence intensity of the endothelial cells. These data correspond to the transfection efficiency calculated by counting EGFP-expressing cells compared with the total cell number ($n = 500$ cells, transfection efficiency of 16 or 8% for ISO-HAS-1 or H441, respectively).

3.6. Immunofluorescent detection of polyethyleneimine uptake and transfection in mono- and coculture

To imitate an inhalative PEI exposure, concentrations of $1.12 \mu\text{g cm}^{-2}$ PEI-OG or PEI-pDNA complex,

apically exposed, were evaluated for their uptake (PEI-OG) or transfection efficiency (EGFP fluorescence) by immunofluorescent microscopy. To achieve this, H441 cells were investigated as a polarized layer in coculture with ISO-HAS-1 cells or in monoculture on μ -slides. An inflammatory alveolar-capillary barrier was simulated by basolateral exposure to TNF- α (300 U ml^{-1}) or TNF- α (300 U ml^{-1}) + IFN- γ (200 U ml^{-1}) for 24 h. To examine in more detail the influence of the nanocarrier on the barrier properties of H441, cell-to-cell contacts were stained immunocytochemically for the TJ protein ZO-1.

For H441 cells in mono- and coculture exposed to PEI-OG or PEI-pDNA ($1.12 \mu\text{g cm}^{-2}$), the expression of ZO-1 was unchanged compared with cells without treatment (data not shown). Non-polarized H441 cells in monoculture on μ -slides showed an enhanced perinuclear and fragmented junctional distribution of ZO-1 (figure 7a,c). PEI-OG labelled almost every H441 cell in monoculture (green dots in figure 7a). The transfection efficiency of the PEI-pDNA complexes in H441 (expression of green fluorescent protein) was 8 per cent as calculated by counting the EGFP-expressing cells within the whole cell population (figure 7e). In contrast to non-polarized H441 cells, apical-basolateral-differentiated (polarized) H441 cells showed a persisting regular ZO-1 staining pattern localized at the apical cell-cell contact areas of adjacent epithelial cells (figure 7b,f). For these H441 cells in coculture, no PEI-OG label (dotted green fluorescence, figure 7b) and no cells expressing EGFP (figure 7f) could be detected 48 h after exposure. PEI-OG was detected following basolateral exposure to TNF- α and

Figure 6. Evaluation of toxicity and uptake/transfection efficiency of 25 kDa PEI formulations in concentrations of 0.14–2.23 $\mu\text{g cm}^{-2}$. (a,c) Mitochondrial activity was quantified by MTS assay for monocultures of epithelial (H441) and endothelial (ISO-HAS-1) cells on 96-well plates, respectively. Cells were exposed to OG 488-conjugated PEI (PEI–OG, open circles), PEI–pDNA complexes (PEI + pDNA, filled triangles) and unlabelled PEI (PEI, filled squares). (b,d) Uptake of PEI–OG (dark grey bars) and EGFP expression (light grey bars) 48 h after exposure of monocultures of H441 and ISO-HAS-1 cells, respectively. The fluorescence signal is expressed as relative fluorescent units (RFU). Results are shown as means $\pm$ s.e. ($n = 3$) of two independent experiments. * $p < 0.05$ versus controls without (w/o) addition of PEI formulations.

Figure 7. Immunofluorescent microscopy of the uptake and transfection efficiency of OG 488-conjugated 25 kDa PEI formulations by the cell line H441. (a–d) Uptake of OG 488-conjugated PEI (1.12 $\mu\text{g cm}^{-2}$ PEI–OG). (e,f) EGFP expression 48 h after exposure to PEI–pDNA complexes (1.12 $\mu\text{g cm}^{-2}$ PEI and 0.88 $\mu\text{g cm}^{-2}$ pDNA). The different culture conditions and treatments are arranged as separate columns: (a,e) H441 in monoculture on μ -slides; (b,f) H441 as polarized monolayer in coculture with ISO-HAS-1; (c,g) basolateral exposure of the coculture to TNF- α (300 U ml^{-1}); (d,h) basolateral exposure of the coculture to TNF- α (300 U ml^{-1}) + IFN- γ (200 U ml^{-1}) for 24 h before PEI treatment. A local disruption of the continuous ZO-1 band is denoted by asterisks. Nuclear counterstain Hoechst 33342. Scale bar, 10 μm .

to a greater extent after exposure to TNF- α + IFN- γ . Here, PEI–OG labelling was predominantly found at those areas with a local disruption of the continuous ZO-1 band. Correlating with this, EGFP-expressing, transfected H441 cells were sparsely found in

cocultures that were basolaterally treated with TNF- α + IFN- γ 24 h before exposure to PEI–pDNA complexes (figure 7h). ISO-HAS-1 interacting with the H441 cells in coculture on the opposite side of the filter membrane failed to demonstrate any

PEI–OG label or any green fluorescent protein expression.

4. DISCUSSION

Effective drug or gene therapy based on nanosize carriers depends on their safe and efficient delivery to the target cells. The alveolar-capillary barrier of the lung is an excellent target for non-invasive drug delivery owing to its large surface area and its accessibility, for example by inhalation spray. In designing appropriate nanoscale carriers to target or traverse the alveolar-capillary barrier, the cellular and physiological characteristics of this barrier must be adequately addressed and the uptake and transport mechanisms understood.

To study nanocarrier interactions at the distal lung, a coculture of alveolar epithelial and endothelial cells seems reasonable considering the close proximity of both cell types *in vivo*. In an attempt to mimic the *in vivo* situation, we developed a bilayer model that permits communication and interaction of alveolar type II-like epithelial (H441) cells and primary isolated HPMECs cultured on opposite sides of Transwell filter growth supports (Hermanns *et al.* 2004). One disadvantage of this model is the need for primary isolated HPMECs. To overcome the donor dependency of primary cells, we investigated the potential of the microvascular endothelial cell line ISO-HAS-1 to substitute the HPMECs in the coculture model. Thus, cytokine effects were compared for H441 in coculture with both microvascular endothelial cell types.

4.1. Generation of an impaired alveolar-capillary barrier in vitro

Damage to the alveolar-capillary barrier is the major critical element occurring during many pathological processes in the lung, e.g. acute inflammatory responses (as reviewed by Lucas *et al.* 2009). As treatment with drugs is often necessary under such pathophysiological conditions, *in vitro* models that mimic an impaired barrier could be useful, and indeed necessary, to predict drug effects on an alveolar epithelium with altered barrier function.

After 10–11 days in coculture with either HPMECs or ISO-HAS-1, the cell line H441 formed a polarized tight monolayer as indicated by TER $400\text{--}550\ \Omega\ \text{cm}^2$ and a regular assembly of both TJs and AJs. This time point was chosen for further treatment with the proinflammatory cytokines TNF- α ($300\ \text{U ml}^{-1} \cong 0.732\ \text{ng ml}^{-1}$) and IFN- γ ($200\ \text{U ml}^{-1} \cong 10\ \text{ng ml}^{-1}$). Upon treatment with TNF- α and IFN- γ , both cocultures reacted in a comparable fashion concerning the effects on TER (figure 2) and the transport of sodium fluorescein (figure 3). When basolaterally (i.e. from the endothelial side) exposed to TNF- α and simultaneously exposed to TNF- α and IFN- γ , an inflamed alveolar-capillary barrier with impaired barrier function was generated. An apical (epithelial) exposure to equal concentrations of the proinflammatory cytokines caused only minor effects on TER and solute movement (paracellular flux of sodium fluorescein). Variable effects of TNF- α and IFN- γ on barrier properties have already been described

for epithelial cells (reviewed by Beaupaire *et al.* in press) and endothelial cells (Oshima *et al.* 2001; Souza & Teixeira 2005) of the intestine and bronchial epithelial cells (Coyne *et al.* 2002; Pohl *et al.* 2009). A recent animal study also discussed a possible role of TNF- α on barrier dysfunction of the distal lung after carrageenan-induced acute lung inflammation in mice (Mazzon & Cuzzocrea 2007). In addition, Angelini *et al.* (2006) demonstrated that TNF- α increases lung capillary endothelial permeability *in vitro*. In addition to permeability changes, effects of the proinflammatory cytokines TNF- α and IFN- γ on the TJ cytoplasmic plaque protein ZO-1 and on TJ structure have been reported for bronchial epithelial cells (Coyne *et al.* 2002; Pohl *et al.* 2009). In our study, immunofluorescence was performed to identify whether the decline of the tight permeability barrier (reduced TER and increased permeability for sodium fluorescein) after basolateral cytokine exposure also correlates with changes of TJ and AJ protein-staining patterns. Contrary to the studies with human bronchial cell types, describing a loss of the structural organization of ZO-1 48 h after costimulatory TNF- α and IFN- γ exposure (Coyne *et al.* 2002; Pohl *et al.* 2009), our study revealed only a mild and focal disruption of ZO-1 labelling. Nevertheless, it should be noted that, compared with our investigation, these studies used cytokine concentrations that were 10-fold higher ($10\ \text{ng ml}^{-1}$ TNF- α and $100\ \text{ng ml}^{-1}$ IFN- γ). Despite the local effects on cell-to-cell junctional contacts, the integrity of the cellular layers after cytokine exposure remained intact. Coincident with our findings after exposure to TNF- α , a paracellular gap formation of HPMECs is also described by Koss *et al.* (2006). Our results indicate that cellular damage in terms of toxicity to the applied concentrations of TNF- α and IFN- γ could be excluded. This finding is also supported by the retained sensitivity of the cocultures to proinflammatory cytokine treatment.

Activation by proinflammatory cytokines *in vivo* causes an inflammatory cascade with secretion of secondary cytokines by alveolar epithelial (Crestani & Aubier 1998; Delclaux & Azoulay 2003) and microvascular endothelial cells (Strieter & Kunkel 1994). Hence, both cell types in coculture responded to proinflammatory cytokine treatment with the release of the secondary cytokines, IL-6, IL-8 and MCP-1. In general, the release patterns were comparable for H441 in the bilayer coculture with either HPMECs or ISO-HAS-1. Under the given conditions of integral barrier properties, the secondary cytokine responses were found to be largely compartmentalized for both the apical (epithelial) and basolateral (endothelial) application. While IFN- γ caused no changes, basolateral exposure to TNF- α and costimulatory exposure to TNF- α + IFN- γ induced an inflammatory cytokine production by the cells of the alveolar-capillary barrier *in vitro* (increased basolateral release of the secondary cytokines IL-6, IL-8 and MCP-1).

4.2. Interaction of the nanocarrier polyethyleneimine with cells of the alveolar-capillary barrier in vitro

Among the non-viral vector systems currently under investigation, we chose the standard polymer-branched

PEI as a potential candidate to investigate gene uptake at the alveolar-capillary barrier *in vitro*. Branched PEI has been reported to be associated with successful pulmonary gene delivery, to initiate *in vivo* transfection and to induce only low levels of cytokine production in the lungs (Gautam *et al.* 2000, 2001a,b). Additionally, PEI-based formulations have proved stable during nebulization and resulted in transfection of a large proportion of bronchial cells in the conducting airways (Gautam *et al.* 2001a).

In our study, the following 25 kDa branched PEI formulations were used: unlabelled PEI, OG 488 (OG)-conjugated PEI (PEI-OG) and PEI complexed with pDNA encoding for green fluorescence protein (PEI-pDNA). H441 and ISO-HAS-1 cells were used in non-polarized monoculture to identify toxicity, adhesion/uptake and transfection efficiency of the PEI formulations. Data obtained by mitochondrial activity testing indicate that unlabelled PEI is most toxic to H441 and ISO-HAS-1 cells followed by PEI-OG or the PEI-pDNA complex. Although the reasons for PEI toxicity are not completely clear, it appears to involve the disruption of the endosome/lysosome complex (Boussif *et al.* 1995; Grosse *et al.* 2007). The lower toxicity of the PEI-pDNA polyplexes compared with PEI is due to the shielding of some of the polymer positive charges by electrostatic interaction with DNA. We anticipate that the lower toxicity of PEI-OG compared with PEI is most probably related to the fact that some of the amines of PEI are used for OG coupling in addition to variations in the hydrophobic character between both polymers.

The fact that EGFP was detected 48 h after transfection with PEI-pDNA complexes indicated that a certain percentage of the particles were taken up with subsequent escape from the endo-lysosomal compartment and DNA translocation to the nucleus. ISO-HAS-1 and H441 cells showed the highest transfection efficiency at concentrations of 0.56 and 1.12 $\mu\text{g cm}^{-2}$ PEI-pDNA, respectively. Although, correlating with an increased toxicity of PEI, transfection efficiency decreased at higher concentrations of PEI-pDNA. The transfection efficiency of 8 per cent gained in H441 is in accordance with that found by other groups for PEI-pDNA complexes in epithelial cells (Doyle & Chan 2007).

An apical (epithelial side) exposure to PEI formulations, mimicking gene delivery via inhalation, was investigated under physiological and impaired barrier conditions (inflammatory conditions). In contrast to monocultures, virtually no PEI-OG or EGFP expression was detectable under the same conditions for the cocultures. These results show that, unlike non-polarized cells with deficient TJ formation, polarized epithelial cells impede the uptake of PEI-OG and PEI-pDNA complexes. Our *in vitro* finding may explain the diffuse transfection in the alveolar lining cells found *in vivo* after PEI gene delivery (Gautam *et al.* 2001a). Owing to the low transfection efficiency of alveolar epithelial cells *in vivo*, several groups have investigated the modulation of TJ gate function as a method to enhance PEI-mediated drug uptake in lungs. Agents that modulate TJ permeability to large molecules, such as EGTA, or sodium caprate (C10), have been shown to increase the permeability of

airway TJs and also enhance gene expression in lung bronchial epithelial cells *in vitro* (Coyne *et al.* 2000; Johnson *et al.* 2003). However, delivery of these agents that alter multiple proteins in the TJs has been linked to inflammation in airways *in vitro* and *in vivo*.

In our study, we were interested in simulating a 'pathophysiological', tight-junction modulation as observed during an inflammatory process *in vivo*. An inflamed barrier was generated by 24 h basolateral co-stimulation with TNF- α and IFN- γ . PEI-OG labelling was predominantly found in those areas, with a local disruption of the continuous ZO-1 band. Hence, PEI might interact with structures at the basolateral membrane of the H441 cells. In accordance with maximum PEI-OG uptake after basolateral coincubation with TNF- α and IFN- γ , sparse EGFP-expressing cells could be detected in polarized H441 cells under the same conditions. However, the transfection efficiency was not comparable to that gained with non-polarized monolayers of H441 cells. Non-polarized epithelial cells are widely used to evaluate the efficiency of gene delivery via nanocarriers. Our results indicate that the data obtained from monocultures are not comparable to those achieved for polarized H441 cells of the alveolar-capillary barrier model. Thus, it is not surprising that some groups refer to a missing *in vivo*-*in vitro* correlation concerning toxic effects of nanoparticulate matter (Sayes *et al.* 2007) when using non-polarized cells. Especially for epithelial cells, which demonstrate an apical-basolateral polarity *in vivo*, an adequate cell differentiation *in vitro* seems to be indispensable in order to study nanocarrier effects. Hence, not only the TJ gate (restriction of paracellular transport) but also its fence function, which sustains asymmetry in protein and lipid composition between apical and basolateral cell surfaces, seems to play an essential role in PEI uptake and transport.

In summary, we developed a coculture model that mimics the structural architecture of the two main cell types at the alveolar-capillary barrier, namely the alveolar epithelium (cell line H441) and the microvascular endothelium (cell line ISO-HAS-1). The described functional alveolar-capillary barrier permits investigation of the effects of nanoparticulate matter on two distinct compartments, the alveolar lumen (apical) and the vascular lumen (basolateral). Data were presented to show that epithelial cells react to nanocarriers in a completely different manner in the coculture setting, compared with the simple monolayer model, as the coculture simulates the interface between the air space and the intravascular compartment in a more *in vivo*-like manner than in the absence of the endothelial cell layer. Moreover, this coculture model allows systematic studies on the adsorption, uptake and trafficking of nanosize carriers at the alveolar-capillary barrier *in vitro* under both physiological and inflammatory conditions. Additionally, this model offers the promise of helping to unravel the mechanisms that determine whether a nanoparticulate (diagnostic or therapeutic) system enters the lung and remains there (intrapulmonary delivery) or actually traverses the cellular bilayer to achieve extra-pulmonary

transport of nanocarriers. Understanding the basic biology of this interface is highly relevant for novel non-invasive strategies in regenerative medicine, so that the coculture model could act as a bridge between simple monolayer screening methods for nanocarrier toxicity and the highly complex situation *in vivo*.

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