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Research Article

Co-culture of human alveolar epithelial (hAELVi) and macrophage (THP-1) cell lines

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Summary
The air-blood barrier is mainly composed of alveolar epithelial cells and macrophages. Whereas the epithelium acts as a diffusional barrier, macrophages represent an immunological barrier, in particular for larger molecules and nanoparticles.
This paper describes a new co-culture of human cell lines representing both cell types. Acquiring, culturing and maintaining primary alveolar epithelial cells presents significant logistical and technical difficulties. The recently established human alveolar epithelial lentivirus immortalized cell line, hAELVi, when grown on permeable filters, form monolayers with high functional and morphological resemblance to alveolar type I cells. To model alveolar macrophages, the human cell line THP-1 was seeded on pre-formed hAELVi monolayers.
The co-culture was characterized regarding cellular morphology, viability and barrier function. Macrophages were homogenously distributed on the epithelium and could be kept in co-culture for up to 7 days. Transmission electron microscopy showed loose contact between THP-1 and hAELVi cells. When grown at air liquid interface, both cells were covered with extracellular matrix-like structure, which was absent in THP-1 mono-culture. In co-culture with macrophages, hAELVi cells displayed similar, sometimes even higher, trans-epithelial electrical resistance than in mono-cultures. When exposed to silver and starch NPs, hAELVi mono-cultures were more tolerant to the particles than THP-1 mono-cultures. The viability in the co-culture was similar to that of hAELVi monocultures. Transport studies with sodium fluorescein in presence/absence of EDTA proved that the co-culture acts as functional diffusion barrier. These data demonstrate that hAELVi-/THP-1 co-cultures represent a promising model for safety and permeability studies of inhaled chemicals, drugs and nanoparticles.

Keywords: *In vitro* model, air-blood barrier, pulmonary drug delivery, nanoparticles, nanotoxicology

1 Introduction

Due to its large surface (Gehr et al., 1978), relatively low enzymatic activity (Forbes et al., 1999; Shoyele and Slowey, 2006) and the possibility to administer drugs in a non-invasive way, the lung represents an attractive route for drug delivery. However, before new drug candidates can enter clinical trials, they have to be evaluated for safety and efficacy (Hittinger et al., 2015). The same applies also for inhaled (nano)materials and other xenobiotics (Wohlleben et al., 2014). In this context, advanced *in vitro* models acknowledging the 3R principle (Replacement, Refinement, Reduction) of Russell and Burch (Russell, 1959) have been applied to evaluate permeability, safety and efficacy of several drug delivery systems/drugs. Nevertheless, there is still a need for relatively simple models of the deep lung that must show essential cellular functions and in particular barrier properties. The air-blood barrier is mainly formed by two epithelial cell types: alveolar type I (ATI) and alveolar type II (ATII) cells. Whereas the cuboidal ATII cells produce surfactant and serve as progenitor cells for ATI cells, the latter themselves are more flattened and responsible for the gas exchange (Williams, 2003). Furthermore, this thin but effective barrier expresses tight intercellular connections and protects the body from outer stimuli, *e.g.* inhaled toxins, particles and microorganisms (Crandall and Matthay, 2001); moreover it also allows transcellular transport of solutes and gases by passive diffusion or active transport (Endter et al., 2009). This fact predestines the air-blood barrier as an versatile route of administration that allows local as well as systemic treatment of diseases such as asthma or diabetes (Mastrandrea, 2010).

So far, several *in vitro* models including mono- and complex co-cultures, have been described to evaluate safety and uptake of nanoparticles (Roggen et al., 2006). Of note, Calu-3, 16HBE14o- (Grainger et al., 2006; Forbes et al., 2003; Foster et al., 2000; Lehmann et al., 2011), NCI-H441 (Sporty et al., 2008; Salomon et al., 2014) or A549 have been frequently used (Foster et al., 1998; Salomon and Ehrhardt, 2011). The latter are cells derived from some adenocarcinoma

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(Giard et al., 1973) and do not express funtional tight junctions which limits their use for permeability studies (Winton et al., 1998). A possible alternaltive to mimic the human air-blood barrier are primary ATI-like cells, which express functional tight junctions when grown on permeable filter substrates (*e.g.* Transwells®) and are, therefore, better suited to evaluate transport of drugs or carriers through the epithelium (Elbert et al., 1999; Daum et al., 2012; Fuchs et al., 2003). These cells were recently combined with alveolar macrophages as an autologous co-culture (Hittinger et al., 2016). Despite the interesting aspect that both cell types originate from the same donor, such a system has several disavantages, such as the limited supply of tissue for isolating primary cells, their short life-spans and inter-individual differences due to different donors. These facts restrict their use for high-throughput screenings of prospective new formulations.

In this context, a new ATI-like cell line expressing functional tight intercellular junctions was recently established in our group, referred to as human alveolar epithelial lentivirus immortalized (hAELVi) cells (Kuehn et al., 2016). Using this cell line, we describe here a more robust co-culture model that mimics the deep lung in terms of cell constitution and physiological conditions (*i.e.* at air liquid interface - ALI). These functions correlate with the site *in vivo* where the apical compartment represents the air-exposed part of the lung, whereas the basolateral compartment mimics the bloodstream. To set up this model, pre-grown hAELVi monolayers were combined with prior differentiated macrophage-like cells (THP-1), derived from monocytes (Tsuchiya et al., 1980), and cultivated under liquid covered conditions (LCC) as well as at ALI. After establishment, the co-culture was characterized regarding cellular morphology, viability and barrier properties. Transport studies were performed to evaluate the functionality as a diffusional barrier. Long-term cultivation up to 7 days was conducted in two individual experiments to determine potential suitability for future studies addressing cancer pathogenesis, inflammation or chronic lung diseases. Silver nanoparticles (Ag NPs), as well as some newly developed starch NPs (Barthold et al., 2016), providing a model for the pulmonary delivery of proteins and peptides, were aerosolized and deposited onto the co-culture in order to evaluate their cytotoxicity and uptake/interactions with this new system.

2 Materials and Methods

2.1 Cell cultures

The establishment and characterization of the established ATI-like cell line hAELVi was recently described in (Kuehn et al., 2016). The clone hAELVi.B (Kuehn et al., 2016) has been used in the present work to set up the co-culture model. hAELVi.B (passages between 25-35) were maintained in fibronectin/collagen-coated cell culture plastic (T75), in SAGM medium containing 1% fetal calf serum (FCS) and 1% penicillin/streptomycin (P/S). When the cells reached about 80-90% confluence, they were used for experiments as described below.
THP-1 monocytes (No. ACC-16, DMSZ) were differentiated to macrophages-like cells with 7.5 ng/mL of phorbol 12-myristate 13-acetat (PMA; Sigma, Germany) (Schwende et al., 1996) incubated for 48 h. To confirm the differentiation into macrophages-like cells, we characterized macrophage marker expression on differentiated THP-1 cells, as described below in the confocal laser scanning microscopy (CLSM) section. Differentiated THP-1 cells were then trypsinized and used in the experiments, as described below. Mono and co-cultures were set up simultaneously, in order to have direct comparison.

Mono-cultures

hAELVi cells ($2x10^5$ cells/cm²) were seeded on fibronectin (1% (v/v); Corning, USA) / collagen (1% (v/v); Sigma, Germany)-coated Transwell® polyester membranes (Corning 3460; growth area 1.12 cm²; pore size 0.4 µm) under LCC. For that the cells were supplied with 500 µL apical and 1.5 mL basolateral of a complex small airway epithelial cell growth medium (SAGM, Lonza), supplemented with 1% (v/v) fetal bovine serum (FBS; Lifetechnologies, Germany) and 1% Penicillin/Streptomycin (P/S; 10.000 U/mL, Gibco Lifetechnologies, USA). One group was kept under LCC, the other was transferred to ALI 2 days' post seeding, *i.e.* the medium from apical compartment was aspirated and the cells were further fed from the basolateral compartment with 500 µL of SAGM. After 14 days some transwells were used to set up the co-culture model while other were kept as monocultures.
THP-1 macrophages (~1.2×10^6 cells/cm^2) were seeded on Transwell® filters (Corning 3460, growth area 1.12 cm²; pore size 0.4 µm) under LCC in RPMI medium containing 10% (v/v) FBS and 1% (v/v) P/S. After 4 h allowing the cells to adhere, they were set on ALI, as described before, by using RPMI in the basolateral side.

Co-cultures

hAELVi mono-culture pre-grown for 14 days were used to set up the co-culture with THP-1 cells. Considering that hAELVi cells have a double time of ~2.5 days (Kuehn et al., 2016), we adjusted the amount of THP-1 to be seeded in order to have a final ration of 3:1 (hAELVi:THP-1)(Stone et al., 1992). The co-culture was set-up as following: differentiated THP-1 cells (~1.2×10^6 cells/cm^2) were resuspended in 6 µL (ALI) or 500 µL (LCC) of RPMI, respectively, and subsequently added on top of the hAELVi cells. In the basolateral compartment, 0.5 mL (ALI) or 1.5 mL (LCC) of SAGM was applied. The co-culture was cultivated for 24 h at 37°C, 5% CO_2. Additionally, in two individual experiments the cells were further co-cultivated for 3 days and 7 days.

2.2 Morphological and ultrastructural analysis

To prepare the cells for morphological and ultrastructural analysis, mono- and co-cultures were cultivated at ALI and under LCC. The samples were evaluated with confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM) or transmission electron microscopy (TEM), following the protocols previously described (Carvalho et al., 2011; Kuehn et al., 2016; Susewind et al., 2015), with minor modifications.

Confocal laser scanning microscopy (CLSM)

The differentiation of THP-1 cells into macrophages-like cells was determined by using FITC anti-human CD11b antibody (BioLegend, 301329), which stains macrophages and FITC anti-human CD14 antibody (BioLegend, 301803), which stains monocytes cells (Mittar et al., 2011). Briefly, differentiated THP-1 cells (1 x10^5 cells/mL) were seeded onto 10-mm diameter#1.5 glass coverslips placed into the a 24-well plate. After 24 h, the cells were stained with CD11b (1:20) or CD14 (1:20) antibodies. For that live cells were incubated with Human IgG 10 µg/mL (Sigma, I 4506) for 15 min, at room temperature, to block nonspecific sites. Thereafter, washed 1x with PBS 37°C, and incubated with CD11b or CD14, for 30 min at 4°C, protected from light. After 2x washing with PBS the cells were fixed with 3% paraformaldehyde (PFA; Stock 16%; 15710-S, Electron Microscopy Sciences, USA), counterstained with DAPI (1:50000) and mounted in DAKO mounting medium (Product No. 85 S302380-2, DAKO, USA).

Macrophage-like cells were pre-labelled with CellTrace™ Far Red (DDAO-SE; C34553; Molecular Probes, USA) following the manufacture's protocol. Mono- and co-cultures were fixed with 3% PFA in phosphate buffered saline (PBS) from basolateral at 4°C overnight, whereas the following steps were performed from apical. The samples were quenched with 150 µL 50 mM NH_4Cl in PBS for 10 min followed by a blocking and permeabilization step using a solution of 0.5% bovine serum albumin(BSA) /0.025% Saponin in PBS for 30 min at room temperature (RT). The primary antibody against occludin (mouse anti-occludin, Catalog No 33-1500, Invitrogen) was diluted in 0.5% BSA/ 0.025% Saponin/PBS-solution (1:200), 150 µL were added in the apical compartment and incubated at 4°C overnight. The secondary antibody (polyclonal Alexa-Fluor 488 conjugated rabbit anti-mouse, Catalog No. A11059, Invitrogen) was diluted in PBS (1:400) and incubated for one hour at RT. The samples were washed with PBS and counterstained with DAPI (1:50000). To visualize the long-term co-culture, THP-1 cells were pre-labeled as previously described. In both, Transwell® membranes were cut out, mounted in DAKO mounting medium.

All samples were analyzed by confocal laser scanning microscopy (Zeiss LSM710, Zeiss, Germany). Lasers at 405 nm (DAPI), 488 nm (FITC) and 633 nm (DDAO-SE) were used for detection. Microscopic images were acquired at 1024 × 1024 resolution, using 63x water immersion objective. Confocal images were analysed using Zen 2012 software (Carl Zeiss Microscopy GmbH) and Fiji Software (Fiji is a distribution of ImageJ[1]).

Scanning electron microscopy (SEM)

Mono- and co-cultures were set-up as previously described and fixed with 3% PFA from basolateral at 4°C overnight after 15 days (hAELVi mono-cultures) or 24 h (THP-1 mono-cultures and co-cultures). Afterwards, the samples were dehydrated with a gradual ethanol row (30-50-60-70-80-90-96-2x100% for 10 min each) followed by hexamethyldisilazane (HDMS) for 10 min. The membranes were then cut with a scalpel, put on a carbon disc and were further sputtered with gold before examined with the SEM. Images were taken with a Zeiss SEM EVO® HD15 (Zeiss, Germany) under high pressure conditions with a secondary electron detector and using 10 kV acceleration voltage.

Transmission electron microscopy (TEM)

Samples were fixed after 15 days (hAELVi mono-cultures) or 24 h (THP-1 mono-cultures and co-cultures) by using 1% final concentration of glutaraldehyde (GA), diluted with 200 mM 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid (HEPES) buffer, pH 7.4, overnight at 4°C. All samples were incubated with GA overnight at 4°C, from both apical and basolateral side. The samples were further processed as described by Susewind *et al*. (Susewind et al., 2015), with minor modifications. Briefly, for epon embedding the samples were postfixed with 2% OsO_4 (EMA, PA, USA) solution containing 1.5% potassium ferricyanide for 1 h on ice, and stained *en bloc* with 1.5% aqueous uranyl acetate (EMS, PA, USA) for 30 min. Cells were then dehydrated at RT using a graded ethanol series (70-80-90-96-(4x) 100% for 10 min each), progressively infiltrated with epoxy resin (50-75-100%) (Sigma-Aldrich; St. Louis/MO, USA). Transwell® membranes with cells were flat embedded and blocks were polymerized overnight at 70°C. Ultrathin sections of 70-80 nm, perpendicular to the filter plane, were cut with a Leica ultra-microtome Ultracut EM UCT (Leica Microsystems, Austria) using an ultra-diamond knife (Diatome, Switzerland) and examined with a JEM-1400 transmission electron microscope (JEOL, USA). The images were taken with a TemCam-F216 camera (Tvips, Germany).

2.3 Barrier properties

Barrier properties were evaluated by measuring the transepithelial electrical resistance (TEER) as described in (Daum et al., 2012; Kuehn et al., 2016; Srinivasan et al., 2015). Briefly, the cells were seeded on prior coated permeable filters with a pore size of 0.4 µm and a growth area of 1.12 cm² (Corning, 3460), at ALI and under LCC. For that, apical compartments of samples grown at ALI were refilled with pre-warmed 500 µL of medium and the basolateral compartments were filled up to a final volume of 1.5 mL. After 1 h of incubation the TEER was measured in all samples by using an epithelial voltohmmeter (EVOM) equipped with chopstick electrodes (both: World Precision Instruments, Sarasota, USA). The electrical resistance was calculated by subtracting the value of blank inserts containing medium (110 Ω) from all samples, and further multiplication with the cultivation area of the inserts (1.12 cm^2).

2.4 Transport studies

Transport experiments were performed using the paracellular marker sodium fluorescein (NaFlu) and ethylenediaminetetraacetic acid (EDTA), as a modulator of the tight junctions, according to (Elbert et al., 1999; Kuehn et al., 2016), with minor modifications. Briefly, hAELVi mono-cultures and co-cultivated with THP-1 were cultured as previously described. 24 h after co-cultivation, the samples were washed once with pre-warmed Krebs-Ringer Buffer (KRB; 142.03 mM NaCl, 2.95 mM KCl, 1.49 mM K_2HPO_4*3H_2O, 10.07 mM HEPES, 4.00 mM D-Glucose, 1.18 mM $MgCl_2$*6H_2O, 4.22 mM $CaCl_2$*2H_2O; pH 7.4). To avoid losing macrophages during the washing steps, the supernatants were centrifuged (4 min, 300

[1] http://fiji.sc

g) and the pellet was further resuspended in 500 µL KRB. After 45 min incubation with KRB, 520 µL NaFlu (10 µg/mL in KRB) ± 16 mM EDTA were added to the apical (donor) and 1.7 mL KRB were put in the basolateral compartment (acceptor). Samples were directly taken from the donor (20 µL) as well as from the acceptor compartment (200 µL) every 30 min for 3 hours and were subsequently transferred into a 96-well plate. Samples taken were replaced with an equal volume of fresh KRB. During the experiment, the plates were placed on a MTS orbital shaker (150 rpm; IKA, Germany) at 37 °C in an incubator (CB 210; Binder, Germany). The samples were then read with a Tecan® plate reader (Tecan Deutschland GmbH, Germany) using wavelength of 488 nm (em) and 530 nm (ex). Epithelial barrier integrity was always determined before and after the experiment.

2.5 Nanoparticle preparation and subsequently application

Before well-characterized Ag NPs (730793; Sigma Aldrich, Germany) and newly developed starch NPs (Barthold et al., 2016), respectively, were deposited onto hAELVi-/THP-1 mono-and co-cultures using the Aeroneb®Lab nebulizer (Aerogen Ltd., Ireland), their colloidal stability upon nebulization was determined in preparation medium including size, size distribution (polydispersity index, PdI) and surface charge (ζ-potential) using the ZetaSizer® Nano ZSP (Malvern Instruments, UK). Particle sizes were intensity based z-average values and standard deviation was of 3 measurements, as previously described in (Barthold et al., 2016). Commercial available Ag NPs were stated by the manufacturer to be 20 ± 4 nm in size, starch NPs were described with a size of 150.6 ± 2.4 nm, a PdI of 0.08 and a ζ-potential of -26.5 ± 1.63 mV.

Further, after 24 h of incubation, cell viability was evaluated by applying the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Mosmann, 1983). Briefly, mono-and co-cultures were washed once with 300 µL Hanks' balanced salt solution buffer (HBSS; 1.12 mM $CaCl_2$, 0.49 mM $MgCl_2*6H_2O$, 0.41 mM $MgSO_4*7H_2O$, 5.33 mM KCl, 0.44 mM KH_2PO_4, 4.17 mM $NaCO_3$, 137.93 mM NaCl, 0.34 mM Na_2HPO_4, 5.55 mM D-Glucose, pH 7.4). To avoid losing macrophages during the washing steps, the supernatants were centrifuged, and the pellets were resuspended in 300 µL HBSS buffer, containing 10% (v/v) MTT reagent (5 mg/mL; Sigma Aldrich, USA), added to the well and incubated for 4 h. After aspirating the reagent, the samples were further incubated with 300 µL DMSO for 20 min. Absorbance was measured with the Tecan Infinite® 200 microplate reader (Tecan Deutschland GmbH, Germany) at 550 nm. During the experiment the plates were placed on a MTS orbital shaker (150 rpm; IKA, Germany) at 37°C in the incubator (CB 210; Binder, Germany). Cell viabilities were calculated in comparison to a positive (untreated cells: 100% cell viability) and a negative control (1% TritonX-100: 0% of cell viability).

The influence of NPs on the epithelial barrier integrity was determined by conducting TEER measurements.

Further, to evaluate the uptake/cellular interactions of starch NPs into mono-and co-cultures, the cationic starch was labeled with a green fluorescent dye (Bodipy® FL C5 NHS Ester; LifeTechnologies, USA) and starch NPs were prepared afterwards from 0.25 mg/mL solutions in a ratio of 1:1. Mono- and co-cultures were cultivated as previously described. After 24 h of co-culture, 250 µL (41.25 µg) of labeled starch NPs were deposited onto mono- and co-cultures with the Aeroneb®Lab nebulizer and further incubated for 24 h. Then, the supernatant was carefully aspirated, and the cells were fixed with 3% PFA from basolateral at 4°C overnight. The following day, 150 µL BSA/Saponin/PBS solution were applied apically to permeabilize the cells, followed by 150 µL anti-rhodamine phalloidin staining (1:100 in PBS; actin; R415; Molecular Probes, USA) for 30 min at RT. After aspirating the staining solution, the samples were counterstained with 150 µL DAPI (1:50000 in PBS; nuclei) and subsequently mounted in DAKO (S302380-2; DAKO, USA). The samples were analyzed using CLSM. Lasers at 405 nm (DAPI), 488 nm (starch NPs), 565 nm (actin) were used for detection. Microscopic images were acquired at 1024x1024 resolution, using 63x water immersion objective. Confocal images were analyzed using ZEN 2012 software (Carl Zeiss Microscopy GmbH) and Fiji software (Fiji is a distribution of ImageJ).

2.6 Statistical Analysis

Data are representative from 2 or 3 independents experiments (as indicated in each legend figure). Graphs represent the mean ± SEM. Two-way ANOVA with Bonferroni´s post hoc test was performed using GraphPad Prism 5 software (GraphPad).

3 Results

hAELVi cells were seeded on fibronectin/collagen-coated polyester membranes with a pore size of 0.4 µm and a growth area of 1.12 cm² under liquid covered conditions (LCC); after 2 days seeding they were set on air liquid interface (ALI), whereas some samples were kept under LCC. After 14 days in culture, hAELVi cells were used to set up the co-culture with differentiated THP-1 cells. The positive staining for CD11b (a macrophage marker) and negative staining for CD14 (a monocyte marker) determined the THP-1 differentiation into macrophage-like cells (Fig. S1[1]). THP-1 macrophages were then added on top of the epithelium and the system was further cultivated for 24 h. Confocal images showed distinct tight junctions in the co-culture after 1 day at ALI (Fig. 1A). This holds also true for LCC, but in contrast to the ALI samples only a few macrophages were observed on top of the epithelial cells (Fig. 1B). Barrier properties were further evaluated by TEER measurements. hAELVi cells maintained the TEER in co-cultures, which was similar or even higher than those observed in mono-cultures at ALI (Fig. 1C), as well as under LCC (Fig. 1D).

In order to have a co-culture system that could potentially be used not only for drug delivery testing but also to study chronic diseases, hAELVi cells and THP-1 were cultured for up to 7 days at ALI; under these conditions both cell types remained viable. After 7 days, however, a decrease in the number of macrophages could be seen (data not shown). In addition, barrier properties were detectable even after 7 days at ALI (data not shown, 2 individual experiments). To prolong

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the co-culture for *e.g.* studying longer exposure times or repeated exposure, one possibility could be to further include more macrophages after 3 days in co-culture.

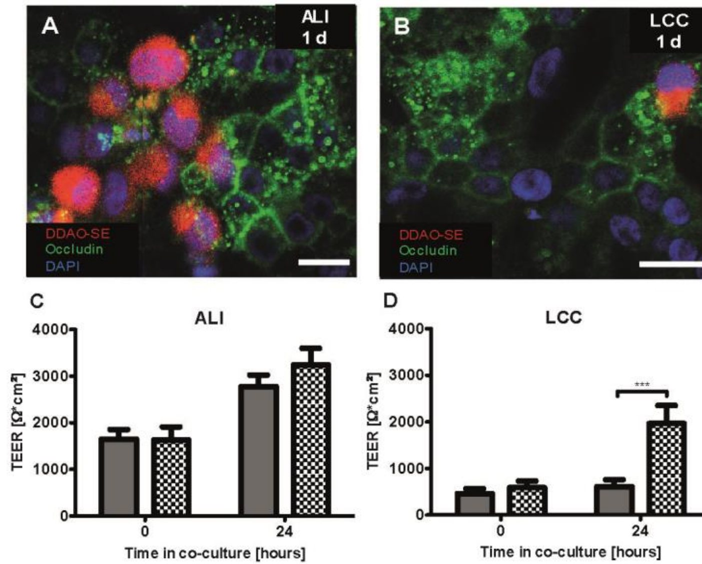

Fig. 1: Morphology and barrier properties of hAELVi/THP-1 co-cultures. A/B: Confocal laser scanning microscopic (CLSM) images of the co-cultures after 24 h, cultivated at ALI (A) and under LCC (B), Bar: 20 µm; ; THP-1 cells (DDAO-SE; red), hAELVi (occludin, green); nuclei are counterstained with DAPI (blue). Bar: 10 µm. C/D: TEER measurements of hAELVi mono-cultures (grey bars) immediately before (0 days) and after (1 day) co-culture with THP-1 (dotted bars), grown at ALI (C) and under LCC (D). A/B: Images are representative for 3 individual experiments; C/D: Data shown here are mean ± SEM (n=9) from 3 independent experiments; ***P<0.001.

The morphology was further evaluated by using SEM, which revealed a homogenous distribution of THP-1 cells on top of the hAELVi cells at ALI (Fig. 2A) and under LCC (Fig. 2B). As shown by parallel ultrastructural analysis, THP-1 macrophages are located on top of the epithelium in co-cultures grown at ALI (Fig. 3A). In co-cultures grown under LCC, no macrophages could be found, presumably because they do not interact strongly enough with epithelial cells to be immobilized by aldehyde fixation. Even at ALI, the two cell types make only loose contact via the microvilli extending from the apical hAELVi cell surface towards the macrophages. Adjacent THP-1 macrophages interacted with pseudopodia (Fig. 3F). Epithelial cells interacted with pseudopodia at their lateral sides and formed tight junctions (TJ) at the top of the epithelium (Fig. 3C). Differences between THP-1 in mono- and co-culture with hAELVi could be observed: whereas in co-culture at ALI THP-1 cells are covered with a layer of extracellular matrix at the surfaces exposed to air (Fig. 3E), this could not be seen when grown in mono-culture (Fig. 3G). The apical surface of epithelial cells is covered with glycocalyx-like structures (Gx) in both conditions, although Gx may have been slightly more prominent at ALI (data not shown).

To evaluate the co-culture for performing toxicity studies, well-characterized silver nanoparticles (Ag NPs) that have been reported to be toxic to mammalian cells (Ahamed et al., 2010), were applied onto the mono- and co-cultures at a dose of 7.25 µg. Additionally, with regard to pharmaceutical relevance, a new drug carrier system based on starch nanoparticles was nebulized with the Aeroneb®Lab nebulizer (Barthold et al., 2016). These NPs are intended for pulmonary drug delivery of proteins and peptides and were already tested on A549 and 16HBE14o- cells applying a dose of 41.25 µg, which was well tolerated. As the co-culture model has both macrophages and functional tight junctions, this new drug delivery system was applied to determine its impact on cell viability and cellular interactions in such a scenario. Before applying to the mono- and co-cultures, particle suspensions were collected before and after nebulization and evaluated regarding size, polydispersity index (PdI) and ζ-potential. Measurements performed with the ZetaSizer® Nano ZSP showed an average size of 26.1 nm of Ag NPs, similar to the manufacturer's information with a size distribution (PdI) of 0.21. Except for a slight increase in ζ-potential, these particles were stable during nebulization. Starch NPs were also stable during nebulization, showing an average size of 115.10 ± 1.21 nm with a narrow size distribution (PdI: 0.11 ± 0.02) and a negative ζ-potential of -26.50 ± 1.63 mV.

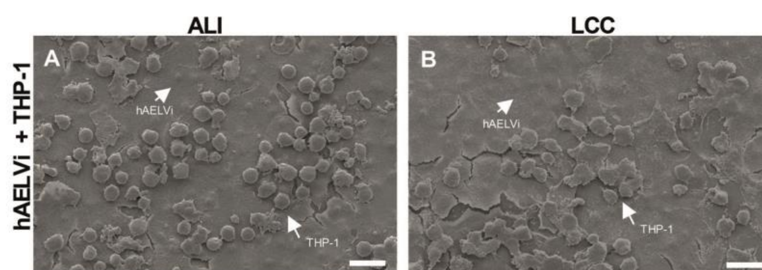

Fig. 2: Morphology of co-cultures. Scanning electron microscopic images of hAELVi cells co-cultured with THP-1, cultivated at ALI (A) and under LCC (B). Bar: 30 µm

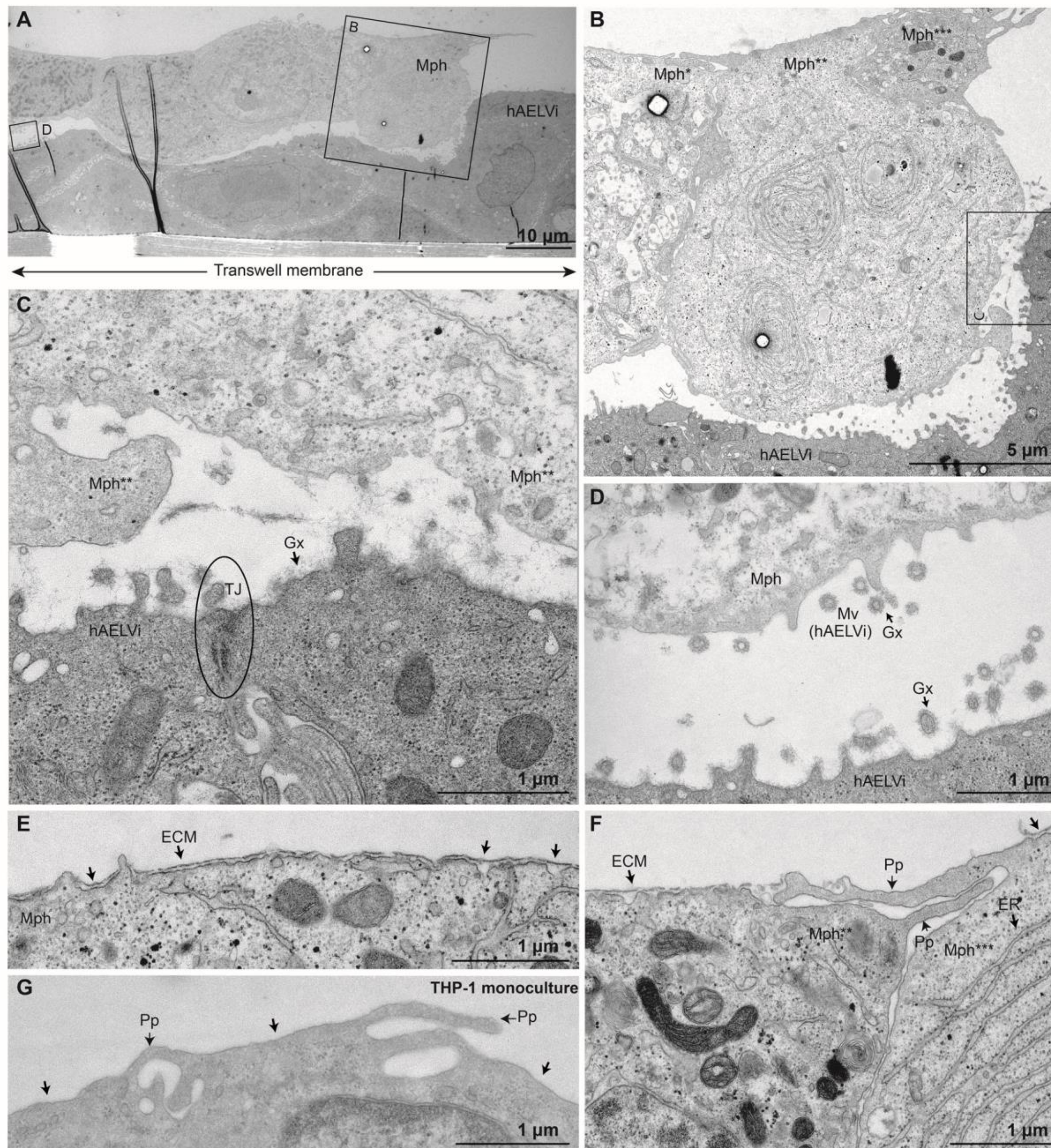

Fig. 3: Ultrastructural analysis of co-cultures at ALI. Transmission electron microscopic (TEM) images of hAELVi cells after 24 h in co-culture with THP-1. A: Overview of several macrophage-like cells on top of epithelial cells. B-D: Space between the two cell types; microvilli (Mv) extending from hAELVi cell surface towards THP-1 cells suggest weak interaction between the two cell types. hAELVi cells form tight junctions (TJ), microvilli (Mv) and glycocalyx (Gx) at the apical cell surface. E, F: In co-culture with hAELVi cells at ALI, the surface of THP-1 cells that is exposed to the air is covered with a layer of extracellular matrix (ECM). Adjacent THP-1 cells interact with pseudopodia (Pp) and have abundant endoplasmic reticulum (ER). G: In mono-culture at ALI, THP-1 cells are not covered with extracellular matrix.

After 24 h in co-culture, doses of 7.25 µg (Ag NPs) and 41.25 µg (starch NPs), respectively, were deposited onto hAELVi-/THP-1 mono-cultures and the co-culture. When exposed to silver and starch NPs, respectively, hAELVi mono-cultures were more tolerant to the particles than the mono-culture of THP-1, which showed only 50% viability. The viability in the co-culture was similar to the one observed for hAELVi monocultures. (Fig. 4A).

To further evaluate the effect of nebulized Ag NPs and starch NPs on the barrier integrity, TEER was measured 24 h after particle incubation. While hAELVi cells in mono-culture displayed ~3900 Ω*cm² (non-treated), the incubation with Ag NPs led to a decrease in TEER down to ~2300 Ω*cm². The untreated co-culture showed a similar TEER value of ~3800 Ω*cm² and displayed a slight and non-significant drop in TEER to ~2500 Ω*cm² with Ag NPs present. Comparing mono- and co-cultures, no difference could be observed as both groups showed the same behavior after NP incubation. The same trend could also be observed in the samples incubated with starch NPs. Two independent experiments showed also a decrease in TEER down to ~2000 Ω*cm² (mono-culture) and ~2500 Ω*cm² (co-culture), respectively (Fig. 4B). However, TEER values > 1000 Ω*cm² represent a non-impaired and tight barrier function in all samples, untreated as well as treated.

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To evaluate functionality of the diffusional barrier within the model in more detail, the transport of sodium fluorescein (NaFlu), a hydrophilic molecule typically used to evaluate paracellular transport, was monitored. Therefore, mono- and co-cultures were cultivated at ALI and under LCC. High TEER is always accompanied with low paracellular transport. This relationship could be verified with the transport of NaFlu across hAELVi-/THP-1 co-cultures at ALI (Fig. 5A) and under LCC (Fig. 5B). After adding 16 mM EDTA, a known modulator of tight junctions, a drop in TEER and subsequent higher transport of NaFlu to the basolateral compartment was observed under both culture conditions. In parallel, transport of NaFlu ± 16 mM EDTA across hAELVi mono-cultures, grown under LCC and at ALI were conducted, yielding similar results as already reported in (Kuehn et al., 2016) (Fig. 5A). Again, the effect of EDTA was most pronounced in case of the co-culture under LCC, presumably because hAELVi cells are in contact with a higher amount of RPMI medium during their cultivation, in conjunction with the higher TEER.

Finally, cellular interactions with the starch NPs as a prospective drug delivery system were also evaluated by CLSM. Z-stacks indicated an internalization of starch NPs into hAELVi mono-cultures (Fig. 6A); as well as into THP-1 mono-cultures after 24 h of incubation (Fig. 6B). In contrast, when doing the same experiments with the co-culture only internalization into macrophage-like cells but no uptake into epithelial cells was observed (Fig. 6C).

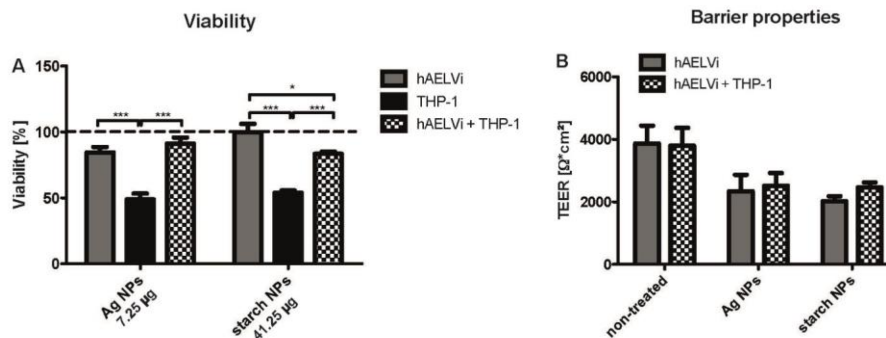

Fig. 4: Evaluation of the effect of nebulized Ag NPs and starch NPs on mono- and co-cultures, cultivated at ALI. A: Cell viabilities of hAELVi-/THP-1 mono- and co-cultures after 24 h of Ag and starch NP incubation. B: TEER-measurements of hAELVi mono-cultures and co-cultivated with THP-1 24 h after nebulization of Ag and starch NPs. Data shown are mean ± SEM (n=9) from 3 independent experiments for Ag NPs and (n=6) from 2 individual experiments for starch NPs, respectively; *P<0.05, ***P<0.001.

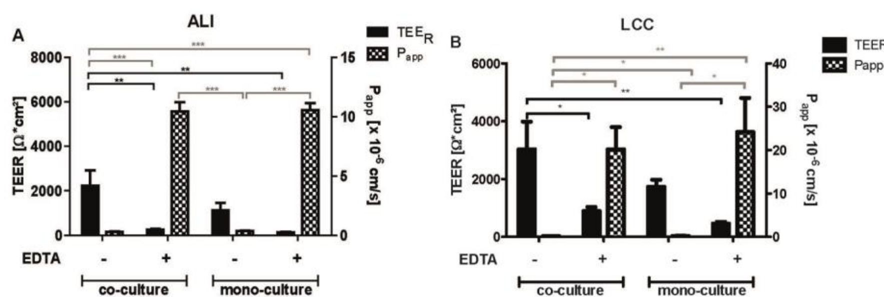

Fig. 5: Permeability assay on mono- and co-cultures grown at ALI and under LCC. Transport of sodium fluorescein ± 16 mM EDTA across hAELVi mono- and co-cultures with THP-1 cultivated at ALI (A) and under LCC (B). The graph combines TEER and P_{app} values. Data are shown in mean ± SEM (n=9) (Fig. 5B) or (n=8) (Fig. 5A) from 3 independent experiments; *P<0.05, **P<0.005, ***P<0.001.

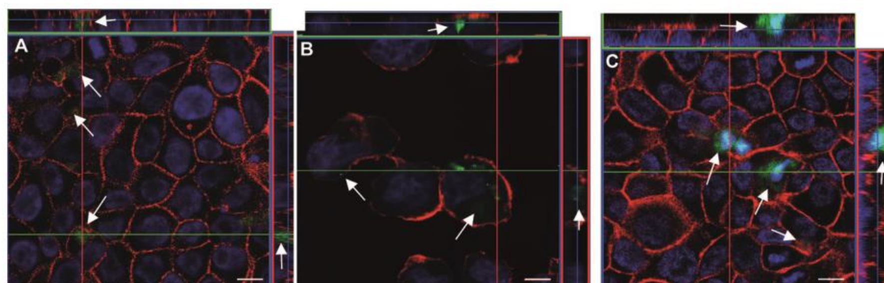

Fig. 6: Uptake/interaction studies of nebulized starch NPs. Confocal laser scanning microscopic images of mono-cultures of hAELVi (A), THP-1 (B) and the co-culture (C) cultivated at ALI after 24 h of incubation with nebulized blank starch NPs. hAELVi / THP-1 mono-cultures: Actin: red, NPs: green. Nuclei were counterstained with DAPI (blue). Images are representative for 2 individual experiments. Arrows indicate starch nanoparticles. Bar: 10 µm.

4 Discussion

This paper reports a new *in vitro* model of the human air-blood barrier consisting of the two essential cell types, i.e. ATI epithelial cells and macrophages. ATI cells make up 70-90% of the alveolar epithelial surface and therefore mainly form the air-blood barrier. They are squamous in shape and therefore highly specialized for the key function of the lung: gas exchange (Williams, 2003). Macrophages, as part of the immune system, are located on top of this epithelial layer and take up inhaled particles, allergens as well as microorganisms that are able to overcome the mucociliary barrier (Crandall and Matthay, 2001). Thus, these two cell types may be considered as essential for developing a predictive *in vitro* model of the deep lung. So far, several models including mono- and complex co-cultures have been described to evaluate nanoparticle uptake, inflammatory responses or disease-relevant mechanisms. For example, Rothen-Rutishauser and colleagues established a triple cell culture system composed of epithelial cells (A549), monocyte derived macrophages and dendritic cells, which cultivated at ALI could show more efficacy in predicting *in vivo* toxicity compared to mono-cultures (Rothen-Rutishauser et al., 2005). Indeed, immune cells play a crucial role in forming an additional barrier due to particle-phagocytosis and inflammatory responses (Muller et al., 2010). The communication and transfer of polyelectrolyte microcapsules within the previous described triple co-culture model could recently be shown by Kuhn et al. (2015). Further, to study the potential toxic effect of particles on the lung, Klein *et al.* established a tetra culture model comprising four different human cell lines including epithelial cells (A549), macrophages (THP-1), mast cells (HMC-1) and endothelial cells (EA.hy 926) (Klein et al., 2013). Moreover, a suitable co-culture model to evaluate adsorption, uptake and trafficking of nanosized carriers was established by Hermanns *et al.* and involves the epithelial cell line NCI-H441 in co-culture with primary isolated human pulmonary microvascular endothelial cells (HPMECs) or endothelial cells (ISO-HAS-1). Within this co-culture, epithelial cells acted differently regarding barrier properties and inflammatory responses (Hermanns et al., 2009). Although the previously described co-culture models show several advantages compared to mono-cultures, they lack ATI cells and therefore are deficient in expressing a functional diffusional barrier.

With this in mind, an autologous co-culture composed of primary ATI-like cells and alveolar macrophages isolated from the same donor has recently been reported by Hittinger *et al.* to evaluate the safety of airborne particles (Hittinger et al., 2016). The primary human ATI cells used in this model form functional tight junctions and exhibit a similar physiology to what is observed *in vivo* (Elbert et al., 1999; Fuchs et al., 2003; Endter et al., 2007). Moreover, the use of primary alveolar macrophages obtained from the same donor provides an additional advantage. Nevertheless, the use of such primary co-culture is limited by the restricted access to primary tissue, short life-spans of primary cells and inter-individual differences. This implicates a limited reproducibility and confines the application of such model in high-throughput assays. These disadvantages, however, can be overcome by replacing primary cells by stable cell lines such as hAELVi (Kuehn et al., 2016) and THP-1 (Tsuchiya et al., 1980) cells.

By keeping the advantage of unlimited access and better reproducibility compared to primary cells the new co-culture described in the present paper features the two functional two important barriers for pulmonary drug delivery, *i.e.* a diffusional epithelial barrier and macrophage-mediated clearance. The co-culture system was set up under LCC and at ALI, and compared to mono-cultures in parallel experiments. In several studies 2D or 3D cell cultures grown under LCC have been used for evaluating toxicity (Roggen et al., 2006), particle interaction (Rothen-Rutishauser et al., 2005; Alfaro-Moreno et al., 2008) or cell infection (Kusek et al., 2014). However, to mimic aerosol deposition in the air space of the lung, LCC is not ideal. An additional problem is that macrophages interact weakly with epithelial cell surfaces under such conditions and are therefore easily removed by aspiration of the medium. This is corroborated by our results, which show less macrophages on top of epithelial cells when grown under LCC conditions compared to ALI.

Besides morphology, mono- and co-cultures mainly differ in their barrier properties. Our results demonstrate an increase in TEER in the co-culture. Chowdhury *et al.* also showed a difference in terms of barrier in a co-culture constituted by 16HBE14o-, a bronchial epithelial derived cell line and human umbilical vein endothelial cells (HUVEC) (Chowdhury et al., 2010). They reported that the alteration in TEER was mediated by soluble endothelial-derived factors. Although our model does not include endothelial cells, hAELVi cells co-cultivated with THP-1 as well as grown in mono-culture with mixed SAGM/RPMI showed an increase in TEER.

Preliminary data show that the co-culture system can be extended up to 7 days, in which hAELVi cells maintain their tight barrier and macrophages remain viable, although the number of macrophage-like cells is decreased. The possibility to further add more macrophages after 3 days in co-culture makes this model appear also suitable to study chronic diseases like chronic bronchitis, asthma and COPD (Adamson et al., 2011). To our knowledge this is the first time a co-culture of lung cells could be cultivated for such a period of time. Nevertheless, more detailed characterization of macrophages regarding receptor expression or cytokines release after long-term co-cultivation should be addressed in future experiments.

To prove the functionality of the diffusional barrier, permeability studies were conducted with the co-culture model by monitoring the transport of sodium fluorescein alone or in combination with EDTA. As tight junctions are able to execute the so-called "fence-function" by mechanically restricting the diffusion of lipids and proteins via the paracellular route, high TEER is always associated with low paracellular transport (Niessen, 2007; Martin and Jiang, 2009). This relationship could be observed in our co-cultures when grown under both culture conditions. After adding EDTA, a drop in TEER, followed by an increase in transport of sodium fluorescein to the basolateral compartment could be observed. Several studies have shown that the removal with chelators of Ca^{2+}, that is known to be involved in tight junction assembly disrupt the tight junctions, resulting in lower transepithelial electrical resistance, coupled with an increase in the permeability to tracers (Bhat et al., 1993; Klingler et al., 2000; Brown et al., 2004; Tria et al., 2013). This effect was more distinct in samples grown under LCC, as hAELVi cells are in contact with a higher amount of RPMI medium during the co-cultivation with THP-1, resulting in higher TEER values and less permeability. Our results suggest that our alveolar co-culture system can serve as a promising model to further evaluate drug absorption. In contrast to hAELVi mono-cultures this model contains macrophages as an additional barrier for NPs, which allows studying transport of new formulations/drug delivery systems in combination with clearance mechanisms.

Ag NPs, previously described to be toxic (Ahamed et al., 2010) were nebulized onto mono-cultures of hAELVi or THP-1 cells as well as onto the co-culture, which were grown at ALI. In THP-1 mono-cultures, already a dose of 7.25 µg reduced the cell viability whereas the hAELVi mono-culture showed a high viability. In the co-culture, cell viability was similar to the one observed for hAELVi monocultures. As the viability assay with MTT does not allow for the discrimination between viable THP-1 or hAELVi, we cannot conclude at this stage that the co-culture is more robust than the monoculture. We have made several efforts towards the determination of a specific cell-type viability response within our co-culture model. As the interaction between THP-1 and hAELVi cells are so weak, all post-staining methods used to stain viable cells failed, since the macrophages were washed out. It is known that multicellular *in vitro* systems show several advantages beyond monocultures, allowing for elucidating cellular responses originated from cell-cell interaction. Nevertheless, up to date there are several drawbacks in terms of determining specific cellular responses in such complex models. A recent work has shown a great progress towards the characterization of cell type response within a lung *in vitro* co-culture model, by using flow cytometry (Clift et al., 2017). Nevertheless, the authors also did not differentiate between the viability of each cell type in their co-culture system, despite they succeeded to detach the cells from the membrane insert and analysed them by flow cytometry. In our model the detachment of hAELVi cells as well as THP-1 cells resulted in a high cell death rate, which would compromise the viability assessment either in treated or untreated cultures and made flow cytometry not the method of choice for such analysis.

TEM images showed some interaction between hAELVi and THP-1 cells and we assume that this interaction may attenuate the toxic effects of Ag NPs. An abundant glycocalyx that covers the epithelial cells and has previously been shown to shield the human airway epithelial cells from virus-mediated gene transfer (Pickles et al., 2000), might somehow extend its protective role to macrophages. Due to the fact that the extracellular matrix layer that covered THP-1 cells in co-culture at ALI could not be observed in THP-1 mono-cultures, we speculate that hAELVi cells secrete factors that modify the surface of macrophage cells or otherwise have influence on these cells. In any event, this might be an evidence of a cross-talk between hAELVi cells and THP-1, that could influence the sensitivity of THP-1 to toxic effects upon NPs exposition. To get more detailed information about the crosstalk between both cell types, further experiments and optimization of methods to allow for cell-type characterization in our model are necessary, as previously discussed.

Kasper *et al.* introduced a co-culture model comprising the epithelial cell line NCI-H441 and ISO-HAS-1 as endothelial cells mimicking the alveolar-capillary barrier to evaluate the impact of silica NPs in the deep lung. They showed that their co-culture was less sensitive regarding toxic effects compared to conventional mono-cultures, but conversely, much more sensitive in terms of the inflammatory response (Kasper et al., 2011). With regard to Ag NPs toxicity, this might be the case for the here described co-culture of macrophages and ATI-like alveolar epithelial cells. Although a slight effect on the permeability of the tight junctions could be observed after the incubation with Ag NPs, the epithelial cells still maintained essentially a tight barrier. The possible induction of inflammation is also an important issue that should always be considered in the context of exposure to NPs. Therefore, experiments for evaluating inflammatory responses after NPs incubation should be included in future studies.

As our model was intended to evaluate the safety of new carrier materials for inhalation pharmaceuticals, starch NPs (Barthold et al., 2016), intended for pulmonary delivery of proteins and peptides, previously tested on A549 and 16HBE14o- were nebulized onto the co-culture system. Again, hAELVi cells showed a tendency to be more robust in respect to the applied starch NPs, whereas THP-1 cells appeared as the more sensitive component in this system. Z-stacks of CLSM indicated an internalization of starch NPs into hAELVi cells when grown in mono-cultures that could already be observed in previous studies conducted with A549 and 16HBE14o- when exposed to these NPs (Barthold et al., 2016). An internalization could also be seen in THP-1 mono-cultures after 24 h. Within the co-culture, however, only an uptake into macrophage-like cells could be observed. Nevertheless, these results indicate the capability of the newly developed starch NPs to be taken up into epithelial cells and macrophages, even if the uptake into the epithelium is significantly reduced when macrophages are present in a co-culture.

In summary, the newly established 3D co-culture model of macrophages and epithelial ATI-like cells with functional tight junctions appears as a valuable tool for both developing inhalation (nano)pharmaceuticals as well as for evaluating the safety of other nanomaterials. Preliminary data indicate that the co-culture can be kept for 7 days during which hAELVi cells maintain their tight barrier and remain viable, but a decreased amount of macrophages could be found. The possibility to prolong the co-culture by further adding more macrophages after 3 days in co-culture should allow the study of longer exposure times or repeated exposure.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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