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Research paper

An *in vitro* triple cell co-culture model with primary cells mimicking the human alveolar epithelial barrier ☆

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ABSTRACT

A triple cell co-culture model was recently established by the authors, consisting of either A549 or 16HBE14o– epithelial cells, human blood monocyte-derived macrophages and dendritic cells, which offers the possibility to study the interaction of xenobiotics with those cells. The 16HBE14o– containing co-culture model mimics the airway epithelial barrier, whereas the A549 co-cultures mimic the alveolar type II-like epithelial barrier. The goal of the present work was to establish a new triple cell co-culture model composed of primary alveolar type I-like cells isolated from human lung biopsies (hAEpC) representing a more realistic alveolar epithelial barrier wall, since type I epithelial cells cover >93% of the alveolar surface. Monocultures of A549 and 16HBE14o– were morphologically and functionally compared with the hAEpC using laser scanning microscopy, as well as transmission electron microscopy, and by determining the epithelial integrity. The triple cell co-cultures were characterized using the same methods.

It could be shown that the epithelial integrity of hAEpC (mean ± SD, 1180 ± 188 Ω cm^2) was higher than in A549 (172 ± 59 Ω cm^2) but similar to 16HBE14o– cells (1469 ± 156 Ω cm^2). The triple cell co-culture model with hAEpC (1113 ± 30 Ω cm^2) showed the highest integrity compared to the ones with A549 (93 ± 14 Ω cm^2) and 16HBE14o– (558 ± 267 Ω cm^2). The tight junction protein zonula occludens-1 in hAEpC and 16HBE14o– were more regularly expressed but not in A549.

The epithelial alveolar model with hAEpC combined with two immune cells (i.e. macrophages and dendritic cells) will offer a novel and more realistic cell co-culture system to study possible cell interactions of inhaled xenobiotics and their toxic potential on the human alveolar type I epithelial wall.

1. Introduction

In general, *in vitro* cell cultures are established to mimic tissues or microenvironments. These models are simplified copies of the organ or tissue of interest and offer the possibility for basic research questions, high throughput screenings and highly reproducible experiments due to the standardized and characterized environment. Furthermore, cell culture models are mainly used in all scientific fields because they allow studies at cellular and subcellular levels, for example on cell–cell interactions, cell growth, or to elucidate metabolism and molecular pathways. Moreover, this approach helps to reduce and replace animal experiments [1].

There are a large number of various *in vitro* models to evaluate the human pulmonary epithelial tissue barrier. They range from simple monocultures [2,3] to highly sophisticated 3D models, involving different cell types [1]. Three-dimensional models represent a more realistic physiological situation [4]. Therefore, monocultures do not sufficiently fulfil the *in vivo* situation of the airway epithelial barrier. *In vitro* models of mucosal surfaces are already in use, particularly to characterize the mechanism of particle sampling by intraepithelial dendritic cells [5–7]. The airway epithelial barrier consists of many different cell types, including macrophages and dendritic cells amongst others, which directly

Abbreviations: AJ, Adherens junctions; EC, Epithelial cells; hAEpC, Human alveolar epithelial cells in primary culture; LSM, Confocal laser scanning microscopy; MDM, Monocyte-derived macrophages; MDDC, Monocyte-derived dendritic cells; NP, Nanoparticle(s); TEM, Transmission electron microscopy; TEER, Transepithelial electrical resistance; TJ, Tight junctions; ZO-1, Zonula occludens-1.

☆ Apart from the isolation of the alveolar primary cells from human lung biopsies which was done at the Department of Biopharmaceutics and Pharmaceutical Technology in Saarbrücken, Germany, all experiments were done at the Institute of Anatomy, University of Bern, Bern, Switzerland.

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communicate *in vivo* through intercellular signalling to control homeostasis [8]. It has been shown that dendritic cells and macrophages collaborate as sentinels against foreign particulate antigens by building a transepithelial interacting cellular network through cytoplasmic processes between epithelial cells across the epithelium, or transmigrate through the epithelium *in vivo* and *in vitro* to take up particles on the epithelial surface [5,9]. These findings showed that the co-culturing of different cells may have an impact on study results. In our group, an *in vitro* triple cell co-culture model of the human airway barrier was developed and characterized to study the cellular interplay and the cellular response of epithelial cells (EC), human blood monocytes-derived macrophages (MDM) and dendritic cells (MDDC) upon exposure to different particles [10–12]. Monolayers either of the epithelial cell line A549, an alveolar type II-like cell line [13], or the 16HBE14o–, a bronchiolar epithelial-like cell line [14,15], are grown on a insert membrane to which MDM are added to the apical side and MDDC to the basal side of the epithelium. The ratio between the three cell types was previously characterized (Blank et al., 2007, Brandenberger et al., 2010). By evaluating toxic responses upon exposure to various types of particles, i.e. combustion-derived and engineered nanoparticles (NP), it could be shown that the triple cell co-cultures responded differently than the monocultures alone [16–18]. Therefore, we assume that there is a synergistic effect due to the interaction of the three cell types (epithelial cells, macrophages and dendritic cells) that reduce the adverse effects of NPs.

The use of the epithelial cell lines provides the comfort of easy handling, high purity and high reproducibility of the cells, as for example the A549 cells described previously. There is ample evidence that immortalized cells, either as cancer or transfected cells, do not resemble primary cells in terms of functionality. The 3D model used with the described epithelial cell lines revealed an architecture corresponding to the *in vivo* situation of the human airway epithelial barrier but not to the alveolar epithelial barrier. For the latter experiments, the A549 cell line was included but those cells form a cuboidal morphology which do not at all reflect the morphology of the thin squamous alveolar type I cells. In addition, epithelial type I cells cover >93% of the alveolar surface, whereas type II epithelial cells cover only ~7% of the alveolar surface [19,20].

Therefore, the aim of the current study was to establish a triple cell co-culture model with primary alveolar type II cells isolated from human lung biopsies, which differentiate into epithelial type I cells to get a step closer to the *in vivo* situation, and to compare the findings with the A549 and 16HBE14o– triple cell co-cultures. This primary alveolar type II cells isolated from human lung biopsies display the alveolar type I-like phenotype after 8–9 days in culture [21,22] and are referred to as human alveolar epithelial cells in primary culture (hAEpC). These primary cells have already been used as monocultures only, in drug transport studies *in vitro* [23,24]. To obtain a functional barrier *in vitro*, one important precondition is the formation of tight junctions (TJ) between the neighbouring cells. TJ separate the epithelium into an apical and basolateral side and control the paracellular transport by preventing macromolecules from easily passing through the epithelium [25]. Therefore, TJ are important in order to preserve the epithelial integrity.

In this study, we evaluated the triple cell co-cultures composed of primary alveolar type II cells isolated from human lung biopsies, MDM as well as MDDC, and compared them with the two epithelial cell line co-cultures. First, monocultures of the epithelial cell lines (A549, 16HBE14o–) were morphologically and functionally compared to the hAEpC by means of laser scanning microscopy (LSM) and transmission electron microscopy (TEM) as well as by determining the integrity by the transepithelial electrical resistance (TEER). The TJ protein zonula occludens-1 (ZO-1) was examined by LSM. Second, the same characterization was performed with the triple cell co-culture model by using the three epithelial cells, 16HBE14o–, A549 and hAEpC. The use of 16HBE14o– and A549 cell lines results in a 3D model of different cell types in which the architecture corresponds to the *in vivo* situation of the human airway epithelial barrier but not to the alveolar epithelial barrier. With the use of hAEpC combined with the MDM, we have the opportunity to perform studies at the alveolar epithelial type I barrier.

2. Methods

2.1. 16HBE14o– and A549 monocultures

The 16HBE14o– bronchial epithelial cell line was used as described in earlier studies [5]. Briefly, 16HBE14o– cells (Passages 2.57–2.86, i.e. meaning 29 passages) were cultured in MEM 1× medium, containing Earle's Salts, 25 mM HEPES without L-glutamine (Gibco BRL Life Technologies Invitrogen AG, Basel, Switzerland), supplemented with 1% L-Glutamine (LabForce AG, Nunningen, Switzerland), 1% penicillin/streptomycin (Gibco BRL) and 10% foetal calf serum (PAA Laboratories, Lucerna-Chem AG, Lucerne, Switzerland). The cells were grown in 25 cm^2 cell culture flasks (TPP, Trasadingen, Switzerland) which were treated with fibronectin coating solution containing bovine serum albumin, 0.1 mg/ml (Sigma, Fluka Chemie GmbH, Buchs, Switzerland) and 1% bovine collagen, Type I (BD Biosciences, Basel, Switzerland) and 1% human fibronectin (BD Biosciences) in LHC Basal Medium (Lucerna Chemie AG) and passaged in turns of every 3 and 4 days, respectively. The 16HBE14o– cells were kindly provided by Dr. Gruenert (Passage number 2.54) (University California, San Fransisco).

The A549 cell line [13] was obtained from the American Tissue Type Culture Collection (LGC Promochem, Molsheim, France). Cells (passage number 20–50) were maintained in RPMI 1640 medium (with 25 mM HEPES, LabForce AG, Nunningen, Switzerland) supplemented with 1% L-glutamine (LabForce AG), 1% penicillin/streptomycin (Gibco BRL, Life Technologies, Basel, Switzerland) and 10% foetal calf serum (LabForce AG). For experimental cultures, 2 ml of cells were seeded at a density of 0.5×10^6 cells/mL on transparent BD Falcon cell culture inserts (surface area of 4.2 cm^2, pores with 3.0 μm diameter, PET membranes for 6-well plates; BD Biosciences). In the case of 16HBE14o–, the cell culture inserts have to be pre-treated with fibronectin coating solution containing bovine serum albumin (details are described earlier). The cells were maintained for 7 days on the inserts and were kept at 37 °C in 5% CO_2 humidified atmosphere.

2.2. Isolation of primary alveolar type II cells from human lung biopsy

The isolation method of human alveolar type II epithelial cells (hAEpC) is described in [24]. The use of human material for primary cell isolation was approved by the local ethical committee (Saarland State Medical Board, Germany).

Briefly, finely chopped lung tissues from biopsy were digested for 40 min at 37 °C using a combination of 150 mg trypsin type I (Sigma, Seelze, Germany) and 0.641 mg elastase (CellSystems, St. Katharinen, Germany) in 30 ml HEPES-buffered balanced salt solution (BSS; 137 mM NaCl, 5.0 mM KCl, 0.7 mM $Na_2HPO_4 \cdot 7H_2O$, 10 mM HEPES (*N*-[2-hydroxy-ethyl]piperazine-*N*′-[2-ethanesulfonic acid]), 5.5 mM glucose and preservatives (penicillin (100 units/ml), and streptomycin (100 μg/ml)), pH 7.4). The human alveolar epithelial type II cell population was purified from the crude cell mixture, using a combination of differential cell attachments, centrifugation with a percoll density gradient and cell sorting with magnetic beads

(anti-HEA (EpCAM) MicroBeads, Miltenyi Biotec, Bergisch Gladbach, Germany). Purified human alveolar epithelial type II cells were then seeded at a density of 600,000 cells/cm^2 on collagen/fibronectin transparent BD Falcon cell culture inserts (surface area of 4.2 cm^2, pores with 3.0 μm diameter, PET membranes for 6-well plates; BD Biosciences), using SAGM small airway epithelial cell growth medium (SAGM Airway Bullet Kit, CC-3118), with supplements including BPE, hydrocortisone, hEGF, epinephrine, insulin, triiodothyronine, transferrin, gentamicin/amphotericin B, retinoic acid, BSA-FAF (Lonza, Verviers, Belgium) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml) and 1% foetal calf serum and were maintained for 14 days until confluence. Cultures were kept at 37 °C in 5% CO_2 humidified atmosphere. Under the chosen culture conditions, the type II pneumocytes transdifferentiate into very thin monolayers of type I-like phenotype cells [22,26]. Throughout this study, these cells are named primary epithelial cells (i.e. hAEpC).

2.3. MDM and MDDC isolated from human peripheral blood monocytes

MDM and MDDC were obtained from human peripheral blood monocytes as described by Sallusto et al. [27]. Briefly, peripheral blood monocytes were isolated from buffy coat (Blood Donation Service, Bern, Switzerland) by density gradient centrifugation on Ficoll-Paque (Amersham Biosciences Europe GmbH, Otelfingen, Switzerland). Peripheral blood monocytes were re-suspended in RPMI 1640 supplemented with 1% L-Glutamine, 1% penicillin/streptomycin and 10% heat-inactivated (pooled) human serum (Blood Donation Service, Bern, Switzerland), and then allowed to adhere for 90 min in 6-well plates (BD Biosciences, Basel, Switzerland). Non-adherent cells were washed away, and adherent cells were cultured in RPMI 1640 medium, supplemented with 1% L-Glutamine, 1% penicillin/streptomycin and 5% heat-inactivated (pooled) human serum. For the generation of MDDC, 34 ng/ml IL-4 (Sigma, Fluka Chemie GmbH, Buchs, Switzerland) and 50 ng/ml GM-CSF (R&D Systems, Oxon, UK) were added to the supplemented medium for 7–8 days. The MDM were only maintained in the supplemented medium for 7–8 days. The cell cultures were kept at 37 °C in 5% CO_2 humidified atmosphere in an incubator.

2.4. Triple cell co-cultures

The co-cultures were prepared as previously described [5,12]. Briefly, epithelial cells (A549, 16HBE14o– and hAEpC) were maintained on transparent BD Falcon cell culture inserts (surface area of 4.2 cm^2, pores of 3.0 μm diameter, PET membranes for 6-well plates; BD Biosciences), in case of 16HBE14o– monocultures, the flasks and membranes were treated with fibronectin coating solution containing bovine serum albumin (for details, see description for monocultures). A549 and 16HBE14o– cells were cultured for 7–8 days, and hAEpC were cultured for 14 days, before MDM were added on top of the epithelial monolayer and MDDC underneath the insert membrane. Before using the triple cell co-cultures, they were kept overnight in medium supplemented with 1% L-Glutamine, 1% penicillin/streptomycin and 5 % heat-inactivated (pooled) human serum at 37 °C in 5% CO_2 humidified atmosphere.

2.5. Measurements of the transepithelial electrical resistance (TEER)

The TEER was measured with the Millicell-ERS system (MERS 000 01; Millipore AG, Volketswil, Switzerland) as described earlier [5,12,28]. Briefly, TEER was measured in the epithelial monocultures as well as in the triple cell co-culture models. The mean of four measurements per insert was determined. The electrical resistance of insert membranes without cells was subtracted from all samples, and the resistance values were multiplied with the surface area of the inserts (4.2 cm^2).

2.6. Cell fixation and labelling for laser scanning microscopy

Cells were labelled and fixed as described in detail [5]. Antibodies were diluted in PBS as follows: mouse anti-human CD14 1:20 (Clone UCHM-1, C 7673, Sigma), mouse anti-human CD86 1:20 (Clone HB15e, 36931A, PharMingen, BD Biosciences), mouse anti-ZO-1 1:100 (Zymed, South San Francisco, California, USA), goat anti-mouse cyanine 2 1:50 (Chemicon, VWR International AG, Life Sciences, Lucerne, Switzerland), goat anti-mouse cyanine 5 1:50 (AP124S, Chemicon, VWR International AG, Life Sciences, Lucerne, Switzerland), goat anti-rabbit cyanine 5 (Chemicon, VWR International AG, Life Sciences, Lucerne, Switzerland), Alexa 488 and phalloidin rhodamine 1:100 (R-415, Molecular Probes, Invitrogen AG, Basel, Switzerland) and DAPI 1:1000 (Chemicon, VWR International AG, Life Sciences, Lucerne, Switzerland).

2.7. Laser scanning microscopy and image restoration

A Zeiss LSM 510 Meta with an inverted Zeiss microscope (Axiovert 200 M, Lasers: HeNe 633 nm, HeNe 543 nm, Ar 488 nm, and Diodenlaser 405 nm) with a 63× objective lens (oil immersion, NA = 1.3) was used. For the detection of the TJ signals, a negative sample containing only the secondary antibodies was scanned first and the detector gain was adjusted so that no fluorescent signal of the specific antibody (such as background) could be detected. The scans of the labelled samples were then acquired using the same detector settings. Image processing and visualization was done using IMARIS, a 3D multi-channel image processing software for confocal microscopic images (Bitplane AG, Zurich, Switzerland).

2.8. Determination of cell number

LSM images were recorded by means of stereology, which means that images were acquired by systematic uniform random sampling.

Images were evaluated with a non-biased counting frame consisting of an inclusion and exclusion line each to a half of the whole frame. All cell nuclei within the frame and touching the inclusion line were counted, whereas those hit by the exclusion line were not considered [29].

2.9. Transmission electron microscopy

For TEM analysis, cells were fixed with 2.5% glutaraldehyde in 0.03 M potassium phosphate buffer, pH 7.4. The cells were post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer and with 0.5% uranyl acetate in 0.05 M maleate buffer. The cells were then dehydrated in a graded series of ethanol and embedded in Epon. Ultrathin sections were cut and transferred onto single-slot grids (2 × 1 mm; Plano GMBH, Wetzlar, Germany), stained with uranyl acetate, counter-stained with lead citrate and observed with a Philips 400 TEM at 60 kV (FEI Company Philips Electron Optics, Zurich, Switzerland).

2.10. Statistics

The values of TEER and cell nuclei count measurements are expressed as mean values with the standard deviation of the mean (SD). The statistical analysis was performed using SigmaStat for Windows (Version 3.10, Systat Software, Inc., Richmond, California, USA) statistical software. To compare more than two groups, an ANOVA on ranks was performed. Two groups were compared using

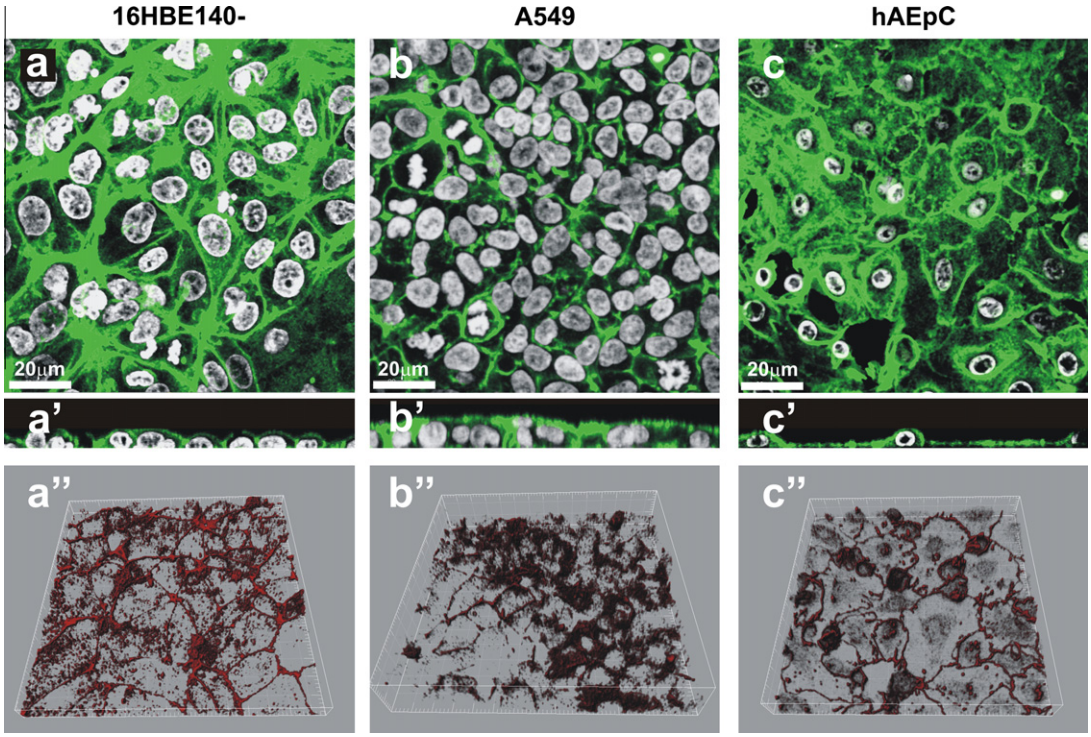

Fig. 1. Characterization and comparison of epithelial monocultures by LSM. Immunofluorescence labelling of F-actin cytoskeleton (in green) and nucleus (in white) in monocultures of 16HBE14o– cells (a), A549 cells (b) and primary hAEpC (c) grown on a insert membrane are presented in the *y–x* projection of single optical sections. The side view of the respective epithelium is shown for each monoculture (a–c′). Appearance of the TJ protein ZO-1 (dark red) in the monocultures is visualized as a 3D reconstruction, presented in normal shading mode, of the same areas as above (a″–c″).

a Mann–Whitney rank sum test. In all cases, $p < 0.05$ was considered to be significant.

3. Results

3.1. Comparison of cellular morphology and cell–cell contacts

For the comparison of monocultures of primary hAEpC, A549 and 16HBE14o– cell lines, the cultures were examined by LSM (Fig. 1a–c). By staining with F-actin, it could be shown that all three cell types grew in the form of a monolayer. This is illustrated by the arrangements of the cell nuclei which are almost set in one plane (Fig. 1a′–c′). Differences in cell shapes were shown in the *y–z* projection (Fig. 1a′–c′). The primary hAEpC cells build a very thin layer with squamous cells, whereas the epithelium of the A549 and the one of the bronchiolar 16HBE14o– represents cuboidal cells. All cell layers normally grew to confluence as shown in Fig. 1. The A549 and 16HBE14o– cell lines always grew to confluence, whereas the primary cells exhibited no confluent cell layer in a few experiments as shown in Fig. 2a and b.

The 3D reconstruction of the TJ label showed that ZO-1 was expressed at the cell–cell contacts and formed the typical belt-like structures of TJ. The distinctive immunostaining of ZO-1 was localized to the cell periphery in a sharp undisrupted band, surrounding each cell at its border. This was true for the 16HBE14o– (Fig. 1a″) and the hAEpC (Fig. 1c″). ZO-1 proteins were also present in the A549 cells (Fig. 1b″) but to a lower extent compared to the other two cell types.

The same findings could be shown by TEM examinations. The 16HBE14o– cells showed a confluent monolayer made from cuboidal cells with the characteristic presence of cell–cell junctions, the TJ, adherens junctions (AJ) and the desmosomes (Fig. 3a and a′). In Fig. 3b′, membrane-bound inclusions, which resemble lamellar bodies of alveolar type II cells, are observable which are specific

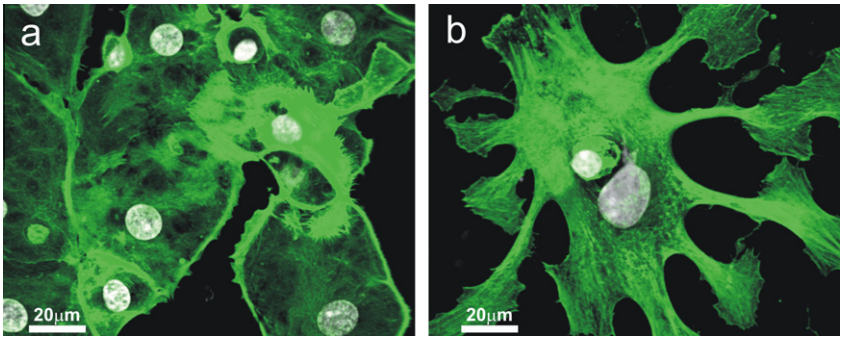

Fig. 2. Growth of the primary hAEpC monolayer. Primary hAEpC are stained for F-actin (green) and nuclei (white) and analysed using LSM. Two illustrations (a and b) of primary hAEpC monolayers which did not grow to confluence.

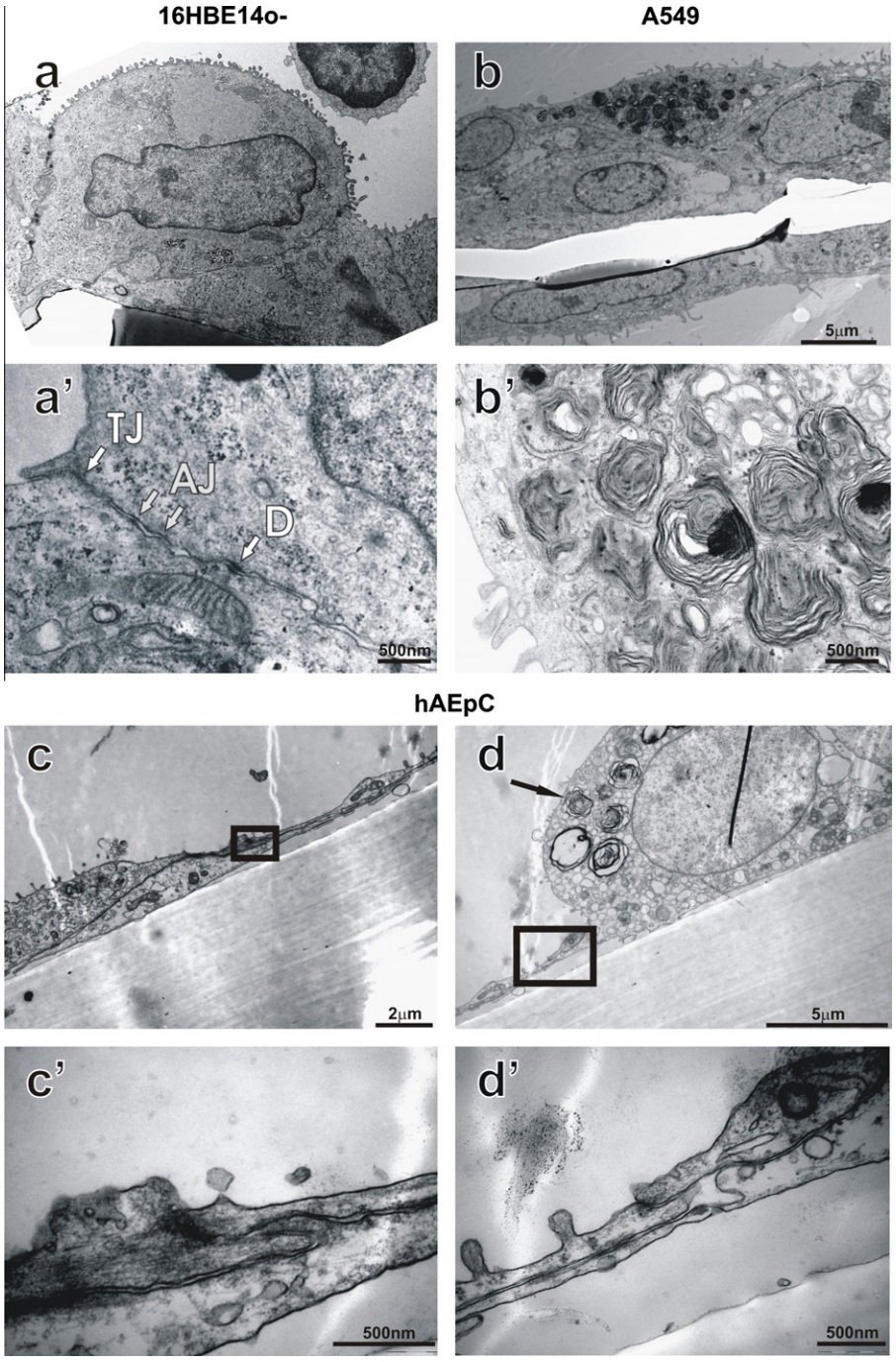

Fig. 3. Characterization and comparison of epithelial monoculture by TEM. TEM micrographs show monocultures of 16HBE14o– (a), A549 (b) and primary hAEpC (c). Cell-specific characteristics are shown in the lower rows. The appearance of well-defined cell contacts as tight junctions (TJ), adherens junctions (AJ) and desmosomes (D) in 16HBE14o– cells (a′); membrane-bound inclusions, which resemble lamellar bodies of type II cells A549 cells (b′). Primary hAEpC are shown in c in a squamous alveolar type I-like cells appearance and in alveolar type II-like cells appearance in d with the lamellar bodies (black arrow). c′ and d′ show a magnification of the square in c and d.

for the A549 cells (Fig. 3b). The thinnest and most squamous monolayer is presented by the primary hAEpC. Actually, the primary hAEpC showed cells with squamous alveolar type I-like as well as cells with an alveolar type II-like appearance with the typically lamellar bodies. The cell layer is confluent and sealed by TJ. All monolayers have a morphologically normal appearance.

3.2. Quantification of epithelial cell numbers

The cell number per mm^2 (Fig. 4) was determined by counting the cell nuclei in a defined area. The values differed between all three cell types. The 16HBE14o– and A549 cell lines showed a significantly higher amount of nuclei per mm^2 compared to the primary hAEpC. Although the 16HBE14o– and the A549 were seeded with exactly the same cell numbers (0.5×10^6 cells/mL), after 7 days, the amount of nuclei of the latter cell type was significantly higher than that of the 16HBE14o–.

3.3. The triple cell co-culture model with various epithelial cells

After the comparison of the epithelial monocultures, the cells were combined in the triple cell co-culture model by adding the

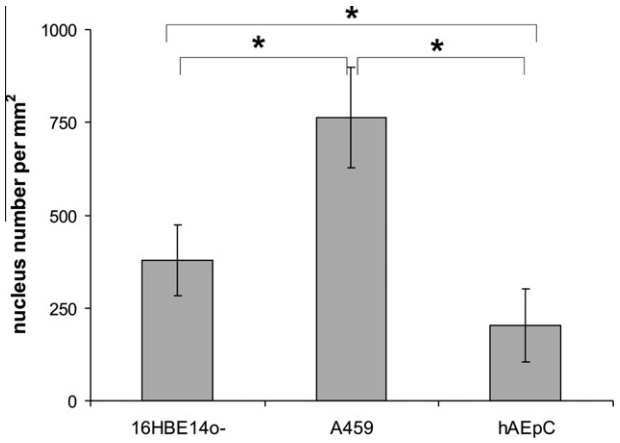

Fig. 4. Cell number per mm^2. By counting the nuclei in a defined area in mm^2, the cell numbers of the three epithelial cells are compared. The data are presented as the mean ± SD. Six different samples of which five areas were counted out in each epithelial cell line by the means of uniformed random sampling, $^*p < 0.05$.

MDM at the apical side of the epithelial cells and MDDC at the basal side of the insert membrane (Fig. 5). The triple cell co-culture made of primary hAEpC (Fig. 5c, c′, f, f′) showed the usual 3D architecture with the appropriate arrangement of the MDM and MDDC as it was shown for the triple cell co-cultures with 16HBE14o– (Fig. 5a, a′, d, and d′) and A549 (Fig. 5b, b′, e, and e′). The MDM are integrated in the monolayer of each epithelial cell type (Fig. 5a–c). The primary hAEpC did not grow to the bottom side of the membrane to the same extent as the A549 and 16HBE14o– cells did.

3.4. The epithelial integrity

TEER values were measured in the monocultures after 7 days for the 16HBE14o– and A549 cells, and after 14 days for the primary hAEpC (Fig. 6, light grey bars). The mentioned period in cultures correlates with the time after which the cell cultures are ready to use for experiments. The 16HBE14o– and hAEpC monolayers showed a significant higher TEER value than the A549 cell layer.

The TEER values of each triple cell co-cultures (16HBE14o–, A549 or hAEpC) were also determined (Fig. 6, dark grey bars), whereas the culture containing primary hAEpC showed the highest integrity followed by the 16HBE14o–. The A549 cultures exhibited a significantly lower TEER value than hAEpC and 16HBE14o–. Comparing the monocultures to the triple cell co-cultures, 16HBE14o– and A549 in the triple cell co-cultures obtained a significantly lower TEER than the respective monocultures, whereas the primary hAEpC in mono- as well as in the triple cell co-culture showed comparable values.

4. Discussion

This paper discusses the possible use of alveolar type I cell types in combination with two immune cells to build a co-culture model of the alveolar epithelial tissue barrier. Up to now, A549 cells have often been used for mimicking the alveolar epithelial barrier; however, since they originate from alveolar type II-like cells, they do not reflect the *in vivo* situation where more than 93% of the alveolar surface is covered by epithelial type I cells [19,20]. The bronchiolar 16HBE14o– cells are only useable to mimic the airway epithelial barrier. Therefore, an appropriate model to study the interaction of inhaled xenobiotics with the alveolar epithelial barrier, built mostly of alveolar type I cells, has not previously been available.

Therefore, in the present study, monocultures of 16HBE14o– and A549 were morphologically and functionally compared to monocultures of primary alveolar type II cells isolated from human lung biopsies (hAEpC) which transdifferentiate into a thin monolayer of alveolar type I-like epithelial cells after 8–9 days in cultures [21,22]. These primary hAEpC were then also combined with MDM and MDDC to produce a triple cell co-culture model representing and mimicking the alveolar epithelial barrier. In turn, this model was compared to the already established models which mimic the respiratory epithelium; the 16HBE14o– cell lines combined within the triple cell co-culture model to mimic the airway epithelial barrier and the A549 cells combined within the triple cell co-culture model to mimic an alveolar epithelial model. Microscopic analysis showed that the three epithelial cells differ in cell shape and cell characteristics. The bronchiolar 16HBE14o– analysis showed cuboidal cells building a very tight monolayer. The formation of cell–cell junctions, TJ, AJ and desmosomes could be verified by means of TEM. Additionally, by immunofluorescence staining, the TJ protein ZO-1 showed nicely developed TJ at the cell borders. The tightness of the monolayer could also be verified by the measure of high TEER values (about 1470 Ω cm^2). These characteristics are consistent with published data from different research groups [14,16,30]. A549 cells also grew to confluence in monolayers, but no continuous staining of ZO-1 was detected by immunofluorescence. Several studies obtained contradictory results and already concluded that A549 cells do not express TJ, or at least not to a high extent, [21,31,32] but TJ could at least be visualized in other studies [12,28]. Although the A549 monolayer always looked very confluent by using LSM and TEM microscopic techniques, the TEER values never reached the values of the 16HBE14o– and primary EC layer. The low TEER values (about 170 Ω cm^2) could be attributed to reduced synthesis of the TJ proteins, as for example ZO-1. The significantly lower TEER values of A549 cells were comparable to studies performed before [1,12,31–33]. Interestingly, the cell number per defined area was the highest for the A549 cells although they were seeded at the same density as the 16HBE14o– cells. The ratio of A549 cells to 16HBE14o– was 2:1 after 7 days in culture. This is in agreement with the result of previous results [5], which showed a ratio of 1.7:1. Lamellar body–like structures which are characteristics for alveolar epithelial type II cells could be visualized with TEM in A549 cells. The results confirm those of [13]. Although the A549 cell line expresses these alveolar type II phenotypical lamellar bodies, these cells do not adequately mimic the alveolar epithelial barrier because the cells themselves are cuboidal and do not represent the squamous phenotype of alveolar type I cells. Therefore, these cells are not ideal for *in vitro* studies at the alveolar epithelial barrier. Monocultures of hAEpC cells were already used in various studies [21,22,24,34]. They developed high barrier resistance (about 1200 Ω cm^2) which could be reproduced within this study. The TEER values were significantly higher than those of the A549 cells and comparable with those of the 16HBE14o–. Confluent monolayers were endowed with TJ, which was shown by LSM and TEM techniques. Moreover, primary hAEpC exhibit characteristics of alveolar type I cells although originating from alveolar type II cells. After isolation from purified alveolar cells, type II cells flatten in culture with monolayer confluence achieved by day 8 or 9 and then, the cells display alveolar type I phenotype [21]. We could show that they build squamous cells with centrally located nuclei. It was found too that the alveolar epithelial monolayer consists of cells which phenotypically belong to alveolar type I as well as to alveolar II cells but it was not investigated as to what extent the two cell types are present. Therefore, it is likely that the differentiation of isolated human alveolar type II-like cells into the monolayers showed characteristics of both cell types [22]. This is a benefit to mimic the alveolar tissue barrier which consists of both cell types *in vivo*. As already mentioned,

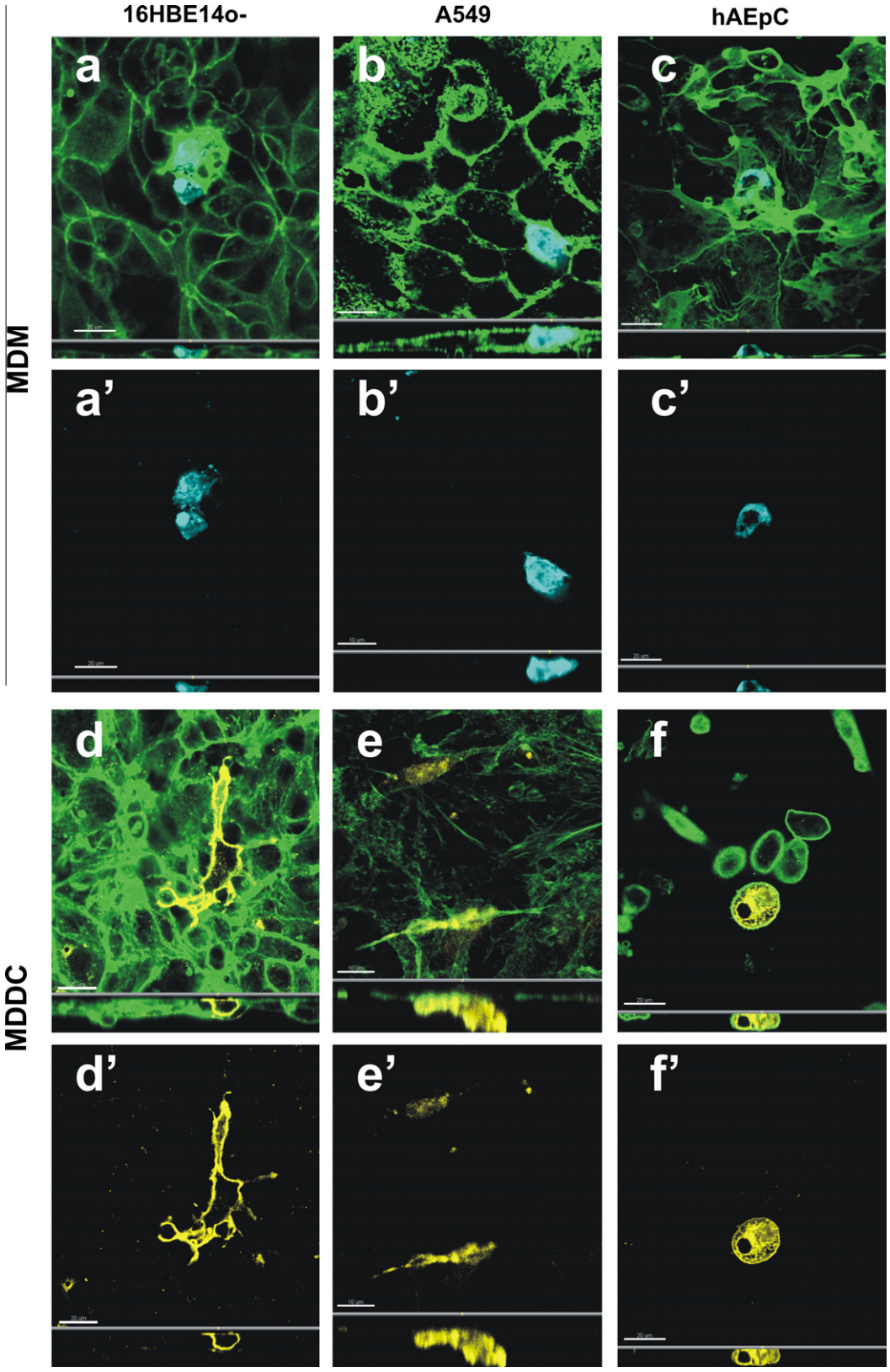

Fig. 5. Triple cell co-cultures with various epithelial cells. LSM images of triple cell co-cultures with 16HBE14o– (a and d), A549 (b and e), and primary hAEpC (d and f) grown on a insert membrane. All cells were stained for F-actin (all cells in green). At the upper side of the insert membrane, the cells were additionally stained for MDM surface marker CD14 (turquoise, a and a′, b and b′, c and c′) and the respective MDM in the co-cultures are presented alone (a′, b′, and c′). Cells of the basal side of the membrane were stained for the MDDC surface marker CD86 (yellow, d and d′, e and e′, f and f′) and the respective MDDC of the co-cultures were presented alone (d′, f′, e′). All images are presented in the *x*–*y* projection as well as the respective side view, directly below each image.

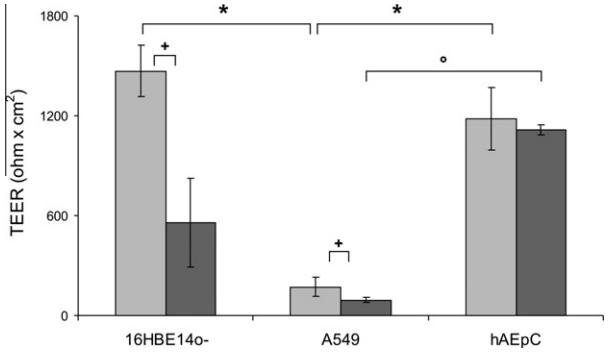

Fig. 6. TEER values of the epithelial monocultures and in combination with the triple cell co-culture model. The values of the TEER of the epithelial monocultures (in light grey) and combined in the triple cell co-cultures (in dark grey) are presented as the mean ± SD. All experiments were performed five times. * and, ° refer to significant difference between the epithelial monoculture and the triple cell co-cultures, respectively; + refers to significant differences in TEER values of the monocultures versus the respective triple cell co-cultures. $p < 0.05$.

these cells were successfully used in studies, for example, in assessments of transport rates of protein and peptides across the monolayer. However, all experiments were performed using monocultures of primary hAEpC only [24]. Therefore, the established co-culture model in this study offers new perspectives and possibilities for studying the alveolar epithelial barrier, including the influence of the cells of the defence system (e.g. macrophages and dendritic cells). In general, the triple cell co-culture model [12] resembles the natural architecture of the *in vivo* epithelial airway and has been used in several studies [1,5,11,28]. The cells of the defence system used in the triple cell co-culture model have similar functional and structural characteristics as the airway/alveolar macrophages and dendritic cells in human lung [35]. Additionally, their number is similar as in the human respiratory tract [5]. It has been shown that the EC, and also MDM and MDDC express the TJ proteins claudin-1, ZO-3, JAM-1 as well as the AJ protein E-cadherin to preserve the epithelial integrity [16,36]. By comparing the different epithelial monocultures to the respective triple cell co-culture models in which the primary EC were combined, the TEER values of the triple cell co-cultures were significantly lower when we used 16HBE14o– and A549 cells. This finding was already published for the triple cell co-culture with 16HBE14o– [16]. The epithelial cells are probably less tightly connected in the monolayer due to the interactions with the cells of the defence system. However, recent findings gave evidence that MDM and MDDC are able to interact in the triple cell co-culture as a transepithelial network, by building cytoplasmic processes (with their pseudopodia) between epithelial cells. It is assumed that MDM and MDDC build TJ-like structures with the epithelial cells to reseal the epithelial layer and to preserve the epithelial integrity [16,36].

It has to be mentioned that the isolation and handling of the primary EC is much more time consuming than the culturing of epithelial cell lines and that therefore cultivation time can vary. Additionally, a careful check of the monolayer's confluence has to be done before using them as monocultures or combined within the triple cell co-culture model. Nevertheless, it is worthwhile to invest the time for the triple cell co-culture model with the primary hAEpC because this model benefits from a very similar architecture and morphology to that of an intact alveolar epithelial barrier. There is a need to study the cell interplay and the cell type-specific role in its environment; therefore, culture models of various cell types better simulate the *in vivo* situation.

This extensively described and characterized triple cell co-culture model with hAEpC and two immune cells builds the basis for additional investigations at the human alveolar epithelial barrier. In further studies, this model will be used to investigate the still unknown mechanisms regarding how inhaled xenobiotics interact with the barrier and the role of each cell type in this complex system.

5. Competing interests

The authors declare that they have no competing financial interest.

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