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A B S T R A C T

Airway epithelial cells provide a barrier to the translocation of inhaled materials. Tight (TJ) and adherens junctions (AJ) play a key role in maintaining barrier functions, and are responsible for the selective transport of various substances through the paracellular pathway. In this study we compared a bronchial cell line (16HBE14o-) and primary bronchial cells (HBEC), both cocultivated with the fibroblast cell line Wi-38, with respect to their structural differentiation and their reaction to cytokine stimulation. HBEC formed a pseudostratified epithelial layer and expressed TJ and AJ proteins after 2 weeks in coculture. Mucus-producing and ciliated cells were found within 24 days. Additionally, a beating activity of the ciliated HBEC (14–19 Hz) could be detected. 16HBE14o- in coculture showed a multilayered growth without differentiation to a pseudostratified airway epithelium. Simultaneous exposure to TNF-α- and IFN-γ-induced significant changes in barrier function and paracellular permeability in the cocultures of HBEC/Wi-38 but not in the 16HBE14o-/Wi-38. In summary, HBEC in coculture mimic the structure of native polarized bronchial epithelium showing basal, mucus-producing and ciliated cells. Our system provides an opportunity to examine the factors that influence barrier and mucociliary function of bronchial epithelium within a time frame of 3 weeks up to 3 months in an *in vivo*-like differentiated model.

Keywords:
Primary bronchial cells
Coculture
Ciliary beat frequency
Lung
Barrier disruption

1. Introduction

Airway epithelial cells fulfil several functions that are involved in protecting the airways from exogenous insults. The formation and maintenance of diffusion barriers is a fundamental requirement for the physiological functioning of the respiratory epithelium [33]. For a number of diseases an important consequence of inflammatory reactions in lung tissue is an impaired epithelial barrier [29]. Altered permeability of the epithelium in the upper respiratory tract has been observed in patients with cystic fibrosis and asthma. As treatment with drugs is often necessary in such disease types, *in vitro* models that mimic an affected barrier could be useful to predict drug effects on epithelium with altered barrier function.

To form a functional barrier, tight junctions (TJ) and adherens junctions (AJ) have to be generated between adjacent cells. Proximal lung epithelial barrier function is regulated by an apical junctional complex (AJC) consisting of tight and adherens junctions. In patients with altered mucosal permeability both tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ), which contribute to the proinflammatory cascade, are elevated. Several studies have examined TNF-α- and IFN-γ-induced changes in epithelial [1,15,16] and endothelial [4,25,28,30] paracellular permeability *in vitro*. Additionally, variable effects of these cytokines on the TJ cytoplasmic plaque protein zona occludens 1 (ZO-1) and TJ structure have already been described for both intestinal and bronchial epithelial cells *in vitro* [8,23].

The epithelium of the proximal airways includes several cell types, among which basal, ciliated and secretory cells are the most abundant [19,33]. Under physiological conditions a pseudostratified epithelium with the formation of cilia and mucus production

Abbreviations: 16HBE14o-, immortalised bronchial epithelial cell line; 16HBE14o-/Wi-38, coculture of 16HBE14o- with Wi-38; AEGM, airway epithelial growth medium; AJ, adherens junction; AJC, apical junctional complex; HBEC, human bronchial epithelial explant-outgrowth cells; HBEC/Wi-38, coculture of HBEC with Wi-38; IFN-γ, interferon gamma; P_{app}, apparent permeability coefficient; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TER, trans-monolayer or bilayer electrical resistance; TJ, tight junction; TNF-α, tumor necrosis factor alpha; ZO-1, cytoplasmic plaque protein zona occludens 1.

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is described for nasal, tracheal and bronchial primary cells of different species [5,14,27,35]. In these studies advantage was taken of the intercellular communication existing between epithelial cells and fibroblasts of the bronchiolar wall. Both cell types seem to exert regulatory control on each other. In addition, air–liquid interface culture conditions seem to have a positive influence on cell differentiation [5].

We compared cocultures out of the immortalised bronchial epithelial cell line 16HBE14o- with the fibroblast cell line Wi-38 and primary isolated human bronchial epithelial cells (HBEC) in coculture with the cell line Wi-38 as bilayer on filter inserts. The examined parameters were the structural differentiation and the reaction to proinflammatory cytokine stimulation of both cocultures. The following study demonstrates that primary human bronchial epithelial cells from organ-outgrowth cultures differentiate in coculture with fibroblasts under air liquid interface conditions, this being in marked contrast to the model with the bronchial cell line 16HBE14o-.

2. Materials and methods

2.1. Chemicals and antibodies

Cell culture media and supplements were purchased from Sigma (Deisenhofen, Germany), culture media for primary cells from Promocell (Heidelberg, Germany). Chemicals and the reagents, TNF-α and IFN-γ, the FITC-conjugated dextrans and the mouse monoclonal antibody against ß-tubulin IV were obtained from Sigma (Deisenhofen, Germany). The rabbit polyclonal antibody to ZO-1 was purchased from Zymed Laboratories (South San Francisco, CA, USA), the rabbit antibody against aquaporin 3 from Chemicon (Schwalbach, Germany), the mouse antibody anti-E-cadherin (uvomorulin, L-CAM) from Monosan (Cellsystems, St. Katharinen, Germany), and the mouse antibodies to CK14, occludin and ß-catenin were purchased from BD Transduction Laboratories (Heidelberg, Germany). Secondary antibodies, Alexa Fluor 488-conjugated anti-mouse IgG and Alexa Fluor 594-conjugated anti-rabbit IgG were obtained from Molecular Probes (MoBiTec, Göttingen, Germany).

2.2. Isolation of primary bronchial cells from the lung tissue

Human bronchial epithelial explant-outgrowth cells (HBEC) were obtained from lungs surgically resected from patients who underwent partial or total lobectomies for early stage lung cancer. The study received approval from the Local Ethics Commission, and was based on informed consent of the individual patients.

HBEC were isolated according to a method by Lechner et al. [21]. Briefly, small bronchi (diameter < 5 mm) were first dissected from the remaining peripheral lung tissue, and were cut into defined pieces from 1 to 2 cm^2 size. The small explants were washed with PBS and then cultivated in tissue-culture flasks coated with 0.01% rat tail collagen type 1 (IBFB, Leipzig, Germany) with the luminal side downwards. The tissue fragments were covered with AEGM (Promocell, Heidelberg, Germany) and incubated in 5% CO_2 at 37 °C. HBEC migrated out of the explants after 1 week.

Upon reaching confluence, cells were passaged using collagenase (Cellsystems, St. Katharinen, Germany) in a ratio of 1:3, and were further cultured in AEGM. In this study, cells from 10 donors at passages 1–4 were used.

2.3. Cell culture

The bronchial cell line 16HBE14o- was obtained from Dr. Gruenert (University of California, Oakland, CA), and the human embryonic lung fibroblast cell line Wi-38 (ATCC-CCL-75) was obtained from the ATCC (Promochem, Wesel, Germany). 16HBE14o- and Wi-38 cells were cultured in Eagle's minimal essential medium supplemented with 10% FCS, 1% penicillin/streptomycin, 2% sodium bicarbonate, 1% Glutamax, and 1% sodium pyruvate using standard cell culture methods. Unless stated otherwise all medium supplements were purchased from Sigma (Deisenhofen, Germany). Wi-38 and 16HBE14o- were split at a ratio of 1:5, and passages 10–30 were used for the current studies.

2.4. Cocultures

Both bronchial models consist of two different cell types as bilayer on opposite sides of HTS 24-Transwell® filter (polycarbonate, 0.4 μm pore size; Costar, Wiesbaden, Germany) membranes. The bronchial models are established as a bilayer coculture of the bronchial cell line 16HBE14o- or primary isolated HBEC with the lung fibroblast cell line Wi-38. For cocultures the upper surface of the Transwells was coated with a solution of collagen type 1 in 0.1 M acetic acid and air-dried at room temperature. Fibroblasts ($1 \times 10^4/cm^2$) were placed on the lower surface of inverted 24-well HTS-Transwell filter membranes, and were incubated for 2 h at 37 °C and 5% CO_2. The filter plates were then inverted and placed in 24-well plates filled with 1 ml medium per well. The bronchial cells (16HBE14o- or primary isolated HBEC ($5 \times 10^4/cm^2$)) were subsequently seeded on the top surface of the HTS 24-Transwell® filter membranes (210 μl/well), and were grown to confluence simultaneously with the corresponding cell type on the lower surface using the same conditions outlined for flasks. After 3 days of coculture both bronchial models were placed at an air–liquid interface and feeded from the basolateral side. Media were replaced twice weekly.

2.5. Measurement of transepithelial electrical resistance (TER)

Electrical resistance across the monolayer and bilayer was measured using an EVOM voltohmmeter (World Precision Instruments, Berlin, Germany) with STX-2 chopstick electrodes. For the measurement the cocultures cultivated at an air–liquid interface were placed in submersed conditions. The medium volumes in the apical and basal compartments were 210 μl and 1 ml, respectively. The cocultures were allowed to adapt to submersed conditions for 2 h at 37 °C and 5% CO_2 to avoid stress and potential changes in TER. After TER reading the primary bronchial models were returned to the air–liquid interface. TER was measured once a week.

The resistance from a blank value obtained from a transwell containing a cell free filter was subtracted from each well to calculate the layer resistance, which was multiplied by the area of the membrane to obtain the final TER value ($\Omega\,cm^2$).

2.6. Electron microscopy

Samples were prepared for both transmission electron microscopy (TEM) and scanning electron microscopy (SEM) using standard procedures. The cocultures on the filter membranes were fixed in 3.7% paraformaldehyde for 20 min. Samples were treated with 1% osmium tetroxide for 1 h, followed by dehydration using graded ethanols. For TEM the filters then were embedded in agar resin (Plano, Wetzlar, Germany) and semithin and ultrathin sections were generated using an ultramicrotom (Leica, Bensheim, Germany) and mounted on copper grids prior to examination in an EM 410 electron microscope (Phillips, Eindhoven, Netherlands). For SEM samples were air-dried and sputtered with gold before study in a DSM962 (Zeiss Inc. Oberkochen, Germany).

2.7. Histology

Cocultures were fixed in 3.7% paraformaldehyde for 20 min. Samples were washed with PBS and then transferred to 70% etha-

nol for paraffin embedding. Sections of 5 μm thickness were deparaffinized in xylene, followed by rehydration in graded alcohols, and were stained for general histology using standard protocols for haematoxylin and eosin (H&E) or alcian blue reaction.

2.8. Transport studies

Primary cocultures were cultivated over 31 days, the 16HBE14o-/Wi-38 model over 10 days before being treated with TNF-α (10 ng/ml) and IFN-γ (100 ng/ml) for 24 or 48 h. The paracellular flux of sodium fluorescein (0.4 kDa) and of FITC-dextrans of varying molecular weight (20 and 70 kDa) at 37 °C was measured. To avoid a long equilibration time with buffer, the dextrans and sodium fluorescein were dissolved in cell culture medium to a concentration of 10 μg/ml for sodium fluorescein and 2.5 mg/ml for the dextrans. To measure the transport from the apical to the basolateral side, samples from the acceptor side (basolateral compartment) were taken every 30 min over a time period of 3 h, and were replaced with an equal volume of fresh media. The samples were diluted 1:5 in NaOH and assayed in a microplate reader (Titertek Multiskan Plus, Labsystems, Frankfurt, Germany) at the excitation and emission wavelengths of 485 and 530 nm, respectively. During the transport studies TER was measured routinely to monitor the integrity of the layer.

The apparent permeability coefficients (P_{app}) were calculated using the following equation:

$$P_{app} = (1/A * C_0) * (dQ/dt)$$

with dQ/dt as the solute flux, A the surface area of the filter (0.33 cm^2) and C_0 the initial concentration.

2.9. Immunocytochemical staining

After exposure with the cytokines the cells from the different models grown on the filters were washed with PBS and fixed in 3.7% paraformaldehyde in CS buffer (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) 0.1 M, ethylene glycol-bis(b-aminoethylether)*N*,*N*,*N*′*N*′-tetraacetic acid (EGTA) 1 mM, 4% polyethylene glycol 800, NaOH 0.1 M) for 20 min, and then washed with PBS again. Cells on the filter membranes were permeabilized with 0.5% Triton X-100 prior to addition of the antibodies against ZO-1, occludin, ß-catenin and E-cadherin and were incubated overnight at 4 °C. Cells were again washed with PBS and the secondary antibodies, Alexa Fluor 488-conjugated anti-mouse IgG and Alexa Fluor 594-conjugated anti-rabbit IgG were added for 3 h at 4 °C. The nuclei were counterstained with a blue fluorescent Hoechst dye (HOE 33342, Sigma, Germany). Filters were then mounted on a slide in Gel/Mount (Biozol, Eching, Germany). Finally, the labeled cells were observed by fluorescence microscopy (Leitz DM RD, Leica, Germany).

2.10. Western blot

For Western blot, protein was extracted from the cocultures treated for 24 or 48 h with TNF-α (10 ng/ml) and IFN-γ (100 ng/ml) under culture conditions as described previously. Two filters of the same treatment were pooled together as one sample in 150 μl APL (buffer containing protease inhibitors, provided from the manufacturer, AllPrep RNA/Protein Kit, Qiagen, Hilden, Germany) lysis buffer. RNA/protein from the combined sides was extracted using an AllPrep RNA/Protein Kit (Qiagen, Hilden, Germany) to obtain protein and RNA from the same samples using the protocol provided by the manufacturer. After lysis and homogenization protein was purified and quantified in a plate reader (Titertek Multiskan Plus, Labsystems, Frankfurt, Germany) using the BCA Kit (Pierce).

For Western blotting 10 μg total protein per lane was used. After separation by SDS–polyacrylamide electrophoresis, proteins were blotted on nitrocellulose membranes. After blocking for 2 h at 4 °C in 5% milk powder dissolved in PBS, the membranes were washed and probed with the antibodies against ZO-1, occludin, ß-catenin and E-cadherin, and were incubated for 2 h at 4 °C and subsequently incubated with the horseradish peroxidase-conjugated secondary antibodies for 30 min. The resulting complexes were visualized using the chemoluminescence detection method according to the manufacturer's protocol (Amersham Biosciences, Munich, Germany), and then were detected by photographic film. Densitometry was performed with TINA 4.0 Image (Raytest, Straubenhardt, Germany) to determine average band intensity. The intensity of lanes containing no bands was set as the background level.

2.11. Ciliary beat frequency

To measure ciliary beat frequency (CBF) cells were cultivated over a period of 3 months. Filters from the 24 transwells were then cut and placed carefully at the borders of the filters on dishes filled with sylgard gel. The dish was filled with 1.5 ml AEGM, and was put on a temperature-controlled stage under a video-optical microscope (BX50Wi, Olympus, Hamburg, Germany). For every time point 1000 video frames were taken at 105 frames/s, and CBF was determined by Fourier transformation using TILLvisION (München, Germany) and Auto Signal v1.7 (Systat Software GmbH, Erkrath, Germany). Each experiment was performed using a minimum of nine filters from three different donors.

2.12. Statistical analysis

From several independent measurements means and standard deviations were calculated. Testing for significant differences between means was carried out using one-way ANOVA and Dunnett's post-test at a probability of error of 5% and 1%.

3. Results

3.1. Morphological studies

For the cocultures primary human bronchial cells or bronchial cell line 16HBE14o- were seeded on the apical side and fibroblasts on the basolateral side (Fig. 1). The transmission electron microscopy was performed on the primary model after 31 days in coculture, and on the permanent cell line model at day 10 in coculture. In coculture on the 24 Transwells the HBEC differentiated to a ciliated pseudostratified airway epithelium. However, this phenomenon was not observed in the coculture with the cell line 16HBE14o-. Independent of the culture time, the latter cells grew in multiple layers on top of each other and gave no evidence of differentiation towards the various bronchial epithelial lineages.

In coculture with the fibroblast cell line Wi-38 the HBEC showed ciliated cells after 24 days in culture (Fig. 2D and higher magnifications E and F). At that time point the HBEC in monoculture merely formed microvilli (Fig. 2A and higher magnifications B and C), but no mature cilia with the typical 9-plus-2 pattern of microtubules could be detected in the monoculture after 24 days in comparison to the coculture (Fig. 2I). With persisting cocultivation time of the HBEC the proportion of ciliated cells slightly increased but the principal finding was that the cilia gained in length and density (Fig. 2G, day 42 and Fig. 2H, day 84). Cilia were motile and beating at a frequency of 14–19 Hz depending on the donor (Table 1). We were unable to stimulate the immortalised cell line 16HBE14o- to differentiate to a pseudostratified airway epithelium, this being the case both for submersion and for air–liquid interface conditions. 16HBE14o- in coculture formed neither cilia, nor even microvilli (data not shown).

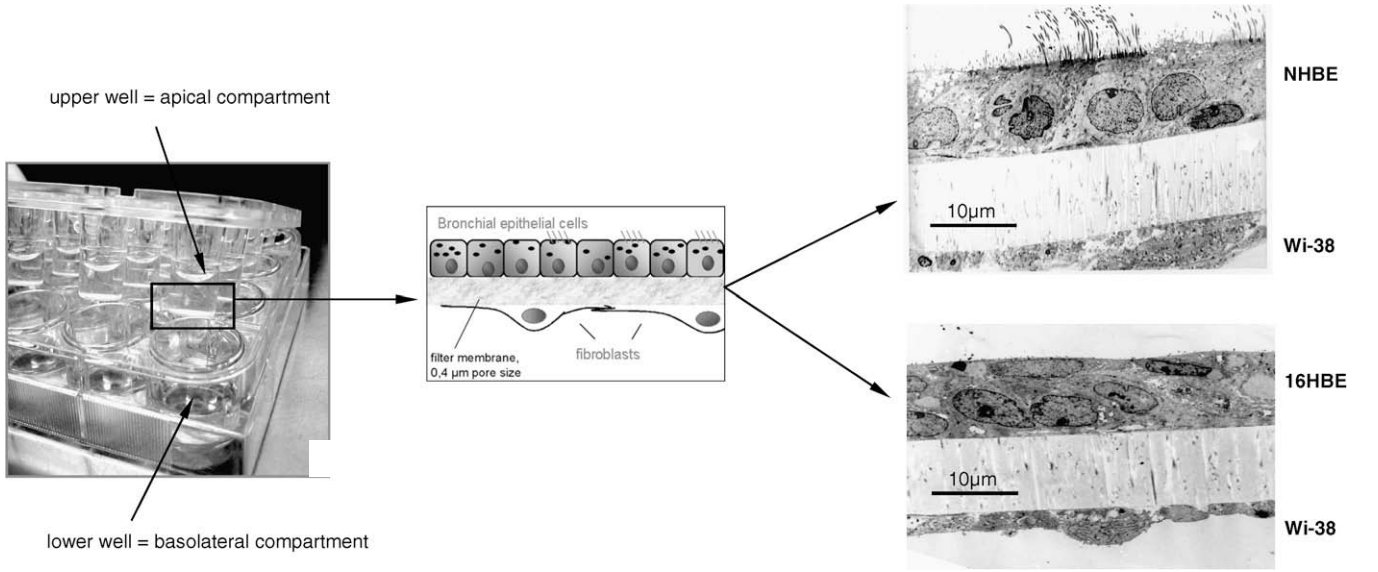

Fig. 1. Constitution of the bilayer cocultures on a 24-well HTS-Transwell filter plate. For the two cocultures the bronchial cell line 16HBE14o- and primary bronchial cells (HBEC) were both cocultivated with the fibroblast cell line Wi-38. Transmission electron microscopy from each model is shown beneath the schematic diagram. Transmission electron microscopy from the primary model was performed after 28 days in coculture, the microscopy from the permanent cell line model at day 10 in coculture.

After explant outgrowth from the small bronchial tissue fragments the HBEC showed cuboidal morphology (Fig. 3A). The outgrowing cells cultured on glass culture slides for 5 days stained positively for the basal cell markers, cytokeratin 14 (CK14) as shown in Fig. 3B and aquaporin 3 (AQP-3) in 3C, respectively. Immunofluorescent staining for different types of mucins (MUC 2, MUC 5AC, MUC 5B) as well as for ß-tubulin, a characteristic structural protein of cilia, was negative at this time point (data not shown).

The differentiation of the primary model was examined at different time points by staining paraffin sections of the cocultures with alcian blue. The latter stains acidic mucosubstances blue. During the first 2 weeks of cocultivation the morphological phenotype of the HBEC changed from a more squamous to a pseudostratified epithelium (Fig. 3D–F). Within 24 days of coculture all three major cell types of the bronchi (basal, ciliated and secretory) could be observed (shown at day 28 in Fig. 3E).

After 4 weeks of coculture a distinct film of acidic mucosubstances was observed on top of the epithelial layer (alcian blue stain, Fig. 3E), which persisted for up to 12 weeks in culture. From 4 weeks (day 28, Fig. 3E) to 12 weeks (day 84, Fig. 3F) in coculture the thickness of the epithelial layer stayed constant but the proportion of ciliated cells and the length of the cilia (see Fig. 3E compared to 3F) increased. After 28 days in coculture a bronchial layer showing the tight junctional protein ZO-1 at the cell–cell contact areas and ß-tubulin within cilia was observed by immunofluorescence (Fig. 3G).

The haematoxylin and eosin (H&E) staining of the model with 16HBE14o-/Wi-38 at day 10 showed an undifferentiated bilayer. Cultures grown at an air–liquid interface (Fig. 3I) built more layers than cells grown under submersed conditions (Fig. 3H).

3.2. Influence of TNF-α and IFN-γ on barrier function

After 14 days in coculture at the air–liquid interface the HBEC developed a detectable transepithelial resistance. TER increased during the cultivation time to a maximum of 600–800 Ω cm^2 (dependent on the donor; 683 ± 148 Ω cm^2, $n = 10$) at day 14–17, and remained relatively constant during long-term cultivation over 3 months (Fig. 4A and B). Neither under submersion nor under air-lift culture conditions HBEC in monoculture were able to build an epithelial layer with verifiable barrier properties (Fig. 4A). The model with the cell line 16HBE14o- showed a TER value of 1000–1200 Ω cm^2 (1123 ± 176 Ω cm^2, $n = 4$) during the cultivation time of 10 days and stayed constant for 4 days (Fig. 4A) before TER decreased. The fibroblasts alone were not able to build a functional barrier (data not shown). TER values were comparable with those from the monoculture of HBEC.

The different cocultures were exposed to the proinflammatory cytokines TNF-α and IFN-γ alone or in combination to determine if cytokines could elicit effects on barrier function and paracellular integrity. Thus, HBEC/Wi-38 were cocultivated over 31 days, and then the cytokines TNF-α (10 ng/ml) and IFN-γ (100 ng/ml) were added to the culture media on the apical (upper well, epithelial side) or on the basolateral (lower well, fibroblast side) side of the filter membrane. The coculture of 16HBE14o-/Wi-38 was cultured over 10 days before the cytokines were added for 24 or 48 h. The given concentrations of TNF-α and IFN-γ were chosen because similar concentrations were found in the mucosa of patients with cystic fibrosis [25].

TER was determined 24 and 48 h after treatment with TNF-α and IFN-γ alone and in combination of both cytokines (HBEC/Wi-38 shown in Fig. 4C and 16HBE14o-/WI-38 shown in 4D). The cytokines had different effects on the cocultures depending on the cells used. In the primary bronchial model no significant effects on TER were measurable with TNF-α and IFN-γ alone after 24 h, independent of an apical or basolateral exposure. However, treatment with both cytokines showed an effect. TER decreased to 67% of non-treated controls under both apical and basolateral exposure. After 48 h treatment with the individual cytokines TER was statistically significantly reduced. With both cytokines TER decreased to 33% of the untreated control. After 48 h treatment with TNF-α and IFN-γ alone the reduction in TER was comparable to that already induced by both cytokines after 24 h. Here, TER significantly decreased to 65% of control without cytokine, and was again independent of an apical or basolateral exposure (Fig. 4C). The cytokines had no effect on the bronchial model with the cell line 16HBE14o- after 24 h and after 48 h whether in combination or alone (Fig. 4D). The TER values stayed constant between 600 and 700 Ω cm^2.

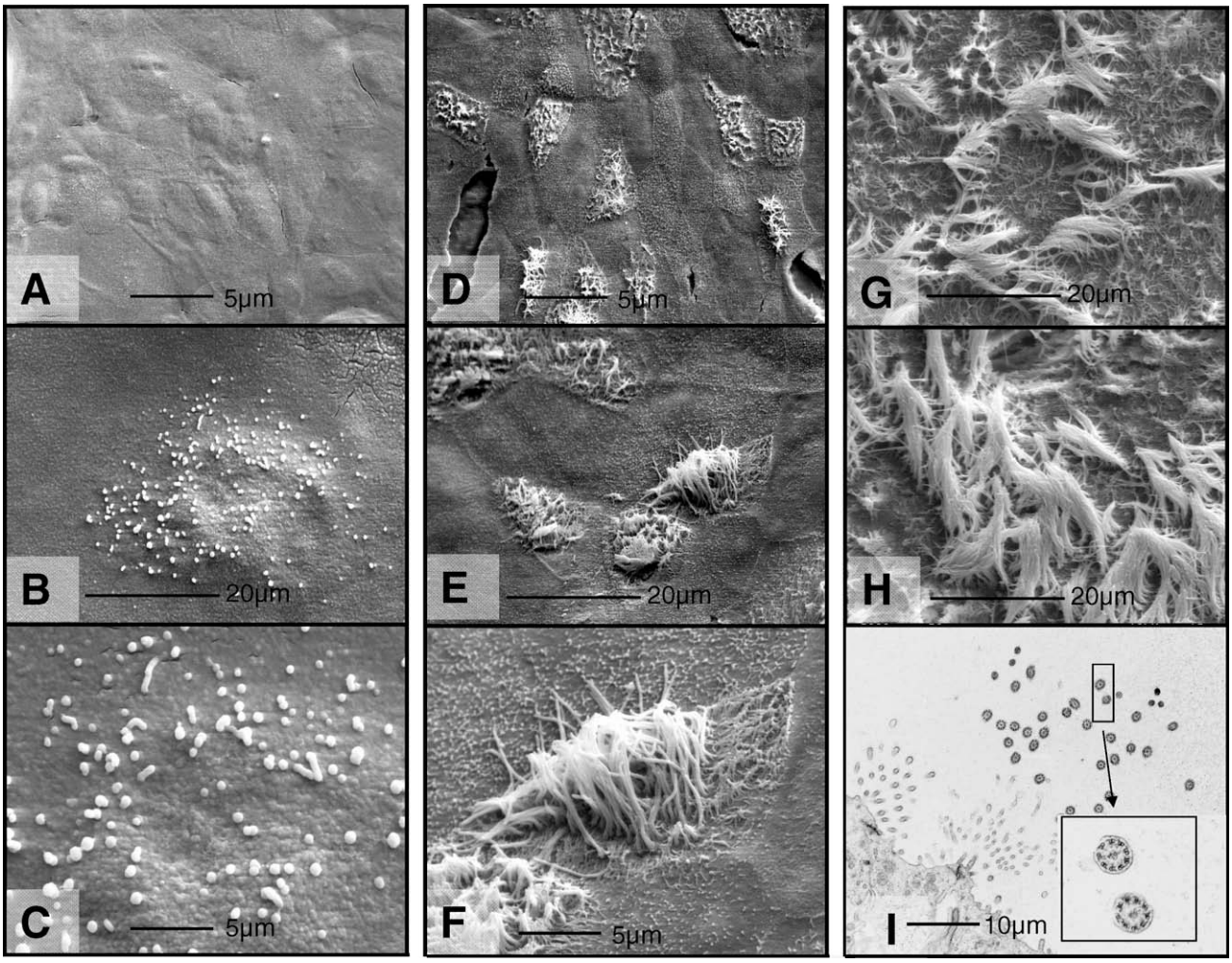

Fig. 2. Scanning electron micrographs of HBEC on transwell filter membranes after 24 days in monoculture (A–C, magnifications 1000×, 2000×, 5000×, resp.) and in coculture with Wi-38 (D–F, magnifications 1000×, 2000×, 5000×, resp.). (G) Scanning electron microscopy after 42 days and (H) after 84 days in coculture (magnification 2000×). While HBEC in monoculture merely formed apical microvilli, in coculture with Wi-38 the proportion of ciliated cells increased and cilia gained in length with increasing cultivation time. (I) A transmission electron micrographs with cuttings through individual cilia. The cilia present their typical structure of nine outer doublet microtubules surrounding the central pair of single microtubules, as found *in vivo*.

Table 1
Ciliary beat frequency

Tissue	Frequency (Hz)	Citation
Bronchial biopsies	9–14	[36,20]
Nasal ciliated epithelium	11–16	[2]
Rat trachea	8–10	[17]
HBEC/Wi-38	14–19	

To assess if the observed decrease in TER was a result of disassembly of the tight junctions in the epithelial layer, we monitored the paracellular transport of the anionic hydrophilic marker sodium fluorescein and FITC-labeled dextrans with molecular weights of 20 and 70 kDa after 48 h treatment with the cytokines. The transport studies were performed under exposure to both cytokines, measuring the apparent permeability coefficient (P_{app}) for the transport from the apical to the basolateral side of the epithelial layer.

A dependency of the P_{app} on the molecular weight of the FITC-dextrans was observed in both untreated and exposed cocultures. Sodium fluorescein as the marker with the lowest molecular weight passed more readily through the cocultures than the dextrans with increased molecular weight. Corresponding to the slight reduction in TER a mild increase in P_{app} after 24 h exposure to the cytokines alone and in combination was detected for sodium fluorescein and all FITC-dextrans in the primary bronchial model (HBEC/WI-38). After 48 h of HBEC/Wi-38 treatment P_{app} increased significantly in parallel to the TER reduction (Fig. 5A and C). The bronchial model with the cell line 16HBE14o- did not show any significant effect in TER, and similarly there were no effects on P_{app} after 24 h or after 48 h cytokine treatment, whether in combination or alone (Fig. 5B and D).

3.3. Influence of TNF-α and IFN-γ on tight junctions and adherens junctions

To determine if the exposure to the cytokines TNF-α and IFN-γ initiates a disrupted organization of TJ- and AJ-associated proteins, we performed immunofluorescence to localise ZO-1, occludin, E-cadherin and ß-catenin. After 31 days in culture primary cells were treated with the cytokines, then fixed and stained with the respective antibodies. As shown in Fig. 6 (left column) in untreated controls all four cell–cell contact proteins were located at the apical borders between adjacent cells. After 24 h exposure to the cytokines TNF-α and IFN-γ in combination, ZO-1 and occludin staining appeared fragmented at the cell–cell contact zones (Fig. 6, second

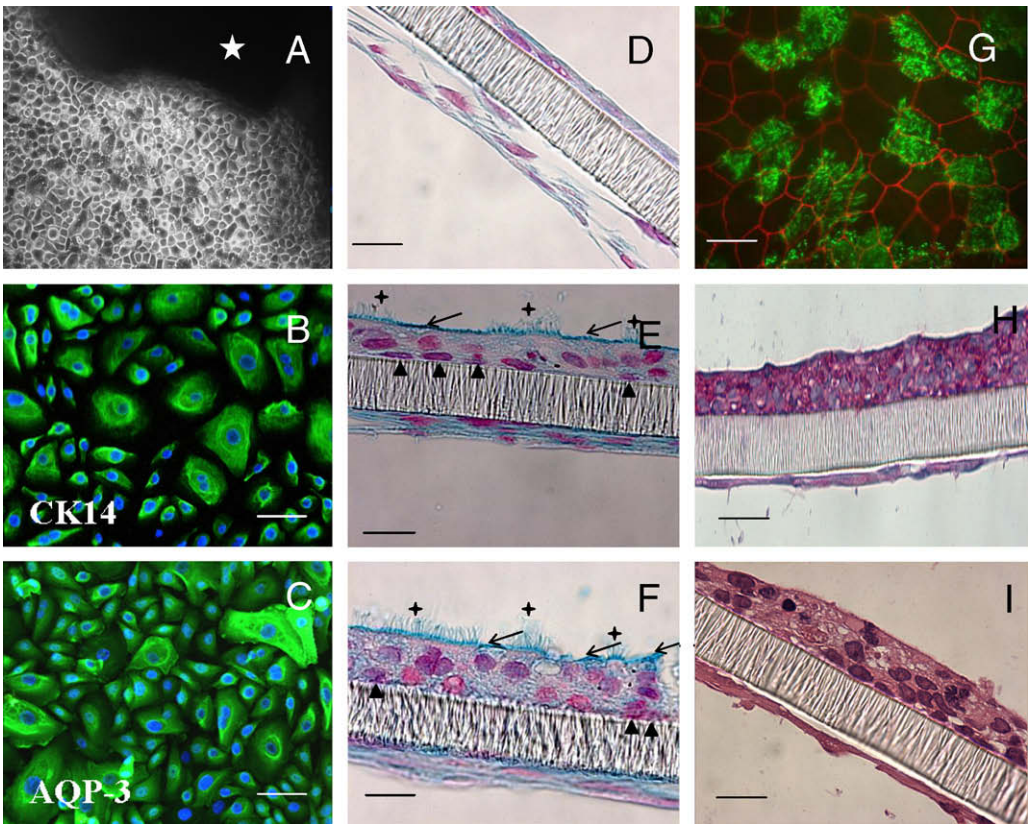

Fig. 3. Morphological phenotype of the normal human bronchial epithelial cells after explant-outgrowth culture (A) as observed in a light microscope without staining. The explant is marked with ★. The expression of cytokeratin 14 (B) and aquaporin 3 (C) is determined by immunofluorescence. Mucociliary differentiation of the primary cells during air–liquid interface culture is shown in semithin sections stained with alcian blue (D–F) prepared after day 7 (D), day 28 (E) and day 84 (F) of coculture. Cilia are marked with ✦, basal cells with ▲ and the mucus layer with ↑. Immunofluorescent staining of the bronchial cells in coculture with Wi-38 on transwell filter membranes with ß-tubulin (green fluorescence) and ZO-1 (red fluorescence) is shown in (G). An apical intercellular TJ complex is present between adjacent ciliated and non-ciliated cells. The alcian blue staining of the model with 16HBE14o-/Wi-38 at day 10 showed many layers on top of each other without differentiation, although cultures grown at an air–liquid interface (I) built more layers than cells grown under submersed conditions (H). Scale bar: 10 μm.

column). Two days after treatment this effect was more pronounced (Fig. 6, third column). ZO-1 and occludin labeling was seen as punctate aggregates. Thus, this staining pattern for both TJ proteins is an evidence for a loss of structural organization, despite the fact that a constant expression of protein was measured in Western blot analysis (Fig. 7). The effect of cytokine exposure on the AJ proteins E-cadherin and ß-catenin was less prominent than that detected for the TJ proteins in immunofluorescence. The HBEC in coculture without cytokine exposure exhibited an intense and continuous staining pattern of ß-catenin and E-cadherin, localised at the lateral borders of the organized epithelium (Fig. 6). One day after simultaneous exposure to TNF-α and IFN-γ the AJ molecule labeling at the cell–cell contact zones showed discontinuities, the appearance of these discontinuities being more distinct after 48 h (Fig. 6, arrows in the pictures). Similar to the TJ proteins the overall expression levels of ß-catenin and E-cadherin remained unchanged as examined by Western blot analysis (Fig. 7). After exposure to TNF-α (Fig. 6, right column) or IFN-γ (data not shown) alone for 24 and 48 h no changes in TJ and AJ protein distribution could be observed. For the 16HBE14o-/Wi-38 model we could not detect any effect either on TJ or on AJ structure, corresponding to the unaltered TER values and P_{app}. The integrity of the 16HBE14o- in coculture with Wi-38 was not influenced by the cytokines.

4. Discussion

Bronchial epithelial tumor cell lines, such as Calu-3, or immortalised primary isolated bronchial epithelial cells, like BEAS-2B and 16HBE14o-, are widely used for toxicological studies of the upper respiratory tract. Compared to *in vivo* studies such cell lines have the benefit of flexibility and specific phenotypic traits [13,18]. However, the epithelial tumor cell lines, typically arising from one genomically altered cell, lack the ability to mimic the differentiated cell types of the bronchial epithelium *in vivo*. An alternative to the use of cell lines is the primary isolated bronchial cells which contain all phenotypes of the bronchial epithelium. Unfortunately, most primary isolated bronchial epithelial cells rapidly lose their differentiated phenotype *in vitro*, possibly caused by the absence of extracellular matrix, growth factors and essential hormones [37]. Epithelial–fibroblast cocultures and air–liquid interface culture conditions have been shown to reconstitute the differentiation of tracheal or bronchial cell phenotypes *in vitro* [6,7,31]. We applied both of these differentiation-inducing conditions to 16HBE14o- as well as to HBEC, that showed typical markers of bronchial basal cells, like CK14 and AQP-3, in primary culture. In contrast to 16HBE14o- cells, which remained phenotypically unchanged, the HBEC showed increasing cellular differentiation over time in coculture with the human lung fibroblast cell line Wi-38. Within 24 days at air–liquid interface in coculture HBEC differentiated to the three main human bronchial cell phenotypes: basal cells as seen in the immunofluorescence staining with CK14 and AQP-3, ciliary cells shown in transmission electron microscopy and in the immunofluorescence staining with ß-tubulin and epithelial cells with secretory activity detected with alcian blue immunohistochemistry. The formed cilia beated with a frequency of 14–19 Hz, which is comparable to that of bronchial epithelial

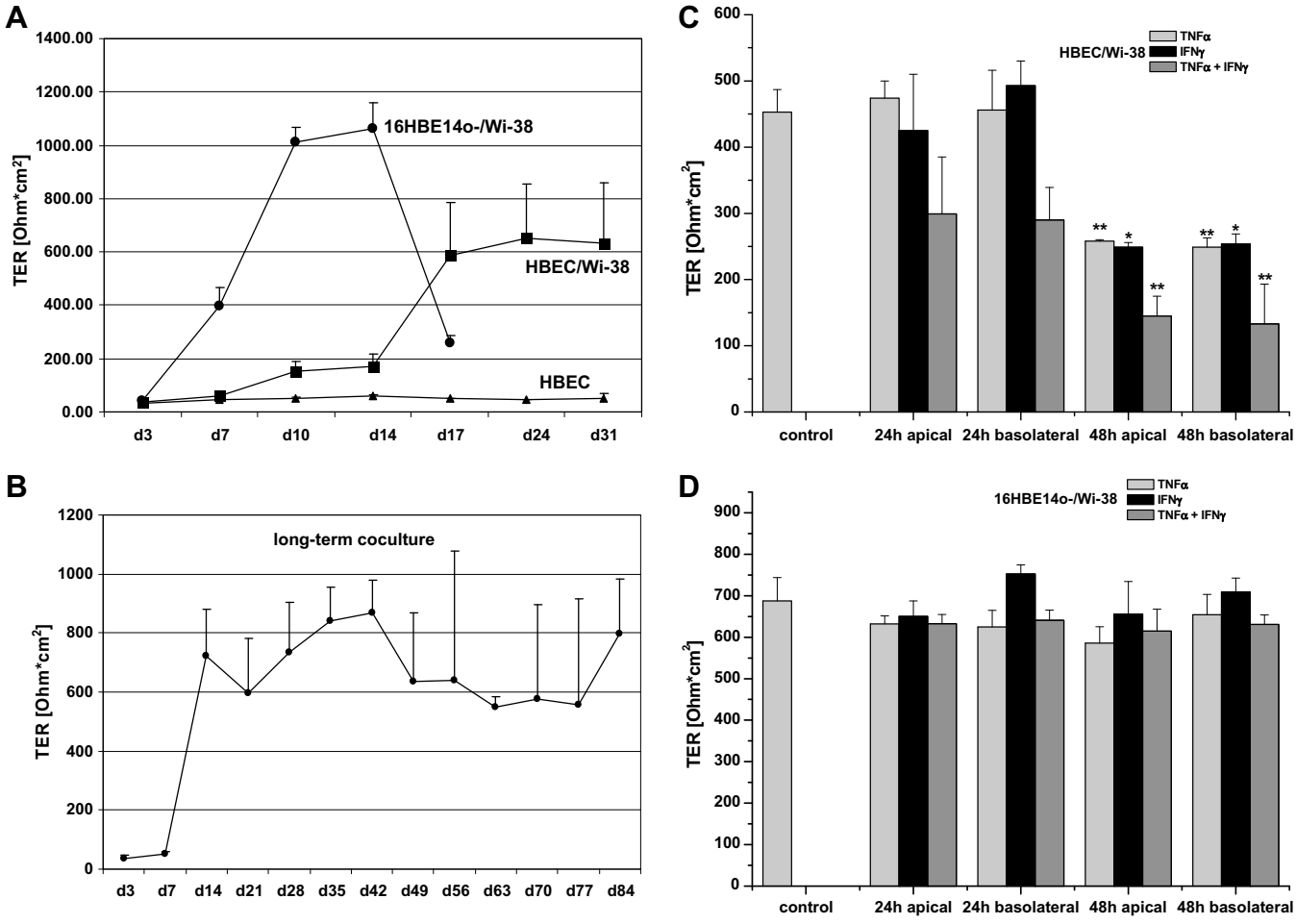

Fig. 4. Measurement of the transepithelial electrical resistance (TER) after treatment with TNF-α and IFN-γ alone and as combined treatment at different time points. (A) TER from monoculture (▲) and the coculture of HBEC/Wi-38 (■) and 16HBE14o-/Wi-38 (●) over a time period of 31 days for the primary model and 17 days for the permanent cell line model. (B) TER from long-term coculture of HBEC and Wi-38 measured over a time period of 3 months. (C) Effects of the cytokines (TNF-α and IFN-γ) in the primary bronchial coculture. (D) TER in cocultures of the bronchial cell line 16HBE14o- with Wi-38. Data are depicted as means ± SD from $n = 3–5$ independent experiments. *Significantly different ($p < 0.05$) or **($p < 0.01$) from the non-treated control.

cells *in vivo*. To our knowledge a ciliary beat activity of human bronchial epithelial cells *in vitro* is so far only described for human tissue-culture preparations [10] or lung slices [9] and could not be sustained for longer than 25 days. In these tissue-culture preparations or lung slices cilia showed a synchronised beating pattern. In our model we could observe a synchronisation between all cilia from one cluster, and the beating activity could be maintained for a period of at least 3 months. The primary bronchial model still has to be modified in order to study mucociliary clearance, but could be very useful to investigate long-term effects of toxic or pharmaceutical substances on the ciliary beat frequency. In our study, cells outgrowing from the bronchial tissue differentiated and assembled into multicellular bronchial epithelial layers sharing similarities to the bronchial epithelium in vivo in terms of their cellular composition and functionality such as the formation of actively beating cilia. This differentiation was only observed in cocultures with fibroblasts, monocultures showed less pronounced differentiation at the same time in culture.

TER of the HBEC in coculture at air–liquid interface increased to a maximum of 600–800 Ω cm^2 within 14–17 days and remained relatively constant during long-term cultivation over 3 months. These data are comparable with previously published TER values of 700–1000 Ω cm^2 in primary cultured tracheal and bronchial epithelial cells of rabbits and humans [3,24]. Under the same conditions 16HBE14o- cells showed maximum TER values of 1000 Ω cm^2 at day 12, which rapidly dropped to 300 Ω cm^2 on day 17. Additionally, 16HBE14o- cells did not form an organized epithelial layer. Although 16HBE14o- cells had been immortalised from primary isolated bronchial epithelial cells they did not have the capacity to differentiate to the three main bronchial phenotypes as was the case for the primary isolated HBEC cells from explant-outgrowth cultures. As described earlier, for 16HBE14o- cells in coculture the air–liquid interface induced a multilayered growth, but no bronchial tissue-like differentiation was seen [11,12]. An additional cocultivation with the fibroblast cell line Wi-38 used in our study also failed to trigger 16HBE14o- cell differentiation. Although the cellular organization of 16HBE14o- was less distinctive the 16HBE14o- in coculture with Wi-38 showed higher maximum TER and lower P_{app}-values than HBEC in coculture. This effect is probably not due to a tighter barrier formed by 16HBE14o- cells but due to the impact of their multilayered growth.

To mimic an affected barrier function both bronchial cell types in coculture were exposed to the proinflammatory cytokines TNF-α and IFN-γ in concentrations found in cystic fibrosis patients [26]. 16HBE14o- cells in coculture were exposed to TNF-α (10 ng/ml) and IFN-γ (100 ng/ml) when they reached maximum TER. As HBEC showed a bronchial tissue-like differentiation after 31 days in coculture, this time point was chosen to study the effects of TNF-α and IFN-γ. For 16HBE14o- cells in coculture TER, paracellular

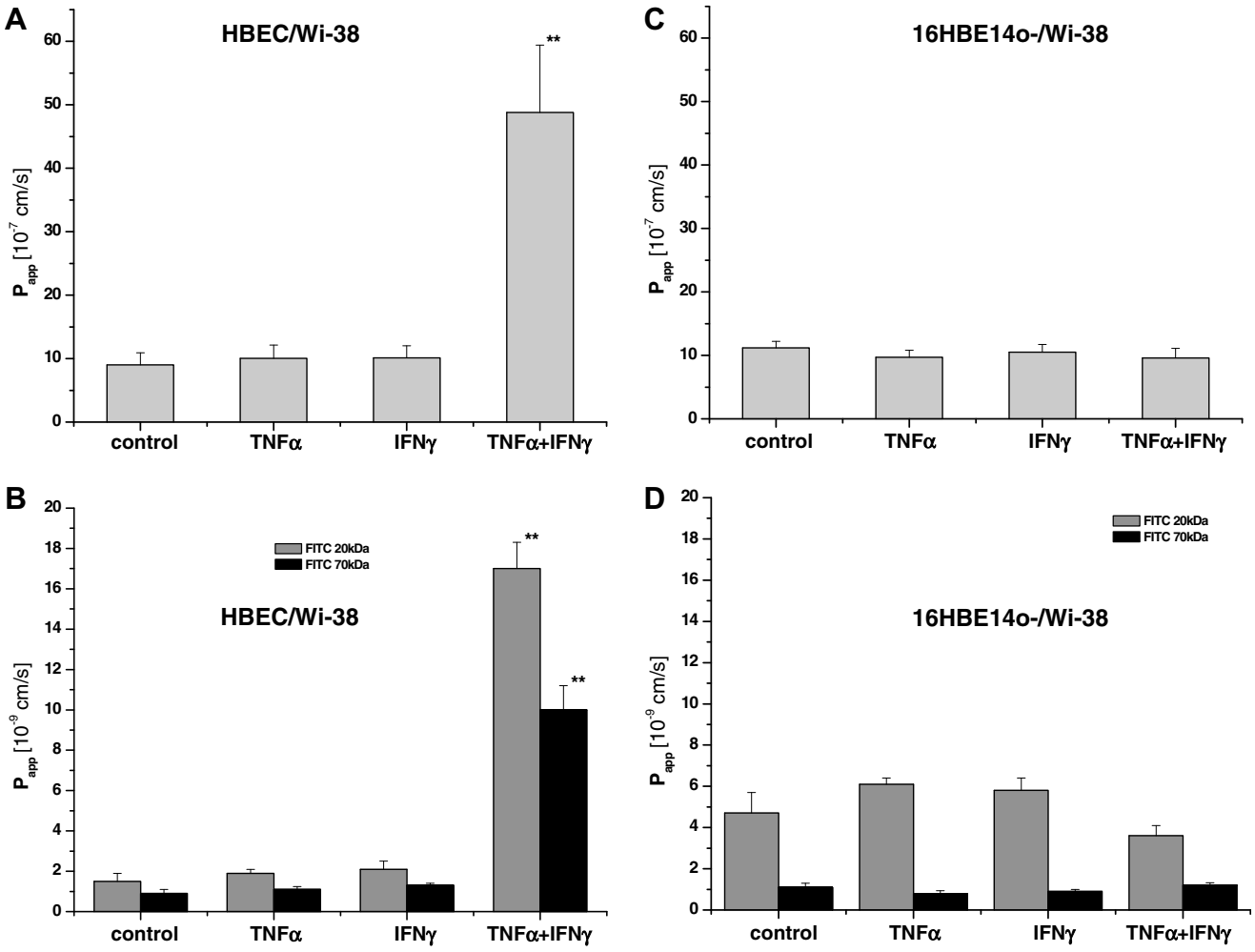

Fig. 5. Comparison of epithelial barrier properties quantified for different *in vitro* models of the respiratory tract, including the cell line 16HBE14o- and primary HBEC. Apparent permeability coefficients (mean ± SD) were determined for the hydrophilic marker compound sodium fluorescein (A and B), and the dextrans of molecular weight 20 and 70 kDa (C and D). The values from the HBEC/Wi-38 model are shown in (A) and (C), values from the coculture of 16HBE14o-/Wi-38 are shown in (B) and (D). Data are depicted as means ± SD from $n = 3–5$ independent experiments. *Significantly different ($p < 0.05$) or **($p < 0.01$) from the non-treated control.

integrity and TJ- and AJ-associated protein expression were not influenced by an exposure to TNF-α and IFN-γ.

In contrast, the primary isolated HBEC in coculture responded to these stimuli with impaired barrier function. After 24 h exposure TNF-α and IFN-γ alone showed no significant decrease in TER, irrespective of an apical or basolateral exposure. After 48 h with either cytokine TER decreased to approximately 65% of the control. The simultaneous exposure with both cytokines showed more than an additive co-stimulatory effect. TER decreased significantly to 33% of the untreated control after 48 h. A similar co-stimulatory effect of TNF-α and IFN-γ on barrier function is also reported in a study using bronchial epithelial cell monolayers [8]. Analogous to our findings Coyne and co-workers [8] showed a reduction of TER values to 60–70% and 30–40% of controls after 24 and 48 h exposure to TNF-α and IFN-γ, respectively.

To assess if the observed decrease in TER was a result of the disassembly of the tight junctions, we monitored the paracellular movement of sodium fluorescein and FITC-labeled dextrans. In previous studies 16HBE14o- cells grown at the air–liquid interface showed P_{app}-values for sodium fluorescein similar to those measured in our untreated coculture model using 16HBE14o- cells [32].

A dependency of the P_{app} on the molecular weight of the FITC-dextrans was observed in both 16HBE14o- and HBEC cocultures. This dependency of the P_{app} on the molecular weight was preserved also in the exposed cocultures up to 24 h exposure. As the bronchial model with the cell line 16HBE14o- did not show any cytokine-induced effect on TER there were also no significant changes detected for the respective P_{app}-values after 24 h or after 48 h exposure. Barrier function of 16HBE14o- cells was not influenced by the used cytokines, indicating that 16HBE14o- cells are not sensitive to TNF-α or IFN-γ stimulation. As several studies have examined TNF-α- and IFN-γ-induced changes in epithelial [1,15,16] paracellular permeability *in vitro* the cell line 16HBE14o- might be unsuitable for the study of the influence of cytokine treatment on bronchial epithelial cells.

In the primary bronchial model P_{app} increased significantly after 48 h exposure to both cytokines, corresponding to the distinct reduction in TER at that time. These data indicate a disassembly of cell–cell contact molecules after 48 h exposure to both cytokines. This finding was confirmed by immunofluorescent location of TJ- and AJ-associated molecules. While HBEC in coculture already showed a fragmented staining of ZO-1 and occludin 24 h after exposure to both cytokines, 48 h after treatment with both cytokines the disassembly of tight junctional molecules was very distinctive. The impact of cytokine exposure on adherens junction proteins was much less marked than that observed for the tight junction proteins occludin and ZO-1. It

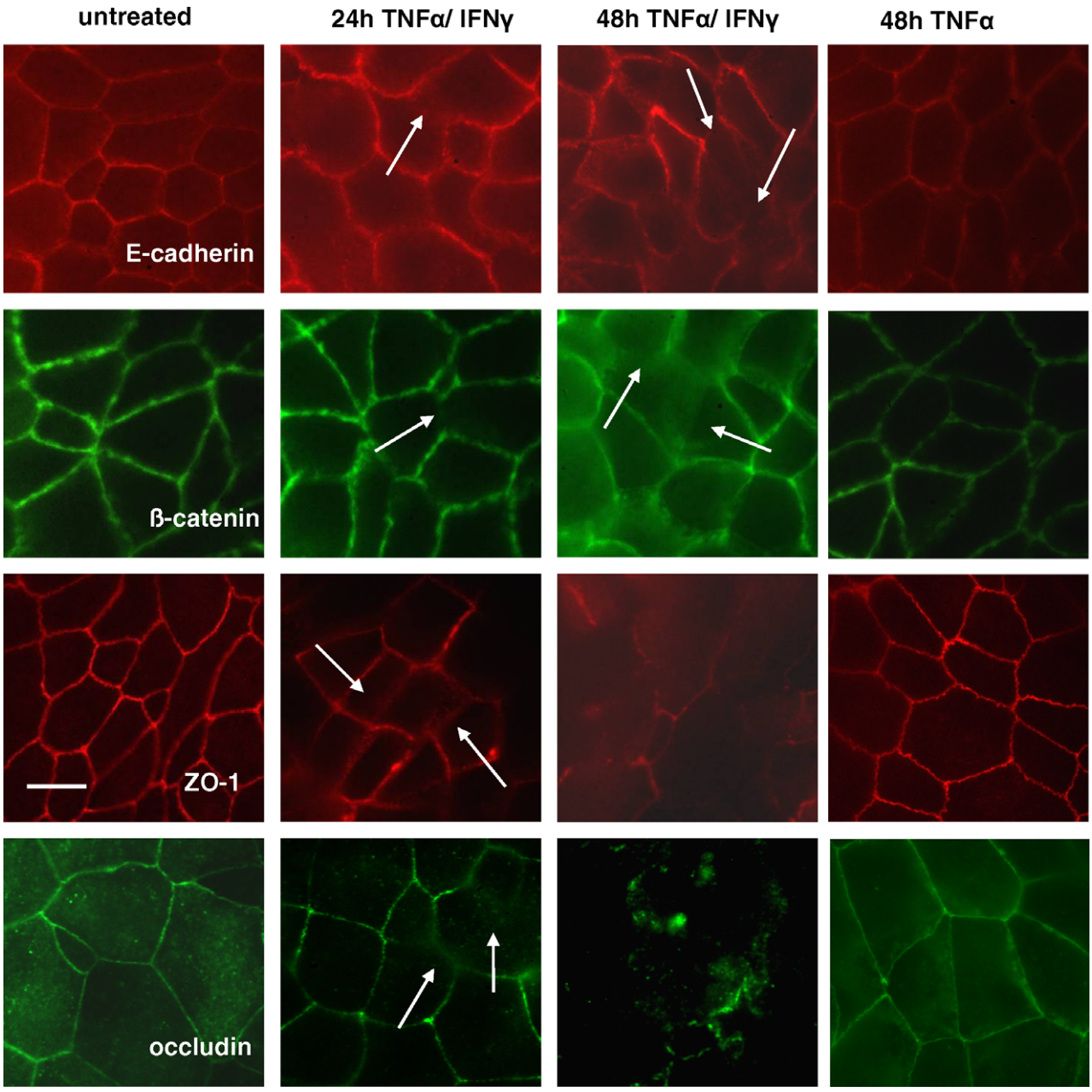

Fig. 6. Immunofluorescent staining of the TJ proteins ZO-1 and occludin and the AJ proteins E-cadherin and ß-catenin. HBEC cells were cocultivated with Wi-38 for 31 days and then treated with the cytokines TNF-α and IFN-γ for 24 and 48 h alone and in combination. The corresponding staining of the individual cell–cell contact proteins is arranged as rows (scale bar = 10 μm). Discontinuities in expression pattern are marked with arrows.

is reported that the claudin family, to which occludin and ZO-1 belong, plays a critical role in maintaining TJ integrity and may be responsible for the passage of ions and molecules through the paracellular space [34]. However, in contrast to Coyne and co-workers [8] who described decreasing levels of the TJ and AJ proteins by immunofluorescence and Western blot, we observed discontinuities of the AJ molecule labeling of the cell–cell contact zones in immunofluorescence but no change at the level of protein concentration in Western blot. While a clear disassembly of the TJ proteins was observed at single cell level the overall expression levels of occludin and ZO-1 were unchanged in our experiments. Hence, investigation at a single cell level presents a more detailed picture of the molecular events in bronchial epithelial cells after stimulation, than that given by the analytical method for total protein detection.

In summary, this paper describes a reproducible human bronchial coculture model which permits epithelial–fibroblast interactions and induces differentiation of the most abundant bronchial epithelial cell types (basal, ciliated and secretory cells). The application of such a coculture model could be valuable for pharmacological studies. Moreover, it can also be used to screen toxicity of different materials relevant for pulmonary exposure. The developed *in vitro* coculture could serve as a model for acute or subacute effects following 4 weeks of differentiation as well as for chronic effects during long-term exposure over a 3-month period [22]. To our knowledge this paper is the first description of a human bronchial model which leads to the appearance of functional cilia, which actually beat for at least 3 months in culture at a frequency observed *in vivo*. In further experiments we will use the established primary coculture model to examine ciliary beat frequency

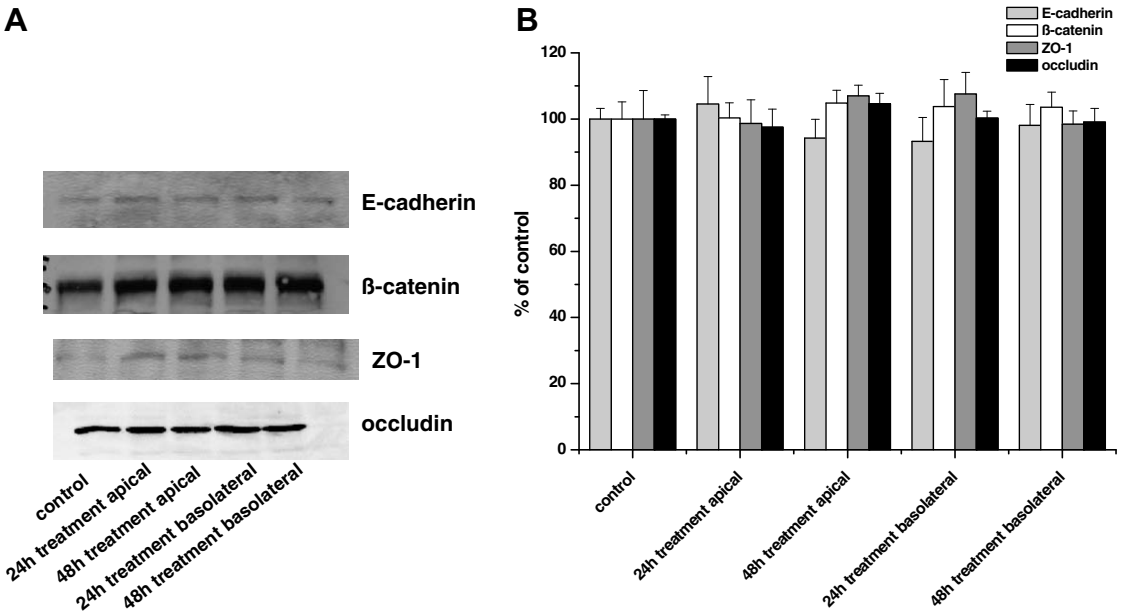

Fig. 7. (A) Effects of TNF-α and IFN-γ on the expression of TJ and AJ proteins. Western blot protein bands were visualized by immunodetection (E-cadherin 120 kDa, ß-catenin 92 kDa, ZO-1 69 kDa and occludin 56 kDa). Protein levels of the TJ and AJ remained unchanged in Western blot. Images are representative of a minimum of $n = 3$ from three different donors. (B) Densitometry of the Western blot protein bands. Protein levels of the cell–cell contacts remained unchanged. Data are depicted as means ± SD from $n = 3$ independent experiments.

modulation by cytokines and elucidate underlying signal transduction pathways.

Acknowledgments

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