Oxidative stress and inflammation response after nanoparticle exposure: differences between human lung cell monocultures and an advanced three-dimensional model of the human epithelial airways

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Combustion-derived and manufactured nanoparticles (NPs) are known to provoke oxidative stress and inflammatory responses in human lung cells; therefore, they play an important role during the development of adverse health effects. As the lungs are composed of more than 40 different cell types, it is of particular interest to perform toxicological studies with co-cultures systems, rather than with monocultures of only one cell type, to gain a better understanding of complex cellular reactions upon exposure to toxic substances. Monocultures of A549 human epithelial lung cells, human monocyte-derived macrophages and monocyte-derived dendritic cells (MDDCs) as well as triple cell co-cultures consisting of all three cell types were exposed to combustion-derived NPs (diesel exhaust particles) and to manufactured NPs (titanium dioxide and single-walled carbon nanotubes). The penetration of particles into cells was analysed by transmission electron microscopy. The amount of intracellular reactive oxygen species (ROS), the total antioxidant capacity (TAC) and the production of tumour necrosis factor (TNF)- α and interleukin (IL)-8 were quantified. The results of the monocultures were summed with an adjustment for the number of each single cell type in the triple cell co-culture. All three particle types were found in all cell and culture types. The production of ROS was induced by all particle types in all cell cultures except in monocultures of MDDCs. The TAC and the (pro-) inflammatory reactions were not statistically significantly increased by particle exposure in any of the cell cultures. Interestingly, in the triple cell co-cultures, the TAC and IL-8 concentrations were lower and the TNF- α concentrations were higher than the expected values calculated from the monocultures. The interplay of different lung cell types seems to substantially modulate the oxidative stress and the inflammatory responses after NP exposure.

Keywords: human epithelial airway model; monocultures; triple cell co-cultures; nanoparticles; reactive oxygen species; inflammation

1. INTRODUCTION

Epidemiological studies have shown an association between exposure to particulate matter with a diameter

less than or equal to 10 μm (PM₁₀) and adverse health effects such as cardiovascular and cardiopulmonary diseases (Samet *et al.* 2000; Brunekreef & Holgate 2002; Pope *et al.* 2004b; Riediker *et al.* 2004). Diesel exhaust particles (DEPs) are an important constituent of PM₁₀ and are the main cause of adverse health effects (Lighty *et al.* 2000; Schwartz 2000). Additionally, *in vitro* studies have shown adverse effects of combustion-derived PM₁₀ in cultures of different cell types. During

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the development of adverse health effects, oxidative stress and inflammatory response play key roles (Brown *et al.* 2001, 2004; Xiao *et al.* 2003; Pope *et al.* 2004a; Donaldson *et al.* 2005). Various studies, performed with human lung cells used as monocultures, have shown that particles, especially DEP, can provoke oxidative stress (Xiao *et al.* 2003; Pan *et al.* 2004), induce inflammatory responses (Becker *et al.* 2005) and increase the cell number of human lung epithelium (Bayram *et al.* 2006). Furthermore, it has been shown that there is a link between exposure to diesel soot and lung cancer (Donaldson *et al.* 2005).

Recent studies indicate a specific toxicological effect of inhaled combustion-derived nanoparticles (NPs; diameter less than or equal to 0.1 μm) (Borm & Kreyling 2004). In addition to the generation of NPs produced by combustion processes in large amounts, there are progressively more manufactured NPs (with at least two dimensions less than or equal to 0.1 μm) released into the air, water and soil every year from other sources, such as nanotechnology (Mazzola 2003; Paull *et al.* 2003). Manufactured NPs have also been shown to cause toxicity (Oberdorster *et al.* 2005; Nel *et al.* 2006).

Owing to the direct contact of the lung surface with the inhaled air and the large surface area of the lungs (approx. 150 m^2 ; Gehr *et al.* (1978)), the effects of inhaled and deposited particles to the lung cells are of particular interest. The surface of the gas exchange area of the lung consists of mainly squamous epithelial cells (type I pneumocytes), which form a tight barrier. These cells are only approximately 0.1 μm in thickness providing a structural lining with a very short diffusion distance for gas exchange between alveolar air and capillary blood. They cover approximately 93 per cent of the alveolar surface, but account for less than 8 per cent of the distal lung cells. Type II epithelial cells are cuboidal in shape and cover approximately 7 per cent of the alveolar surface while making up 16 per cent of the cells in the distal lung (Crapo *et al.* 1982; Stone *et al.* 1992; Ochs & Weibel 2008). In addition to the epithelial cells, there is a population of macrophages (Brain 1988; Lehnert 1992) on the apical side and a population of dendritic cells underneath the airway epithelium (Holt *et al.* 1990). Macrophages are phagocytotic cells, whereas dendritic cells, as the most potent antigen-presenting cells, can initiate the adaptive immune response (Blank *et al.* 2008). Recently, it has been shown that the two immune cell types interact directly with each other as sentinels against fine particulate antigens (Blank *et al.* 2007), or in a paracrine way (Fujii *et al.* 2002). To obtain a more realistic assessment of the effects of nanosized particles on human lung cells, the current study simulated this situation with a co-culture model for the human airway composed of different cell types and not only of monocultures (Tao & Kobzik 2002; Ishii *et al.* 2005; Roggen *et al.* 2006; Alfaro-Moreno *et al.* 2008; Rothen-Rutishauser *et al.* 2008a).

The aim of this work was to compare the cellular responses upon particle exposure in monocultures of human epithelial cells (A549 epithelial type II cell line), human monocyte-derived macrophages (MDMs) as well as human monocyte-derived dendritic cells

(MDDCs) and in triple cell co-cultures composed of all three cell types (Rothen-Rutishauser *et al.* 2005). By using this cell model, the individual cellular response and the interaction between different cell types could be studied. The study used combustion-derived NPs (DEP) and manufactured NPs (single-walled carbon nanotubes (SWCNTs) and titanium dioxide (TiO_2)). All these NPs have previously been used in our laboratory, and various publications resulted from these experiments (Rothen-Rutishauser *et al.* 2006, 2007a, 2008b; Wick *et al.* 2007; Helfenstein *et al.* 2008). The intracellular localization of all three particles was assessed by transmission electron microscopy (TEM), and the potential of these particles to induce oxidative stress and an inflammatory reaction was studied in the exposed cell cultures using different cell assay kits. The expected values (such as oxidative stress and inflammatory responses) for the triple cell co-cultures were calculated theoretically from the observed values in the monocultures and compared with the observed values.

2. MATERIAL AND METHODS

2.1. A549 monocultures

The A549 epithelial cell line (Lieber *et al.* 1976) from the American Tissue Type Culture Collection (LGC Prochem, Molsheim, France) was used. The cells (passage numbers 5–38) were handled as described by Rothen-Rutishauser *et al.* (2005). They were maintained in standard tissue culture flasks (25 cm^2 , 60 ml, with filter screw cap, sterile; TPP AG, Trasadingen, Switzerland). Cell cultures were kept at 37°C under a 5 per cent CO_2 humidified atmosphere using medium RPMI-1640 (with 25 mM HEPES; Labforce AG, Nunningen, Switzerland) with 10 per cent foetal calf serum (PAA Laboratories, Lucerna-Chem AG, Lucerne, Switzerland), 1 per cent L-glutamine (200 mM stock solution; LabForce AG) and 1 per cent penicillin/streptomycin (10 000 U ml^{-1} penicillin G and 10 000 $\mu\text{g ml}^{-1}$ streptomycin sulphate in 0.85% saline; Gibco BRL, Invitrogen AG, Basel, Switzerland). For splitting and seeding, the cells were detached from the flask with trypsin–EDTA (0.5 g l^{-1} trypsin, 0.2 g l^{-1} EDTA.4Na in Hanks' balanced salt solution (HBSS); Gibco BRL, Invitrogen AG). The cells were counted with a Neubauer counting chamber and diluted to obtain a density of 0.5×10^6 cells ml^{-1} . Two millilitres of the cell suspension was added to cell culture inserts (surface area of 4.2 cm^2 , pores with 3.0 μm in diameter, high pore density PET membranes for six-well plates; BD Falcon, BD Biosciences, Basel, Switzerland). The inserts were placed in six-well tissue culture plates (BD Biosciences). Three millilitres of the medium was added individually to the lower chamber of each insert. The medium was changed every 3 days. The cells were kept a total of 7–9 days in culture.

2.2. Monocyte-derived macrophage and monocyte-derived dendritic cell monocultures

The MDMs and MDDCs were obtained from human blood monocytes as described by Rothen-Rutishauser

et al. (2005). First, peripheral blood monocytes were isolated from buffy coats (blood donation service, Bern, Switzerland) by density gradient centrifugation on Ficoll-Plaque (Amersham Biosciences Europe GmbH, Otelfingen, Switzerland). The monocytes were re-suspended in RPMI-1640 with 1 per cent L-glutamine, 1 per cent penicillin/streptomycin and 10 per cent heat-inactivated human serum (blood donation service). After 2 h in two-chamber slides (Lab-Trek, VWR International AG, Life Science, Lucerne, Switzerland) to allow for adhesion, the non-adherent cells were washed away. The adherent monocytes were cultured in 2 ml RPMI-1640 with 1 per cent L-glutamine, 1 per cent penicillin/streptomycin and 5 per cent heat-inactivated human serum. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 were added to the medium for the generation of MDDCs. For the generation of macrophages, no supplement was added. The monocytes were kept for 7 days in the respective medium to allow for differentiation.

2.3. Triple cell co-cultures

The triple cell co-cultures were prepared as described by Rothen-Rutishauser *et al.* (2005). Briefly, A549 cells were cultured for 7 days on the membranes in inserts. Medium was removed from the upper and lower chambers, and the inserts with the established epithelial cell layer were turned upside down and deposited in sterile Petri dishes (BD Biosciences). During the 7 days in culture, epithelial cells grown in monolayers could traverse the membrane and grow on the bottom side of the membrane. Therefore, the epithelial cells at the bottom side were abraded carefully with a cell scraper. The cell suspension was removed, and the bottom side of the membrane was washed once with RPMI-1640 medium. MDDCs were harvested and 300 μl of the cell suspension was added to the basal side of the inserts turned upside down. The Petri dishes were incubated for 1.5 h before the non-adherent MDDCs were removed and the inserts placed back into the tissue plates with 3 ml of RPMI-1640 supplemented with 1 per cent L-glutamine, 1 per cent penicillin/streptomycin and 5 per cent heat-inactivated human serum. MDMs were harvested, and 500 μl of the cell suspension was added to the apical surface of the epithelial monolayer. Cells were allowed to attach for 1.5 h, non-adherent cells were washed away and 2 ml of RPMI-1640 supplemented with 1 per cent L-glutamine, 1 per cent penicillin/streptomycin and 5 per cent human serum was added to the upper chamber. The triple cell co-cultures were kept for 2–24 h at 37°C under a 5 per cent CO₂ humidified atmosphere.

2.4. Particle suspensions and exposures

Particle suspensions were prepared as described in detail by Helfenstein *et al.* (2008). Briefly, SWCNTs produced by arc-discharged evaporation of graphite rods filled with nickel and yttrium powder (diameter approx. 20 nm) were purchased from Yangtze Nanotechnology (Shanghai, China) and handled as described by Wick *et al.* (2007). The SWCNT stock suspensions

were then suspended in water containing 40 $\mu\text{g ml}^{-1}$ Tween-80 (Tween-80, cell culture tested, Sigma-Aldrich Chemie GmbH, Steinheim, Germany). After centrifugation, two different fractions of SWCNT suspensions were obtained: CNT supernatant (bundles of SWCNTs) and CNT pellet (containing mostly amorphous carbon and catalyst residues such as nickel and yttrium) (Wick *et al.* 2007). These fractions were then added to the cell cultures at a concentration of 30 $\mu\text{g ml}^{-1}$.

The DEPs (diameter of 15–300 nm with a mode at approx. 60 nm) were collected at a test bench of EMPA (Dübendorf, Switzerland). They were derived from a state-of-the-art heavy-duty engine with six cylinders and a maximum power of 390 kW. The engine lacks any after-treatment systems. This type of engine is typically installed in construction machines. The particle sample was collected at the constant operation mode at 1800 r.p.m. producing a torque of 200 Nm. A small part of the exhaust gas was directed to a dilution tunnel where particle-free air of ambient temperature was added. This aerosol sample diluted by a factor of about 5 was sucked through a glass fibre filter (Pall, T60A20, 70 mm diameter), which retained the particles. The temperature on the filter was approximately 60°C. After conditioning and weighing, the filter was transferred to a sterile 15 ml tube. Sterilized, high-purity water supplemented with 30 mg ml^{-1} Tween-80 was added (2 ml mg^{-1} DEP on the filter). The tube was vortexed, sonicated for 1.5 h and then the filter was removed. To calculate the DEP concentration of the stock solution, a drop of the solution was transferred to a 200-mesh uncoated copper grid and this sample was compared with samples of known DEP concentrations using TEM. The determined concentration of the stock solution was 250 $\mu\text{g ml}^{-1}$. The DEP suspension was added to the cell cultures at a concentration of 125 $\mu\text{g ml}^{-1}$.

TiO₂ NPs (titanium (IV) oxide, anatase; Alfa Aesar, Johnson Matthey GmbH, Karlsruhe, Germany; diameter of 20–30 nm) were suspended in sterilized, high-purity water at a concentration of 2.5 mg ml^{-1} TiO₂ and vortexed. TiO₂ NPs were added to the cell cultures at a concentration of 2.5 $\mu\text{g ml}^{-1}$ diluted with serum-free medium.

Before adding the suspensions to the cell cultures, they were sonicated for 2 min and heated to 37°C. For all cultures, the medium in the well or in the upper chamber was removed and 1 ml of the diluted particle suspension was added.

2.5. Transmission electron microscopy and energy-filtered transmission electron microscopy

TEM analysis was performed as described by Rothen-Rutishauser *et al.* (2006). Briefly, cells were fixed with 2.5 per cent glutaraldehyde in 0.03 M potassium phosphate buffer, pH 7.4. The cells were post-fixed with 1 per cent osmium tetroxide in 0.1 M sodium cacodylate buffer and with 0.5 per cent uranyl acetate in 0.05 M maleate buffer. Cells were then dehydrated in a graded series of ethanol and embedded in Epon.

Ultra-thin (less than or equal to 80 nm) sections were cut, transferred onto 200-mesh uncoated copper grids, stained with uranyl acetate, counter-stained with lead citrate and observed with a Philips 300 TEM at 60 kV (FEI Company Philips Electron Optics, Zurich, Switzerland).

For energy-filtered TEM (EFTEM) analysis, the ultra-thin sections were mounted onto 600-mesh uncoated copper grids and observed unstained with a LEO 912 transmission electron microscope (Zeiss, Oberkochen, Germany) using electron energy loss spectroscopy.

2.6. Reactive oxygen species detection

To detect the reactive oxygen species (ROS) in the cells, an ROS detection kit was used (Image-iT LIVE Green Reactive Oxygen Species Detection Kit, Molecular Probes, Invitrogen AG). The kit was applied as described in the manufacturer's protocol. Briefly, intracellular, unspecific ROS were marked with carboxy- H_2DCFDA solution and the cell nuclei with Hoechst 33342 solution. After washing the cells with HBSS (Gibco BRL, Invitrogen AG), the cells were fixed. Medium alone was used as a negative control and with tert-butyl hydroperoxide (TBHP, at a concentration of 100 μM) as a positive control, incubated for the same duration as the samples.

After ROS and cell nuclei labelling had been performed, the cell cultures were fixed by incubating them for 15 min at room temperature in paraformaldehyde (3% in phosphate-buffered saline (PBS; 10 mM, pH 7.4, 130 mM NaCl, Na_2HPO_4 , KH_2PO_4)). The paraformaldehyde was removed and PBS was added.

The cells were finally mounted in PBS : glycerol (2 : 1) containing 170 mg ml⁻¹ Mowiol 4-88 (Calbiochem, VWR International AG, Life Sciences, Dietikon, Switzerland) on object holders covered with cover slips (Rothen-Rutishauser *et al.* 2005).

The cell culture samples were imaged using a Leitz DMDR fluorescence microscope (Leica Microsystems (Schweiz) AG, Glattbrugg, Switzerland) with an Olympus Digital Camera (C-3000 Zoom, with the objective C3030-ADU). The ROS signal was taken with a standard fluorescein isothiocyanate (FITC) filter. The Hoechst signal of the cell nuclei was imaged with a 4', 6-diamidino-2-phenylindole (DAPI) filter.

The imaging and the processing were done with analySIS software (Olympus Soft Imaging Solutions GmbH, Münster, Germany) and included the overlapping of the single photos taken with the FITC filter and the DAPI filter.

2.7. Total antioxidant capacity

The total antioxidant capacity (TAC) (including vitamins, proteins, lipids, glutathione and uric acid) was detected with the Antioxidant Assay Kit (Cayman Chemical, Chemie Brunschwig AG, Basel, Switzerland) as described in the manufacturer's protocol with some small adaptations. Briefly, the cells were washed three times with PBS, scraped off from the inserts and transferred to Eppendorf tubes. After centrifugation

(Eppendorf Minispin Plus, 3000 r.p.m., 10 min, 6°C), the supernatant was removed, 100 μl cold buffer was added and the cell solution was stored on ice. Afterwards, the samples were vortexed for a short time and sonicated for 2 min in ice water. Then the samples were centrifuged for a second time at 12 200 r.p.m. at 6°C for 15 min. The supernatant was finally transferred to another Eppendorf tube and the samples were frozen at -70°C.

The test followed the manufacturer's instructions. In brief, the reagents and the standard solutions were prepared. The prepared kit solutions, metmyoglobin and chromogen, were added to each well of the 96-well plate. As standard solutions, different concentrations of Trolox (a water-soluble tocopherol analogue) were used. The samples were added to the wells. Each sample and standard was measured as a duplicate. At the end, hydrogen peroxide was added as fast as possible with a multichannel pipette. The plate was covered and incubated on a shaker at room temperature. Exactly 5 min after the addition of hydrogen peroxide, the measurement in a microplate reader (Benchmark Plus Microplate Spectrophotometer; BioRad, Hemel Hempstead, UK) was initiated.

The resulting TACs are indicated in Trolox equivalents. High values of TAC represented upregulated antioxidative defence systems, indicating an induction of oxidative stress. The test was repeated twice for each sample.

2.8. Cytokine/chemokine detection

Following particle incubation, the supernatants of the cell cultures out of the two-chamber slides (MDM and MDDC monocultures) or of the upper and lower chamber (A549 monocultures and triple cell co-cultures) were collected separately and stored at -70°C. After centrifugation, the cytokine tumour necrosis factor (TNF)- α and the chemokine IL-8 concentration were quantified by a commercially available DuoSet ELISA Development kit (R&D Systems, catalogue number: DY 210, respectively, DY 208, Oxon, UK) performed according to the manufacturer's recommendations. The assay was repeated twice, each in duplicate. An aliquot of 100 μl of the diluted capture antibody (mouse anti-human TNF- α /IL-8, concentration of 4 μg ml⁻¹ PBS) was incubated overnight in a 96-well immunoassay plate (NUNC, MaxiSorp) at room temperature. Differing from the producer's protocol, the plate was blocked with PBS supplemented with 1 per cent bovine serum albumin (BSA), 5 per cent sucrose and 0.05 per cent NaN_3 for 1 h at room temperature. After washing with buffer, supernatants from samples and the standards (0–10 ng ml⁻¹ recombinant human TNF- α and 0–2 ng ml⁻¹ recombinant human IL-8) were pipetted into the wells and incubated at room temperature for 2 h. After washing, the detection antibody (biotinylated goat anti-human TNF- α /IL-8) diluted in reagent diluent was added. The plate was covered with an adhesive strip and incubated again for 2 h. The anew washing was followed by the addition of horseradish peroxidase-conjugated streptavidin to the plates and incubation for 20 min at room temperature in the dark. Finally,

Figure 1. EFTEM analysis of yttrium as a constituent of the CNT pellet catalyst in an epithelial cell. (a) Overview of one A549 epithelial cell in the triple cell co-cultures with intracellular CNT pellet. (b) EFTEM detection of yttrium (red points).

the substrate solution (tetramethylbenzidine/ H_2O_2 ; R&D Systems, catalogue number: DY 999) was added. After 20 min in darkness, the colour development was stopped by adding 1 M H_2SO_4 and the plate was put on the shaker (differing from the protocol) for 10 min. The absorbance was then read at 450 nm using an ELISA reader (SpectraMax 340 PC or Benchmark Plus Microplate Spectrophotometer). The concentration of the cytokine or chemokine was determined by comparing the absorbance of the samples with standard samples.

2.9. Calculation of the expected triple cell co-culture values

To calculate the expected values for the triple cell co-cultures, the amount of TAC, TNF- α and IL-8 per cell in each type of monoculture was determined (observed values). These values were multiplied by the number of each cell type in the triple cell co-culture model (Blank *et al.* 2007) and summarized to result in expected values.

2.10. Statistical analysis

The data are expressed as mean values with the standard deviation from at least three independent experiments with at least two internal replicates, with each in duplicate. The statistical analysis was performed using Excel for Windows and SigmaStat for Windows (v. 3.10, SIGMASTAT Software, Inc.). Different groups were compared using the ANOVA on ranks test followed by Dunn's test in case of significance. p -values less than 0.05 were considered to be statistically significant.

Owing to the variability of cytokine/chemokine and oxidative stress production, the results of the single cultures are presented as percentages of the concentrations of the unexposed control. In order to evaluate the interplay of the different cell types in the real triple cell co-culture, the cytokine and oxidative stress values of the triple cell co-cultures were compared with an expected toxicity value gained from the results of the monocultures. The expected and observed values were compared using the rank-sum test.

3. RESULTS

3.1. Particle penetration into cells

When particles are added to cell cultures as a suspension, it is important to show whether particles are inside the cells or whether they are attached to the cell surface. Therefore, the cells were analysed after particle exposure by EFTEM or conventional TEM. Titanium from the TiO_2 NP as well as yttrium, a residual of the SWCNT, can be identified by EFTEM. TiO_2 NPs were found in all cell types of the triple cell co-cultures (data not shown; see also Rothen-Rutishauser *et al.* (2007a)). Yttrium of the CNT pellet suspension was identified in epithelial cells by EFTEM (figure 1). For EFTEM, the sections were not post-treated with uranyl acetate; therefore, the contrast of the cells was not as strong as in conventional TEM. By applying this method, CNT could be identified in the cytoplasm, not membrane bound. Additionally, bundles of the CNT pellet were found in all three cell types of the triple cell co-cultures by conventional TEM (figure 2). DEPs were found by TEM as agglomerates in all cell types of the triple cell co-cultures (figure 3). By conventional TEM, it was not possible to detect single SWCNTs or DEPs. All three particle types were also found in the cells of monocultures (data not shown).

3.2. Qualitative analysis of reactive oxygen species production

After it had been shown that DEPs and the two manufactured NPs penetrated the cells, the potential of these particles to induce oxidative stress in the mono- and triple cell co-cultures was compared. ROS production was induced in all cell culture types after exposure to TBHP, a substance that is known to induce oxidative stress at the concentration used. In epithelial cells and MDM monocultures, ROS production was induced by all particle types (figure 4a,b). MDCC monocultures showed almost no production of ROS (figure 4c). In the triple cell co-cultures, the majority of the cells were ROS positive after exposure to all particle types (figure 4d). As far as the particle type is concerned, it can be concluded that CNT pellet and DEPs induced most ROS, followed by TiO_2 NPs, while CNT supernatant produced the least.

Figure 2. TEM pictures of the triple cell co-cultures with intracellular CNT pellet. Overview of the cell (left) and details of the part in white frame (right). (a) A549 epithelial cell, (b) MDM and (c) MDDC.

3.3. Quantitative analysis of oxidative stress and inflammation reactions

For quantitative analysis of cellular responses, the TAC and the release of $\text{TNF-}\alpha$ as well as IL-8 were determined.

3.3.1. A549 monocultures. In the A549 cultures, a TAC level of 0.22 ± 0.07 mM Trolox was detected in the negative control; this value was then taken as 100 per cent. The IL-8 concentration in the control cultures was 16.38 ± 5.9 ng ml⁻¹ (100%). Compared with the controls, the A549 monocultures exposed to CNT pellet and DEPs showed an increased TAC value of

124 per cent (s.d. 56%) and 130 per cent (s.d. 51%), respectively, and a moderate increase in IL-8 release of 106 per cent (s.d. 11%) and 110 per cent (s.d. 11%), respectively (figure 5a). In the supernatant of A549 monocultures, no $\text{TNF-}\alpha$ could be measured in particle-exposed cultures or in the negative or positive control (data not shown).

3.3.2. Monocyte-derived macrophage monocultures. In the MDM control cultures, the TAC level was 0.30 ± 0.09 mM Trolox (100%) and the concentration of IL-8 was 33.10 ± 4.9 ng ml⁻¹ (100%). Again no $\text{TNF-}\alpha$

Figure 3. TEM pictures of the triple cell co-cultures with intracellular DEPs. Overview of the cell (left) and details of the part in white frame (right). (a) A549 epithelial cell, (b) MDM and (c) MDDC.

could be detected in any of the cultures (data not shown). The TAC of MDMs exposed to TBHP (75%, s.d. 28%) and TiO_2 (82%, s.d. 31%) was reduced and the TAC of MDMs exposed to DEPs was higher than the negative control (119%, s.d. 44%) (figure 5b).

3.3.3. Monocyte-derived dendritic cell monocultures. A level for TAC of 0.24 ± 0.06 Mm Trolox (100%), a $\text{TNF-}\alpha$ concentration of $0.95 \pm 0.11 \text{ ng ml}^{-1}$ (100%) and a concentration of $21.82 \pm 9.09 \text{ ng ml}^{-1}$ IL-8 (100%) were measured in the control cultures. The differences induced by the incubation with particles were not statistically significant and were inconsistent (figure 5c). TBHP-exposed MDDC monocultures

showed increased TAC (115%, s.d. 35%) and $\text{TNF-}\alpha$ levels (143%, s.d. 38%), but no enhancement of IL-8. Exposure to TiO_2 induced an increase in TAC (152%, s.d. 23%), $\text{TNF-}\alpha$ (124%, s.d. 1.5%) and IL-8 levels (106%, s.d. 9.5%), but the latter only weakly. CNT supernatant increased the TAC level (118%, s.d. 31%) and decreased $\text{TNF-}\alpha$ (84%, s.d. 51%) as well as IL-8 levels (75%, s.d. 40%). CNT pellet induced a small increase for all three parameters, such as 113 per cent (s.d. 32%) for TAC, 109 per cent (s.d. 17%) for $\text{TNF-}\alpha$ and 106 per cent (s.d. 13%) for IL-8.

3.3.4. Triple cell co-cultures. In the triple cell co-cultures, the control reached a TAC level of

Figure 4. ROS production in cell cultures exposed to different nanosized particles for 24 h. Blue staining shows the cell nuclei and green staining shows the ROS. All signals were compared with the negative control (medium) and the positive control (TBHP). (a) A549 epithelial cells. TiO_2 , DEP, CNT supernatant and CNT pellet induce a high production of ROS. (b) MDM. TiO_2 , DEP, CNT supernatant and CNT pellet induce a high production of ROS. (c) MDDC. DEP and CNT supernatant induce a high production of ROS. (d) Triple cell co-cultures. TiO_2 , DEP, CNT supernatant and CNT pellet-exposed cells show a higher production of ROS.

0.22 ± 0.09 mM Trolox (100%), a TNF- α concentration of 0.73 ± 0.55 ng ml $^{-1}$ (100%) and an IL-8 concentration of 27.85 ± 3.84 ng ml $^{-1}$ (100%). The particle-exposed cells showed only small and not statistically significant different values compared with the negative control (figure 5d). The TAC levels of TiO_2 -exposed cells were statistically significantly higher than the levels of CNT supernatant and in the DEP-exposed cell cultures (figure 5d).

3.4. Expected versus observed oxidative stress and cytokine/chemokine concentrations in the triple cell co-cultures

As all three cell types from the monocultures are combined in the triple cell co-culture system, it was hypothesized that all the effects observed in the individual cell type cultures could be summarized in a weighted way, and this should result in the values observed in the co-cultures. In order to test this hypothesis, the summarized values of the monocultures (expected

values) were compared with the values of the triple cell co-cultures (observed values).

3.4.1. Reactive oxygen species. As there were only qualitative ROS results, it was not possible to calculate an expected ROS value from the monocultures for the triple cell co-cultures. However, from the fluorescence shown in the micrographs, it seemed that there were no or only small differences between cells in monocultures when compared with cells in triple cell co-cultures.

3.4.2. Total antioxidant capacity. The comparison of expected and observed TAC levels showed statistically significant higher than expected values in cells exposed to medium only, CNT supernatant, CNT pellet and DEPs. For TBHP and TiO_2 exposure, the differences were not statistically significant, but higher (figure 6a).

Figure 5. TAC equivalent, TNF- α and IL-8 concentrations in monocultures and triple cell co-cultures following exposure to different nanosized particles for 24 h. All values are expressed as a percentage of control. (a) A549 epithelial cell monocultures show no statistically significant enhancement of TAC or IL-8 release compared with the negative control and do not produce TNF- α . (b) MDM monocultures produce no TNF- α . For TAC and IL-8, no differences between the negative control and particle exposure are detected. (c) In MDDC monocultures, no statistically significant differences between control and particle exposure could be observed. (d) The levels in triple cell co-cultures were not enhanced by particle exposure; however, the TAC levels of TiO₂-exposed cells were statistically significantly ($P < 0.05$) higher than the levels in CNT supernatant and DEP-exposed cell cultures. Dark grey, TAC; light grey, TNF- α ; unfilled, IL-8.

3.4.3. Tumour necrosis factor- α . All observed TNF- α concentrations in the triple cell co-cultures (observed values) were higher than the expected values calculated from the monocultures, except for the cultures exposed to TiO₂ NPs, although these findings were not statistically significant (figure 6b).

3.4.4. Interleukin-8. The observed concentrations of IL-8 in the triple cell co-cultures were, under all conditions, lower than the expected values estimated from the monocultures, but not statistically significant (figure 6c).

4. DISCUSSION

Many studies investigating the toxicity of particles in lung cells give a good basis for the evaluation of the toxic potential of particles and, in particular, of NPs (Donaldson *et al.* 2005, 2006; Oberdorster *et al.* 2005). In most studies, cell lines or primary cell cultures were used as monocultures (Amakawa *et al.* 2003; Shvedova *et al.* 2003; Cheng *et al.* 2004; Brunner *et al.* 2006; Kagan *et al.* 2006; Mundandhara *et al.* 2006; Limbach *et al.* 2007; Wick *et al.* 2007; Mitschik *et al.* 2008).

However, the histological composition of the lung is not restricted to one single cell type. It is a rather complex network of various cell types (Brain 1988; Holt *et al.* 1990; Lehnert 1992; Nicod 1997). In fact, the lung consists of more than 40 different and highly specialized cell types (Ochs & Weibel 2008). This complexity can never be mimicked by artificial cell cultures, but the use of co-cultures consisting of various cell types realistically mimics the situation in the human lung and gives a more reliable toxicological evaluation than studies with cell monocultures (Roggen *et al.* 2006; Rothen-Rutishauser *et al.* 2008a). In the present work, it was found that all the NPs used including TiO₂, DEP and SWCNT can penetrate into epithelial cells, MDMs and MDDCs (figures 1–3). In addition, the differences of cellular responses between monocultures of epithelial cells, MDMs and MDDCs and triple cell co-cultures composed of these three cell types after exposure to different NPs in suspension were investigated and compared.

4.1. Nanoparticle penetration into cells

The penetration of different NPs into human lung cells *in vivo* and *in vitro* has been shown in many studies

Figure 6. Comparison of the observed versus expected cytokine/chemokine and oxidative stress values in the triple cell co-cultures. The expected co-culture values were calculated by summing up the amounts produced in the monocultures, adjusted for cell number. The expected TAC (a) and IL-8 (c) values were higher than the observed values. The observed TNF- α (b) concentrations are higher for all conditions, but not for TiO₂ NPs. Unfilled bar, expected; filled bar, observed.

(Shvedova *et al.* 2003; Geiser *et al.* 2005; Limbach *et al.* 2005; Wick *et al.* 2007). However, the penetration mechanisms are still not known (Rothen-Rutishauser *et al.* 2007b). It is not only endocytic pathways, which all include vesicle formation, that are discussed to account for the translocation of NPs. In many *in vitro* and *in vivo* studies, NPs were found to be non-membrane bound (Kapp *et al.* 2004; Geiser *et al.* 2005; Rothen-Rutishauser *et al.* 2007a). This supports the theory of

NPs to enter cells by a non-endocytic mechanism that is supported by a study performed with human red blood cells (Rothen-Rutishauser *et al.* 2006).

The current study found TiO₂ NPs in all cell types of the monocultures and in the triple cell co-cultures, which is in agreement with previously published results (Stearns *et al.* 2001; Rothen-Rutishauser *et al.* 2007a). The titanium of the TiO₂ NPs has been identified in all three cell types by EFTEM, and the particles have

been detected as agglomerates, which were membrane bound or as small aggregates free in the cytoplasm (Rothen-Rutishauser *et al.* 2007a). TiO₂ NPs were also detected by EFTEM in A549 monocultures in another study (Stearns *et al.* 2001).

The identification of SWCNTs in cells is difficult as the tubes are built from a graphene layer that cannot be distinguished from the cellular structures that also consist of carbon. There are studies showing intracellular CNTs by conventional TEM; however, in these studies, only larger aggregates of SWCNTs were detected (Shvedova *et al.* 2003; Worle-Knirsch *et al.* 2006; Pulskamp *et al.* 2007). In the present work, yttrium, a residual of the SWCNTs, was identified—to our current knowledge—for the first time by EFTEM inside the cells (figure 1). Using this method, single SWCNTs or small aggregates could be shown to be free within the cytoplasm. In another study (Davoren *et al.* 2007), intracellular SWCNTs in A549 cells by conventional TEM could not be found, which might be explained by the fact that single SWCNTs or small aggregates cannot be identified by conventional TEM methods.

DEPs were found in every cell type of the triple cell co-cultures, but only as aggregates in vesicles and not as single particles (figure 3). It is not possible to state that there are no single particles inside the cells, because they might not be detectable. The uptake of DEPs by human epithelial cells by formation of vesicles was previously shown in other studies (Boland *et al.* 1999), but, to our knowledge, never in A549 cells. In other studies, the uptake of DEPs by a human fibroblast-mutant Chinese hamster ovary hybrid cell (Bao *et al.* 2007), the internalization of NPs and fine particles in alveolar macrophages (Beck-Speier *et al.* 2005) and in primary cultures of human bronchial epithelial cells (Reibman *et al.* 2002) were presented. As it is not possible to verify DEPs inside cells by elemental analysis (EFTEM), it may not be possible to detect single particles in cells as was done by other researchers using conventional TEM (Bao *et al.* 2007).

4.2. Oxidative stress: reactive oxygen species production and total antioxidant capacity

In both assays, TBHP, a potent inducer of oxidative stress, was used as a positive control. ROS formation was observed, but the TAC levels were surprisingly low. Since the dose was used according to the company's manual, it might be that the activity in the cell culture medium decreased. Dringen *et al.* (1998) measured a half-time of 24 min for TBHP at 37°C in a similar culture medium to the one currently used. In all cultures, it was observed that exposure to DEPs, TiO₂ NPs and CNT pellet as well as to CNT supernatant induced the production of ROS (figure 4). This was in accordance with other studies for DEPs (Jacobsen *et al.* 2008a), TiO₂ (Long *et al.* 2006; Rothen-Rutishauser *et al.* 2008b) and CNTs (Pulskamp *et al.* 2007; Jacobsen *et al.* 2008b). CNT supernatant induced less ROS-positive cells than the other particle types. This is in agreement with other studies that showed that CNT supernatant suspension is less toxic than

CNT pellet in a mesothelioma cell line MSTO-211H (Wick *et al.* 2007) and in RAW 264.7 macrophages (Kagan *et al.* 2006). MDDCs in monocultures seem to react less to all NPs used in the present study than MDMs or epithelial cells. Instead, there is an observable tendency for an upregulation of the TAC. This may suggest that MDDCs are better at adapting their defence systems against ROS production than the other cell types. MDDCs play an important role during the immune response against antigens, and often ROS are involved in a non-pathogenic but physiological way during cellular signalling (Matsue *et al.* 2003). This could be a reason for an optimized defence system against environmental stressors and thus a lower production of ROS and a higher level of TAC in MDDCs when compared with the other cell types.

The triple cell co-cultures showed ROS production at a similar level to the monocultures of epithelial cells, and there is no evidence for a synergistic or alleviative effect by the interplay of the different cell types. Only for the triple cell co-cultures treated with CNT pellet there appears to be more ROS-positive cells than in the monocultures. However, for the ROS production, it was not possible to calculate an expected value owing to the qualitative form of the analysis. When comparing the expected and observed levels of TAC in the triple cell co-cultures, higher expected values than observed values are seen under all exposure conditions (figure 6). The cellular interplay of the different cell types seems to help the cells dealing with oxidative stressors.

4.3. Inflammation reaction: release of tumour necrosis factor- α and interleukin-8

In the current experiments, a higher release of TNF- α and IL-8 into the supernatants owing to particle exposure was not observed in all cultures (figure 5). The differences between control and exposed cells were small and inconsistent. Various studies have also shown that CNTs, TiO₂ or DEPs do not enhance the release of TNF- α and IL-8 (Tao & Kobzik 2002; Lindbom *et al.* 2006; Pulskamp *et al.* 2007; Veranth *et al.* 2007; Rothen-Rutishauser *et al.* 2008b). However, there are also contradictory studies that found inflammatory reactions induced by DEPs and TiO₂ particles in epithelial cells, macrophages and dendritic cells (Boland *et al.* 1999; Tao & Kobzik 2002; Lindbom *et al.* 2006; Porter *et al.* 2007; Veranth *et al.* 2007). Finally, it has to be considered that cell cultures, DEP compositions and collection methods as well as the suspension preparations of the other particles used in different studies might vary, and therefore a direct comparison can be difficult.

No detectable increase in TNF- α and IL-8 concentrations could be a consequence of cytokine or chemokine binding by particles, as described in a previous study (Kocbach *et al.* 2008). However, there is no indication for a binding of cytokines to the applied particles, as the present work has used three completely different particle types and the fact that all three NP types bind to the chemokines/cytokines to the same extent seems to be unrealistic.

When comparing the expected and observed TNF- α concentrations in the triple cell co-cultures, higher values in the triple cell co-cultures than expected from the calculations based on the monoculture exposures (figure 6) were observed. As only TNF- α was measured in the monocultures of MDDCs and not in MDMs or epithelial cells, it is assumed that, in the triple cell co-cultures, either the MDDCs stimulate the MDMs or the epithelial cells to produce TNF- α , or the interplay of the cell activates the MDDCs to release more TNF- α . It is thought that, for this modulation, MDDCs play the key role. Since in a previous study with co-cultures of A549 epithelial cells, macrophages (THP-1 cell line) and mast cells (HMC-1 cell line), but without dendritic cells, the opposite observation was seen, namely that the TNF- α concentrations were higher for the expected values than for the observed values (Alfaro-Moreno *et al.* 2008).

Regarding the IL-8 concentrations, the opposite effect was seen. The expected concentrations were higher than the observed concentrations for all conditions, but not to a statistically significant extent. This result is again in contradiction to the results of a previous co-culture study by Alfaro-Moreno *et al.* (2008), who found higher observed IL-8 concentrations than expected. The current result was not expected because it is known that the pro-inflammatory chemokine TNF- α stimulates other cells to release IL-8 (DeForge *et al.* 1992; Nukada *et al.* 2008). Therefore, higher observed values, compared with the expected values calculated from the values of the monocultures, were expected.

4.4. The potential of the three-dimensional epithelial airway model to mimic the interplay of various cell types

Based on the current findings, it is hypothesized that there is a synergistic effect between the different cell types (epithelial cells, MDMs and MDDCs), owing to the interaction of the three cell types that modulate the TAC levels as well as the release of cytokines and chemokines, as was observed in the present study. There is a need for the inclusion of different cell types in cell culture models. Co-culture models are better at simulating the real situation in the lung than monocultures. This is particularly important for toxicological studies including oxidative stress and inflammatory reactions in lung cell culture models upon NP exposure.

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