A 4D lung multi-culture system mimicking alveolar cellular organization to study the toxic potential of airborne particles

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Introduction

Exposure to small ambient particles like particulate matter (PM) has remained constant in last years in many areas of the world and the production and commercialization of engineered nanoparticles may further increase human exposure to particles lading to increased risk for respiratory diseases.

We developed a complex multicellular system that is composed of four cell types alveolar type-II cell line (A549), differentiated macrophage-like cells (THP-1), mast cells (HMC-1) and endothelial cells (EA.hy 926), seeded in a 3D orientation on microporous membranes.

The system resembles most of the *in vivo* conditions of the alveolar region, including the production of surfactant, which allows the cells to grow and survive for several days at the air–liquid-interface. The system is designed and optimized for coupling to a native aerosol exposure system (Vitrocell™ chamber). The complete system is able to react in different ways to oxidative stress, when compared to single or two-cell-types coculture, meaning that there is cross-talk and interaction between the cells.

The use of this tetraculture system will lead to a more realistic risk assessment of new ENPs.

Material and Methods

- The spatial distribution of the cells in the tetraculture was analysed by confocal laser scanning microscopy (CLSM)
- Expression of target genes was evaluated by qRT-PCR followed by statistical analysis
- To evaluate the response to oxidative stress, the DCFH-DA assay was used together with AAPH as inducer of oxidative stress
- To evaluate cell viability, we used the Alamar Blue assay in an adapted version for the transwell inserts
- The tetraculture was exposed to an aerosol of 50 nm SiO_2-Rhodamine NPs in PBS using the Vitrocell™ system
- The distribution of the NPs in the tetraculture after exposure was evaluated by CLSM (Zeiss LSM 510 META)
- Digital image restoration and evaluation was done using ImageJ and Zen 2011 (Zeiss)

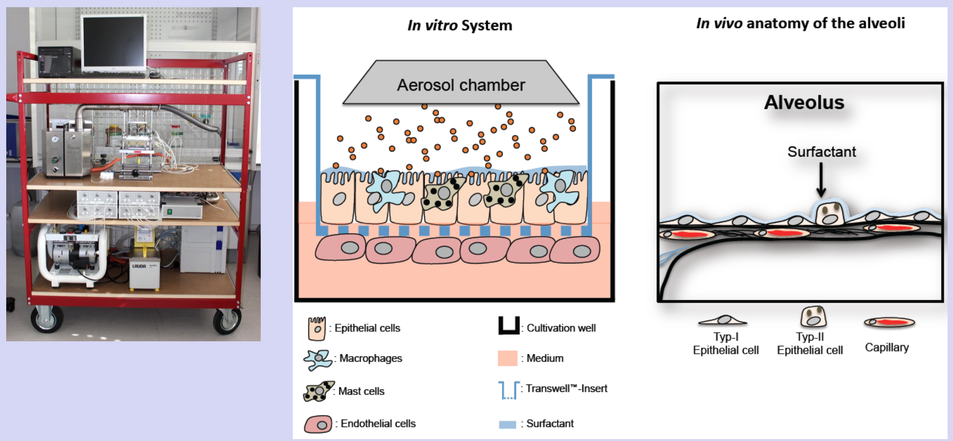

Figure 1: A: Setup to expose cells to particles in suspension or to chemicals**. B:** Organization and composition of the *in vitro* system compared to the *in vivo* anatomy of the alveolar barrier (**C**). The presence of immune cells on the alveolar surface was not considered in the *in vivo* scheme. Adapted and modified from Klein et al., 2011.

References

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Klein, S.G., Hennen, J., Serchi, T., Blömeke, B., Gutleb, A.C., 2011. Potential of coculture *in vitro* models to study inflammatory and sensitizing effects of particles on the lung. Toxicol. *In Vitro* 25, 1516-1534.

Results

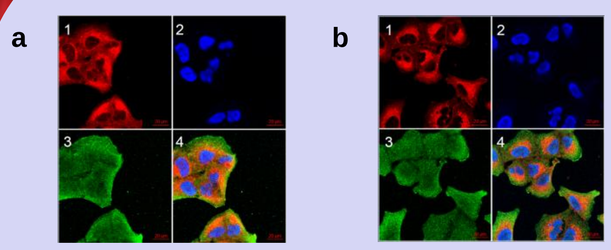

Figure 2: Confocal laser scanning experiments to characterise the qualities of the *in vitro* system.

Immunohistochemistry staining of surfactant protein A (**a**), and C (**b**) in A549 cells. A549 cells were grown in Labtek-II chambers for 48 h. Afterwards, cells were fixed, permeabilized and stained for cellular membranes, nuclei and surfactant protein**. 1:** membranes stained with cell mask deep red. **2:** Nuclei stained with DAPI. **3:** Surfactant protein stained with anti-surfactant-protein antibody (1:200). **4:** Overlay.

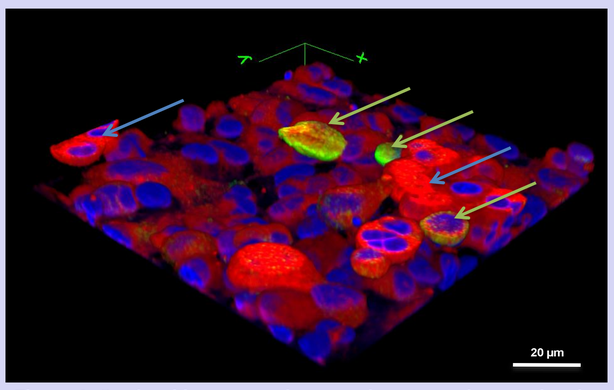

Figure 4: Confocal laser scanning experiments to characterise the qualities of the *in vitro* system

The distribution of A549, differentiated THP-1, HMC-1 and EA.hy 926 cells in the tetraculture was analysed via CLSM. Cellular membranes are stained with cell mask deep red dye (red) and nuclei are stained with DAPI (blue); Macrophage-like cells are counterstained with an anti-CD11b-antibody. 3D reconstruction of the triculture based on the results from a z-stack image series. THP-1 (green arrows) and HMC-1 (blue arrows) cells are found on top of the epithelial cells.

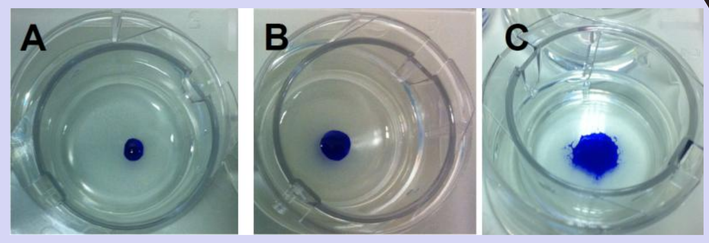

Figure 3: Results for the surfactant droplet test. In figure 2, the production of surfactant proteins in A549 cells was evaluated. By using the surfactant droplet test, the secretion of surfactant can be visualized indirectly as the secretion lowers the surface tension of the epithelial layer.

Briefly, the medium was removed from the apical compartment of the transwell and the cells were cultivated at the air-liquid-interface for 24 h. Controls were kept under submerged conditions.

In order to determine the surface tension, DMP/O droplets were placed on the cell surface. Droplet diameters, d0 and d, were measured before and after deposition. A large diameter indicates a high surface tension, e.g. of cells grown under submerged conditions. A small diameter of the drop indicates a lower surface tension, e.g. of cells grown under ALI conditions with the possibility to secrete surfactant.

A: A549 cell exposed for 24h at the air-liquid-interface; **B:** A549 kept under submerged conditions; **C:** EA.hy 926 cells kept under submerged conditions

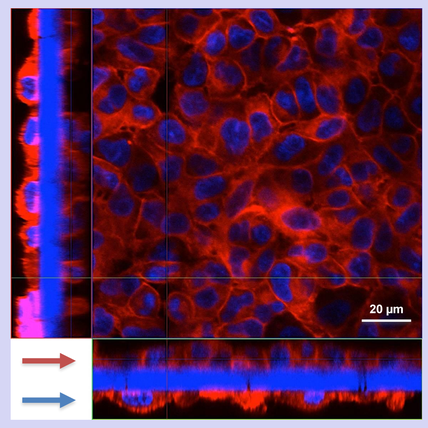

Figure 5: Confocal laser scanning experiments to characterise the qualities of the *in vitro* system.

Confocal laser scanning micrograph of the three-dimensional distribution of A549 (red arrow) and EA.hy 926 (blue arrow) cells above and below the transwell membrane.

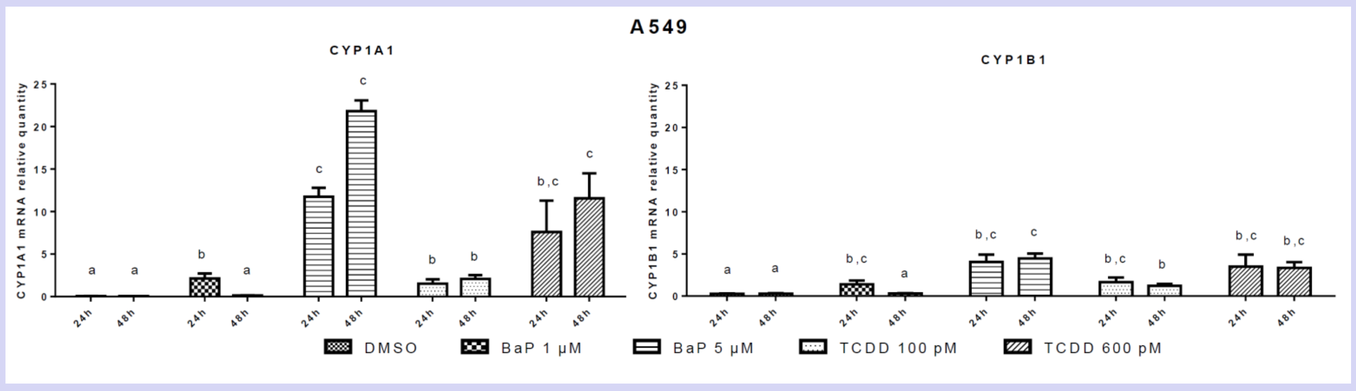

Figure 6: Relative expression of CYP1A1 and CYP1B1 in A549 cells exposed to BaP or TCDD for 24 h and 48 h. DMSO was used as a negative control. Data represents the mean ± S.E.M (n=3). Groups that are sharing the same letters are not significantly different (P > 0.05).

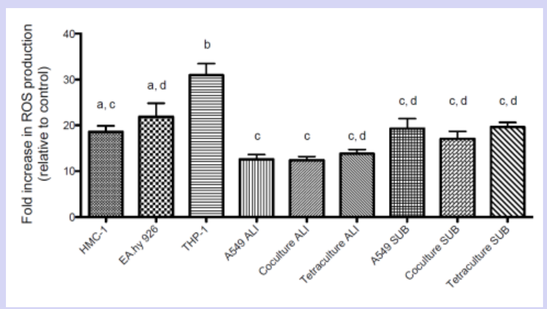

Figure 7: DCFH-DA assay to analyse the behaviour of the different cultures in response to an oxidative stress inducer. Cultures were preloaded with DCFH-DA dye and subsequently exposed to 20 mM AAPH in medium for 2 h. Data represents the mean of four independent transwell inserts ± standard error of mean. Groups that are sharing the same letters are not significantly different (P > 0.05).

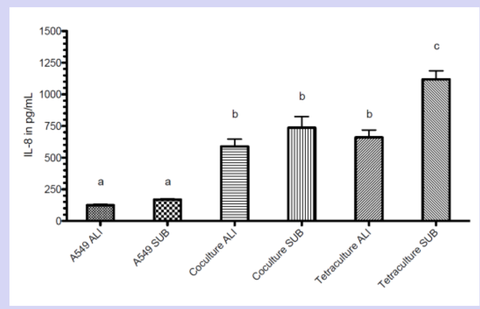

Figure 8: Evaluation of the levels of IL-8 after treatment with AAPH. Cultures were exposed to 20 mM AAPH in medium for 2 h. Afterwards samples of the undernatant were collected and analysed to evaluate the amounts of IL-8. Data represents the mean of at least four independent transwell inserts ± SEM. Groups that are sharing the same letters are not significantly different (P > 0.05).

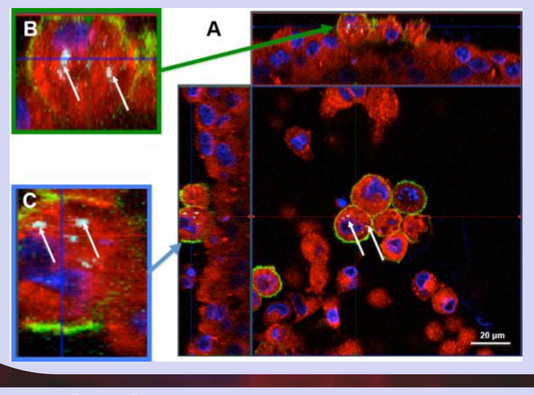

Figure 9: Z-stack image series to analyse the phagocytic activity of THP-1 macrophage-like cells in the triculture present in the apical compartment of the system after exposure to 50 nm SiO_2-Rhodamine nanoparticles with the Vitrocell™ system and submerged exposure.

A: Tetracultures of A549, differentiated THP-1, HMC-1 and EA.hy 926 exposed to an aerosol of 50 nm SiO_2-Rhodamine particles for 30 minutes using the Vitrocell™ aerosol exposure system. Signals of ingested SiO_2-Rhodamine particles were detected in differentiated THP-1 cells (white arrows), but not in A549 or HMC-1 cells. The image shows an x–y projection with the respective side views. Cellular membranes stained with cell mask deep red dye (red) and nuclei with DAPI (blue) are shown. Macrophage-like cells are counterstained with an anti-CD11b-antibody.

B and **C** show a macrophage-like cell in a higher magnification.

Conclusions

- The surfactant layer can be considered as an important source of proteins with which NPs can interact *in vivo.* A549 cells grown under submerged conditions produce surfactant, but the secretion is lower than under ALI conditions (Figure 2 and 3).
- A549 cells show high induction of CYP1A1 expression when incubated with AhR ligans, such as BaP (Figure 6).
- The interplay of model cells for the immune system (THP-1 and HMC-1) with A549 epithelial cells influences the behaviour of the system, resulting in an lower effect for oxidative stress compared to the monocultures (Figure 7).
- Macrophage-like THP-1 cells are efficiently intercepting the SiO_2-Rhodamine NPs (Figure 9).
- The system can be used in conjunction with a native aerosol exposure system and may finally lead to a more realistic judgement about the hazard of new compounds and/or new nano-scaled materials in the future.

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