An alveolar airway *in vitro* co-culture model to determine the potential (pro-)inflammatory impact of engineered nanomaterial exposure

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Rationale and Aims:

Engineered nanomaterials (ENM) pose an inevitable health risk to humans through long-term, repetitive, low-dose exposures. We aim to develop a system that allows prediction of the long-term impact of ENM exposure, achieved by an advanced *in vitro* system using short-term exposures *via* optimising the alveolar lung model with *in vivo* extrapolated dosing strategies, resembling realistic ENM lung exposures, combined with specific biological endpoint measurements.

Characterisation of epithelial layer, addition of macrophage-like cells and implementation of established dosing strategy to enable both single and repeated ENM exposures

Submerged cultures = Sub
Air-Liquid Interface = ALI

Characterisation of A549 cells

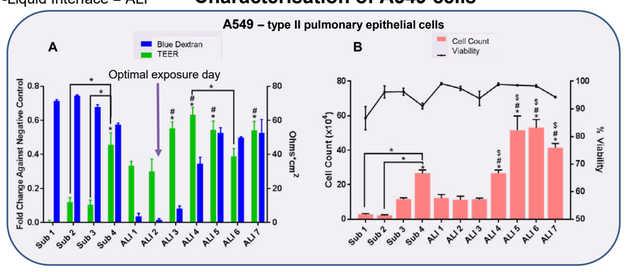

Figure 1. Epithelial cell culture characterisation of A549 cells. Membrane integrity measured by trans-epithelial membrane resistance (TEER) and blue dextran (200kDa – measures the translocation of the blue dye from the apical to the basal side of the membrane in a normal culture method. Higher fold values against the negative control represents lower membrane integrity). **(A)** Membrane integrity of the culture (**B**) Cell count and viability of the cells over the life of the culture. N=6 with all assays performed in triplicate. The data is presented as the mean ±SEM. Significance is denoted as the following: compared to either Sub or ALI day 1 p<0.05(*); compared to Sub or ALI day 2 *p*<0.05(#); and compared to Sub or ALI day 3 *p*<0.05($).

Addition of Macrophages (dTHP-1)

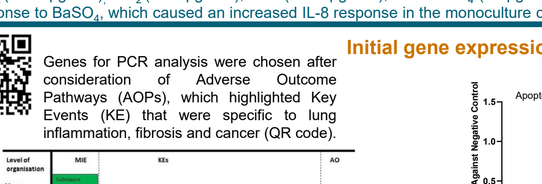

Figure 2. Addition of dTHP-1 macrophages to an epithelial cell culture. Epithelial cells were initially grown on transwell inserts before being switched to an air-liquid interface (ALI). THP-1 cells were differentiated (dTHP-1) using PMA (20nM) for 48 hours ((Risby *et al*., (in prep)) and allowed to recover for 48 hours. dTHP-1 cells were then seeded onto the apical side of the culture and allowed to adhere for 2 hours before switching the whole culture to an ALI.

Particles, exposure time and method

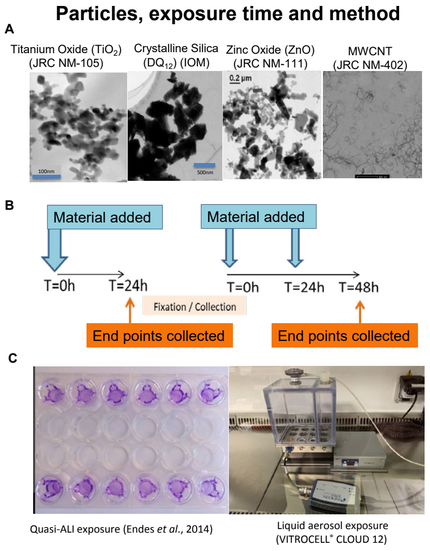

Figure 3. Particles and exposures used. (A) Particles used within this work. Concentrations used were extrapolated from characterisation and historical *in vivo* data (IVIVE method). Images are taken from the JRC data repository. **(B)** Exposure time used for materials determined after initial experimental work to establish the optimum exposure time and duration. Cells within the Quasi-ALI system (Endes *et al*., 2014) were exposed to the material twice over 48 hours (repeated dose) before the endpoints were analysed at 48 hours. Cells within the VitroCell Cloud12 system were exposed and analysed 6, 24 and 72 hours post-exposure. **(C)** Currently work has been completed using both the Quasi-ALI exposure method and the VitroCell Cloud12. Focus will be to the Quasi-ALI with initial Vitrocell exposure data also being demonstrated.

Summary

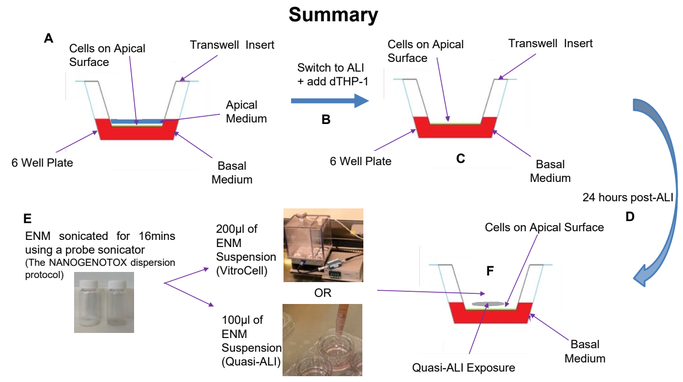

Figure 4. Summary of both Quasi-ALI and VitroCell exposure protocol. (A) A549 cells are grown on the apical side of the membrane for 4 days before **(B)** adding dTHP-1, and allowing them to adhere for two hours. The apical medium is then removed **(C)** and the model switched to an ALI. The model is then allowed to equilibrate for 24 hours **(D)** before the ENM of interest is sonicated following the NANOGENOTOX dispersion protocol **(E)** and 100µl of the ENM solution applied to the apical side of the membrane or for the VitroCell exposures the 100µl exposure is replaced with the aerosol exposure **(F)**.

Summary:

- Characterisation of A549 determined that the cells are grown in submerged conditions (for 4 days) before being switched to ALI, and exposure 24 hours post-ALI.
- THP-1 cells were differentiated (dTHP-1) for 48 hours with PMA (20nM), 24 hours in media and 24 hours at ALI.
- DQ_{12} (0-100µg/cm^2), TiO_2 (0-5.2µg/cm^2), ZnO (0-100µg/cm^2), and $BaSO_4$ (0-6µg/cm^2) were exposed at a Quasi-ALI repeated for 48 hours before analysis.
- TiO_2 ZnO, Carbon Black (CB) (0-31.19µg/cm^2) and MWCNTs (0-31.19µg/cm^2) were then exposed using the VitroCell Cloud12 for and analysed at 6, 24, 72 hours post-exposure.

48 hour repeated exposures using a Quasi-ALI method

A549 Monoculture

A549+dTHP-1 co-culture

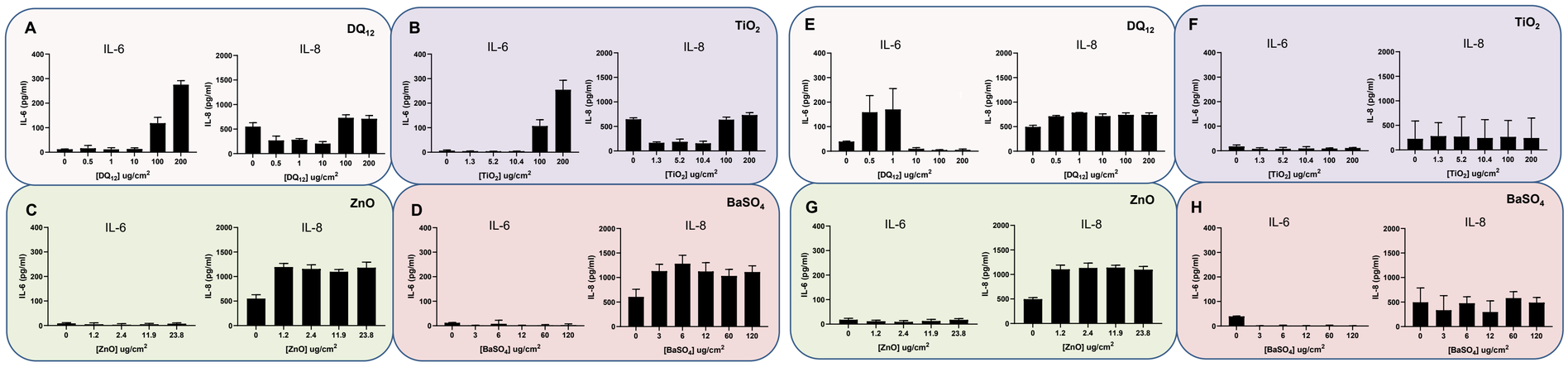

Figure 5. Repeated (over 48 hours) DQ_{12},TiO_2, ZnO and $BaSO_4$ Quasi-ALI exposures on both A549 monocultures and A549+dTHP-1 co-cultures. IL-8 and IL-6 concentrations measured in the basal compartment of the ALI culture after a repeated particle exposure (onto the apical side) to either an A549 monoculture (left hand side) or an A549+dTHP-1 co-culture (right hand side). **(A) and (E)** are measurements after repeated DQ_{12} exposures; **(B) and (F)** are measurements after repeated TiO_2 exposures; **(C) and (G)** are measurements after repeated ZnO exposures; and **(D) and (H)** are measurements after repeated $BaSO_4$ exposures Concentrations were measured after exposure. N=3 with all assays performed in triplicate. The data is presented as the mean ± SD. Significance is denoted as the following: compared to a negative control *p*<0.01(*).

Summary:

- DQ_{12} (0-100µg/cm^2), TiO_2 (0-5.2µg/cm^2), ZnO (0-100µg/cm^2), and $BaSO_4$ (0-6µg/cm^2) were exposed at a Quasi-ALI repeated for 48 hours. These exposures indicated that the co-culture was more sensitive to the effects of the ENM (increase in IL-6 and IL-8), except the response to $BaSO_4$, which caused an increased IL-8 response in the monoculture compared to the co-culture. Within the co-culture models, DQ_{12} elicited the biggest IL-6 response, with ZnO causing the highest IL-8 response when compared to the medium control.

Initial gene expression changes 6, 24 and 72 hours after exposure to TiO_2 (using the VitroCell Cloud12 aerosol exposure)

Genes for PCR analysis were chosen after consideration of Adverse Outcome Pathways (AOPs), which highlighted Key Events (KE) that were specific to lung inflammation, fibrosis and cancer (QR code).

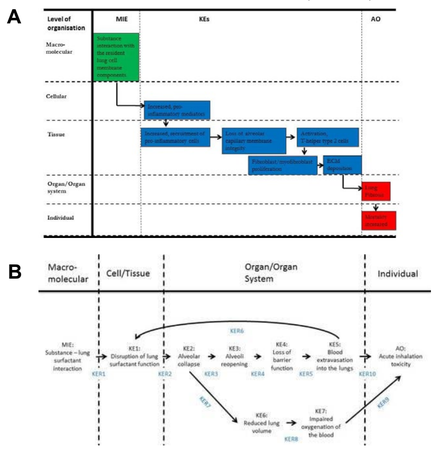

Figure 6. Key Event Pathways (KEPs) used to determine potential target genes for PCR analysis. Specifically AOP 173 **(A)** and 302 **(B)** were identified as important (https://training.aopwiki.org/aops).

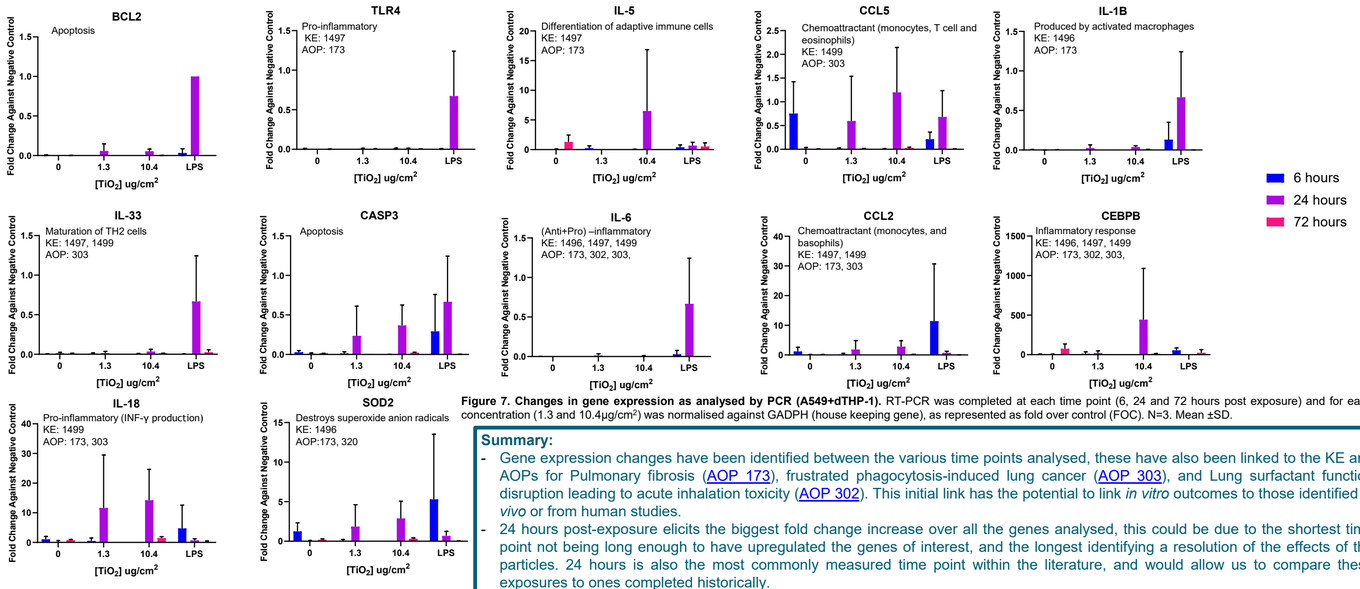

Figure 7. Changes in gene expression as analysed by PCR (A549+dTHP-1). RT-PCR was completed at each time point (6, 24 and 72 hours post exposure) and for each concentration (1.3 and 10.4µg/cm^2) was normalised against GADPH (house keeping gene), as represented as fold over control (FOC). N=3. Mean ±SD.

Summary:

- Gene expression changes have been identified between the various time points analysed, these have also been linked to the KE and AOPs for Pulmonary fibrosis (AOP 173), frustrated phagocytosis-induced lung cancer (AOP 303), and Lung surfactant function disruption leading to acute inhalation toxicity (AOP 302). This initial link has the potential to link *in vitro* outcomes to those identified *in vivo* or from human studies.
- 24 hours post-exposure elicits the biggest fold change increase over all the genes analysed, this could be due to the shortest time point not being long enough to have upregulated the genes of interest, and the longest identifying a resolution of the effects of the particles. 24 hours is also the most commonly measured time point within the literature, and would allow us to compare these exposures to ones completed historically.

Overall Summary

- Characterisation completed indicated that the optimal time to switch the A549+dTHP-1 culture to ALI was 4 days after seeding, with 24 hours at an ALI before any exposures. It was also identified that the co-culture was more sensitive than the monoculture used (*i.e.* there was significant increases in both IL-6 and IL-8 secretion from the co-culture when compared to the monoculture, however no changes in viability or membrane integrity between the two culture systems were observed).
- Single and repeat exposures over 24, 48 and 72 hours indicated that a repeat exposure over 48 hours elicited the biggest (pro-)inflammatory response, indicating that this exposure approach was ideal to elicit a response from this system.
- Using a quasi-ALI approach it was identified than none of the ENM used induced a significant change in cellular viability or (pro-)inflammatory response, although exposure to ZnO, and DQ_{12} did cause an increase in IL-8 secretion. Using an aerosol exposure approach (VitroCell) it was identified that 24 hours post-exposure related to the majority of gene expression changes, although 6 hours and 72 hours post-exposure also indicated changes in expression for specific genes (using the KE could identify potential downstream effects of these exposures).
- Using the end points of 6, 24 and 72 hours for the PCR analysis will allow us to identify potential long term effects exposure to these ENMs on the cellular model when compared to the more commonly used short term exposures (24 hours), and therefore potentially relate this to environmental human exposures and the effects of these ENM.

Future Outlook

- Complete PCR analysis on both TiO_2 and ZnO exposures after aerosol exposures to allow comparison between a soluble and in-soluble ENM using the same exposure method.
- Further comparisons will then be completed at the 24 and 72 hour time points between the quasi-ALI and VitroCell exposure methods for both ZnO and TiO_2 to determine methodological difference between the two systems.

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