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Advanced Tools for NanoSafety Testing

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A dynamic *in vitro* model approach towards deducing the hazard of long-term nanomaterial exposure to the alveolar epithelial barrier

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Introduction:

- Exposure to engineered nanomaterials (ENM) poses an inevitable health risk to both humans and the environment through long-term, repetitive, low-dose exposures.
- The focus of this part of the study was to develop and expose an advanced lung model.
- Current *in vitro* lung models have both advantages and disadvantages (Table 1.)

Table 1. Advantages and disadvantages of current *in vitro* models. Not one model is perfect!

	Heterogeous Cell Population	3D Conformation	Chemical Cues	Mechanical Stimulus	Low Cost	Easy use	Low Equipment/ Facilities
2D plastic cell culture	✓	✗	✗	✗	✓	✓	✓
Inserts	✓	✗	✗	✗	✓	✓	✓
Organoids	✓	✓	✓	✗	✓	✓	✓
Microfluids	✗	✗	✗	✓	✗	✗	✗
Synthetic Scaffolds	✗	✓	✓	✗	✓	✓	✓
Biological Scaffolds	✗	✓	✓	✗	✓	✓	✓
3D Bioprinting	✗	✓	✓	✓	✗	✗	✗

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Current *in vitro* models advantages (✓) and disavantages (✗).

Aims:

We aim to develop systems to allow key long-term studies to be achieved *in vitro,* by optimising lung models and establishing dosing strategies to enable long-term, repeated nanomaterial exposures.

Methods:

Characterise and determine optimal exposure time point for both A549 and NCI-H441 epithelial cells. Additional of macrophages (dTHP-1 (Risby *et al.,* (in prep), THP-1 cells were differentiated using PMA (20nM) for 48hours with a 48hour recovery period) to the A549 cells.

Initial exposure to a standard ENM (DQ_{12}) for single and repeat exposures over a range of time points (24hours-72hours) in both monocultures of A549 and co-cultures of A549+dTHP-1.

- Grow cells at an air-liquid interface (ALI)
- Expose them to standard ENMs that are commercially available (Figure 1.).

Measure:

- Membrane integrity (blue dextran),
- cell counts and viability (trypan blue method)
- IL-8 and IL-6 basal concentrations measured

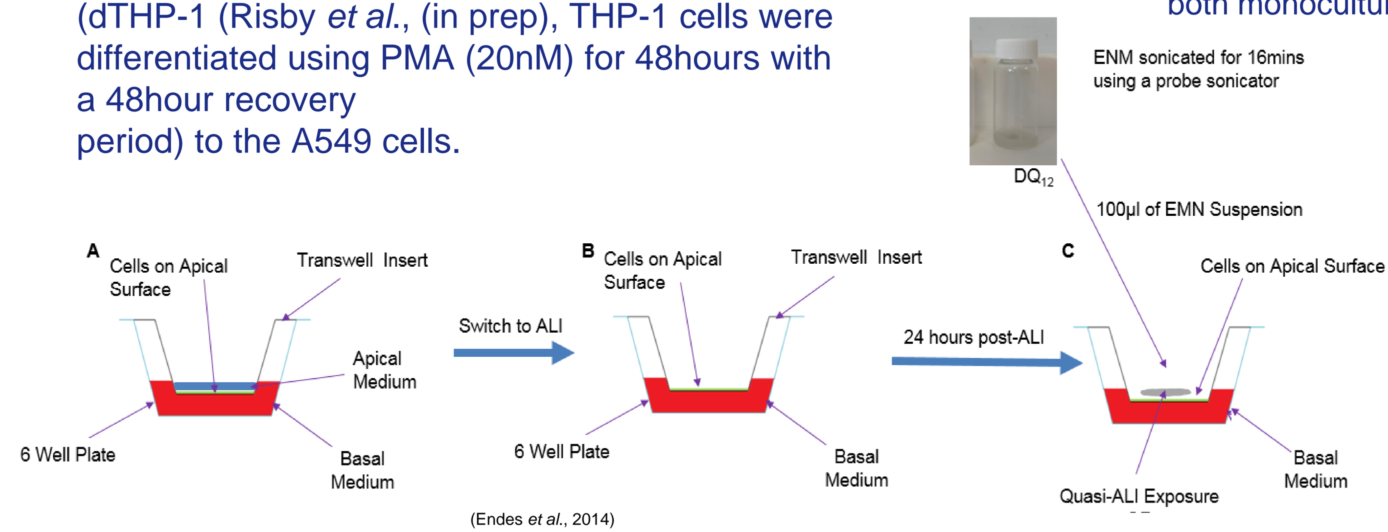


Figure 1. Exposure schematic A. Cells initially grow in submerged conditions. B. Cells witched to an ALI and allowed to equilibrate for 24 hours before C. exposure to the ENM via a quasi-ALI exposure.

Epithelial Cell Culture Characterisation

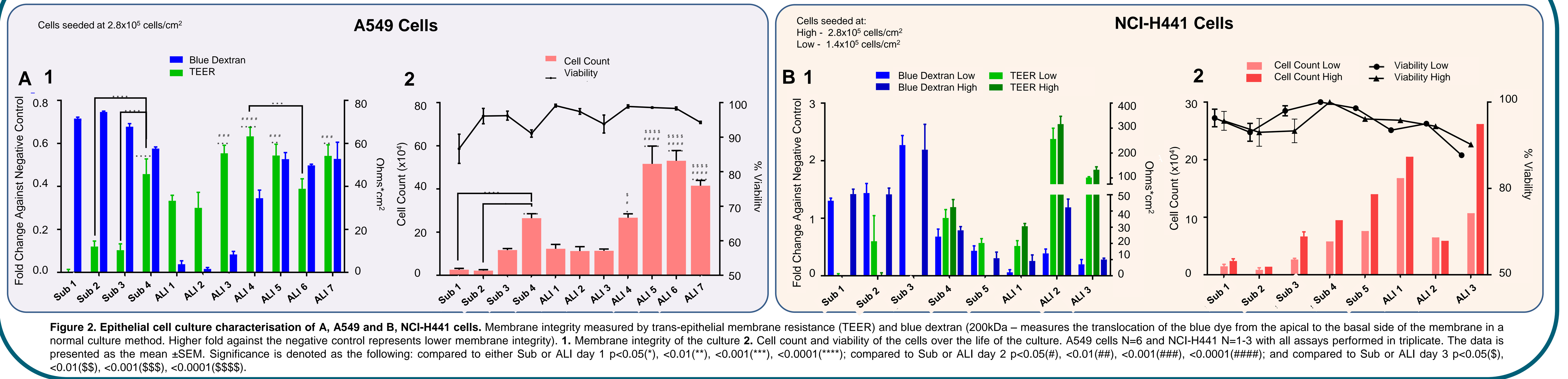
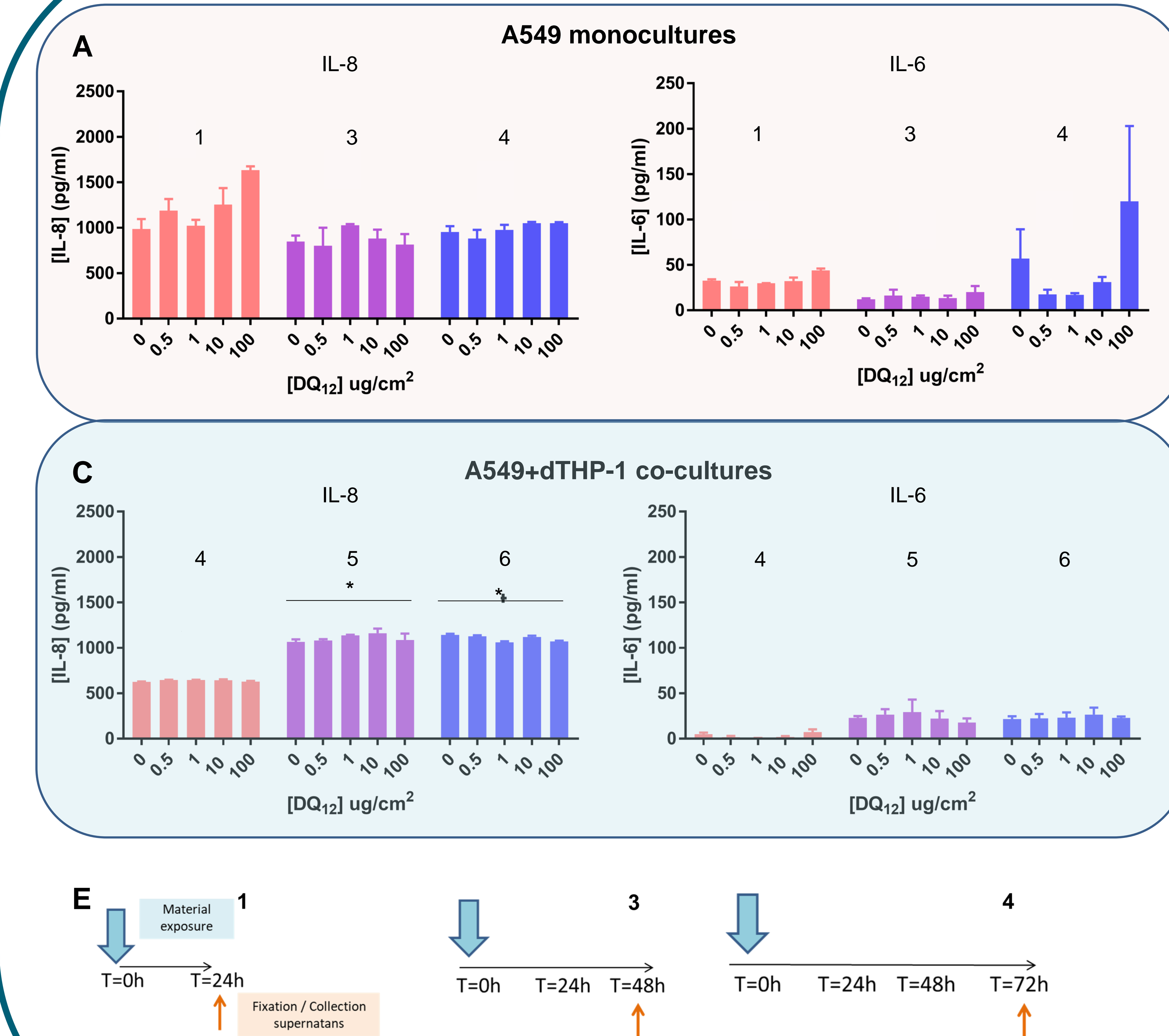


Figure 2. Epithelial cell culture characterisation of A, A549 and B, NCI-H441 cells. Membrane integrity measured by trans-epithelial membrane resistance (TEER) and blue dextran (200kDa – measures the translocation of the blue dye from the apical to the basal side of the membrane in a normal culture method. Higher fold against the negative control represents lower membrane integrity). **1.** Membrane integrity of the culture **2.** Cell count and viability of the cells over the life of the culture. A549 cells N=6 and NCI-H441 N=1-3 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(*), <0.01(**), <0.001(***), <0.0001(****); compared to Sub or ALI day 2 p<0.05(#), <0.01(##), <0.001(###), <0.0001(####); and compared to Sub or ALI day 3 p<0.05($), <0.01($$), <0.001($$$), <0.0001($$$$).

Single DQ_{12} Exposures



Repeat DQ_{12} Exposures

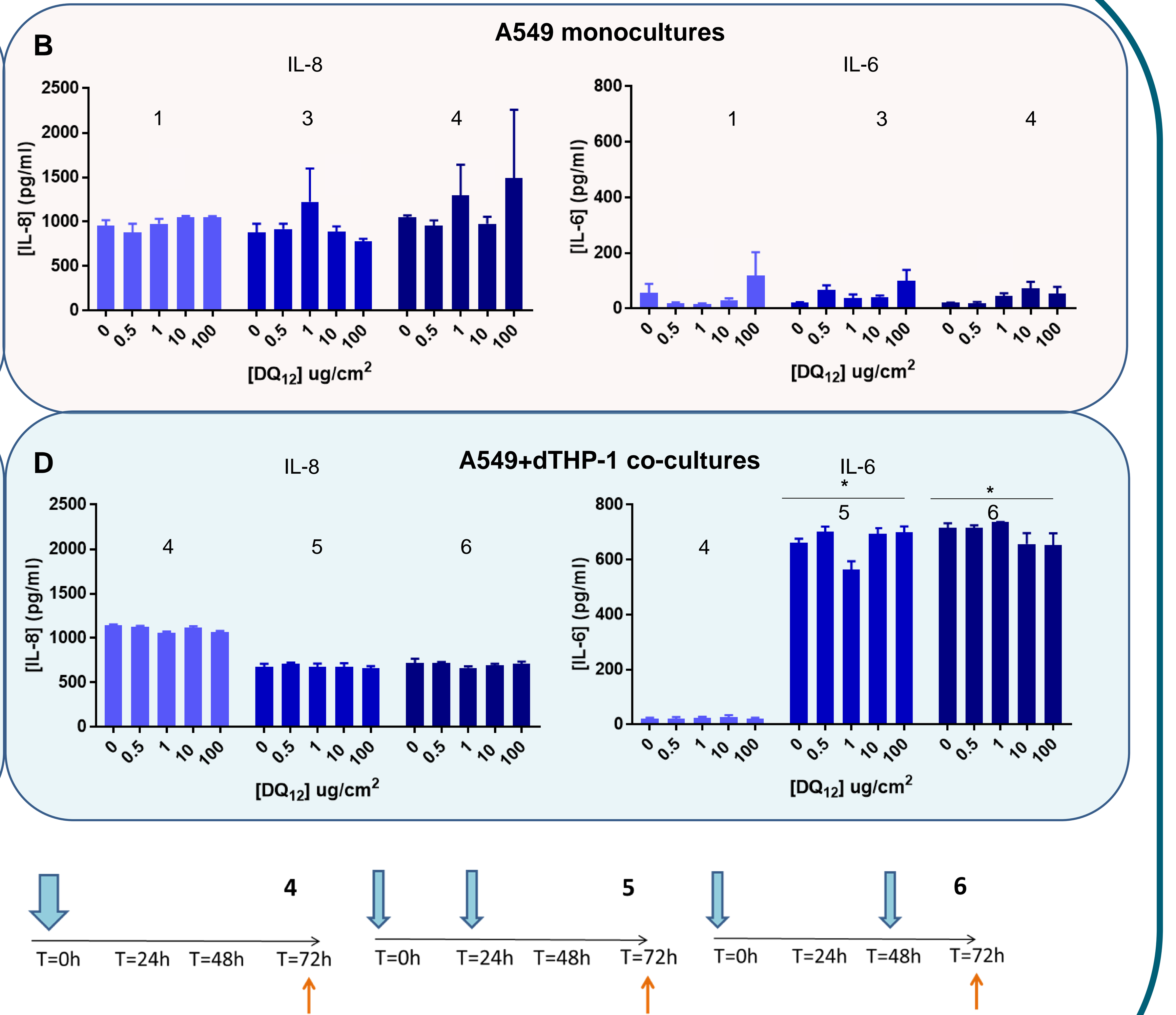


Figure 3. Single and Repeat DQ_{12} exposures in both A549 monocultures and A549+dTHP-1 co-cultures. IL-8 and IL-6 concentrations measured in the basal compartment of the ALI culture after a single DQ_{12} exposure **(A)** to either an A549 monoculture or an A549+dTHP-1 co-culture **(C)** or after repeat DQ_{12} exposures to either an A549 monoculture **(B)** or an A549+dTHP-1 co-culture **(D)**. Concentrations were measured after a pre-determined exposure time (24-72 hours post exposure) indicated by the various exposure scenarios **(E)**. N=3 with all assays performed in triplicate. The data is presented as the mean ±SEM. Significance is denoted as the following: compared to a single exposure p<0.01(*).

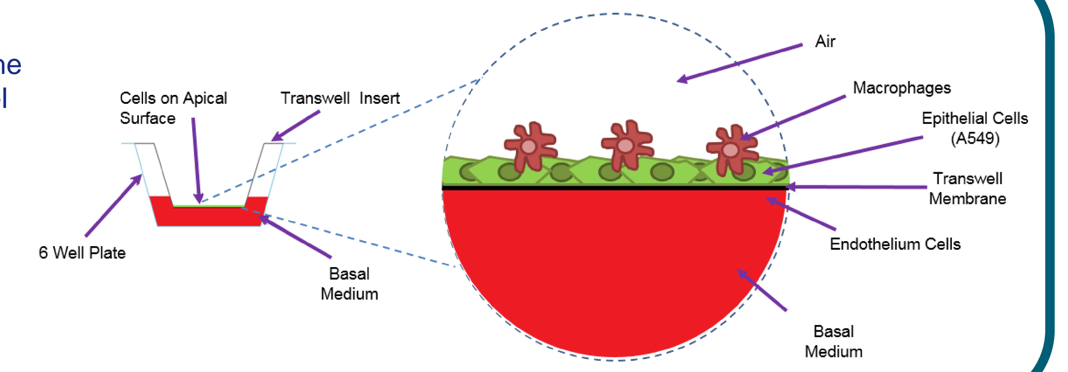
Summary of key findings

- Switching the A549 cells and NCI-H441 cells to ALI after 4 days and 3 days submerged and exposure after 24hours at ALI was found to be the optimal time point.
- Initial characterisation of the models indicates they are most stable at ALI day 2 for exposures, which agrees with the literature.
- There was no change in the cell viability or membrane integrity after any of the exposure scenarios.
- The addition of macrophages (dTHP-1) to the model caused differences in IL-8 and IL-6 concentrations.
- Repeat exposures did not appear to cause any changes in IL-8 and IL-6 concentration in the A549 monoculture, but in the co-culture there was differences in the responses seen when comparing a single exposure to repeated exposures over 72 hours.
- These effects were also shown in NCI-H441 monocultures (data not shown) and NCI-H441+dTHP-1 co-culture exposures are currently being complete in order to complete the comparison.

This indicates that there is the potential for the exposure length and repitition to influence the endpoints of these cell models and should be taken into consideration when evaluating the potential toxicity of EMNs (as the majority of sudies only investigate an acute or chronic efect and not repeaterd exposures).

Further Development of the Model

- Addition of endothelial cells to the basal side of the insert in order to further replicate an *in vivo* model closer
- Exposures using other routes – including dry powder and aerosol exposures
- Addition of flex to the model (to mimic breathing)
- Addition of fluidic flow to the model
- Addition of lung surfactant to the model
- Further development of a type II inflammatory model



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