

## Transferability and reproducibility of exposed air-liquid interface co-culture lung models

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### ABSTRACT

**Background:** The establishment of reliable and robust *in vitro* models for hazard assessment, a prerequisite for moving away from animal testing, requires the evaluation of model transferability and reproducibility. Lung models that can be exposed *via* the air, by means of an air-liquid interface (ALI) are promising *in vitro* models for evaluating the safety of nanomaterials (NMs) after inhalation exposure. We performed an inter-laboratory comparison study to evaluate the transferability and reproducibility of a lung model consisting of the human bronchial cell line Calu-3 as a monoculture and, to increase the physiologic relevance of the model, also as a co-culture with macrophages (either derived from the THP-1 monocyte cell line or from human blood monocytes). The lung model was exposed to NMs using the VITROCELL® Cloud12 system at physiologically relevant dose levels.

**Results:** Overall, the results of the 7 participating laboratories are quite similar. After exposing Calu-3 alone and Calu-3 co-cultures with macrophages, no effects of lipopolysaccharide (LPS), quartz (DQ12) or titanium dioxide (TiO<sub>2</sub>) NM-105 particles on the cell viability and barrier integrity were detected. LPS exposure induced moderate cytokine release in the Calu-3 monoculture, albeit not statistically significant in most labs. In the co-culture models, most laboratories showed that LPS can significantly induce cytokine release (IL-6, IL-8 and TNF- $\alpha$ ). The exposure to quartz and TiO<sub>2</sub> particles did not induce a statistically significant increase in cytokine release in both cell models probably due to our relatively low deposited doses, which were inspired by *in vivo* dose levels. The intra- and inter-laboratory comparison study indicated acceptable interlaboratory variation for cell viability/toxicity (WST-1, LDH) and transepithelial electrical resistance, and relatively high inter-laboratory variation for cytokine production.

**Conclusion:** The transferability and reproducibility of a lung co-culture model and its exposure to aerosolized particles at the ALI were evaluated and recommendations were provided for performing inter-laboratory comparison studies. Although the results are promising, optimizations of the lung model (including more sensitive read-outs) and/or selection of higher deposited doses are needed to enhance its predictive value before it may be taken further towards a possible OECD guideline.

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## 1. Introduction

Inhalation is considered an important route of human exposure to nanomaterials (NMs). There are concerns that following long-term inhalation exposure, some NMs might cause chronic inflammation progressing towards fibrosis and cancer, which has been observed in rodent studies (Halappanavar et al., 2020; Dong and Ma, 2019; Vlachogianni et al., 2013; Braakhuis et al., 2021; Lee et al., 1985; Muhle et al., 1989; Reuzel et al., 1991; Muhle et al., 1995). Given the wide variety of NMs, it is not feasible to test them all *in vivo*, and one of the goals within the nanotoxicology community is to develop *in vitro* assays that are predictive of *in vivo* outcomes to prioritize NMs that need further testing. Several *in vitro* lung models have been developed, differing in their complexity. Some models are based on cell-lines, whereas others are based on primary cells or co-cultures of different cell types (Lacroix et al., 2018; Hiemstra et al., 2018; Barosova et al., 2020a; Diabaté et al., 2020; Diabate et al., 2008; Hermanns et al., 2009; Hermanns et al., 2004; Huh et al., 2010; Klein et al., 2011; Lehmann et al., 2011; Luyts et al., 2015; Alfaro-Moreno et al., 2008; Cappellini et al., 2020; He et al., 2021; Papazian et al., 2016). It is generally assumed that increasing the complexity and physiological relevance of the models enhances their predictivity (Lacroix et al., 2018). In addition, lung cells can be exposed *via* the air-liquid interface (ALI). There is consensus that testing NMs at the ALI is more relevant and realistic compared to traditional submerged exposure approaches, since the physiological conditions of the lung are better reflected (Lacroix et al., 2018; Paur et al., 2011). Furthermore, ALI exposure allows for the physicochemical characteristics of the pristine NMs to not be affected by the components of the cell culture medium (*i.e.*, protein corona formation) (Lynch et al., 2007; Walczyk et al., 2010). The currently published lung models are mainly being used in a research environment and are not validated for regulatory purposes. For regulatory acceptance of *in vitro* models, the robustness, reproducibility and predictivity need to be demonstrated (Hiemstra et al., 2018; Barosova et al., 2021). Only few interlaboratory comparison studies using *in vitro* lung models have been reported and they are based on immortalized cell-lines cultured as a monolayer (Barosova et al., 2021; Xia et al., 2013). In the study of Xia et al., the NIEHS NanoGO Consortium (8 participating labs) tested the potential effects of zinc oxide (ZnO), titanium dioxide (TiO<sub>2</sub>) and multi-walled carbon nanotubes (MWCNTs) in two different human lung epithelial cell lines and a human monocyte cell-line. They observed large interlaboratory variation in interleukin-1 $\beta$  (IL-1 $\beta$ ) release by the THP-1 monocytes. Interlaboratory variation could be decreased by harmonizing protocols (Xia et al., 2013). Barosova et al., investigated variability of the A549 human alveolar cell line in two labs by culturing the cells both submerged and at the ALI. They also observed large variations between the two labs, especially in cytokine release, that could be reduced by using the same batch of serum and using the positive control lipopolysaccharide (LPS) from the same company (Barosova et al., 2021).

Within the EU-funded project PATROLS, human lung models were developed and optimized for testing repeated exposure to NMs, to mimic long-term exposure to aerosols. Different physiologically-anchored lung models were optimized within the project (PATROLS, 2022), including models based on cell-lines (Barosova et al., 2021; Braakhuis et al., 2020), co-culture models (He et al., 2021) and models based on primary cells (Barosova et al., 2020a). Yet, despite such advances, there remains the need to harmonise such *in vitro* lung models and exposure approaches. Therefore, within PATROLS, we aimed to contribute to the standardisation of lung models for NM toxicity testing, through testing the transferability and reproducibility of a next-level *in vitro* lung cell system, *via* an interlaboratory comparison study. To achieve this, we selected a lung model based on the human bronchial cell line Calu-3 both as a monoculture and as a co-culture with macrophages (either derived from the THP-1 monocyte cell line or from human blood monocytes), to increase the relevance of the model. The reason for choosing Calu-3 bronchial cells is their suitability to be cultured and

exposure at the ALI for a longer period of time - up to 2 months - without affecting their viability and barrier integrity (He et al., 2021; Braakhuis et al., 2020). Macrophages are essential for detecting particle toxicity and they are not confined to the alveoli; they are present throughout the respiratory system (Brain, 1988; Tang et al., 2022; Bosso et al., 2022) and are therefore included in the lung model in the current study.

To our knowledge, this is the first time a co-culture lung model has undergone an interlaboratory comparison. Both the mono-culture model and the co-culture model were exposed to the positive control lipopolysaccharide (LPS) and to two particles: DQ12 and TiO<sub>2</sub> NMs *via* the ALI using the commercially available VITROCELL® Cloud12 system (Lenz et al., 2014). DQ12 and TiO<sub>2</sub> were selected as there is evidence from animal studies that these materials can induce toxicity after inhalation exposure. In rats, DQ12 induced chronic inflammation and fibrosis after 90 days inhalation exposure (Reuzel et al., 1991), and cancer after 2-years inhalation exposure (Muhle et al., 1995). TiO<sub>2</sub> induced chronic inflammation and cancer after 2-years inhalation exposure in rats (Braakhuis et al., 2021; Lee et al., 1985; Heinrich et al., 1995).

In total, 7 laboratories participated in the comparison study hereby described. The results of this study provide insight in the variation of a co-culture lung model *vs.* a mono-culture model and provide options for improving transferability and reproducibility of *in vitro* lung models to enhance their regulatory acceptance. This is a first step towards standardisation and validation of an ALI lung cell model.

## 2. Materials and methods

All SOPs can be found in the PATROLS SOP Handbook (PATROLS, 2022). Details of the aerosolisation using the VITROCELL® Cloud12 system are specified in the PATROLS SOPs “Guidance Document for ENMs Aerosolization using VITROCELL® Cloud System” (#3601); and “Vitrocell dry powder system” (#3206; see also (Bannuscher et al., 2022)). Details of the materials and methods for testing transferability and reproducibility of the Calu-3 model with and without macrophages are given in the PATROLS SOPs “Guidance Document for co-culture of a lung epithelial cell-line (Calu-3), and macrophages derived from peripheral blood monocytes” (#3603) and “Guidance Document for co-culture of a lung epithelial cell-line (Calu-3), and macrophages derived from a monocytic cell line (dTHP-1)” (#3604). In addition, the Calu-3 lung cell monoculture for testing NMs exposure is published online (Braakhuis et al., 2020). Details on the co-culture models are published by He et al. (He et al., 2021). Details on the culture of Calu-3 cells, macrophages derived from THP-1 cells, and macrophages derived from primary human monocytes are provided in the PATROLS SOPs: “Guidance Document for cell culture of lung epithelial cell-line (Calu-3)” (#3104), “Guidance Document for macrophage differentiation from THP-1 cells” (#3108), and “Guidance Document for the isolation and differentiation of peripheral blood monocytes and further assembly into co-culture models with epithelial cells” (#3109), respectively.

### 2.1. Participating labs

In this interlaboratory testing, 7 partners participated: 1) National Institute for Public Health and the Environment (RIVM), The Netherlands, 2) Adolphe Merkle Institute (AMI), Switzerland, 3) Swansea University (SU), Wales, UK 4) BASF SE, Germany, 5) National Institute of Occupational Health (STAMI), Norway, 6) Helmholtz Center Munich - German Research Center for Environmental Health (HMGU), Germany, 7) Luxembourg Institute of Science and Technology (LIST), Grand Duchy of Luxembourg.

### 2.2. Materials

A list of all chemicals, reagents and equipments is given in the Supplementary materials. LPS was purchased from Sigma (L4391).

DQ12 was received from the Institute of Occupational Medicine (IOM), UK; TiO<sub>2</sub> NM-105 was provided by the Fraunhofer Institute for Molecular Biology and Applied Ecology (Fraunhofer IME, Germany). The characteristics of TiO<sub>2</sub> NM-105 can be downloaded from <https://publications.jrc.ec.europa.eu/repository/handle/JRC86291>. All participants used the same batch of nanomaterials.

### 2.3. (Nano-)material dispersion

Particles were dispersed according to the Nanogenotox protocol for toxicity testing ([https://www.rivm.nl/sites/default/files/2018-11/NANoREG\\_Guidance\\_Document.pdf](https://www.rivm.nl/sites/default/files/2018-11/NANoREG_Guidance_Document.pdf)) in a volume range between 4 mL and 10 mL in ultrapure water, but without bovine serum albumin (BSA) to prevent formation of a protein corona surrounding the particles and to prevent agglomeration effects that lead to blocking of the nebulizer and impede reproducibility and reliability of the Quartz Crystal Microbalance (QCM) data. Briefly, for example, 10.24 mg of the material powder were weighted into glass vials, pre-wetted with 20 µL ethanol and mixed with water to a stock concentration of 2.56 mg/mL. The solution was sonicated using an ultrasonic tip device to reach a total power of 7056 J (7.35 W for 16 min). During the sonication, the glass vial was kept on ice to prevent excess heating of samples during sonication. The stock was further diluted with ultrapure water to the final concentration of 500 µg/mL. All suspensions were sonicated for 10 min in an ultrasonic bath and vortexed prior to usage.

### 2.4. Cells and cell culture conditions

The reason for choosing the Calu-3 cell line is that these cells form a tight monolayer with a high trans-epithelial electrical resistance (TEER) (He et al., 2021), they can be cultured at the ALI without a decrease in TEER for weeks (Braakhuis et al., 2020), and they are easy to handle. For the macrophages, we included both primary human monocyte-derived macrophages (MDM) from human buffy coats and the human monocyte cell-line THP-1 that can be differentiated into macrophage-like cells. In this way, we could compare the response between primary macrophages, and macrophages differentiated from a cell line.

Human bronchial epithelial cells (Calu-3) and human acute monocytic leukemia cells (THP-1) were purchased from American Type Culture Collection (ATCC, Rockville, USA). Calu-3 cells were cultured in MEM + GlutaMAX supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% Non-Essential Amino Acids (NEAA) solution. THP-1 cells were cultured in RPMI medium supplemented with 10% FCS, 1% L-glutamine, 100 U/ml penicillin, and 100 µg/mL streptomycin. The cells were incubated at 37 °C in a 100% humidified atmosphere containing 5% CO<sub>2</sub>. The medium was renewed every 2–3 days.

Monocytes were isolated from human buffy coats and differentiated to monocyte-derived macrophages (MDM) for 6 days (Barosova et al., 2020b). MDM were cultured in RPMI medium supplemented with 10% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine. THP-1 cells were differentiated to macrophage-like cells (dTHP-1) by incubation with 100 ng/ml PMA (Sigma P8139) for 5 days, followed by a recovery (without PMA) for 2 days.

### 2.5. Preparation of the lung models

Calu-3 cells were seeded at a density of 100,000 cells/cm<sup>2</sup> on Transwell® inserts (12-well inserts, 1.12 cm<sup>2</sup>, 0.4 µm pores; Corning CLS 3460). The cells were cultured under submerged conditions for 7 days. After 7 days, the apical medium was removed, and the cells were cultured at the ALI for an additional 7 days. On day 14, either MDM or dTHP-1 were added to obtain a co-culture model.

A schematic overview of culturing and exposing the cell model is given in Fig. 1.

### 2.6. Co-culture with MDM

The isolation of primary blood monocytes (PBM) from human buffy coats was previously described (Barosova et al., 2020b). After isolation, PBM were differentiated into MDM using 10 ng/mL macrophage colony-stimulating factor (M-CSF; Miltenyi 130-096-485) for 6 days. After 6 days, MDM were scraped off the wells, counted and added on top of the Calu-3 monolayer. As not all MDM attach to the Calu-3 cells, 39,200 MDM were added in a volume of 0.2 mL to achieve a final concentration of 25,000 MDM/cm<sup>2</sup> (He et al., 2021). After 4 h, the apical medium was removed, and the co-culture was kept at 37 °C overnight. Monocytes were derived from different donors for each biological repetition.

### 2.7. Co-culture with THP-1 macrophage-like cells

To differentiate the THP-1 monocytes into macrophages, the cells were seeded at a density of 500,000 cells/ml in the presence of 30 ng/mL LPMA for 5 days. At day 5, the differentiation medium was removed and replaced by fresh cell culture medium for 48 h. dTHP-1 were detached using accutase, counted and added on top of the Calu-3 monolayer. 14,000 cells were added in a volume of 0.1 mL to achieve a concentration of 12,500 THP-1 cells/cm<sup>2</sup>. After 2 h, the apical medium was removed, and the co-culture was incubated at 37 °C overnight.

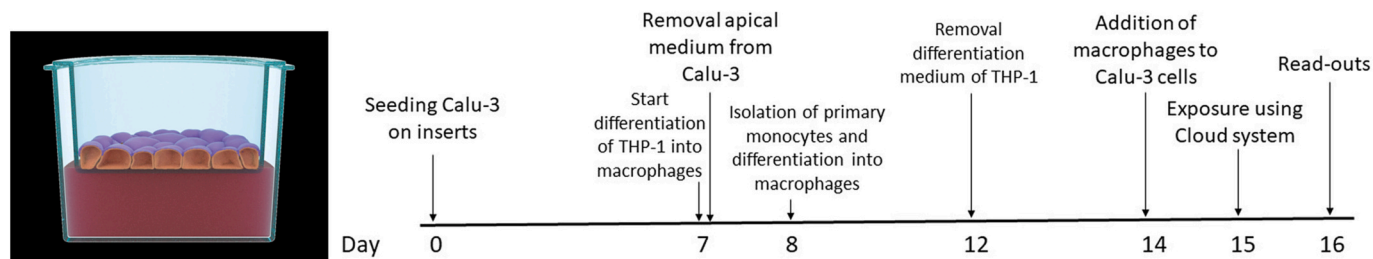
### 2.8. NM exposure

For aerosolized NM exposure at the ALI, the VITROCELL® Cloud12 system was used. The dosimetrically accurate NM deposition using the Vitrocell® Cloud12 system has been established among the different partners in a first phase of this interlaboratory study (Bannuscher et al., 2022). This first phase comprised the same laboratories as the ones performing the interlaboratory study described in the present manuscript (second phase). The current interlaboratory effort was focused on the comparison of the effects induced by the two NMs in the Calu-3 cell model with and without macrophages after the exposure performed by using the VITROCELL® Cloud12 system.

All partners used a VITROCELL® Cloud12 system (VITROCELL® Systems GmbH, Germany) equipped with a Quartz Crystal Microbalance (QCM; VITROCELL® Systems GmbH, Germany) and an Aeroneb® Lab nebulizer (4–6 µm pore size, Aerogen, Ireland). Details on the principle and setup of the QCM, VITROCELL® Cloud system and nebulization process can be found in (Ding et al., 2020).

Operation of the VITROCELL® Cloud12 system has been harmonized among the laboratories based on a SOP “Guidance Document for ENMs Aerosolization using VITROCELL® Cloud System” (#3601). In brief, the Cloud system was heated to 37 °C before use. QCM data acquisition was started and the QCM stability was measured for 1 min. For blank/sham correction, 200 µL ultrapure water spiked with 1% isotonic (i.e. 9 mg/mL) NaCl (90 µg/mL NaCl final concentration) was nebulized to check proper operation of the VITROCELL® Cloud12 system (QCM values should be <250 µg/cm<sup>2</sup> to indicate the absence of residual NMs in the nebulizer). Then, 250 µL particle suspension was combined with 2.5 µL isotonic NaCl solution. 200 µL of the mixture was immediately used for nebulization. After 6 min, the cover of the chamber was lifted to dry the QCM for 1 min. Afterwards, it was placed back to prevent ventilation and temperature effects that could influence the QCM signal. Data acquisition was stopped after an additional 3 min to assure a stable QCM signal. For data evaluation, the mean of the values recorded in the last 30 s was calculated. For cleaning, the reservoir of the nebulizer was rinsed with water prior to each usage. As a check for proper cleaning, deposition of water with 1% isotonic NaCl was additionally measured (QCM signal of <250 µg/cm<sup>2</sup> indicated “clean” conditions).

The cells were exposed to LPS, DQ12 and TiO<sub>2</sub> using the VITROCELL® Cloud12 system as described above. For LPS, a concentration of 175 µg/mL was prepared in ultrapure water and nebulized to achieve about 0.25 µg/cm<sup>2</sup> after a single nebulization. For DQ12 and TiO<sub>2</sub>, a



**Fig. 1.** Schematic overview of culturing and exposing the co-culture lung model consisting of Calu-3 cells and macrophages seeded on the apical side.

concentration of 500  $\mu\text{g}/\text{mL}$  was prepared to achieve a deposition of about 1  $\mu\text{g}/\text{cm}^2$  after two 200  $\mu\text{L}$  nebulizations.

## 2.9. Measurements of transepithelial electrical resistance (TEER)

Transepithelial electrical resistance (TEER) was measured using the Evom2 Voltohmmeter equipped with a chopstick electrode (World Precision Instruments, Inc., FL, USA). TEER was measured before exposure and at 24 h after exposure. As TEER can fluctuate depending on temperature, measurements were performed within a 10-min period after getting the cells from the incubator. The TEER values were obtained by subtracting the resistance of a cell-free Transwell® insert and multiplying by the surface area of the insert.

## 2.10. Membrane integrity

Lactate dehydrogenase (LDH) release was measured in the basolateral cell culture medium using an LDH kit (No. 11644793001, Roche Diagnostics GmbH, Mannheim, Germany). Briefly, at 24 h after exposure, 100  $\mu\text{L}$  of sample was taken off and incubated with 100  $\mu\text{L}$  of reaction mix for 15–20 min in the dark. After adding the stop solution, absorbance was measured at a wavelength of 490 nm. All measurements were corrected for the background of the cell culture medium and normalized to the maximum LDH release after lysis of the control cells.

## 2.11. Mitochondrial metabolic activity

Cell mitochondrial activity was evaluated using the WST-1 cell Proliferation Reagent (No. 11644807001, Roche Diagnostics GmbH, Mannheim, Germany). At 24 h after exposure, and after collection of supernatants for cytokine analysis (see paragraph below), WST-1 reagent was added to the apical side of the inserts for 1 h. Subsequently, 100  $\mu\text{L}$  of supernatant of each insert was transferred to a 96-wells plate in duplicate. Absorbance was measured using a spectrophotometer at a wavelength of 440 nm and a reference wavelength of 620 nm.

## 2.12. (pro)inflammatory cytokines

At 24 h after exposure, 500  $\mu\text{L}$  cell culture medium was added to the apical side of the inserts. After 10 min, the apical and basolateral supernatant were collected separately for cytokine analysis. The supernatants from AMI and HMGU were shipped to RIVM for cytokine analysis. Release of inflammation markers tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-6 and IL-8 were measured in the supernatants using ELISA kits (eBioscience, Inc.), Milliplex (HCYTOMAG-60 K), or Bio-Plex Human Cytokine assays (Bio-Rad Laboratories, Inc.). Samples were collected from three independent experiments. Cytokine analysis was performed according to the manufacturer's instructions. For ELISA, absorbance was measured using a spectrophotometer at a wavelength of 450 nm and a reference wavelength of 570 nm. For Milliplex, multiplex beads were measured using a Luminex® 200™ Multiplexing Instrument (Luminex, Austin, TX, USA) or a Bio-Plex MAGPIX multiplex reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

## 2.13. Macrophage staining

To visualize the macrophage attachment in the co-culture models, before adding the macrophages to the Calu-3 cells they were labeled using Vybrant Dil dye (Thermo Fisher Scientific Inc.) according to (Septiadi et al., 2018). Pictures were taken using a fluorescence microscope.

## 2.14. Statistical analysis

For statistical analyses, GraphPad Prism 9.1.0 software (San Diego, CA, USA) was used. For TEER and cytokine measurements, absolute values were used for the statistical analyses. For LDH release and WST-1 conversion, normalized values were used for the statistical analyses. Most participating laboratories performed 3 independent replicate experiments (RIVM, AMI, SU, HMGU and LIST) using 3 or 4 inserts per condition. Other laboratories performed 2 independent replicate experiments (BASF) or a single experiment (STAMI) using 3 or 4 inserts per condition.

Replicates of the experiments were used for calculating the standard deviation (SD) and mean values. To assess the variation in the data, the coefficient of variation (%CV) was calculated by  $\text{SD}/\text{mean}$ . 2-way ANOVA followed by Tukey correction was used to assess statistically significant differences between exposures.

## 3. Results

### 3.1. (Nano-)material characterization

Characterization data for DQ12 and TiO<sub>2</sub> NM-105 are well-described in the literature and selected key properties are summarized in Table 1 (Barosova et al., 2020a; Robock, 1973; Driessen et al., 2015; Bannuscher et al., 2020).

#### 3.1.1. Deposition of DQ12 and TiO<sub>2</sub> NM-105

For the nebulization of the particles, a concentration of 500  $\mu\text{g}/\text{mL}$  was chosen to achieve a deposited target dose of about 1  $\mu\text{g}/\text{cm}^2$  (as measured by quartz microbalance (QCM)). This deposited dose corresponds to doses that induce pulmonary inflammation *in vivo* (Braakhuis et al., 2021; Lee et al., 1985; Reuzel et al., 1991; Muhle et al., 1995; Heinrich et al., 1995).

The measured deposited mass of aerosolized DQ12 and TiO<sub>2</sub> NM-105 ranged between ca. 0.5 and 1.3  $\mu\text{g}/\text{cm}^2$  independent of particle type as shown in Fig. 2.

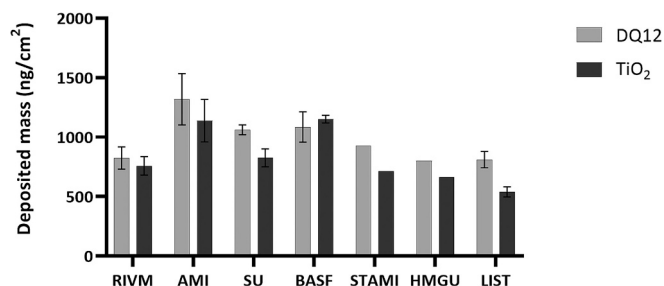
### 3.2. Variation in the Calu-3 mono-culture cell model

#### 3.2.1. TEER and cell viability

LDH release was measured directly after exposure as a measure of membrane integrity. WST-1 conversion was measured 24 h after exposure as a measure of mitochondrial activity of the cells. Trans-Epithelial electrical resistance (TEER) was measured in Calu-3 monocultures before and 24 h after exposure to the vehicle control, LPS, DQ12 or TiO<sub>2</sub>

**Table 1**Overview of the characteristics of DQ12 and TiO<sub>2</sub> NM-105.

Material	Name	Composition/Structure	Primary particle diameter	Surface area (BET)	Hydrodynamic diameter D50 (water)	Zeta potential (pH 7.4)
			[nm]	[m <sup>2</sup> /g]	[nm]	[mV]
Quartz CAS 14808-60-7	DQ12	Quartz, 87% crystalline and 13% amorphous SiO <sub>2</sub>	≤5 μm	11	338	-39
Titanium dioxide CAS 13463-67-7	TiO <sub>2</sub> NM-105	85% anatase and 15% rutile	21	61	478	-17



**Fig. 2.** Deposited mass of DQ12 and TiO<sub>2</sub> (minus vehicle control - water with 1% isotonic NaCl solution), using the VITROCELL® Cloud12 at 7 different labs. Bars show mean ± SD of N = 3 experiments (RIVM, AMI, SU, BASF and LIST) and values of single experiments (STAMI and HMGU).

NM105. In the monolayer of Calu-3 cells, there was no effect of LPS, DQ12 and TiO<sub>2</sub> on membrane integrity, mitochondrial activity, or TEER (Fig. 3).

The intra- and interlaboratory variation in LDH release, WST-1 conversion and TEER measurements was relatively low (Fig. 4). For LDH release the interlaboratory variation was below 13%, for WST-1 conversion below 12% and for TEER below 40%. This may suggest that the Calu-3 cells are a stable model showing acceptable interlaboratory variations of LDH release and WST-1 conversion, while TEER shows a clearly higher interlaboratory variation possibly due to the high sensitivity of TEER to both exact positioning of the electrodes and even slight corrosion levels (Fig. 4). The TEER may, however, be useful as a control for assay performance within a specific lab.

### 3.2.2. Pro-inflammatory cytokine release

For the Calu-3 cells, IL-6 and IL-8 could be measured, whereas TNF-α was below the detection limit of 0.01 pg/ml for all laboratories. Cytokine release was measured both at the apical side, from the apical wash, and in the basolateral medium. As Calu-3 cells form a barrier, cytokines that are released at the apical side are thought not to diffuse to the basolateral side. The cytokine release at the apical side was about 10 times higher compared to the basolateral side (supplementary files).

The results from the apical side show that in all laboratories exposure to LPS induces an increase in IL-6 and IL-8, although in only one laboratory the increase is statistically significant (Fig. 5). For STAMI, there is a clear increase in IL-6 release after LPS exposure, although not statistically significant because it is data from a single experiment. Exposure to DQ12 and TiO<sub>2</sub> NM-105 did not induce IL-6 or IL-8 release in Calu-3 cells.

The intra- and interlaboratory variation indicates that for IL-6 release, there is a high interlaboratory variation after LPS exposure (200% CV), which might be related to differences in LPS activity. For IL-8 release, the interlaboratory variation is lower (around 45% CV) and thus in the range of typical intra-laboratory variation. There is, however, substantial intra-laboratory variation (about 100% CV) in some samples from RIVM and AMI (Fig. 6).

### 3.3. Variation in the Calu-3 + macrophage co-culture models

There was little to no effect on the Calu-3 cells of the aerosol exposure to LPS, DQ12 and TiO<sub>2</sub> NM-105 (see Figs. 3 and 5). It is known that macrophages play an essential role in the immune response to inhaled particles. Therefore, our next step was to include macrophages in the *in vitro* lung model. From the 7 participating labs, 2 labs (RIVM and AMI) used primary monocyte-derived macrophages (MDM) and 5 labs (SU, BASF, STAMI, HMGU and LIST) used macrophage-like cells derived from the human monocyte cell-line THP-1 (dTHP-1).

#### 3.3.1. Macrophage attachment

The lung models were stained to check cell morphology and macrophage attachment (Fig. 7). In both co-culture models, macrophages (stained with Vybrant-Dil Dye) are visible in green on top of the Calu-3 monolayer. The pictures confirm the presence of macrophages in the co-culture models.

#### 3.3.2. TEER and cell viability

The membrane integrity, mitochondrial activity, and TEER of the co-cultures showed no differences of LPS- and particle-treated cells relative to vehicle control (Supplementary files). Similar to the results for the Calu-3 cells, the variation in the co-culture model is below 16% for LDH release, below 14% for WST-1 conversion and below 42% for TEER (Fig. 8), indicating that the addition of macrophages did not induce additional variation in these parameters.

#### 3.3.3. Pro-inflammatory cytokines release

Fig. 9 depicts the cytokine concentration measured at 24 h after exposure at the apical side of the co-culture model. LPS exposure induced an increase in IL-6, although in only one lab this increase was statistically significant. LPS exposure induced a significant increase in IL-8 release in 5 of the 7 labs. In addition, TNF-α levels were above the detection limit (0.01 pg/ml) in all labs and stimulation with LPS significantly increased TNF-α in 3 of the 5 labs with elevated IL-8 levels. Calu-3 cells alone did neither release TNF-α nor IL-8 upon particle exposure. This indicates that the macrophages are important for the cellular interplay and subsequent cytokine release. The increase in cytokines with LPS exposure was observed in both labs using MDM as well as in 3 of the 5 labs using dTHP-1 cells. Compared to LPS exposure, cytokine increase induced by particle exposure was very low; no significant increase could be detected (except for a moderate 2.5-fold IL-8 increase after treatment with DQ12 in the AMI lab) (Fig. 10).

When looking into the variation within (intra) and between (inter) labs, there is a relatively large variation in IL-6 release after LPS exposure between different laboratories (240% CV). This could be due to differences in the potency of the LPS batch, albeit that an identical supplier and product number was used. The intra- and interlaboratory variation for IL-8 release is relatively small when compared to the other cytokines (about 45% CV), suggesting that the co-culture model is quite robust for measuring this cytokine. In contrast, for TNF-α the co-culture model shows large intra- and interlaboratory variation independent of sample type (about 100% CV), which is probably due to the low absolute

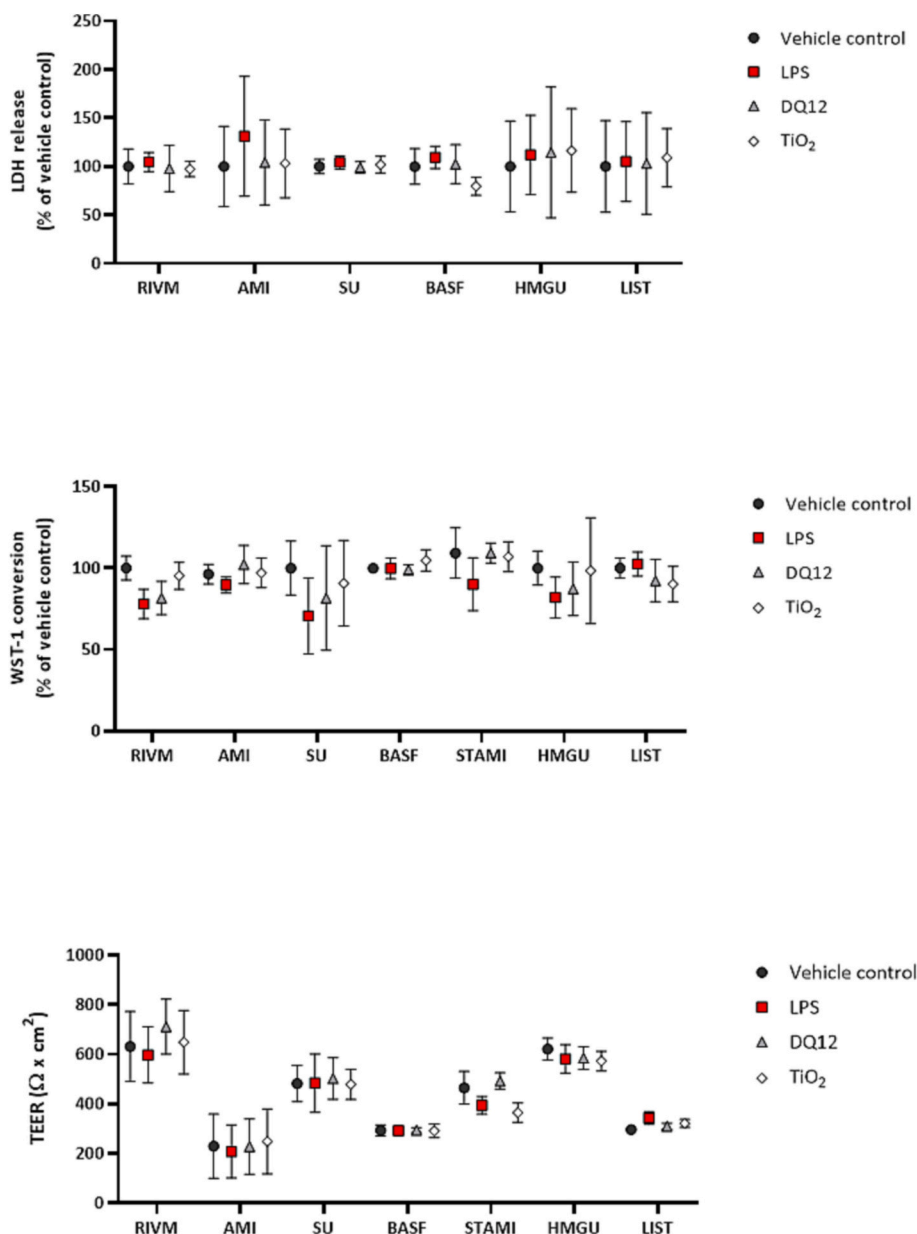


Fig. 3. Overview of membrane integrity, mitochondrial activity, and TEER of Calu-3 cells after ALI exposure to LPS, DQ12 and TiO<sub>2</sub>. The exposures had no effect on LDH release (top), WST-1 conversion (middle) and TEER (bottom). Mean  $\pm$  SD of  $N = 3$  experiments (RIVM, AMI, SU, HMGU, LIST), of  $N = 2$  experiments (BASF) and of a single experiment (STAMI).

cytokine levels, near and in some occasions below the detection limit.

#### 4. Discussion

The establishment of reliable and robust *in vitro* models for hazard assessment, moving away from animal testing, requires the evaluation of model transferability and reproducibility. Lung models that can be exposed to inhalable toxins – here NMs - *via* the air by means of an ALI are promising *in vitro* models for evaluating the safety of NMs upon inhalation exposure. We presented here the results of an interlaboratory comparison study to evaluate the transferability and reproducibility of a mono- and co-culture lung model and their exposure to NMs using the VITROCELL® Cloud12 system at physiologically relevant dose levels. To the best of our knowledge this is the first time an ALI exposed co-culture lung model has been tested across different laboratories. Our results show that the lung models can be transferred to different laboratories, while the reproducibility of the obtained results can be improved,

especially regarding cytokine release.

Overall, the results obtained by the 7 participating laboratories are quite similar. After exposing Calu-3 monolayers and Calu-3 co-cultures with macrophages, no effects of LPS, DQ12 or TiO<sub>2</sub> NM-105 on the cell viability (measured *via* membrane integrity (LDH) and mitochondrial activity (WST-1)) and on the TEER were detected. In the Calu-3 monolayers, LPS exposure induced IL-6 and IL-8 release, although not statistically significant in most labs. The co-culture model was more sensitive as most laboratories showed that LPS can significantly induce cytokine release; 5 out of 7 laboratories reported a significant increase in IL-8 release, while 3 laboratories (that all reported a significant increase in IL-8) reported a significant increase in TNF- $\alpha$ . The other laboratories observed an increased IL-8 and TNF- $\alpha$  release after LPS exposure, however not statistically significant. In addition, we quantified the intra- and inter-laboratory variability for the cell viability assays, TEER measurements, and cytokine measurements. We discuss this variability below.

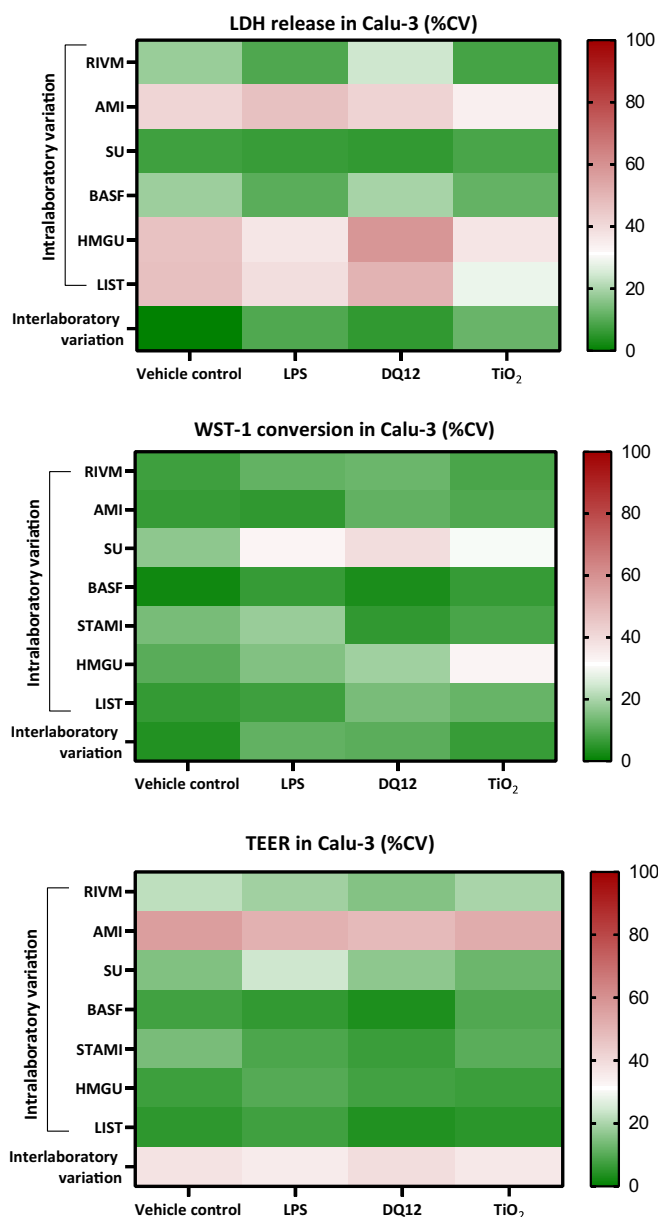


Fig. 4. Coefficient of variation (%CV) for measurements of LDH release (top), WST-1 conversion (middle) and TEER (bottom) in Calu-3 cells. Data are from  $N = 3$  experiments (RIVM, AMI, SU, HMGU, LIST),  $N = 2$  experiments (BASF) and from a single experiment (STAMI).

Cytotoxicity is a crucial endpoint for the final interpretation of toxicological results to differentiate between specific and unspecific effects (Escher et al., 2020). Therefore, we applied two different assays in parallel to identify the impact of the substances on cell integrity and viability. The LDH assay represents the membrane integrity, while the WST-1 assay represents the mitochondrial activity by measuring the mitochondrial dehydrogenase activity. Our data show that none of the substances significantly impair the cell integrity/viability. The WST-1 assay showed lower variability than the LDH assay. Both assays show relatively low interlaboratory variation, indicating they are robust and reproducible. It should not be forgotten that nanomaterials might interfere with the readout, therefore a careful selection of the appropriate assay should be done beforehand (Kroll et al., 2011).

TEER measurements were conducted to evaluate the Calu-3 tissue barrier properties. The measurement of the resistance was performed by a common chopstick electrode method, used in all laboratories. To get

robust and reproducible data, it is very important to follow a detailed and harmonized SOP considering the time point of measurement after bringing the samples out of the incubator into the hood, the temperature of the buffer solution that is added to the cells to dip in the chopsticks, and the handling of the chopsticks themselves. Moreover, variable levels of corrosion on the electrodes may significantly affect the observed TEER values. Hence, our data show that absolute values might differ between laboratories but the intra-laboratory consistency is relatively high since handling and operational differences are mainly relevant for inter-laboratory consistency (see Figs. 4 and 8). This variability is also described in literature (Wiese-Rischke et al., 2021). Considering this inter-laboratory variability, the TEER values are applicable for cell culture characterization by each individual laboratory but not appropriate as a strict absolute endpoint for barrier integrity.

As a measure of inflammation potential, we included measuring the release of the pro-inflammatory cytokines IL-6, IL-8 and TNF- $\alpha$ . The selection of these cytokines is based on results from previous studies. Ma-Hock et al. for example recommend the measurements of several inflammatory mediators in 5-day inhalation studies, including IL-6, IL-8 and TNF- $\alpha$  (Ma-Hock et al., 2009). Also other studies report release of inflammatory mediators after inhalation exposure to TiO<sub>2</sub> (Noël et al., 2013; Wang et al., 2021; Scarino et al., 2012; Lehotska Mikusova et al., 2023). RNA expression levels of TNF- $\alpha$  and IL-6 were increased after pulmonary exposure to silica-coated TiO<sub>2</sub> NMs in mice (Leppänen et al., 2015). *In vitro*, exposure to TiO<sub>2</sub> NMs can also induce the release of pro-inflammatory cytokines, including IL-6, IL-8 and TNF- $\alpha$  in alveolar cells (Sweeney et al., 2015), and in co-cultures of epithelial cells and THP-1 macrophages (Dekali et al., 2013). DQ12 exposure induced a significant increase in TNF- $\alpha$  in mouse macrophages *in vitro* (Boyles et al., 2018). A co-culture of Calu-3 cells with EA.hy926 and THP-1 showed induction of IL-6, IL-8 and TNF- $\alpha$  upon particle exposure (Zhang et al., 2019). A co-culture of Calu-3 with THP-1 and HMC-1 cells showed induction of IL-6, IL-8 and TNF- $\alpha$  after exposure to disinfectant aerosols (Kim et al., 2016). Submerged exposure of Calu-3 cells to silica nanoparticles induced IL-6 and IL-8 (McCarthy et al., 2012). Yet, most of these observations were obtained for higher doses than used here. In addition, SARS-CoV infection of Calu-3 cells increased the production of IL-6, IL-8, and CXCL10 (Yoshikawa et al., 2009). In that study a dose- and time-dependency was shown for IL-6 and IL-8. For a range of 14 other cytokines, no or minute amounts of cytokines were detectable in both control and infected Calu-3 cells. We therefore chose IL-6 and IL-8 release for monitoring exposure effects on Calu-3 cells.

We observed quite some variation in the absolute levels of cytokines released after 24 h of exposure, between the participating labs, especially in IL-6 release after LPS exposure (in both cell models) and in TNF- $\alpha$  release in the co-culture. This was already observed in previous studies investigating cytokine intra- and inter-laboratory variation (Barsova et al., 2021; Xia et al., 2013; Piret et al., 2017). In these studies, when dTHP-1 cells were used as representative for macrophages, the release of IL-1 $\beta$  and TNF- $\alpha$  after submerged exposure to LPS and silver NMs varied greatly (Piret et al., 2017), and also the release of IL-1 $\beta$  after submerged exposure to TiO<sub>2</sub> showed high variation (Xia et al., 2013). In A549 alveolar cells, the induction of IL-6 and IL-8 varied between 2 labs after exposure to LPS and TNF- $\alpha$  (Barsova et al., 2021). When using biological fluids, e.g. human plasma, measurements of cytokines can also vary greatly within and between laboratories (Fahey et al., 2000). Interestingly, some laboratories show less variation compared to others. The low intra-laboratory variation might be due to an extensive optimization of cytokine measurements by investigating optimal dilution of the samples and by assigning the performance of the assays to a single person. From the cytokines measured, apical IL-6 and IL-8 showed the lowest %CV and are therefore the markers of choice in this model. Although TNF- $\alpha$  has a clearly higher %CV and is therefore less suitable, it has the advantage over IL-6 and IL-8 of being produced only by the macrophages. It may thus be considered as a marker for macrophage functionality. We observed a higher TNF- $\alpha$  release in the co-culture

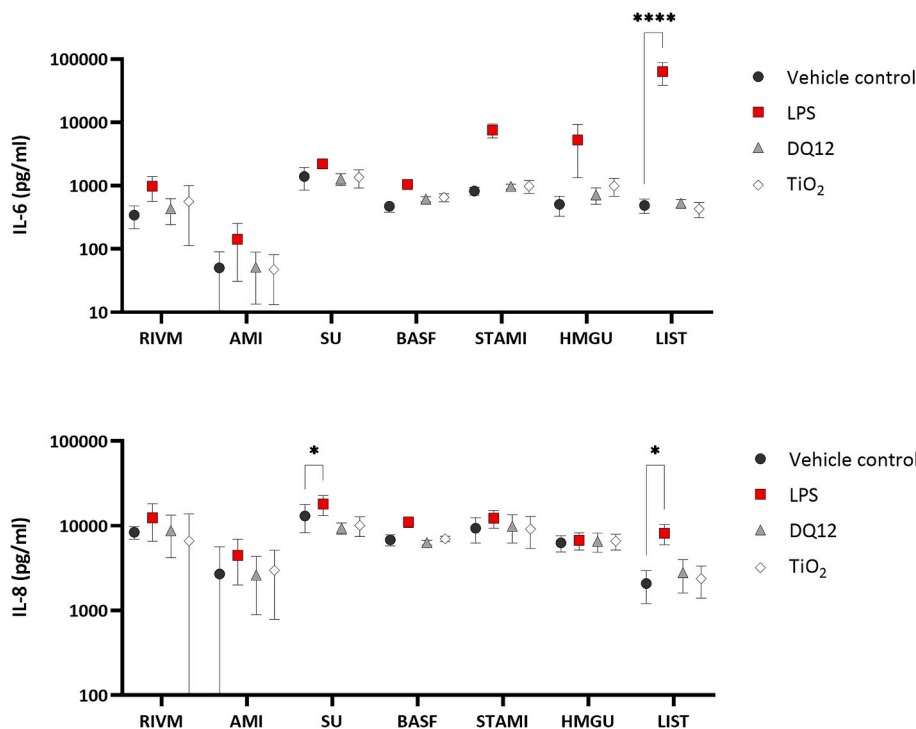


Fig. 5. IL-6 and IL-8 release in Calu-3 cells measured at the apical side. Mean  $\pm$  SD of  $N = 3$  experiments (RIVM, AMI, SU, HMGU, LIST), mean  $\pm$  SD of  $N = 2$  experiments (BASF) and mean  $\pm$  SD of a single experiment (STAMI). Note that the Y-axes are on a logarithmic scale.

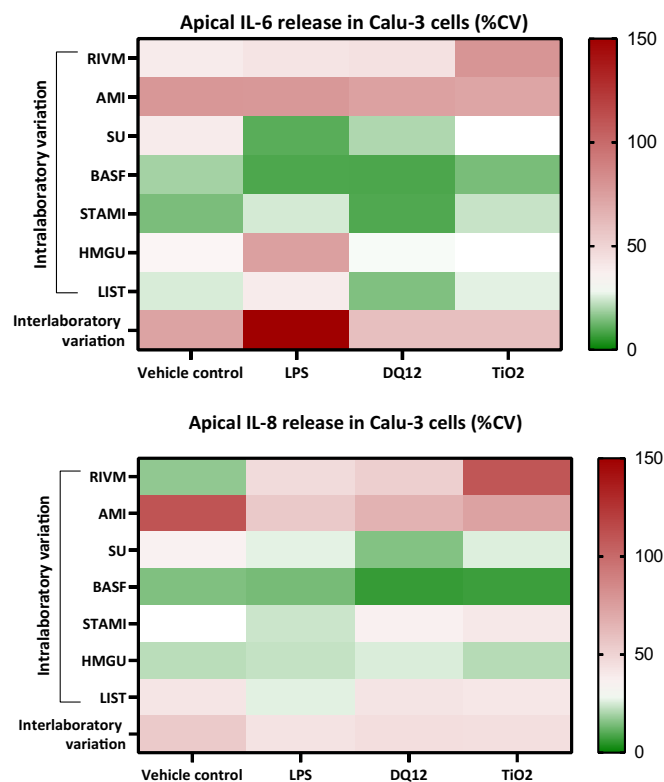


Fig. 6. Coefficient of variation (%CV) for measurement of IL-6 release (left) and IL-8 release (right) in Calu-3 cells. Data are from  $N = 3$  experiments (RIVM, AMI, SU, HMGU, LIST),  $N = 2$  experiments (BASF) and from a single experiment with 4 technical replicates (STAMI).

model with MDM compared to the co-culture model with dTHP-1, indicating that MDM might be more sensitive. It should be noted that the absolute concentration values for TNF- $\alpha$  are much lower than those measured for IL-6 and IL-8, which means that a small variation in the measured concentrations for TNF- $\alpha$  corresponds to a bigger variation in the %CV. In conclusion, we propose to measure all three cytokines, IL-6, IL-8, and TNF- $\alpha$ .

Aerosol exposure via an ALI gives rise to multiple sources of variation between laboratories. First, variation can be introduced by differences in the deposited doses of LPS, DQ12 and TiO<sub>2</sub> NM-105 between the labs. Bannuscher et al. (Bannuscher et al., 2022) describe the variation between labs in the deposited dose using the VITROCELL® Cloud12 system and its accurate real-time determination using a quartz crystal microbalance (QCM). Improvements in the SOP for performing ALI exposures using the VITROCELL® Cloud12 system have decreased the variation between the labs. Nevertheless, they do observe differences in the deposited doses that might have contributed to the observed differences in the cellular response. The advantage of using the VITROCELL® Cloud system is the opportunity to measure the deposited dose of the substance under investigation through the integrated QCM while performing the cell exposure. Variation in the lung co-culture models can be introduced by differences in the number of macrophages that remain attached on top of the Calu-3 cells and by differences in the activation state of the macrophages between the labs. It is known that THP-1 cells can vary between batches, passage number, and labs (Xia et al., 2013; Piret et al., 2017; Sakaguchi et al., 2010). Primary cells (MDM) are known for their donor-to-donor variation. Second, the use of different cell batches, and different FCS manufacturers probably has introduced variation. Barsova et al. showed that interlaboratory variation can be decreased by using the same FCS batch (Barsova et al., 2021). Third, differences in the personnel performing the assays and the equipment used may have introduced variation. In some labs, a single person was responsible for performing all assays, which might have had a positive effect on the results, by decreasing the intra-laboratory variation.

One can debate if in inter-laboratory comparison studies the same batches of cells and reagents should be used, as this would decrease the



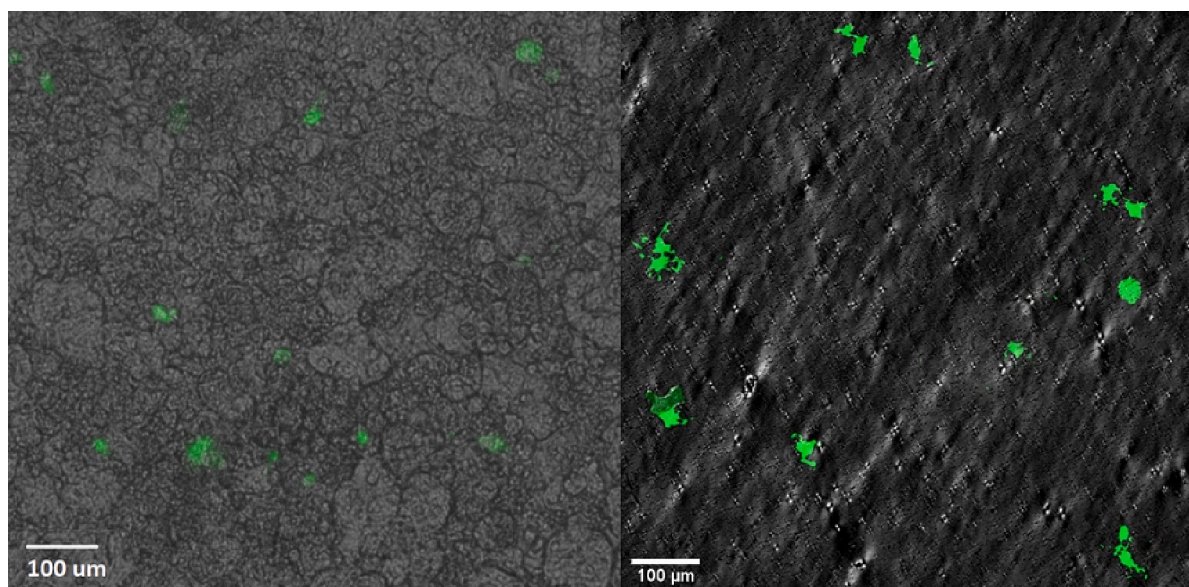


Fig. 7. Representative fluorescence microscope pictures from the co-culture models with the macrophages stained green. Calu-3 with dTHP-1 (left) and Calu-3 with MDM (right). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

variability. However, this approach cannot be replicated in common practice, since in future tests other labs will not have access to exactly the same batches of cells and reagents. Therefore, testing the variability and reproducibility of results under more realistic conditions (using different batches of cells and FCS) helps to understand the robustness of the model. Even though we did not use the same batches of cells and FCS in the current inter-laboratory comparison study, we did observe agreement in the results across all labs.

A difficulty of the present study is that the deposited doses of DQ12 and TiO<sub>2</sub> NM-105 probably were too low to induce detectable cellular responses. The deposited dose was about 1 μg/cm<sup>2</sup>, which corresponds to doses that induce effects *in vivo* (Braakhuis et al., 2021; Reuzel et al., 1991; Muhle et al., 1995; Heinrich et al., 1995; Monteiller et al., 2007; van Ravenzwaay et al., 2009; Borm et al., 2018). However, usually higher doses are used *in vitro* to detect toxicity of NMs, especially when exposing cells under submerged conditions when concentrations up to 100 μg/mL or even milligrams are tested which corresponds to ca. 33 μg/cm<sup>2</sup> or more for an assumed 0.3 ml/cm<sup>2</sup> of cell culture medium (Jang et al., 2021). The use of such high concentrations under submerged cell culture conditions may induce formation of unrealistically large NM agglomerates, particle-assay interference, and non-NM specific cytotoxicity due to e.g. nutrient depletion of the cells being completely covered with a thick layer of NM. In the current study, we aimed to test realistic doses under realistic exposure conditions using aerosol-based ALI exposure and the primary aim of this study was to compare the results among the different laboratories and not necessarily to induce a toxic response.

The lack of a detectable cellular response may also be due to the specific *in vitro* lung model used, or possibly *in vitro* lung models in general. The Calu-3 cells are relatively robust compared to other epithelial cells (He et al., 2021) making them suitable for repeated exposure at the ALI while at the same time less suitable for detecting (nano)particle toxicity. By adding the macrophages, the sensitivity of the model is enhanced. The model sensitivity could be further enhanced by including more sensitive read-outs such as transcriptomics or metabolomics. In addition, multiple doses and higher doses could have been tested. Using VITROCELL® Cloud nebulization, a dose-range can be tested by either increasing the concentration in the nebulized particle suspension or by applying repeated nebulization to increase the deposited dose (e.g. by comparing a single, double and triple dose). Future studies could include a dose-range to allow dose-response analysis and

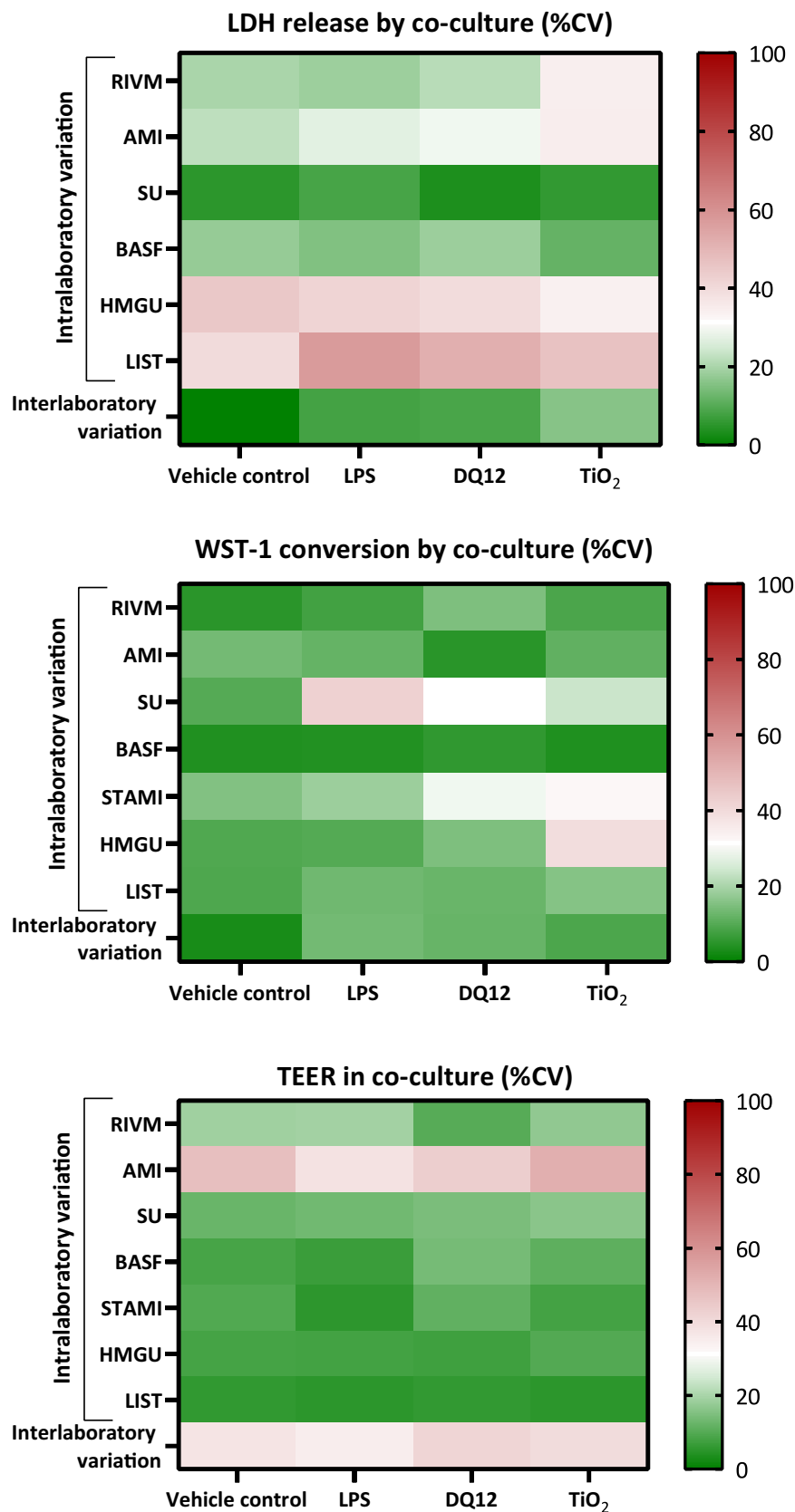
comparison of potencies between different materials.

Both bronchial epithelial and alveolar epithelial cell models are viable options for *in vitro* toxin exposure under ALI conditions, especially NMs, since both the tracheobronchial and alveolar regions are supposed to receive NMs on their surface. Deposition of NMs is not limited to the alveoli, and with decreasing air velocity, substantial deposition can also occur on the terminal bronchial epithelium (Braakhuis et al., 2014). In addition, macrophages are important in detecting particle toxicity (Wang et al., 2021) and they are not confined to the alveoli; they are present throughout the respiratory system (Brain, 1988; Tang et al., 2022; Bosso et al., 2022) and are therefore included in the lung model in the current study. Nonetheless, to comprehensively evaluate inhalation toxicity of NMs *in vitro*, it is warranted to study their effects both in bronchial epithelial cell models and alveolar epithelial cell models. Therefore, a transferability/reproducibility study should also be performed on a selected alveolar model, including a co-culture model.

An additional topic for discussion is the difference in cellular response between the Calu-3 cells and the macrophages. In the co-cultures, the observed response is from both cell types and one cannot discriminate between the two. Therefore, we cannot be certain that the macrophages were unaffected by the exposures and remained viable during the exposure period. As the deposited doses were chosen to be relatively low (realistic *in vivo* doses are low compared to submerged exposure) decreased viability of the macrophages is not expected, however, this was not verified in the current interlaboratory experiment. Moreover, one can debate that for screening purposes using macrophages alone might be the preferred option as they show higher sensitivity towards (nano)particle exposure.

Performing inter-laboratory comparison studies is challenging. Based on our experience with this (and earlier) inter-laboratory comparison studies, we would like to recommend the following for future comparison studies:

1. Use a biphasic pre-validation study, the first phase focusing on transferability with two to three labs (naïve and experienced), with SOPs being optimized, and the second phase focusing on inter-laboratory reproducibility with a larger number of participating labs. Of course, all participating labs should have trained personnel, adequate equipment and if possible be certified, e.g., ISO 17025.
2. Train personnel on-site. Unfortunately, due to COVID-19, this was not possible for the current inter-laboratory study. We used



**Fig. 8.** Coefficient of variation (%CV) for measurements of LDH release (left), WST-1 conversion (middle) and TEER (right) in the co-culture model (Calu-3 + macrophages). Data are from  $N=3$  experiments (RIVM, AMI, SU, HMGU, LIST),  $N=2$  experiments (BASF) and from a single experiment with 4 technical replicates (STAMI).

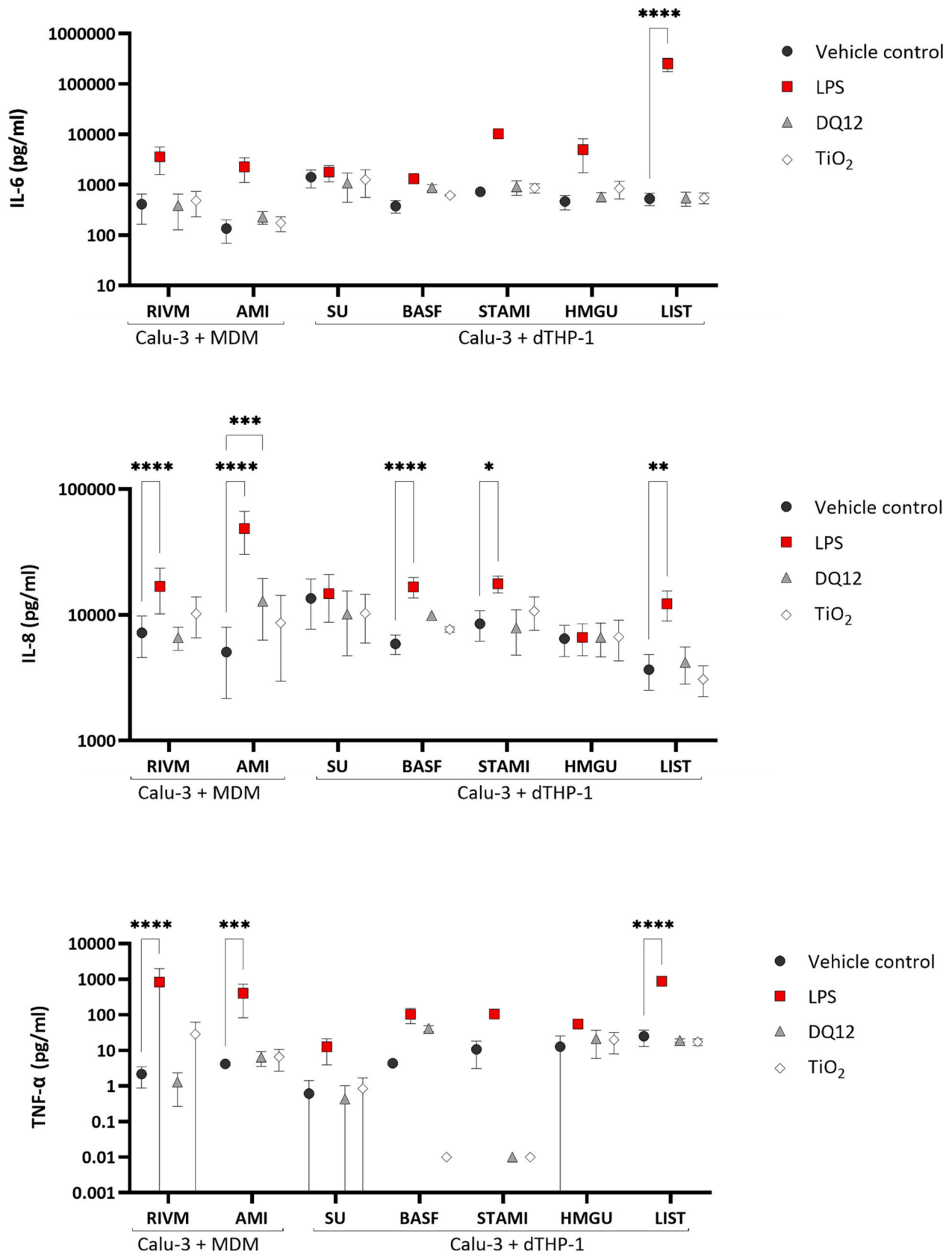
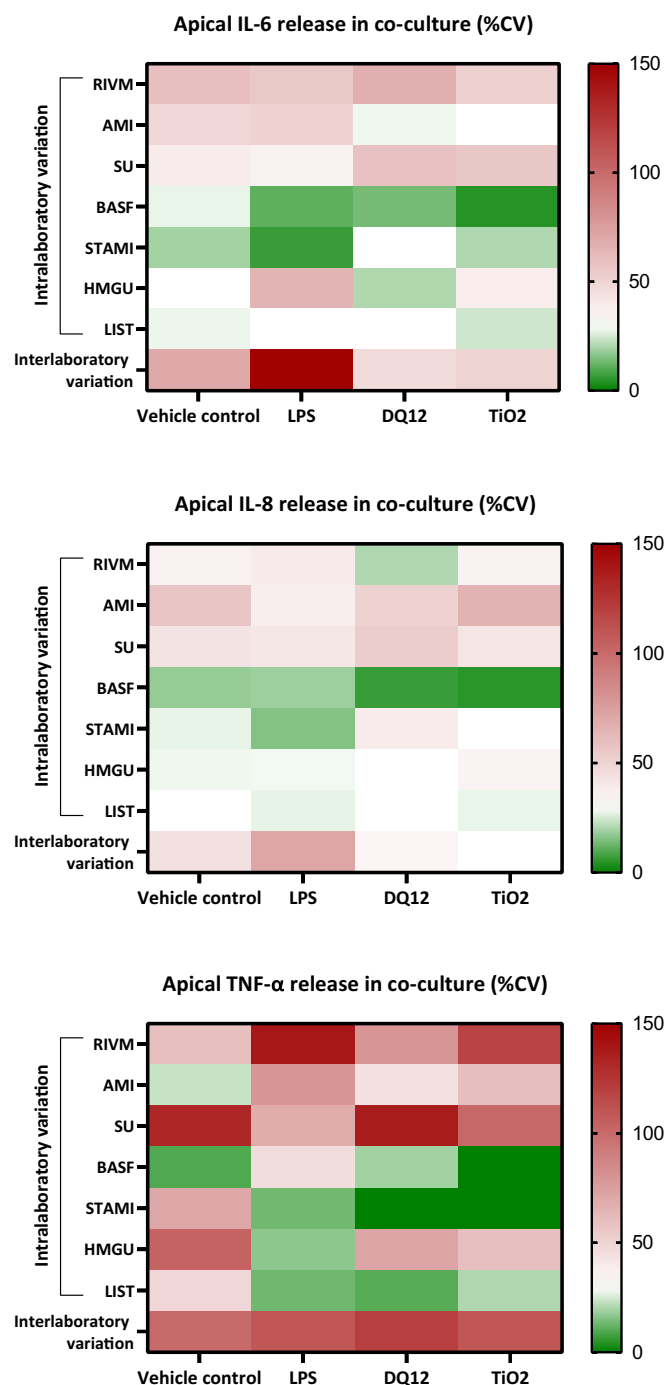


Fig. 9. IL-6, IL-8 and TNF-α release by the Calu-3 / macrophage co-cultures measured at the apical side at 24 h after exposure. Mean ± SD of N = 3 experiments (RIVM, AMI, SU, HMGU, LIST), mean ± SD of N = 2 experiments (BASF) and mean ± SD of a single experiment (STAMI). TNF-α levels were below the detection limit for the DQ12- and TiO<sub>2</sub>-exposed cells at STAMI. Note that the Y-axes are on a log scale.



**Fig. 10.** Coefficient of variation (%CV) for measurement of IL-6 (left), IL-8 (middle), and TNF- $\alpha$  release in co-culture models (Calu-3 and macrophages). Data are from N = 3 experiments (RIVM, AMI, SU, HMGU, LIST), N = 2 experiments (BASF) and from a single experiment with 4 technical replicates (STAMI).

interactive webinars to let all partners acquire knowledge on the models. Information on hands-on work with the model was also obtained from the published video (Braakhuis et al., 2020). In addition, information on replacing and cleaning the quartz of the QCM was obtained from a video (VITROCELL, 2022).

- SOPs, prepared in line with OECD GIVIMP rules, need to be very detailed to avoid miscommunication.
- Establishment of the cell culture model with characterization of morphology, viability and cell-type specific markers is required. In

the co-culture model, attachment of the macrophages to the Calu-3 cell layer is a critical issue. It is advised to check macrophage attachment via staining of macrophages followed by microscopy both before and after exposure.

- Aerosolized NM application to ALI cell culture models is a complex and critical step in the SOP. Sufficient time and effort should be reserved for method development of nebulizing different test materials. When using the VITROCELL® Cloud, especially the 12-well model, the QCM that is used to measure ENM deposition, is an intricate tool. In addition to the training provided by VITROCELL Systems, it is advised to read background information on the QCM (Ding et al., 2020), and on using the VITROCELL® Cloud system with integrated QCM for real-time monitoring of NM deposition on the cells (Bannuscher et al., 2022).
- A dose finding experiment is recommended to identify a dose level where at least one of the investigated response parameters shows a statistically significant response.
- TEER can show high variations among laboratories, but should still be used as a quality control and sensitive response parameter to assess the lung tissue barrier properties in one laboratory before and after particle exposure.
- When measuring cytokine release, optimizing sample dilution and performance of the assays by a single person is recommended.
- Before exposure of the models to the NMs, the response to LPS should be checked and optimized. This includes checking the biological activity of LPS and the number of viable macrophages in the co-culture (He et al., 2021).

To conclude, we evaluated the transferability and reproducibility of a lung co-culture model and its aerosolized exposure to two types of NM via the ALI. Detailed SOPs for culturing and exposing the lung models are available via PATROLS <https://patrols-h2020.eu/publications/sops/index.php>. We are confident that, based on these detailed SOPs and 1) using a stepwise approach for a new round of interlaboratory comparison as described above and 2) increasing the deposited doses of NMs or using NMs with higher mass-specific toxicity e.g. CuO, and 3) including more sensitive read-outs (e.g. RNA sequencing) the predictive value of these models can be enhanced and that they may be taken into consideration for a possible OECD guideline.

#### CRediT authorship contribution statement

**Hedwig M. Braakhuis:** Conceptualization, Methodology, Formal analysis, Resources, Writing – original draft, Visualization, Supervision. **Eric R. Gremmer:** Investigation. **Anne Bannuscher:** Investigation. **Barbara Drasler:** Investigation. **Sandeep Keshavan:** Investigation. **Barbara Rothen-Rutishauser:** Conceptualization, Writing – review & editing, Funding acquisition. **Barbara Birk:** Conceptualization, Writing – review & editing. **Andreas Verlohner:** Writing – review & editing. **Robert Landsiedel:** Writing – review & editing. **Kirsty Meldrum:** Investigation, Writing – review & editing. **Shareen H. Doak:** Funding acquisition. **Martin J.D. Clift:** Writing – review & editing, Funding acquisition. **Johanna Samulin Erdem:** Writing – review & editing. **Oda A.H. Foss:** Investigation. **Shanbeh Zienolddiny-Narui:** Writing – review & editing. **Tommaso Serchi:** Writing – review & editing. **Elisa Moschini:** Investigation. **Pamina Weber:** Investigation. **Sabina Burla:** Investigation. **Pramod Kumar:** Investigation. **Otmar Schmid:** Writing – review & editing. **Edwin Zwart:** Investigation. **Jolanda P. Vermeulen:** Investigation. **Rob J. Vandebriel:** Conceptualization, Writing – review & editing, Supervision.

#### Declaration of Competing Interest

Barbara Birk, Andreas Verlohner and Robert Landsiedel are employees of BASF. This model might be used in the future for the registration of BASF products. Otmar Schmid and Pramod Kumar are

employees of the Helmholtz Center Munich, which receives license fees for the VITROCELL Cloud technology. Otmar Schmid, a co-patent holder of the underlying ALICE Cloud technology, receives a share of these license fees. All other authors declare no competing financial interests.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.impact.2023.100466>.

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