

# Downscaling of a Three-Dimensional Alveolar *In Vitro* Model for High-Throughput Screening of Inhalable Respiratory Sensitizers in the Food Industry

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## RESEARCH ARTICLE

### Abstract

Allergic diseases represent an increasing global health problem, among which food and respiratory allergies play an important part. Inhalation of sensitizers from food sources primes the immune system for an exacerbated response upon subsequent exposure via the ingestion route. With the increasing emergence of novel food additives and proteins and growing morbidity in both food and respiratory allergies, the hazard assessment of the allergenicity of food components is of paramount importance. A three-dimensional (3D) alveolar *in vitro* model for respiratory sensitization prediction was downscaled from the 6- to the 24-well plate insert format, to meet the industry's high-throughput (HTS) screening requirements. The resemblance of the cellular architecture of the downscaled model to the alveolar region of the lung was evaluated through microscopy techniques and the functionality of the *in vitro* model was assessed following air-liquid interface (ALI) exposure to ammonium persulfate (APS). The model represents a promising *in vitro* tool to screen for the respiratory sensitization potential of food components in a fast and reliable approach.

**Keywords:** alveolar, air-liquid interface, cell surface markers, cytokines, *in vitro*


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## INTRODUCTION

Allergic diseases represent a growing public health problem, as the prevalence and incidence are increasing worldwide (Loh and Tang, 2018), posing a considerable socio-economic impact. It is acknowledged that allergies affect the quality of life of the concerned individuals, and represent a significant burden for the healthcare systems (Papadopoulos et al., 2012; Fox et al., 2013; Pawankar, 2014; Peniamina et al., 2016). All allergies develop in two phases: sensitization or induction and elicitation phase. In the sensitization phase, exposure of a susceptible individual to a sensitizer triggers an adaptive immune response which primes the immune system for lasting elevated responsiveness to the sensitizer. On a secondary exposure to the sensitizing agent, via a relevant route, which in

the case of food allergy is by ingestion and in respiratory allergies by inhalation, an inappropriate immune response is generated, resulting from a local reaction, with the possibility to develop a systemic inflammatory response. This represents the elicitation phase (Krutz et al., 2020).

Food allergy is defined as an adverse health effect arising from a nontoxic, IgE-mediated mechanism that occurs reproducibly on oral exposure to a given food (WHO, 2022). Respiratory allergies, such as rhinitis, rhinoconjunctivitis, and asthma are induced by exposure to airborne sensitizers through immune-mediated mechanisms (Frew, 1996). The allergic reaction to foods can be induced in sensitized subjects following exposure to animal and vegetable high molecular weight (HMW) proteins and enzymes or low molecular weight (LMW) chemicals such as food additives (Jeebhay et al., 2019; Witkowski et al., 2022).

Allergies are generally considered to be organ-specific (Pucci et al., 2008), but their systemic nature is substantiated by two factors: (1) the possibility of developing the allergy at a different location in the human body, than at the location of the route of exposure in the sensitization phase; (2) manifestation of allergy symptoms at a different level, than the organ of exposure, such as acute respiratory symptoms following food allergens ingestion (Nowak-Węgrzyn et al., 2014).

Food hypersensitivity reactions following oral ingestion have been the center of attention, while allergies caused by exposure to food allergens by non-ingestant routes were probably under-recognized and under-reported. Lately, the reactions caused by skin contact and inhalation have slowly gained interest due to the life-threatening potential of some food allergens. Hypersensitivity to food allergens may also develop through non-gastrointestinal exposure, by inhalation and skin exposure (Asero and Antonicelli, 2010; Ramirez and Bahna, 2009; Roberts and Lack, 2003; van Bilsen et al., 2017), and can impact not only the gastrointestinal tract, but can affect other organ systems (Salvatori et al., 2008), leading to mild local reactions or severe systemic effects.

Some food allergens can induce reactions via the inhalation route only, localized in the respiratory tract, yet be well-tolerated following ingestion. One peculiar case of such allergens are those from the bakery industry, such as flour proteins, additives and enzymes, inducing Baker's asthma, which is one of the most common causes of occupational asthma (OA) worldwide. Interestingly, patients with Baker's asthma usually ingest wheat products without developing any allergic manifestations (Brisman, 2002; Cartier, 2010).

Food additives are defined in the Regulation (Ec) No 1333/2008 Of The European Parliament and of the Council of 16 December 2008 on food additives as "any substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food, whether or not it has nutritive value, the intentional addition of which to food for a technological purpose in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food results, or may be reasonably expected to result, in it or its by-products becoming directly or indirectly a component of such foods". One of the general conditions for use of food additives is its safety regarding human health and consumer protection at the level of use proposed. The increasing number of additives used to ameliorate the qualities of food products and the emergence of new proteins deems the allergenicity and safety assessment of such food components necessary (Krutz et al., 2020; Mullins et al., 2022). The sensitization potential of food additives should be correctly evaluated, as inhalation of food components can be a leading cause of allergic sensitization which may result in OA and food allergy (Cartier et al., 2010).

Ammonium persulfate (APS) is a LMW inorganic compound with strong oxidizing properties, used in various industries. APS is used as an oxidizing agent in personal care products, such as hair bleaching and hair coloring cosmetics, while in the food industry it is used as a food preservative (antimicrobial) and additive (modification of starch). It is also used in food contact materials, as component of adhesives, paper and paperboard materials of food packaging (US FDA; Warshaw et al., 2022). Despite its successful application in many industrial sectors, APS is also a chemical agent inducing OA in hairdressers (Macan et al., 2022).

A three-dimensional (3D) alveolar *in vitro* model was developed for the identification of respiratory sensitizers (Chary et al., 2019). The model can correctly identify LMW and HMW allergens, being therefore a promising model to evaluate the sensitizing potential of a large variety of potentially sensitizing agents. The model was originally designed for 6-well plate cell culture inserts. With the emergence of novel materials which ameliorate the quality of food and consumer products in general, the biotechnology field is challenged by the need to scale up *in vitro* experimentation to meet industrial needs. The opposing force is the necessity to downscale *in vitro* models to reach high-throughput screening (HTS) (Goers et al., 2014).

This study aims to meet the industry needs for HTS testing, by downscaling the 3D alveolar *in vitro* model from the 6- to the 24-well plate insert format. The cell densities and cell culture medium volumes were optimized for the downscaled format, as these are two crucial parameters when establishing physiologically relevant *in vitro* models. The downscaled 3D alveolar *in vitro* model was then characterized and challenged using APS and relevant controls, and respiratory sensitization endpoints were assessed to validate the successful downscaling of the *in vitro* model.

## MATERIALS AND METHODS

All reagents, unless otherwise specified, were purchased from Sigma-Aldrich (Overijse, Belgium). Cell culture media and UltraPure™ DNase/RNase-Free Distilled Water were purchased from Gibco (Erembodegem, Belgium) and 2-

mercaptoethanol from Bio-Rad (Temse, Belgium).

## Mammalian cell culture

### Cell lines

The *in vitro* model employs the use of three human cell lines: type II alveolar epithelial cell line (A549) (Lieber et al., 1976), endothelial cell line (EA.hy926) (Edgell et al., 1983) and monocyte cell line (THP-1) (Tsuchiya et al., 1980). The cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in culture using respective complete cell culture media presented in Table 1.

**Table 1.** Complete cell culture media for the cell lines and the build-up of the *in vitro* model.

Monocultures			
Cell line	Medium	Fetal Bovine Serum (FBS)	Supplement
A549	DMEM		25 mM HEPES
EA.hy926		10% (v/v)	
THP-1	RPMI 1640		50 $\mu$ M $\beta$ -ME
Co-cultures			
Condition	Medium	FBS	Supplement
Submerged (cell growth and proliferation)	75% HEPES-buffered	10% (v/v)	
Air-liquid interface (maintenance)	DMEM; 15% RPMI; 10% IMDM	1% (v/v)	25 mM HEPES
Exposure			

Note: DMEM=Dulbecco's Modified Eagle's Medium; RPMI-1640=Roswell Park Memorial Institute 1640; IMDM=Iscove's Modified Dulbecco's Medium; FBS=Fetal Bovine Serum Superior. All media contained GlutaMAX™ (L-alanine-L-glutamine) instead of L-glutamine.

### Identification of cell lines

The correct identity of each cell line used in this study was confirmed through the Human STR profiling cell authentication service provided by ATCC. A 40  $\mu$ L volume of cell suspension (containing at least  $1 \times 10^6$  cells) of each cell line was placed on the Sample Collection Card. The sample collection cards were allowed to dry at room temperature and then shipped to ATCC for the genotyping of the cell lines. The correct identity of all three cell lines was confirmed by ATCC.

### Three-dimensional (3D) alveolar *in vitro* model build-up

The original 3D alveolar *in vitro* model was developed by Chary et al., 2019. The model was designed using the 6-well plate hanging cell culture insert. The 6-well plate insert presents a high pore density Polyethylene Terephthalate (PET) membrane with an effective growth surface area of 4.5 cm<sup>2</sup> and 5  $\mu$ m diameter pores. For the downsized model, the 24-well plate hanging cell culture insert was used, fitted with a membrane that has an effective growth area of 0.3 cm<sup>2</sup>. The well of the 6-well plate has an effective area of 9.6 cm<sup>2</sup> and the well of the 24-well plate an effective surface of 1.9 cm<sup>2</sup>. These values of surface areas were considered when establishing the cell seeding densities and volumes, presented in Table 2.

**Table 2.** Cell type, seeding density and cell culture medium volume.

Cell type	Seeding density (cells/cm <sup>2</sup> )	Seeding volume ( $\mu$ L)	Insert membrane/Plate surface area (cm <sup>2</sup> )
Endothelial (EA.hy926)	$3.33 \times 10^4$	60	0.3
Epithelial (A549)	$6.66 \times 10^4$	200	0.3
Dendritic-like (DC-THP-1)	$1.05 \times 10^4$	150	0.3
Macrophage-like (M $\Phi$ -THP-1)	$12 \times 10^4$	200	1.9

The 3D alveolar *in vitro* model was assembled as previously described (Klein et al., 2013) with some modifications (Chary et al., 2019). On day 0, THP-1 cells were seeded in T175 flasks at a density of  $4 \times 10^5$  cells/mL in complete cell culture medium and stimulated to differentiate into macrophage-like THP-1 cells (MΦ-THP-1) by addition of phorbol-12-myristate-13-acetate (PMA) at a concentration of 20 ng/mL, for 48 hours in a humidified incubator at 37 °C and 5% CO<sub>2</sub>. The PMA-supplemented cell culture medium was removed on day 2 by aspiration and replaced by fresh THP-1 complete cell culture medium. Differentiation of THP-1 cells into MΦ-THP-1 represents a modification of the protocol for the build-up of the *in vitro* model proposed by Chary et al., 2019, as higher differentiation rates have been observed with 48h PMA stimulation compared to the 24h PMA stimulation. The MΦ-THP-1 were left to rest for another 4 days. PMA was prepared as a stock solution (10 mg/mL) in ultrapure absolute ethanol. Stocks were kept in the freezer, at -20 °C. MΦ-THP-1 cells were rinsed with Dulbecco's modified phosphate buffered saline (DPBS) and detached using accutase to harvest them (day 6). On day 2, plates with hanging cell culture inserts were prepared and inverted to seed the human endothelial cells, EA.hy926, on the basolateral side of the insert semipermeable membrane. Upon attachment of cells to the membrane, the plate with inserts was turned back to its original orientation and A549 epithelial cells were seeded in the cell culture inserts, at the apical side. Medium in the basolateral compartment was adjusted to a final volume of 960 μL and epithelial and endothelial cells were grown to confluency for three days, at 37 °C and 5% CO<sub>2</sub> in a humidified atmosphere. On day 5 the cell culture medium was removed from the apical and basolateral compartments by aspiration, and fresh co-culture medium supplemented with 10% FBS (v/v) was added to both apical and basolateral compartments, to ensure the proliferation of epithelial and endothelial cells. On day 6, DC-THP-1 cells were seeded in the wells of new 24-well plates, and the inserts were transferred from the old plates to the new plates. MΦ-THP-1 cells were seeded in the apical compartment, on the surface of the A549 cells monolayer, to complete the tetra-culture *in vitro* model. The medium for the complete *in vitro* model contained only 1% FBS to avoid extensive proliferation of DC-THP-1 cells. The attachment of MΦ-THP-1 cells to the epithelial monolayer was verified by light microscopy, 4 h post-seeding. Upon attachment of MΦ-THP-1, the medium was removed from the apical compartment by gentle aspiration, the *in vitro* model was cultivated for additional 24 h, at the air-liquid interface (ALI), prior to exposure.

## Exposure

### *Lipopolysaccharide (LPS) and thymic stromal-lymphopoietin cytokine (TSLP)*

LPS, an endotoxin shown to induce a strong pro-inflammatory response in lung cell models (Bisig et al., 2019), and TSLP, an epithelium derived mediator of allergic reaction known to activate antigen-presenting cells such as dendritic cells (Paplinska-Goryca et al., 2020), were used as positive controls. 10 μL of a freshly mixture prepared in co-culture medium supplemented with 1% FBS containing LPS resuspended in DPBS (final concentration: 10 μg/mL) and TSLP resuspended in DPBS (final concentration: 20 ng/mL) were added in the apical compartment of the positive control inserts.

### *Submerged exposure*

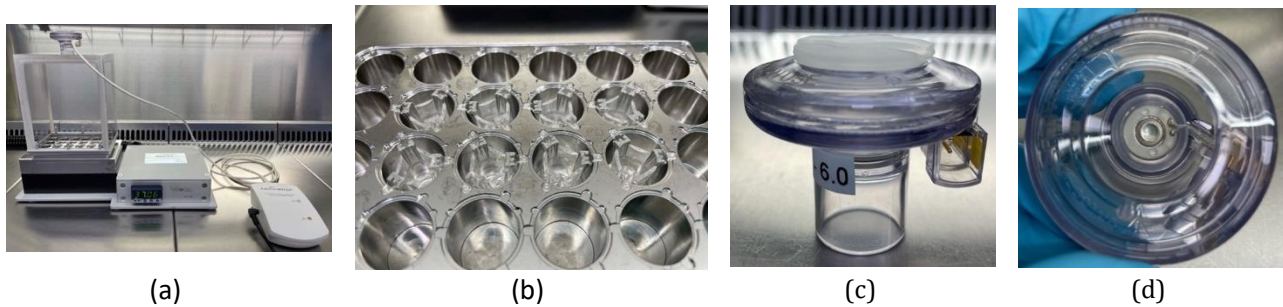
The *in vitro* model was exposed in submerged conditions to determine the optimal doses for the air-liquid interface (ALI) exposure. Briefly, the stock solution of ammonium persulfate (APS) was prepared in UltraPure™ DNase/RNase-Free Distilled Water and a series of dilutions of APS was prepared in co-culture medium 1% FBS. The *in vitro* model was exposed to 10 μL of the co-culture medium 1% FBS containing increasing concentrations of APS and 10 μL of the cell co-culture medium 1% FBS without chemical treatment as vehicle control. The 10 μL volume is optimal to cover the apical surface of the insert and at the same time to establish the range of APS solution concentrations able to decrease the viability of cells in the *in vitro* model.

**Table 3.** Exposure doses (μg/cm<sup>2</sup>) and concentrations (mg/mL) applied at the ALI and in submerged conditions, respectively.

APS exposure	Vitrocell® Cloud-24	Submerged
	μg/cm <sup>2</sup>	mg/mL
E1	300	9
E2	500	15
E3	750	22.5
E4	800	24
E5	850	25.5
E6	900	27

### *Air-liquid interface (ALI) exposure*

The Vitrocell® Cloud-24 exposure system (Vitrocell®, Waldkirch, Germany) was used for the exposure of the *in vitro* model at the ALI, to test the influence of the selected chemical compound (APS) compared to the vehicle control (Figure 1, A). The exposure system allows the simultaneous exposure of up to 24-well plate cell culture inserts (Figure 1, B). A stock solution of APS and serial dilutions of the stock solution were prepared in UltraPure™ DNase/RNase-Free Distilled Water. Exposure solutions were prepared by mixing 50% of the APS solution dilutions with 50% of DPBS (v/v), to produce an aerosol out of the APS solution. The exposure system was used with an Aerogen® nebulizer provided with a vibrating membrane (Figure 1, C, D) that generates aerosols from liquid solutions and suspensions in a closed chamber. The aerosolized solutions of APS settled down on the base plate (Figure 1, B), which is heated up to 37 °C (Figure 1, B). The nebulized cloud allows a dose-controlled and spatially uniform aerosol deposition in the apical compartment of the inserts.



**Figure 1.** Vitrocell® Cloud-24 exposure system (A), base plate allowing the simultaneous exposure of up to 24-well plate inserts (B) and Aerogen® nebulizer (C) fitted with a vibrating mesh membrane (D)

### **3D alveolar *in vitro* model characterization**

#### *Electron microscopy (EM): sample preparation and imaging*

Cell culture medium was removed from inserts on day 7 of the workflow, and the apical and basolateral compartments were washed twice with DPBS. Cells were fixed with 4% (v/v) formaldehyde in DPBS for 15 minutes at room temperature. Inserts containing the fixed cells were washed three times with DPBS and post-fixed with 1% osmium tetroxide (OsO<sub>4</sub>) in DPBS for 1 h at room temperature, in the dark. Cells were dehydrated in ethanol of ascending concentrations (30, 50, 70, 90, and 100%), for 10 minutes per concentration, and later with acetone 100% for 2 hours. Insert membranes were detached from the hanging cell culture insert supports and embedded in Spurr resin (Polysciences, Hirschberg, Germany). Sections with a thickness of 300 nm were obtained with an ultramicrotome (Leica, Vienna, Austria) and mounted on silicon wafers. The electron microscope (EM) images were acquired with a focused ion beam scanning electron microscope (FIB-SEM) (Scios DualBeam, ThermoFisher, Eindhoven, The Netherlands) using a back-scattered electron (BSE) detector. Several images of various zones of each sample were taken at 5kV and 0.4 nA of probe current. Images were recorded as a matrix of 1536 x 1024 image points with a counting time of 5 μs/pixel.

#### *Confocal microscopy: cell fixation, labelling and image restoration*

Inserts with endothelial, epithelial and macrophage-like THP-1 cells were prepared. The inserts were washed with DPBS prior to staining. Samples were fixed with 4% (v/v) formaldehyde in DPBS (15 minutes at room temperature) and washed three times with DPBS. Samples were permeabilized using a 0.1% (v/v) Triton X-100 in DPBS (10 minutes at room temperature) and then blocked with a 10% (w/v) BSA in DPBS solution (1 hour, room temperature, on a horizontal shaker). Samples were incubated with the primary antibody, Mouse anti-CD11b (1:10 dilution in 1% (v/v) BSA in DPBS solution) at room temperature, gently shaking on a horizontal shaker, for 1h. All the following steps were performed in the dark, to avoid alteration of fluorescent probes. Fluorescently tagged secondary antibody, AlexaFluor488 Cross-Adsorbed (Invitrogen, Eugene, OR, USA) (diluted 1:500 in 1% v/v BSA in DPBS) was used. Nuclei were counterstained with Hoechst 33342 dye (Invitrogen, Eugene, OR, USA) using a 1:10000 dilution in DPBS (at room temperature, gently shaking on a horizontal shaker for 15 minutes). Samples were imaged using a Zeiss LSM 880 laser scanning confocal inverted microscope (Zeiss, Jena, Germany). Image processing and visualization was performed using the Zeiss Software ZEN 2011.

#### *Surfactant droplet test*

The presence of pulmonary surfactant was determined via the DMP/O droplet test performed as previously described (Schürch et al., 1978; Rothen-Rutishauser et al., 2008; Klein et al., 2013). A 4:1 (v:v) solution of dimethyl

phthalate and octanol (DMP/O) was prepared, and 4 mg/mL of crystal violet were added for visual inspection. Droplets of DMP/O solution containing crystal violet were pipetted on the cells from the apical compartment kept at the ALI, to determine the surface tension.

### Cell viability assessment

Following 24 h exposure to the increasing concentrations (mg/mL) and doses ( $\mu\text{g}/\text{cm}^2$ ) of APS (Table 3) and the corresponding negative control (vehicle control), the cell viability of the 3D alveolar *in vitro* model was evaluated using the resazurin assay. Co-culture medium containing 1% FBS (v/v) was supplemented with 20 mM resazurin (sodium salt) stock solution prepared in DPBS to reach a working concentration of 400  $\mu\text{M}$  resazurin. 200  $\mu\text{L}$  of the working solution of resazurin was added in the apical compartment of the insert (A549+M $\Phi$ -THP-1 cells). As the basolateral compartment of the insert contains adherent endothelial cells (EA.hy926) and DC-THP-1 cells in suspension, 3  $\mu\text{L}$  of 20 mM resazurin stock solution were added to the 150  $\mu\text{L}$  co-culture 1% FBS medium present in the well of the plates and then mixed to reach a homogenous solution of 400  $\mu\text{M}$  resazurin. The plates with inserts were incubated at 37 °C, 5% CO<sub>2</sub>, in the dark. After 1 h, medium was sampled from the apical and the basolateral compartments and moved to a 96-well plate. The fluorescence intensity of resorufin, the metabolized resazurin, was measured at 530 nm excitation and 590 nm emission using a multi-mode microplate reader (Spark 20M, Tecan, Mechelen, Belgium).

### Flow cytometry measurements

After 24 h exposure to the increasing concentrations of APS (50, 100, 200, 300, 500  $\mu\text{g}/\text{cm}^2$ ), LPS and TSLP mixture (positive control) and the corresponding negative control (vehicle control), DC-THP-1 cell suspension was collected from the wells of the plate and was centrifuged for 5 minutes at 300 x g. The supernatant of each exposed sample was then collected and stored at -80 °C for further analysis. The DC-THP-1 cell pellet was resuspended and washed in DPBS at room temperature. The cells were centrifuged for 5 minutes at 300 x g, the DPBS wash was discarded and the cells were resuspended in DPBS containing 2% FBS (v/v) to avoid the unspecific binding of antibodies. Cells were stained at 4 °C for at least 30 minutes using commercially available fluorescent-labelled monoclonal antibodies: allophycocyanin (APC)-anti TSLPr (clone 1F11), Brilliant™ Blue 515 (BB515)-anti CD54 (clone HA58) and Phycoerythrin (PE)-anti CD86 (clone 2331 [FUN-1]), and isotype-matched controls (mouse IgG1). Fluorescent-labelled monoclonal antibodies and isotype-matched controls were purchased from BD Pharmingen (Heidelberg, Germany). 1  $\mu\text{M}$  Sytox™ Blue Nucleic Acid Stain (Invitrogen, Carlsbad, NM, USA) was used as marker for non-viable cells. Surface marker expression was measured using the BD FACSCelesta™ Cell Analyzer (BD Biosciences Erembodegen, Belgium) instrument provided with the BD FACSDiva™ Software for data acquisition. 10000 events were acquired per sample and further analysis was performed by using FLOWJO Software V10 (FlowJo LLC, Ashland, OR, USA). Relative geometric mean fluorescence intensities (rMFI) were expressed as % compared to the vehicle control and calculated using the equation:

$$\frac{\text{MFI of the test item exposed sample} - \text{MFI of the test item exposed isotype control sample}}{\text{MFI of the vehicle control sample} - \text{MFI of the vehicle isotype control sample}} \times 100$$

### Cytokine release following exposure to APS and LPS

Respiratory sensitizer- and LPS-induced pro-inflammatory effects were investigated by quantifying the levels of GM-CSF, IFN- $\gamma$ , IL-6, IL-7, IL-10, IL-12, MCP-1, MIP-1 $\beta$  and RANTES in the cell culture medium collected from the basolateral compartment of the *in vitro* model exposed to vehicle (negative control), LPS and TSLP mix (positive control) and APS (25, 50, 100, 200  $\mu\text{g}/\text{cm}^2$ ). Cell culture medium containing the DC-THP-1 cells in suspension were collected at 24 h post-exposure from the wells of the plate. The collected medium was centrifuged twice, the supernatant was distributed in 96-well plates and stored at -80 °C until analysis was performed. The concentrations (pg/mL) of the selected cytokines were determined using a Bio-Plex 3D Suspension Array System (Bio-Rad Laboratories, USA) with the Human Bio-Plex Cytokine Magnetic 9-Plex Panel kit (Bio-Rad, USA) according to the manufacturer's recommendations. Biological triplicates were analyzed in technical duplicates and concentrations in range were accepted according to the Bio-Plex 3D Manager 6.1 software (Bio-Rad, Hercules, USA).

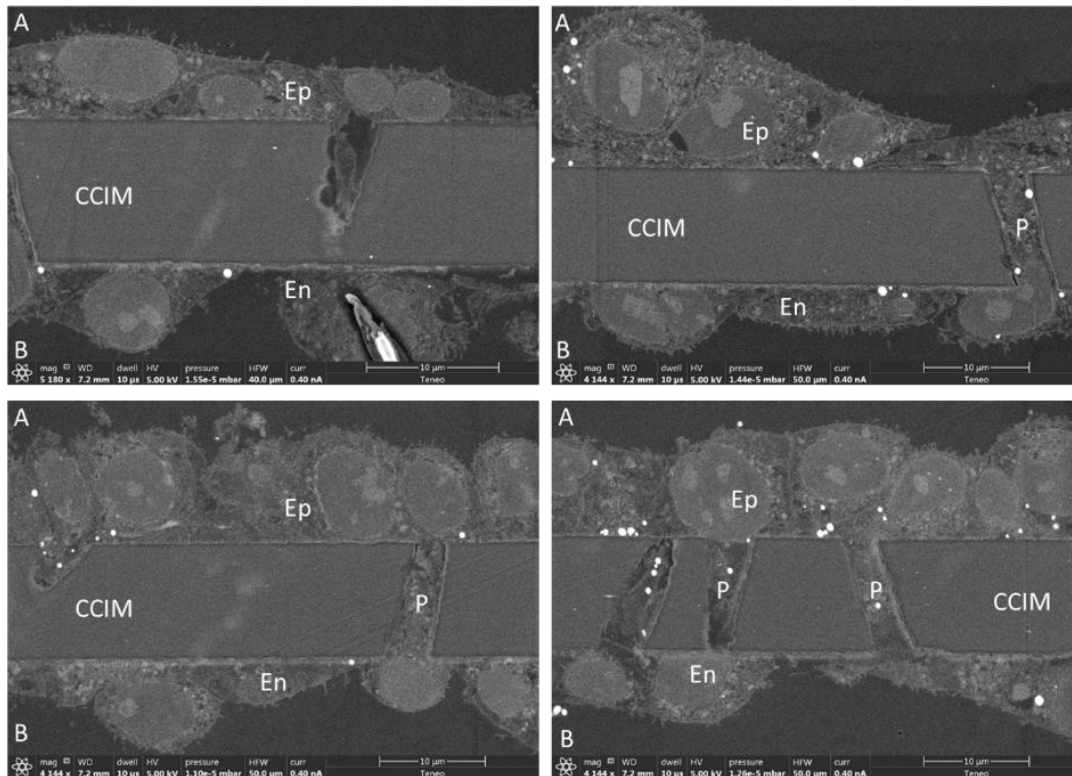
### Statistical Analysis

Data are presented as mean + standard deviation (SD) or standard error of mean (SEM). Data were analyzed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). Statistical and post-hoc tests, and the number of technical replicates (n) and biological replicates (N) are indicated on the description of the figures. Differences between groups were considered statistically significant when \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ; \*\*\*\* $P \leq 0.0001$ .

## RESULTS AND DISCUSSIONS

### 3D alveolar *in vitro* model cell architecture – Structure of the epithelial and endothelial cell monolayers

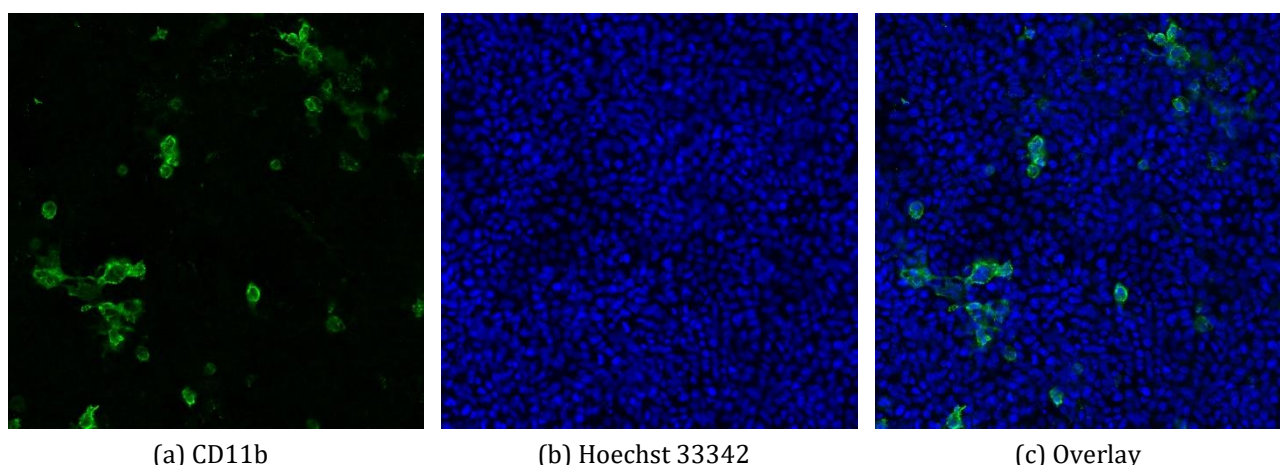
To produce a physiologically relevant alveolar region in our *in vitro* model, the endothelial and epithelial cells were grown to full confluency in submerged conditions and then cultured at the ALI to mimic the native lung-specific environment. The EM images (Figure 2) show that the 3D alveolar *in vitro* model built-up in 24-well plate inserts recapitulates the cellular architecture of the alveolar barrier. The epithelial (A549) and the endothelial (EA.hy926) cells grew in monolayers. Moreover, the 5  $\mu\text{m}$  pore diameter of the membrane of the insert allowed the direct contact and communication between the two cell types present on the opposite sides of the insert membrane.



**Figure 2.** Electron microscopy (EM) images illustrating the *in vitro* model. A – apical compartment; B – basolateral compartment; CCIM – cell culture insert membrane; En – endothelial cells (EA.hy926) monolayer; Ep – epithelial cells (A549) monolayer; P – cell culture insert membrane pore (Scale bar 10  $\mu\text{m}$ ).

### Distribution of macrophages on the epithelial cells surface

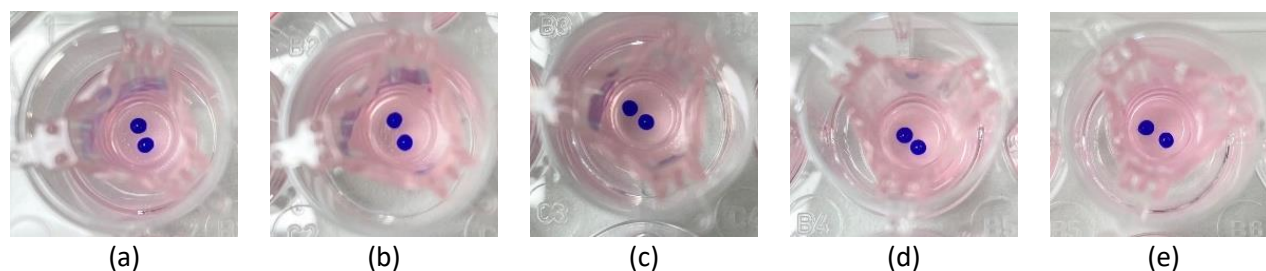
Alveolar macrophages are present on the surface of the human alveolar epithelium *in vivo* and their main role is to guard the lungs from foreign inhaled material, such as microorganisms (bacteria and viruses) and particulate matter. In addition, macrophages play a significant role in the maintenance of homeostasis of a tissue constantly exposed to xenobiotics (Draijer and Peters-Golden, 2017; Hirayama et al., 2018). THP-1 cells are a well-characterized and widely used surrogate of dendritic-like cells *in vitro* (Chanput et al., 2014), which can be differentiated into macrophage-like cells using synthetic chemicals, such as vitamin D3 (Hmama et al., 1999) and PMA (Tsuchiya et al., 1982). The upregulation of the surface receptor CD11b was described in literature as a marker for differentiated macrophages (Schwende et al., 1996). The expression of CD11b on the surface of THP-1 cells was verified by immunocytochemistry and confocal microscopy. THP-1 cells were differentiated into M $\Phi$ -THP-1 cells using PMA. As M $\Phi$ -THP-1 cells adhered to the cell culture flask, they were detached, collected, and seeded in inserts on the apical compartment at a high cell density, as the cell culture medium containing the M $\Phi$ -THP-1 cells is removed by aspiration to lift the inserts at the ALI. Cells were fixed and a specific antibody against the CD11b receptor was used to highlight, in green, the M $\Phi$ -THP-1 cells. The nuclei were counterstained in blue with Hoechst 33342. M $\Phi$ -THP-1 cells seeded in the apical compartment of the inserts attached stably to the epithelial monolayer of A549 cells and expressed CD11b on their surface, as shown in Figure 3.



**Figure 3.** Confocal laser scanning microscopy images illustrating the presence of (c) differentiated THP-1 (MΦ-THP-1) cells attached to the epithelial monolayer of A549 cells. The cells in the *in vitro* model were fixed and MΦ-THP-1 cells were stained for (a) CD11b marker (green); (b) nuclei of MΦ-THP-1 and A549 cells were counterstained with Hoechst 333422 (Magnification 20x).

### Surfactant production and ALI maintenance

To demonstrate the ability of A549 cells to secrete a functional surfactant layer (Nova et al., 2020), while maintaining the ALI in different exposure conditions, the unexposed 3D alveolar *in vitro* model was compared with the exposed model to two different volumes of two vehicles used in *in vitro* testing for preparation of stock solutions of test items. Presence of the pulmonary surfactant and ALI maintenance were assessed using the DMP/O droplet test, which allows a direct evaluation of the surface tension of a cellular layer. The smaller the size of the DMP/O droplet, the lower the surface tension of the epithelium, which is an indication of the presence of surfactant. Exposure to two different volumes (250 and 500  $\mu\text{L}$ ) of the aerosolized vehicle solutions, water and DMSO, resulted in droplets with diameters approximately similar to the droplets obtained by pipetting DMP/O in the control inserts. This confirms the presence of surfactant, and it also points out that aerosolized volume did not impact the function of surfactant secretion of A549 cells (Da Silva et al., 2021). In addition, the ALI condition was not influenced by the highest volume of aerosolized solution used in this study.



**Figure 4.** Surfactant production and ALI maintenance following exposure of the *in vitro* model evaluated using the DMP/O droplet test. (a) unexposed (control) *in vitro* model; (b) 250  $\mu\text{L}$  and (c) 500  $\mu\text{L}$  H<sub>2</sub>O:DPBS=1:1 (v/v) exposed, respectively; (d) 250  $\mu\text{L}$  and (e) 500  $\mu\text{L}$  DMSO:DPBS=1:1 (v/v) exposed, respectively. All inserts were maintained for 24 h post-exposure and showed comparable DMP/O droplet diameters.

### Cell viability

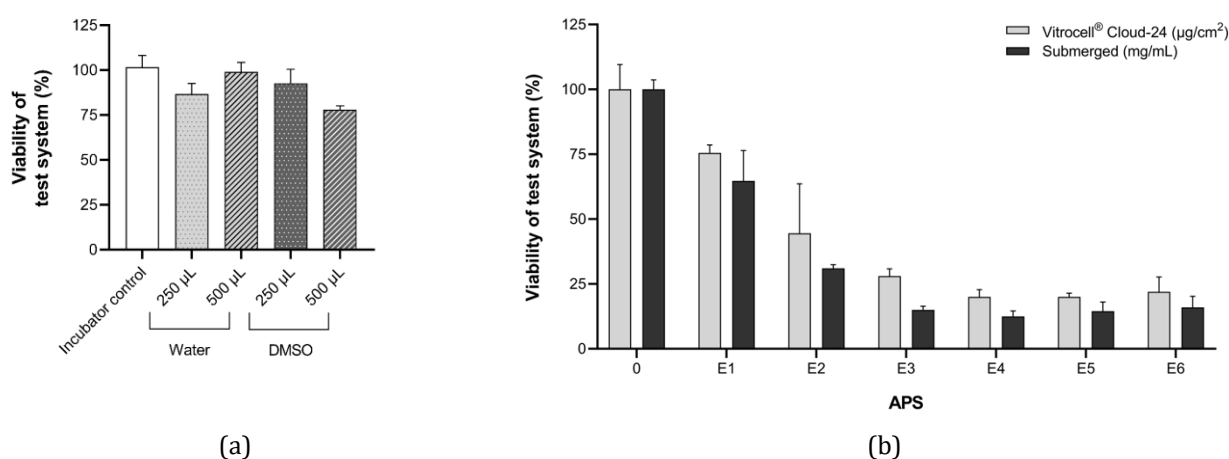
Resazurin assay was performed to determine the metabolic activity of the cells, as a quantitative measurement of cell viability. This assay was chosen for its applicability to assess the viability of cells grown in cell culture inserts as it is a simple, fast, non-toxic for the cells and reliable method (O'Brien et al., 2000; Rampersad, 2012). The cell viability was assessed for the cells from the two compartments of the alveolar model, apical and basolateral, corresponding to the different cell types, A549 and MΦ-THP-1 cells, and EA.hy926 and DC-THP-1, respectively.



## Effect of aerosolized vehicle solution composition and volume on cell viability

In the experimental design for toxicity studies, water, phosphate buffered saline (i.e. DPBS) and growth cell culture medium are the standard vehicles for the evaluation of biological responses (endpoints) following exposure to the investigated test items. Chemical substances with a low n-Octanol/Water Partition coefficient (Kow) are hydrophilic and therefore easy to test in respect to their solubility in aqueous solutions and cell culture medium. For stock solution preparation of test items with a high Kow (polar and non-polar compounds which are poorly soluble in water), DMSO is a widely used vehicle in toxicology and pharmacology in *in vitro* cell culture studies, as an organic polar aprotic molecule. Both positive and negative impacts of DMSO on biologically relevant endpoints have been reported in the literature (Timm et al., 2013; Verheijen et al., 2019). In the current study, the effect on the cellular viability of the 3D alveolar *in vitro* model potentially induced by water and DMSO was evaluated by exposing the model to nebulized solutions of H<sub>2</sub>O:DPBS=1:1 (v/v) and DMSO:DPBS=1:1 (v/v), applying two different volumes for each mixture (250 and 500  $\mu$ L). The 3D alveolar *in vitro* model was kept at the ALI in the incubator for 24 h prior to exposure and for an additional 24 h post-exposure. Post-exposure, the absence of bacterial contamination was verified by light microscopy and the resazurin assay was performed to assess cell viability. This endpoint was measured in the apical compartment, for the A549 epithelial cell monolayer and M $\Phi$ -THP-1 cells present on the surface of A549 cells, and separately in the basolateral compartment for the endothelial cell monolayer and DC-THP-1 cells. Unexposed 3D alveolar *in vitro* model kept in the incubator at the ALI for 24 h was used as incubator control.

The cell viability was not significantly decreased following exposure to the tested vehicle solutions, as compared to the incubator control. The cell viability of the 3D alveolar *in vitro* model was not decreased when the highest volume of vehicle solution (500  $\mu$ L) was used, as shown in Figure 5, A. Since the ALI condition was not compromised upon exposure to an aerosol generated using 500  $\mu$ L of vehicle solution and the viability of the *in vitro* model was not impacted, all the following experiments were carried out using 500  $\mu$ L of test item solution mixed with DPBS for aerosol generation to perform ALI exposure using the Vitrocell® Cloud-24 system.



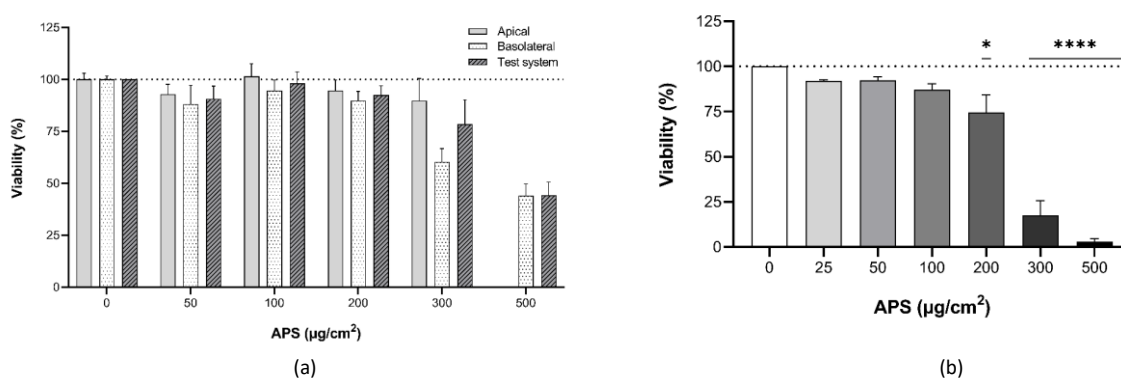
**Figure 5.** (a) Cell viability of the *in vitro* model following exposure to water and DMSO, relative to the incubator control. Data are expressed as mean + SEM, of n=2, N $\geq$ 3. P value was determined by one-way ANOVA, Dunnett's post-hoc.  $P=0.1663$ . (b) Cell viability screening of the *in vitro* model following exposure to APS (Table 3) at the ALI using Vitrocell® Cloud-24 exposure system (gray bars) and in submerged conditions (black bars), relative to the vehicle control (0  $\mu$ g/cm<sup>2</sup>; 0 mg/mL). Data are expressed as mean + SD, of n=2, N=1.

### Submerged and ALI exposure

The estimated settling time of aerosol representing a single dose of a test item in the Vitrocell® Cloud-24 system is of 15 minutes for water-soluble compounds, and of up to 45 minutes for DMSO-soluble compounds. To reduce the timeframe in generating a dose-response curve for the cell viability endpoint, a submerged exposure was performed in parallel with an exposure at the ALI using APS. The cell viability dose-response curve (Figure 5, B) shows that APS solution concentrations can be converted to exposure doses at the ALI, and different exposures, expressed as concentration (mg/mL) or dose ( $\mu$ g/cm<sup>2</sup>), lead to a similar pattern in decreasing the cell viability of the *in vitro* model. A higher decrease of cell viability was observed for the submerged exposure, as the test item is 100% delivered to the cells in the *in vitro* model. In the case of ALI exposure, aerosol deposition efficiency is below 100% and the reported doses are nominal doses and not cell-delivered doses (Bannuscher et al., 2022). As the first exposure dose (300  $\mu$ g/cm<sup>2</sup>) reduced the cell viability of the *in vitro* model by approximately 25%, lower exposure

doses were used to generate the final dose-response curve.

The cell viability of A549 and MΦ-THP-1 cells in the apical compartment, EA.hy926 and DC-THP-1 cells in the basolateral compartment, and the combined viability of the four cell types present in the 3D alveolar *in vitro* model, was determined using the resazurin assay. The *in vitro* model was exposed to increasing doses of APS (50, 100, 200, 300 and 500  $\mu\text{g}/\text{cm}^2$ ) and the viability was measured at 24 h post-exposure. The results are expressed as percentage relative values to the negative vehicle control (0  $\mu\text{g}/\text{cm}^2$ ). A similar trend of cell viability decrease in the apical and basolateral compartments was observed when the 3D alveolar *in vitro* model was exposed to APS, with lower viability values measured in the basolateral compartment, when the same exposure dose of APS was delivered (Figure 6, A). The cell viability of DC-THP-1 cells was additionally assessed by flow cytometry, to derive a dose-response curve. This step is essential for the evaluation of respiratory sensitization endpoints, as the expression of TSLPr, CD54 and CD86 markers should be measured on the surface of DC-THP-1 cells with a residual cell viability of minimum 75% (CV75) (Chary et al., 2019). The flow cytometry data shows a higher cytotoxic effect of APS on the DC-THP-1 cells alone at 300 and 500  $\mu\text{g}/\text{cm}^2$ , by comparison to the resazurin assay (Figure 6, B).



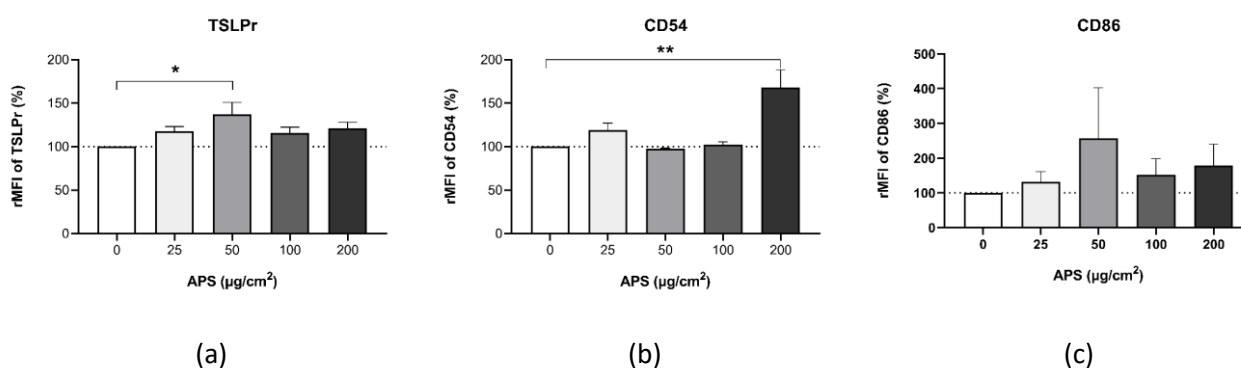
**Figure 6.** (a) Cell viability dose-response curve of the *in vitro* model following APS exposure at the ALI, assessed by resazurin assay. The viability was measured at 24 h post exposure to 50, 100, 200, 300 and 500  $\mu\text{g}/\text{cm}^2$  APS, relative to the vehicle control (0  $\mu\text{g}/\text{cm}^2$ ). Data are expressed as mean + SEM, of n=2, N $\geq$ 3. (b) Cell viability dose-response curve of DC-THP-1 following APS exposure at the ALI, assessed by flow cytometry using SytoxBlue™ dead cell staining. The viability was measured at 24 h post exposure to 25, 50, 100, 200, 300 and 500  $\mu\text{g}/\text{cm}^2$  APS, relative to the vehicle control (0  $\mu\text{g}/\text{cm}^2$ ). Data are expressed as mean + SEM, of n=1, N=3. P values were determined by one-way ANOVA, Dunnett's post-hoc. \* $P\leq 0.05$ ; \*\*\*\* $P\leq 0.0001$ .

### Cell surface markers

CD54 and CD86 are established markers for the prediction of skin sensitizers in the human cell line activation test (h-CLAT) (OECD Guidelines for the Testing of Chemicals, 2018). Respiratory sensitizers, such as chloramine-T (Ponder et al., 2022), maleic anhydride, and ethylenediamine (Ponder et al., 2022; Sadekar et al., 2021) showed increase of at least one of the two markers in the h-CLAT assay (Ashikaga et al., 2010). TSLP is an epithelial-derived cytokine, whose receptors comprise TSLPr and IL-7R $\alpha$ , which are expressed on monocytes, myeloid-derived DCs and B cells (O'Shea et al., 2019). TSLP is involved in the mechanism of respiratory allergic diseases, as it induces the maturation of DCs towards a T helper 2 (T<sub>H</sub>2) phenotype (Ziegler, 2012). TSLPr has been identified as a promising marker for respiratory sensitization (Chary et al., 2019) which could possibly differentiate respiratory from skin sensitizers.

In this study, the up-regulation of CD54, CD86 and TSLPr cell surface makers was measured by flow cytometry, following exposure of the 3D alveolar *in vitro* model to increasing doses of APS, at the ALI, using the Vitrocell® Cloud-24 exposure system. A statistically significant increase in the expression of the cell surface marker CD54 of up to 168% was observed for the 200  $\mu\text{g}/\text{cm}^2$  exposure dose, compared to vehicle control (grey dotted line) (Figure 7, B). None of the exposure doses induced an up-regulation of the CD54 cell surface marker above the threshold of 200% set for the h-CLAT assay to predict a test item as positive. Exposure of the *in vitro* model to the respiratory sensitizer APS induced an up-regulation of the CD86 and TSLPr markers expression on the surface of DC-THP-1 cells at all exposure doses. None of the exposure doses induced a statistically significant up-regulation of the CD86 marker by comparison to the vehicle control (grey dotted line) (Figure 7, C). Only the 50  $\mu\text{g}/\text{cm}^2$  exposure dose leads to an up-regulation of the CD86 cell surface marker above the threshold of 150% set for the h-CLAT assay to predict a test item as positive. Exposure of the *in vitro* model to the respiratory sensitizer APS at an exposure dose of 50  $\mu\text{g}/\text{cm}^2$  led to a statistically significant up-regulation in expression of the TSLPr marker on the surface of DC-THP-1 cells

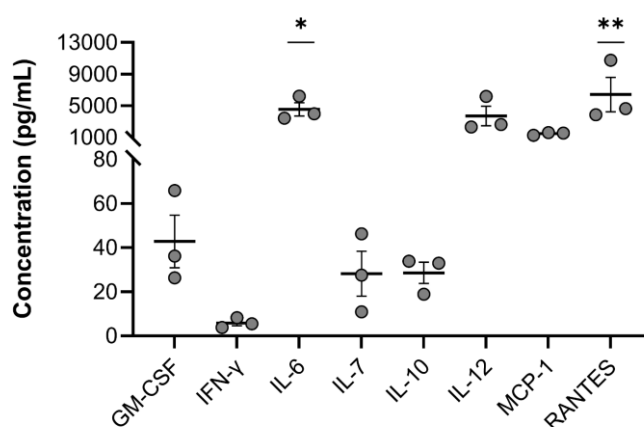
(137%), by comparison to the vehicle control (grey dotted line) (Figure 7, A). Only the rMFI values of TSLPr corresponding to the exposure doses of 50 and 200  $\mu\text{g}/\text{cm}^2$  are above the threshold of 120%. The rMFI values of the investigated cell surface markers corresponding to the 300 and 500  $\mu\text{g}/\text{cm}^2$  exposure dose are not shown, as these exposure doses decreased the cell viability of DC-THP-1 cells under 75%.



**Figure 7.** Expression of (a) TSLPr, (b) CD54 and (c) CD86 markers measured on the surface of DC-THP-1 cells following APS exposure of the *in vitro* model at the ALI, assessed by flow cytometry. The rMFI (%) was measured at 24 h post exposure to 25, 50, 100 and 200  $\mu\text{g}/\text{cm}^2$  APS, relative to the vehicle control (0  $\mu\text{g}/\text{cm}^2$ ). Data are expressed as mean + SEM, of  $n=1$ ,  $N=3$ . P values were determined by one-way ANOVA, Dunnett's post-hoc. \* $P\leq 0.05$ ; \*\* $P\leq 0.01$ .

### Cytokine release

The LPS endotoxin is used as a positive control in toxicological studies assessing immune responses of lung cell models, as it exacerbates the respiratory inflammation and allergic responses (Bisig et al., 2019). LPS exposure has been previously shown not to affect cellular viability in the tetra-culture model for respiratory sensitization (Chary et al., 2019). To evaluate the responsiveness of the downsized 3D alveolar *in vitro* model, LPS-induced cytokine release was measured. LPS stimulation led to an increased release of IL-6 (5800 times), IL-10 (12 times), MCP-1 (20 times) and RANTES (27 times) in the *in vitro* model (Figure 8). For the cytokines GM-CSF, IFN- $\gamma$ , IL-7 and IL-12 the fold increase and statistical significance could not be calculated, as the values for the control were below the range of detection. The cytokine MIP-1 $\beta$  was above the range of detection following LPS exposure (data not shown).



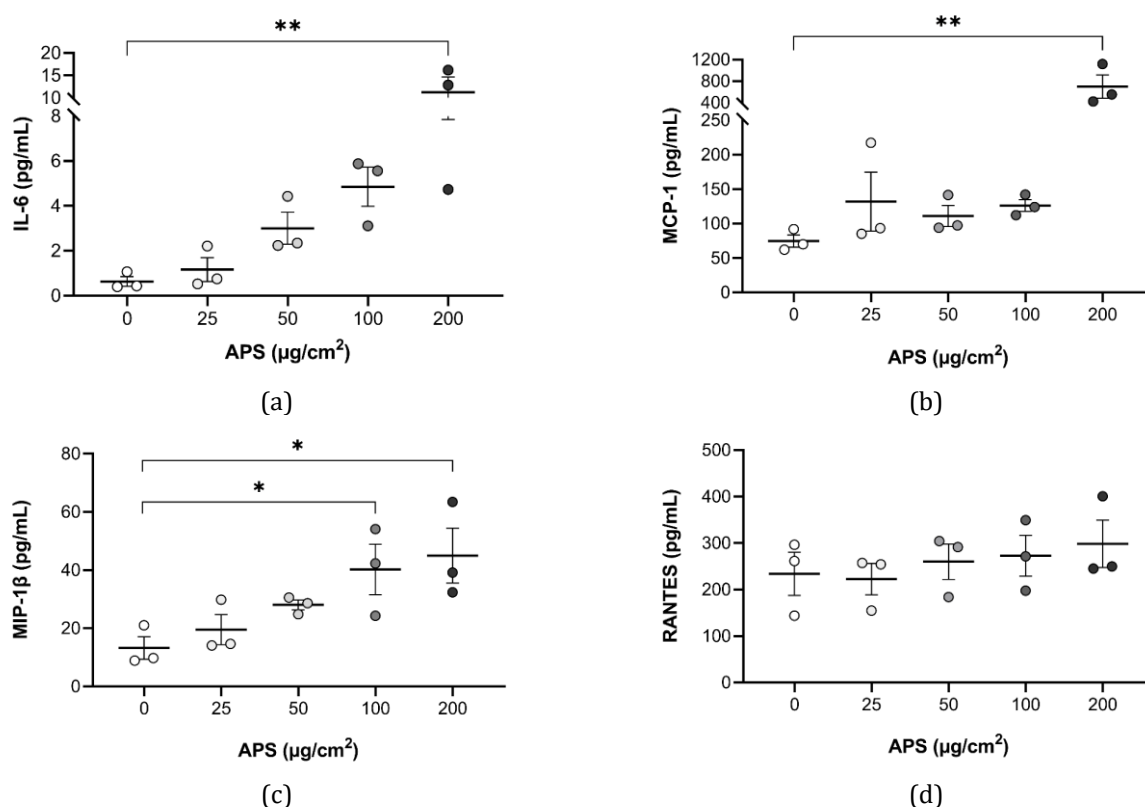
**Figure 8.** GM-CSF, IFN- $\gamma$ , IL-6, IL-7, IL-10, IL-12, MCP-1 and RANTES cytokines release following LPS exposure of the *in vitro* model. The concentration (pg/mL) of cytokines was measured at 24 h post exposure. Data are expressed as mean  $\pm$  SEM, of  $n=1$ ,  $N=3$ . P values were determined by one-way ANOVA, Tukey's post-hoc. \* $P\leq 0.05$ ; \*\* $P\leq 0.01$ .

The cytokine secretion in the *in vitro* model is dependent on the APS exposure dose, revealing a dose-response profile for IL-6 and MIP-1 $\beta$ . The concentrations (pg/mL) of secreted IL-6 and MCP-1 measured in cell culture medium were significantly different from vehicle control, when a dose of 200  $\mu\text{g}/\text{cm}^2$  APS was delivered to the 3D alveolar *in vitro* model. Exposure to APS induced the secretion of MIP-1 $\beta$  cytokine at both 100 and 200  $\mu\text{g}/\text{cm}^2$  exposure doses, while none of the exposure doses induced a statistically significant secretion of the RANTES cytokine.

Interleukin 6 (IL-6) is a pro-inflammatory cytokine involved in the immunologic activity regulation of the alveolar tissue, and it has been shown to be secreted by alveolar type II (ATII) A549 epithelial cell line following appropriate stimulation, monocytes and endothelial cells (Crestani et al., 1994). IL-6 enhances the expression of CD54 cell surface marker (Duits et al., 1992). In our experimental design, the 200  $\mu\text{g}/\text{cm}^2$  exposure dose of APS induced a statistically significant increase in secretion of IL-6 cytokine, and CD54 cell surface marker was significantly up-regulated following exposure to the same exposure dose of APS.

Monocyte chemoattractant protein-1 (MCP-1), a member of the C-C chemokine family, represents a potent chemotactic factor for monocytes. MCP-1 is expressed at high levels in  $T_H2$  immune-mediated diseases, such as asthma (Deshmane et al., 2009), and might be a suitable cytokine to identify respiratory sensitizers using *in vitro* models (Gibb and Sayes, 2022) and to discriminate respiratory from skin sensitizers (Huang et al., 2013). In the 6-well plate model for respiratory sensitization, exposure to the trimellitic and phthalic anhydride respiratory sensitizers did not render a significant increase of MCP-1 secretion (Chary et al., 2019), while the highest dose of APS was found to upregulate the secretion 9.4-fold, by comparison to the vehicle control in the 3D alveolar *in vitro* downscaled model.

Macrophage inflammatory protein 1 $\beta$  (MIP-1 $\beta$ /CCL4) is a chemokine secreted by dendritic cells of lymphoid and myeloid origin (Penna et al., 2002), which was found to be significantly activated in bronchoalveolar lavage (BAL) cells of patients with corticosteroid-resistant asthma patients (Goleva et al., 2008). MIP-1 $\beta$  secretion was investigated in a study to define a novel marker for the prediction of skin sensitization. Data shows that inorganic tested compounds (nickel sulfate hexahydrate, cobalt sulfate heptahydrate, ammonium tetrachloroplatinate) induced the highest production of MIP-1 $\beta$  by THP-1 cells (Hirota and Moro, 2006). APS exposure of the downscaled 3D alveolar *in vitro* model resulted in a significant increase of MIP-1 $\beta$  secretion at 100 and 200  $\mu\text{g}/\text{cm}^2$ . Additional organic and inorganic compounds which are known to sensitize the respiratory tract should be tested for induction of MIP-1 $\beta$  secretion in the 3D alveolar *in vitro* model, as some sensitizing agents that do not fulfill the definition of organic low molecular weight compounds, could act through different mechanisms and require a separate adverse outcome pathway (AOP) (Sullivan et al., 2017).



**Figure 9.** (a) IL-6, (b) MCP-1, (c) MIP-1 $\beta$  and (d) RANTES cytokines release following APS exposure of the *in vitro* model at the ALI, assessed by multiplexing using the Bio-Plex 3D Suspension Array System. The concentration (pg/mL) of cytokines was measured at 24 h post exposure to 25, 50, 100 and 200  $\mu\text{g}/\text{cm}^2$  APS and vehicle control (0  $\mu\text{g}/\text{cm}^2$ ). Data are expressed as mean  $\pm$  SEM, of n=1, N=3. P values were determined by one-way ANOVA, Dunnett's post-hoc. \*P $\leq$ 0.05; \*\*P $\leq$ 0.01.

The secretion profile of chemokine (C-C motif) ligand 5 (CCL 5), also known as RANTES (regulated on activation, normal T cell expressed and secreted) was evaluated in *in vitro* 3D human models of the respiratory tract, in a dose- and time-dependent manner (Huang et al., 2013) and at subtoxic levels of compounds (Hermanns et al., 2015). Higher levels of RANTES secretion were induced by respiratory sensitizers in both studies, indicating CCL5 as a marker to differentiate respiratory from skin sensitizers and respiratory irritants. In our study, RANTES secretion was measured at a single time-point, at 24 h post exposure, and data show a dose-dependent secretion profile, with a modest 1.3-fold increase at the highest exposure dose of APS (200 µg/cm<sup>2</sup>).

## CONCLUSIONS

The 3D alveolar *in vitro* model designed for the identification of potentially sensitizing agents upon inhalation was successfully downscaled. The 24-well plate format 3D alveolar *in vitro* model was thoroughly characterized, so it can be used as an alternative testing method to animal experimentation for hazard classification of HMW and LMW potentially sensitizing agents of the respiratory tract. The downscaled model resembles the cellular architecture of the complex *in vivo* alveolar barrier, similarly to the original 3D alveolar *in vitro* model built-up using the 6-well plate hanging cell culture insert as support. The response of the downscaled model to a recognised respiratory sensitizer, ammonium persulfate (APS) and positive controls (LPS and TSLP), was evaluated through several endpoints. Air-liquid interface (ALI) exposure using the Vitrocell Cloud-24 exposure system to increasing doses of APS has shown to decrease the cell viability of the *in vitro* model in a dose-response curve manner. Cytokine release by the cells used for the build-up of the *in vitro* model of the alveolar barrier and activation of dendritic-like THP-1 cells (DC-THP-1), shown by statistically significant increase in the expression of the TSLPr cell surface marker prove the functionality of the downscaled *in vitro* model.

The 24-well plate insert sized 3D alveolar *in vitro* model presents a series of advantages: (1) use of less biological material and therefore a reduction in use of cell culture consumables (i.e. flasks, tubes) and reagents (i.e. FBS, cell culture media), meeting the needs of sustainability in research; (2) generation of more data in a shorter time (i.e. exposure of 4 times more inserts for the 24-well plate insert format in one nebulization step in a Vitrocell® Cloud-24 exposure system, by comparison to the 6-well plate insert format); (3) meeting the requirements of multiple industries, among which food industry is a prominent sector, for HTS screening of inhalable respiratory sensitizers; (4) possibility to combine the 3D alveolar *in vitro* model with other *in vitro* models in microfluidics for systemic studies.

**Author Contributions:** S.B., A.C. and E.M. conceived and designed the experiments; S.B. performed the investigations, collected the data and interpreted the results; C.B.A.S. performed the electron microscopy sample preparation and imaging; S.B. wrote the original manuscript; A.C.G. and T.S. supervised the experimental work; A.C.G., M.A.S. and C.S. revised the original draft of the manuscript. All authors commented on the manuscript.

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## Conflicts of Interest

The authors declare that they do not have any conflict of interest.

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