Positive controls use for performance evaluation of an *in vitro* test system designed for the prediction of chemical respiratory sensitizers

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INTRODUCTION

BACKGROUND: Respiratory sensitizers are considered substances of very high concern (SVHC) under EU REACH legislation, hazard which should be assessed in the early phases of development of novel molecules and compounds with applications in various industries. Respiratory sensitization is associated with tissue inflammation, as indicated by the second key event (KE2) from the proposed adverse outcome pathway (AOP:39). Measuring relevant biomarkers for respiratory sensitization in physiologically relevant *in vitro* models is crucial to anticipate the sensitizing potential of chemicals. Simultaneously, it is very important to evaluate the consistency of the *in vitro* method's responsiveness concurrently with the test articles. Incorporating positive controls is of paramount importance, as it enhances the identification of both false-positive and false-negative results. Lipopolysaccharide (LPS), an endotoxin shown to induce a strong pro-inflammatory response in lung cell models, and thymic stromal-lymphopoietin cytokine (TSLP), an epithelium derived mediator of allergic reaction known to activate antigen-presenting cells such as dendritic cells, are often employed as positive controls to assess the pro-inflammatory responses in *in vitro* test systems representative for the respiratory tract.

OBJECTIVE: The objective of the study was to evaluate the responsiveness of an *in vitro* test system designed for the prediction of chemical respiratory sensitizers – ALIsens® (Figure 3f) (Chary et al., 2019). The cellular arrangement enables the development of a tissue microenvironment (Figure 1) achieved by direct communication between cells (Figure 2) and indirectly via secreted messenger molecules. Additionally, it facilitates the exposure to respiratory sensitizers in a realistic approach, at the air-liquid-interface (ALI), as well as in submerged conditions.

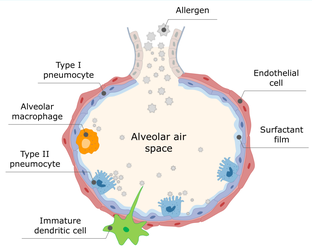
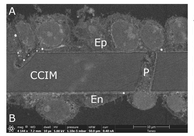


Figure 1: Structure of a human *in vivo* alveolus



A – apical compartment
B – basolateral compartment
CCIM – cell culture insert membrane
En – endothelial cells monolayer
Ep – epithelial cells monolayer
P – cell culture insert membrane pore (scale bar 10 µm)

Figure 2: Electron microscopy image of the alveolar *in vitro* model - ALIsens®

MATERIALS and METHODS

TEST SYSTEM: The ALIsens® model was built on the semipermeable membrane of a hanging cell culture insert by seeding human alveolar type II epithelial cells (A549) in the apical compartment and endothelial cells (EA.hy926) on the basolateral side. Macrophage-like cells (MΦ-THP-1), phorbol-12-myristate-13-acetate (PMA)-differentiated THP-1 cells, and dendritic-like cells (DC-THP-1) were distributed on the luminal surface of the epithelial, respectively beneath the endothelial cell monolayers.

Day 0:

a. THP-1 cells PMA differentiation to MΦ-THP-1 cells

Day 2:

b. PMA stimulation removal from MΦ-THP-1
c. EA.hy926 cells (red) seeding
d. A549 cells (blue) seeding

Day 6:

ALIsens® 3D alveolar *in vitro* model tetraculture:
e. Seeding of DC-THP-1 (green) and MΦ-THP-1 (orange)
f. Switch to ALI

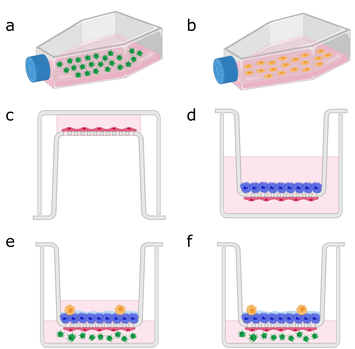


Figure 3: Alveolar *in vitro* model – ALIsens®

EXPOSURE Day 7:

TREATMENT:

Mixture of LPS from *Escherichia coli* O26:B6 – 10 µg/mL and TSLP human recombinant – 20 ng/mL prepared in coculture supplemented with 1% FBS medium; a volume of 250 µL of treatment was added in the apical compartment of ALIsens® model

CONTROL:

Exposure at the ALI using Vitrocell® Cloud 6 system to 500 µL liquid mix in the nebulizer: chemical in vehicle:PBS = 1:1 (vol/vol)

ENDPOINTS Day 8:

Figure 4: BD FACSCelesta™ cell surface markers measured on DC-THP-1 cells: TSLPr, CD54, CD86

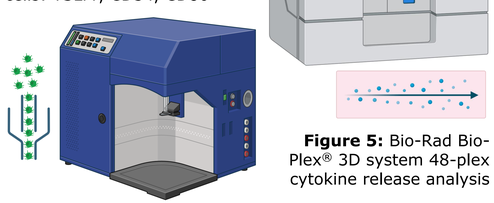


Figure 5: Bio-Rad Bio-Plex® 3D system 48-plex cytokine release analysis

RESULTS

CELL SURFACE MARKERS

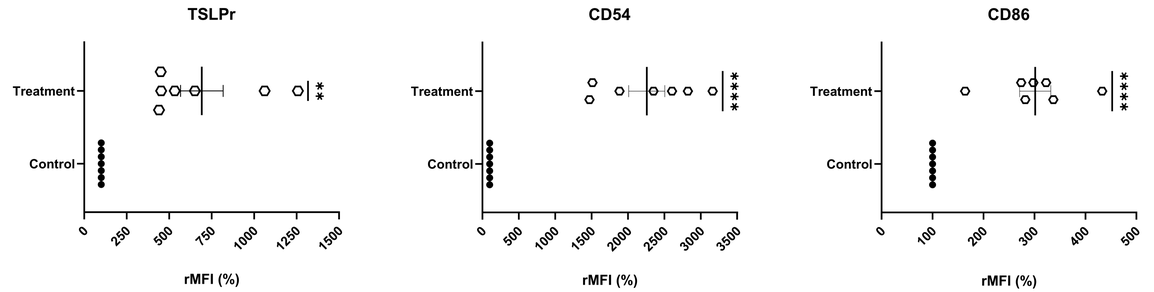


Figure 6: TSLPr, CD54 and CD86 cell surface markers expression on DC-THP-1 cells in the ALIsens® model after 24h exposure to treatment and to control expressed as relative mean fluorescence intensity (rMFI %). Mean ± SEM, n=7; ** $P \leq 0.01$; **** $P \leq 0.0001$

PRO-INFLAMMATORY SECRETED MARKERS

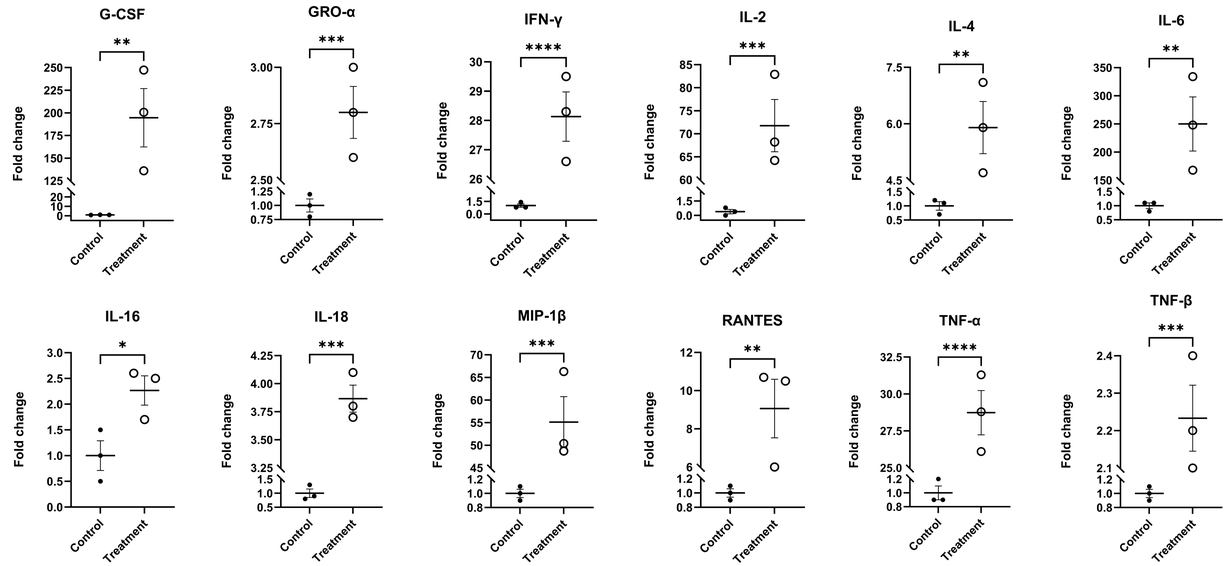


Figure 7: Release of pro-inflammatory markers G-CSF, GRO-α ((CX)CL1), IFN-γ, IL-2, IL-4, IL-6, IL-16, IL-18, MIP-1β (CCL4), RANTES (CCL5), TNF-α and TNF-β in the ALIsens® model 24h after exposure to treatment and to control expressed as fold change. Mean ± SEM, n=3; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$

CONCLUSIONS

- The data show that LPS and TSLP are meaningful positive controls for the induction of pro-inflammatory secreted markers, cytokines and chemokines (Figure 7) and can successfully induce the activation of dendritic-like cells (Figure 6).
- LPS and TSLP can be successfully used for the responsiveness check of *in vitro* models envisaged for the evaluation of respiratory inflammation and sensitization, based on a combination of the human cell lines A549, EA.hy926 and THP-1.

REFERENCE Chary, A., et al. (2019). ALTEX 36(3), 403-418. DOI:10.14573/altex.1901241

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