

A novel approach to assess irritant or respiratory allergenic potential of chemicals *in vitro*

Singal M.¹, Vitale D.¹, Smith L.¹, Czollner K.², Farmer L.², Höpfner C.², Satzer H-P.², Schoeffmann S.², Schwartz B.², Weber E.²

¹ Research Institute for Fragrance Materials, Inc., Woodcliff Lake, NJ, USA ; ² Seibersdorf Labor GmbH, Seibersdorf, Austria

ABSTRACT

There are currently no validated methods for the identification of chemical respiratory allergens, neither *in vivo* nor *in vitro*. Due to increasing health concerns associated with occupational or chemically-induced asthma, and impending directives on the regulation of respiratory sensitizers, standardized test methods are urgently required to identify respiratory allergens.

To establish an *in vitro* test system for the identification of chemical respiratory sensitizers, an immunocompetent, 3D triple cell co-culture system, representing the proximal alveolar region of the human lung, was developed. It is comprised of immature dendritic cells, derived from human peripheral blood monocytes, human lung alveolar epithelial-like cells (A549 cells) and macrophages differentiated from U937 cells.

Employing the Vitrocell® system, two well-known respiratory allergens, toluene-2,4-diisocyanate (TDI) and trimellitic anhydride (TMA), as well as, two irritants, acrylic acid and acetaldehyde, were applied to the model at the air/liquid interface, mimicking the *in vivo* situation in the lung. Since, this system allows for the delivery of test materials in their native form, liquid aerosols (TDI, acrylic acid, acetaldehyde) or particle aerosols (TMA) were generated to mimic a real exposure scenario. Aerosol particle sizes and the amount of test substance applied were quantified.

After a 4 hour exposure scenario, cellular viability was evaluated in dose response studies using flow cytometric evaluation of cell cycle analysis. Cytokine release and dendritic cell maturation was investigated to identify whether chemical exposure induces specific inflammatory mediators, and/or cellular changes, in order to identify predictive endpoints and biomarkers that may be indicative of potential respiratory allergens *in vivo*.

INTRODUCTION

Currently, there are no accepted methods for the evaluation of respiratory sensitizers, neither *in vivo* nor *in vitro*. Therefore, the development of tests is of great concern, especially considering the legislation of chemicals by the European Union (REACH). Attention should be directed to *in vitro* models since the REACH guidelines clearly state that tests on animals should be avoided and a complete ban on animal testing for cosmetic ingredients will be implemented in Europe by 2013 under the EU Cosmetics Directive.

To establish an *in vitro* test system for the identification of chemical respiratory sensitizers, an immunocompetent, three-dimensional co-culture system representing the proximal alveolar region of the human lung was developed. The model contains alveolar type II epithelial cells, macrophages, and monocyte-derived dendritic cells as antigen-presenting cells. Employing the Vitrocell® system in combination with different inhalation tools, 1 well-known contact sensitizer, d-limonene, and one irritant, ammonia (NH₃), were applied to the model at the air/liquid interface, mimicking the *in vivo* situation in the lung, in which various endpoints were analyzed.

METHODS

Triple cell co-culture model exposure

Inserts with ECs and AMs were exposed to d-limonene or NH₃ for 4 hours, employing the Vitrocell® system in combination with an aerosol generator (aerosol nozzle, TSE systems) and a syringe pump (NH₃) or with an ultra-sonic nebulizer (TSE systems) (d-limonene). Afterwards, inserts were placed on top of immature DCs in cell culture wells for 24 hours.

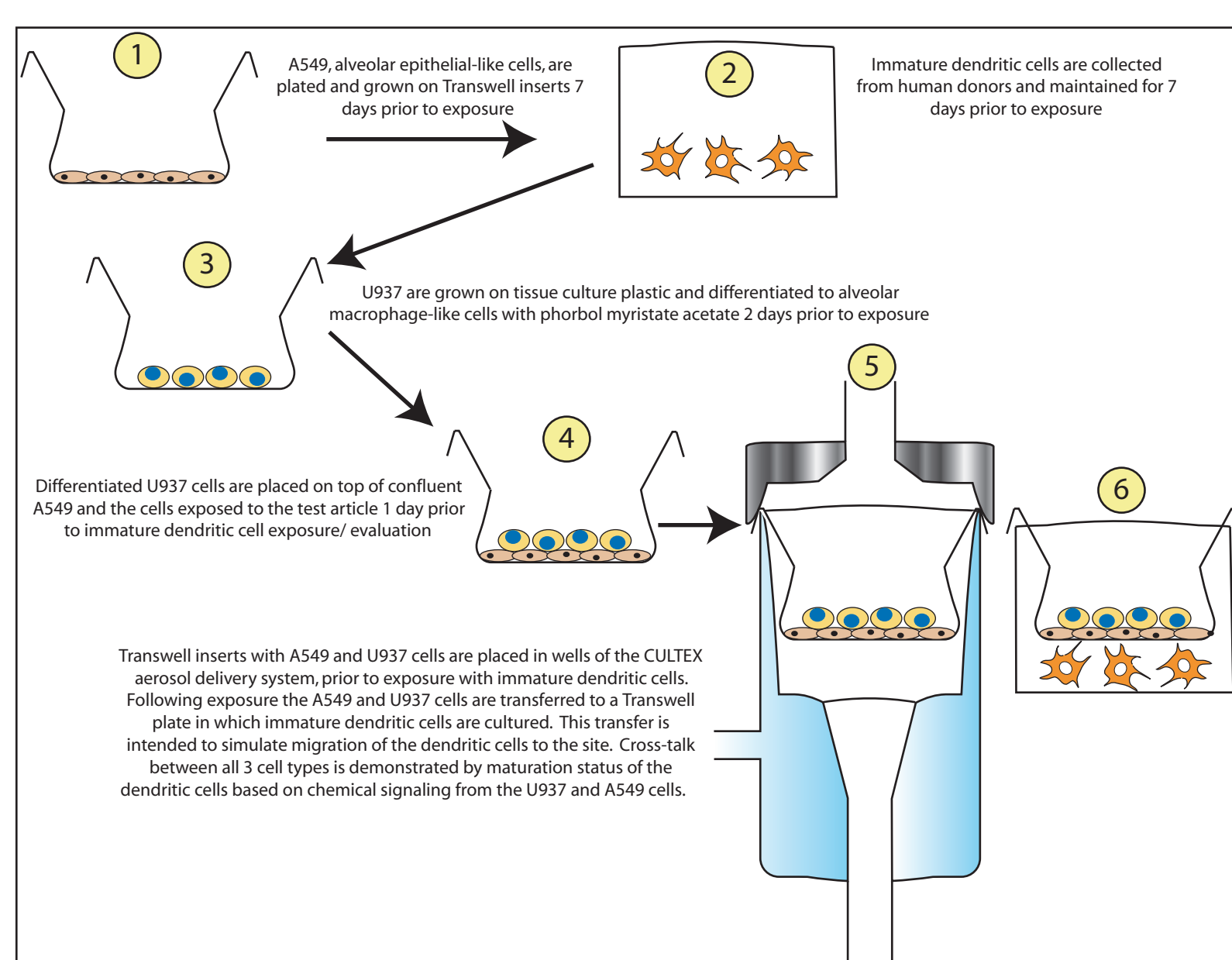


Figure 1: Generation and exposure of the triple cell co-culture system.

Endpoints

- Cytotoxicity (Propidium iodide, PI)
- Oxidative burst (Dihydrorhodamine assay, DHR)
- Pro- and anti-inflammatory cytokines IL-2, IL-8, IL-10, IL-13, CD54, TNF- α , IFN- γ , MCP-1, RANTES (Cytometric Bead Array, CBA)
- Staining of dendritic cell maturation marker CD86

RESULTS

Dose-response experiments

NH₃ and d-limonene were applied to ECs and AMs on inserts in decreasing concentrations for 4 hours and cytotoxicity was measured by propidium iodide (PI) dye exclusion. For NH₃, a concentration of 0.32 mg/L resulting in 14% cytotoxicity was chosen for further experiments. Exposure with 1.75 mg/L and 0.55 mg/L d-limonene yielded 43% and 9% cytotoxicity, respectively. Both concentrations were analyzed in further experiments.

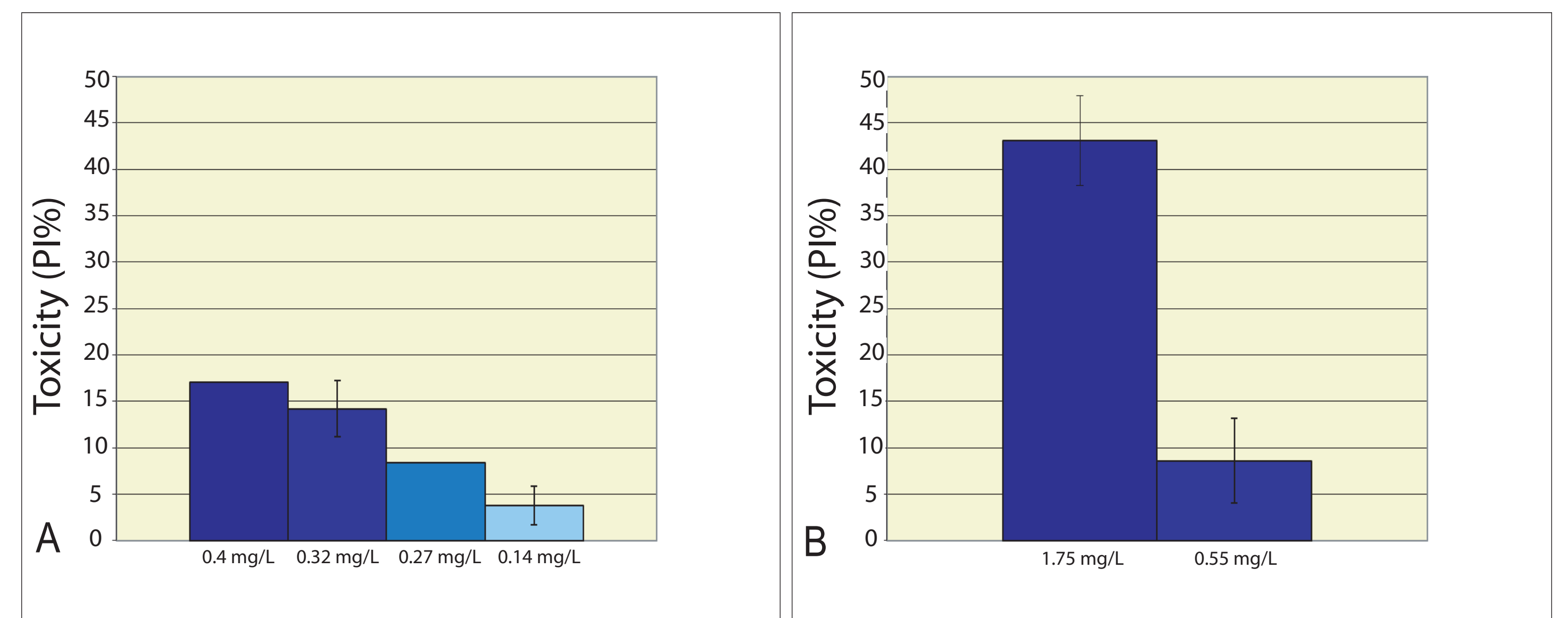


Figure 2: Dose-response experiments for NH₃ (A) and d-limonene (B). Values represent mean values based on 4 replicates, +/- standard deviation.

Analysis of dendritic cell maturation

Exposure of ECs and AMs with the contact sensitizer d-lim and the irritant NH₃ and subsequent transfer of the inserts on immature DCs did not induce maturation of DCs (measured as increase of CD86+ cells). However, if d-limonene was applied directly to immature dendritic cells in comparable concentrations, maturation was induced.

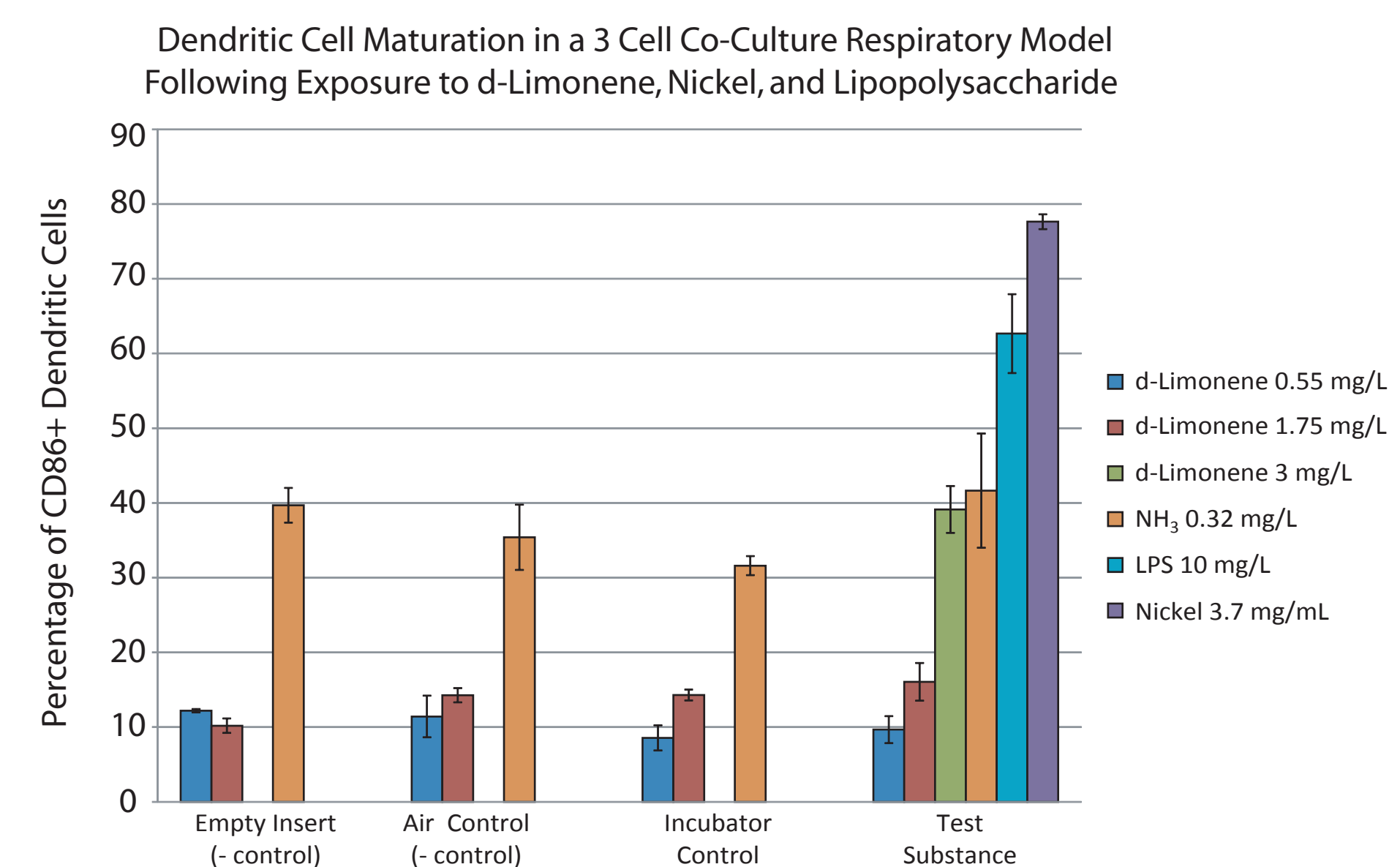


Figure 3: Dendritic cell maturation, as measured by CD86 marker, following 4 hour exposure to d-limonene (0.55, 1.75, and 3 mg/L), NH₃ (0.32 mg/L), LPS (10 mg/L), and nickel (3.7 mg/mL). n = 4 and values are presented +/- standard deviation.

Analysis of cytokines

IL-8, MCP1, RANTES and CD54 were above the detection limit of 10 pg/mL. 6A: the irritant NH₃ decreases IL-8 and increases RANTES produced during the exposition. 6B,C: After the co-culture with DCs an increase of IL-8 and CD54 was induced by d-limonene in the upper and lower compartment. Different results of the higher concentration of d-limonene are attributed to the high toxicity. NH₃ showed an increase of CD54 and a decrease of IL-8, MCP-1 and RANTES in the upper compartment.

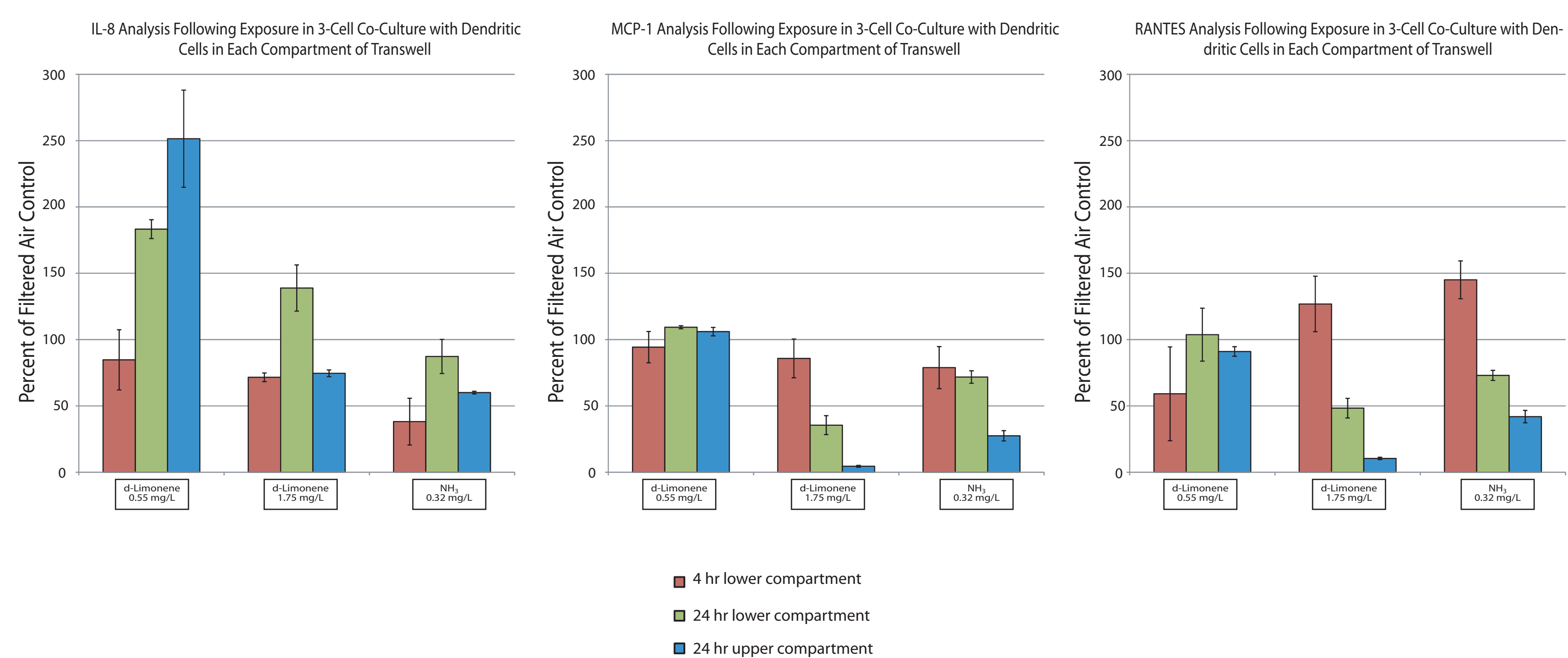


Figure 4: Analysis of IL-8 (A), MCP-1 (B), and RANTES (C) following exposure for either 4 hours (with DCs in the lower compartment) or 24 hours after co-culture with DCs in the lower compartment and upper compartment. Data are shown as percent of negative control data (negative control = 100%) +/- standard deviation.

CONCLUSIONS

The results of this study suggest that the triple cell co-culture model developed here is feasible and has the potential to analyze effects of chemical aerosols at the air/liquid interface. Analysis of DC maturation, after exposure to d-limonene, showed that the triple cell culture model produces an effect similar to NH₃, a known respiratory irritant. Analysis of cytokines allows the possibility of discrimination between irritants and sensitizers. These data indicate that the final test assay, developed with this methodology, may be able to discriminate between respiratory and contact sensitizers.