Non-Combustible Next Generation Products induce lower toxicity than Combustible Tobacco in an Alveolar-Immune coculture model

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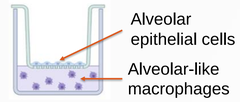
INTRODUCTION

Immune cells are a critical component of the lower airways, however there is currently a lack of *in vitro* models available which can assess the interaction of immune cells with alveolar cells and model their combined responses to external stressors. Here we assess a commercially available co-culture model, ImmuLUNG™ (ImmuONE Ltd), which utilises alveolar epithelial and alveolar macrophage-like cells [1].

We exposed the ImmuLUNG™ model to combustible tobacco smoke or non-combustible next generation nicotine delivery product (NGP) aerosol fractions and determined any resulting biological responses. ImmuLung cultures were treated and subsequently stained with antibodies / dyes to investigate potential impact on macrophage health and morphology using high content screening technology. Additional endpoints assessed included macrophage phagocytic activity and alveolar epithelial barrier properties.

ImmuLUNG Schematic [1]



METHODS

Test Articles

- 1R6F Reference Cigarette (University of Kentucky)
- NGP: E-Vapor product (EVP), "myblu" EU Tobacco 1.6% Nicotine
- NGP: Heated Tobacco Product (pHTP), "Pulze" with "iD stick": Balanced Tobacco

Smoke / Aerosol Extract Generation method

Smoke and aerosol from test products was generated with a Vitrocell VC10s (Vitrocell, Munich, Germany) smoking machine. Smoke or aerosol extracts were prepared by bubbling the sample aerosol into 3 in-line Impingers each containing 10 mL Phosphate Buffered Saline (PBS) solution (See Figure 1). A total stock solution of 30 mLs per test article was used: 1.8 puffs per mL for 1R6F cigarette and 3 puffs per mL for the NGPs.

Figure 1: Bubbling smoke/vapor exposure system

Trapped nicotine and carbonyls were quantified within the aerosol and smoke bubbled PBS (bPBS) samples (See Table 1). Nicotine was quantified using liquid chromatography with tandem mass spectrometry (LC-MS/MS) with an AB Sciex API 6500 QTRAP (SCIEX, Framingham, MA, USA) using nicotine-d4 as the internal standard. For the analysis of Carbonyls, bPBS samples were diluted with 2,4-dinitrophenylhydrazine (DNPH). The carbonyl-DNPH derivates were then quantified using high performance liquid chromatography with a diode-array detector (HPLC-DAD, Agilent Technologies 1100 Series).

***In Vitro* Toxicological assessment**

ImmuLung cultures were treated with 5 or 10% v/v bPBS per test article for either 24 or 48 hours. For the cell health and morphology assessment, cells were stained with a dye cocktail containing Hoechst 33342 (nuclei), MitoTracker Red (active mitochondria), Image-It Dead Green (membrane integrity) and Cell Mask Deep Red (cytoplasm to identify vacuoles). Images were captured with a 40x objective in standard 2D imaging mode. Each sample was imaged using 36 fields representing in total between 100 to 4000 cells per well. Macrophage phagocytic activity was determined at the end of treatment period. The media containing 1 µm Carboxylate-Modified Microspheres (Invitrogen, Renfrewshire, UK) was added for 2 h and cells were incubated at normal culture conditions. Cells were harvested by gentle scraping and the cell fluorescence (Ex/Em: 505/515) was measured using the Guava EasyCyte flow cytometer (Guava EasyCyte 8HT, Millipore, UK). Cells were identified from free particles and cellular debris by their forward and side scatter. Phagocytic activity was assessed by processing the green fluorescence (525 ± 30 nm) of cells and comparison with untreated control. At least 5000 cells were counted for each sample. Finally, the alveolar epithelial barrier properties were determined using transepithelial electrical resistance (TEER) measurement. This was measured using EVOM2 TEER meter with probe attachment (WPI, Hertfordshire, UK).

Statistical Analysis

Data are expressed as mean of n = 3 replicates ± standard deviation (SD) about the mean. Results are compared to PBS vehicle control and expressed as a ratio (fold change). Statistical significance was determined using one-way ANOVA with Dunnett's multiple comparison test. Statistical significance is marked as follows: * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ and **** indicates $p < 0.0001$.

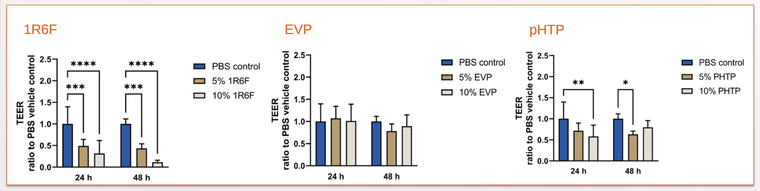
RESULTS

Table 1: Nicotine and carbonyl levels in bubbled PBS extracts

Test article	Nicotine [µg/mL]	Formaldehyde [µg/mL]	Acetaldehyde [µg/mL]	Acetoin [µg/mL]	Acrolein [µg/mL]	Propionaldehyde [µg/mL]	Crotonaldehyde [µg/mL]	2-Butanone (MEK) [µg/mL]	n-Butyraldehyde [µg/mL]
E-Vapor Product	263.8	0.34	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Heated Tobacco Product	203.7	0.93	40.49	3.82	0.44	1.94	0.58	0.69	1.52
1R6F Reference cigarette	206.3	8.40	156.76	20.56	2.65	7.37	3.06	3.76	2.26

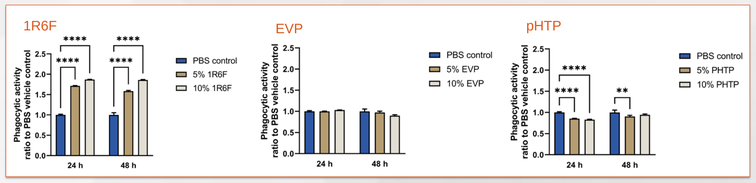
For each test article, PBS from all three impingers was combined to generate 30mls stock for analysis. In keeping with past studies [2], nicotine trapping in PBS was highest for e-vapor and lowest for HTP (Table 1). Carbonyl levels were highest in PBS samples with 1R6F cigarette smoke bubbled through it with marked reductions in carbonyl levels recorded for HTP and e-vapor aerosol extracts.

Figure 2: Alveolar epithelial barrier integrity



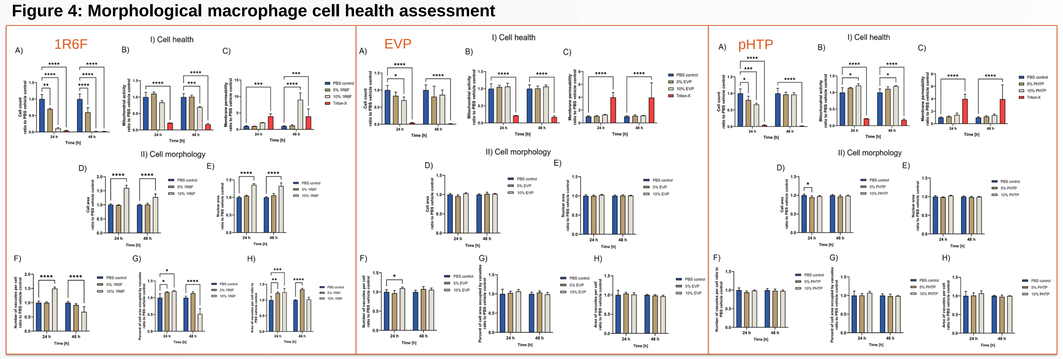
Cigarette (1R6F) smoke extract significantly decreased TEER in a time and dose dependent manner. Significantly lower TEER was also reported with 10% v/v HTP aerosol extract after 24 h treatment and with 5% v/v concentration after 48 h; there was no significant difference in treatments with e-vapor aerosol extracts.

Figure 3: Macrophage phagocytic activity



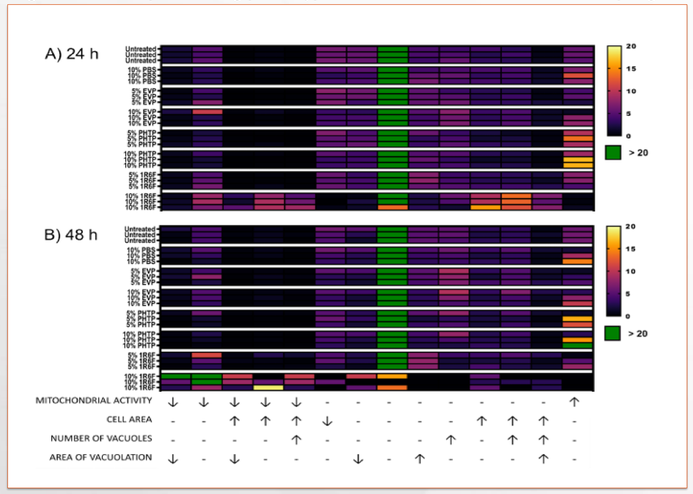
A significant increase in phagocytosis, in a time and dose dependent manner, was noted with cigarette smoke extract treatment. Significantly decreased phagocytic activity was observed after 24 h exposure to 5% v/v and 10% v/v HTP and this remained impaired after 48 h but only with the 5% v/v PHTP exposure. There was no significant difference in phagocytic activity of cells exposed to e-vapor aerosol extract in comparison with the PBS control.

Figure 4: Morphological macrophage cell health assessment



Cigarette smoke extracts at 10% v/v affected alveolar-like macrophage health by reducing the number of viable cells, decreasing their mitochondrial activity, and increasing their membrane permeability. Cell morphology was also changed, manifested in the presence of larger cells (increased cell and nuclear area) with initially increased number of vacuoles and vacuolation area, followed by decreased number of vacuoles upon longer exposure. HTP aerosol extracts increased mitochondrial activity at higher concentrations without affecting cell membrane integrity, suggesting that this is most likely an adaptative response; cell morphology was not altered. The e-vapor aerosol extract did not affect alveolar-like macrophage health, morphology nor functionality.

Figure 5: Heatmap indicating phenotypic assessment of alveolar-like macrophages



Four cell characteristics (mitochondrial activity, cell area, vacuole number per cells and area of cell occupied by vacuoles) were expressed at three levels generating 81 possible phenotypes. Each square represents the % of the cell population with that given phenotype in each replicate of one experiment. The colour gradient sets the lowest value for each given parameter in the heat map (black, 0%), highest value (green, above 20%) and mid-range values (yellow, 20%) with a corresponding gradient between these extremes.

Exposure to cigarette smoke altered the cell phenotype profile by reducing the mitochondrial activity with or without elevated vacuolation pattern (foamy macrophage phenotype). The phenotype profile for EVP and HTP exposures was similar to that of the PBS vehicle control samples. At the tested concentrations and time points, perturbations in phenotype distribution were deemed to be minor regarding macrophage cell heath and morphology.

CONCLUSION

- Cigarette smoke extracts significantly affected macrophage health by reducing the number of viable cells, decreasing mitochondrial activity and increasing their membrane permeability. Additionally, cigarette smoke extracts increased macrophage vacuolation and phagocytic activity, whilst reducing alveolar membrane integrity.
- In contrast to cigarette smoke extracts, there was limited to no impact of HTP or e-vapor aerosol extracts on macrophage health or morphology. HTP aerosol extracts caused slight impairment to phagocytic activity, and alveolar membrane integrity with increasing concentrations, whilst e-vapor aerosol extracts did not alter either endpoint at both concentrations under the conditions of the test. The results presented supports these products' placement on the relative risk scale.
- Future studies will assess a broader range of non-cytotoxic exposures and further measurements of phagocytosis with assessment of potential phospholipid accumulation in alveolar macrophages.

REFERENCES

[1] Hutter V, Hopper S, Skamarauskas J, Hoffman E. High content analysis of in vitro alveolar macrophage responses can provide mechanistic insight for inhaled product safety assessment. Toxicol In Vitro. 2023 Feb;86:105506. doi: 10.1016/j.tiv.2022.105506. Epub 2022 Oct 27. PMID: 36330929.
[2] Simms L, Yu F, Palmer J, Rudd K, Sticken ET, Wieczorek R, Chapman F, Czekala L, Stevenson M and O'Connell G (2022) Use of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes to Predict the Cardiotoxicity Potential of Next Generation Nicotine Products. Front. Toxicol. 4:747508