UTILISATION OF HUMAN 3D BRONCHIAL TISSUES FOR E-CIGARETTE ASSESSMENT

IMPERIAL BRANDS SCIENCE

Visit our Scientific Research website
www.imperialbrandscience.com

21st International Congress ESTIV 21st – 25th November 2022

Matthew Stevenson[1], Lukasz Czekala[1], Roman Wieczorek[1], Edgar Trelles Sticken[1], Sarah Jean Pour[1], Fiona Chapman[1], Liam Simms[1], [1]Imperial Brands PLC, 121 Winterstoke Road, Bristol, BS3 2LL, UK,
Corresponding author's e-mail: Matthew.Stevenson@uk.imptob.com

1. INTRODUCTION

With the rise in popularity of e-cigarettes amongst adult smokers as a potentially less harmful alternative to combustible cigarettes, there is a need for better understanding of the potential biological impact of these products. *In vitro* techniques fulfil that need, allowing rapid and robust assessments. Here we describe the results from a range of studies utilising human 3D bronchial tissues for the assessment of e-cigarette aerosols.

2. METHODS

Study	Test Articles	Exposure	Cell system	Endpoints
1	- Commercial cigarettes - blu PLUS+™ e-cigarette, 2.4% nicotine, blueberry flavour - Blu PLUS+™ e-cigarette, 2.4% nicotine, no flavour	Using the VITROCELL VC1 manual smoking machine (VITROCELL Systems GmbH) tissues were exposed in triplicate to 9, 27 or 45 puffs of whole smoke generated from commercial cigarettes or to 80, 240 or 400 puffs of aerosol from blu PLUS+ e-cigarettes.	EpiAirway™ (MatTek Corp. Ashland, MA, USA) produced from a disease-free, non-smoking 23-year old, male Caucasian donor	Viability and barrier integrity (MTT Assay & transepithelial electrical resistance (TEER) using a EVOM2 voltohmmeter, cytokine secretion (IL-6 and IL-8) and oxidative stress (8-isoprostane) (both using ELISA) and tissue morphology (H&E). Endpoints were measured 24 hours after exposure.
2	- 3R4F reference cigarette - *my*blu™ e-cigarette, 2.4% nicotine, blueberry flavour	Using the VITROCELL VC1 manual smoking machine tissues were exposed to a single sub-cytotoxic exposure of 3R4F smoke and the equivalent nicotine delivered dose from *my*blu e-cigarette.	MucilAir™ (Epithelix Sarl) produced from a pathology-free, non-smoking 41-year old, male Caucasian donor	TEER using EVOM Epithelial Ohm Meter, cytotoxicity (Adenylate Kinase (AK), and cell viability (WST-8), RNA Sequencing using IlluminaTruSeq® Stranded Total RNA Library Prep Gold Kit and Gene set enrichment (fast-pre-ranked gene set enrichment analysis (fgsea) R package). Endpoints were measured 4 or 48 hours after exposure.
3	- 3R4F reference cigarette - *my*blu™ e-cigarette, 1.6% nicotine, tobacco flavour	Using Imperial Brands' Smoke Aerosol Exposure In Vitro System (SAEIVS) tissues were repeatedly exposed (3 times per week) at the ALI for 4 weeks to either 30, 60 or 90 puffs of aerosol/smoke/filtered humidified air control. 1R6F smoke was diluted with air 1:17 times whilst *my*blu aerosol was undiluted.	MucilAir™ (Epithelix Sarl) produced from a pathology-free, non-smoking 41-year old, male Caucasian donor	TEER using a EVOM2 voltohmmeter, cilia beat frequency (CBF) and active area (AA) (4x; Olympus IX53P1F inverted; and Sisson-Ammons Video Analysis software), LDH release (CytoTox 96® assay), pro-inflammatory mediator release (IL-1β, IL-6, IL-8, TNF-α, MMP-1, MMP-3 & MMP-9) and tissue morphology (H&E, MUC-5AC and FOX-J1 staining).

3. RESULTS

Study 1: Acute blu PLUS+ whole aerosol exposure up to 400 puffs did not significantly alter barrier function, cellular viability or cytokine secretion compared to air matched controls[1]

Figure 1. Tissue viability

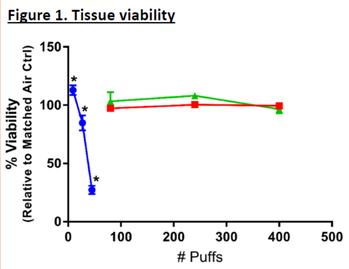
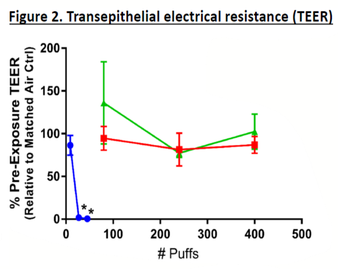


Figure 2. Transepithelial electrical resistance (TEER)



←Figures 1 & 2: Under the experimental conditions, cigarette smoke impaired barrier function (TEER) and reduced cell viability to approximately 30% after exposure to 45 puffs and induced secretion of inflammatory cytokines. blu PLUS+ aerosol up to 400 puffs did not alter barrier function or tissue viability compared to air matched controls.

Figure 3. Cytokine secretion: IL-6

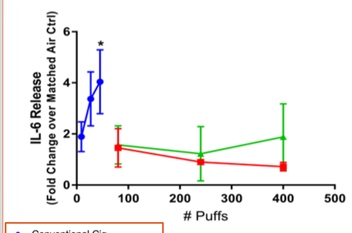
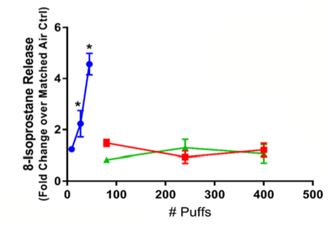


Figure 4. The oxidative stress response

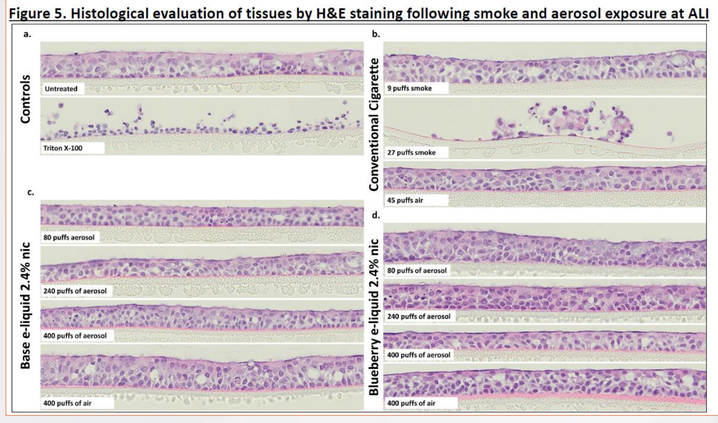


Conventional Cig
Base e-Liquid, 2.4% nic
Blueberry e-Liquid, 2.4% nic

Mean ± s.d. is shown (n = 3, *p-value ≤0.05 comparing smoke/aerosol to matched air exposure at each dose)

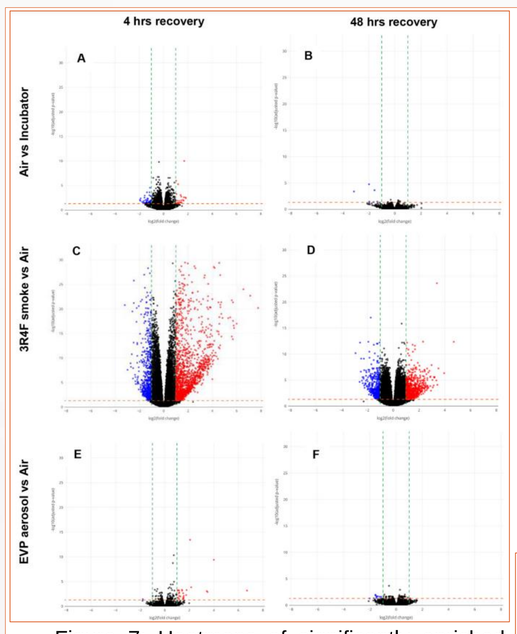
←Figures 3 & 4: Exposures of 27 and 45 puffs of 3R4F smoke, caused statistically significant increases in IL-6 and 8-isoprostane release. IL-6 levels remain largely unaffected by blu PLUS+ aerosols (except slight, non- significant increases for the highest dose, 400 puffs of flavoured e-liquid). blu PLUS+ aerosol exposures did not significantly change oxidative stress (8-isoprostane) levels compared to the matched air controls at any of the doses tested.

Figure 5. Histological evaluation of tissues by H&E staining following smoke and aerosol exposure at ALI

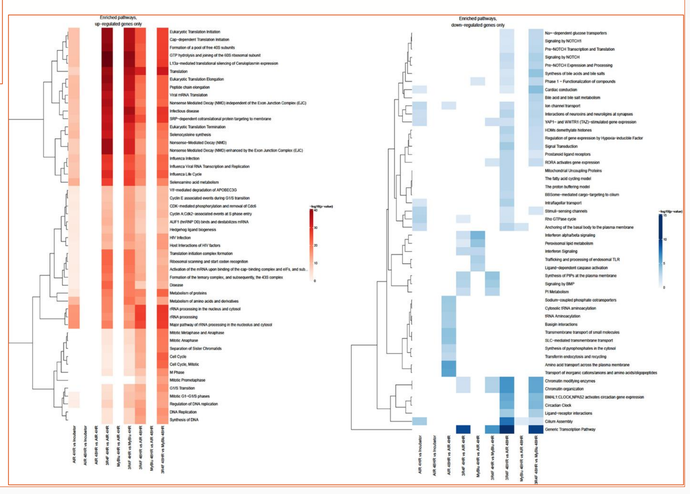


←Figure 5: No tissue morphology differences were observed between 9 puffs of cigarette smoke and match air control. EpiAirway tissue exposed to 27 and 45 (not shown) puffs of cigarette smoke demonstrated disruption to tissue architecture. blu PLUS+ aerosols up to the highest dose tested (400 puffs) did not significantly alter tissue morphology. H&E staining results correspond with the measured TEER values and cell viability (See Figures 1 and 2).

Study 2: Acute *my*blu whole aerosol exposure to 3D human bronchial tissue resulted in minimal transcriptomic responses when compared to cigarette smoke[2]

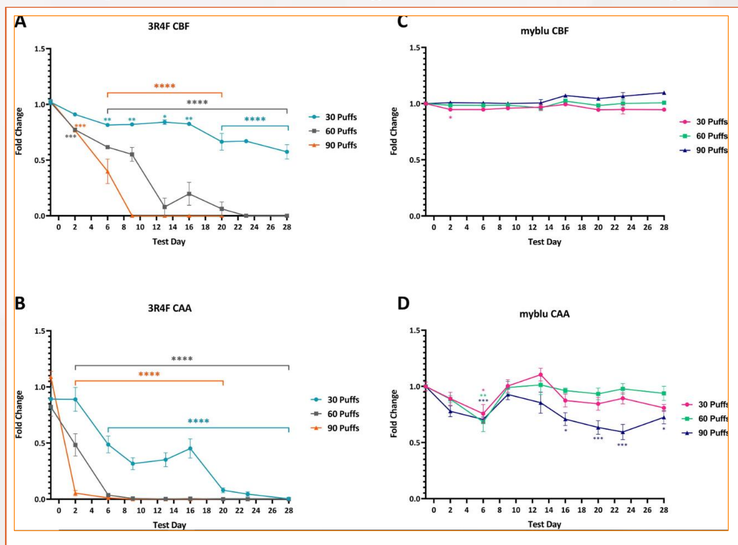


← Figure 6: Volcano plots for six RNA-Seq comparisons showing significance (log10 adjusted p-values against log2 fold change (FC)). Differential Expressed Genes, up-regulated (red dots), down-regulated (blue dots) and representative subsample of full data set (black dots). At 4 hours recovery, 3R4F smoke significantly induced 2 FC expression of 2199 genes (up- and down-regulated) and at 48 hours, 1103 genes were up- and down-regulated. *my*blu aerosol exposure resulted in 28 and 12 significantly ($p < 0.05$ FDR, FC≥2) expressed genes at 4 hours and 48 hours respectively. There was also a high degree of similarity in the differential gene expression response between 3R4F smoke and *my*blu aerosol and 3R4F smoke and air. At 4 hours recovery there was 64% similarity in the gene expression profile, which at 48 hours remained high at 54%. This may suggest a degree of similarity between the cell cultures' genetic response to e-cigarette aerosol and that following air exposure.

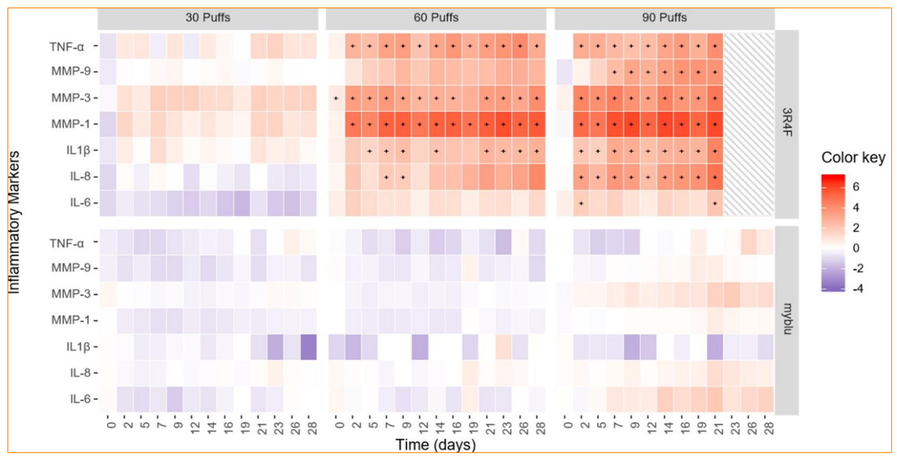


→ Figure 7: Heatmaps of significantly enriched Reactome pathways (an online database of biological pathways). Significant genes (at adjusted p-value <0.05 and fold change >2) from each comparison were analysed for enrichment of top 50 Reactome pathways. 3R4F had large effects across a broad range of pathways and were mainly found to significantly affect pathway-specific genes after 48 hours of recovery, including oxidative stress and inflammation. *my*blu aerosol exposed tissues had little change in gene expression, which largely occurred at 4h post exposure and largely resolved at 48h.

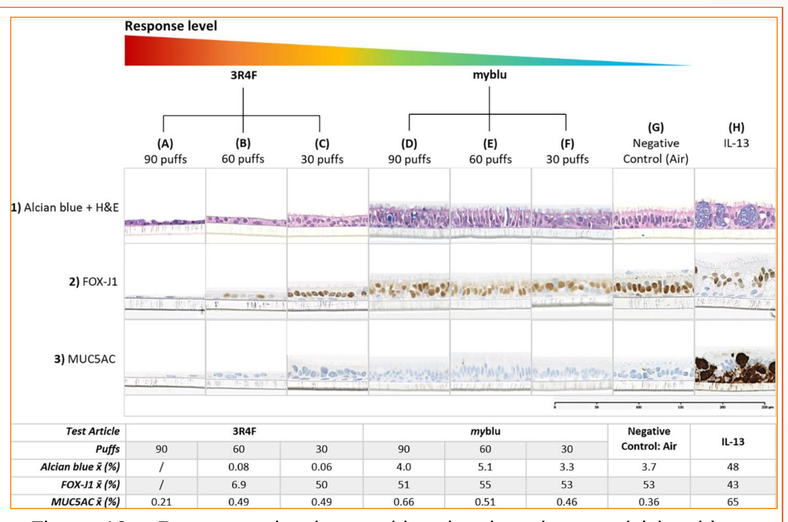
Study 3: Repeated *my*blu whole aerosol exposure resulted in little to no ciliated cell, inflammatory marker or tissue morphology disruption, whilst cigarette smoke significantly altered all endpoints[3]



↑ Figures 8 A-D: Fold change in CBF and CAA over time following repeated exposures to 3R4F smoke (A & B) and *my*blu aerosol (C & D) compared to matching air controls. The 3R4F smoke caused a dose dependent decrease of both CBF and CAA over the exposure period. The CBF and AA was not markedly affected by *my*blu aerosol. *p ≤0.05, **p ≤0.01, ***p ≤0.005, ****p ≤0.0001. Error bars = SEM.



↑ Figure 9: Heat-maps of the differences in expression of inflammatory markers, calculated using the difference between log intensity of the test product and the control (log fold change). The significance threshold in the statistical test was adjusted to 5% ($p-value < 0.05$) and significant changes are indicated by '+'. The *my*blu aerosol at all doses tested did not significantly alter cytokine secretion in comparison to matched air control, whilst the 3R4F cigarette significantly altered the secretion of cytokines at 60 and 90 puff exposures.



Test Article	3R4F			myblu			Negative Control: Air	IL-13
Puffs	90	60	30	90	60	30		
Alcian blue x̄ (%)	/	0.08	0.06	4.0	5.1	3.3	3.7	48
FOX-J1 x̄ (%)	/	6.9	50	51	55	53	53	43
MUC5AC x̄ (%)	0.21	0.49	0.49	0.66	0.51	0.46	0.36	65

↑ Figure 10: Representative immunohistochemistry images (alcian blue + H&E, FOX-J1 and MUC5AC) of 3D tissues after exposure to test articles or negative control (humidified filtered air). Exposure to cigarette smoke led to dramatic changes in tissue morphology and ciliated cell numbers over the experimental period at all doses tested but did not appear to induce goblet cell hyperplasia. *my*blu aerosol exposed tissues were indistinguishable from air control.

4. CONCLUSIONS

- The 3D organotypic tissues used in these studies can be utilised for a range of different endpoints and exposures, and therefore may be considered as a key component of inhaled product assessment strategy.
- These results show that e-cigarettes have a marked reduction in cellular and transcriptomic responses, adding to the growing body of evidence that e-cigarettes are likely to be considerably less harmful than combustible cigarettes.

REFERENCES

[1] Czekala L, Simms L, Stevenson M, Tschierske N, Maione AG, Walele T, Toxicological comparison of cigarette smoke and e-cigarette aerosol using a 3D in vitro human respiratory model. Regul Toxicol Pharmacol. 2019 Apr;103:314-324.1.; [2] Phillips G, Czekala L, Behrsing HP, Amin K, Budde J, Stevenson M, Wieczorek R, Walele T, and Simms L. "Acute electronic vapour product whole aerosol exposure of 3D human bronchial tissue results in minimal cellular and transcriptomic responses when compared to cigarette smoke. Toxicol. Res. Appl. 2021;5:1–19; [3] Czekala L, Wieczorek R, Simms L, Yu F, Budde J, Trelles Sticken E, Rudd K, Verron T, Brinster O, Stevenson M, Walele T, Multi-endpoint analysis of human 3D airway epithelium following repeated exposure to whole electronic vapor product aerosol or cigarette smoke, Current Research in Toxicology, Volume 2, 2021, Pages 99-115.