Multiparameter characterisation of human alveolar macrophage-epithelial co-culture (ImmuLUNG™) following exposure to whole smoke

labcorp

ImmuONE™
Lung & Immune *In Vitro* Assay Systems

M. Hollings[1], E. Rothwell[1], R. Bedford[1], N. Haydare[2], B. Ghiot[2], L. Scott[2], R. Mahendran[2] and V. Hutter[2]; [1]Labcorp, Harrogate, UK; [2]ImmuONE Ltd, Stevenage, UK

Summary

ImmuLUNG™ cultures were exposed to whole smoke (WS) generated from 1R6F Kentucky Reference using a Vitrocell® VC10® smoking robot at varying airflows. The effects of WS on cytotoxicity, oxidative stress, epithelial barrier permeability, cytokine secretion, gene expression and individual cell phenotype via high content imaging analysis were assessed. The ImmuLUNG™ epithelial component exhibited high TEER values. Concentration-dependent viability and oxidative stress responses were observed, as indicated by 8-isoprostane/LDH release. Gene expression analysis revealed relevant macrophage RNA markers and upregulation of genes controlling macrophage production, differentiation, and function. Proteomic analysis showed increased expression of inflammatory signaling proteins and protein expression changes associated with oxidative stress control. High-content image analysis of ImmuLUNG™ macrophages revealed morphological changes, indicating assessment of macrophage endpoints are compatible with WS exposure.

Introduction

Conventional inhalation safety assessment of aerosols often relies on immortalized cell lines or primary lung models at the air-liquid interface (ALI). While valuable in many contexts, these models frequently lack immune components, exhibit species differences, and demonstrate donor variability, raising concerns about reproducibility and relevance. Multiple endpoints are linked to better mechanistic understanding of toxicity and inflammation, which informs safety.

ImmuLUNG™, a human epithelial-macrophage co-culture, addresses these issues by allowing multiplexing at the ALI to predict aerosol-induced toxicological and immune responses in human lungs. This study aimed to validate ImmuLUNG™ as a tool for investigating the toxicological and immunological impacts of whole cigarette smoke on alveolar epithelial-macrophage co-culture.

Methods

3D cell model: ImmuLUNG™ tissues supplied by ImmuONE Ltd., UK and cultured following manufacturer's guidelines **(Figure 1)**

Test articles: 1R6F reference test cigarette (University of Kentucky, USA)

Controls: ALI control for treatment was exposed to 0.2 L/min flowing air. Untreated tissues were used as INC controls. Blank transwells were used as negative controls for the transepithelial electrical resistance (TEER), WST-8 (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and LDH (Takara-Bio, Göteborg, Sweden) assays. Triton X-100 at 1%, Staurosporine and PMA (100 ng/mL)/Ionomycin (2 µg/mL) were treated basolatterally as a positive control.

Whole aerosol generation: Vitrocell® VC10® Smoking Robot (serial number VC10/301118) was used to generate whole smoke (WS). WS was then diluted with different flow rates of flowing air. 1R6F- 10, 8, 6, 4 L/min. A vacuum rate of 5 mL/min was used. 1R6F was smoked according to ISO 20768 smoking regime (55 mL puff, 2 sec duration, 30 sec frequency), 100% vent blocked. Two 1R6F were smoked per airflow for a total of 8 minutes.

TEER: Trans Epithelial electrical resistance (TEER) measurements were conducted pre- and post-exposure with EVOM2 epithelial volthometer (World Precision Instruments Ltd., Hitchin, UK).

WST-8 and LDH Release: WST-8 and lactate dehydrogenase (LDH) assay was performed according to the manufacturer's instructions. WST-8 was determined by measuring optical density at 450nm and LDH activity at 490 nm.

Gene expression: RNA isolation performed with RNAqueous™ Total RNA Isolation Kit. cDNA performed with QuantiTect® Reverse Transcription reagent and gene expression performed using custom TaqMan™ Gene Expression 96-well plates all as per manufacturer's instructions.

Nicotine determination: Dosimetry was performed via the analytical determination of nicotine in PBS from the smoke/aerosol exposed liquid trap. Sub-samples of each well were diluted with a basified acetate buffer/acetonitrile solution prior to quantification using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) using an AB SCIEX® API 4000™ coupled to Waters® Acquity UPLC.

Cytokine/chemokine analysis: The Proteome Profiler Human Cytokine Array kit was purchased from R&D Systems (Abingdon, Oxfordshire, UK) and used for the parallel determination of the relative levels of 36 selected human cytokines and chemokines.

Morph_ONE, high content image analysis (HCIA): Fluorescent probes were obtained from Invitrogen, Renfrewshire, UK. For the cell morphology, assessment cells were stained with a dye cocktail containing Hoechst 33342 (nuclei) and Cell Mask™ Deep Red (cytoplasm to identify vacuoles).

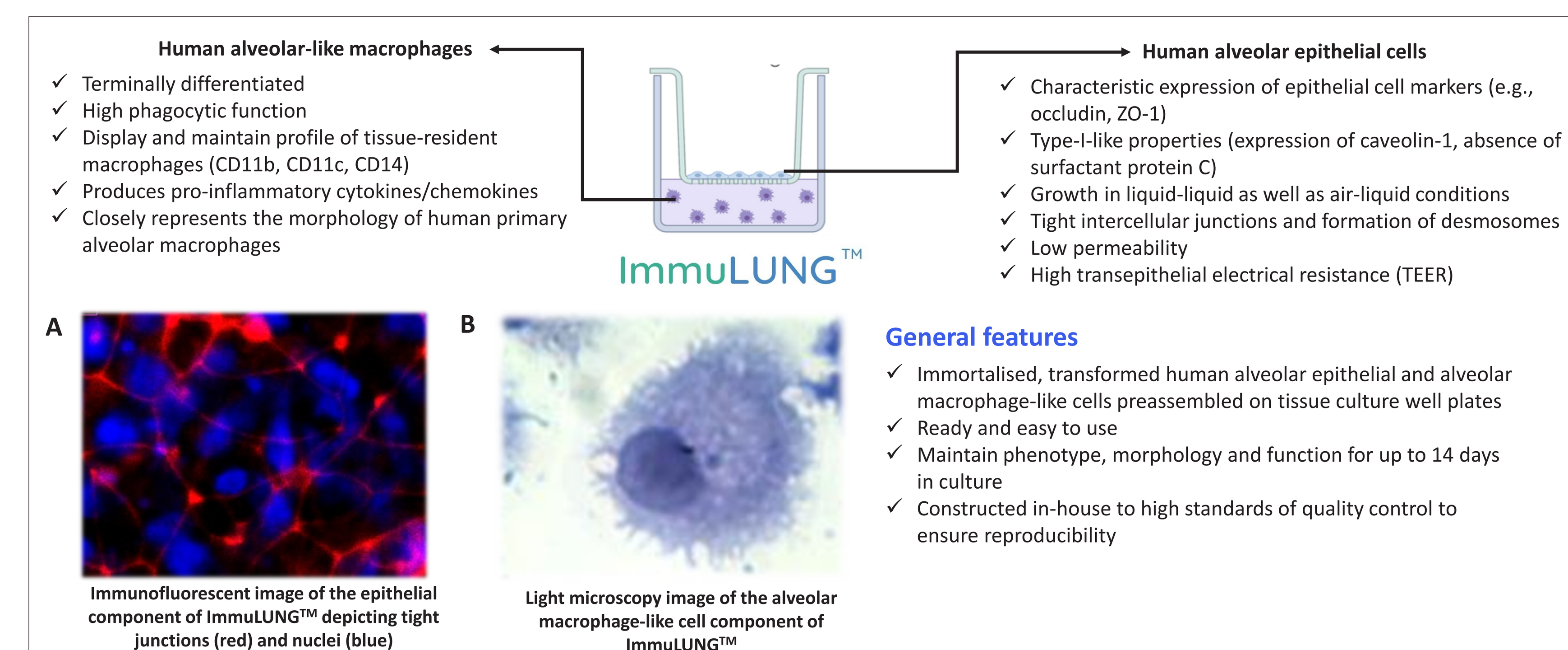

Figure 1. Schematic and characterization of ImmuLUNG™ system used in this study.

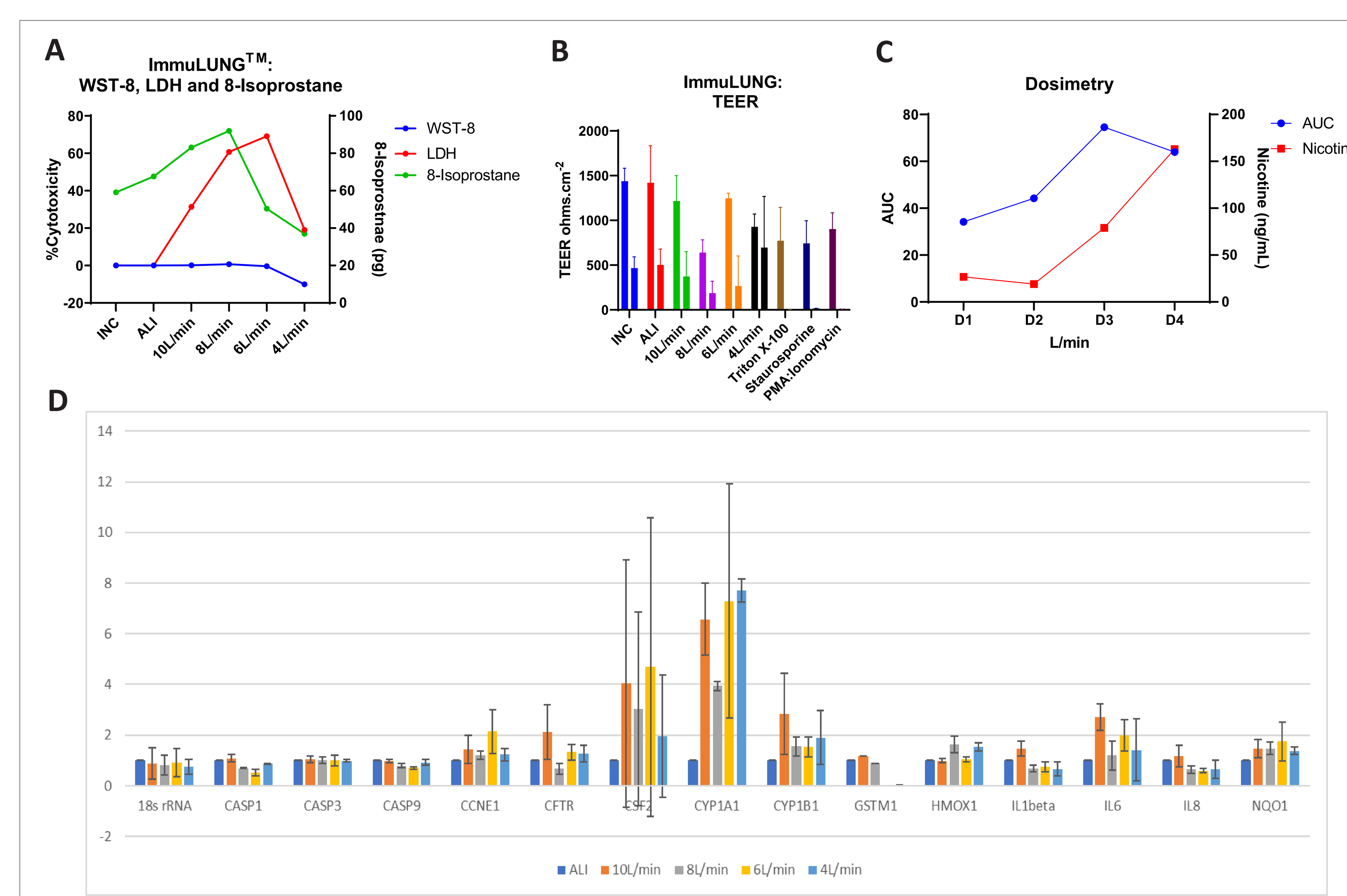

Figure 2. (A) WST-8 and LDH results as a % cytotoxicity of ALI, at each dose. 8-isoprostane was measure in both module and recovery media and converted to total (pg) 8-isoprostane release. **(B)** Barrier integrity was measured pre- and post-exposure and was measured by transepithelial electrical resistance (TEER). Mean ± SD. **(C)** Photometer Area Under Curve values and nicotine determination confirming exposure. **(D)** Gene expression data post 24-hour recovery, RNA expression was quantified by qPCR using the ΔΔCt method and results were normalised first to the GAPDH gene control and then to the ALI air control.

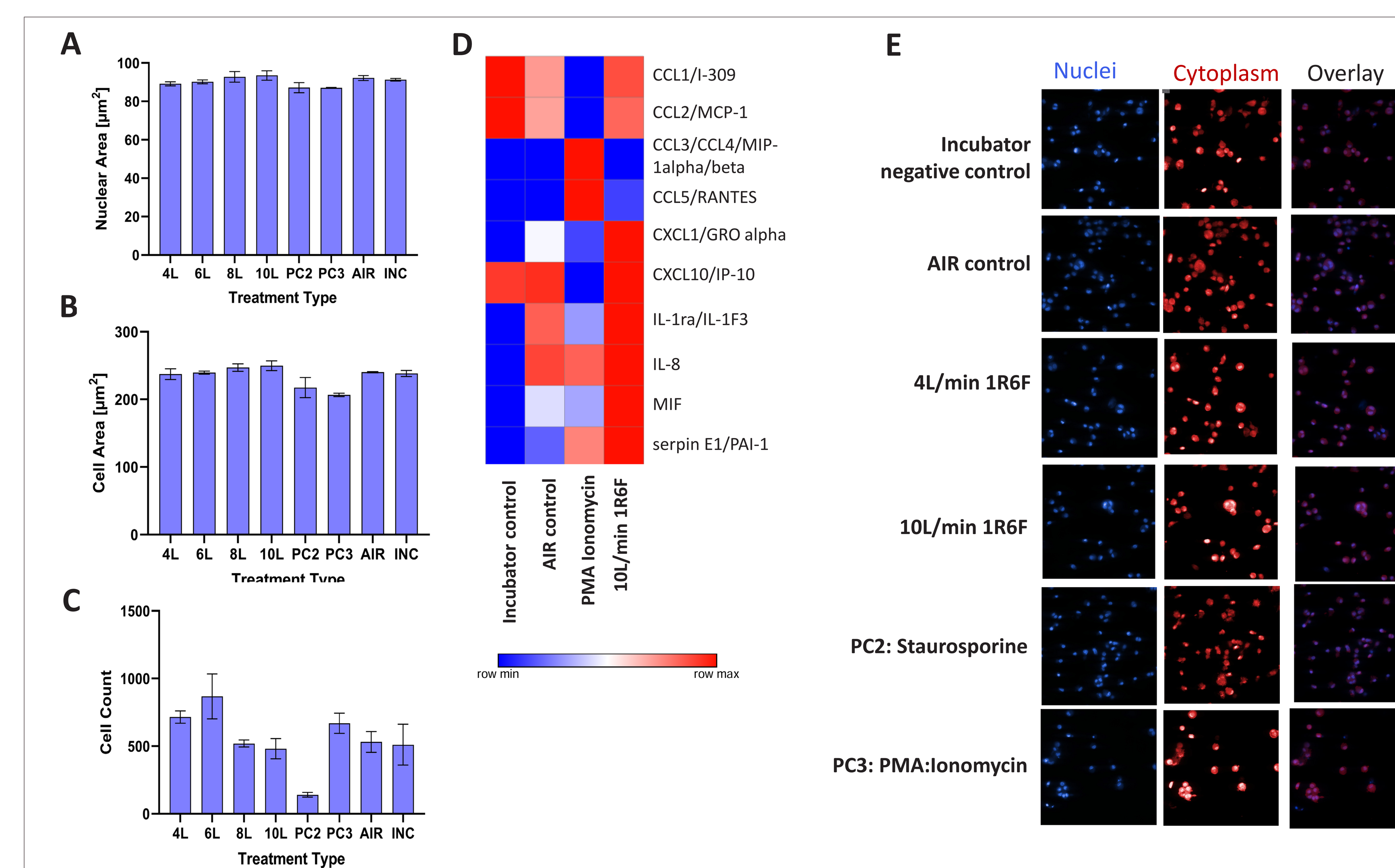

Figure 3. Assessment of immune responses using ImmuLUNG™ system. (A-C) Macrophage morphology including nuclear and cellular area and cell count were quantified using high content imaging. Data were expressed as **(D)** Evaluation of panel of human cytokine/chemokine panel secretion for ImmuLUNG™ after exposure to air control, PMA Ionomycin or whole aerosol cigarette smoke. Data were expressed as a percentage of the maximum pixel density for each cytokine among all treatments to determine the relative change in cytokine levels between samples. **(E)** Representative images of macrophage component of ImmuLUNG™. Cells were fixed, stained with Hoechst 33342 for nuclei (blue) and Cell Mask Deep Red for cytoplasm (red), harvested and imaged. Images were captured using IN Cell Analyzer 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.

Results

- No cytotoxicity as measured by WST-8. Concentration dependent increase in LDH release (membrane integrity) and 8-isoprostane (oxidative stress) **(Figure 2A)**.
- Epithelial cell survival post transit was good (TEER >1000 ohms.cm^{-2}) **(Figure 2B)**.
- Expression of relevant macrophage RNA markers (CSF2) **(Figure 2D)**.
- Concentration dependent response for IL-1β, IL-6 and IL-8 RNA expression. HMOX-1 and NQO-1 correlated with 8-isoprostane release. Increase in CYP genes confirm xenobiotic metabolism and confirm 1R6F exposure **(Figure 2D)**.
- Morphometric changes in macrophages corresponded with toxicity of treatment **(Figure 3A-C)**.
- Cytokine and chemokine analysis revealed pro-inflammatory phenotypes upon treatment with 1R6F **(Figure 3D)**.
- HCIA combines alveolar macrophage morphology/health endpoints as an *in vitro* tool for inhaled safety assessment. Multiparameter data analysis provides high-throughput profiling of drug-impact on numerous features of macrophage cell, focusing on cell vacuolation pattern. **(Figure 4).**
- Morphometric endpoints (cell size, vacuolation pattern) could be directly correlated with *in vivo* observation.

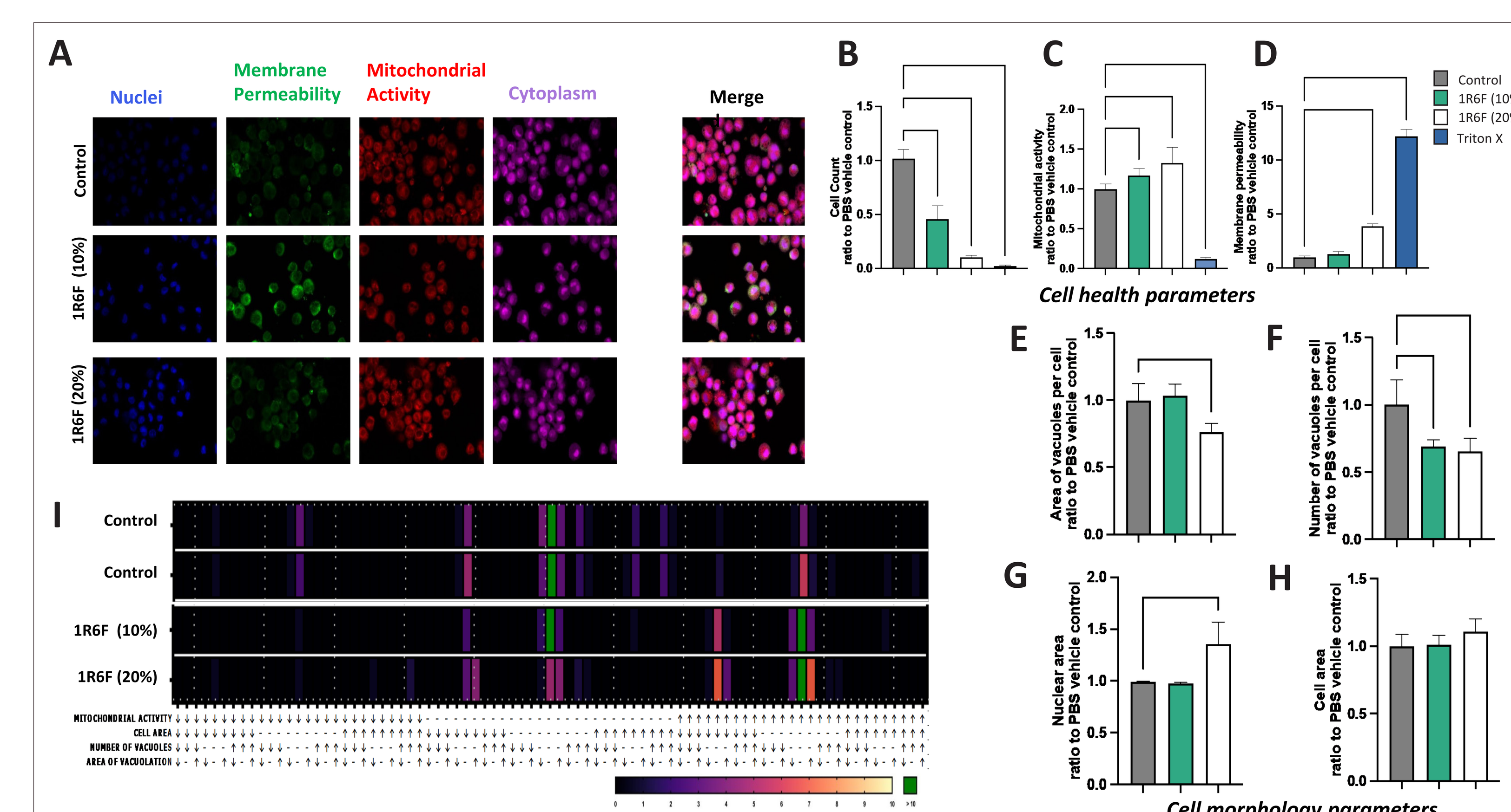

Figure 4. High content image analysis of macrophage component of ImmuLUNG™ exposed to cigarette smoke 1R6F. (A) Representative images of alveolar macrophages stained with Hoechst 33342 for nuclei (blue), Cell Mask™ Deep Red for cytoplasm (purple), MitoTracker™ Red for mitochondrial activity (red) and Image-It™ Dead Green™ for membrane permeability. Images were captured using IN Cell Analyzer with a 40x objective in standard 2D imaging mode. **(B-H)** Cell health and morphology parameters were quantified from images and presented as mean ± SD. **(I)** Heatmap indicating phenotypic assessment of alveolar macrophages. ImmuPHAGE™ model (an *in vitro* cell model of human alveolar macrophages) was exposed to test compounds A1-A4 and controls for 48 h. 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 one experiment (n=7). 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.

Conclusions

- ImmuLUNG™ is a suitable model for assessing cytotoxicity and mechanistic insights following exposure to whole smoke and potentially for next generation products (NGPs).
- This valuable tool for collaborative research is compatible with aerosol exposure studies, and equipped with multiple endpoints to elucidate mechanistic toxicity, particularly lung inflammation.
- The system is suitable for high content imaging providing detailed individual cell data for better characterisation of different types of immune responses.
- Our findings underscore its utility in assessing the toxicological and immunological effects of whole smoke on alveolar macrophages, highlighting its potential for inhaled and systemic product safety assessment.
- This form of analysis may be useful in identifying new inhaled drug liabilities in *in vitro* environment, before processing to *in vivo* studies and to facilitate informed decision making.

Reference

1. Xiang, L. 2016. *In vitro* toxicity testing of cigarette smoke based on the air-liquid interface exposure: A review. Toxicology *in vitro*, 36, 105-113.

CORESTA Congress; October 13-17, 2024; Edinburgh, Scotland

©2024 Labcorp. All rights reserved.