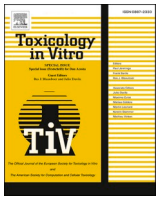
Toxicology in Vitro 86 (2023) 105506

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit



High content analysis of *in vitro* alveolar macrophage responses can provide mechanistic insight for inhaled product safety assessment

V. Hutter [a,b,*], S. Hopper [c,d], J. Skamarauskas [b], E. Hoffman [a]

[a] ImmuONE Ltd, Science Building, College Lane, Hatfield, Herts AL10 9AB, UK
[b] Centre for Topical Drug Delivery and Toxicology, University of Hertfordshire, College Lane Campus, Hatfield, Herts AL10 9AB, UK
[c] Thornton & Ross Ltd, Linthwaite, Huddersfield HD7 5QH, UK
[d] School of Clinical and Applied Sciences, Leeds Becket University, City Campus, Woodhouse Lane, Leeds LS1 3HE, UK

ARTICLE INFO

Editor: Martin Leonard

Keywords:
Alveolar macrophage
High content analysis
Inhalation toxicology
In vitro
E-liquid

ABSTRACT

Assessing the safety of inhaled substances in the alveolar region of the lung requires an understanding of how the respired material interacts with both physical and immunological barriers. Human alveolar-like macrophages *in vitro* provide a platform to assess the immunological response in the airways and may better inform the understanding of a response to an inhaled challenge being adaptive or adverse. The aim of this study was to determine if a morphometric phenotyping approach could discriminate between different inhaled nicotine products and indicate the potential mechanism of toxicity of a substance. Cigarette smoke (CS) and e-liquids extracted into cell culture medium were applied to human alveolar-like macrophages in mono-culture (ImmuONE™) and co-culture (ImmuLUNG™) to test the hypothesis. Phenotype profiling of cell responses was highly reproducible and clearly distinguished the different responses to CS and e-liquids. Whilst the phenotypes of untreated macrophages were similar regardless of culture condition, macrophages cultured in the presence of epithelial cells were more sensitive to CS-induced changes related to cell size and vacuolation processes. This technique demonstrated phenotypical observations typical for CS exposure and indicative of the established mechanisms of toxicity. The technique provides a rapid screening approach to determine detailed immunological responses in the airways which can be linked to potentially adverse pathways and support inhalation safety assessment.

1. Introduction

Currently, *in vitro* inhalation toxicology is mostly limited to epithelial cell culture models of the conducting airways (Faber and McCullough, 2018). Constructed of human primary cells or established cell lines cultured at the air-liquid interface these provide an adequate representation of the barrier function, ciliated and goblet cells found in the upper airways (Faber and McCullough, 2018; Upadhyay and Palmberg, 2018). Previously, this type of model for pre-clinical inhalation safety assessment has largely been satisfactory with many inhaled medicines targeting the bronchi or upper bronchiolar regions of the lung (Faber and McCullough, 2018; Lacroix et al., 2018). However, in recent years, with better understanding of inflammatory airways disease and particle deposition in the lungs, targeting inhaled substances to the small airways and alveolar region now presents a challenge for preclinical inhalation toxicity assessment (Forbes et al., 2014; Movia et al., 2020). Furthermore, with infectious and communicable lung diseases demonstrating pathophysiology within the small airways understanding immune responses and interplay with epithelial cells within the alveolar region has become paramount (Bissonnette et al., 2020; Hewitt and Lloyd, 2021).

Over the past decade, it has become well established that alveolar macrophages are the first line of defence against inhaled substances in the lower airways (Jones and Neef, 2012). Whilst their roles in alveolar homeostasis, infection and disease are relatively well understood, their responses linked to inhaled toxicity are poorly defined (Forbes et al., 2014). For example, one third of candidate inhaled medicines fail in pre-clinical *in vivo* rodent studies due to the presence of abnormal alveolar macrophage morphology, whereby the cell takes on a highly vacuolated or 'foamy' appearance (Forbes et al., 2014). The extent to which this response is adaptive or adverse is poorly understood and as such results in the failure of candidate compounds to progress into phase 3 trials,

* Corresponding author at: ImmuONE Ltd, Science Building, College Lane, Hatfield, Herts AL10 9AB, UK.
E-mail addresses: victoria.hutter@immuone.com, v.hutter@herts.ac.uk (V. Hutter).

https://doi.org/10.1016/j.tiv.2022.105506
Received 6 June 2022; Received in revised form 11 October 2022; Accepted 23 October 2022
Available online 27 October 2022

despite not knowing if these observations are relevant to human health (Forbes et al., 2014; Jones and Neef, 2012; Nikula et al., 2014). Whilst the pharmaceutical industry is required to undergo rigorous safety assessment of inhaled products, there are many other industries with aerosolised products on the market which are known to be deposited in the alveolar region which either historically have not required detailed assessment to reach the market or are forbidden from performing *in vivo* assessment (Clippinger et al., 2018). As such, the need for human relevant, immunocompetent models of the alveolar region is becoming increasingly urgent (Krewski et al., 2020; Movia et al., 2020).

Previously, we have demonstrated that high content image analysis of both rat and human alveolar macrophages in monoculture can be used to reproducibly identify differences in cell morphology between healthy or untreated cells and those induced to a well-established example of a 'foamy' macrophage response by treatment with amiodarone to induce phospholipidosis (Hoffman et al., 2017; Patel et al., 2019). Furthermore, this detailed morphological assessment could also identify differences between different types of foamy macrophage response (apoptosis and phospholipidosis) as well as tracking the development and resolution of these morphological characteristics over time (Hoffman et al., 2020) and potential link to macrophage activation (Hoffman et al., 2021). Whilst there are other functional endpoints that could be considered *in vitro* for the assessment of the mechanism of response of an alveolar macrophage, morphological assessment is the focus of this study due to the direct link with standard alveolar macrophage pathology reporting in *in vivo* studies (*e.g.* course/fine vacuolation properties and changes to cell size) (Forbes et al., 2014; Nikula et al., 2014).

Whilst previous work has established the technique is suitably reproducible and able to reliably detect morphological changes in cells exposed to substances in comparison with an untreated control, each characteristic has been reported independently. This study was based on the hypothesis that combining multiple morphometric and cell stress descriptors to gain a phenotypic profile of each individual cell could help elucidate the potential mechanism of toxicity of a substance that could be ultimately applied as part of a screening approach for discovery and/or early product development. A second hypothesis was that the presence of alveolar epithelial cells within the *in vitro* system would influence the alveolar macrophage response to support the differentiation of adverse and non-adverse responses.

The toxicological profile of inhaled cigarette smoke has been well established *in vivo* and *in vitro*, however the long-term implications on lung health of inhaling e-liquids is largely unknown (Gotts et al., 2019). Whilst both these products are established to deposit in the alveolar region, there is limited literature available reporting their impact on human alveolar health *in vitro* and specifically their interaction with alveolar macrophages and potential adverse, toxicological or immunological responses (Jasper et al., 2021). As such, the aim of this study was to establish if alveolar macrophage phenotypic responses to a known inhaled toxicant (cigarette smoke) differed when cultured alone or as part of a human *in vitro*, 3D immunocompetent model of the alveolus and compare these responses to e-liquid products. Ultimately, the goal was to ascertain if this approach could provide a more accurate and mechanistic-driven approach to inhaled drug safety assessment.

2. Materials and methods

2.1. Preparation of cigarette and e-cigarette samples

Smoke from Embassy No. 1 cigarettes (Imperial Tobacco, Bristol, UK) and two commercial flavours of e-liquid (Green Apple and Tutti Frutti) with a base ratio of 50:50 PG/VG and 1.8% nicotine (Totally Wicked, Blackburn Lancashire, UK) were used in this investigation. *E*-liquids were individually vaped using a Tornado V5 e-cigarette (Totally Wicked, Blackburn, Lancashire, UK). Both e-liquid vapes and cigarette smoke were extracted into 40 mL of complete cell culture medium (ImmuPHAGE™ growth medium, ImmuONE, Hatfield Herts, UK) using a Ceti 8 smoking machine with button activation (Cerulean, Milton Keynes, UK) using a square wave puff profile with 3 s draw, 45 mL volume and 30 s interval. Samples were vaped at a 45° angle until approximately 1.0 g of e-liquid had been used. A control sample was produced under the same conditions without being attached to an e-liquid or cigarette which was used as an experimental air control for comparison of all samples.

2.2. Cell culture and sample exposure

ImmuPHAGE™ (human alveolar macrophage-like cells) and ImmuLUNG™ (3D alveolar epithelial-macrophage co-culture model) were provided by ImmuONE (Hatfield, Herts, UK). Cells were authenticated prior to experimentation and validated for macrophage and epithelial biochemical and physical characteristics, cell surface markers and functionality (ImmuONE, Hatfield, Herts, UK). Both cell culture models ($n = 12$ mono-culture, $n = 9$ co-culture) were exposed to either ImmuPHAGE™ growth medium control, smoke or e-liquid samples at a volume of 1 mL / 1×10^5 immune cells and incubated at 37 °C, 5% *v*/v CO_2 in a humidified atmosphere for 4 h, 24 h and 48 h (ImmuPHAGE™) or 48 h (ImmuLUNG™).

2.3. Fluorescence staining and imaging

Alveolar macrophage-like cells were processed and assessed for cell health and morphology as previously described (Hoffman et al., 2017). In brief, cells were stained with a dye cocktail containing Hoechst 33342 10 μg/mL, MitoTracker Red 300 nM and Image-It Dead Green 25 nM (Invitrogen, Paisley, Renfrewshire, UK) for 30 min. Cells were washed once with 100 μL PBS and centrifuged at 380*g* before fixation with 3.7% *w/v* paraformaldehyde for 15 min. Fixed cells were stained overnight with Cell Mask Deep Red (Invitrogen, Renfrewshire, UK) diluted 1:1000 (according to the manufacturer's protocol). Cells were washed once with PBS as described above before imaging. Samples were stored at 4 °C in the dark for up to 7 days prior to sample acquisition. Images were captured using the In Cell Analyser 6000 (GE Healthcare, Little Chalfont, Bucks, UK) with a 40× objective in standard 2D imaging mode with an exposure time of 0.1 s.

2.4. Quantitative high content image analysis

Image analysis was performed as previously described (Hoffman et al., 2017) using In Cell Developer Toolbox v 1.9.2, Level 3 analysis (GE Healthcare, Little Chalfont, Bucks, UK). In brief, the cell nuclear dye Hoechst 33342 was used to identify nucleated cells, while Cell Mask Deep Red staining highlighted cytoplasm and allowed quantification of vacuoles based on negative staining. Vacuoles within cells were identified based on negative staining and quantified using a grey scale threshold of 19,000 to minimize any background noise and to include only true vacuole features. MitoTracker Red and Image It Dead Green were used to identify the extent of mitochondrial activity (cell stress) and cell viability respectively. Each sample was imaged using 12 fields representing in total between 500 and 1500 cells per well. Quantitative measurements for each cell were generated from the image analysis for cell area, vacuole number per cell and vacuole area per cell.

2.5. Post-analysis cell phenotyping

A phenotypic analysis approach was adopted using three descriptors of cell morphology (cell area, number of vacuoles and total area of vacuolation) and one for cell stress (mitochondrial activity). These descriptors were selected to enable separation of the cell population and identify different mechanisms of cellular response. Each descriptor was assigned a classification level (low <10th centile; moderate 10th – 90th centile; high >90th centile) resulting in 81 potential phenotypes. Cells

were sorted into these 81 phenotypes and data expressed as the percentage of the population with the given phenotype.

2.6. Statistical analysis

A two-way ANOVA analysis with Dunnett's multiple comparison post-hoc tests were used to assess the statistical significance between the different cell treatments. Statistical significance was evaluated at a 99.9% confidence level ($P < 0.001$). All statistical tests were performed using GraphPad Instat® version 9.0.0 (GraphPad Software, San Diego, CA).

3. Results

3.1. Cell health characterisation

Individual cell data for each parameter related to cellular health (cell count, membrane permeability and mitochondrial activity) were derived from the images and plotted relative to the air control (Fig. 1). The relative cell count (number of cells counted during analysis) was significantly reduced ($P < 0.001$ co-culture) after 48 h exposure to cigarette smoke (Fig. 1a). In comparison, relative membrane permeability was significantly increased ($P < 0.001$) after 48 h exposure to cigarette smoke in the alveolar macrophage mono-culture model (ImmuPHAGE™) only (Fig. 1b). Relative cell count provides a more accurate assessment of cell health as dead cells are removed during sample preparation. Mitochondrial activity per cell was significantly reduced ($P < 0.001$) for cells exposed to cigarette smoke in comparison with air control samples at all time points assessed and for both alveolar macrophage-like cells in mono- and co-culture models (Fig. 1c). In comparison, no significant change ($P > 0.05$) in cell number, membrane permeation or mitochondrial activity was noted for cells exposed to either of the e-liquid samples. This suggests the viability and cell stress of alveolar macrophage-like cells were unaffected by exposure to e-liquid samples.

3.2. Characterisation of morphological features

No significant differences ($P > 0.05$) were observed in gross morphological features (cell area and nuclear area) for alveolar-macrophage-like cells in mono- or co-culture models exposed to e-liquids or cigarette smoke samples in comparison with air control (Fig. 2). The number of vacuoles per cell was significantly reduced ($P < 0.001$) for alveolar macrophage-like cells exposed for 24 h or greater to cigarette smoke in both mono-culture and co-culture systems (Fig. 3b). Whilst no significant differences ($P > 0.05$) were observed for the number of vacuoles for e-liquid samples. The area occupied by vacuoles within each cell was not significantly different ($P > 0.05$) among samples (Fig. 3a). This indicates that cells exposed to cigarette smoke have increased numbers of small vacuoles with no changes in their size within the cell.

3.3. Phenotyping multiple individual cell responses

Whilst the data presented in Figs. 1-3 provides valuable insight to the individual cell health and morphology of the alveolar macrophage-like cell population, assessing individual parameters in isolation does not provide the full breadth of cellular responses to a substance. Therefore, a phenotyping approach was adopted which combined four characteristics (mitochondrial activity, cell area, number of vacuoles and cell area occupied by vacuoles) separated into three levels to generate 81 potential phenotypes (Fig. 4). Alveolar macrophage-like cells exposed to the air control consistently displayed 12 key phenotypes, each occupying >1% of the cell population which were replicated in all timepoints for both mono-culture and co-culture systems (Fig. 4a), supporting the reproducibility of the baseline alveolar macrophage-like characteristics. Exposure to cigarette smoke significantly altered the cell phenotype profile by reducing the mitochondrial activity (Fig. 4b), whilst the phenotype profile for e-liquid exposure was similar to that of the air control samples (Fig. 4c,d).

The 27 phenotypes which each represented >1% of the population in all samples were selected for more detailed comparison (Fig. 5). For alveolar macrophage-like cells exposed to the air control, three phenotypes were identified as significantly different between mono-culture and co-culture systems ($P > 0.001$) however the difference in the values was <1.5 fold changes (Fig. 5a) supporting the reproducibility of alveolar macrophage-like cell morphology and mitochondrial activity at rest in both mono- and co-culture models. For cells exposed to cigarette

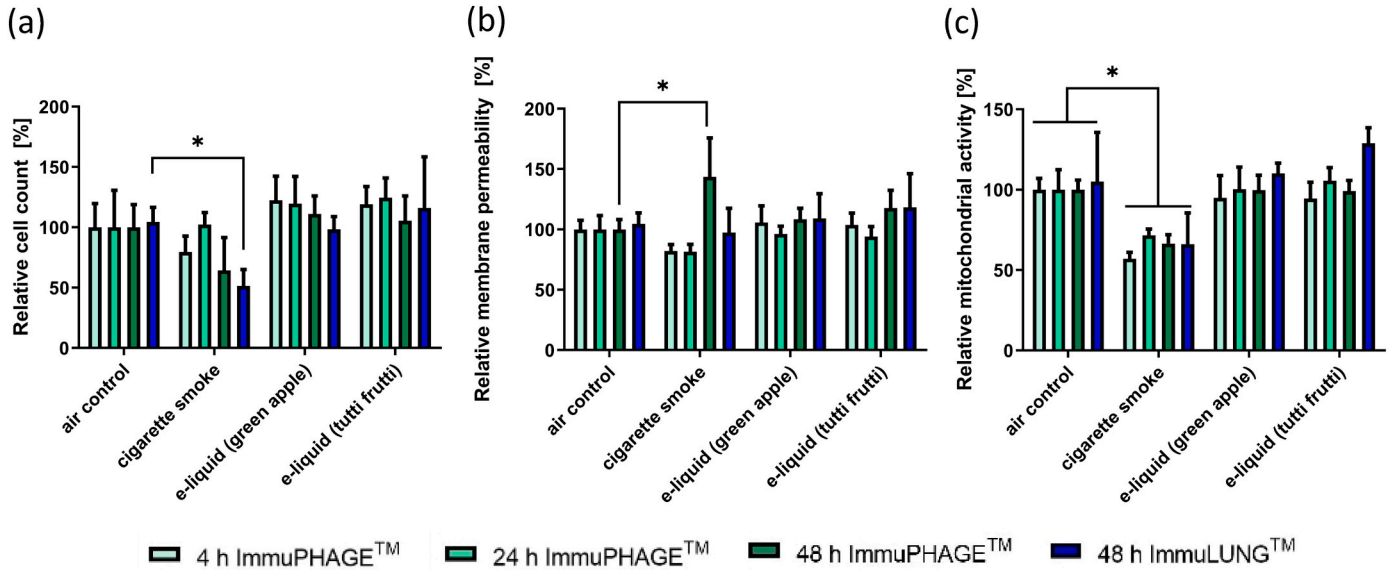


Fig. 1. Assessment of e-liquids and cigarette smoke on the cell health of alveolar-macrophage-like cells *in vitro*. ImmuPHAGE™ (mono-culture) and ImmuLUNG™ (co-culture) models were exposed to e-liquids (green apple or tutti frutti) or cigarette smoke and expressed as the average of 500–1500 individual cell responses relative to air control for cell count (a), membrane permeability (b) and mitochondrial activity (c) by high content image analysis. Each data point represents the mean ± SD ($n = 12$ mono-culture; $n = 9$ co-culture). * indicates $P < 0.001$; two-way ANOVA with Dunnett's multiple comparison test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

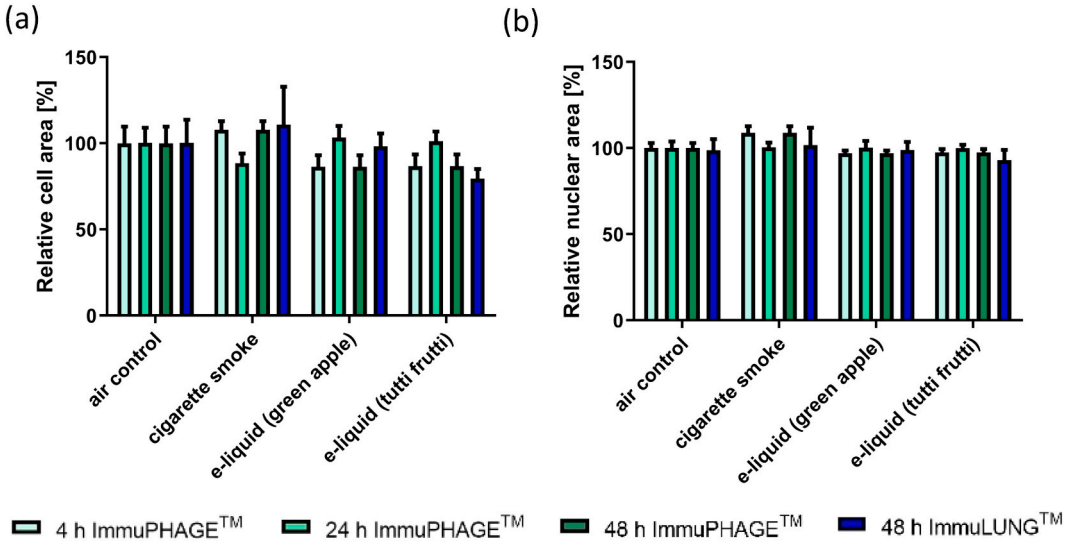


Fig. 2. Assessment of e-liquids and cigarette smoke on the gross morphology of alveolar-macrophage-like cells *in vitro*. ImmuPHAGE™ (mono-culture) and ImmuLUNG™ (co-culture) models were exposed to e-liquids (green apple or tutti frutti) or cigarette smoke and expressed as the average of 500–1500 individual cell responses relative to air control for cell area (a), nuclear area (b) by high content image analysis. Each data point represents the mean ± SD (n = 12 mono-culture; n = 9 co-culture). * indicates $P < 0.001$; two-way ANOVA with Dunnett's multiple comparison test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

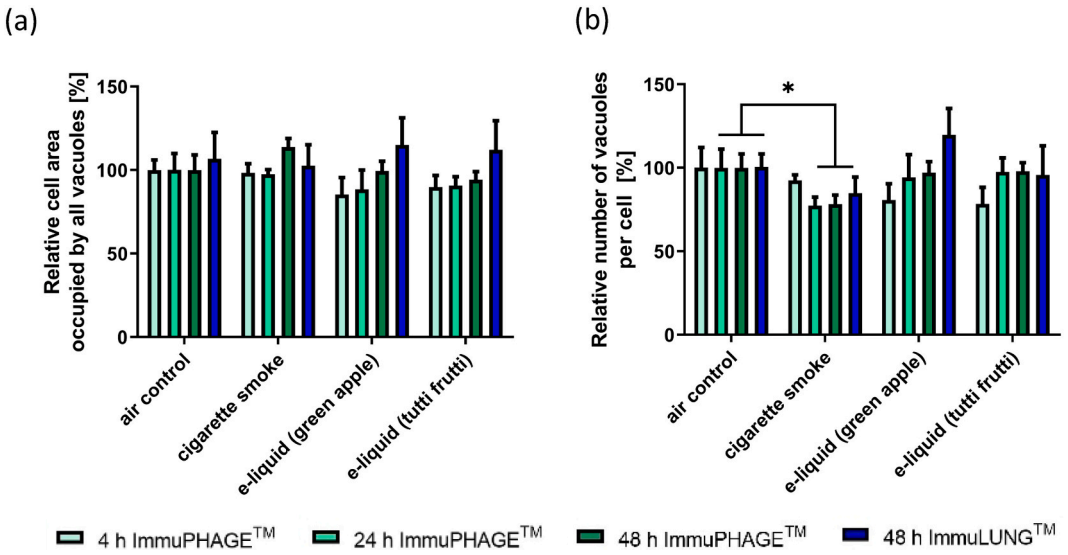


Fig. 3. Assessment of e-liquids and cigarette smoke on the detailed morphology of alveolar-macrophage-like cells *in vitro*. ImmuPHAGE™ (mono-culture) and ImmuLUNG™ (co-culture) models were exposed to e-liquids (green apple or tutti frutti) or cigarette smoke and expressed as the average of 500–1500 individual cell responses relative to air control for the number of vacuoles per cell (a) and cell area occupied by all vacuoles (b) by high content image analysis. Each data point represents the mean ± SD (n = 12 mono-culture; n = 9 co-culture). * indicates $P < 0.001$; two-way ANOVA with Dunnett's multiple comparison test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

smoke, the phenotypes associated with reduced mitochondrial activity alongside increased cell area with moderate or increased vacuole number were significantly elevated ($P < 0.001$) in the co-culture model with a > 10-fold increase (Fig. 5b). Similarly, for both e-liquid flavours tested, significantly higher ($P < 0.001$) populations of cells were identified in the co-culture system in comparison with the mono-culture system for phenotypes with altered mitochondrial activity and elevated cell area with moderate or increased vacuolation characteristics (Fig. 5c and d). This data suggests that some alveolar-macrophage phenotypic responses are independent of the alveolar epithelial response whereas other phenotypic responses, particularly those with elevated cell area with/without changes in vacuolation were dependent on the presence of alveolar epithelial cells.

3.4. Elucidating the mechanisms of response for alveolar macrophages

Individual phenotypes were considered in more detail to link to the mechanism of macrophage response. A significant increase ($P < 0.001$) in the percentage of macrophages with enhanced mitochondrial activity only (unchanged cell area and vacuole characteristics) was observed for all cells exposed to cigarette smoke in both mono and co-culture models and at all timepoints (Fig. 6). Conversely, a significant reduction ($P < 0.001$) in the percentage of cells displaying a phenotype with increased mitochondrial activity was observed for cells in both mono- and co-culture exposed to cigarette smoke for 48 h. This suggests that cigarette smoke induces changes in mitochondrial activity from a direct mechanism from exposure to macrophages and in line with established

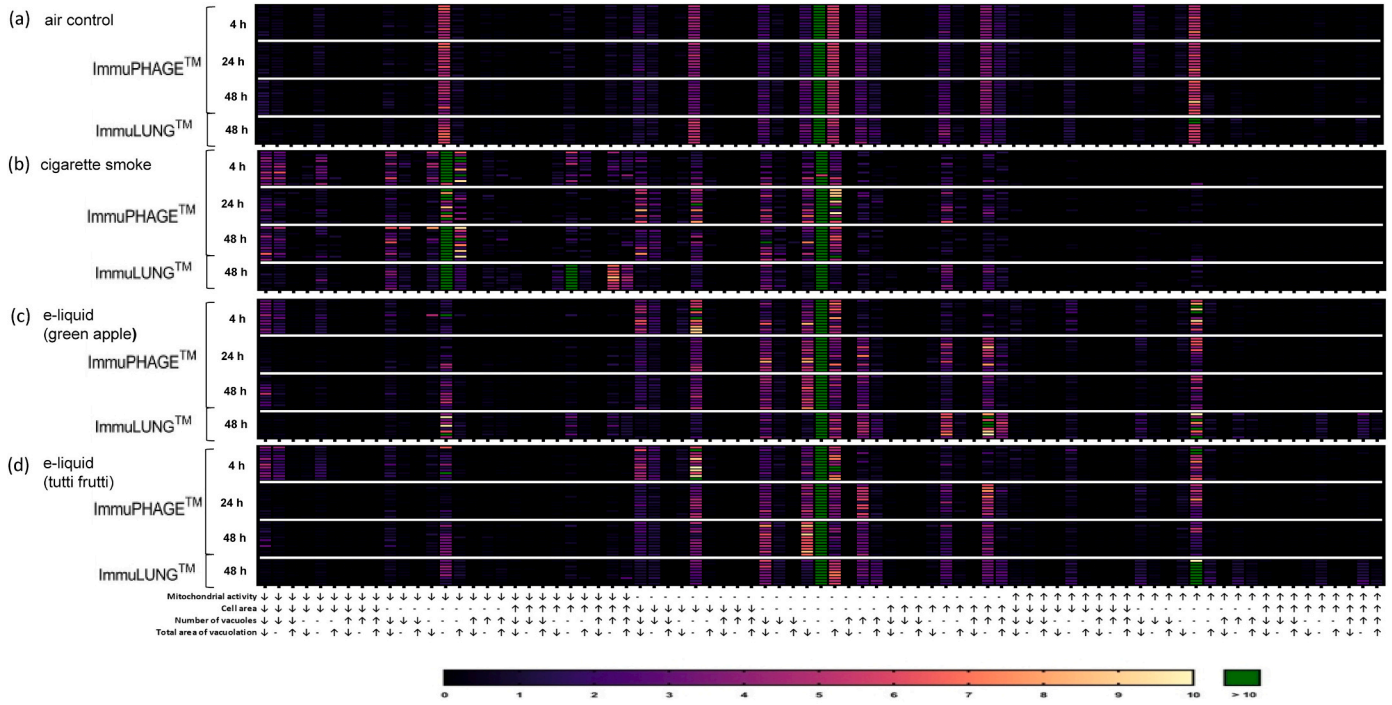


Fig. 4. Heatmap indicating phenotypic assessment of alveolar macrophage-like cells. ImmuPHAGE™ (mono-culture) and ImmuLUNG™ (co-culture) models were exposed to air control (a), cigarette smoke (b), e-liquids (green apple (c) or tutti frutti (d)). Four cell characteristics (mitochondrial activity, cell area, vacuole number per cells and area of cell occupied by vacuoles) were expressed at three levels generating 72 possible phenotypes. Each square represents the % of the cell population with that given phenotype in one experiment (n = 12 mono-culture; n = 9 co-culture). The colour gradient sets the lowest value for each given parameter in the heat map (black 0%), highest value (green 50%) and mid-range values (yellow 20%) with a corresponding gradient between these extremes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mechanisms of oxidative stress (van der Vaart et al., 2004).

A significant increase ($P < 0.001$) in the macrophage phenotypes with reduced mitochondrial activity and enhanced cell area with and without enhanced vacuole characteristics was observed after 4 h in monoculture but had resolved after 24 h and 48 h timepoints (Fig. 7a, b and c). In comparison, this phenotype was significantly elevated ($P < 0.001$) for the macrophages in the ImmuLUNG™ co-culture model after 48 h exposure and at a significantly higher level ($P < 0.001$) than that observed for monoculture exposure after 4 h. This suggests that this initial direct response of macrophages to cigarette smoke which resolves after 24 h is likely to be a different mechanism to the same phenotypic observed in the co-culture model where the macrophage response is likely to be driven indirectly by the response of the epithelial cells to cigarette smoke.

In contrast to cigarette smoke, the only significant phenotypic profile changes for altered mitochondrial activity alone were a significant increase ($P < 0.001$) in the elevated mitochondrial activity phenotype after 48 h exposure to the tutti frutti e-liquid in the ImmuLUNG™ co-culture model only (Fig. 6b). Whilst no significant differences ($P > 0.05$) were observed for the tutti-frutti phenotypes with enhanced cell area with/without vacuolation changes, significant ($P < 0.001$) observations were made for the green apple e-liquid (Figs. 7a-d). Whilst there appeared to be no direct effect on the phenotypes with increased cell size in monoculture, there was a significant ($P < 0.001$) increase in the low mitochondrial phenotypes associated with increased cell area with/without increased vacuolation albeit, this response was 4–5 fold lower than that observed for cigarette smoke.

4. Discussion

Alveolar macrophage responses are becoming increasingly crucial in understanding both short- and long-term safety of inhaled substances in the respiratory airways (Bissonnette et al., 2020; Movia et al., 2020). The responses of alveolar macrophages to inhaled substances in animal preclinical *in vivo* studies are driving decision making despite being poorly understood and without having fully evaluated the implications for humans (Forbes et al., 2014; Jones and Neef, 2012). Therefore, characterising the human alveolar macrophage response *in vitro*, linking this to the mechanism and likelihood of adversity in human lungs is key to advancing inhalation safety assessment. This study aims to demonstrate how *in vitro* alveolar macrophage models (in both monoculture and in co-culture with epithelial cells) can be utilised to ascertain mechanistic information to more accurately assess cellular responses which may in turn support safety decision making. Whilst a plethora of biochemical assessment strategies can be attempted *in vitro* to elucidate a mechanism, many of these endpoints can not be directly compared with *in vivo* data which is predominantly pathological descriptions from lung slices (Forbes et al., 2014). Therefore, this study aimed to use morphological characteristics such as cell size and size and extent of cellular vacuolation as translatable endpoints.

Many standard approaches for *in vitro* toxicity assessment rely on reporting a single cell characteristic which is often reported as an average of a cell population (*e.g.* extracellular lactate dehydrogenase concentration). Whilst this can be informative for some *in vitro* safety strategies, the approach is limited to indicating acute, severe cellular toxicity and is unable to differentiate the response of individual cells/subpopulations or the mechanism of response (Hoffman et al., 2017). In particular, this approach is unsuitable for alveolar macrophage responses where characterising and discerning the impact of changes in cell morphology, functionality and biochemical perturbations and the implications to humans is required (Hoffman et al., 2017).

High content image analysis or 'cell painting' has been applied successfully in drug discovery programmes for several years using phenotyping of cell responses to inform candidate drug selection in a high throughput approach (Bray et al., 2016; Willis et al., 2020). This approach has been applied to toxicological assessments with similar

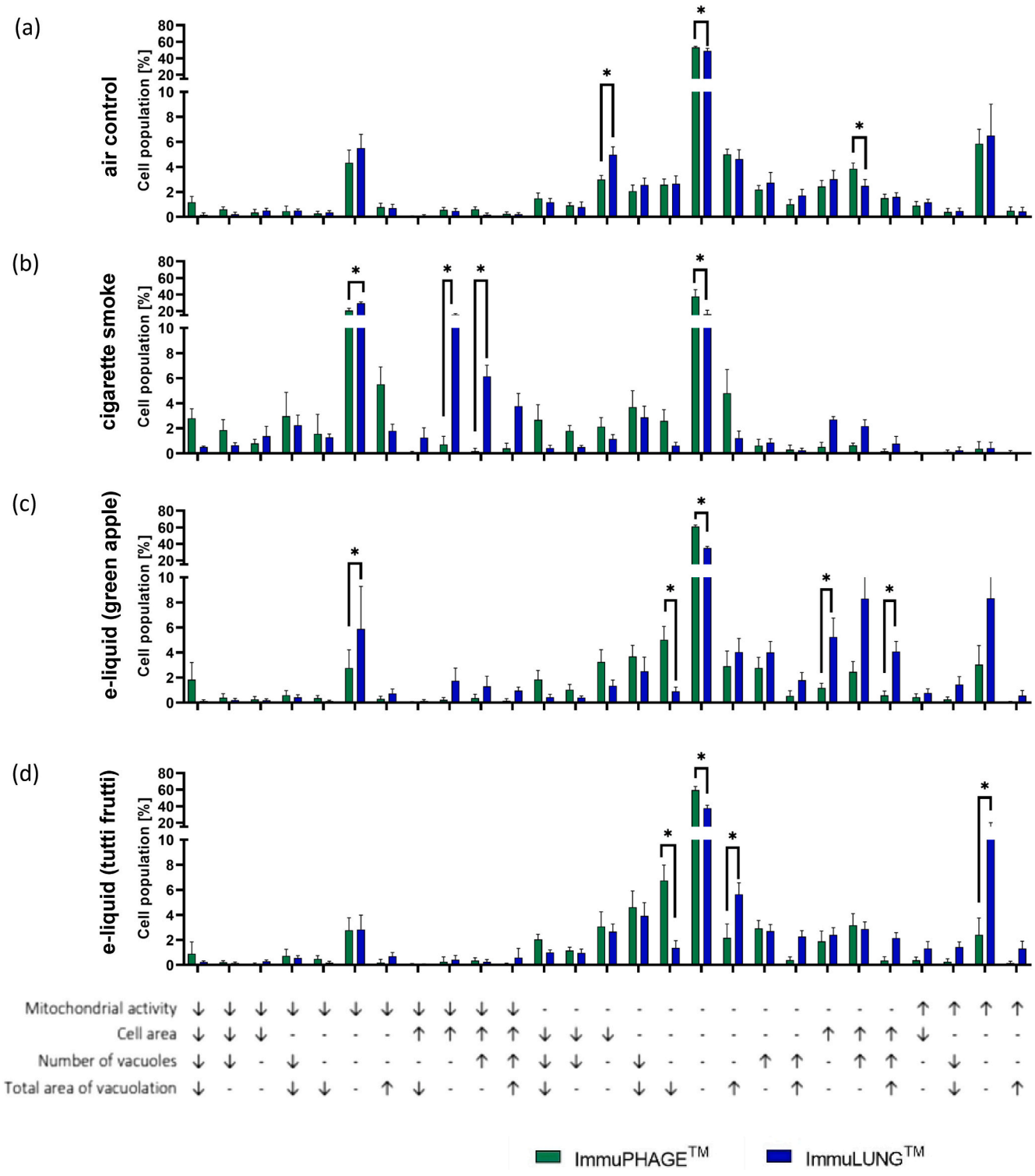


Fig. 5. Comparison of alveolar macrophage-like phenotypes in mono-culture and co-culture. ImmuPHAGE™ (mono-culture) and ImmuLUNG™ (co-culture) models were exposed to air control (a), cigarette smoke (b), e-liquids (green apple (c) or tutti frutti (d)). The 27 phenotypes which occupy >1% of the total population are displayed as mean ± SD (n = 12 mono-culture; n = 9 co-culture). * indicates $P < 0.001$ (comparison between alveolar macrophage-like cells in mono-culture *vs* co-culture). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

success with a significant enhancement in improving both the sensitivity and specificity of toxicity prediction in comparison with standard high throughput techniques. (Li and Xia, 2019; O'Brien, 2014; Uteng et al., 2014). Characterising multiple endpoints enables a mechanistic approach to be undertaken in an attempt to classify a response as adverse or adaptive (Nyffeler et al., 2021). For example, it is widely accepted that an increase in mitochondrial activity is an adaptive cellular response on exposure to a substance (Meyer et al., 2018). Alone, this is not an accurate marker of toxicity, but in combination with altered cell morphology or biochemical markers the response can be linked to a mechanism which supports decision making as to whether the response is adaptive or adverse (Meyer et al., 2018; O'Brien, 2014).

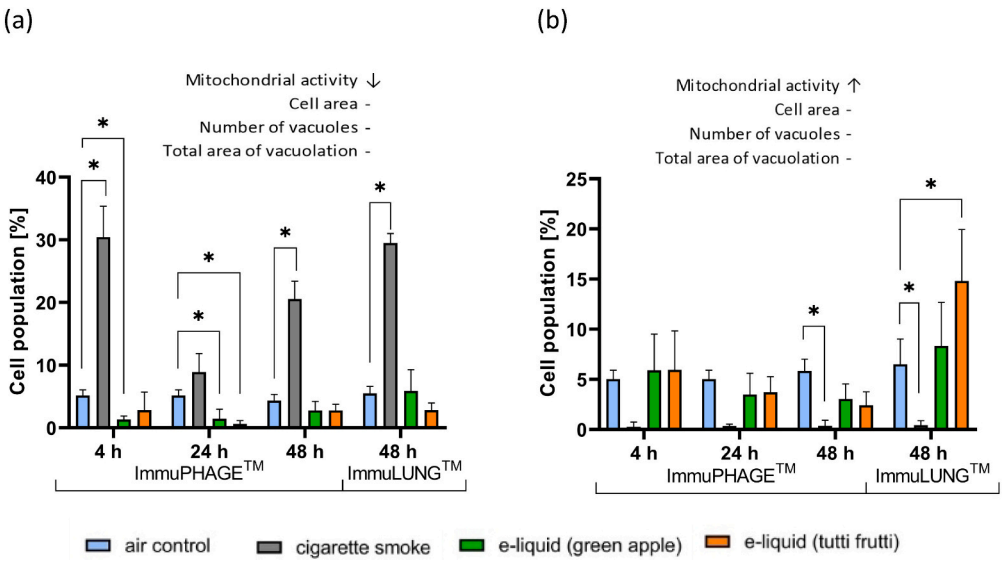


Fig. 6. Changes in selected macrophage phenotypes upon treatment with e-cigarettes and traditional smoke. The phenotypes are displayed as mean ± SD (n = 12 mono-culture; n = 9 co-culture). *$P < 0.001$; two-way ANOVA with Dunnett's multiple comparison test.

This study used cigarette smoke and e-liquids as model substances to determine the significance of epithelial cell cross-talk in the alveolar macrophage response and demonstrate how a combinational approach of morphological and biochemical assessment strategies can inform the mechanism of toxicity. It is well established that cigarette smoke causes oxidative stress in acute *in vitro* toxicity assessment (van der Vaart et al., 2004; Aridgides et al., 2019) and this was observed with the reduction of mitochondrial activity, providing a clear differential between alveolar macrophage responses to cigarette smoke and e-liquids. Other well established adverse effects reported include an accumulation of autophagic vesicles in human alveolar macrophages *ex vivo* (Kono et al., 2021; Lugg et al., 2022; Monick et al., 2010). Autophagy is an intracellular catabolic pathway involving the formation of autophagosomes for the degradation of contents within the cell (Painter et al., 2020). Cells undergoing autophagy are typically larger in size, associated with increased vacuolation within the cell and reduced mitochondrial activity (Jung et al., 2020; Monick et al., 2010; Neufeld, 2012). As such, it could be hypothesised that the cell phenotypes described in Fig. 7a-c with these cellular features could be related to an autophagic response. There is evidence that autophagy is regulated by communication between epithelial and macrophage cells in other tissues and these phenotypes were only observed for the immune-epithelial co-culture model exposed to cigarette smoke and not in the alveolar macrophage-like monoculture system in this study, however the specific mechanisms have yet to be fully elucidated for the lung (Aridgides et al., 2019; Ortiz-Masiá et al., 2014; Painter et al., 2020; Yuan et al., 2012).

In contrast, e-liquids are established to generate very different cellular responses in comparison with cigarette smoke. In this study, macrophages exposed to the e-liquids tested produced minor cell perturbations in their phenotypic profiles in comparison with cigarette smoke. Morphological and mitochondrial activity responses of alveolar macrophage to e-liquids and cigarette smoke were not the same, indicating different mechanisms of action. In line with other published reports, no effect on cell viability was observed (Gómez et al., 2020). There were observations of elevated vacuolisation with the green apple e-liquid which could link with lipid-laden macrophages observed with e-cigarette exposure in other studies (Shields et al., 2020) however further characterisation of the vacuole contents would be required to confirm this hypothesis.

This work has identified that the majority of macrophage responses to cigarette smoke and e-liquids used within this study can be observed from a monoculture system and their response becomes more physiologically-relevant in combination with alveolar epithelial cells. This is demonstrated where epithelial-dependent phenotypic changes indicative of an autophagic response were observed in the immune-epithelial co-culture system but absent in the alveolar macrophage-like cells alone. This highlights the need to consider the impact of complexity of the *in vitro* system and an appreciation of the mechanism and cellular interplay within a tissue to select the most appropriate *in vitro* model. Indeed, other studies have indicated as the complexity of an *in vitro* cell model is increased cell types become more physiologically relevant (Marescotti et al., 2019) supporting the application of co-culture systems in the move towards human-relevant mechanistic-driven toxicity assessment.

Whilst high content image analysis is emerging within the field of toxicology the majority of analytical approaches rely on reporting individual cell characteristics and use a mathematical approach to cluster compounds of similar responses of individual cell readouts. In this study, we have combined multiple cell features to describe a cell phenotype and link this to their established mechanism of toxicity *in vivo*. This combinatorial approach integrating morphology and mitochondrial parameters to describe a phenotype was able to clearly distinguish the differing alveolar macrophage responses to cigarette smoke and e-liquids which in turn could be linked to a mechanistic driven hypothesis of the type of cellular processes occurring in the cells. Whilst morphological descriptors are not associated with a specific mechanistic pathway (as opposed to a biochemical approach) for this study they were selected with a view to generating meaningful *in vitro* data with both the potential to correlate directly with *in vivo* descriptors generated from animal studies (Patel et al., 2019) and to inform a mechanistic understanding of cell perturbations (Hoffman et al., 2017, 2020). Alveolar tissue and specifically alveolar macrophage morphology are key endpoints for respiratory *in vivo* assessments (Nikula et al., 2014) and the *in vitro* models and assessment tools described in this approach have the potential to support interpretation of *in vivo* findings and drive forward a mechanism-based approach by which inhaled drug and product safety could be conducted.

Funding statement

This work was funded by ImmuONE Ltd who were supported by Innovate UK grant number 105374.

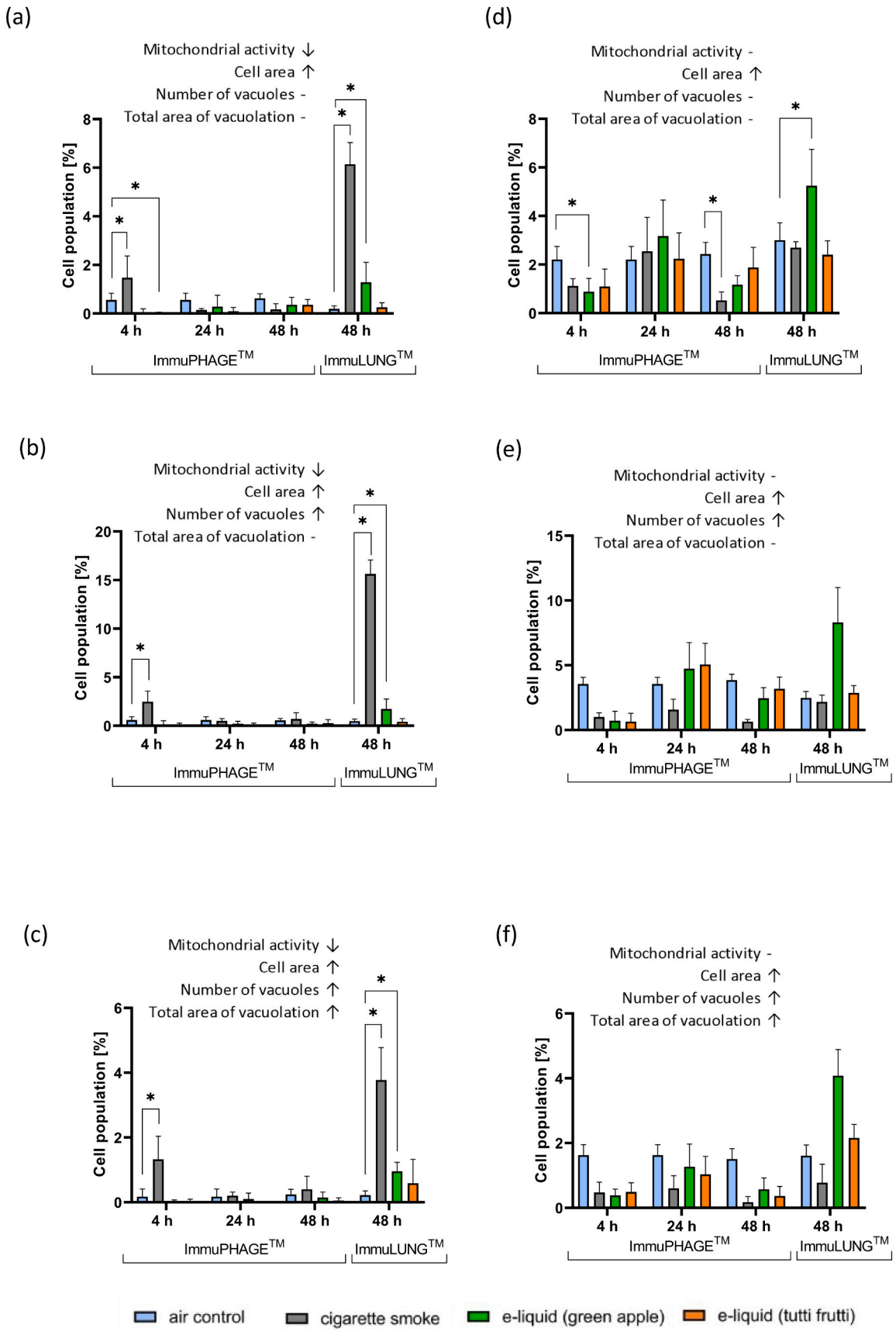


Fig. 7. Changes in selected macrophage phenotypes upon treatment with e-cigarettes and traditional smoke. The phenotypes are displayed as mean ± SD (n = 12 mono-culture; n = 9 co-culture). *$P < 0.001$; two-way ANOVA with Dunnett's multiple comparison test.

Conflicts of interest

All testing and analysis of these samples was performed independently at ImmuONE Limited. ImmuONE manufactures, validated and provided all *in vitro* cell culture models used in this assessment. Cigarette smoke and e-liquid samples were prepared at Thornton & Ross Ltd.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Victoria Hutter reports a relationship with ImmuONE Limited that includes: board membership, consulting or advisory, employment, equity or stocks, and funding grants. Ewelina Hoffman reports a relationship with ImmuONE Limited that includes: employment and funding grants. Sarah Hopper reports a relationship with Thornton and Ross Limited that includes: employment. Victoria Hutter has patent #GBP7810X pending to Licensee.

Data availability

Data will be made available on request.

References

Aridgides, D.S., Mellinger, D.L., Armstrong, D.A., Hazlett, H.F., Dessaint, J.A., Hampton, T.H., Atkins, G.T., Carroll, J.L., Ashare, A., 2019. Functional and metabolic impairment in cigarette smoke-exposed macrophages is tied to oxidative stress. Sci. Rep. 9 (1), 9624. https://doi.org/10.1038/s41598-019-46045-7.

Bissonnette, E.Y., Lauzon-Joset, J.-F., Debley, J.S., Ziegler, S.F., 2020. Cross-talk between alveolar macrophages and lung epithelial cells is essential to maintain lung homeostasis. Front. Immunol. 11 https://doi.org/10.3389/fimmu.2020.583042.

Bray, M.-A., Singh, S., Han, H., Davis, C.T., Borgeson, B., Hartland, C., Kost-Alimova, M., Gustafsdottir, S.M., Gibson, C.C., Carpenter, A.E., 2016. Cell painting, a high-content image-based assay for morphological profiling using multiplexed fluorescent dyes. Nat. Protoc. 11 (9), 1757–1774. https://doi.org/10.1038/nprot.2016.105.

Clippinger, A.J., Allen, D., Jarabek, A.M., Corvaro, M., Gaça, M., Gehen, S., Hotchkiss, J. A., Patlewicz, G., Melbourne, J., Hinderliter, P., Yoon, M., Huh, D., Lowit, A., Buckley, B., Bartels, M., BéruBé, K., Wilson, D.M., Indans, I., Vinken, M., 2018. Alternative approaches for acute inhalation toxicity testing to address global regulatory and non-regulatory data requirements: an international workshop report. Toxicol. in Vitro 48, 53–70. https://doi.org/10.1016/j.tiv.2017.12.011.

Faber, S.C., McCullough, S.D., 2018. Through the looking glass: *in vitro* models for inhalation toxicology and Interindividual variability in the airway. Appl. In Vitro Toxicol. 4 (2), 115–128. https://doi.org/10.1089/aivt.2018.0002.

Forbes, B., O'Lone, R., Allen, P.P., Cahn, A., Clarke, C., Collinge, M., Dailey, L.A., Donnelly, L.E., Dybowski, J., Hassall, D., Hildebrand, D., Jones, R., Kilgour, J., Klapwijk, J., Maier, C.C., McGovern, T., Nikula, K., Parry, J.D., Reed, M.D., Wolfreys, A., 2014. Challenges for inhaled drug discovery and development: induced alveolar macrophage responses. Adv. Drug Deliv. Rev. 71, 15–33. https://doi.org/10.1016/j.addr.2014.02.001.

Gómez, A.-C., Rodríguez-Fernández, P., Villar-Hernández, R., Gibert, I., Muriel-Moreno, B., Lacoma, A., Prat-Aymerich, C., Domínguez, J., 2020. E-cigarettes: effects in phagocytosis and cytokines response against mycobacterium tuberculosis. PLoS One 15 (2), e0228919. https://doi.org/10.1371/journal.pone.0228919.

Gotts, J.E., Jordt, S.-E., McConnell, R., Tarran, R., 2019. What are the respiratory effects of e-cigarettes? BMJ 366, l5275. https://doi.org/10.1136/bmj.l5275.

Hewitt, R.J., Lloyd, C.M., 2021. Regulation of immune responses by the airway epithelial cell landscape. Nat. Rev. Immunol. 21 (6), 347–362. https://doi.org/10.1038/s41577-020-00477-9.

Hoffman, E., Patel, A., Ball, D., Klapwijk, J., Millar, V., Kumar, A., Martin, A., Mahendran, R., Dailey, L.A., Forbes, B., Hutter, V., 2017. Morphometric characterization of rat and human alveolar macrophage cell models and their response to amiodarone using high content image analysis. Pharm. Res. 34 (12), 2466–2476. https://doi.org/10.1007/s11095-017-2176-5.

Hoffman, E., Murnane, D., Hutter, V., 2020. Investigating the suitability of high content image analysis as a tool to assess the reversibility of foamy alveolar macrophage phenotypes *in vitro*. Pharmaceutics 12 (3). https://doi.org/10.3390/pharmaceutics12030262.

Hoffman, E., Napieralska, P., Mahendran, R., Murnane, D., Hutter, V., 2021. High content image analysis as a tool to morphologically distinguish macrophage activation and determine its importance for foamy alveolar macrophage responses. Front. Immunol. 12 https://doi.org/10.3389/fimmu.2021.611280.

Jasper, A.E., Sapey, E., Thickett, D.R., Scott, A., 2021. Understanding potential mechanisms of harm: the drivers of electronic cigarette-induced changes in alveolar macrophages, neutrophils, and lung epithelial cells. Am. J. Phys. Lung Cell. Mol. Phys. 321 (2), L336–L348. https://doi.org/10.1152/ajplung.00081.2021.

Jones, R.M., Neef, N., 2012. Interpretation and prediction of inhaled drug particle accumulation in the lung and its associated toxicity. Xenobiotica 42 (1), 86–93. https://doi.org/10.3109/00498254.2011.632827.

Jung, S., Jeong, H., Yu, S.-W., 2020. Autophagy as a decisive process for cell death. Exp. Mol. Med. 52 (6), 921–930. https://doi.org/10.1038/s12276-020-0455-4.

Kono, Y., Colley, T., To, M., Papaioannou, A.I., Mercado, N., Baker, J.R., To, Y., Abe, S., Haruki, K., Ito, K., Barnes, P.J., 2021. Cigarette smoke-induced impairment of autophagy in macrophages increases galectin-8 and inflammation. Sci. Rep. 11 (1), 335. https://doi.org/10.1038/s41598-020-79848-0.

Krewski, D., Andersen, M.E., Tyshenko, M.G., Krishnan, K., Hartung, T., Boekelheide, K., Wambaugh, J.F., Jones, D., Whelan, M., Thomas, R., Yauk, C., Barton-Maclaren, T., Cote, I., 2020. Toxicity testing in the 21st century: progress in the past decade and future perspectives. Arch. Toxicol. 94 (1), 1–58. https://doi.org/10.1007/s00204-019-02613-4.

Lacroix, G., Koch, W., Ritter, D., Gutleb, A.C., Larsen, S.T., Loret, T., Zanetti, F., Constant, S., Chortarea, S., Rothen-Rutishauser, B., Hiemstra, P.S., Frejafon, E., Hubert, P., Gribaldo, L., Kearns, P., Aublant, J.M., Diabaté, S., Weiss, C., de Groot, A., Kooter, I., 2018. Air-liquid Interface *in vitro* models for respiratory toxicology research: consensus workshop and recommendations. Appl. In Vitro Toxico. 4 (2), 91–106. https://doi.org/10.1089/aivt.2017.0034.

Li, S., Xia, M., 2019. Review of high-content screening applications in toxicology. Arch. Toxicol. 93 (12), 3387–3396. https://doi.org/10.1007/s00204-019-02593-5.

Lugg, S.T., Scott, A., Parekh, D., Naidu, B., Thickett, D.R., 2022. Cigarette smoke exposure and alveolar macrophages: mechanisms for lung disease. In: Thorax (Vol. 77, Issue 1, pp. 94–101). BMJ Publishing Group. https://doi.org/10.1136/thoraxjnl-2020-216296.

Marescotti, D., Serchi, T., Luettich, K., Xiang, Y., Moschini, E., Talikka, M., Martin, F., Baumer, K., Dulize, R., Peric, D., Bornand, D., Guedj, E., Sewer, A., Cambier, S., Contal, S., Chary, A., Gutleb, A.C., Frentzel, S., Ivanov, N.V., Peitsch, M.C., Hoeng, J., 2019. How complex should an *in vitro* model be? Evaluation of a complex 3D alveolar model with transcriptomic data and computational biological network models. ALTEX 36 (3), 388–402. https://doi.org/10.14573/altex.1811221.

Meyer, J.N., Hartman, J.H., Mello, D.F., 2018. Mitochondrial toxicity. Toxicol. Sci. 162 (1), 15–23. https://doi.org/10.1093/toxsci/kfy008.

Monick, M.M., Powers, L.S., Walters, K., Lovan, N., Zhang, M., Gerke, A., Hansdottir, S., Hunninghake, G.W., 2010. Identification of an autophagy defect in Smokers' alveolar macrophages. J. Immunol. 185 (9), 5425. https://doi.org/10.4049/jimmunol.1001603.

Movia, D., Bruni-Favier, S., Prina-Mello, A., 2020. *In vitro* alternatives to acute inhalation toxicity studies in animal models—a perspective. Front. Bioeng. Biotechnol. 8 https://doi.org/10.3389/fbioe.2020.00549.

Neufeld, T.P., 2012. Autophagy and cell growth – the yin and yang of nutrient responses. J. Cell Sci. 125 (10), 2359–2368. https://doi.org/10.1242/jcs.103333.

Nikula, K.J., McCartney, J.E., McGovern, T., Miller, G.K., Odin, M., Pino, M., Reed, M.D., 2014. STP position paper: interpreting the significance of increased alveolar macrophages in rodents following inhalation of pharmaceutical materials. Toxicol. Pathol. 42 (3), 472–486. https://doi.org/10.1177/0192623313507003.

Nyffeler, J., Haggard, D.E., Willis, C., Setzer, R.W., Judson, R., Paul-Friedman, K., Everett, L.J., Harrill, J.A., 2021. Comparison of approaches for determining bioactivity hits from high-dimensional profiling data. SLAS Discov. 26 (2), 292–308. https://doi.org/10.1177/2472555220950245.

O'Brien, P.J., 2014. High-content analysis in toxicology: screening substances for human toxicity potential, elucidating subcellular mechanisms and in vivo use as translational safety biomarkers. Basic Clin. Pharmacol. Toxicol. 115 (1), 4–17. https://doi.org/10.1111/bcpt.12227.

Ortiz-Masiá, D., Cosín-Roger, J., Calatayud, S., Hernández, C., Alós, R., Hinojosa, J., Apostolova, N., Alvarez, A., Barrachina, M.D., 2014. Hypoxic macrophages impair autophagy in epithelial cells through Wnt1: relevance in IBD. Mucosal Immunol. 7 (4), 929–938. https://doi.org/10.1038/mi.2013.108.

Painter, J.D., Galle-Treger, L., Akbari, O., 2020. Role of autophagy in lung inflammation. Front. Immunol. 11 https://doi.org/10.3389/fimmu.2020.01337.

Patel, A., Hoffman, E., Ball, D., Klapwijk, J., Steven, R.T., Dexter, A., Bunch, J., Baker, D., Murnane, D., Hutter, V., Page, C., Dailey, L.A., Forbes, B., 2019. Comparison of oral, intranasal and aerosol administration of amiodarone in rats as a model of pulmonary phospholipidosis. Pharmaceutics 11 (7). https://doi.org/10.3390/pharmaceutics11070345.

Shields, P.G., Song, M.-A., Freudenheim, J.L., Brasky, T.M., McElroy, J.P., Reisinger, S. A., Weng, D.Y., Ren, R., Eissenberg, T., Wewers, M.D., Shilo, K., 2020. Lipid laden macrophages and electronic cigarettes in healthy adults. EBioMedicine 60, 102982. https://doi.org/10.1016/j.ebiom.2020.102982.

Upadhyay, S., Palmberg, L., 2018. Air-liquid interface: relevant *in vitro* models for investigating air pollutant-induced pulmonary toxicity. Toxicol. Sci. 164 (1), 21–30. https://doi.org/10.1093/toxsci/kfy053.

Uteng, M., Germano, D., Balavenkatraman, K.K., Pognan, F., Wolf, A., 2014. High content imaging approaches for *in vitro* toxicology. In: Bal-Price, A., Jennings, P. (Eds.), Vitro Toxicology Systems. Springer, New York, pp. 377–397. https://doi.org/10.1007/978-1-4939-0521-8_17.

van der Vaart, H., Postma, D.S., Timens, W., Ten Hacken, N.H.T., 2004. Acute effects of cigarette smoke on inflammation and oxidative stress: a review. Thorax 59 (8), 713–721. https://doi.org/10.1136/thx.2003.012468.

Willis, C., Nyffeler, J., Harrill, J., 2020. Phenotypic profiling of reference chemicals across biologically diverse cell types using the cell painting assay. SLAS Discov. 25 (7), 755–769. https://doi.org/10.1177/2472555220928004.

Yuan, K., Huang, C., Fox, J., Laturnus, D., Carlson, E., Zhang, B., Yin, Q., Gao, H., Wu, M., 2012. Autophagy plays an essential role in the clearance of Pseudomonas aeruginosa by alveolar macrophages. J. Cell Sci. 125 (2), 507–515. https://doi.org/10.1242/jcs.094573.