Optimization and utilization of \textit{in vitro} airway models to establish cellular specificity and toxicity of airway toxicants

Thesis

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OPTIMIZATION AND UTILIZATION OF *IN VITRO* AIRWAY MODELS TO ESTABLISH CELLULAR SPECIFICITY AND TOXICITY OF AIRWAY TOXICANTS

A thesis submitted to The Open University for the degree of Doctor of Philosophy in the School of Life, Health and Chemical Sciences

Affiliated Research Centre:
UK Health Security Agency

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2022

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Director of Studies: Dr. Martin Leonard

Third Party monitor: Dr. Ken Raj
DECLARATION OF ORIGINALITY

I declare that this thesis titled “optimization and utilization of in vitro airway models to establish cellular specificity and toxicity of airway toxicants” and the experiments described are a record of independent research performed by myself under supervision and guidance of Dr Martin Leonard.

Signed: [Signature]

Date: 23-06-2022
Abstract

The human small airways have a unique position, morphology and cellular composition within the lung resulting in regional susceptibility to toxicant induced injury. Current in vitro airway models generally lack regional organotypic differentiation. Therefore, this work aimed to optimize in vitro models relevant for small airway toxicity testing and to determine the cellular specificity of small airway toxicants with the overall aim to improve in vitro testing capabilities.

Primary small airway epithelial cell (SAEC) models were optimized for regional specific differentiation. DCI differentiated primary SAEC showed higher levels of club cell markers compared to basal and PneumaCult™-ALI differentiated SAEC and were thus identified as most applicable to small airway regional specific toxicity testing. iPSC derived SAEC models did not show sufficient expression of mature airway markers within this thesis and were not further explored. Macrophages and dendritic cells play an important role in small airway responses to toxicants. Therefore, CD34+ and iPSC derived macrophage and dendritic cell models were established and thoroughly characterized using single cell sequencing. Although these models likely represent inflammatory phenotypes, they are relevant for airway toxicity testing.

Chemical exposures were used to further characterize the in vitro models and identify cellular specificity of exposures. Paraquat (PQ) exposure showed cellular specific effects, as demonstrated by specific gene activation in differentiated primary SAEC and CD34+ derived dendritic cells. Both Basal SAEC and iPSC derived macrophages and dendritic cells did not show a profound effect upon PQ exposure, indicating adequate differentiation is required to detect PQ effects and further efforts are needed for the iPSC models developed within this thesis to be applicable to toxicity testing.

Overall, this demonstrates the importance and feasibility of organotypic differentiation to establish in vitro models that are applicable to in vitro small airway toxicity testing.
ACKNOWLEDGEMENTS

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<tr>
<td>3R</td>
<td>Replace, Reduce and Refine</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AbSeq</td>
<td>Antibody Sequencing</td>
</tr>
<tr>
<td>ADME</td>
<td>Absorption, Distribution, Metabolism and Excretion</td>
</tr>
<tr>
<td>AEC</td>
<td>Airway epithelial cells</td>
</tr>
<tr>
<td>AFE</td>
<td>Anterior Foregut Endoderm</td>
</tr>
<tr>
<td>Al₂O₃</td>
<td>Aluminium Oxide</td>
</tr>
<tr>
<td>ALI</td>
<td>Air-Liquid Interface</td>
</tr>
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<td>AM</td>
<td>Amiodarone</td>
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>AO</td>
<td>Adverse Outcome</td>
</tr>
<tr>
<td>AOP</td>
<td>Adverse Outcome Pathway</td>
</tr>
<tr>
<td>ASL</td>
<td>Airway Surface Liquid</td>
</tr>
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<td>AT1</td>
<td>Alveolar Type I</td>
</tr>
<tr>
<td>AT2</td>
<td>Alveolar Type II</td>
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<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar Lavage</td>
</tr>
<tr>
<td>BBE</td>
<td>Bovine Brain Extract</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
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<tr>
<td>BP</td>
<td>Biological Process</td>
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<td>cAMP</td>
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<td>Club Cell Secretory Protein</td>
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<tr>
<td>cDC</td>
<td>Myeloid Dendritic Cell</td>
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<tr>
<td>CeO₂</td>
<td>Cerium Dioxide</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic Fibrosis Transmembrane Conductance Regulator</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
</tr>
<tr>
<td>cPAS</td>
<td>Combination Probe Anchor Synthesis</td>
</tr>
<tr>
<td>CSE</td>
<td>Cigarette Smoke Extract</td>
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<td>Cytochrome P450</td>
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<td>DC&lt;sup&gt;cd34&lt;/sup&gt;</td>
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<td>DNA nano balls</td>
</tr>
<tr>
<td>EB</td>
<td>Embryonic Body</td>
</tr>
<tr>
<td>EC50</td>
<td>Effective Concentration 50</td>
</tr>
<tr>
<td>ECHA</td>
<td>European Chemicals Agency</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assays</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic Stem Cells</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>F.O.C.</td>
<td>Fold Over Control</td>
</tr>
<tr>
<td>FB-like</td>
<td>Fibroblast-like</td>
</tr>
<tr>
<td>FC</td>
<td>Fold Change</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>FOXI1</td>
<td>Forkhead Box I1</td>
</tr>
<tr>
<td>FOXJ1</td>
<td>Forkhead Box Protein J1</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced Vital Capacity</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-Macrophage Colony-Stimulating Factor</td>
</tr>
<tr>
<td>GO</td>
<td>Gene Ontology</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-Transferase</td>
</tr>
<tr>
<td>HDM</td>
<td>House Dust Mite</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic Stem Cell</td>
</tr>
<tr>
<td>IBMX</td>
<td>Isobutylmethylxantine</td>
</tr>
<tr>
<td>iDC</td>
<td>Immature Dendritic Cell</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon Gamma</td>
</tr>
<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>iMC</td>
<td>Immature Macrophage</td>
</tr>
<tr>
<td>iPSC</td>
<td>induced Pluripotent Stem Cells</td>
</tr>
<tr>
<td>KE</td>
<td>Key Event</td>
</tr>
<tr>
<td>KRT</td>
<td>Cytokeratin</td>
</tr>
<tr>
<td>LC50</td>
<td>Lethal Concentration 50</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>LP</td>
<td>Lung Progenitor</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LSD</td>
<td>Least Significant Difference</td>
</tr>
<tr>
<td>M0</td>
<td>Unpolarized Macrophage</td>
</tr>
<tr>
<td>M1</td>
<td>Pro-Inflammatory Macrophage</td>
</tr>
<tr>
<td>M2</td>
<td>Anti-Inflammatory Macrophage</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>MC&lt;sup&gt;cd34&lt;/sup&gt;</td>
<td>CD34+ derived Macrophage</td>
</tr>
<tr>
<td>MC&lt;sup&gt;IPSC&lt;/sup&gt;</td>
<td>iPSC derived Macrophage</td>
</tr>
<tr>
<td>MC-like</td>
<td>Macrophage-like</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage Colony-Stimulating Factor</td>
</tr>
<tr>
<td>MF</td>
<td>Molecular Factor</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MIE</td>
<td>Molecular Initiating Event</td>
</tr>
<tr>
<td>MoA</td>
<td>Mode of Action</td>
</tr>
<tr>
<td>Mo-DC</td>
<td>Monocyte-derived Dendritic Cell</td>
</tr>
<tr>
<td>Mon&lt;sup&gt;IPSC&lt;/sup&gt;</td>
<td>iPSC derived Monocytes</td>
</tr>
<tr>
<td>MPS</td>
<td>Microphysiological System</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MSCA-ITN</td>
<td>Marie-Skłodowska-Curie Action – Innovative Training Network</td>
</tr>
<tr>
<td>MUC</td>
<td>Mucin</td>
</tr>
<tr>
<td>NHBE</td>
<td>Normal Human Bronchial Epithelial cell</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
</tr>
<tr>
<td>NOX</td>
<td>NADPH Oxidase</td>
</tr>
<tr>
<td>OECD</td>
<td>Organisation for Economic Co-operation and Development</td>
</tr>
<tr>
<td>Padj</td>
<td>Adjusted P-value</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic Aromatic Hydrocarbon</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic Acid-Schiff</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PB(P)K</td>
<td>Physiologically Based (Pharmaco)kinetic</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCLS</td>
<td>Precision Cut Lung Slices</td>
</tr>
<tr>
<td>PCR</td>
<td>Polynucleotide Chain Reaction</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid Dendritic Cell</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipidosis</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear</td>
</tr>
<tr>
<td>PNEC</td>
<td>Pulmonary Neuroendocrine Cells</td>
</tr>
<tr>
<td>POR</td>
<td>Cytochrome P450 Oxidoreductase</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome Proliferator-Activated Receptor γ</td>
</tr>
<tr>
<td>PQ</td>
<td>Paraquat</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polynucleotide Chain Reaction</td>
</tr>
<tr>
<td>QSAR</td>
<td>Quantitative structure-activity relationship</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic Acid</td>
</tr>
<tr>
<td>REACH</td>
<td>Registration, Evaluation, Authorization and Restriction of Chemicals</td>
</tr>
<tr>
<td>Ri</td>
<td>Rho Associated Protein Kinase Inhibitor</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive Nitrogen Species</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho Associated Protein Kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RPE</td>
<td>Random Primer Extension</td>
</tr>
<tr>
<td>RPKM</td>
<td>Reads per Kilobase per Million mapped reads</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-Time Polynucleotide Chain Reaction</td>
</tr>
<tr>
<td>SAE</td>
<td>Small Airway Epithelium</td>
</tr>
<tr>
<td>SAEC</td>
<td>Small Airway Epithelium Cells</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem Cell Factor</td>
</tr>
<tr>
<td>SCGB1A1</td>
<td>Uteroglobin</td>
</tr>
<tr>
<td>ScSeq</td>
<td>Single Cell Sequencing</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of Mean</td>
</tr>
<tr>
<td>SFTP</td>
<td>Surfactant Protein</td>
</tr>
<tr>
<td>SHH</td>
<td>Sonic Hedgehog</td>
</tr>
<tr>
<td>SiO₂</td>
<td>Silicon Dioxide</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
</tr>
<tr>
<td>SR-1</td>
<td>StemRegenin 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>TCT</td>
<td>Tissue Culture Treated</td>
</tr>
<tr>
<td>TEER</td>
<td>Transepithelial Electrical Resistance</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor Beta</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper</td>
</tr>
<tr>
<td>THT</td>
<td>Tetrahydrothiophene</td>
</tr>
<tr>
<td>TiO₂</td>
<td>Titanium Dioxide</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>TP63</td>
<td>Transformation-related Protein 63</td>
</tr>
<tr>
<td>TPO</td>
<td>Thrombopoietin</td>
</tr>
<tr>
<td>TPP3</td>
<td>Tubulin Polymerization Promoting Protein Family Member 3</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded Protein Response</td>
</tr>
<tr>
<td>URT</td>
<td>Upper Respiratory Tract</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>ZnO</td>
<td>Zinc Oxide</td>
</tr>
</tbody>
</table>
CHAPTER 1: GENERAL INTRODUCTION
1.1. Introduction

Airway toxicity testing is required to ensure safe use of chemical substances, such as pharmaceuticals, pesticides, food additives and nanomaterials. Toxicological assessments can also be used to gain insights into mechanism of action of for instance environmental pollutants. Furthermore, it allows for classification of hazardous materials and the identification of toxicological properties essential for the evaluation of risks upon human exposure. Such risk assessments form part of safety programs within the pharmaceutical industry and environmental protection efforts. In addition, initiatives such as the European Union (EU) Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) program, have increased the need for toxicological evaluations for consumer products (Arts et al., 2008). Currently, airway toxicological evaluations rely primarily on tests performed in animals (in vivo models), based on lethal concentration 50 (LC50; concentration that causes deaths in 50% of animals) or evident toxicity, as outlined by the Organisation for Economic Co-operation and Development (OECD) test guidelines (Arts et al., 2008; Bakand et al., 2005; Movia et al., 2020).

However, species differences between animals and humans within the airways, including airway dimensions, chemical clearance, differences in cellular composition and other structural and physiological parameters reduce the translational confidence of toxicity testing when using animal models. Moreover for inhalation exposures, differences in deposition patterns due to airflow dynamics further hamper extrapolation between animal and human data (Kolanjiyil et al., 2019; F. Miller et al., 1993). In addition, differences in chemical metabolism are observed between species, further obstructing extrapolation of airway toxicity data for compounds that undergo bioactivation or detoxification (Oesch et al., 2019).

Apart from difficulties in extrapolating animal data, ethical considerations have also led to regulatory efforts to advance approaches that replace, reduce and refine (3R) animal use in regulatory testing (Krewski et al., 2020). Article 4 of Directive 2010/63/EU on the Protection of Animals Used for Scientific Purposes (EU, 2010) incorporates the 3R concept and instructs
member states to use alternatives to replace animal testing wherever possible. Furthermore, article 18 of the cosmetics regulation (EC) 1223/2009 prohibits the use of animal testing for cosmetic products and ingredients (EC, 2009), highlighting the need to develop suitable alternatives to animal testing.

One way to reduce animal testing consists of the application of in silico models to predict the toxicity of chemicals based on structural properties. For instance, further understanding of the relationships between chemical molecular structures and biological targets allows the development of quantitative structure-activity relationship (QSAR) models, as chemicals with similar properties potentially are likely to have similar effects (Madden et al., 2020). Ultimately, the integration of adverse outcome pathways (AOP) and physiologically based kinetic (PBK) models utilizing advanced in vivo, in vitro and in silico models have the potential to improve toxicological evaluations and risk assessment in the future.

Several in vitro models for airway toxicity testing, such as cell culture and ex-vivo precision-cut lung models (G. Liu et al., 2019; Rayner et al., 2019), have been described in the literature and will be further discussed in section 1.6. Although alternatives to animal testing for certain endpoints, such as mutagenicity and skin sensitization, are available for regulatory purposes (Almeida et al., 2017), currently no alternatives are approved for airway inhalation toxicity as a stand-alone replacement. Nevertheless, in vitro toxicity assays can be valuable as “non-guideline” models to support and reduce animal testing in risk assessment and drug development (Movia et al., 2020).

Cell lines are commonly used for in vitro airway toxicity assessments as these cells have an extended life span and availability. However, cell lines generally lack many in vivo airway characteristics that are required for competent toxicological assessments, including mucociliary differentiation and xenobiotic metabolism (Hiemstra et al., 2018). In contrast, primary cells show normal cell morphology and maintain in vivo markers and functions (Pan et al., 2009). Limited life span, availability and difficulty in culturing of these cells limit their applicability to
larger scale toxicity screenings (Lauenstein et al., 2014). Induced pluripotent stem cell (iPSC) cultures on the other hand, may be a viable alternative for primary cells in *in vitro* toxicity testing, as these cells form an in theory unlimited supply generated from easily accessible somatic cells (Okita et al., 2007). However, iPSC approaches for toxicity testing are still in their infancy and many cell types still require optimization of their differentiation status.

1.1.1. Regional and cellular specificity of airway toxicants

Regional differences in airway morphology and function affect susceptibility to chemical and particle exposures (J. Yang et al., 2017). Overall, current *in vitro* airway models still lack regional specific differentiation and require further optimization to detect regional susceptibilities of toxicants within the airways. An understanding of how these regions differ can allow for a better modelling and optimisation approach to in vitro model development of airway cells.

The human respiratory system consists of three major regions; the extrathoracic region, the tracheobronchial region and the pulmonary region (Figure 1-1) (Gomes et al., 2013). The extrathoracic region, also referred to as the upper respiratory tract (URT), consists of the nose, mouth, pharynx and larynx. The tracheobronchial region, also referred to as the conducting airways, include the trachea, bronchi and bronchioles up to the terminal bronchioles. The pulmonary regions include the respiratory bronchioles, alveolar ducts, alveolar sacs and alveoli (Gomes et al., 2013).

Different airway regions and cell types respond differently to systemic and inhalation toxicant exposures, due to differences in morphology and function, such as metabolism and cellular transport (Baskoro et al., 2018). For instance, larger sized (>10 µm) particles mainly deposit in the nasal regions, attributing to increased susceptibility to airway irritants such as dust within this region (Schachter et al., 2001). Furthermore, highly water soluble chemicals, such as the URT irritant formaldehyde, are mainly absorbed within the upper airways, and thus do not reach more distal regions (Schachter et al., 2001; Wilhelmsson & Holmström, 1992). Nevertheless, the small airways, consisting of both bronchioles and respiratory bronchioles, are thought to be
especially susceptible to a large number of toxicant exposures due to their unique location and morphology within the respiratory tract (Fransen & Leonard, 2022).

![Regions with the human respiratory system](image.png)

**Figure 1-1 Regions with the human respiratory system.** Adapted figure (Majid & Madl, 2011).

1.1.2. Small airway disease and susceptibility to chemical and particle injury

Within the respiratory tract, the small airways are especially relevant for *in vitro* inhalation and systemic toxicity testing as these are morphologically different in animals, difficult to reach through biopsies in humans and established targets for toxicity of chemicals and nanomaterials (Bergmann, 1998; Warheit & Hartsky, 1990). It is for these reasons we have focussed to this part of the lung in this thesis.

Small airways are commonly defined as non-cartilaginous airways with an internal diameter smaller than 2mm and/or as being located between the 6th and the 23rd generation of airway
branching (Carr et al., 2017; Crystal et al., 2008; Evans & Green, 1998; Jain & Sznajder, 2007; Peake & Pinkerton, 2015). The small airways function as pathways of low pulmonary resistance that move air from the bronchi to the alveoli, are located in the tracheobronchial and pulmonary regions and consist of both non-respiratory and respiratory bronchioles (Weibel, 1963). The level of alveolarization increases upon airway branching, the most distal non-respiratory bronchioles being the terminal bronchioles.

The small airways are often referred to as the “silent zone of the lung”, as a large part of this airway compartment can be damaged before symptoms occur. Consequently, small airway disease can develop over a long period of time, without being noticed (King et al., 2018; Lipworth et al., 2014; Mead, 1970). Even though the resistance in small airways is low in normal physiology, this part of the lung forms the major site of obstruction in small airway disease (Burgel et al., 2013; Hogg et al., 1968). Small airway disease is a common heterogeneous disease that involves recruitment of inflammatory cells in the small airway epithelium (SAE) (Burgel et al., 2013), but is not well defined (Rice & Nicholson, 2009). Small airway disease can result from environmental exposures to pollutants, chemicals and allergens as well as fine particles (<2.5 µm), as these remain suspended in inhaled air until they reach the small airways and deposit there (Hogg et al., 2017).

Small airway disease plays an important role in conditions such as chronic obstructive pulmonary disease (COPD) (Crisafulli et al., 2016). Toxic particles and gases, such as cigarette smoke, can cause damage to the SAE, disruption of tight junctions and increasing release of inflammatory mediators, leading to COPD (Hogg et al., 2017; Zhao et al., 2018). In COPD the small conducting airways show epithelial disruption, goblet cell metaplasia, disappearance and loss of function of ciliated cells (D. Singh, 2017), as well as inflammatory immune cell infiltration, thickening of smooth muscle cells and fibrosis (Hogg et al., 2004). The increased production and viscosity of mucus and reduced clearance results in mucus accumulation (Hogg et al., 2017), causing narrowing of the small airway lumen, terminal bronchiolar loss and susceptibility to infections.
General introduction

(Hogg et al., 2013). Patients with COPD show reduced numbers of club cells accompanied by an increased number of goblet cells and a reduced production of club cell secretory protein (CCSP) in small airways (Gamez et al., 2015).

Small airways play an important role in both detoxification and inflammatory responses (Hackett et al., 2012; Macklem, 1998) and are thought to be especially susceptible to epithelial injury after particle inhalation and chemical exposure, due to morphology, deposition patterns, uptake mechanisms, an absence of a protective mucus barrier as well as the presence of metabolically active secretory club cells (Boucher et al., 1988; Castell et al., 2005; Gloede et al., 2011). The small airways are the primary site of smoking induced pathogenesis and increased airway resistance for smoking related chronic obstructive pulmonary disease (COPD) (Beasley, 2010). Interestingly, cigarette smoke exposure results in re-patterning of the small airways, causing a more proximal phenotype, characterized by a loss of club cells and an increase in mucus-producing goblet cells (J. Yang et al., 2017). Furthermore, industrial use of flavourings such as diacetyl have been linked to bronchiolitis obliterans, selectively in the small airways (Harber et al., 2006). These observations highlight the importance of regional specific models for airway toxicity assessment.

Club cells are thought to play a major role in small airway toxicity, due their high metabolic capacity, the presence of cytochrome p450 (CYP450) enzymes and their ability to differentiate into other cell types (Zuo et al., 2018). Mice studies have shown that different chemical exposures, such as furan, naphthalene and styrene, can selectively target and damage club cells within the terminal bronchioles attributed to CYP450 mediated bioactivation mechanisms (Cruzan et al., 2009; L. Li et al., 2011; Tǎbǎran et al., 2019). However, it has been shown that humans are 100-fold less sensitive to styrene induced lung tumours than mice, due to species differences in bioactivation of this compound (Sarangapani et al., 2002). In addition, relatively little is known about human club cells. In contrast to many mammalian species where club cells are present throughout the airways (Coppens et al., 2007; Pack et al., 1980; Plopper et al., 1987;
Seidel et al., 2013), human club cells are localized in the small airway epithelium, possibly explaining the susceptibility of this part of the airway to toxic response (Boers et al., 1999). It is important that *in vitro* small airway epithelial cell models contain functional and metabolically active club cells to accurately demonstrate toxicity of compounds undergoing bioactivation or detoxification *in vivo*.

Apart from airway epithelial cells, which will be discussed further in section 1.2, the immune system forms an important part of the small airways and consists of, amongst others, alveolar macrophages, dendritic cells, and lymphocytes (Bienenstock, 1984; Lambrecht & Hammad, 2003). Macrophages and dendritic cells play an important role in innate immune responses in the airways, as these cells are able to interact with allergens, pollutants and pathogens to elicit an immune responses (Iwasaki et al., 2017); these cells will be further discussed in sections 0 and 0.
1.2. **Airway epithelium**

The population of cells that covers the airway surface, the airway epithelium, forms a physical barrier to the environment and is thus on the frontline for inhaled toxicant exposures (Kojima et al., 2013; Rajasekaran et al., 2001). Furthermore, these cells have been suggested as targets for systemic exposures of several chemicals, due to specific uptake mechanisms (Dinis-Oliveira et al., 2008). The conducting airway epithelium consists of at least seven specialised cells (Figure 1-2) with critical functions, including the regulation of fluid balance, metabolism and mucociliary clearance, as well as regulation of other cells such as inflammatory and smooth muscle cells (Knight & Holgate, 2003). The airway epithelium is morphologically different in different airway regions, being pseudostratified in large airways and columnar or cuboidal in the small airways (Crystal et al., 2008). Furthermore, the small airways are mainly characterized by a higher proportion of club cells accompanied by lower proportions of goblet and basal cells compared to more proximal airways (Hackett et al., 2012). Understanding the functions and characteristics of airway epithelium cell types is important for *in vitro* model development and optimization.

![Diagram of common and rare human airway epithelial cells](image)

*Figure 1-2 Common and rare human airway epithelial cells.* Adapted figure (Reid et al., 2019).
1.2.1. Basal cells

Basal cells, the primary epithelial stem cells within the bronchial airways, are capable of self-renewal and differentiation towards goblet, club, brush, and ciliated cells, pulmonary neuroendocrine cells (PNEC) and pulmonary ionocytes (Boers et al., 1998; Hajj et al., 2007; Montoro et al., 2018). These cuboidal cells are present throughout the airway; however, numbers decrease with decreasing airway size. Basal cells form 31% of the epithelial cells in the bronchi and decrease in numbers upon airway branching to 6% in the respiratory bronchioles (Boers et al., 1998; Mercer et al., 1994). Cytokeratin 5 (KRT5) and 14 (KRT14) are typically expressed in basal cells, as well as transformation-related protein 63 (TP63) (Rock et al., 2010). Basal cells are firmly attached to the basement membrane and their abundant cytoskeletal, junctional and adhesive proteins help anchor other epithelial cell types (Rock et al., 2010).

1.2.2. Club cells

Human club cells, also referred to as bronchiolar exocrine cells, are smooth cuboidal-shaped cells with a distinctive dome-shaped luminal surface (Boers et al., 1999). Classically, club cells have been characterized by their ultrastructural features and expression of uteroglobin (SCGB1A1), also known as CCSP (Plopper et al., 1980). Based on these classical descriptions, club cells are thought to be absent in proximal airways in humans and to form ±10% of the airway epithelium in the terminal bronchioles and ±20% of the airway epithelium in the respiratory bronchioles (Boers et al., 1999; Lumsden et al., 1984), these numbers being significantly lower in the bronchioles and respiratory bronchioles of smokers (Lumsden et al., 1984; J. Yang et al., 2017).

SCGB1A1 has been widely used as club cell marker (Boers et al., 1999; Hackett et al., 2012; A. Miller et al., 2017; Zhu et al., 2018; Zuo et al., 2018). Indeed, SCGB1A1 is the most highly expressed gene in the small airways, the proposed location of club cells (Hackett et al., 2012). However, cells that express SCGB1A1, but do not fit with the classical definitions of club cells were shown to be present throughout the human respiratory tract from the trachea to the
terminal bronchioles (Lynch et al., 2019; Okuda et al., 2018). SCGB1A1-reactive cells in the human bronchi showed, for instance, a lower proliferative capacity and higher expression of mucins (MUC5AC, MUC5B) compared to club cells in the small airway epithelium (Barth et al., 2000; Okuda et al., 2018).

The primary function of club cells is thought to be secretory. Club cells secrete a large quantity of lipids and proteins that line the respiratory bronchioles (Knight & Holgate, 2003; Zuo et al., 2018), primarily secretoglobins (SCGB1A1, SCGB3A1, SCGB3A2) (Shijubo et al., 2005). These proteins have protective and anti-inflammatory properties (Alessandrini et al., 2010; G. Singh et al., 1990; Tokita et al., 2014; Weitnauer et al., 2016). There are indications that club cells have an important role in regulating the pulmonary immune system, for instance, club cells express cytokines that attract dendritic cells and neutrophils (Elizur et al., 2007; Reynolds & Malkinson, 2010; Zuo et al., 2018).

Club cells are capable of cell renewal upon injury and differentiation towards other airway epithelial cell types (Boers et al., 1996; Rawlins et al., 2009; Wong et al., 2009). Sub-populations of human club cells that express basal cell, ciliated cell or goblet cell markers have been identified by single cell sequencing and are likely intermediate type cells that are transitioning towards pure ciliated or goblet cell populations ( McCauley, Alysandratos, et al., 2018; Zuo et al., 2018). Furthermore, lineage tracing has shown that club cells can differentiate towards alveolar type 2 and alveolar type 1 cells in mice after alveolar injury (Guha et al., 2017; Zheng et al., 2017) and in vitro differentiation has shown the same in human cells upon cellular injury (Ling et al., 2006).

Club cells express many mitochondrial encoded genes, suggesting a high metabolic activity (Zuo et al., 2018). Club cells also contain a high number of CYP450 enzymes and oxidases (Boei et al., 2017; Saarikoski et al., 1998), therefore these cells are referred to as the main metabolizing cells of the lung (Hukkanen et al., 2002). Most knowledge on club cell metabolism however originates from animals studies; metabolic enzymes in human club cells are thought to be different (Castell
et al., 2005; Cruzan et al., 2018; Hukkanen et al., 2002). More studies are needed to define levels of metabolic enzymes in human club cells.

1.2.3. Goblet cells

Goblet or mucous cells, as identified by the presence of periodic acid-Schiff (PAS)-positive mucous vacuoles in the cytoplasm, form 11% of the epithelial cells in the human bronchi, decrease in prevalence upon airway branching, and are absent in respiratory bronchioles (Boers et al., 1999). Their main function is to secrete mucus in order to trap foreign objects in the airway lumen. Mucus produced by goblet cells consist of several components such as electrolytes, metabolites, fluids, antimicrobials and mucins such as MUC5B and MUC5AC (Whitsett, 2018). Goblet cells are capable of self-renewal and further differentiation towards ciliated cells (Knight & Holgate, 2003).

As previously discussed by Lynch et al, (2019), a typical goblet cell morphology is not usually observed in healthy human bronchi (Duclos et al., 2019; Goldfarbmuren et al., 2020; Lynch et al., 2019; Okuda et al., 2018; Vieira Braga et al., 2019), whereas goblet cell hyperplasia accompanied by a reduction in club cells is a common phenomenon in diseases such as asthma and COPD and in response to cigarette smoke exposure (Lynch et al., 2019; Vieira Braga et al., 2019; J. Yang et al., 2017). This could suggest that club cells differentiate towards goblet cells upon injury or in disease development. Ruiz García et al. (2019) have described goblet cells as “hyperactive” club cells as single cell sequencing showed higher levels of MUC5AC, MUC5B and SCGB1A1 in goblet cells compared to club cells (Ruiz García et al., 2019). Indeed, goblet cells can be detected after interleukin 13 (IL13) treatment of club-like cells in vitro (Laoukili et al., 2001), indicating they could be an inflammatory state of secretory club cells.

1.2.4. Ciliated cells

Columnar ciliated cells are present throughout the airways and account for over 50% of all airway epithelial cells (Knight & Holgate, 2003). Their main function is to regulate mucus secretion and to move mucus towards the upper pharynx through the rhythmic beating of cilia,
commonly referred to as mucociliary clearance (Breeze & Wheeldon, 1977). The transcription factor forkhead box protein J1 (FOXJ1) is required for cilia formation and thus a specific marker for ciliated cells (M. Guo et al., 2019). Ciliated cells can be derived from basal, club or goblet cells dependent on Notch signalling pathways (McCauley, Alysandratos, et al., 2018; Ruiz García et al., 2019). Recently, single cell sequencing has identified “deuterosomal cells”, intermediate precursors for ciliated cells expressing mature ciliated cell markers, basal cell markers as well as specific cell markers such as deuterosome assembly protein 1 (DEUP1) (Ruiz García et al., 2019).

1.2.5. Rare cells

Rare cells populating the conducting airway epithelium PNECs, ionocytes and brush cells (Goldfarbmuren et al., 2020). PNECs, key communicators between nervous and immune systems, are rare cells that form 0.33 and 0.49% of the epithelial cells in small airways and larger airways respectively (Boers et al., 1996). In vitro and animal studies suggest that PNEC play a role in oxygen sensing, smooth muscle tone and immune responses (Branchfield et al., 2016; Linnoila, 2006). These specialised epithelial cells secrete a variety of biogenic amines and peptides to stimulate immune responses (Knight & Holgate, 2003). Furthermore, these cells can form neuroendocrine bodies that closely resemble chemoreceptors and act as hypoxia sensitive airway sensors (Youngson et al., 1993).

Heterogenous populations of rare ionocytes and brush cells have recently been identified in mouse and human airways (Deprez et al., 2020; Montoro et al., 2018), however, their specific localization and functions within the airways are still unclear. Ionocytes are estimated to comprise less than 2% of the airway epithelium, express forkhead box I1 (FOXI1) and CF transmembrane conductance regulator (CFTR) and are explored as possible targets for cystic fibrosis treatments (Montoro et al., 2018; Plasschaert et al., 2018). Brush cells, also known as tuft cells or microvillous cells, require POU domain class 2 homeobox 3 (POU2F3) for differentiation, secrete specific molecules such as IL25, acetylcholine and eicosanoids and are
thought to have chemosensory, neuronal and immunological functions (J. D. Davis & Wypych, 2021; Schneider et al., 2019).

1.2.6. Alveoli

The conducting airways transport the air from the trachea to the alveoli where gas exchange with the blood takes place. The human lung contains circa 500 million alveoli (Ochs et al., 2004). In contrast to the epithelium lining the conducting airways, the alveolar epithelium comprises only two cell types: alveolar type I (AT1) and AT2 cells. Squamous AT1 cells cover circa 90% of the alveolar airway epithelium and form a narrow barrier between air and blood compartments, allowing for gas exchange (Dobbs & Johnson, 2007; Whitsett & Alenghat, 2015). Cuboidal AT2 cells play a role in synthesis, secretion and reuptake of proteins that play a role in surfactant homeostasis such as surfactant protein A (SFTPA), SFTPB, SFTPC, SPFPD, required to reduce surface tension and regulate gas exchange (Whitsett et al., 2010). Furthermore, AT2 cells function as alveolar progenitor cells, as these are capable of self-renewal and differentiation towards AT1 cells upon cellular injury (Barkauskas et al., 2013).

1.2.7. Non-epithelial airway cells

Apart from differences in airway epithelial cells, there are structural differences between the airway regions. Cartilage and submucosal glands are present within the proximal airways but absent in the small airways. Collagen and proteoglycan-rich cartilage functions to keep the tracheal and bronchial airways open during ventilation cycles (Roberts et al., 1997), whereas collagen and elastic fibres form three-dimensional basket-like structures around and help stabilise the alveoli and small airways (C. Young et al., 1980). Submucosal glands contribute to the secretion of mucus in the larger airways in humans (Wine, 2004). Furthermore, submucosal glands could function as a reservoir of stem cells that can regenerate the airway after injury (Tata et al., 2018). The mucus layer in larger conductive airways is circa 5µm thick, however, thickness of the mucus layer decreases with airway size to 0-1 µm in the terminal bronchioles (Mauroy et al., 2015). The airway epithelium is richly innervated, some of which are in direct
contact with PNECs (Knight & Holgate, 2003). Smooth muscle cells form a circular band around the small airway epithelium and are regulated by the autonomic nervous system and mediators that are released from mast, inflammatory and neuroendocrine cells (Nomellini & Chen, 2012). The immune system forms an important part of the airways (Bienenstock, 1984; Lambrecht & Hammad, 2003); airway macrophages and dendritic cells will be discussed in more detail in the following sections.
1.3. **Airway macrophages**

Macrophages, present throughout the human body, play an important role in innate immune responses and are the most abundant immune cells within the respiratory tract (Joshi et al., 2018). As these cells are frontline responders to inhalation toxicant exposure, *in vitro* macrophage models are highly important for airway toxicity assessment. Understanding the functions and characteristics of airway macrophages is important for successful modelling of these cells in *vitro*.

Pulmonary macrophages form a heterogeneous population of immune cells with specific functions, including phagocytosis of cellular debris and surfactants, immune surveillance, microbial clearance, and responses to infection (Joshi et al., 2018). Lung resident macrophages originate from either embryonic precursors or blood monocytes derived from hematopoietic stem cells in the bone marrow (Epelman et al., 2014). In adult unchallenged mice, and as a consequence of clean living conditions in the laboratory, most airway resident macrophages are derived from embryonic precursors and self-renewal (Hashimoto et al., 2013; Yona et al., 2013). Although the origin of human airway macrophages is poorly understood, there are indications that the majority of adult airway macrophages originate from hematopoietic precursors, due to continuous exposure to allergens, microbes and pollutants and consequent depletion of embryonic precursor derived alveolar macrophages over time (Figure 1-3) (Byrne et al., 2020).

Airway macrophages within the human airways are classified in two major categories: alveolar macrophages and interstitial macrophages (Schyns et al., 2018). A humanized mouse model showed that human CD34+ hematopoietic stem cells are able to generate both macrophage populations (Evren et al., 2021). Within adult humans, most alveolar macrophages are localized in the diffusing airspaces (95%), however, this is mostly due to the larger volume of alveoli compared to small conducting airways as the alveolar macrophage density is more similar (1.6 million macrophages/cm³ in the small airway lumen compared to 4.7 million macrophages/cm³ in the alveolar lumen) in both smokers and non-smokers without a history of pulmonary disease.
Interstitial macrophage density follows a similar pattern with 15 million macrophages/cm$^3$ in the alveoli and 11 million macrophages/cm$^3$ in the small airway walls (Hume et al., 2020). However, observations of immune cells with an alveolar macrophage phenotype in the airway interstitium and of interstitial macrophages migrating into airway lumen however limits a clear distinction of alveolar and interstitial macrophages (Holt et al., 1985; Sabatel et al., 2017).

1.3.1. Alveolar macrophages

Alveolar macrophages reside in the conducting airways and the alveoli, on the epithelial surface in direct contact with air in the airway lumen and are thus strategically located to respond to invading pathogens and toxicants (Joshi et al., 2018). Alveolar macrophages are essential for regulating surfactants, as depletion results in accumulation of surfactants within the alveoli (Trapnell et al., 2019), and regeneration of injured tissues through production of repair factors (Minutti et al., 2019).

As discussed previously, the majority of human alveolar macrophages are derived from blood monocytes, whereas the majority of unchallenged murine alveolar macrophages are derived
from embryonic progenitors (Joshi et al., 2018). Alveolar macrophages from both origins respond to changes in the local environment during lung injury, however both qualitative and quantitative transcriptomic changes were shown to be more prominent in monocyte-derived alveolar macrophages in mice and human studies (Joshi et al., 2018; Mould et al., 2017). Whereas in mice alveolar macrophages are identified as CD45⁺, CD11c⁺, Siglec-F⁺, CD64⁺, I-Aβ⁺/−, F4/80⁻, and CD11b⁺, human alveolar macrophages isolated from bronchoalveolar lavage (BAL) are characterized as CD45⁺, CD11b⁺, HLA-DR⁺, CD163⁺, and CD206⁻ cells within the same study (Nayak et al., 2018). This observation could imply further functional differences between species, which are likely relevant for human toxicity assessment.

In steady state, murine alveolar macrophages adhere closely to airway epithelial cells, produce few inflammatory cytokines and show poor phagocytic activity in order to avoid damage to airway epithelial cells, as shown by the downregulation of the phagocytic receptor CD11b (Garn et al., 2006). Furthermore, alveolar macrophages actively suppress the adaptive immune system through interaction with dendritic cells and T cells. Indeed, human alveolar macrophages have been shown to be poor at antigen presenting to peripheral blood T lymphocytes, even though more than 90% of alveolar macrophages express HLA-DR antigens, due to lack of co-stimulatory molecules such as CD86 (Hussell & Bell, 2014; Lipscomb et al., 1986). Furthermore, alveolar macrophages were shown to produce immunosuppressive prostaglandins as well as transforming growth factor-β1 (TGFβ1), indicating that alveolar macrophages are able to suppress T cell activation (Roth & golub, 1993). However, alveolar macrophages were shown to be able to specifically stimulate the proliferation of already activated T cells, indicating a role in maintaining inflammatory responses (Lipscomb et al., 1986).

Pulmonary injury leads to a loss of contact of alveolar macrophages with airway epithelial cells, resulting in inactivation of TGFβ and consequent macrophage activation, as shown by secretion of pro-inflammatory cytokines (tumour necrosis factor (TNF), IL10 and IL6) and an increase in phagocytic activity (Garn et al., 2006). Alveolar macrophages are prone to undergo regulated
cell death following toxicant exposures, which is essential in triggering the recruitment of circulating monocytes and neutrophils and the initiation of inflammatory responses (Fan & Fan, 2018). It has been shown that recruited monocytes can adopt the suppressive resident alveolar macrophage phenotype within days (Bilyk & Holt, 1995).

If airway epithelial cells are depleted upon toxicant exposure, alveolar macrophages lose an important feedback mechanism, which results in excessive production of pro-inflammatory cytokines as well as TGFβ, which might result in prolonged activation of fibroblasts and consequently pulmonary fibrosis (Lambrecht, 2006).

1.3.2. Interstitial macrophages

Interstitial macrophages reside in the airway interstitium and are observed in lungs from both healthy subjects (Crapo et al., 1982) and diseased patients (G. S. Davis et al., 1978). Interstitial macrophages were first considered as a transition state between blood monocytes and alveolar macrophages (Landsman & Jung, 2007). However, as interstitial macrophages were shown to be smaller and more heterogeneous cells with a lower phagocytic activity, a higher peroxidase activity and higher expression of surface makers such as HLA-DR compared to alveolar macrophages (Chandler et al., 1986; Fathi et al., 2001; Ferrari-Lacraz et al., 2001), interstitial macrophages were further described as a macrophage population that is morphologically and functionally distinct from alveolar macrophages (Schyns et al., 2018).

Mouse interstitial macrophages can be divided into 3 subtypes based on CD11c and Major Histocompatibility Complex (MHC)-II expression (IM1: CD11c<sub>lo</sub>MHCII<sub>lo</sub>, IM2: CD11c<sub>lo</sub>MHCII<sub>hi</sub>, and IM3: CD11c<sub>hi</sub>MHCII<sub>hi</sub>), likely representing a continuum of differentiation from blood monocytes to more differentiated interstitial macrophages (Gibbings et al., 2017). Single-cell mRNA sequencing data have identified human macrophages similar to the murine IM2 and IM3 populations indicating that human interstitial macrophages follow a similar classification (Chakarov et al., 2019).
In humans, interstitial macrophages have been shown to secrete a higher level of both pro-inflammatory cytokines IL6 and TNF-α (Hoppstädter et al., 2010) and anti-inflammatory cytokine IL10 compared to alveolar macrophages (Lehnert et al., 1985). IL10 expression of interstitial macrophages increases, in both mice and humans, upon exposure to immune stimulants such as lipopolysaccharides (LPS) or house dust mite (HDM) (Liegeois et al., 2018; Toussaint et al., 2013), indicating an anti-inflammatory response and an important role in maintaining homeostasis. Bronchial biopsies of asthmatic patients showed a lower level of IL10+ interstitial macrophages compared to healthy subjects, indicating that interstitial macrophages might be functionally impaired in asthma (Draijer et al., 2017). However, human interstitial macrophages are likely to consist of different sub-populations with specialized functions. Due to limited access to healthy human samples, characterization of interstitial macrophages within human airways has proven difficult and requires further study with for instance single-cell transcriptomic analyses (Schyns et al., 2018). This lack of availability is also paralleled by a lack of *in vitro* models of interstitial macrophages and reveals an opportunity that new approaches such as iPSCs may help develop.
1.4. Airway dendritic cells

Dendritic cells are professional antigen presenting cells and form a link between innate and adaptive immunity. They are essential for immune responses to environmental toxicants, such as chemical sensitizers and various microbial exposures (Mizoguchi et al., 2017). Dendritic cells are found in lymphoid organs, blood and within different tissues within the human body, including the lung throughout the alveolar and conducting airways (Figure 1-4) (Sertl et al., 1986). Dendritic cells are mainly characterized by the presence of dendrites, used to sample antigens from the environment. However, morphological evaluations are not sufficient to describe dendritic cell subsets, as these subsets structurally resemble each other and other mononuclear phagocytes such as macrophages and monocytes (Collin & Bigley, 2018).

In homeostasis, dendritic cells recognize self-antigens and express low amounts of costimulatory molecules, inducing weak T cell stimulation, resulting in a rise of regulatory T cells and immune tolerance in homeostasis (Lanzavecchia & Sallusto, 2001). In response to pathogens, tissue injury or toxicant exposure, dendritic cells can undergo maturation, resulting in an increased expression of antigen presentation (MHC) and co-stimulatory molecules (CD80, CD86, CD40) as well as increased production of cytokines (Hubo et al., 2013). Dendritic cell activation (e.g. via toll-like receptors (TLRs)) and maturation, leads to proliferation and activation of T-cells and B-cells. Dendritic cells are able to capture antigens within the airway lumen (by phagocytosis or receptor-mediated endocytosis), degrade them into smaller peptides and present these peptides onto MHC class I and MHC class II molecules expressed on the cell surface (Ahmad et al., 2017). Thereafter, dendritic cells can migrate to lymph nodes via the lymphatics, mediated by the CCR7 ligands CCL19 and CCL21, to present these antigens to T-cells (Clatworthy et al., 2014). MHC molecules together with co-stimulatory factors can activate naïve T-cell differentiation towards CD8+ or CD4+ T cells. CD8+ T cells further differentiate towards cytotoxic T cells that kill microbial infected cells, whereas CD4+ T cells can differentiate towards Th1, Th2,
Th17 or Tregs to regulate the inflammatory state of immune cells such as macrophages and importantly, direct antibody production in B-cells (Merad et al., 2013).

Thorough characterization using cell surface marker expression or single cell transcriptomics is typically required to identify dendritic cell subsets, and have primarily been described for human blood dendritic cells (Collin & Bigley, 2018; Villani et al., 2017). Blood derived immune cells have also been used as in vitro models of dendritic cell function (Lundberg et al., 2013). Although extensively used, these cells may not truly represent normal airway dendritic cell phenotype and function (Patel et al., 2017). We will describe what is known regarding airway dendritic cells as a basis to establish characterisation parameters to use in the optimisation of in vitro cultures for this cell type.

Figure 1-4 Airway dendritic cells. Schematic overview of dendritic cell localization, activation, and sensing within airway epithelium. Adapted figure (Kool et al., 2012)

1.4.1. Airway resident dendritic cells

Single cell sequencing efforts have shown dendritic cell presence in distal, intermediate, tracheal and nasal areas within the normal human airway epithelium (Deprez et al., 2020), including both non-inflammatory (CLEC10A+, CD1C+, CD14-) and inflammatory dendritic cells (CLEC10A+,
CD1C-, CD14+) have been identified (Vieira Braga et al., 2019). Due to the tendency of airway resident dendritic cells to migrate to the spleen and lymphoid system, as well as their rapid turnover rate, differences between airway resident and blood dendritic cells are difficult to determine (J. I. Gray & Farber, 2022). Further characterization is therefore needed to more accurately determine dendritic cell subtypes within both healthy and diseased human airway epithelium (Deprez et al., 2020). Nevertheless, airway resident dendritic cells consist of several sub-populations with similar profiles to those observed within the blood, such as plasmacytoid and myeloid dendritic cells (Schupp et al., 2020). Therefore, insights into characterization of the more extensively studied blood dendritic cells can be useful to explore dendritic cell differentiation markers relevant for in vitro small airway dendritic cell model development.

1.4.2. Blood dendritic cell characterization and functions

Previously three major blood dendritic cell subsets were described in humans: plasmacytoid DC, myeloid/conventional DC1 and myeloid/conventional DC2 (Ziegler-Heitbrock et al., 2010). However, single-cell sequencing analysis of immune cells isolated from healthy blood donors has identified six dendritic cell sub types (Table 1-1) (Villani et al., 2017). Dendritic cells have a limited life span (days to weeks) and must be continuously replenished by haematopoiesis. Therefore, a pre-DC population exists in between CD34+ progenitors and mature dendritic cells (See et al., 2017). These precursor cells do not express mature dendritic cell phenotype markers and are identified as DC5 and characterized by CD123 and CD303 expression (Table 1-1) (Villani et al., 2017).

Plasmacytoid dendritic cells (pDCs) mainly function to respond to viral infections through production of type I and III interferon and cytokines, and expresses surface markers involved with this function, such as CD303, CD304, CD85K and FceR1 (Collin & Bigley, 2018). Furthermore, these cells retain the pre-DC marker CD123 and lack myeloid antigen expression such as CD11c, CD33, CD11b and CD13 and are identified as DC6 (Table 1-1) (Villani et al., 2017).
cDC2 form the major population of myeloid dendritic cells (cDCs) in human blood and tissues and are characterized using expression of CD1c, CD2, FcεR1, as well as the myeloid antigens CD11b, CD11c, CD13 and CD33 (Collin & Bigley, 2018). Single-cell sequencing has characterized two subtypes of cDC2: DC2 and DC3 (Villani et al., 2017). In this study DC3 cells showed higher expression of inflammatory genes such as CD14, S100A9 and S100A8, whereas DC2 cells showed higher levels of MHC class II genes (Villani et al., 2017). In vitro studies have shown that cDC2 are potent activators of T-helper 1 (Th1), Th2, Th17 and CD8+ T-cells, indicating involvement in a wide range of immune responses (Di Blasio et al., 2016).

DC1 corresponds to the dendritic cell subtype previously characterised as human myeloid cDC1, best characterized by high expression of CLEC9A as shown in Table 1-1 (Villani et al., 2017). Like cDC2, these cells express CD13 and CD33, however they have low CD11b and CD11c expression. There are suggestions that DC1 are more highly enriched in tissues compared to blood, however limitations regarding characterization hamper comparisons (Collin & Bigley, 2018). Myeloid cDC1 have a high capacity to present antigens to CD8+ T-cells via MHC class I and to promote Th1 and natural killer responses (Jongbloed et al., 2010).

DC4 corresponds to a population previously identified as CD16+ non-classical monocytes. These cells are considered as DC by some authors and as “true” non-classical monocytes by others. DC4 most likely consist of a subset of CD16+ non-classical monocytes expressing SLAN, together with lower CD11b, CD14 and CD36 (Collin & Bigley, 2018).

Monocyte-derived DCs (mo-DCs), also known as inflammatory DCs (Table 1-1), were previously described as macrophage-like due to the overlap in mo-DC and monocyte-derived inflammatory macrophage expression patterns (Collin & Bigley, 2018). Depending on the site of inflammation, mo-DCs show expression of CD11c, CD1c, FcεR1, CD206 and IRF4. Mo-DCs secrete IL1, TNFα, IL12, IL13 and CCR7 indicating their ability to stimulate CD4 and CD8 T-cells (Segura et al., 2013).
### Table 1-1 Dendritic cell sub-populations (Villani et al., 2017)

<table>
<thead>
<tr>
<th>DC subtype</th>
<th>Main markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloid cDC1 (DC1)</td>
<td>CD141, CLEC9A</td>
</tr>
<tr>
<td>Myeloid cDC2 (DC2/3)</td>
<td>CD1c, CD11c, CD11b</td>
</tr>
<tr>
<td>Non-classical monocyte (DC4)</td>
<td>CD16, CX3CR1, SLAN</td>
</tr>
<tr>
<td>Pre-DC (DC5)</td>
<td>CD123, CD303</td>
</tr>
<tr>
<td>Plasmacytoid DC (DC6)</td>
<td>CD123, CD303, CD304</td>
</tr>
<tr>
<td>Mo-DC (inflammatory DC)</td>
<td>CD11c, CD1c, CD206</td>
</tr>
</tbody>
</table>
1.5. Pulmonary susceptibility to toxicant exposure

Regional differences in airway morphology, cellular composition and airflow dynamics affect susceptibility to inhaled toxicants, including particles and chemicals. Furthermore, differences in bioactivation, detoxification and transporters affect airway susceptibility not only to inhaled toxicants but also systemic chemical exposures. These differences highlight the need for specific and organotypic airway models for \textit{in vitro} toxicity testing to capture differentiation and regional dependent susceptibilities relevant for human exposure. Therefore, regional differences in pulmonary susceptibility to particle and chemical exposures will be further explored. The small airways and small airway resident immune cells are particularly interesting for \textit{in vitro} assay development, as these cells play an important role in the mechanism of action of toxicant exposures and will therefore be the main focus within this section.

1.5.1. Regional particle susceptibility

The small airways are thought to be especially sensitive for epithelial injury after particle inhalation, due to morphology, deposition patterns and the absence of a protective mucus barriers (Boucher et al., 1988). Several studies have linked particle exposure to small airway disease in humans. Agricultural dust was shown to accumulate in the bronchioles and to be associated with wall thickening and remodelling in the respiratory bronchioles, whereas conducting airways showed little agricultural dust accumulation and pathological changes (Schenker et al., 2009). Furthermore, case reports showed thickening of the bronchioles upon silica dust exposure causing small airway disease (Bernard et al., 1994; Choudat et al., 1990; Tsuchiya et al., 2017). Asbestos workers showed distinctive lesions in the respiratory bronchioles and alveolar ducts as well as wall thickening in the bronchioles (Bellis et al., 1989; Wright & Churg, 1984; X. Yang et al., 2017). Patients with asbestosis show obstructive defects in the small airways and decreased small airway function, as shown by a decrease in Forced Vital Capacity (FVC) (Hjortsberg et al., 1988). These findings indicate that the small airways are indeed more susceptible to injury caused by the inhalation of particles compared to more proximal airways.
1.5.1.1. Particle deposition

Coarse particles (2.5 - 10 µm) mainly deposit by impaction in the first 10 generations of the bronchi as these particles develop sufficient momentum, their course will not follow the direction of airflow, resulting in collision with the walls in the upper airways (Figure 1-5) (Darquenne, 2012). Fibre particles, due to their elongated shape, deposit upon contact with the airway wall (Lippmann et al., 1980). Fine particles with an aerodynamic diameter between 0.5 – 2.5 µm deposit due to gravity in the smaller bronchi and larger bronchioles. Particles with an aerodynamic diameter below 0.5 µm are able to reach the distal airways and will deposit through diffusion, as the gas they are dissolved in will diffuse faster than the particle itself according to Einstein’s work on Brownian motion, resulting in a greater concentration of fine particles deposition in the small airways (Hogg & Hackett, 2018; Lippmann et al., 1980).

![Particle deposition in the lungs](image)

**Figure 1-5 Particle deposition in the lungs.** Schematic diagram showing the three main mechanisms of particle deposition related to airway region and particle size (Williams et al., 2011).

So far, a limited amount of human exposure studies on ultrafine particles deposition in human small airways is available. Usmani et al. (2004), showed a higher peripheral lung deposition of 1.5 µm sized albutarol aerosols (25%) compared to 6 µm aerosols (10%) in asthmatic patients (Usmani et al., 2005). Several animal studies were performed to analyse the regional deposition of inhaled aerosols and nanoparticles. These studies indicate that fine particles mainly deposit
in the terminal bronchioles, with a limited amount reaching the alveolar regions (Brody & Roe, 1983; C. Guo et al., 2018; Kuehl et al., 2012; Silva et al., 2014). However, the morphology of the small airways in animals is significantly different to humans, making extrapolation of deposition dosages challenging (Bergmann, 1998; Warheit & Hartsky, 1990). Therefore, *in silico* and *in vitro* studies are useful in predicting the regional airway deposition of nanoparticles within humans. Several computational models have indicated that fine particles mainly deposit within the terminal bronchioles and alveolar entrance ring in humans (Islam et al., 2017; Kolanjyil & Kleinstreuer, 2013; Manojkumar et al., 2019; Riebeling et al., 2016). A true-scale experimental *in vitro* model of small airway regions to study trajectories of inhaled smoke particles (0.1 – 2 µm in diameter) also showed a higher particle deposition in the terminal bronchioles and proximal alveolar regions compared to more distal alveolar regions independent of particle size (Fishler et al., 2015). Overall, these studies indicate that fine particles deposit in the small airways, whereas coarse particles with an aerodynamic diameter below 10 nm deposit mainly in the proximal lung and upper airways.

### 1.5.1.2. Mucociliary clearance

After deposition, chemicals and particles are retained, absorbed, degraded, or cleared through mucociliary clearance or phagocytosis. Insoluble particles can also be trapped in the mucus layer covering the airway epithelium and cleared due to cilia movement.

The mucus layer in larger conductive airways is circa 5µm thick. The thickness of the mucus layer decreases with airway size to 0-1 µm in the terminal bronchioles (Mauroy et al., 2015). The lack of a mucus barrier might contribute to small airway susceptibility to chemicals and nanoparticles (Manke et al., 2013). Indeed, Okuda et al. (2018) showed that terminal bronchioles lack MUC5B and MUC5AC mRNA and protein staining (Okuda et al., 2018). More proximal small airways contained MUC5B, but not MUC5AC mRNA and protein staining (Okuda et al., 2018). MUC5B is necessary to sustain mucociliary clearance, whereas MUC5AC has been recognized as a “response mucin”, providing innate immune functions during airway stresses (Roy et al., 2014;
The absence of both mucins in terminal bronchioles and the absence of MUC5AC in small airways may contribute to why these regions are specifically vulnerable to chemical and nanoparticle induced injury. Möller et al. (2007) showed rapid mucociliary clearance of radiolabelled 100 nm carbon particles in the proximal airways (25% in 24 hours), compared to negligible clearance in the small airways in healthy non-smokers, asymptomatic smokers and COPD patients (Möller et al., 2008). Indeed, the ciliary beat frequency was shown to be 35% lower in bronchioles compared to the larger bronchi (Clary-Meinesz et al., 1997), which could result in a longer time of exposure in the bronchioles resulting in an increased dose.

The smaller diameter of small airways results in increased susceptibility to airway obstruction and consequent airway disease in this area. In small airway disease the mucus layer in the bronchioles is significantly thicker, narrowing the lumen of the airway. This results in increased susceptibility to accumulation of particles due to impaction and turbulent diffusion in small airway disease (Fahy & Dickey, 2010).

Interesting observations in pig proximal tracheal airways indicate that the luminal epithelium is mainly absorptive for airway surface liquid (ASL) and the liquid is produced and secreted from the submucosal gland (Flores-Delgado et al., 2016). This is suggested as different in the small airways where a mixture of secretory and absorptive ASL cells are indicated according to folds manifesting on inhalation/exhalation cycles. These inherent differences in cell types may also manifest in different responses to toxic insult (Flores-Delgado et al., 2016).

1.5.1.3. Phagocytosis

As the alveolar regions lack mucociliary clearance mechanisms, deposited particles can only be cleared through phagocytosis by alveolar macrophages (Labiris & Dolovich, 2003). Alveolar macrophages are also shown to be present in the small airways, although the density is slightly lower in this area (Hume et al., 2020), which could result in increased susceptibility to particle exposures in small airway areas with limited mucociliary clearance.
1.5.2. Regional chemical susceptibility

Regional uptake of chemicals is dependent on airflow rates, blood flow rates, chemical concentrations, solubility, tissue thickness, metabolism rate and partition coefficients, as has been established for acrolein and diacetyl airflow simulation models for example (Corley et al., 2012; Gloede et al., 2011; Morris & Hubbs, 2009).

The small airways susceptibility to chemical toxicity is suggested as a consequence of airflow patterns, uptake mechanisms and the increased presence of metabolically active club cells (Castell et al., 2005; Gloede et al., 2011). High levels of chemical uptake were observed in bronchial and bronchiolar areas where changes occur in airflow direction and velocity, as well as in the distal bronchiolar regions due to a higher level of metabolism (Corley et al., 2012).

The small airways are especially sensitive to chemicals present in cigarette smoke, such as polycyclic aromatic hydrocarbons (PAHs) and nitrosamines. The small airways react to low levels of cigarette smoke, as shown by up- and down regulation of smoking related genes in the SAE of subjects where nicotine or its metabolites are detectable in the urine at any level (Buro-Auriemma et al., 2013; Harvey et al., 2006; Hübner et al., 2009; Strulovici-Barel et al., 2010; Tilley et al., 2009, 2011). Cigarette smoking results in re-patterning of the small airways, causing a more proximal phenotype, characterized by a loss of club cells and an increase in mucus-producing goblet cells (J. Yang et al., 2017). Other studies have previously indicated small airway involvement in cigarette smoke toxicity, characterized by lower club cell numbers and progressive pathological changes (Cosio et al., 1980; Lumsden et al., 1984). No quantitative comparison between responses of small airways and more proximal airways to cigarette smoke exposure have been made. Nonetheless, in smoking associated lung diseases, such as COPD, both pathological changes and airway obstruction mainly occur in the small airways (Hogg et al., 1968, 2013, 2017; Niewoehner et al., 1974), indicating small airway susceptibility to cigarette smoke exposure.
1.5.2.1. Bioactivation and detoxification

Metabolic transformation is a fundamental defence mechanism for detoxification of toxic chemicals. However, in some cases metabolism results in the generation of more active and toxic metabolites. Phase I and II metabolism is mediated through enzymes including CYP450, aldehyde dehydrogenase and glutathione transferase enzymes (Hukkanen et al., 2002). Differential expression of these enzymes exists in different cell types and may therefore confer tissue specific metabolic influence and toxic potential. A human ex vivo study of lung explants demonstrated that freshly isolated metabolically active fractions of different airway regions have different levels of lung enzymes related to bioactivation, indicating that inherent epithelial differences exist in the human lung may underlie region specific susceptibilities to toxicity and disease (Iba & Caccavale, 2013).

CYP450 enzymes are involved in metabolic activation of many organic toxicants (Castell et al., 2005). A relatively limited number of studies have examined expression of CYP enzymes along the human respiratory tract. CYP1A1 and CYP1B1 are involved in bioactivation of PAHs and nitrosamines present in for instance diesel exhaust and cigarette smoke (Burczynski & Penning, 2000). CYP1A1 and CYP1B1 expression were shown to be induced by exposure to cigarette smoke and absent in non-smokers (Anttila et al., 1991; J. Kim et al., 2004; Mclemore et al., 1990; Saarikoski et al., 1998; Willey et al., 1997). CYP1A1 was shown to be highly expressed in the cuboidal epithelium of the terminal bronchiole of smokers and was not expressed in the epithelium of bronchioles larger than 1 mm in diameter (Anttila et al., 1991; Saarikoski et al., 1998), indicating regional susceptibility and/or responsiveness to cigarette smoke. Furthermore, when human small airway epithelial cells and rat lung slices were exposed to AHR ligands, the expression of CYP1A1 and CYP1B1 enzymes were localised to SCGB1A1 positive club cells (Chang et al., 2006), further supporting regional specific effects.

The analysis of metabolic enzymes, in particular CYP expression, and their functional consequences has been examined more extensively in rodents. For example, cytochrome B5,
important for specific CYP450 activities (e.g. CYP2A6), is restricted to the bronchiolar areas and not expressed in larger bronchial or smaller alveolar structures in mice (Ménoret et al., 2012). The toxicity of a combustion product furan has been investigated in mice and rats and found to have specific bronchiolar injury attributable to CYP2E1 expression in club cells (Tăbăran et al., 2019). It has also long been recognized that chemicals such as styrene, permethrin and Fluensulfone (Cruzan et al., 2017; Strupp et al., 2016; Yamada et al., 2017) are specifically toxic to the terminal bronchioles in mice, attributable to the high number of club cells in this region enriched for CYP2F2 activity (Cruzan et al., 2009). This enzyme metabolises these chemicals to toxic metabolites, which induce toxicity and tumour formation. While the implications of this mechanism for most chemicals metabolised by CYP2F2 are considered irrelevant for human toxicological risk assessment due to species specific enzyme activity (Cruzan et al., 2009), the concept of regional specific CYP expression mediated effects may have functional consequences for other types of human exposures. This is likely to become a lot clearer, when characterisation of human bronchiolar responses to toxic exposure is carried out. At the moment, this is hampered by a lack of appropriate models of the terminal and respiratory bronchioles.

A humanised transgenic mice model, where the human CYP2F2 orthologues CYP2F1 and CYP2A13 were expressed on a mouse CYP2F2 knock-out (KO) background was used to examine Naphthalene toxicity; a chemical commonly found in combustion products such as tobacco smoke and diesel exhaust (Marr et al., 1999; Witschi et al., 1997) and used experimentally to deplete club cells in mice (L. Li et al., 2011). This study demonstrated that inhaled naphthalene toxicity showed some degree of regional specificity, as CYP2A13 expression in the olfactory mucosa was associated with greater toxicity than CYP2F1 mediated toxicity in the lower respiratory tract (L. Li et al., 2017). A more complete understanding of such will come with a better characterisation of human metabolism and detoxification gene localisation and functions within the airways.
1.5.2.2. Uptake of chemical exposures

Apart from chemical metabolism, regional differences in uptake is dependent on chemical solubility, tissue thickness, airflow rates, blood flow rates and uptake mechanisms such as transmembrane transporters are likely to affect regional toxicity (Gloede et al., 2011).

Injury along the airways as a result of gaseous chemical inhalation exposures is suggested to be proportional to the degree of chemical solubility in that airway region. Irritant gases with high solubility such as formaldehyde (Schachter et al., 2001) will be absorbed in the upper airways and will not reach high concentrations in the bronchioles but rather concentrate in the proximal airways, while gases such as phosgene or nitrogen dioxide have the potential to exert more distal toxicological effects (Diller, 1985; Weiss & Lakshminarayan, 1994).

Paraquat, a redox cycling herbicide, preferentially accumulates in the lung upon systemic exposure, possibly due to the presence of polyamine transporters, which are expressed on the membranes of club cells, AT1 and AT2 cells (Dinis-Oliveira et al., 2008), indicating susceptibility of small airways and alveolar regions.

1.5.2.3. Airway dimensions

The combined surface areas of the small airways (±24.000 cm²) are much lower than that of the alveolar regions (±143.000 cm²) (Gehr et al., 1978; Yeh & Schum, 1980). Therefore, the alveolar regions are suspected to be less susceptible to inhalation toxicity compared to the small airways, as the large surface area results in lower local concentrations.

1.5.2.4. Repair

Although differences in dosage and uptake explain part of the observed regional differences in airway toxicity, the ability of epithelial cells to repair toxicant induced damage differs between airway regions. A recent study demonstrated that acute inhalation of chlorine in a rabbit exposure model resulted in similar levels of acute injury in the small and large airways but that during the recovery period, there was more inflammation and less repair of the epithelium in
bronchioles compared to the bronchi (Musah et al., 2017). The authors suggest that basal cells present in the pseudostratified proximal airways but absent in distal bronchioles allowed for faster repair. This mechanism may also contribute to altered susceptibility of the small airways to injury and disease particularly in chronic conditions or exposures. Finally, it is also possible that inherent differences in the epithelial cells of the airways may underlie small airway susceptibility to injury. As discussed before (section 1.2.2), club cells in the small airways differ from SCGB1A1-expressing cells in the larger airways. Therefore, it is interesting to speculate as to what impact such differences in cellular composition of the airways may have on region susceptibilities to injury. This is particularly important since club cells are suggested as the primary cells responsive to chemical effects.

1.5.3. Macrophage responses to airway toxicants

Monocyte derived macrophages that are accumulated in the lung after toxicant induced injury, as well as resident alveolar and interstitial macrophages, can become activated by exposure to toxicants or mediators released by injured tissues (Bezold et al., 2019). These may be classified as inflammatory macrophages. Characterization of macrophages according to their activation state allows identification of M0 (unpolarized), pro-inflammatory M1 (classically activated) and anti-inflammatory M2 (alternatively activated) macrophages (Figure 1-6) (Bezold et al., 2019). This M1/M2 characterization has played an important part in our historical understanding of the functions of macrophages. However, it comprises a simplistic view of macrophage diversity and function. Macrophages in vivo exist as a dynamic continuum of M1 and M2 markers with overlapping functions (Laskin et al., 2019). Macrophages are highly plastic cells that rapidly change phenotype upon changes in micro-environment. However, understanding of M1/M2 characterization is relevant for in vitro macrophage model development, as activation state is likely to impact their response to toxicants.
Pro-inflammatory M1 macrophages (characterized by expression of CD86, MARCO, NOS2, MHCII) develop in response to pro-inflammatory mediators, such as LPS and interferon gamma (IFN\(\gamma\)), and release proinflammatory cytokines, reactive oxygen species (ROS), reactive nitrogen species (RNS), proteolytic enzymes, and bioactive lipids, potentially exacerbating tissue damage induced by pulmonary toxicants (Laskin et al., 2019; Stöger et al., 2012). Interestingly, several studies have shown that when the pro-inflammatory activity of M1 macrophage is blocked using anti-inflammatory steroids, lung damage induced by toxicants such as silica (DiMatteo & Reasor, 1997), hydrogen sulfide (Geng et al., 2018) and bleomycin (F. Chen et al., 2006) was reduced in rodents, suggesting an important role of M1 macrophages in toxicant induced lung injury. This is further supported by the observations that pre-treatment of rodents with M1 macrophage activators such as LPS, exacerbated lung injury induced by toxicants such as bleomycin and endotoxins (Chyczewska et al., 1993; Tasaka et al., 1995).

Anti-inflammatory M2 macrophages balance M1 activity by downregulating inflammation, supressing Th1 and M1 polarization and promoting tissue repair (Martinez et al., 2009; Stöger et al., 2012). These macrophages can be divided into four subtypes (M2a, M2b, M2c and M2d) based on \textit{in vitro} experiments, although it is unclear whether these subtypes are expressed in...
humans *in vivo* (Abdelaziz et al., 2020; Roszer, 2015). Alternative activated M2a macrophages are induced by IL4 and IL13 and express high levels of CD206, CD163, MHCII, TGM2 and IL1R2. M2a macrophages are suggested to promote wound healing and mediate fibrosis (X. Huang et al., 2018). M2b macrophages, activated by immune complexes and toll-like receptor (TLR) ligands, are the only M2 subtype that releases proinflammatory cytokines such as IL1β, IL6 and TNFα. However, this subtype secretes low amounts of IL12 indicating they do not induce a Th1 response and high amounts of IL10 indicating immunoregulatory functions. M2b are characterized by high levels of CD206, CD86 and MHCII expression as well as secretion of IL1β, IL6, TNFα, IL10 (X. Huang et al., 2018). M2c macrophages that play an important role in phagocytosis of dead cells and tissue remodelling, are activated by glucocorticoids, IL10 and TGFβ and characterized by high CD206, C1QA, CD163, MRC1, IL10 and IL1R2 expression (Ehrchen et al., 2007). M2d, activated by IL6 and adenosines express high vascular endothelial growth factor (VEGF) and IL10 levels, inducing angiogenesis as well as tumour growth (X. Huang et al., 2018; Roszer, 2015).

In pulmonary fibrosis, excess deposition of collagen and other extracellular matrix (ECM) components lead to scarring of the airways, due to imbalance in M1 and M2 macrophages after prolonged inflammation leading to dysregulated wound repair (Laskin et al., 2019). M2 macrophages are suggested to promote fibrosis through release of pro-fibrotic mediators such as TGFβ, CCL18, TNFα, IL1, IL10, IL13, IL33, fibroblast growth factor (FGF) and fibronectin, stimulating fibroblast proliferation and collagen synthesis (Pulichino et al., 2008; Wynn & Vannella, 2016). The importance of M2 macrophages in the development of pulmonary fibrosis is supported by observations that pulmonary toxicity upon exposure to fibrogenic toxicants, such as bleomycin, is exacerbated when M2 macrophage activation is increased and reduced when M2 macrophages are depleted in rodents (Luzina et al., 2013; Pilling et al., 2007).

Identification of the activation state of *in vitro* differentiated macrophages is valuable for assessing the capability of the model to detect toxicant responses appropriate for small airway
exposures. Much less is known regarding how macrophage phenotype contributes to pulmonary susceptibility to injury. This is an active area of study and shall be explored within this thesis.

1.5.4. Dendritic cells responses to airway toxicants

Dendritic cells play an important role within the airway immune system, as these cells take up, transport and present antigens to T-cells within the lymph nodes (Sullivan et al., 2017). In addition to airway resident dendritic cells present throughout the respiratory system, airway inflammation allows for recruitment of both pDC and cDC (de Heer et al., 2004). These cells have an essential immunoregulatory role in driving either tolerance or an immune response to inhaled materials (de Heer et al., 2004). Airway sensitizers, but not irritants, cause maturation and migration of dendritic cells to the lymph nodes and a consequent adaptive immune response (Rees et al., 2011). These presence of these cells within the airways therefore confers susceptibility to certain chemical inhalation exposures.

Dendritic cells are also suggested to be sensitive to environmental toxicants such as lead (D. Gao et al., 2007) and atrazine (Pinchuk et al., 2007). Observations that environmental exposure to lead results in increased immunoglobulin E (IgE) production and consequent increase in asthma in children indicate that chemical exposures might influence the adaptive immune system. Indeed, both in vivo and in vitro studies suggest that lead exposed dendritic cells promote activation of T-cells to Th2 cells inducing a type II immune response (D. Gao et al., 2007). Cigarette smoke induced oxidative stress also induces pro-inflammatory dendritic cell responses (Vassallo et al., 2008), indicating susceptibility to redox cycling compounds. Indeed, increased levels of oxidative stress have been shown to increase dendritic cell capacity to activate CD4+ and CD8+ T cells at levels that do not affect cellular proliferation or cytotoxicity (Batal et al., 2014). Furthermore, gold nanoparticles have been shown to affect dendritic cell maturation and cytokine secretion, depending on size, shape and surface chemistry (Ahmad et al., 2017), indicating dendritic cell susceptibility to nanoparticle exposures.
Both nanoparticles and redox cycling chemicals have the potential to affect dendritic cell activation and maturation state. It is important that \textit{in vitro} dendritic cell models are able to capture activation and maturation responses in order to detect small airway toxicants.
1.6. *In vitro* models of airway toxicity testing

The small airways play an important role in detoxification and inflammatory responses (Burgel et al., 2013) and are considered a target for pulmonary toxicity of small particles and chemicals (Fishler et al., 2015; Möller et al., 2008). Since these airways are morphologically different in animals, alternative models are required to evaluate toxicological responses as well as to model disease development and processes. *Ex vivo* approaches such as precision cut lung slices (PCLS) and isolated perfused lungs allow for structural and functional integrity in complex 3D structures (G. Liu et al., 2019). However, due to limited life span and availability, these models are less applicable for high throughput screening of chemicals (Lauenstein et al., 2014) and more longer term exposure scenarios. It is important that *in vitro* alternatives accurately model the function and morphology of different airway regions. As discussed previously, club cells in the small airways differ from SCGB1A1 expressing cells in the upper airways, with differential mucin and metabolic enzyme expressions (Okuda et al., 2018). As bioactivation and detoxification process play an important role in chemical toxicity, it is important that the model accurately captures these capacities of the small airways in humans (Castell et al., 2005). Furthermore, in contrast to upper airways, the small airways lack a substantial protective mucus layer *in vivo*, affecting for instance particle movement and retention properties (Okuda et al., 2018).

Several tumour or immortalized cell lines modelling airway epithelium are available. Cell lines have an extended life span and availability, require less expensive culture medium, are easy to handle and are thus applicable for high-throughput screening (Hiemstra et al., 2018). Cell lines are available for submerged and air-liquid interface (ALI) cultures. The tumour cell lines Calu-3 and NCI-H322 and immortalized bronchial epithelial cell line BEAS-2B are widely used for *in vitro* toxicology testing (Hiemstra et al., 2018). Calu-3, derived from a human lung adenocarcinoma, expresses mucins and some cilia after several weeks in ALI culture, resembling bronchial epithelium characteristics. These cells show a reasonable barrier activity and presence of tight junctions and transporter genes (Kreft et al., 2015; Stewart et al., 2012). However, Calu-3
consists of only one cell type with an abnormal karyotype (Sanchez-Guzman et al., 2021). NCI-H322 structurally resembles human bronchiolar club cells and contain some metabolic enzymes, such as glutathione S-transferase (Kiefer et al., 1988; Wiebel et al., 1986). CYP1A1 protein and mRNA expression can be induced in this cell line (Ueng et al., 2000). The adenovirus 12-simian virus 40 hybrid virus immortalized bronchial epithelial cell line BEAS-2B resembles airway basal cells that do not differentiate or form a tight barrier (Stewart et al., 2012). However, SCGB1A1 protein expression can be induced in this cell line (X. Yao et al., 1998). Human bronchial epithelial cells can be immortalized by forced expression of telomerase (hTERT) and cell cycle protein cdk4 to prevent senescence (Ramirez et al., 2004). Vaughan et al. (2006) showed that these hTERT transformed immortalized human bronchial epithelial cells maintain mucociliary differentiation capacity on ALI (Vaughan et al., 2006). A similar approach was used to generate immortalized small airway epithelial cells that are able to differentiate towards club cells (B. Gao et al., 2009). In general, deregulation of differentiation and cell cycle regulation in cell lines results in a lack of in vivo characteristics such as a combination of mucociliary differentiation, club cell differentiation, barrier formation and xenobiotic metabolism (Hiemstra et al., 2018).

Macrophages and dendritic cells form heterogenous populations of immune cells that can direct innate immune responses and integrate adaptive immune signals to regulate appropriate inflammmatory pathways to maintain tissue homeostasis. These cells play an important role in both inhaled and systemic airway toxicity and thus also important targets to assess for respiratory toxicity testing. Several human monocytic cell lines with the ability to differentiate towards macrophage and dendritic cell phenotypes exist for in vitro toxicity studies. THP-1, U937 and Mono Mac 6 human monocytic cell lines are frequently used, however, represent relatively immature cells (Åbrink et al., 1994). For instance, THP-1 cells can be differentited towards a macrophage-like phenotype after exposure to phorbol-12-myristate-13-acetate (PMA) or a dendritic-like phenotype using IL4, granulocyte-macrophage colony-stimulating factor (GM-CSF), TNFα and ionomycin (Chanput et al., 2015). THP-1 has been proven useful as a simplified model to study macrophage polarization upon food-derived compound exposures (Chanput et
In comparison to THP-1 and U937 monocytic cell lines, Mono Mac 6 expresses more mature monocyte markers and contains the ability to phagocytose and thus considered more suitable to study functional characteristics of macrophages (Ziegler-Heitbroc et al., 1988). THP-1, HL-60, MUT-Z3 are human myeloid leukemia cell lines with monocyte-like characteristics used to study dendritic cells *in vitro*. Both THP-1 and HL-60 express MHC class I molecules while lacking MHC class II molecules and can be induced to express costimulatory molecules CD80 and CD86 (Berges et al., 2005; van Helden et al., 2008). MUTZ-3 can be differentiated towards an inflammatory / mo-DC phenotype using GM-CSF and IL4 and further matured using LPS or cytokine stimulation to resemble primary activated DCs (Masterson et al., 2002). Although MUTZ-3 cells are more heterogeneous and difficult to culture than the other two cell lines, their differentiation and maturation more accurately reflects dendritic cell development (van Helden et al., 2008).

Although cell lines are useful in high-throughput *in vitro* studies, they might lack the complete set of *in vivo* characteristics necessary to model chemical and nanoparticle toxicity responses as they would occur in humans. Furthermore, the abnormal phenotype of immortalized cells, characterised by abnormal karyotypes, might reduce the validity of the model depending on the study purpose. Both primary and iPSC models may prove to be more valuable alternatives to cell lines for *in vitro* toxicity testing and will be discussed in the following sections (1.6.1 and 1.6.2).

### 1.6.1. Primary models

Primary cells are directly isolated from human tissue and typically display better organotypic phenotype and function compared to transformed and tumour cell lines (Hiemstra et al., 2018)

#### 1.6.1.1. Airway epithelium

Primary human bronchial airway epithelial cells and primary small airway epithelial cells (SAEC) can be grown in submerged cultures or on filter inserts at the ALI (Figure 1-7). Submerged cultures lack some physiological characteristics and biological relevant exposure mechanisms (Pezzulo et al., 2011). ALI cultures can differentiate towards a polarized mucociliary airway
epithelial cell layer that more closely resembles the airway epithelium \textit{in vivo}, as shown by morphology and gene expression studies (Dvorak et al., 2011). Primary bronchial epithelial cells have basal cell properties and show differentiation towards goblet, ciliated and basal cells when cultured in ALI, but lack neuroendocrine and club cell differentiation (A. S. Davis et al., 2015). Furthermore, expression of biotransformation genes, such as CYP2B6, CYP2F1, CYP4B1, CYP4X1, ALDH1A1, increased when bronchial epithelial cells were cultured on ALI in comparison to submerged cultures (Baxter et al., 2015; Boei et al., 2017). Small airway epithelial cells (SAEC) have basal cell properties and can be differentiated towards club, ciliated and goblet cells in ALI cultures (Zuo et al., 2018). SAEC club cells express several metabolic enzymes, such as GSTP1, FMO2, ALDH2, CYP2F1, CYP4B1, CYP51A1, CYP2B6, and CYP2J1 (Zuo et al., 2018). To develop \textit{in vitro} models that represent the specific airway regions, it is necessary to take into account localization of metabolic enzymes along the airways as well as regional cellular differences to optimize differentiation (Castell et al., 2005).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Air-liquid face (ALI) differentiation of primary airway epithelial cells}
\end{figure}

A major drawback of primary airway epithelial cells is their limited life span and availability, due to altered phenotypical and functional characteristics after four passages (Rayner et al., 2019). In the past, feeder cells have been used to enhance the life span of primary airway epithelial cells (de Jong et al., 1993). Life span of primary epithelial cells can also be enhanced by inhibition of Rho associated protein kinase (ROCK) in combination with TGFβ and bone morphogenetic protein (BMP) inhibition to prevent spontaneous differentiation (Mou et al., 2016). This method
may also be applied to primary airway epithelium cultures in future *in vitro* models, for instance in the development of co-cultures (Yonker et al., 2017).

Although more challenging to culture, primary airway cells show normal cell morphology and maintain markers and functions as seen *in vivo* and thus form a promising alternative to animal testing (Pan et al., 2009). Another advantage of primary airway cells is the possibility to study specific demographics or patient populations, as primary epithelial cells from patients with severe asthma and COPD maintain pathological characteristics *in vitro* when cultured on ALI (Amatngalim et al., 2017; Gras et al., 2012).

1.6.1.2. Airway macrophages and dendritic cells

Human macrophages directly isolated from tissues *in vivo* are difficult to obtain in sufficient amounts for application in toxicity testing. Furthermore, these macrophages do not proliferate and their morphology and activation state might change rapidly upon isolation and culture (Groot-Kormelink et al., 2012), limiting their applicability to *in vitro* toxicity assays. Primary alveolar macrophages can be obtained via BAL from healthy human volunteers, however cannot be expanded *in vitro* and are therefore substituted by other macrophage models for *in vitro* studies (Groot-Kormelink et al., 2012). Monocytes, isolated from blood, are more readily available, can be maintained for up to 6 weeks *in vitro* and can differentiate towards M1/M2 macrophages *in vitro* (Bennett et al., 1992; Grotenhuis et al., 2013). However, monocytes differentiated towards macrophages using macrophage colony stimulating factor (M-CSF) show a transcriptionally different macrophage phenotype compared to alveolar macrophages isolated from BAL from the same healthy donor (J. Li et al., 2007). Primary dendritic cells can be directly isolated from blood (<1 % of blood leukocytes), however, like macrophages, these are unable to proliferate *in vitro* limiting their applicability to toxicity studies (Langhoff et al., 1991). Mo-DCs can also be derived from monocytes. However, monocyte derived macrophages and dendritic cells show inflammatory phenotypes, and might not accurately represent tissue resident or non-monocyte derived immune cells with specific functions (e.g. cDC1 and cDC2) (Vogel et al., 2018).
Hematopoietic stem cells (HSC) have the potential to self-renew and differentiate towards all lineages of blood cells and are typically positive for the CD34 antigen. HSC can be isolated from peripheral blood (0.15%), cord blood or bone marrow and expanded in vitro (Herbein et al., 1994). Macrophages and dendritic cells (cDC1, cDC2, pDC, mo-DC) can be differentiated from CD34+ hematopoietic progenitor cells in vitro (Figure 1-8), following in vivo differentiation trajectories typically observed in inflammatory disease states (Caux et al., 1996; Clanchy & Hamilton, 2013; Rosenzwajg et al., 1996; J. W. Young et al., 1995).

![Hematopoietic stem cell differentiation](image)

**Figure 1-8 Hematopoietic stem cell differentiation.** Hematopoietic stem cells (HSC) give rise to both myeloid and lymphoid cell lineages. Myeloid progenitor cells can differentiate towards several cell types including myeloid DCs (myDC) and plasmacytoid DCs (pDC). Monocytes derived from myeloid progenitors can differentiate towards macrophages or dendritic cells (MDDC) upon injury. Adapted figure (Rocamonde et al., 2019).

HSC derived macrophages and dendritic cells are considered primary cell cultures. When HSC differentiation towards macrophages is guided via monocyte differentiation, HSC derived macrophages do not differ from blood monocyte derived macrophages with regards to cell surface markers or phagocytotic potential, indicating an inflammatory phenotype (Vogel et al.,...
CD34+ HSC derived dendritic cells also show an inflammatory phenotype. Factors released by airway epithelium have been shown to reduce the pro-inflammatory phenotype in dendritic cells, indicating that the use of co-cultures or the addition of growth factors could prevent the inflammatory phenotype and guide cell populations towards a differentiation state observed in normal human tissue (Gallucci et al., 1999).

1.6.2. Induced pluripotent stem cells (iPSC)

Pluripotent stem cells may offer a promising alternative in vitro method to primary airway epithelial cells and cell lines. Embryonic stem cells (ESC) can be differentiated towards lung progenitor cells (Longmire et al., 2012). Ethical issues however limit the availability of these cells from human sources. iPSC are somatic cells that are reprogrammed to gain multi-potent-like features by introducing four pluripotency associated genes, oct4, sox2, cMyc, and Klf4 (Okita et al., 2007). They lack many of the ethical issues associated with ESC use.

Several studies have described the differentiation of iPSC towards monocytes, macrophages and dendritic cells (Cao et al., 2019; Senju et al., 2011; Takata et al., 2017; Vaughan-Jackson et al., 2021; Yanagimachi et al., 2013; H. Zhang et al., 2015), including a protocol aiming to efficiently generate iPSC derived monocytes with the ability to differentiate towards macrophages, which has been applied for drug screening (Gutbier et al., 2020; van Wilgenburg et al., 2013). Furthermore CD14 sorted iPSC derived monocytes could be differentiated towards macrophage and dendritic cell populations, functionally similar to those derived from CD34+ cells (Monkley et al., 2020).

iPSC can also be differentiated through different stages of lung development using a combination of growth factors, inhibitors, and other factors to generate lung progenitor cells (S. Huang et al., 2014). Published protocols have shown feasibility for this approach to generate alveolar and airway epithelial cells in both organoids and monolayers (Dye et al., 2015; Firth et al., 2014; Ghaedi et al., 2014; Gotoh et al., 2014; Hawkins et al., 2017; Jacob et al., 2017; Konishi et al., 2016; McCauley, Hawkins, et al., 2018; A. Miller et al., 2017; Mitchell et al., 2017; Serra et al., 2018).
General introduction

al., 2017; Suzuki et al., 2016; Tamo et al., 2018; C. Wang et al., 2016; Wong et al., 2012; Yamamoto et al., 2017). Currently however, there is no protocol available for the generation of iPSC derived small airway epithelium that is directly applicable to inhalation toxicity testing (Hawkins & Kotton, 2015; Hiemstra et al., 2018).

The main advantage of iPSC is in theory an unlimited supply of donor specific cells that are generated from easily accessible somatic cells. The ability to generate such differentiated cells from a variety of genetic backgrounds is also an advantage for the study of population responses as well as specific genetic susceptibilities. However, this approach is still labour intensive, expensive and requires optimization for each cell line (Hiemstra et al., 2018). Interestingly, the work presented in this thesis is part of the Marie-Sklodowska-Curie Action – Innovative Training Network (MSCA-ITN) project called “in3”, aimed to drive the development of well-characterized iPSC models for human brain, liver, kidney, lung and vascular cells in order to delineate tissue and cell type specific effects of chemical and nanoparticle exposures, highlighting the potential of using iPSC in in vitro toxicity testing. Future development of iPSC derived small airway models could advance the development of co-cultures, as material from the same donor could be used for all cell types, creating a donor specific model (Mertens et al., 2017).

1.6.3. Toxicological assessment and interpretation

In vitro models that test the effects of toxicants on cultured cells or tissues can be used to reduce the number of animals needed for toxicity testing in early stages of drug development or risk assessment. Airway epithelial cells cultured on ALI can be exposed on the basolateral side to mimic systemic exposures or on the apical side to mimic inhalation exposures. Macrophages and dendritic cells are commonly exposed by adding the toxicant directly to the culture medium, however, co-cultures with airway epithelium cells would allow to model more physiologically relevant exposures. Depending on the type of toxicants, humans are exposed acutely or over a prolonged period (e.g., environmental pollutants), which can be reproduced in in vitro cultures by either acute exposure, long term exposures or repeated dose exposures.
1.6.3.1. Cytotoxicity

Cytotoxicity assays are used to identify the ability of a compound to induce cell death, because of dysregulation of cellular functions. These assays are a first step for these type of studies but are also necessary to identify the concentration range to be used for more detailed mechanistic toxicological studies. Moreover, cytotoxicity assays can be used to quantitatively compare responses of compound e.g., by identify the dose at which 50% of the cells are damaged (EC50).

Furthermore, some correlation was found between 68 in vitro cytotoxicity responses and acute toxicity in humans (Clemedson et al., 1996), indicating they may be applicable for human risk assessment.

Several well characterized methods are available to analyse cytotoxicity upon compound exposure, including assessments of mitochondrial function, cellular morphology, cell proliferation and permeability of a cellular monolayer. Cellular lysis as a form of uncontrolled cell death can be measured by the release of intracellular enzymes (such as lactate dehydrogenase (LDH)) into the supernatant or dye exclusion (e.g., Trypan blue) (H. Kim et al., 2009) and is typically reflective of necrotic processes. Lactate is produced by glycolysis and the detection of L-lactate in culture medium can be used to measure a switch in mitochondrial metabolism, which often precedes cell death processes (Limonciel et al., 2011). Resazurin (AlmarBlue®), MTT, MTS and WST assays are colorimetric proliferation assays commonly used to measure cellular reductase activity mainly within the mitochondria and are often used as a surrogate for cellular viability assessment (Jennings et al., 2007). Furthermore, quantification of intracellular adenosine triphosphate (ATP) by luciferin-luciferase reactions can be used to estimate cellular viability (Cree & Andreotti, 1997). Within this thesis, cytotoxicity assays will focus on cellular permeability (LDH) and viability (Resazurin). These will be further discussed in the methods section (chapter 2).
1.6.3.2. **Cellular function and mechanistic toxicology assessment**

Effects of toxicants are commonly evaluated by their impact on gene expression and protein profile changes as a first line approach to understanding mechanisms of toxicity. They also offer a more sensitive assessment of cellular perturbations, that occur before overt toxicity manifests. Transcriptomics (mRNA expression patterns) and proteomics are mainly used to identify differences in gene expression and translation pattern upon toxicant exposures. Although inflammatory responses can be up- or downregulated by post-transcriptional regulation by microRNAs, they still follow upregulation of inflammatory gene expression (Mann et al., 2017).

Enzyme-linked immunosorbent assays (ELISA), western blot, antibody arrays and mass spectrometry (MS) methods are widely used to measure and detect specific proteins and cytokines and have applications for e.g., the analysis of up- and down regulation of pro-inflammatory cytokines upon compound exposure. However, in general, proteomics assays are more complex and thus prone to error compared to gene expression. Furthermore, antigen binding affinity differs between proteins, limiting reliability of high throughput antibody arrays for the simultaneous detection of a large number of proteins (Heijne et al., 2005).

Establishing the changes in mRNA levels upon compound exposure can predict changes in protein levels and cellular activity. mRNA expression patterns can be analysed using reverse transcriptase followed by real time polymerase chain reaction (RT-PCR) or northern blotting techniques. These methods allow the quantification of low levels of individual mRNAs (Bustin, 2000), however limit the number of genes that can be analysed at the same time. Microarrays can be used to analyse a larger number of gene profiles with a known sequence and have been shown to relate to mechanism of toxicity (Waring et al., 2001). However, mRNA-Sequencing shows better specificity compared to microarrays, can detect novel transcripts and is more sensitive in detecting differential expression of low expressed genes upon compound exposure (Sirbu et al., 2012). Targeted mRNA-Sequencing allows the analysis of specific transcripts of interest in a large number of samples and requires low input amounts. TempO-Seq™ technology
can be used to identify differential expression of genes with particular interest to toxicology and has shown similar outcomes compared to RNA-Sequencing or Microarray methods (Bushel et al., 2018).

Within this thesis, functional changes upon compound exposure are explored using mRNA expression differences as measured by RT-PCR, TempO-Seq or (single cell) mRNA-Sequencing and these methods will be further discussed in the methods section (chapter 2).

1.6.3.3. Applications of in vitro models

In vitro outcomes need to be converted to in vivo dose-response relationships in order to be relevant for human risk assessment. PBK models can be used to link in vitro and in vivo data to predict the behaviour of compounds in humans, based on absorption, distribution, metabolism and excretion (ADME) characteristics (Louisse et al., 2017). Furthermore, the use of (quantitative) adverse outcome pathways (AOPs) to describe toxicological events could help develop models for in vitro toxicity testing for regulatory purposes, as it identified endpoints required to link a molecular initiation event (MIE) via key events (KEs) to an adverse outcome (AO) (Spinu et al., 2020). AOPS have been applied to regulatory decision making for several acute toxicity endpoints such as skin irritation, skin corrosion and skin sensitization (Tollefsen et al., 2014), however future work is needed to increase the confidence in AOPs for other endpoints.
1.7. Aims and outline of the thesis

Toxicological safety assessment is essential to ensure safe exposure levels of chemicals and nanomaterials. Animal models are generally used as gold standard for legislation, however, species differences and 3R considerations demonstrate the need for *in vitro* alternatives (Kolanjiyil et al., 2019; Krewski et al., 2020). Although region specific susceptibility to chemical and nanoparticle exposures has been described within the human airways, currently used *in vitro* airway models generally lack regional specific differentiation (Luengen et al., 2020). The human small airways are especially susceptible to several chemical and nanoparticle exposures. Therefore, *in vitro* models of the small airways, including airway epithelial cell, macrophage, and dendritic cell models, will be explored within this thesis. Primary cells can be used as *in vitro* models, as these cells maintain normal cell morphology and functions (Pan et al., 2009). However, their limited life span and availability limits their use in larger scale toxicity assessments (Lauenstein et al., 2014). iPSC derived models could be an interesting alternative to primary cell cultures, as these cells form an in theory unlimited supply derived from easily accessible somatic cells (Okita et al., 2007). Ultimately, evaluation of chemical responses within the *in vitro* models will aid in determining both the cellular specificity of these compounds as well as the applicability of the *in vitro* small airway models to tissue or cell type specific toxicity.

The aims of this thesis (Figure 1-9), presented in 7 chapters are:

1) to establish and refine models for *in vitro* airway toxicity testing,

2) to determine cellular specificity and toxicity of airway toxicants,

3) to investigate mechanisms of action of airway toxicants.

These aims are structured to address the central hypothesis:

- “*in vitro* primary and iPSC cultures of small airway epithelial and immune cells can be optimised and applied to regional specific small airway toxicity testing.”
Figure 1-9 Schematic overview of thesis chapters
CHAPTER 2: MATERIALS AND METHODS
2.1. Cell culture

2.1.1. Reagents

Table 2-1 Reagents and tissue culture plates used for cell culture protocols

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
<th>Catalogue #</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05% Trypsin/0.53 mM EDTA</td>
<td>Sigma</td>
<td>T3924</td>
</tr>
<tr>
<td>A83-01</td>
<td>Sigma</td>
<td>SML0788</td>
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<tr>
<td>Accutase</td>
<td>Sigma</td>
<td>A6964</td>
</tr>
<tr>
<td>Activin A</td>
<td>R&amp;D Systems</td>
<td>338-AC</td>
</tr>
<tr>
<td>All trans retinoic acid (ATRA)</td>
<td>Sigma</td>
<td>R2625</td>
</tr>
<tr>
<td>B27</td>
<td>ThermoFisher</td>
<td>17504-044</td>
</tr>
<tr>
<td>B27 w/o retinoic acid</td>
<td>ThermoFisher</td>
<td>12587001</td>
</tr>
<tr>
<td>2-mercaptoethanol (BME)</td>
<td>ThermoFisher</td>
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</tr>
<tr>
<td>BMP4</td>
<td>R&amp;D Systems</td>
<td>314-BP</td>
</tr>
<tr>
<td>BMS 453</td>
<td>Sigma</td>
<td>SML1149</td>
</tr>
<tr>
<td>Bovine Brain Extract (BBE)</td>
<td>Lonza</td>
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<td>BSA</td>
<td>ThermoFisher</td>
<td>15260-037</td>
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<td>Calcitriol</td>
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<tr>
<td>cAMP</td>
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<td>CHIR66021</td>
<td>Torcis</td>
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<td>Sigma</td>
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<td>ThermoFisher</td>
<td>41965039</td>
</tr>
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<td>DMEM/F12</td>
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<tr>
<td>DMSO</td>
<td>Sigma</td>
<td>D2650</td>
</tr>
<tr>
<td>Dorsomorphin</td>
<td>Torcis</td>
<td>3093</td>
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<td>EDTA (0.02% Versene)</td>
<td>ThermoFisher</td>
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</tr>
<tr>
<td>EGF</td>
<td>R&amp;D Systems</td>
<td>2028-EG</td>
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<tr>
<td>EGF inhibitor</td>
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</tr>
<tr>
<td>Endo-IWR-1</td>
<td>Tocris</td>
<td>3533</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>Sigma</td>
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</tr>
<tr>
<td>Ethanolamine</td>
<td>Sigma</td>
<td>E9508</td>
</tr>
<tr>
<td>F12</td>
<td>Sigma</td>
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<tr>
<td>FBS (hESC-quality)</td>
<td>ThermoFisher</td>
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<td>Fetal Clone II</td>
<td>HyClone</td>
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<td>FGF10</td>
<td>R&amp;D Systems</td>
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<td>Fibronectin</td>
<td>Sigma</td>
<td>F0895</td>
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<td>Flt-3</td>
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<td>GM-CSF</td>
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<td>Human serum replacement 3</td>
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<td>S2640</td>
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<td>Hydrocortisone</td>
<td>Sigma</td>
<td>H0888</td>
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<tr>
<td>IBMX</td>
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<tr>
<td>IL13</td>
<td>R&amp;D Systems</td>
<td>213-ILB</td>
</tr>
<tr>
<td>IL3</td>
<td>R&amp;D Systems</td>
<td>203-IL-010</td>
</tr>
<tr>
<td>IL4</td>
<td>R&amp;D Systems</td>
<td>204-IL</td>
</tr>
<tr>
<td>IL6</td>
<td>R&amp;D Systems</td>
<td>7270-IL</td>
</tr>
<tr>
<td>IMDM</td>
<td>ThermoFisher</td>
<td>42200014</td>
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<tr>
<td>Insulin</td>
<td>Sigma</td>
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<tr>
<td>Isoproterenol</td>
<td>Sigma</td>
<td>I5627</td>
</tr>
<tr>
<td>ITS</td>
<td>Sigma</td>
<td>I3146</td>
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<td>IWP2</td>
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<td>3533</td>
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<td>KGF</td>
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<td>Sigma</td>
<td>L2020</td>
</tr>
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<td>L-ascorbic acid</td>
<td>Sigma</td>
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<td>LHC basal</td>
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<td>MCDB-201</td>
<td>Sigma</td>
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<td>M-CSF</td>
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<td>216-MCC</td>
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<td>mTeSR</td>
<td>StemCell</td>
<td>85850</td>
</tr>
<tr>
<td>MTG</td>
<td>Sigma</td>
<td>M6145</td>
</tr>
<tr>
<td>N2</td>
<td>ThermoFisher</td>
<td>17502-048</td>
</tr>
<tr>
<td>Non-essential amino acids</td>
<td>ThermoFisher</td>
<td>11140050</td>
</tr>
<tr>
<td>PBS wo Ca2+ and Mg2+</td>
<td>Gibco</td>
<td>14190-144</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>ThermoFisher</td>
<td>15140122</td>
</tr>
<tr>
<td>Phosphoryl ethanolamine</td>
<td>Sigma</td>
<td>P0503</td>
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<tr>
<td>Rock inhibitor (Y-27632)</td>
<td>Abcam</td>
<td>Ab120-129</td>
</tr>
<tr>
<td>RPMI</td>
<td>ThermoFisher</td>
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<td>SB431542</td>
<td>Tocris</td>
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<td>SCF</td>
<td>R&amp;D Systems</td>
<td>7466-SC</td>
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</tr>
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<td>Stemspan</td>
<td>StemCell</td>
<td>9600</td>
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<tr>
<td>T3</td>
<td>Sigma</td>
<td>T5516</td>
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<td>TGF-b</td>
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<td>TPO</td>
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<td>Transferrin</td>
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<td>Ultrorser G</td>
<td>Pall</td>
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<td>VEGF</td>
<td>R&amp;D Systems</td>
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<td>Wnt3a</td>
<td>R&amp;D Systems</td>
<td>5036-GMP</td>
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<tr>
<td>XAV939</td>
<td>Sigma</td>
<td>X3004</td>
</tr>
<tr>
<td>X-VIVO 15</td>
<td>Lonza</td>
<td>LZE02</td>
</tr>
<tr>
<td><strong>Tissue culture plate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 well – TCT</td>
<td>Corning</td>
<td>3516</td>
</tr>
<tr>
<td>12 well – TransWell</td>
<td>Corning</td>
<td>9460</td>
</tr>
<tr>
<td>24 well – low attachment</td>
<td>Corning</td>
<td>3473</td>
</tr>
<tr>
<td>24 well – TCT</td>
<td>Corning</td>
<td>3527</td>
</tr>
<tr>
<td>24 well – non-TCT</td>
<td>Falcon</td>
<td>351147</td>
</tr>
<tr>
<td>24 well – TransWell</td>
<td>Corning</td>
<td>3470</td>
</tr>
<tr>
<td>48 well – TCT</td>
<td>Corning</td>
<td>3524</td>
</tr>
<tr>
<td>48 well – non-TCT</td>
<td>Falcon</td>
<td>351178</td>
</tr>
<tr>
<td>96 well – round bottom low attachment</td>
<td>Corning</td>
<td>CLS7007-24EA</td>
</tr>
<tr>
<td>96 well – TCT</td>
<td>Corning</td>
<td>3598</td>
</tr>
</tbody>
</table>

2.1.2. iPSC maintenance

Induced pluripotent stem cells (iPSC; StemBANCC, SBAD2 and SBAD3) were obtained from NewCells Biotech (Newcastle, UK), under conditions of use outlined in the in3 MSCA-ITN project licence agreement. iPSC were cultured as previously described (Wilmes et al., 2017). Cells were stored in cryovials (ThermoFisher) in liquid nitrogen before being thawed in a warm water bath at 37 °C. Once only a small ice crystal remained, cells were transferred to 4 mL of warm mTeSR medium (StemCell), as prepared according to the manufacturer’s instructions, in 15 mL Falcon tube (Greiner) and centrifuged for 5 minutes at 300 x g in a centrifuge (Sorvall Legend RT,
TermoFisher) at room temperature. The supernatant was removed, and the cell pellet was resuspended in fresh mTeSR medium supplemented with 10 μM rock inhibitor (Ri) and 100 U/mL Pen/Strep and ±100,000 cells were transferred to 6 well plates (Greiner Bio-One CellStar) pre-coated with Matrigel. Matrigel coating was carried out at a concentration of ~ 18 μg/cm² Matrigel in 1 mL DMEM/F12 per well for 1 hour at 37 °C, 5% CO₂ in a humidified atmosphere. mTeSR medium was changed daily and cells were passaged at a 1:3 ratio every 3-4 days when 80% confluency was reached using EDTA/versene. Cell culture medium was aspirated and cells washed with phosphate buffered saline (PBS) without calcium and magnesium before EDTA/versene was added. Cells were incubated for 5 minutes at 37 °C or until they detached from each other but not the plastic. EDTA/versene was aspirated and attached cells were washed of the plastic using 3 x 1 mL of cold mTeSR medium per well and passaged to freshly Matrigel coated wells or frozen. Before freezing, cryovials were labelled with cell line name, passage number and date and chilled at -20 °C. Cells from all wells were pooled and mixed with ice-cold freezing medium (10% DMSO/90% FBS (hESC quality)) and aliquoted 1 mL per cryovial before being transferred to CoolCell™ freezing container (Corning) and placed at -80°C overnight before being transferred to liquid nitrogen storage.

2.1.3. iPSC differentiation towards small airway epithelium

2.1.3.1. 2D endoderm differentiation (iPSC airway protocol 1)

iPSC differentiation follows biological developmental stages, therefore differentiation was guided through definite endoderm (DE) and anterior foregut endoderm (AFE) to achieve airway differentiation. A high purity of DE (96%), was achieved using a 2-dimensional (2D) differentiation protocol (Mitchell et al., 2017; Ninomiya et al., 2015). We tested this protocol, further referred to as iPSC airway protocol 1. Briefly, on day -1, iPSC were seeded at 4*10⁵ cells/cm², 400 μl per well on Matrigel pre-coated 24-well or 6-well plates in mTeSR medium supplemented by 5 μM Ri. Cells were grown at 37 °C, 5% CO₂, 99% humidity at atmospheric O₂. iPSC were differentiated towards DE and AFE by refreshing differentiation medium daily, with
cell culture medium formulations recapitulating sequential signalling events of early development (Table 2-2). Concentrations displayed in this table are the final concentrations within the differentiation media.

### Table 2-2 Differentiation media at different stages of iPSC airway Protocol 1 (Mitchell et al., 2017).

<table>
<thead>
<tr>
<th>Medium</th>
<th>1A</th>
<th>1B</th>
<th>1C</th>
<th>1D</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Days</strong></td>
<td>0 – 1</td>
<td>1 - 3</td>
<td>3 - 4</td>
<td>4 - 5</td>
</tr>
<tr>
<td><strong>Base medium</strong></td>
<td>DMEM</td>
<td>DMEM</td>
<td>DMEM</td>
<td>DMEM</td>
</tr>
<tr>
<td>B27 W/O retinoic acid</td>
<td>2%</td>
<td>2%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Human serum replacement</td>
<td>2%</td>
<td>2%</td>
<td>2%</td>
<td>2%</td>
</tr>
<tr>
<td>Non-essential amino acids</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
</tr>
<tr>
<td>Glutamax</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
</tr>
<tr>
<td>BSA</td>
<td>0.1%</td>
<td>0.1%</td>
<td>0.1%</td>
<td>0.1%</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>100 U/ml</td>
<td>100 U/ml</td>
<td>100 U/ml</td>
<td>100 U/ml</td>
</tr>
<tr>
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<td>5 µM</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LY294002</td>
<td>2.5 µM</td>
<td>2.5 µM</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Activin A</td>
<td>10 ng/mL</td>
<td>10 ng/mL</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Dorsomorphin</td>
<td>-</td>
<td>-</td>
<td>1 µM</td>
<td>-</td>
</tr>
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<td>SB4315420</td>
<td>-</td>
<td>-</td>
<td>2 µM</td>
<td>2 µM</td>
</tr>
<tr>
<td>Endo-IWR1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>200 nM</td>
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</table>

#### 2.1.3.2. 3D endoderm differentiation (iPSC airway protocol 2)

In addition to the 2D differentiation protocol, endoderm differentiation can also be achieved through 3D differentiation, as previously described in detail (S. Huang et al., 2015). This protocol will be further referred to as iPSC airway protocol 2. iPSC cultured in 6 well plates, were washed twice with 2 mL of PBS without calcium and magnesium and once with 1 mL of EDTA/versene per well. Thereafter, 1 mL of EDTA/versene was added and cells were incubated at 37 °C for 5-6 minutes. Once cells detached from each other but not from the plastic, EDTA/Versene was aspirated and the loosened cells were washed off with 3 times 1 mL of mTeSR medium using a p1000 pipette. Collected cells were pooled in a 15 mL Falcon tube and 9 mL of D0-D1 medium was added for a
total of 12 mL. Cells were transferred to low attachment 6-well plates (Costar), 2 mL per well, to allow for embryonic body (EB) formation (3D step). After 24 hours, EBs were collected in a 15 mL tube. 1 mL of medium 2B (Table 2.3) was added to the low attachment plates while the EBs were allowed to settle for 15 minutes at room temperature. The supernatant was aspirated and EBs resuspended in 12 mL of medium 2B per 6-well plate. 2mL was added back to the low-attachment 6 well plates per well for a total volume of 3 mL before returning to 37 °C, 5% CO2 and atmospheric O2. On day 3, half of the medium 2B was removed and 1.5 mL of fresh medium 2B was added. On day 5, EBs were collected in a 15 mL Falcon tube, medium was aspirated and 10 mL of warm 0.05% Trypsin was added per 6 well plate for ±3 minutes while flicking the tube gently every 15 seconds. After, the tube was flicked with a continuous medium force for 5-10 seconds. Roughly half of the EBs became dissociated while the supernatant turned cloudy. The remaining EBs were allowed to settle for 20 seconds before 8 mL of the cloudy supernatant was transferred to a 50 mL tube containing 1:1 IMDM/FBS. The tube containing the remaining EBs was flicked again with medium force until they were dissociated and 1:1 IMDM/FBS was added. An excess of IMDM medium was added and cells were pelleted at 300g for 5 minutes. Supernatant was aspirated and cells were resuspended in medium 2C (Table 2.3) and plated at a density of 5-7.5*10^4 cells/cm² in 0.2% fibronectin pre-coated 24 well plates and incubated at 37 °C, 5% CO2 and atmospheric O2. On day 6, medium 2C was removed and medium 2D was added. On day 7, medium 2D was removed and medium 2E was added, medium was refreshed every other day until day 15.
**Table 2-3** Differentiation media at different stages of iPSC airway protocol 2 (S. Huang et al., 2015)

<table>
<thead>
<tr>
<th>Medium</th>
<th>2A (0-1)</th>
<th>2B (1-5)</th>
<th>2C (5-6)</th>
<th>2D (6-7)</th>
<th>2E (7-15)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Base medium (1:1)</strong></td>
<td>IMDM/F12</td>
<td>IMDM/F12</td>
<td>IMDM/F12</td>
<td>IMDM/F12</td>
<td>IMDM/F12</td>
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<tr>
<td>Glutamax</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
</tr>
<tr>
<td>BSA</td>
<td>7.5%</td>
<td>7.5%</td>
<td>7.5%</td>
<td>7.5%</td>
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<td>N2</td>
<td>0.5%</td>
<td>0.5%</td>
<td>0.5%</td>
<td>0.5%</td>
<td>0.5%</td>
</tr>
<tr>
<td>B27</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>100 U/ml</td>
<td>100 U/ml</td>
<td>100 U/ml</td>
<td>100 U/ml</td>
<td>100 U/ml</td>
</tr>
<tr>
<td>L-Ascorbic acid</td>
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<td>50 µg/ml</td>
<td>50 µg/ml</td>
<td>50 µg/ml</td>
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<tr>
<td>MTG</td>
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<td>0.04 µg/mL</td>
<td>0.04 µg/mL</td>
<td>0.04 µg/mL</td>
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<td>Rock inhibitor</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Wnt3a</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>BMP4</td>
<td>10 ng/ml</td>
<td>0.5 ng/ml</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FGF2</td>
<td>-</td>
<td>2.5 ng/ml</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Activin A</td>
<td>-</td>
<td>-</td>
<td>2 µM</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SB431542</td>
<td>-</td>
<td>-</td>
<td>10 µM</td>
<td>10 µM</td>
<td>-</td>
</tr>
<tr>
<td>IWP2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1 µM</td>
<td>-</td>
</tr>
<tr>
<td>CHIR99021</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3 µM</td>
<td>-</td>
</tr>
<tr>
<td>KGF</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10 ng/ml</td>
</tr>
<tr>
<td>FGF10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10 ng/ml</td>
</tr>
<tr>
<td>BMP4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10 ng/ml</td>
</tr>
<tr>
<td>EGF</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20 ng/ml</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>50 nM</td>
</tr>
</tbody>
</table>

**2.1.3.3. Basal cell and ALI differentiation (iPSC airway protocol 3)**

Another 2D endoderm differentiation protocol and subsequent airway epithelial cell (AEC) differentiation will be further referred to as iPSC airway protocol 3. iPSC were seeded in Matrigel pre-coated 12 well plates (Split ratio of 1 well of 6 well plate into 6 wells of 12 well plate) in mTeSR medium on day -1 and incubated at 37 °C, 5% CO₂ and atmospheric O₂. On day 0, mTeSR medium was removed and medium 3A was added (Table 2-4). On days 1, 3, 4 and 6 cells were washed with PBS and medium was changed in accordance with Table 2-4. Medium 3E was refreshed every other day until day 15. On day 15, cells were digested with warm 0.05% Trypsin for 1 minute in order to remove any possible neuronal tissue growth (S. Huang et al., 2015). Trypsin was removed, medium 3E was added and cells were mechanically detached using 100 µL barrier tips and transferred to 15 mL Falcon tube. The suspension was pipetted up and down
with medium force before clumps of cells were allowed to settle for a few minutes. The cloudy supernatant was removed, and cell clumps were resuspended in medium 3F. Cells were passaged to Matrigel pre-coated 12 well plates in 1:3 ratio and medium was refreshed every other day until day 25. On day 25, confluent wells were passaged as single cells using 0.05% Trypsin and medium 3G on Collagen IV coated 12 well plates. These cells could be maintained to, at least, day 40 and survive cell freezing and storage. Cells were frozen in 9:1 FBS/DMSO using a CoolCell™ freezing container.

**Table 2-4 Differentiation media at different stages of iPSC airway protocol 3**

<table>
<thead>
<tr>
<th>Medium</th>
<th>3A</th>
<th>3B</th>
<th>3C</th>
<th>3D</th>
<th>3E</th>
<th>3F</th>
<th>3G</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Days</strong></td>
<td>0-1</td>
<td>1-3</td>
<td>3-4</td>
<td>4-6</td>
<td>6-15</td>
<td>15-25</td>
<td>25+</td>
</tr>
<tr>
<td><strong>Base medium</strong></td>
<td>DMEM/ MCDB</td>
<td>DMEM/ MCDB</td>
<td>DMEM/ MCDB</td>
<td>DMEM/ MCDB</td>
<td>IMDM/F12</td>
<td>IMDM/F12</td>
<td>KSFM</td>
</tr>
<tr>
<td>LA-BSA</td>
<td>0.25x</td>
<td>0.25x</td>
<td>0.25x</td>
<td>0.25x</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ITS</td>
<td>0.25x</td>
<td>0.25x</td>
<td>0.25x</td>
<td>0.25x</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>100 U/ml</td>
<td>100 U/ml</td>
<td>100 U/ml</td>
<td>100 U/ml</td>
<td>100 U/ml</td>
<td>100 U/ml</td>
<td>100 U/ml</td>
</tr>
<tr>
<td>L-Ascorbic acid</td>
<td>100 nM</td>
<td>100 nM</td>
<td>100 nM</td>
<td>100 nM</td>
<td>50 µg/mL</td>
<td>50 µg/mL</td>
<td>-</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>1 µM</td>
<td>1 µM</td>
<td>1 µM</td>
<td>1 µM</td>
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<td>-</td>
<td>-</td>
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<td>50 µM</td>
<td>50 µM</td>
<td>50 µM</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Wnt3a</td>
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<td>5 µg/mL</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Activin A</td>
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<td>100 ng/mL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SB431542</td>
<td>-</td>
<td>-</td>
<td>1 µM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IWP2</td>
<td>-</td>
<td>-</td>
<td>5 µM</td>
<td>5 µM</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BSA</td>
<td>-</td>
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<td>-</td>
<td>0.05%</td>
<td>0.05%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glutamax</td>
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<td>-</td>
<td>-</td>
<td>1%</td>
<td>1%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.5%</td>
<td>0.5%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B27</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1%</td>
<td>1%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MTG</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.04 µg/mL</td>
<td>0.04 µg/mL</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CHIR99021</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3 µM</td>
<td>3 µM</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KGF</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10 ng/ml</td>
<td>10 ng/ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BMP10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10 ng/ml</td>
<td>10 ng/ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BMP4</td>
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<tr>
<td>EGF</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20 ng/ml</td>
<td>-</td>
<td>0.5 ng/mL</td>
<td>-</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>50 nM</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Human Albumin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>25 µg/mL</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A83-01</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1 µM</td>
<td>-</td>
</tr>
<tr>
<td>Y-27632 (RI)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5 µM</td>
<td>-</td>
</tr>
<tr>
<td>BMS 453</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>50 nM</td>
<td>-</td>
</tr>
<tr>
<td>BPE</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>30 µg/mL</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3 µM</td>
<td>-</td>
</tr>
</tbody>
</table>
To further differentiate cells, after day 25, cells were passaged onto 5 μg/ml fibronectin, 5 μg/ml laminin and 60 μg/ml collagen IV coated 24 well TransWell inserts (Corning) in medium 3G using 0.05% Trypsin. Once wells reached confluency, apical medium was removed and basolateral medium was changed to PneumaCult™-ALI differentiation medium, prepared according to manufacturer’s instructions. Alternatively, cells were transferred a differentiation medium containing Dexamethasone, cAMP and IBMX (DCI) compounds, based on Firth et al. (2014) (Table 2-5). Basolateral medium was refreshed every other day and cells were washed weekly on the apical side using 30 μL of respective medium.

Table 2-5 DCI differentiation medium (Firth et al., 2014)

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM/F12</td>
<td>1x</td>
</tr>
<tr>
<td>Ultroser G</td>
<td>2%</td>
</tr>
<tr>
<td>Fetal Clone II</td>
<td>2%</td>
</tr>
<tr>
<td>Insulin</td>
<td>2.5 μg/ml</td>
</tr>
<tr>
<td>Bovine Brain Extract</td>
<td>22.5 μg/ml</td>
</tr>
<tr>
<td>Transferrin</td>
<td>2.5 μg/ml</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>20 nM</td>
</tr>
<tr>
<td>T3</td>
<td>500 nM</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>1.5 μM</td>
</tr>
<tr>
<td>Retionic acid</td>
<td>10 nM</td>
</tr>
<tr>
<td>Phosphoryletanolamine</td>
<td>250 nM</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>250 nM</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>100x</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>25 ng/mL</td>
</tr>
<tr>
<td>cAMP</td>
<td>0.1 MM</td>
</tr>
<tr>
<td>IBMX</td>
<td>0.1 mM</td>
</tr>
</tbody>
</table>

2.1.3.4. iPSC differentiation towards macrophages and dendritic cells

In addition to airway epithelium, a protocol has previously been described to efficiently obtain monocytes from iPSC (van Wilgenburg et al., 2013). These monocytes could then be further differentiated towards iPSC derived macrophage like (MC\textsuperscript{iPSC}) and dendritic like cells (DC\textsuperscript{iPSC}). iPSC grown on Matrigel coated 6 well plates were removed with accutase for 5 minutes at 37 °C as described in section 2.1.3., and plated at a density of 1*10\textsuperscript{5} cells / mL in 100 μL per well of a 96
well U-bottom ultra-low adherence plates (Corning) in mTeSR medium supplemented with 50 ng/mL BMP4, 20 ng/mL SCF, 50 ng/mL VEGF and 10 µM Rock inhibitor, in order to form EBs. 75% of the medium was refreshed, without rock inhibitor supplement, daily for 3 days. On day 5, EBs were transferred to a 6 well plate in X-VIVO 15 medium supplemented with 100 ng/mL M-CSF, 25 ng/mL IL3, 2 mM Glutamax, 100 U/mL Pen/Strep, and 0.055 mM β-mercaptoethanol (BME). Medium was then refreshed weekly and from week 3 onwards, monocytes were collected weekly from the EB cultures using a 40 µm reversible cell strainer (StemCell, cat # 27250). Monocytes were further differentiated towards DC\textsuperscript{iPSC} and MC\textsuperscript{iSPC} in tissue culture treated (TCT) and non-TCT 48 well plates, respectively in RPMI medium supplemented with 10% low IgG FBS, 2 mM glutamax, pen/strep and also containing MC and DC supplements as outlined in Table 2-6. Medium was refreshed after 3 days and cells treated and/or harvested after 7 days.

Table 2-6 iPSC macrophage and dendritic cell differentiation medium supplements

<table>
<thead>
<tr>
<th></th>
<th>MC</th>
<th>DC</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>50 ng/ml</td>
<td>20 ng/ml</td>
</tr>
<tr>
<td>IL-6</td>
<td>10 ng/ml</td>
<td>10 ng/ml</td>
</tr>
<tr>
<td>M-CSF</td>
<td>50 ng/ml</td>
<td>-</td>
</tr>
<tr>
<td>TGFb1</td>
<td>2 ng/ml</td>
<td>-</td>
</tr>
<tr>
<td>FLT3 Ligand</td>
<td>-</td>
<td>100 ng/ml</td>
</tr>
<tr>
<td>SCF</td>
<td>-</td>
<td>20 ng/ml</td>
</tr>
<tr>
<td>IL4</td>
<td>-</td>
<td>20 ng/ml</td>
</tr>
</tbody>
</table>

2.1.4. Primary SAEC culture and differentiation

Primary SAEC isolated from biopsies from the distal lung from 6 different donors without reported pathologies were obtained from Epithelix in a cryogenic vial containing more than 1 million viable cells in a volume of 1 mL of freezing medium. These SAEC were used throughout experiments.

2.1.4.1. Thawing, maintaining and freezing cells

Cells were stored in liquid nitrogen, thawed at 37 °C in a warm water bath and transferred to 35 mL of 9:1 DMEM/FBS (low IgG) and centrifuged at 2000 rpm for 7 minutes. Supernatant was aspirated and cells resuspended in 15 mL KSFM medium supplemented by 0.5 ng/mL EGF, 30
µg/mL BPE, 25 µg/mL rHuman Albumin, 1 µM A83-01, 5 µM Ri, 3 µM isoproterenol and 3 µM BMS 453. The cells were then plated onto a collagen IV coated 75 cm² flasks (Greiner) at incubated at 37 °C under a 5% CO₂ humidified atmosphere. Medium was refreshed every 2-3 days until 80% confluency was reached. Cells were washed with 15 mL warm PBS without calcium and magnesium before adding 2.5 mL of 0.25% Trypsin/EDTA and incubated at 37 °C until most of the cells detached. Cells were collected in a 50 mL tube containing 35 mL of 9:1 DMEM/FBS and centrifuged at 2000 rpm for 7 minutes. Supernatant was aspirated and cells were re-suspended in 1 mL of the supplemented KSFM culture medium using a 1 mL pipette or, to freeze cells, 1 mL of 9:1 FBS/DMSO in cryovials. 14 mL of supplemented KSFM culture medium was added to tube containing 1 mL of cell suspension in culture medium and transferred to collagen IV coated 75 cm² flasks (split ratio of 1:3).

For cytotoxicity curves, cells were seeded in collagen IV coated 48 well plates and differentiated for 7 days using DCI differentiation medium (Table 2-5) with cell culture medium replaced daily.

### 2.1.4.2. Differentiating cells on air-liquid interface (ALI)

To achieve a mature differentiated airway epithelium, cells between passage 2-4 were passaged to 5 µg/ml laminin, 5 µg/ml fibronectin, 60 µg/ml collagen IV coated 12 well Transwell inserts (Corning) using 0.25% Trypsin/EDTA and supplemented KSFM medium. Once confluency was reached after ± 4 days, apical medium was removed and basolateral medium was changed to differentiation media described in Table 2.7, PneumaCult™-ALI medium prepared according to manufacturer’s instructions or DCI differentiation medium as described in Table 2-5. Differentiation medium was changed every 2-3 days and cells were treated and/or harvested at 21 days post differentiation.
Table 2-7 Primary airway differentiation media

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM/LHC basal (50:50)</td>
<td>1x</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>rHuman Albumin</td>
<td>25 µg/ml</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>EGF</td>
<td>0.5 ng/ml</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>P/S</td>
<td>1X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>BPE</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Transferrin</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
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<td>X</td>
</tr>
<tr>
<td>T3</td>
<td>10 nM</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
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<td>X</td>
<td>X</td>
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<td>X</td>
<td>X</td>
<td>X</td>
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<td>X</td>
<td>X</td>
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</tr>
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<td>X</td>
<td>X</td>
<td>X</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>EGF Inhibitor</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>IL-13</td>
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<tr>
<td>Ascorbic acid</td>
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<td>X</td>
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<td>X</td>
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</tr>
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<td>X</td>
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<td>X</td>
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</tr>
<tr>
<td>8-bromo-cAMP</td>
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<td>X</td>
<td>X</td>
<td>X</td>
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</tr>
<tr>
<td>IBMX</td>
<td>100 µM</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>RA inhibitor</td>
<td>50 nM</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
<td>High Calcium</td>
<td>1 mM</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

2.1.4.3. Transepithelial Electrical Resistance

The transepithelial electrical resistance (TEER) was measured to establish the airway epithelial monolayer confluence using the EVOM2 Epithelial Voltohmmeter (World Precision Instruments). Briefly, culture medium was warmed to 37 °C and 2 mL was added to an Endohm chamber suitable for 12 well plate inserts (World Precision Instruments). 1.5 mL of medium was added to the Transwell insert apically before the insert was transferred to the Endohm chamber and measuring the TEER.

2.1.5. Primary CD34+ HSC culture and differentiation towards macrophages and dendritic cells

Bone marrow derived CD34+ HSC from 5 different donors were obtained from StemCell (Grenoble, France). Cells were stored in liquid nitrogen and thawed at 37 °C in a warm water
bath. Once thawed, cells were added to 20 mL of LHC medium containing 10% low IgG FBS and spun down at 300g for 7 minutes. CD34+ cells were expanded in Stemspan medium supplemented with human serum albumin (0.05%), pen/strep, FLT3L (50 ng/mL), thrombopoietin (TPO; 50 ng/mL), StemRegenin 1 (SR-1; 1 µM), stem cell factor (SCF; 50 ng/mL), IL6 (20 ng/mL) and IL3 (20 ng/mL) in 24 well low attachment plates (Corning), 50,000 cells per mL, 1 mL per well, for 1 week. Medium was refreshed when medium colour changed. After 7 days, cells were spun down at 300g for 7 minutes to remove medium and either frozen down in 9:1 FBS/DMSO or replated at a density of 50,000 cells per mL in low attachment 24 well plates in Stemspan medium supplemented with human serum albumin (0.05%), pen/strep and week 2 MC or DC medium supplements (Table 2-8). On day 14, cells were spun down at 300g for 7 minutes and replated at a density of 100,000 cells per mL in either non tissue culture treated (MC<sup>cd34</sup>) or tissue culture treated (DC<sup>cd34</sup>) 48 well plates (Falcon) in RPMI supplemented with low IgG FBS (10%), Glutamax (2 mM) and pen/strep and MC or DC differentiation supplements respectively (Table 2-8). On day 17, 500 µL of respective medium was added to each well. Cell cultures were treated with chemicals or nanoparticles at day 21.

### Table 2-8 CD34+ DC and MC differentiation media

<table>
<thead>
<tr>
<th></th>
<th>MC week2</th>
<th>DC week 2</th>
<th>MC</th>
<th>DC</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLT3 Ligand</td>
<td>-</td>
<td>100ng/ml</td>
<td>-</td>
<td>100ng/ml</td>
</tr>
<tr>
<td>SCF</td>
<td>50ng/ml</td>
<td>100ng/ml</td>
<td>-</td>
<td>20 ng/ml</td>
</tr>
<tr>
<td>IL-3</td>
<td>10ng/ml</td>
<td>20ng/ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-6</td>
<td>10ng/ml</td>
<td>20ng/ml</td>
<td>10ng/ml</td>
<td>10ng/ml</td>
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<tr>
<td>M-CSF</td>
<td>25ng/ml</td>
<td>-</td>
<td>50 ng/ml</td>
<td>-</td>
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<tr>
<td>GM-CSF</td>
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<td>-</td>
<td>50 ng/ml</td>
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<tr>
<td>TGFb1</td>
<td>-</td>
<td>-</td>
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<tr>
<td>IL4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20 ng/ml</td>
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</tbody>
</table>

2.1.6. Chemical and particle exposures

To evaluate cell type specific responses and/or compound specific toxicity, cells were exposed to the compounds displayed in Table 2-9. Chemicals were diluted in culture medium to desired concentrations and cells were exposed for 24 hours. Nanoparticle stock solutions were suspended in distilled water and sonicated at 4.2*10⁵ kJ/m³ (QSonica Sonicators) one hour
before exposure. ALI airway cultures were exposed apically to CSE, DEPE, HDM, CeO$_2$ and SiO$_2$ treatments in 50 µL of differentiation medium for 24 hours (Quasi-ALI exposure). Concentrations of AM, BUS and PQ exposures were determined based on relevant plasma concentrations observed in vivo (Lafuente et al., 2009; McCune et al., 2000; Senarathna et al., 2009) and cytotoxicity curves performed in submerged DCI differentiated primary SAEC (Supplemental figure 9-1). Concentrations that precede active cytotoxic processes were used for transcriptomic analysis to prevent cell death and degradation of mRNA. The same concentration was used in in vitro cell models for chemical exposures, whereas the same amount per cm$^2$ was used for nanoparticle and HDM exposures. After exposures, medium was removed and stored at -80°C for LDH assays and RLT lysis buffer (Qiagen) or TempO-Seq Lysis buffer (Bioclavis) was added and lysed cells were stored at -80°C until further processed for qPCR, RNA-Sequencing and TempO-Seq analysis.

Table 2-9 Chemicals and particles used for toxicity testing

<table>
<thead>
<tr>
<th>Compound</th>
<th>Abbreviation</th>
<th>Supplier</th>
<th>Catalogue #</th>
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<tr>
<td>Aluminium oxide nanoparticles</td>
<td>Al$_2$O$_3$</td>
<td>Sigma</td>
<td>544833</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>AM</td>
<td>Sigma</td>
<td>A8423</td>
</tr>
<tr>
<td>Busulfan</td>
<td>BU</td>
<td>Sigma</td>
<td>B2635</td>
</tr>
<tr>
<td>Carbon nanoparticles</td>
<td>C</td>
<td>Sigma</td>
<td>633100</td>
</tr>
<tr>
<td>Cerium dioxide nanoparticles</td>
<td>CeO$_2$</td>
<td>Sigma</td>
<td>544841</td>
</tr>
<tr>
<td>Cigarette smoke extract</td>
<td>CSE</td>
<td>University of Kentucky</td>
<td>3R4F</td>
</tr>
<tr>
<td>Diesel exhaust particle extract</td>
<td>DEPE</td>
<td>NIST</td>
<td>SRM 1975</td>
</tr>
<tr>
<td>House dust mite soluble extracts</td>
<td>HDM</td>
<td>Stallergenes Greer</td>
<td>XPB82D3A25</td>
</tr>
<tr>
<td>Paraquat dichloride</td>
<td>PQ</td>
<td>Sigma</td>
<td>36541</td>
</tr>
<tr>
<td>Silicon dioxide nanoparticles</td>
<td>SiO$_2$</td>
<td>Sigma</td>
<td>637246</td>
</tr>
<tr>
<td>Titanium dioxide nanoparticles</td>
<td>TiO$_2$</td>
<td>Sigma</td>
<td>637254</td>
</tr>
<tr>
<td>Zinc oxide nanoparticles</td>
<td>ZnO</td>
<td>Sigma</td>
<td>677450</td>
</tr>
</tbody>
</table>
2.2. PCR

Polynucleotide chain reactions (PCRs) were performed to characterize differences in gene expression upon differentiation and/or chemical and particle exposures in iPSC and primary cultures.

2.2.1. mRNA extraction

Approximately $5\times 10^5$ cells were lysed using 350 µL RLT buffer (Qiagen) and homogenized using a QIA Shredder (Qiagen) at 2000 RPM for 2 minutes. mRNA was isolated using a RNeasy Mini Kit (Qiagen) in accordance with the manufacturer’s instructions. Briefly, one volume of ethanol (70%) was added to the lysates, transferred to RNeasy spin-columns and centrifuged at 8000g for 15 seconds. Flow through was discarded and 700 µL of RW1 buffer was added to the spin columns before samples were centrifuged at 8000g for 15 seconds. Flow through was discarded and 500 µL of RPE buffer was added and columns were centrifuged at 8000g for 15 seconds. Flow through was again discarded and 500 µL of RPE buffer was added before centrifuging at 80000g for 2 minutes. RNeasy spin columns were placed in new collection tubes and mRNA was eluted by adding 30 µL RNase-free water and centrifuging at 8000g for 1 minute. Concentration and purity of mRNA samples were established using the NanoDrop 2000 Spectrophotometer (Thermofisher scientific). After extraction, mRNA was stored at -80 °C. The RNA degradation level was not checked.

2.2.2. cDNA synthesis

RNA was reverse transcribed to cDNA using a random hexamer-based protocol and Maxima reverse transcriptase (Thermofisher scientific) in accordance with the manufacturer’s descriptions. Briefly, 500 ng mRNA was mixed with Random Hexamer primers (100 pmol) and dNTP Mix (0.5 mM) in nuclease free water up to a total volume of 14.5 µL in PCR reaction tubes (VWR). Tubes were centrifuged, incubated at 65 °C for 5 minutes and chilled on ice. Thereafter 5X RT buffer (4 µL), RNase Inhibitor (20 U) and Maxima Reverse Transcriptase (200 U) were added, totalling a volume of 20 µL. PCR tubes were centrifuged briefly and incubated at 25 °C.
for 10 minutes followed by 50 °C for 30 minutes. The reaction was terminated at 85 °C for 5 minutes. cDNA was stored at -20 °C.

2.2.3. RT-PCR

Gene expression was analysed by using SYBR green based real-time quantitative PCR (RT-qPCR). cDNA samples were diluted with RNase free water (1:10) and 10 µL of diluted samples were added into 384 well plates (Bioscience). A negative control was added for each primer. The plates were sealed and centrifuged at 250g for 2 minutes. 1 µL of 2 µM primer mix containing forward and reverse primers was mixed with 9 µL of Power SYBR Green PCR Master Mix (Applied Biosystems) and added to the 384 well plates containing cDNA samples, totalling a sample volume of 20 µL. Plates were sealed, inverted and centrifuged at 250g for 2 minutes. Plates where then analysed using the Quantstudio™ 6 Flex Real-Time PCR System (Applied Biosystems). The PCR conditions entailed enzyme activation at 95 °C for 10 minutes followed by 40 cycles of denaturation at 95 °C for 15 seconds each and data collection at 60 °C for 1 minute. A melting curve was performed at the end of the PCR cycles to ensure specificity of reactions. Several commonly used reference genes (β actin, B2M, GAPDH, HPRT, RPL13A and 18S) were examined and GADPH was chosen as most consistent between samples and treatments (data not shown). GAPDH is a key enzyme for energy metabolism and glycolysis (Nicholls et al., 2012). The impact of chemical treatments on GAPDH mRNA levels was explored as these could impact glycolysis pathways and thereby GAPDH levels. No significant differences in GAPDH transcript levels upon chemical treatments were found (Supplemental table 9.1), therefore GAPDH was used as a housekeeper gene for normalisation of qPCR data throughout this thesis. Undifferentiated samples or unexposed controls were used to establish the baseline expression. Results were calculated as delta delta CT (ΔΔCT) values as average of 3 replicates ± standard error of mean (SEM).
2.3. Cytotoxicity assays

2.3.1. Resazurin

A resazurin assay was used to establish the cell viability after chemical and particle exposures (Koppelstaetter et al., 2004). Dark blue coloured resazurin solutions can be directly added to the cell cultures where reduction of resazurin by viable cells reduces the amount of its dark blue oxidized form to pink and fluorescent resorufin, indicating the viability of the cell culture (Riss et al., 2004). Cytotoxicity caused by toxicant exposures reduces the number of viable cells and thus proportionally resazurin reduction. Resazurin stock solution (880 µM) was prepared by dissolving 0.011g resazurin sodium salt (Sigma, cat # R7017), in 1 mL 0.1 N sodium hydroxide (NaOH) and diluted to 50 mL with PBS. pH was adjusted to a range of 7.8-11 and resazurin stock was sterile filtered (0.2 µm) under laminar flow. 22.5 hours after exposure, medium was collected from the wells and resazurin (Sigma) diluted in culture medium to 44 µM was added to the cell cultures. Cells were incubated for 1.5 hours before 100 µL of medium containing resazurin was transferred to a clear bottom 96 well plate (Thermofisher). A blank well with no cells was included in the assay to determine background resazurin Fluorescence. Fluorescence was measured at 540 nm excitation and 590 nm emission (Koppelstaetter et al., 2004) using a Synergy HT plate reader (BioTek). Blank values were subtracted, and results were shown as average of 3 replicates ± standard error of mean (SEM) of percentage of unexposed controls.

2.3.2. Lactate dehydrogenase (LDH)

A lactate dehydrogenase (LDH) assay was performed to analyse cell death and lysis upon chemical and particle exposure by analysing the LDH levels in the medium released from damaged cells using the Pierce LDH Cytotoxicity Assay kit (Thermofisher Scientific). Medium, as collected in section 2.1.6, was thawed and 50 µL of each sample was added to a clear bottom 96 well plate (Thermofisher). Reaction mixture solution was prepared according to manufacturer’s instructions and 50 µL was added per well. Samples were incubated for 30 minutes, protected from light at room temperature. Reactions were stopped by adding 50 µL of
stop solution to each well. Absorbance was measured at 490 nm and 680 nm using a Synergy HT plate reader (BioTek). Wells containing culture medium without cells were used as negative control and lysed cells (10X lysis buffer) were used as positive control. To determine levels of LDH, 680 nm absorbance values were subtracted from 490 nm absorbance values and results were shown as mean of 3 replicates ± standard error of mean (SEM) fold over control (F.O.C.) of unexposed cells.
2.4. Bulk RNA Sequencing

Bulk RNA-sequencing of the transcriptome was performed to characterize differentiation of primary CD34+ and SAEC cultures as well as chemical exposures in primary airway epithelium. RNA was lysed using an RLT buffer (Qiagen) and isolated using a RNeasy Mini Kit (Qiagen) in accordance with the manufacturer’s instructions (see section 2.2.1). RNA concentration and purity were determined using the NanoDrop 2000 Spectrophotometer (Thermofisher scientific). Sample processing and pair ended (150 PE) sequencing was performed externally using DNBSeq™- T7 platform (BGI, Hong Kong) (Q. Li et al., 2019).

DNBSeq combines DNA nano balls (DNB) technology, high density patterned arrays and combination probe anchor synthesis (cPAS) technology. The cPAS incorporates a fluorescent probe to a DNA anchor on the DNB, followed by high resolution digital imaging to capture the fluorescent signals. DNB uses rolling circle replication to amplify small amounts of RNA. The combination of linear cPAS amplification and DNB technology reduces error rates and enhances signals (Q. Li et al., 2019).

At BGI, image data was transferred into sequence data via base calling and saved as FASTQ files. These FASTQ files contain detailed read sequences and quality information. Obtained quality scores of samples were averaged at 97.5%. Obtained data contained up to 50 million clean reads per sample which were analysed using CLC Genomics Workbench (CLCBio). Paired files were trimmed to remove low-quality reads, remaining adapters and other variable sequences and annotated using the Hg38 Human reference genome assembly build (REFSeq Transcript ID).

RNA-Seequencing analysis was performed as previously described (Mortazavi et al., 2008). RPKM (Reads per Kilobase per Million mapped reads) values, FDR P-values (corrected for multiple testing) and Fold Change were determined and used to identify significantly differentially expressed genes between samples. Significant differences were visualized on volcano plots using the EnhancedVolacano package within R (Bilighe et al., 2018).
Gene ontology (GO) enrichment pathway analysis was performed on significantly differentially expressed genes using g:Profiler Toolkit (Raudvere et al., 2019). g:GOSt performs over-representation (ORA) analysis on a list of genes and maps genes to known functional information sources (e.g., KEGG Reactome, WikiPathways, GO) resulting in the detection of statistically significantly enriched terms.
2.5. TempO-Seq

Targeted RNA sequencing, TempO-Seq was used to characterize iPSC and CD34+ derived macrophages and dendritic cell responses to chemical and particle exposures. This method allows for simultaneous detection of hundreds to thousands of genes in high throughput. The input for this method can be as little as 10 pg of total RNA, maximizing the utilization of limited samples (Yeakley et al., 2017). Based on BioSpyder Technologies’ propriety Template Oligo Detection Assay, TempO-Seq avoids the need of RNA purification or reverse transcription. This can be achieved by targeting RNAs with detector oligos and ligating and amplifying only correctly hybridized detector oligos through primer landing sites shared among gene probes (Yeakley et al., 2017). This method allows for high throughput multiplexing, as only two PCR primers are required for each sample, reducing primer cross-hybridization and competition which is often a limitation for multiplex PCR (Figure 2-1) (Yeakley et al., 2017).

**Figure 2-1 TempO-Seq workflow.** TempO-Seq methods targets sample RNAs by annealing to detector oligos containing target specific sequences (green). Primer landing sites (red, yellow) are shared across all detector oligos. Excess oligo removal is achieved by 3’ exonuclease. Correctly hybridized oligos are ligated and amplified using primers containing sample tag sequences (purple, orange) and adaptors required for sequencing. Finally, amplified products are pooled, concentrated and purified before sequencing (Yeakley et al., 2017).

CD34+ and iPSC cultures were lysed using 100 µL TempO-Seq Lysis buffer (BioClavis) and samples frozen at -80 °C. The samples were sent to BioClavis Technologies Ltd. (Glasgow, UK) and were
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Processed for TempO-Seq quantification of 3565 gene probes (EU-ToxRisk v2.1 panel) representing 3257 genes involved in toxicological responses. The number of probes was higher than the number of genes as multiple probes were included for several genes.

Raw TempO-Seq read count data of the data passing standard QA/QC tests were obtained by BioClavis using TempO-SeqR software packages. FASTQ files containing reads and quality scores for each sample were aligned using STAR algorithm (Dobin & Gingeras, 2015). The obtained gene count matrix table was used for further analysis and aligned to a metadata file containing information on cell model, differentiation state and exposure analysis. Counts were analysed for differential expression using DESeq2 version 1.30.0 within the R environment, as previously described (Love et al., 2014). Bar plots of raw count numbers, principal component analysis and Poisson distribution analysis were used to detect outliers within the samples (data not shown).

Normalized counts were obtained using EstimatedSizeFactors function within the DeSeq2 package, using median ratio methods (Love et al., 2014). The normalized read counts were used for differential expression analysis using the DESeq2 function, which generated fold change values, p-values, base mean expression values and adjusted p-values (Padj). These metrics were used as measures of statistical significance between cell culture models and exposures. To obtain Padj, p-values obtained using the Wald test were corrected for multiple testing using the Benjamini and Hochberg method within the DESeq2 function (Love et al., 2014). Probes which had a low or zero read count were detected by an automatic independent filtering method within DESeq2 and their corresponding Padj were set to NA. To detect significant differences, cut-offs were set to 0.05 for Padj and 2 for fold change. Significant differences were visualized on volcano plots using the EnhancedVolcano package within R (Blighe et al., 2018). GO pathway analysis was performed on significantly differentially expressed genes as described in section 2.5.
2.6. Single cell transcriptome and surface marker sequencing

iPSC and CD34+ hemopoietic stem cell derived macrophages and dendritic cells were further characterised using single cell transcriptome (Sc) and cell surface marker antibody (Ab) sequencing. Cells were first removed from plates and separated with 5 mM EDTA for 30 minutes on ice to create single cell suspensions and were subsequently resuspended in cold sample buffer (BD).

2.6.1. Antibody and sample tag labelling

Human BD Fc block (BD, cat # 564219) was 1:20 diluted in stain buffer (BD) and used to block non-specific antibody binding to CD16/CD32. For each sample, cells were pelleted, resuspended in 110 µL diluted Fc block and incubated at room temperature for 10 minutes. 20 µL of Sample Tags from BD Human Single-Cell Multiplexing Kit (BD, Cat # 633731) were mixed with 80 µL of BD AbSeq-Oligos (Table 2-10) combined and diluted 1:40 in Stain buffer. 100 µL of the cell suspension was added to the mixture containing a different sample tag for each sample and incubated on ice for 45 minutes. To remove residual unbound antibodies, samples were transferred to 5 mL Falcon tubes (Corning) containing 2 mL stain buffer and centrifuged at 400g for 5 minutes. Supernatant was removed, cells were washed again using 2 mL stain buffer and resuspended in 620 µL cold sample buffer (BD). In order to count the cells in each sample, 3.1 µL of 2 mM Calcein AM (Thermofisher) and 3.1 µL of 0.3 mM Draq7 (BD) were added to the vials containing the labelled cells and gently pipet-mixed before being incubated at 37 °C for 5 minutes. 10 µL of the suspensions were loaded into disposable haemocytometers (INCYTO), analysed using the BD Rhapsody™ Single-Cell Analysis System and samples containing >1000 cells/µL were diluted using cold sample buffer. The sample calculator function was used to obtain sample volumes to pool the samples into a cell suspension totalling 650 µL.
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Table 2-10 BD AbSeq Human Oligos

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Catalogue #</th>
</tr>
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<tbody>
<tr>
<td>CCR7 (CD197)</td>
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<td>940394</td>
</tr>
<tr>
<td>CD14</td>
<td>MPHIP9</td>
<td>940005</td>
</tr>
<tr>
<td>CD16</td>
<td>3G8</td>
<td>940006</td>
</tr>
<tr>
<td>CD11b</td>
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</tr>
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<td>CD11c</td>
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</tr>
<tr>
<td>CD163</td>
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<tr>
<td>HLA-DR</td>
<td>G46-6</td>
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</table>

2.6.2. Cell capture

To capture single cells, a BD rhapsody Cartridge was primed using 100% ethyl alcohol and wash buffers (BD), loaded with the cell suspension obtained in section 2.6.1. using the BD Rhapsody™ Single-Cell Analysis System, and incubated at room temperature for 15 minutes. The cartridge “Cell load” was then imaged using the BD Rhapsody™ scanner. Cell capture beads (BD) were loaded into the cartridge and incubated at room temperature for 3 minutes. The cartridge was then washed twice using cold sample buffer before lysis buffer supplemented with DTT (BD) was loaded into the cartridge and incubated at room temperature for 2 minutes. Cell capture beads were then collected in 5 mL of Lysis buffer in a 5 mL LoBind tube (Eppendorf). The Lysis buffer was removed using a large magnet (V&P Scientific), beads were washed twice using cold bead wash buffer (BD) and resuspended in 1 mL cold Bead Wash buffer.

2.6.3. cDNA synthesis

Cell capture beads were resuspended in 200 µL cDNA mix containing RT buffer (40 µL), dNTP (20 µL), RT 0.1 M DTT (10 µL), bead RT/PCR enhancer (12 µL), RNase inhibitor (10 µL), reverse transcriptase (10 µL) and nuclease-Free water (98 µL and incubated on a SmartBlock Thermoblock 1.5 mL on thermomixer (Eppendorf) at 1200 rpm and 37 °C for 20 minutes. Supernatant was removed using a 1.5 mL tube magnet (New England Biolabs) and cell capture beads were resuspended in 200 µL Exonuclease I mix containing 10X exonuclease I buffer (20
µL), exonuclease I (10 µL) and nuclease-Free Water (170 µL). The suspension was incubated on the thermomixer at 1200 rpm and 37 °C for 30 minutes, followed by 0 rpm and 80 °C for 20 minutes. Supernatant was removed and beads were resuspended in cold bead resuspension buffer (BD).

2.6.4. Random priming and extension

First, random priming products were generated from the cell capture beads with cDNA obtained in section 2.6.3. Exonuclease I treated cell capture beads were resuspended in 75 µL elution buffer (BD) and placed in a heat block at 95 °C for 5 minutes. Supernatant was removed using a 1.5 mL magnet and kept for AbSeq and Sample Tag library preparation. The cell capture beads were resuspended in 200 µL elution buffer and supernatant was removed. Beads were resuspended in 174 µL random primer mix containing 20 µL WTA Extension buffer, 20 µL WTA extension primers and 134 µL nuclease-free water and incubated in a heat block at 95 °C for 5 minutes, followed by thermomixer at 1200 rpm and 37 °C for 5 minutes and 1200 rpm and 25 °C for 15 minutes. Thereafter, 26 µL of extension enzyme mix containing 10 mM dNTP (8 µL), bead RT/PCR enhancer (12 µL), WTA extension enzyme (6 µL) was added for a total volume of 200 µL. Beads were incubated on the thermomixer at 1200 rpm and 25 °C for 10 minutes, followed by 1200 rpm and 37 °C for 15 minutes, 1200 rpm and 45 ºC for 10 minutes, and 1200 rpm and 55 °C for 10 minutes. Supernatant was removed and beads resuspended in 205 µL elution buffer and incubated in a heat block at 95 °C for 5 minutes followed by 1200 rpm at room temperature for 10 seconds. 200 µL of the supernatant was transferred to a new LoBind tube and labelled as random primer extension (RPE) product.

Primer dimers and other small molecular weight by-products were removed using a single-sided AMPure clean-up, obtaining “purified RPE product”. In order to generate multiple copies of each random-primed molecule, PCR amplification of the RPE product was performed: 40 µL of purified RPE product was added to 80 µL of RPE PCR Mix containing 60 µL PCR MasterMix, 10 µL universal oligo and 10 µL WTA amplification primer in a PCR tube. PCR tubes were incubated at
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95 °C for 3 minutes, followed by 13 PCR cycles consisting of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 1 minute, and extension at 72°C for 1 minute. A final extension was performed at 72°C for 2 minutes before the tube was cooled down to 4 °C.

A single-sided AMPure clean-up was performed to purify the RPE PCR amplification products, obtaining “RPE PCR product”. The quantity and quality of the RPE PCR product were determined using the Qubit Fluorometer dsDNA HS Assay and the Agilent 4200 TapeStation using Agilent High Sensitivity D1000 ScreenTape assay according to manufacturer’s instructions. RPE PCR product was stored at 4 °C or -20 °C until further processed.

2.6.5. Sample tag and AbSeq library preparation

The AbSeq/Sample Tag products were amplified through PCR: 67 µL of the supernatant obtained in section 2.6.4 was mixed with 133 µL AbSeq/Sample Tag PCR1 reaction mix containing 100 µL PCR MasterMix, 10 µL Universal Oligo, 12 µL Bead RT/PCR enhancer, 1 µL Sample Tag PCR1 Primer and 10 µL BD AbSeq primer in a PCR tube. PCR tubes were incubated at 95 °C for 3 minutes, followed by 14 PCR cycles consisting of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72°C for 1 minute. A final extension was performed at 72°C for 5 minutes before the tube was cooled down to 4 °C. A single-sided AMPure cleanup was performed to remove primer dimers from these AbSeq/Sample Tag PCR1 products, obtaining “purified PCR1 product”, which were analysed with the Agilent 4200 TapeStation system using Agilent High Sensitivity D1000 ScreenTape assay.

PCR1 products were diluted to 0.5 ng/µL and amplified through PCR: 5 µL of PCR1 products were mixed with Sample Tag PCR2 reaction mix containing 25 µL PCR master mix, 2 µL universal oligo, 3 µL Sample Tag PCR2 primer and 15 µL nuclease-free water in PCR tubes. PCR tubes were incubated at 95 °C for 3 minutes, followed by 10 PCR cycles consisting of denaturation at 95 °C for 30 seconds, annealing at 66 °C for 30 seconds, and extension at 72°C for 1 minute. A final extension was performed at 72°C for 5 minutes. Primer dimers were removed from these PCR2 products using single-sided AMPure clean-up, to obtain Sample Tag “PCR2 product”.

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The concentration of PCR2 product was estimated using the Qubit dsDNA HS Assay Kit on the Qubit Fluorometer. PCR2 products were diluted to 0.5 ng/µL using elution buffer and 3 µL was mixed with 47 µL Sample Tag index PCR mix containing 25 µL PCR MasterMix, 2 µL library forward primer, 2 µL library reverse primer 1 and 18 µL nuclease-free water in PCR tubes. PCR tubes were incubated at 95 °C for 5 minutes, followed by 7 PCR cycles consisting of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72°C for 30 seconds. A final extension was performed at 72 °C for 1 minute. These Sample Tag index PCR products (50 µL) were mixed with 50 µL AMPure beads and incubated at room temperature for 5 minutes. Beads were washed twice with 80% ethyl alcohol and airdried at room temperature for 3 minutes. 30 µL of elution buffer was added to the beads and incubated at room temperature for 2 minutes. The tube was placed on a magnet and the eluate (30 µL) was transferred to a new 1.5 mL LoBind tube labelled “Sample Tag Sequencing Library”. The concentration and quality were estimated using the Qubit dsDNA HS Assay Kit on the Qubit Fluorometer and Agilent 4200 TapeStation using Agilient High Sensitivity D1000 ScreenTape Assay following manufacturer’s instructions.

PCR1 products were diluted to 0.5 ng/µL. 3 µL of PCR1 products were mixed with 47 µL of AbSeq index PCR mix containing 25 µL PCR MasterMix, 2 µL library forward primer, 2 µL library reverse primer 1 and 18 µL nuclease-free water. PCR tubes were incubated at 95 °C for 5 minutes, followed by 7 PCR cycles consisting of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72°C for 1 minute. A final extension was performed at 72 °C for 1 minute. 40 µL of AMPure magnetics beads were added to 50 µL of AbSeq Index PCR products and incubated at room temperature for 5 minutes. Beads were washed twice with 80% ethyl alcohol and dried at room temperature for 3 minutes. 30 µL of elution buffer was added to the tubes and incubated for 2 minutes. Eluate (30 µL) was transferred to a new 1.5 mL LoBind tube labelled “AbSeq sequencing library”. Concentration and quality of this library was estimated using the Qubit dsDNA HS Assay Kit on the Qubit Fluorometer and Agilent 4200 TapeStation.
using Agilent High Sensitivity D1000 ScreenTape Assay according to manufacturer’s instructions.

2.6.6. mRNA Whole Transcriptome Analysis library preparation

RPE PCR products obtained in section 2.1.6 were diluted in elution buffer so that the 200-600 bp peak, as analysed with the Agilent TapeStation, is 2 nM. 10 µL of diluted RPE PCR products were mixed with 40 µL index PCR mix, containing 25 µL PCR MasterMix, 5 µL Library Forward primer, 5 µL Library Reverse primer 1 and 5 µL Nuclease-free water. PCR tubes were incubated at 95 °C for 3 minutes, followed by 8 PCR cycles consisting of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72°C for 30 seconds. A final extension was performed at 72 °C for 1 minute. To ensure that this library is an appropriate size for Illumina sequencing, double-sided AMPure clean-up was performed, obtaining “WTA Index PCR”. Concentration and quality of this sample was estimated using the Qubit dsDNA HS Assay Kit on the Qubit Fluorometer and Agilent 4200 TapeStation using Agilent High Sensitivity D1000 ScreenTape Assay following manufacturer’s instructions.

2.6.7. Sequencing and analysis

WTA index PCR and Sample Tag and AbSeq sequencing libraries were pooled and outsourced for 2x 151bp pair ended sequencing using Illumina HiSeq™ X platform (BGI, Hong Kong), using PCR-free preparations. Data was analysed externally using BD WTA Multiplex Rhapsody Analysis Pipeline Version 1.8, leaving 85% of reads after quality filtering and at least 40,000 reads per cell.

Single cell sequencing data was analysed using SeqGeq (FlowJo) version 1.7.0 software. The library size was plotted against the number of the total genes expressed for all samples in order to remove outlier events such as empty wells or doublets and keeping >97.5% of cells as quality cells. Dimensionality reduction was performed using PCA on normalized genes. UMAP plots were created based on machine learning algorithms within SeqGeq based on highly dispersed genes between samples, genes typically differentially expressed between MC<sup>cd34</sup> and DC<sup>cd34</sup>
cultures as identified with bulk RNA Sequencing (117 genes, section 2.4) and 9 antibodies as mentioned in section 2.6.1. k-means clustering was used to identify cell clusters. Cells were also annotated to sample tags. Differentially expression analysis was performed to identify significantly different genes between samples. Significant genes were determined using cut offs of >2 for fold change and 0.05 for q-value (adjusted p-value) within SeqGeq software.
2.7. Nanoparticle characterization

The sizes of the nanoparticles used in this thesis (Table 2-9) were determined. The dynamic light scattering (DLS) method, using Brownian motion to determine the size of particles, was used to establish the size of nanoparticles suspended at 1 mg/mL in distilled H₂O, culture medium (RPMI) and RPMI supplemented with HDM. Particles were sonicated at 4.2*10⁵ kJ/m³, 1 hour before measurement. Before measurements, particles were diluted to 10⁸-10⁹ particles per mL and added to disposable folded capillary cuvettes (Malvern). The Z-average size was determined using the Zetasizer Nano-ZS (Malvern). Results are shown as average over 3 replicates ± SEM.
2.8. Statistical analysis

Statistical analysis for PCR and cytotoxicity assays were performed using one-way analysis of variance (ANOVA) and Fisher’s Least Significant Difference (LSD) tests in GraphPad Prism 8 software. Statistical significance was set at a p-value of <0.05.
CHAPTER 3: OPTIMIZATION AND CHARACTERIZATION OF *IN VITRO* SMALL AIRWAY MODELS
3.1. Introduction

The development of organotypic in vitro small airway models as an alternative to animal models for toxicity testing and as an approach to establish specific mechanisms of toxicity for the lung, is an important research gap, not yet fully established. To develop the use of in vitro testing for toxicological evaluation and regulatory purposes, thorough characterisation of in vitro models is essential to establish and optimize the extent to which the models are comparable to in vivo counterparts, human responses and relevant for toxicity assessments. As discussed previously, airway epithelial cells as well as resident immune cells play an important role in small airway barrier function and toxicological responses. Both cell types provide exclusive and complementary functions within the airway, underpinning the importance of developing relevant models for each. Although iPSC derived airway cells can form an attractive alternative to primary cells, iPSC models of the small airway (epithelial and immune cell) have not been adequately achieved to date and efforts to progress this area will significantly advance in vitro airway toxicological assessment potential.

3.1.1. Models of the primary airway epithelium

Despite their limitations, primary airway epithelial cultures are the model of choice for many toxicological evaluations. Understanding their origin, proliferative capabilities and differentiation characteristics are an important reference and guide to developing iPSC derived models. Conducting airway epithelial cells of the lung, isolated from human tissue, can be differentiated on ALI to establish a polarized mucociliary epithelial cell layer which morphologically resembles in vivo human airway epithelium (Karp et al., 2002). Transcriptomic analysis has demonstrated that ALI cultured human primary airway epithelial cells closely resemble human airway epithelium when compared to cell-lines grown at the ALI (such as Calu-3) or primary cells grown in submerged culture (Pezzulo et al., 2011).

The pseudostratified conducting airway epithelium consists of basal, ciliated, and secretory cells as well as rare cells such as ionocytes, PNECs and brush cells. The relative proportion of these
cells differs within different regions of the airway (Vieira Braga et al., 2019). Historical use of conducting airway primary cell models, however, rarely attempt to differentiate cells to the appropriate proportions. Understandably this is a difficult task with only recent advances in culture conditions allowing some progress. Previously, normal human bronchial epithelial (NHBE) or SAEC were cultured under submerged cultures or ALI to model proximal bronchial and distal small airway regions respectively (Bhowmick & Gappa-Fahlenkamp, 2016). While the use of submerged conditions is not representative of normal airway conditions within the lung, it does highlight mechanisms of regional differentiation are highly influenced by environmental conditions. Indeed, components within differentiation cell culture media can greatly impact the proportion of airway cell types (Sachs et al., 2003). These are arguably more important than the regional origin of the primary basal cells in establishing a model with specific cell types (Luengen et al., 2020). In order to further optimise the conditions for organotypic culture, this work also investigated the impact of different molecular pathway modulators on small airway differentiation.

3.1.2. iPSC derived airway epithelium

Previous investigation of iPSC differentiation towards airway epithelium follows human development through DE, AFE and lung progenitor (LP) stages (S. Huang et al., 2014). In order to differentiate iPSC towards a functional airway epithelium, exposure of cells to different growth factor, molecular pathway inhibitors and activators in a sequential and timed manner are used to recapitulate different stages of embryonic development. The first step in development of the respiratory system is a localized domain of NKX2.1 expression in the ventral wall of the anterior foregut (Lazzaro et al., 1991; Morrisey & Hogan, 2010), regulated by the dose and timing of signals from the surrounding mesoderm (Serls et al., 2005). BMP signalling gradients are essential for dorsoventral patterning, separating the oesophagus from the trachea (Pizette et al., 2001). BMP4 is suggested to indirectly promote NKX2.1 expression through repression of SOX (Domyan et al., 2011). Localized expression of FGF10 drives cell proliferation and lung bud outgrowth and requires retinoic acid (RA) signalling as well as TGFβ inhibition (F.
Chen et al., 2007). After differentiation of iPSC to lung progenitor cells, modulation of Notch and Wnt signals results in distinct cell populations as summarized in Figure 3-1 (McCauley et al., 2017). Wnt inhibition after lung progenitor differentiation will guide cells towards airway progenitors, a subsequent inhibition of the Notch pathway will increase multiciliated cell differentiation.

Figure 3-1 Differentiation of human pluripotent stem cells (hPSCs) by modulation of Wnt and Notch signalling pathways (McCauley et al., 2017).

Several protocols are published for iPSC differentiation into airway epithelium cells; however, none are directly applicable to in vitro airway toxicity testing as they predominantly developed cultures as organoids. For the protocol to be relevant for inhalation testing, the cells must be consistently cultured as a polarized mucociliary epithelium at a monolayer on ALI. Green et al. (2011) first demonstrated the possibility to differentiate iPSC through DE to AFE stages by inhibition of BMP and TGFβ. They attempted to differentiate these cells further towards lung progenitor and mature airway epithelium, however purity of lung progenitors and maturity of differentiated cells were both limited (Green et al., 2011). In 2012, Wong et al. published a protocol for the generation of mature airway epithelium at the air liquid interface from human iPSC. This group used a different approach for AFE differentiation using sonic hedgehog (SHH) and FGF2. They reported 78% of cells expressing NKX2.1 (Wong et al., 2012), however this result
could not be reproduced by another group (S. Huang et al., 2014), indicating difficulties in reproducing efficiency of generating NKX2.1 expressing cells between laboratories and iPSC lines.

Nevertheless, several studies have achieved differentiation of iPSC towards proximal airway epithelial cells at the ALI (Firth et al., 2014; Green et al., 2011; Hawkins et al., 2017; S. Huang et al., 2015; Konishi et al., 2016; Serra et al., 2017; Suzuki et al., 2016). One of these studies demonstrated feasibility of generating an iPSC derived polarized epithelial cell layer, resembling the airway epithelium on ALI consisting of club, ciliated, goblet cells and basal cells (Firth et al., 2014). However, the unwanted presence of a mesenchymal layer on the basolateral side and the requirement to culture at the ALI from day 0 still limit the use of this protocol in toxicity studies. The Snoeck group developed an iPSC airway differentiation protocol that allows for freezing of lung progenitor or basal-like airway epithelium cells that can be further differentiated toward an airway epithelium (S. Huang et al., 2014, 2015). However, the initial steps of this protocol require optimization for every cell line. Given the variability and inconsistencies surrounding differentiation of iPSCs towards conducting airway cells at the ALI, further work is necessary to successfully establish these cultures. An additional challenge to differentiate such cultures towards small airway epithelial cells also remains and has not been addressed in any substantial way to date.

3.1.3. Models of airway macrophages and dendritic cells

Airway resident macrophages and dendritic cells can interact with allergens, pollutants and pathogens to elicit an immune responses within the small airways and are thus relevant to include small airway toxicity assessments (Iwasaki et al., 2017). As discussed before in section 2.1.5, primary alveolar macrophages and dendritic cells directly isolated from the small airways are difficult to obtain and culture, therefore CD34+ HSC derived models will be used as primary models for macrophages and dendritic cells within the small airways.
CD34+ HSC have been used as a renewable source for different immune cell populations, including monocytes, macrophages, dendritic and mast cells. Normally residing within bone marrow in the adult, they can be a source of lung immune cell populations in homeostasis and disease (Evren et al., 2020). Importantly, primary CD34+ HSC can also be used as a source of immune cells for toxicity testing purposes. Maintenance of myeloid pluripotency and proliferation potential in vitro typically involves a combination of cytokines and growth factors including Flt-3 ligand, TPO, SCF, IL6 and IL3 (Bedke et al., 2020; Gammaitoni et al., 2003). Macrophages and dendritic cells can then be selectively differentiated from CD34+ hematopoietic progenitor cells for in vitro testing purposes. The phenotype of these lineages typically align to in vivo differentiation trajectories normally observed in inflammatory disease states (Caux et al., 1996; Clanchy & Hamilton, 2013; Rosenzwajg et al., 1996; J. W. Young et al., 1995).

Under homeostatic conditions in the lung, alveolar and interstitial macrophages are resident members of the airway epithelial layer, including the small airway epithelium (Hume et al., 2020). In disease states or as a consequence of adverse exposures, monocyte derived macrophages can replenish depleted resident populations. Differentiation of blood monocytes towards alveolar macrophages is dependent on GM-CSF produced by epithelial cells (Schneider et al., 2014). GM-CSF induces peroxisome proliferator-activated receptor γ (PPARγ) a key regulator of lipid metabolism in alveolar macrophages (Schneider et al., 2014). Furthermore, rodent studies have indicated that further upregulation of PPARγ by TGFβ is essential for alveolar macrophage development (Yu et al., 2017). It has been previously shown that serum is required for increased M-CSF induced macrophage elongation and CD163 expression (Rey-Giraud et al., 2012). Therefore, addition of GM-CSF and TGFβ in addition to M-CSF in serum-containing medium during macrophage differentiation may help direct HSC differentiation towards a more airway myeloid phenotype as present within the small airways. Differentiation trajectories of CD34+ HSC towards an alveolar macrophage phenotype might pass interstitial macrophages as an intermediate differentiation state, therefore differentiation of CD34+ HSC
toward alveolar macrophages is likely to produce a combination of alveolar and interstitial macrophages (Evren et al., 2021). CD34+ differentiation towards dendritic cells has been demonstrated using a combination of IL4 and GM-CSF to positively regulate dendritic cell development while inhibiting macrophage differentiation (Bedke et al., 2020; Rougier et al., 1998). These factors will be important aspects to consider for the differentiation of CD34+ HSC and iPSC towards airway macrophage and dendritic cells.

3.1.4. iPSC macrophages and dendritic cells

During development, resident lung macrophage populations arise from foetal liver and yolk sac, which migrate to the lung (Ginhoux & Guilliams, 2016). Although, these populations can be supplemented from monocytes via CD34+ HSC intermediaries during a lifetime, the use of such precursors as a source for in vitro culture may not fully capture the unique capabilities of resident airway immune cells as they occur in vivo. Derivation of cells from iPSC may therefore have the advantage in capturing some of these properties. This, in addition to the unrestricted supply of iPSC and the difficulties in acquiring airway macrophages and dendritic cells in humans, make the prospect of iPSC derived airway immune cells for toxicity testing an attractive goal. Several studies have previously described the differentiation of iPSC towards monocytes, macrophages and dendritic cells (Cao et al., 2019; Monkley et al., 2020; Senju et al., 2011; Takata et al., 2017; Vaughan-Jackson et al., 2021; Yanagimachi et al., 2013; H. Zhang et al., 2015). This includes a protocol of iPSC derived monocytes with the ability to differentiate towards macrophages with drug screening applications (Gutbier et al., 2020; van Wilgenburg et al., 2013). This protocol involves the initial formation of EBs consisting of all three germ layers, using BMP4, VEGF and SCF. These EBs can then be further differentiated along the myeloid lineage using IL3 and M-CSF to form “myeloid factories”, from which non-adherent monocyte-like cells can be collected on a weekly basis (Gutbier et al., 2020). As monocyte-like cells can be collected from the same culture for a prolonged period of time and are generated under feeder-free and serum-free conditions, it gives the opportunity to create a large number of monocytes with a high level of reproducibility, highly applicable for toxicity testing applications. Subsequent
Optimization and characterization of in vitro small airway models
differentiation of iPSC derived monocyte-like cells towards macrophages using M-CSF and
towards dendritic cells using GM-CSF and IL4 has been previously achieved (Monkley et al.,
2020). Modifications to these differentiation protocols to achieve airway macrophage
phenotypes remains a challenge.

3.1.5. Overall aims

The human small airways differ from those within animal models and are particularly susceptible
to toxicant exposure. Current models for in vitro toxicity assessment are lacking. Further
optimising the differentiation conditions of primary cells and the use of iPSC derived cells have
the potential to bring considerable improvements and sustainability. Specific aims of this
chapter to address this goal are described below and summarised in Figure 3-2.

Aims:

- Establish, optimise, and characterise human primary small airway epithelial in vitro ALI
differentiation.
- Establish and characterise iPSC derived airway epithelial cells for ALI toxicity testing
- Establish and characterise CD34+ derived macrophage and dendritic cell populations
- Establish and characterize iPSC differentiation towards macrophages and dendritic cell
trajectories.

Figure 3-2 Overview of Chapter 3
3.2. Optimisation of culture conditions for primary airway epithelial cell differentiation

Primary human SAEC were differentiated on ALI for 21 days using different combinations of media components known to impact airway epithelial cell differentiation as described in Table 2-7 (section 2.1.5). Examination of differentiation characteristics reflective of small airway phenotype was then carried out. This involved measurement of TEER for barrier integrity and levels of marker genes for different airway cell phenotypes using PCR analysis. There was a high level of variability across the 6 donors used, however some general conclusions may be drawn. Media F, containing dexamethasone, cAMP and IBMX (DCI) compounds was observed to have a high level of TEER (Supplemental figure 9-2B), while also having lower levels of basal, goblet, alveolar and oxidative stress markers (Supplemental figure 9-2C,D). This media formulation may reflect conditions more favourable for small airway differentiation, but conclusive findings require additional experimental work.

We did however further explore the DCI component of this media formulation for its ability to differentiate towards the small airway and club cell phenotype. Interestingly, DCI formulation has been previously used to induce small airway maturation (McCauley, Alysandratos, et al., 2018). A base medium developed by Firth et al. (2014) supplemented with DCI compounds (DCI medium, Table 2-5) was compared to a commercially available differentiation medium, PneumaCult™-ALI for their ability to differentiate basal small airway epithelial cells (Figure 3-3A). PneumaCult media is typically used for upper airway differentiation and was used as a possibility to drive the proximal small airway phenotype.

Basal airway epithelial cells (basal AEC, day 0), from the small airway were differentiated for 3 weeks on ALI using DCI or PneumaCult™-ALI differentiation medium and examined for differences in morphology (Figure 3-3B). Basal AEC showed a cuboidal cell phenotype. DCI differentiation resulted in a monolayer with no visible ciliated cells as observed by cilia beating under a microscope (data not shown). PneumaCult™-ALI differentiation resulted in a
pseudostratified layer with a substantial mucus layer and functional ciliated cells. Furthermore, the TEER increased upon differentiation with both differentiation media, indicating that the cell layers formed a tight barrier (Figure 3-3C). Interestingly, the TEER was slightly higher in DCI differentiated airway epithelium compared to PneumaCult™-ALI.

Figure 3-3 Primary airway epithelium differentiation on air-liquid interface. Primary airway epithelium cells (basal cells, day 0) were differentiated on air-liquid interface (ALI) using DCI or PneumaCult™-ALI differentiation media for 3 weeks (A). Morphological differences between the undifferentiated and differentiated cells were assessed using microscopy images (100x) (C). The integrity of the cellular layer was determined using transepithelial electrical resistance (TEER) measurements in Basal (day 0) DCI (day 21) and PneumaCult™-ALI (day 21) differentiated cells, data is shown as mean ± SEM (N=6 different donors, significance shown as *, P<0.05 compared to basal cells) (B).
qPCR analysis was performed to characterise basal AEC, DCI and PneumaCult™-ALI differentiated cells (Supplemental figure 9-3) and showed downregulation of basal cell markers (KRT14) and upregulation of club (SCGB1A1), goblet (MUC5AC), multiciliated (FOXJ1), PNEC (FOXN4), ionocyte (FOXI1) and CYP enzyme mRNA expressions upon differentiation. Quantification of mRNA was carried out using RNA-Seq. Both DCI and PneumaCult™-ALI differentiation demonstrated significant upregulation of the club cell markers SCGB1A1, SCGB3A2 and BPIFA1 as well as upregulation of goblet cell (MUC5AC), ciliated cell (FOXJ1), inflammation (CXCL2, CXCL8) and metabolic enzyme (CYP2A6, CYP26A1) markers (Figure 3-4A,B). Further comparison of undifferentiated basal AEC, DCI and PneumaCult™-ALI conditions demonstrated highest expression of basal cell markers (KRT5, KRT14, KRT16) in basal AEC, highest expression of club cell markers (SCGB1A1, SCGB3A1, BPIFA1) in DCI and highest expression of multiciliated marker (FOXJ1, PIFO, TUB1A1) in PneumaCult™-ALI differentiated cells. Direct comparison of DCI and PneumaCult™-ALI differentiation shows significant higher expression of the club cell markers SCGB3A1 and BPIFA1 in DCI-differentiated cells and significantly higher expression of ciliated cell marker FOXJ1 in PneumaCult™-ALI differentiated cells (Figure 3-4A). Similar levels of secretory cell markers (MUC5B, MUC5AC) were observed in both differentiated cell types (Figure 3-4C).
Figure 3-4 Characterisation of primary airway differentiation using RNA-sequencing. Primary airway epithelium cells (basal cells, day 0) were differentiated on air-liquid interface (ALI) using DCI or PneumaCultTM-ALI differentiation media for 21 days. Cultures were examined for differences in gene expression using RNA-sequencing analysis and displayed as volcano plots. Significantly up- or downregulated genes are highlighted as red (log₂ fold change >2 and FDR P-value <0.05) (N=6) (A). The most significantly differentially expressed genes between the populations, as well as alveolar cell markers (AQP5, SFTPA, SFTPC and SFTPD; non-significant) are displayed in a heatmap of log2 RPKM values (B). Selected genes are displayed as bar graphs of RPKM values (mean ± SEM) in Basal, DCI and PneumaCult™-ALI differentiated cells (n=6) (C).
Furthermore, differentiation resulted in an increased expression of phase I and phase II metabolic enzymes compared to undifferentiated basal cell populations (Figure 3-5). Particularly phase II metabolic enzymes (GSTA1, GSTA2, ALOX15) showed higher expression in PneumaCult\textsuperscript{TM}-ALI compared to DCI differentiated cells, whereas CYP2A6 and CYP2F1 mRNA levels were slightly higher in DCI differentiated cells (Figure 3-5). These results represent mRNA levels and may not precisely reflect protein levels within cells. As the presence of mRNA strongly indicates protein expression, while the absence of mRNA does not indicate an absence of protein, there is a confidence that increased metabolic gene expression upon differentiation translates to functional metabolic activity within these cells.

![Figure 3-5](image.png)

**Figure 3-5 Differentiation effects on metabolic enzyme expression in primary airway epithelium.** RPKM values of significantly differentially expressed phase I (A) and phase II (B) metabolic enzyme expression (N=6) between basal cells and 3 weeks differentiation with DCI or PneumaCult\textsuperscript{TM}-ALI medium from RNA-sequencing analysis. Data are presented as mean RPKM value ± SEM.
A comparison of human small airway brushing transcriptome to other airway regions revealed a selection of marker genes (Hackett et al., 2012). Analysis of levels of these genes between different media conditions in our datasets demonstrated that several genes are upregulated in DCI (SCGB1A1, SCGB3A1), whereas others are more highly expressed in PneumaCult™-ALI differentiated cells, including Tubulin Polymerization Promoting Protein Family Member 3 (TPP3) (Figure 3-6). These results further indicate DCI differentiated AEC show a more secretory small airway phenotype, whereas PneumaCult™-ALI differentiated cells represent a more ciliated phenotype.

Figure 3-6 Expression of top 10 small airway enriched genes in primary airway epithelium. Log2 RPKM values from RNA-sequencing analysis in Basal, PneumaCult™-ALI and DCI differentiated cells (N=6), genes represent top 10 genes preferentially expressed in human small airway epithelium, obtained from Hackett et al. (2012).
3.3. Differentiation of iPSC towards airway epithelial cells

Induced pluripotent stem cell lines SBAD2 and SBAD3 (iPSCs) were differentiated using several protocols (section 2.1.3) in order to successfully establish a small airway epithelium model at the ALI. The first approach involved a protocol with an initial 2D endoderm differentiation step (iPSC airway protocol 1) (Mitchell et al., 2017). Differentiation toward DE was observed from increased FOXA2 and downregulation of pluripotency marker OCT4 expression (Supplemental figure 9-4). However, there was clear cell death after 5 days as indicated by cellular detachment. Further investigation indicated high levels of oxidative stress with upregulation of NQO1 expression (Supplemental figure 9-4). This protocol was not pursued.

The second approach (iPSC airway protocol 2) used a protocol developed by the Snoeck group (S. Huang et al., 2015) (Supplemental figure 9-5). Although differentiation resulted in progression through DE development and production of LP-like cells expressing lung progenitor / basal cell markers NKX2.1 and TP63, this protocol had limited feasibility, as 3D differentiation in early stages showed limited reproducibility between replicates (data not shown). Furthermore, LP cells could not be successfully frozen or further differentiated on ALI (data not shown), limiting feasibility of this approach.

As 3D endoderm differentiation resulted in decreased reproducibility of results due to critical points in trypsinization of EBs, a further 2D endoderm differentiation protocol developed for hepatic differentiation (Roelandt et al., 2013) was then explored (iPSC airway protocol 3) (Figure 3-7A). In contrast to iPSC airway protocol 1, this protocol did not result in substantial cellular death after initial endoderm differentiation and could be further differentiated to basal-like single cells (Figure 3-7B). qPCR analysis showed that differentiation resulted in downregulation of pluripotency marker OCT4 after AFE differentiation stages and upregulation of endoderm marker FOXA2, LP marker NKX2.1 and basal cell marker TP63 after LP differentiation (Figure 3-7C). Furthermore, slight increases in club cell marker SCGB1A1 and secretory marker MUC5AC expression were observed in LP cells (Figure 3-8C).
Figure 3-7 iPSC airway lung progenitor differentiation. iPSC were differentiated to airway basal-like cells via anterior foregut endoderm (AFE) and lung progenitor (LP) as displayed (A). Morphological differences (SBAD2) upon differentiation are shown as microscopy images (100x) (B). RT-PCR mRNA expression changes (N=2, SBAD2, SBAD3) of selected genes upon differentiation are shown as fold change over undifferentiated iPSC, corrected for GAPDH expression (mean ±SEM).
LP cells derived from iPSC protocol 3 could be expanded in a proliferation medium containing Ri, A83-05, BMS234 and EGF, allowing LP cells to be passaged and be frozen down as “basal” cells expressing higher levels of LP marker NKX2.1 and basal cell marker TP63 compared to undifferentiated iPSC (Figure 3-8). Furthermore, the endoderm marker FOXA2 showed an initial increase in expression but decreased upon basal cell passages. LP marker NKX2.1 and club cell marker SCGB1A1 slightly increased in expression upon progressive basal cell passages. Together these results indicate that further passage of basal-like cells may result in increased LP differentiation.

*Figure 3-8 iPSC airway basal cell proliferation.* iPSC-derived airway basal-like cells were maintained in culture medium for 40 days. RT-qPCR analysis of selected genes during basal cell proliferation over 4 passages (N=2, SBAD2 and SBAD3) shown as fold change over undifferentiated iPSC, corrected for GAPDH expression (mean ±SEM).
These basal-like cells were further differentiated towards small airway epithelium on ALI using DCI and PneumaCult™-ALI differentiation as used in section 3.2. Differentiation resulted in morphological changes (Figure 3-9A) and a tight epithelial layer for PneumaCult™-ALI but not DCI differentiated cells (Figure 3-9B). qPCR analysis showed that basal-like, PneumaCult™-ALI and DCI differentiation cells resulted in a downregulation of pluripotency markers OCT4 and SOX2 mRNA levels. Basal cell markers TP63, KRT14 and club cell marker SCGB1A1 were significantly upregulated in DCI differentiated cells, whereas CYP26A1 was shown to be upregulated in PneumaCult™-ALI differentiated cells. Neither cell type showed upregulation of ciliated cell marker FOXJ1, or alveolar cell marker AQP5 expression compared to undifferentiated iPSC. Although PneumaCult™-ALI differentiation of iPSC basal-like cells did not show an upregulation of epithelial cell marker expression, this medium showed repeatability with regards to monolayer formation, whereas DCI differentiation from basal cells did not form a monolayer in every replicate (data not shown). Furthermore, differentiation of primary basal SAEC cells resulted in an upregulation of genes such as SCGB1A1 with fold change values ranging between 1000-10000 as measured by qPCR (Supplemental figure 9-3). In comparison, DCI differentiation of iPSC cells showed a SCGB1A1 expression fold change value of several orders of magnitudes lower using the same method (Figure 3-9C), further illustrating the lack of mature differentiation within the iPSC models. Therefore, iPSC small airway epithelium models require further optimization to be applicable to in vitro toxicity testing.
Figure 3-9 iPSC airway differentiation on air liquid interface. iPSC-derived airway basal-like cells were differentiated on air-liquid interface (ALI) using DCI or Pneumacult™ differentiation media for 3 weeks, as shown morphologically using microscopy images (100x) (A). The integrity of the cellular layer was determined using transepithelial electrical resistance (TEER) measurements in Basal (day 0) DCI (day 21) and Pneumacult™-ALI (day 21) differentiated iPSC, data is shown as mean ± SEM (N=3, significance shown as *, P<0.05) (B). RT-qPCR analysis of selected genes upon ALI differentiation using DCI and Pneumacult™ media was performed and shown as fold change over undifferentiated iPSC, corrected for GAPDH (N=3, SBAD2, significance shown as * P<0.05, ** P<0.005, *** P<0.0005) (C).
3.4. Characterisation of CD34+ HSC derived macrophages and dendritic cells

Human primary bone marrow derived CD34+ HSC from three separate donors were differentiated to macrophages (MC^{cd34}) and dendritic cell (DC^{cd34}) lineages according to protocols outlined in Figure 3-11A and section 2.1.6. MC^{cd34} differentiation was induced by a combination of IL-6, M-CSF, GM-CSF and TGF-β, while DC^{cd34} differentiation was driven by FLT3L, SCF, GM-CSF and IL-4. Morphological differences were observed between the two cell populations, with MC^{cd34} showing a more adherent and elongated profile compared to a more non-adherent state in DC^{cd34} cultures (Figure 3-10B). MC^{cd34} differentiation over time was analysed using qPCR of specific markers (Supplemental figure 9-6) and showed a temporal increase in macrophage specific genes (CD14, CHI3L1, MARCO and MRC1) upon differentiation and a decrease in CD34 mRNA levels after 3 weeks of differentiation. RNA-sequencing was used to further characterise the mRNA profiles of CD34+ cells, MC^{cd34} and DC^{cd34} (Figure 3-10C). Comparison of differentiated MC^{cd34} and DC^{cd34} cultures to CD34+ stem cell cultures demonstrated a reduction of CD34 expression, indicating a reduction in stem cell population. Among the most highly differentially expressed genes in MC^{cd34} compared to CD34+ cells were CHI3L1, CD14 and MARCO, similar to qPCR analysis. Similarly, DC^{cd34} demonstrated an increased expression of CHI3L1, CD86 and CD1E compared to CD34+ cells, the later markers important for antigen presentation a key function of dendritic cells. A comparison was also performed to investigate differences between MC^{cd34} and DC^{cd34} cultures (Figure 3-10C; right panel). This revealed differential expression of macrophage (e.g. MARCO, CD163, ORM1) and dendritic cell (e.g. CD1B, CD1E) markers within MC^{cd34} and DC^{cd34} cultures respectively. Visualisation of differentially expressed genes as a heatmap (Figure 3-10D) further shows similarities (e.g. MRC1, MMP9 and MMP12 upregulation and CD34 downregulation) and differences between MC^{cd34} and DC^{cd34} cultures. Interestingly there was a reduction in several plasma immunoglobulin and globin genes such as IGKC and
IGLC2 upon myeloid differentiation (Figure 3-10D), indicating the presence of mixed stem cells or pluripotency potential towards lymphoid lineages within the CD34+ stem cell population.

**Figure 3-10** Characterisation of CD34 macrophage and dendritic cell differentiation using RNA-sequencing. CD34+ hemopoietic stem cells were differentiated towards macrophages (MC_{CD34}) and dendritic cells (DC_{CD34}) (A). Morphological differences are shown using microscopy images (B). CD34, MC_{CD34} and DC_{CD34} cultures (N=3) were examined for gene expression and significantly differentially expressed genes (Log₂ fold change > 2 and RPKM P-value <0.05) are highlighted in red in volcano plots (C). Selected significantly differentially expressed genes are shown in a heatmap (log₂ RPKM values) (D). Bar graphs further visualize RPKM values (mean ± SEM) of selected differentially expressed genes from RNA-Sequencing analysis (N=3) (E)
Single cell transcriptome and surface marker antibody sequencing was performed using the BD Rhapsody platform to further characterize the MC\textsuperscript{cd34} and DC\textsuperscript{cd34} cultures differentiated according to Figure 3-10A. Single cell sequencing allows for the identification of sub-populations within the cultures. Highly dispersed genes as identified by RNA-Sequencing were used to create a highly dispersed marker population (ScSeq (117) and AbSeq (9)) from which UMAP dimensionality reduction plots of single cells were generated (Figure 3-11A). Both MC\textsuperscript{cd34} and DC\textsuperscript{cd34} populations were analysed together by single cell sequencing to reveal similarities and differences between the differentiation trajectories. There was a complete separation of both cell types (Figure 3-11A, left panel) further confirmed as macrophage and dendritic cell lineages by macrophage marker (CD14 and CD163) and dendritic marker (CD1A and CD1C) antibody staining respectively (Figure 3-11B). k-means clustering using selected genes and antibodies as define above was used to define sub-populations of cells and mapped onto the UMAP plot (Figure 3-11A, right panel). Five subpopulations of cells were subsequently identified and analysed for whole genome differential expression within the dataset to fully characterise their likely phenotype. The top cluster specific genes were visualised in a heatmap (Figure 3-11C).

Calls were made as to cell phenotype as follows. 1) CD34\textsuperscript{+} polymorphonuclear (PMN) – like cells express a higher level of neutrophil markers such as LTF, MPO and AZU1. 2) CD34\textsuperscript{+} immature macrophages (iMC) express macrophage markers such as APOE and SPP1, but to a lower extent compared to CD34\textsuperscript{+} macrophage (MC) – like cells. Further evidence for an immature phenotype is indicated by higher levels of CD14 and CD163, more highly expressed on monocytes from which macrophages develop. 3) CD34\textsuperscript{+} MC-like cells show high expression of macrophage markers MMP9, SPP1, CHI3L1 and APOE which indicates that these cells are more mature macrophages compared to the CD34\textsuperscript{+} iMC. 4) CD34\textsuperscript{+} immature dendritic cells (iDC) and 5) dendritic (DC)-like cells cluster closely together and are only separated by a higher expression of major histocompatibility complex (MHC) related genes (e.g., HLA-DR) in the CD34\textsuperscript{+} DC-like cells.

Further exploration of the differences between populations within a bubble plot (Figure 3-11D) shows that PMN-like cells uniquely express high levels of LTF and MPO, but in a relatively low
portion of the cells within the cluster, indicating a mixed population, this population overlaps MC_{cd34} and DC_{cd34} cultures, indicating it might form a common early point in differentiation for all populations. Furthermore iMC population shows higher expression of CYBB, LYZ and C1QA mRNA. In contrast, MC-like populations showed higher expression of typical macrophage markers such as SPP1, MMP7 and CHI3L1 compared to the other cell types, indicating a more mature macrophage-like phenotype. iDC and DC-like populations did not show substantial differences in gene expression, however antigen presentation related genes such as HLA-DRA and CD74 were more highly expressed in the DC-like clusters, indicating a more mature population of dendritic cells within this cluster.

Overall, the CD34 differentiation to macrophage and dendritic cell populations was successful based on ScSeq characterisation data.
Figure 3-11 Characterisation of CD34+ macrophage and dendritic cell using Abseq and single cell mRNA sequencing. CD34+ derived macrophages (MC\textsuperscript{cd34}) and dendritic cells (DC\textsuperscript{cd34}), differentiated as previously described, were characterized using single cell antibody sequencing. Distribution of cells was visualized using UMAP based on selected genes (102) (A). Anti-bodies were visualized on the UMAP plot (B) and used, in addition to selected genes, to cluster and identify cell populations (A). Population enriched genes were visualized in a heatmap of log\textsubscript{2} count values (C) and average count values as well as % of cells expressing selected genes are shown in a bubble plot (D).
3.5. Characterisation of iPSC derived macrophages and dendritic cells

iPSC (SBAD3) were differentiated towards macrophage (MC\textsuperscript{iPSC}) and dendritic cell (DC\textsuperscript{iPSC}) populations via monocyte (Mon\textsuperscript{iPSC}) intermediary stage (van Wilgenburg et al., 2013) according to the differentiation protocol outlined in Figure 3-12A and section 2.1.4. Morphological evaluation showed unattached cells in the Mon\textsuperscript{iPSC} population and a combination of attached and unattached in the MC\textsuperscript{iPSC} and DC\textsuperscript{iPSC} populations (Figure 3-12B). TempO-Seq targeted gene expression analysis of mRNA levels was performed for both iPSC and CD34\textsuperscript{+} derived macrophage and dendritic cells in order to provide initial characterisation of iPSC differentiation success and relationship to primary cells. Poisson sample distance distribution and PCA dimensionality reduction shows that iPSC and CD34\textsuperscript{+} derived macrophage and dendritic cells do not cluster closely together (Figure 3-12C,D). However, differentiated iPSC cluster differently from undifferentiated iPSC. Indeed, differentiation resulted in downregulation of pluripotency markers including POU5F1 and SOX2, and iPSC monocytes demonstrated expression of monocyte/macrophage markers CD14 and CD163 (Figure 3-12E). Further differentiation to iPSC derived macrophage and dendritic lineages was not reflected by identification of MC and DC markers in the heatmap in Figure 3-12E. It did however reveal an upregulation of mesenchymal cell markers including CCDC80 and COL1A1, indicating an incomplete or divergent differentiation from myeloid cells. Several common macrophage and dendritic cell markers were however absent from the TempO-Seq probe set and indicates this type of analysis is inadequate to fully characterise myeloid cell differentiation. Therefore, qPCR analysis of these common markers was performed and showed significant upregulation of ITGAM (CD11b), ITGAX (CD11c) and MRC1 upon macrophage differentiation in both MC\textsuperscript{CD34} and MC\textsuperscript{iPSC} (Figure 3-13F). However, MARCO was only significantly upregulated in MC\textsuperscript{CD34}, showing a non-significant increase in expression in MC\textsuperscript{iPSC}. The dendritic cell maturation marker CCR7 was significantly upregulated in DC\textsuperscript{iPSC} and not DC\textsuperscript{CD34}. However, ITGAM, ITGAX and MRC1 were significantly upregulated in DC\textsuperscript{CD34} and not in DC\textsuperscript{iPSC} (Figure 3-12F).
Figure 3-12 Characterizing iPSC differentiation towards macrophages and dendritic cells. iPSC were differentiated towards macrophages (MC\textsuperscript{iPSC}) and dendritic cells (DC\textsuperscript{iPSC}) as shown (A). Morphological differences are shown using microscopy images (B). Analysis of mRNA transcripts was carried out using TempO-Seq and shown as Poisson sample distances (C) and PCA-plot (D), comparing iPSC to CD34\textsuperscript{+} derived macrophages and dendritic cells. Top significantly differentially expressed genes (adjusted p-value <0.05 and fold change >2) are shown as log\textsubscript{2} normalized counts of undifferentiated (UND), monocytes (MON), macrophages (MAC) and dendritic cells (DC) (E). RT-qPCR analysis was carried out for selected macrophage and dendritic cell marker genes in iPSC and CD34\textsuperscript{+} derived cells, data are visualized as fold change over undifferentiated iPSC or CD34, corrected for GAPDH, mean ± SEM (N=3). Significance P<0.05 is shown as * (F).
As qPCR analysis indicates some macrophage and dendritic cell specific differentiation within the iPSC populations and TempO-Seq analysis indicated presence of other cell types, single cell Abseq and mRNA sequencing was next performed to provide a more complete analysis of cell types and differentiation success. MC\textsuperscript{iPSC}, DC\textsuperscript{iPSC}, MC\textsuperscript{cd34} and DC\textsuperscript{cd34} cells were included within the same experiment such that direct comparison between cell types could be achieved. Clustering and UMAP dimensionality reduction visualisation of protein level Abseq surface staining levels and mRNA level data were carried out as described in section 2.6.10. These plots clearly separate the different cell differentiation protocols, MC\textsuperscript{iPSC}, MC\textsuperscript{cd34}, DC\textsuperscript{iPSC} and DC\textsuperscript{cd34} (Figure 3-13A, left panel) and cell sub-population clusters using k-means clustering (Figure 3-13A, right panel).

Sub-population clusters were examined for whole genome transcriptomic signatures and visualised in a heatmap (Figure 3-13B). The CD34+ PMN-like, iMC, MC-like, IDC and DC-like populations were discussed in section 3.4. iPSC fibroblast (FB) – like cells express a higher level of LUM, COL3A2 and COL1A1 compared to the other clusters, indicating a fibroblastic or mesenchymal differentiation state. iPSC derived iMC show lower levels of typical macrophage markers (e.g. APOE, MMP9) compared to CD34+ MC-like cells, apart from SPP1. Overall, their expression pattern seems to resemble the CD34+ iMC more closely than the CD34+ MC-like cells.

iPSC derived IDC show a lack of expression of MHC genes (e.g., HLA-DRA), however higher expression of monocyte like markers CD14 and CD163 compared to CD34+ DC-like cells, indicating a more monocyte-like or immature dendritic cell phenotype. However, typical dendritic cell markers such as CD1C are highly expressed within the iPSC IDC population indicating some level of dendritic cell lineage differentiation.

Cell surface protein marker expression is classically used to define immune cell sub-types. Protein levels of the monocyte/macrophage markers CD14 and CD163, and dendritic cell markers CD1C and CD1A, within the ScSeq dataset were detected by Abseq analysis and displayed as a UMAP plot (Figure 3-13C). Quantification of these protein levels (Figure 3-13D) shows that CD14 and CD163 are upregulated in CD34+ iMC, iPSC iMC and CD34+ MC-like cells.
compared to CD34+ PMN-like, iPSC PMN-like and iPSC FB-like cell populations. Furthermore, the levels of these two markers within iPSC iDC are higher than in CD34+ iDC or CD34+ DC-like cells. CD1C and CD1A protein levels were shown to be upregulated within CD34+ iDC and CD34+ DC-like cells, whereas within iPSC iDC only CD1C was highly expressed.

Figure 3-13 Single cell sequencing characterization of iPSC differentiation towards macrophages and dendritic cells. iPSC derived macrophages (MC\textsuperscript{iPSC}) and dendritic cells (DC\textsuperscript{iPSC}), differentiated as previously described, were characterized using single cell antibody sequencing. Distribution of these cells was visualized using UMAP based on selected genes, comparing iPSC to CD34+ derived cells (A). Population enriched genes are visualized on a heatmap of log\textsubscript{2} count values (B). Anti-bodies were visualized on this UMAP plot (C) and used, in addition to selected genes, to cluster and identify cell populations (A). The average levels of antibodies (ABs) within the populations are visualised on bar graphs (Mean ± SEM) (D). Quantification of average mRNA expression levels of selected genes within the cell populations (Mean ± SEM) are visualized on bar graphs (E).
Quantitative transcriptomic analysis was also performed within the Scseq dataset to provide further detail on differentiation profiles and success of the iPSC differentiation process. Overall, expression patterns of differentiated cells were similar between CD34+ and iPSC derived cells for immune cell specific genes such as MMP12, C1QA, MRC1 and FCGR3A mRNA (Figure 3-13E). Furthermore, both CD34+ MC-like and iPSC iMC showed macrophage marker SPP1 and CD14 mRNA upregulation and both CD34+ DC-like and iPSC iDC showed upregulation of dendritic cell marker CD1C mRNA (Figure 3-13E).

As iPSC iMC and iDCs are most similar to CD34 iMC and iDCs, these populations were further compared using volcano plots (Figure 3-14). Comparing iPSC iMC to CD34 iMC shows higher expression of macrophage genes such as MMP12 and SPP1 in the iPSC population and higher expression of other macrophage lineage specific genes such as MARCO and HLA-DRA in the CD34 population. This indicates that, although there are similarities between the populations as previously described, these populations most likely represent different sub-types of macrophages. Similarly, comparing iPSC iDC to CD34+ iDC shows higher expression of dendritic markers such as CD1C and TGM2 in the iPSC population and higher expression of dendritic markers such as HLA-DRA and CD74 in the CD34+ population. However, comparing iPSC iDC to iPSC iMC shows higher expression of macrophages markers such as SPP1, CD14 and CD163 in the iMC population and higher expression of dendritic cell markers such as CD1C, TGM2 and CD1B in the iDC population, indicating that differentiation followed the expected lineages.
Overall, the iPSC differentiation to macrophage and dendritic cell populations was relatively successful based on ScSeq characterisation data.

Figure 3-14 Differences between iPSC and CD34+ derived macrophages and dendritic cells. Significantly up-or downregulated (fold change > 2 and p-value <0.05) genes from single cell sequencing analysis described before, between CD34+ immature macrophages (green) or dendritic cells (orange) and iPSC macrophages (cyan) or dendritic cells (purple) are visualized in volcano plots.
3.6. Discussion

For In vitro models to be applicable for small airway toxicity testing, they require thorough characterization. This chapter covered the optimization and characterization of primary and iPSC small airway epithelial and immune cell models. Thorough characterization of primary airway, macrophage and dendritic cell model serves both as a starting point for compound toxicity testing and as reference for iPSC differentiations.

3.6.1. Primary human small airway epithelial cell differentiation

Human primary small airway epithelial cells were cultured under different media conditions and examined for cell type differentiation status. Under normal conditions within the lung small airway epithelial cells have a higher number of secretory club cells typified by expression of the marker SCGB1A1 (Boers et al., 1999). Two media formulations, DCI and PneumaCult™ displayed the most optimal differentiation as shown by an increase in membrane integrity (TEER value) and increased levels of differentiation marker expression. This was also accompanied by decreased levels of basal stem cell makers in comparison to undifferentiated basal cell cultures. Interestingly, DCI differentiation showed higher levels of club cell specific markers (SCGB1A1, BPIF1A) whereas PneumaCult™-ALI differentiation showed higher levels of ciliated cell markers (FOXJ1, TUB1A1) and the undifferentiated basal AEC cultures showed high levels of basal cell markers (KRT5, KRT14). We can therefore assert with some level of confidence that the three culture conditions represent an undifferentiated basal-like small airway epithelial cell type (basal AEC), a more distal small airway epithelium with higher proportions of club cells (DCI differentiation) and a more proximal ciliated small airway epithelium (PneumaCult™-ALI). Furthermore, PneumaCult™-ALI differentiated cells demonstrated a thicker layer of mucus and a larger amount of functional multiciliated cells (as observed with microscope, data not shown) compared to DCI differentiated cells, which is also observed in more proximal small airways in the human lung (Mercer et al., 1994; Okuda et al., 2018). Furthermore, TEER values were slightly higher in DCI differentiated compared to PneumaCult™-ALI differentiated cells. This was also
previously observed in SmallAir™ model compared to proximal airway cultures differentiated using PneumaCult™-ALI (Bovard et al., 2020) further supporting DCI differentiation towards a more distal phenotype.

In addition, both DCI and PneumaCult™-ALI differentiation resulted in an increase in phase I (e.g. CYP2A6, CYP2F1, CYP4B1) and phase II (e.g. GSTA1, GSTA2, ALOX15) metabolic enzyme gene expression, indicating these differentiated cultures are more suitable for determining the toxicity of compounds that undergo bioactivation or detoxification in airway epithelium. Another study, using primary human bronchial epithelial cells also showed increased levels of e.g. GSTA1, CYP4B1, and CYP2F1 gene expression upon mucociliary differentiation in a pseudostratified epithelial cell layer (Boei et al., 2017). Furthermore, a commercial bronchial airway epithelium model, MucilAir™, also showed metabolic activity of e.g. CYP2A6 as measured by coumarin 7-hydroxylation activity (Baxter et al., 2015). This study also demonstrated that immature differentiated primary bronchial epithelial cells did not show metabolic activity, indicating prolonged differentiation (21-28 days) is required for an organotypic culture (Boei et al., 2017). Gene expression of metabolic enzymes correlated with their activity in mature ALI differentiated models (Boei et al., 2017), which is likely also the case within our models.

Studies utilizing primary SAEC for toxicity studies, generally use commercially available differentiation medium, such as S_ALI from Lonza, to differentiate SAEC to a mature airway epithelium on ALI (Zuo et al., 2018). More recent studies have used a newly developed formulation from StemCell, PneumaCult™-ALI-S differentiation medium specifically developed for small airway differentiation from SAEC. This media, similar to our own DCI formulation displays higher levels of club cell marker SCGB1A1 expression when compared to PneumaCult™-ALI differentiation (Bluhmki et al., 2020; Gindele et al., 2020; Wohnhaas et al., 2021). It is interesting to speculate, whether DCI form part of the active components within PneumaCult™-ALI-S media and whether further improvements towards organotypic could be made in the future through further alterations in media formulation.
Several commercially available models using primary airway epithelial cells from healthy donors and disease conditions are available, for instance, EpiAirway™, a mucociliary tissue model derived from human tracheobronchial epithelial cells (BéruBé et al., 2010) and SmallAir™, derived from distal lung tissue (S. Huang et al., 2017). The EpiAirway™ model was shown to be more resistant to toxicological impacts than cell lines, potentially due to biologically relevant protective mechanisms (Balharry et al., 2008; Zavala et al., 2016). However, the EpiAirway™ cells showed less ciliated and more basal cell types compared to in vivo large airway epithelial cells (Dvorak et al., 2011). SCGB1A1 protein was highly expressed in SmallAir™ and ±15% of cells resembles club cells (S. Huang et al., 2017). There are however major drawbacks of using commercially available models such as cost and limited flexibility in study designs (Hiemstra et al., 2018). Therefore, the use of in house differentiated cultures, has the benefit of flexibility and reduced costs and in our hands basal, DCI and PneumaCult™ protocols offers the opportunity to explore a range of airway cell types and regional differentiation from the same genetic background, offering a more comprehensive assessment of small airway toxicity in vitro. This will be explored in more detail in chapter 4.

3.6.2. iPSC airway epithelium differentiation

Several protocols for iPSC differentiation towards DE, AFE, LP and mature (small) airway epithelium were explored in this chapter. iPSC airway protocol 1 previously described iPSC differentiation towards DE (96% efficiency) and AFE (97% efficiency) (Mitchell et al., 2017; Ninomiya et al., 2015). However, we were unable to replicate this and observed high levels of cell detachment and stress after initial DE differentiation. iPSC airway protocol 2 showed some initial successes in differentiation of SBAD2 and SBAD3 cells to lung LP populations, indicated by NKX2.1 and TP63 expression, however, this differentiation showed limited reproducibility of early differentiation steps (3D endoderm formation). Furthermore, these LP-like cells could not be recovered from cryopreservation or further differentiated on ALI using DCI or PneumaCult™-ALI differentiation medium.
As 3D endoderm differentiation showed limited reproducibility between attempts, another 2D endoderm differentiation protocol originally described for hepatic differentiation (Roelandt et al., 2013) was used for initial DE differentiation (iPSC airway protocol 3). Unlike, the protocol described by Mitchell et al. (2017), this did not result in a high level of oxidative stress or cell detachment and allowed further differentiation to lung progenitor cells as described by Huang et al. (2015). This could be due to addition of the antioxidant L-ascorbic acid within the 2D endoderm differentiation protocol as described by Roelandt et al. (2013). However, as multiple compounds are different between the two 2D endoderm differentiation protocols, the precise mechanism of the increased yield in the latter protocol cannot be determined.

The iPSC derived basal-like cells derived using iPSC airway protocol 3 showed increased expression of NKX2.1 and TP63 and low expression of hepatic or neuronal markers (data not shown). These lung progenitor cells could be expanded and successfully recovered from cryopreservation. Furthermore, these basal-like cells formed a monolayer on TransWell inserts and could be differentiated using the DCI and PneumaCult™-ALI differentiation medium on ALI as described for primary small airway epithelium cells. However, DCI differentiation did not form a functional epithelial barrier nor showed reproducibility in differentiation attempts. However, when three inserts that did form a monolayer were analysed using qPCR, they did show upregulation of typical club cell differentiation marker SCGB1A1. In contrast, PneumaCult™-ALI differentiation was more reproducible and resulted in higher TEER values but did not show upregulation of typical small airway differentiation markers. Despite some level of differentiation observed with both media formulations, when compared to primary cells differentiated under the same conditions, the levels of small airway differentiation markers were much lower in iPSC derived cells. This indicates only a small percentage of cells within these cultures as having the capacity to differentiate towards airway cells from a low starting number of appropriate lung progenitor cells. Strategies to purify NKX2.1 positive progenitor cells (McCauley, Alysandratos, et al., 2018), or using NKX2.1 and TP63 dual fluorescent reporter systems using cell sorting analysis has proven successful (Hawkins et al., 2021) in preparing
functional cultures of airway cells. A strategy to purify these progenitors for subsequent differentiation would no doubt have improved the yield.

Apart from progenitor cell purification, optimisation of ALI differentiation medium could also improve iPSC differentiation. Recently, addition of notch inhibition using DAPT between day 10 and 14 to PneumaCult™-ALI differentiation medium was shown to increase the generation of ciliated cells from iPSC derived basal airway cells (Djidrovski et al., 2021). Furthermore, addition of DAPT from day 1 of ALI culture was observed to increase the ciliated cell marker FOXJ1 in iPSC derived airway epithelium (Firth et al., 2014). We investigated this strategy in part but initial experiments investigating DAPT and DCI exposure during ALI differentiation of primary SAEC did not show significantly different results (data not shown). Furthermore, addition of a Wnt inhibitor XAV939 after iPSC differentiation towards NKX2.1+ lung progenitor cells has been previously demonstrated to increase club cell marker SCGB1A1 expression within mature airway cultures (McCauley, Alysandratos, et al., 2018). However, XAV939 did not result in increased SCGB1A1 expression within our primary SAEC models either. These pathways were therefore not further explored within our iPSC small airway differentiation strategies. With more time however, these approaches could provide future directions to improve iPSC differentiation towards a mature small airway epithelium.

Overall, these results indicate that additional work is required with regards to optimization of iPSC differentiation towards a mature airway epithelium on ALI and that development of a small airway epithelial cell differentiation ALI model from iPSC for toxicity testing remains to be established.
3.6.3. CD34+ HSC macrophage and dendritic cell differentiation

CD34+ HSC were differentiated towards macrophage (MC$^{\text{cd34}}$) using IL6, M-CSF, GM-CSF and TGFβ and dendritic cell (DC$^{\text{cd34}}$) populations using FL3TL, SCF, GM-CSF and IL4, recapitulating in vivo differentiation trajectories. Bulk mRNA-Sequencing analysis showed a loss of CD34 expression upon macrophage and dendritic cell differentiation accompanied by an increase in MRC1, MMP9 and MMP12 expression, indicating a common myeloid differentiation trajectory. Furthermore, MC$^{\text{cd34}}$ cells showed increased expression of typical macrophage markers MARCO, CD163, CAMP ORM1 whereas DC$^{\text{cd34}}$ cells showed increased expression of dendritic cell specific markers CD1B, CD1C, CD1E, and CLEC10A. Single cell Abseq analysis for cell surface protein levels confirmed these differentiation trajectories using CD14 and CD163 for the MC$^{\text{cd34}}$ population and CD1A and CD1C for the DC$^{\text{cd34}}$ population. Exploring the single cell mRNA expression patterns within the cultures revealed an additional common PMN-like cell population expressing MPO, LTF and ELANE could be identified within both MC$^{\text{cd34}}$ and DC$^{\text{cd34}}$. While common to both, it is unlikely a common progenitor but a differentiation trajectory that happens in both protocols.

Furthermore, iMC and MC-like populations could be detected within the MC$^{\text{cd34}}$ culture, with MC-like cells showing higher expression of mature macrophage markers APOE, SPP1, CHI3L1 compared to iMC which express high levels of monocyte markers CD163, CYBB and LYZ, indicating a more monocyte-like cell population in the iMC population. However, the iMC population also expresses low levels of CLEC7A and a combination of CD163 and CLEC7A expression is commonly used as a marker for anti-inflammatory M2 Macrophages (Abdelaziz et al., 2020; Roszer, 2015). Overall, this population likely forms a more immature inflammatory macrophage population. The MC-like population demonstrated high levels of mature macrophage marker expression, however, showed lower levels of typical human alveolar macrophage markers such as HLA-DR, CD163 and CD206 (Nayak et al., 2018). However, this population expressed high levels of two matrix metalloproteinases, MMP7 and MMP9, which are expressed in human alveolar M2 macrophages (Hussell & Bell, 2014) and have been shown
to be induced upon addition of LPS stimuli in monocyte-derived macrophages (Moin et al., 2021). Furthermore, we have shown that these macrophages cluster most closely to monocyte-derived macrophage-like cells differentiation using M-CSF (Fransen & Leonard, 2021). As these inflammatory monocyte-derived macrophages play an important role in immune processes within diseased airway epithelium, as observed in for instance asthma (Jiang & Zhu, 2016), MC<sup>cd34</sup> in vitro culture, while possessing macrophage functionality may be better aligned at investigating responses in the context of inflammatory disease conditions. An in vitro study utilizing CD14+ monocyte derived macrophages has previously shown increased inflammatory phenotypes following in vitro differentiation trajectories using GM-CSF compared to M-CSF (Hohenhaus et al., 2013). Polarized in vitro macrophages using GM-CSF (M1) or M-CSF (M2) can however undergo reversible functional changes depending on the micro-environment (Xu et al., 2013), as in our study a combination of GM-CSF and M-CSF was used, modulating the relative concentrations could improve differentiation of a more alveolar-like macrophage phenotype. Overall, these results indicate that more efforts are required to differentiate CD34+ cells in a pure alveolar macrophage cell population, for instance, by co-culture with mature airway epithelium on ALI.

In contrast to the macrophage differentiation protocol, iDC and DC-like populations were induced when the DC<sup>cd34</sup> culture protocol was used. These two populations show a largely overlapping gene expression pattern on single cell sequencing analysis, but a slightly higher level of mature dendritic cell markers such as HLA-DR, CD1c, CD74 and CLEC7A within the DC-like population compared to the iDC population. Within the human airways, three dendritic cell populations are known, myeloid DC type 1 (CD1c+HLA-DR+), myeloid DC type 2 (CD141+HLA-DR+) and plasmacytoid DC (CLEC4C+CD123+) (Demedts et al., 2005). Compared to these populations, our iDC and DC-like dendritic cells most accurately reflect a myeloid DC type 1 population as shown by high expression of CD1c and HLA-DR. However, clustering analysis showed that the DC<sup>cd34</sup> culture also resembled monocyte-derived cells, indicating an inflammatory monocyte dendritic cell phenotype (Fransen & Leonard, 2021; Villani et al., 2017).
Indeed, the iDC population showed a higher expression of inflammatory markers such as CCL22 and CCL13, indicating a more inflammatory phenotype. Importantly, monocyte derived dendritic cells have been previously shown to be inadequate representatives of airway resident dendritic cells based on whole transcriptomic profiling (Patel et al., 2017). Further optimization of dendritic lineage differentiation is required to develop an airway resident dendritic cell model observed within the normal human lung. To the best of our knowledge, no in vitro model for airway specific dendritic cell populations has been developed so far. Factors released by airway epithelium have been shown to reduce the pro-inflammatory phenotype in dendritic cells, indicating that the use of co-cultures or the addition of relevant stabilising factors could prevent the inflammatory phenotype (Gallucci et al., 1999).

Overall, these results indicate a mixed population of mature and immature inflammatory myeloid cell types within the MC\textsuperscript{cd34} and DC\textsuperscript{cd34} cultures. Therefore, further optimization of culture medium or cell sorting to improve the organotypic nature of the cultures could further increase their applicability to toxicity studies. As an assessment method for broad toxicological responses attributable to myeloid lineage macrophage and dendritic cells, these CD34 derived cultures are considered a useful starting point.

3.6.4. iPSC derived macrophage and dendritic cells show similarities and differences compared to CD34+ derived counterparts

iPSC derived monocytes were differentiated towards macrophage (MC\textsuperscript{iPSC}) and dendritic cell (DC\textsuperscript{iPSC}) populations using the same final differentiation media supplements used for CD34+ differentiation. We have used TempO-Seq transcriptomic analysis, qPCR and single cell mRNA and Abseq analysis to characterize these cell populations. TempO-Seq analysis showed that the iPSC derived monocyte-like cell population expressed high levels of monocyte markers CD14 and CD163 compared to the other cultures. Further differentiation towards MC\textsuperscript{iPSC} and DC\textsuperscript{iPSC} populations resulted in a loss of CD14 and CD163 expression, which would be expected but there was also an increase in several genes including mesenchymal differentiation markers (COL12A1,
CCDC80) indicating broad mixed cell type populations. Importantly, however, typical myeloid differentiation markers such as MRC1, ITGAM, ITGAX, MARCO and CCR7 were not included within the TempO-Seq probe set and therefore explored using qPCR methods. These showed an increase in MRC1, ITGAM and ITGAX expression in MCiPSC, comparable to MCEx34 and an increase in the dendritic cell maturation marker CCR7 within the DCiPSC cultures.

Single cell Abseq of cell surface protein markers was also used to further characterise the iPSC derived populations. This demonstrated FB-like, PMN-like, iDC and iMC populations. The presence of the FB-like population expression COL1A1, COL3A2 and CCDC80 shows that iPSC derived monocyte-like cells did not differentiate into pure macrophage and dendritic cell populations but also resulted in an increased level of mesenchymal cell differentiation lineages compared to undifferentiated iPSC. As these mesenchymal cell populations were not observed within the CD34+ differentiated macrophages and dendritic cells, the iPSC derived immune cells form more heterogenous populations in comparison. Progenitor cell sorting is a possible strategy to improve the protocol and remove contaminating mesenchymal/fibroblastic cells. A recent study however, using CD14+ microbeads sorting also showed a significant higher level of collagen expression (e.g., COL1A2 and COL3A1) in iPSC derived macrophages and dendritic cells as compared to monocyte derived primary cells (Monkley et al., 2020). This would indicate an additional cell sorting step or culture medium optimization may still be required to fully prevent mesenchymal differentiation within the iPSC populations. Another possibility, although unproven is the functional significance of this mesenchymal differentiation from monocyte like precursors in conditions of tissue injury and infection, a capacity perhaps lost in more mature progenitor CD34+ populations.

iPSC derived iMC and iDC populations showed similar levels of MMP12, C1QA, MRC1 and FCGR3A gene expression compared to primary counterparts, indicating appropriate immune lineage differentiation. Furthermore, iPSC iMC showed similar levels of SPP1 and CD14 mRNA compared to primary CD34 iMC and MC-like cells, whereas iPSC iDC showed similar levels of
CCL18 and CD1C compared to primary CD34 iDC and DC-like cells. Interestingly, the iPSC derived iMC and iDC populations were grouped more closely to their primary CD34 iMC and iDC on an UMAP plot, indicating these iPSC populations represent more immature immune cells. However, iPSC iMC showed slightly higher levels of MMP12, SPP1 and CD14 expression compared to CD34 iMC. Similar findings have been observed previously when comparing iPSC derived macrophages to primary monocyte derived macrophages. This study found increased MMP12 and CD14 expression within the iPSC compared to the primary macrophages (Monkley et al., 2020), indicating the presence of a more monocyte-like immature macrophage phenotype within the iPSC population. Furthermore, both dendritic cell marker CD1c and macrophage / monocyte markers CD14 and CD163 were more highly expressed in iPSC iDC compared to CD34 iDC, as shown by antibody and mRNA expression. This is consistent with iPSC iDC displaying a more immature monocyte derived dendritic cell population (Collin & Bigley, 2018). As CD1c is also used as a marker for inflammatory monocyte derived dendritic cells (Villani et al., 2017), the iPSC iDC may also capture a more inflammatory phenotype. Further work is needed to clearly define these differentiated cell types. Furthermore, iPSC iDC showed lower levels of major histocompatibility complex (MHC) related genes compared to CD34 DC-like or iDC cells and higher levels of CCR7. Indeed, it was also observed that increased CCR7 and decreased MHC related gene expression was present in iPSC derived dendritic cells compared to primary monocyte derived counterparts (Monkley et al., 2020).

Overall, published studies describe some successes in iPSC differentiation towards macrophages and dendritic cells (Monkley et al., 2020; van Wilgenburg et al., 2013), which we also observed in our own work. It still remains however, that iPSC derived macrophage and dendritic cells likely represent a more underdeveloped and embryonic phenotype when compared to primary counterparts. Indeed, our study compared the iPSC and CD34+ derived macrophages and dendritic cells on a single cell level and showed both similarities and differences, indicating appropriate lineage differentiation but different subtypes or levels of maturity of immune cells. A limitation to this work is that initial steps of the protocol only demonstrated feasibility for
SBAD3 and not SBAD2 iPSC line, indicating additional efforts may be required to use this protocol for other iPSC lines. Overall, additional efforts are required to differentiate iPSC into more mature and purer macrophage and dendritic cell cultures, for instance by using cell sorting techniques to allow for more pure monocyte populations. Furthermore, co-culturing of mature iPSC derived macrophages and dendritic cells with primary or iPSC derived airway epithelial cells could aid in guiding differentiation towards more airway resident phenotypes. However, these iPSC (SBAD3) derived iDC and iMC described here are still relevant for in vitro toxicity testing.

3.6.5. Conclusions and future directions

This chapter described the optimization and characterization of several in vitro models with the aim to reflect small airway phenotypes for toxicity testing. We established primary airway epithelium cells protocols for undifferentiated basal AEC, a DCI differentiated inflammatory distal small airway and a PneumaCult™-ALI differentiated proximal small airway epithelium. Furthermore, CD34+ derived macrophages (MC<sup>cd34</sup>) and dendritic cell (DC<sup>cd34</sup>) cultures were also successfully achieved. It is clear however that further work is needed to bring such differentiated cultures closer to in vivo steady state tissues.

iPSC can be obtained from patient-specific populations and edited using genome editing methods, such as CRISPR-Cas9 to develop, for instance, disease specific models. Furthermore, the use of iPSC has the advantage of the possibility to generate several organ models from the same donor origin, allowing future development of donor specific models through co-cultures and advanced in vitro systems (Mertens et al., 2017). The iPSC derived small airway epithelium model requires further optimization to be able to differentiate a mature airway epithelium on ALI, for instance by creating more pure progenitor cells by cell sorting for basal cell (NK2.1 / TP63) markers or optimizing ALI differentiation medium. The iPSC derived macrophage (MC<sup>iPSC</sup>) and dendritic cell (DC<sup>iPSC</sup>) differentiation protocols, however, were successful in generating immature cell type specific cultures and demonstrated feasibility for the SBAD3 but not SBAD2 iPSC line.
Overall, the primary small airway epithelial cell models and CD34+ and iPSC macrophage and dendritic cell models showed promising results and will thus be further examined for toxicological responses in chapters 4 and 5 (Figure 3-15), whereas the iPSC small airway epithelial cell model will not be explored further.

Figure 3-15 Schematic overview of Chapter 3 outcomes
CHAPTER 4: PRIMARY SMALL AIRWAY EPITHELIAL CELL RESPONSES TO CHEMICAL EXPOSURES
4.1. Introduction

Initial plans for this PhD project were to compare the toxicological responses of iPSC derived airway epithelium with primary counterparts to establish their usefulness for small airway toxicity testing. Unfortunately, however, and as discussed in the section 3.3, we were unable to establish an adequate iPSC derived airway epithelium model at the ALI. We did however recognise that primary small airway in vitro models to date, do not take into consideration the range of differentiation states within the bronchioles, when considering specific chemical toxicities that can be cell type and thus airway regional specific. Therefore, the aim of this chapter was to explore how such differentiated states representing different cell types and compartments within the small airways respond to chemical insult. Such approaches have the potential to provide a more comprehensive in vitro assessment of small airway toxicity.

Three primary airway epithelial cell differentiation models, all derived from the same small airway basal stem cell population were established as described in section 3.2 and chosen to represent different cellular states and compartments of the small airways. Basal small airway cell cultures (basal AEC) are an undifferentiated proliferative cell state, mainly observed in proximal small airway regions and after injury to the epithelium. DCI differentiated SAEC at the ALI represent a distal small airway-like epithelium, while PneumaCult™-ALI differentiated cells show a more proximal small airway-like phenotype (section 3.2).

Insight into the mechanisms of toxicity of various pulmonary toxicants can also be investigated by comparing responses from all three culture conditions. This is particularly important where cell and tissue type expression of transporters, metabolic genes and other phenotypic properties result in susceptibility to toxicant injury. Differentiation on ALI using DCI or PneumaCult™-ALI media, results in organotypic expression of transporters and phase I/II metabolic enzymes (section 3.2), which can result not only in detoxification of chemicals but also the creation of more toxic metabolites and toxic susceptibilities (Hukkanen et al., 2002).
In terms of small airway susceptibility to toxicant injury, club cells have been highlighted as of particular interest as described in section 2.2.2. For example, cigarette smoke and diesel exhaust exposure have been shown to specifically target small airway club cells (Murphy et al., 1999; J. Yang et al., 2017). In addition to these environmental complex chemical mixtures, numerous other chemical exposures can damage the lungs and small airway regions. These include pharmaceuticals such as amiodarone (AM), busulfan (BUS) and the herbicide paraquat (PQ). Understanding the mechanisms of toxicity of these and other compounds is important not only to understand how these exposures may be managed better, but also to understand small airway susceptibility features and the refinement of in vitro airway toxicity models.

4.1.1. Amiodarone

Approximately 5-15% of patients that are prescribed the class III anti-arrhythmic drug AM develop pulmonary toxicity (Camus et al., 2004; Martin & Rosenow, 1988). Damage is mainly observed for distal airway regions including the alveolus and small airways. Specific cellular and regional targets for injury and dysfunction are not fully established but it is thought to involve distal airway epithelial cells including bronchiolar, alveolar type II cells, immune cells, and endothelial cells (Papiris et al., 2010). Difficulty in understanding the precise mechanisms of toxicity are compounded by a patient population that is already susceptible to pulmonary disease (Feduska et al., 2021). In vitro alveolar cell line and in vivo hamster models have indicated that epithelial to mesenchymal transition plays a role in AM induced pulmonary fibrosis, as shown by an increased levels of TGFβ upon AM exposure (Card et al., 2003; Weng et al., 2020). Furthermore, accumulation of AM within fatty tissues and its relatively long half-life are suggested as important factors for injury (Kuhlman et al., 1990). Within the lung, accumulation within foamy macrophages in alveolar spaces is observed in AM treated patients with and without observed pulmonary toxicity, further supporting a relationship to lipid accumulation (J. Myers et al., 1987; Santucci et al., 2016). At a molecular level, toxicity is thought to involve accumulation of phospholipids, free radical induced cytotoxic injury or altered immunological reactions (Papiris et al., 2010; Reasor & Kacew, 1996). Whether the molecular
mechanism of toxicity within airway epithelial cells and immune cells involves modulation of lipid accumulation remains to be fully explored. It can be concluded that the overall mechanism of action of AM induced pulmonary toxicity remains poorly understood and requires further investigation.

4.1.2. Busulfan

BUS (1,4-butanediol dimethanesulfonate) is a cytotoxic alkylating chemotherapeutic agent used prior to hematopoietic stem cell transplantation to treat hematologic malignant diseases. However, prolonged or high-dose BUS treatment is known to cause fibrotic lung disease in small airways and alveolar spaces, also referred to as “busulfan lung” (Burns et al., 1970; Oliner et al., 1961; Pearl, 1977). Indeed, pulmonary disease was observed in 21% of all childhood cancer survivors after 35 years of follow-up (Kasteler et al., 2018), which could be at least partially attributed to BUS exposure.

BUS contains two methane sulfonate groups that can be hydrolysed to produce two reactive carbonium ions that can alkylate and damage DNA molecules (A. Myers et al., 2017). BUS interacts with guanosine and was shown to cause lesions in G-A and G-G sequences and cross-links in G-A sequences in DNA chains in leukaemia HL-60 cells in vitro, resulting in decreased proliferation and viability (Iwamoto et al., 2004). Furthermore, BUS can bind to the cysteine molecules of histones resulting in DNA-protein binding (Hartley & Fox, 1986). Glutathione (GSH) conjugation of BUS via Glutathione S-Transferase (GST) enzymes is the first step in BUS metabolism and is suggested to result in toxic BUS metabolites, increased active cellular transport and/or a disruption of intracellular redox equilibriums due to GSH depletion and thereby an increased level of oxidative stress (Hassan & Andersson, 2013; A. Myers et al., 2017). After GSH conjugation BUS might be further metabolized towards known and unidentified metabolites, which may play a role in the pulmonary toxicity that is observed after BUS treatment (A. Myers et al., 2017). Evidence to support this mechanism of toxicity comes from a study examining occupational exposure to the BUS downstream metabolite.
tetrahydrothiophene (THT), which was related to the incidence of COPD (Baur & Bittner, 2009). The precise mechanism of BUS induced pulmonary toxicity is currently unknown but thought to be related to injury and inflammation of distal airway epithelial cells (Littler et al., 1969). Pulmonary BUS toxicity has not yet been studied extensively in vitro, therefore, mechanisms of cellular susceptibility are largely unknown (A. Myers et al., 2017). The use of small airway in vitro models has the potential to help progress this open question, through exploration of mechanisms and pathways of cellular responses.

4.1.3. Paraquat

PQ (1,1'-dimethyl-4,4'-bipyridinium dichloride) is a chemical herbicide known to be highly toxic to humans (Dinis-Oliveira et al., 2008; Subbiah & Tiwari, 2021). Upon ingestion, PQ uptake results in systemic toxicity manifesting as acute mortality due to multiple organ failure and delayed mortality due to pulmonary fibrosis (Subbiah & Tiwari, 2021). Within the human airway epithelium, small airway club cells and alveolar type I and II cells are suggested to be particularly susceptible to PQ exposure, due to a higher level of polyamine transporters (Dinis-Oliveira et al., 2008; Hoet & Nemery, 2000). However, while in vitro studies have demonstrated competitive inhibition of PQ uptake by polyamines in distal AEC (Hoet & Nemery, 2000; Silva et al., 2015), some in vivo work suggest unknown additional mechanisms may also be involved (Dunbar et al., 1988).

The molecular mechanism of toxicity of PQ comprises the NADPH-dependent reduction of PQ$^{2+}$ ions as an initial step in redox cycling, generating superoxide anions (Figure 4-1). Reduction of PQ to generate superoxide within human cells requires enzymatic activity, mediated via mitochondrial complex I (Cochemé & Murphy, 2008), NADPH oxidases (NOX1-2) (Cristóvão et al., 2009), nitric oxide synthase (NOS) (Day et al., 1999), and/or cytochrome P450 oxidoreductase (POR) (Han et al., 2006). Redox cycling catalysts may be different in different cell types. A CRISPR-based knockout library screen has identified POR as the primary mediator for
PQ induced superoxide generation and consequent toxicity in immortalized human T lymphocyte cells (Reczek et al., 2017).

![Schematic overview of paraquat redox cycling toxicity](image)

**Figure 4-1** Schematic overview of paraquat redox cycling toxicity. PUS; polyamine uptake system, SOD; superoxide dismutase, CAT; catalase, GPx; Glutathione peroxidase, Gred; Glutathione reductase, HMP; hexose monophosphate pathway, HWR; haber-weiss reaction, FR; fenton reaction. Adapted figure (Dinis-Oliveira et al., 2008).

As a consequence of redox cycling, PQ induced generation of reactive oxygen species (ROS) results in cellular injury due to oxidation of lipids, proteins or DNA (Dinis-Oliveira et al., 2008). Furthermore, the oxidation of NADPH results in a reduction of the levels of this essential electron donor within the lung. Inflammatory responses due to the activation of redox-sensitive signalling pathways such as NF-κB and mitogen-activated protein kinase (MAPK) also play an important role in PQ induced pulmonary toxicity (Subbiah & Tiwari, 2021). Despite, the advances in understanding PQ toxicity, uncertainty remains including precise mechanisms of airway epithelial regional and cell type specific susceptibilities. These processes are important to understand why the lung is particularly sensitive to this toxicant.
4.1.4. Overall aims

The aims in this chapter were to use different differentiated primary airway epithelial cell models established in section 3.2 to evaluate their use for \textit{in vitro} small airway toxicity testing. It was also the aim to investigate the molecular mechanisms of toxicity for chemicals where little information exists for airway specific effects. The comparison of differentiation dependent effects is a key component in addressing airway epithelial regional specific effects. To address these aims, measurements of toxicity were used, including standard Resazurin and LDH, together with mRNA changes using PCR and RNA-Seq.

**Specific Aims:**

- Establish \textit{in vitro} model differentiation dependent and regional airway specificity effects, through analysis of responses to known small airway toxicants CSE and DEPE.
- Identify basal AEC and DCI and PneumaCult\textsuperscript{TM}-ALI differentiation specific responses to understudied airway toxicants (AM, BUS and PQ).
- Understand the mechanisms through which airway cell types respond to toxicant exposure, further revealing molecular susceptibilities important for regional specific airway epithelial toxicity.

Figure 4-2 Schematic overview of Chapter 4
4.2. Airway epithelial differentiation and cell type dependent responses to toxicant exposure

To investigate whether undifferentiated basal AEC, DCI differentiated cells and PneumaCult™-ALI differentiated cells respond differentially to toxicant exposures, all three cultures were exposed (Quasi ALI) apically (50 µL) to cigarette smoke extract (CSE) and diesel exhaust particles (DEPE). These toxicants have previously been shown to specifically target the small airway epithelium and club cells (J. Yang et al., 2017). Cells were exposed for 24 hours to allow for comparisons within the in3-MSCA-ITN project. Apical CSE (4 µg/cm² and 20 µg/cm²) and DEPE (4 µg/cm² and 20 µg/cm²) exposures were initially tested for 24 hours in basal (N=2) and DCI differentiated (N=3) AEC models. 4 µg/cm² CSE and DEPE exposures did not significantly alter CYP1A1 mRNA levels in either model, whereas 20 µg/cm² CSE and DEPE exposures resulted in significantly increased levels of CYP1A1 mRNA in both models (data not shown). Therefore, only 20 µg/cm² CSE and DEPE exposures were further explored in basal, DCI and PneumaCult™-ALI differentiated AEC models.

4.2.1. Impact of CSE and DEPE on primary airway epithelial cell viability and cytotoxicity levels

Figure 4-3A shows that both resazurin reduction (cell viability) and supernatant LDH levels (necrotic injury) were not significantly altered upon 24 hours of apical CSE (20 µg/cm²) or DEPE (20 µg/cm²) exposure in basal, DCI or PneumaCult™-ALI differentiated cells. Apical DEPE but not CSE exposure showed a non-significant decrease in resazurin reduction in basal SAEC cultures. Furthermore, both CSE and DEPE exposures resulted in a non-significant decrease in resazurin reduction in PneumaCult™-ALI differentiated cultures. Supernatant LDH levels were not increased upon CSE or DEPE exposure in any of the airway epithelial cell models at the tested concentration. As gene expression changes involved in stress response pathways typically precede changes in cytotoxicity or viability levels, the same concentration of 20 µg/cm² was also used to explore changes in mRNA.
Figure 4-3 Differentiation effects on responses to cigarette smoke extract (CSE) and diesel exhaust (DEPE). Undifferentiated basal and DCI or Pneumacult™-ALI differentiated cells were exposed apically to 20 µg/cm² CSE or DEPE in small droplets of medium (30 µl) for 24 hours. Viability was measured using resazurin reduction assay (N=4, mean ±SEM) and cytotoxicity was analysed using supernatant LDH measurements (N=4, mean ±SEM) (A). mRNA expression changes of NQO1, SLC7A11 and CYP1A1 upon CSE or DEPE exposure were analysed using RT-qPCR, corrected for GAPDH, and shown as fold change over control (mean ± SEM). Significant differences compared to control values are shown as * (P<0.05) (B).

4.2.2. Primary airway epithelial cell differentiation alters CSE and DEPE effect on NQO1, SLC7A11 and CYP1A1 mRNA expression

Gene expression differences upon apical CSE and DEPE exposures were analysed using qPCR methods. CYP1A1 is involved in the bioactivation of PAHs and nitrosamines in CSE and DEPE and was previously shown to be induced by cigarette smoke exposure, especially in club cells (Chang et al., 2006). Furthermore, NQO1 and SLC7A11 are genes regulated by the NRF2 pathway involved in oxidative stress induced by CSE or DEPE (Shin et al., 2017). Detailed methods are described in chapter 2. NQO1 mRNA levels were significantly upregulated upon CSE exposure in undifferentiated basal AEC, but not in either differentiated culture (Figure 4-3B). SLC7A11 was upregulated upon CSE exposure in DCI differentiated cells, but not the other cell types (Figure
4-3B). Furthermore, CYP1A1 mRNA was induced in all three cell types upon CSE exposure, but average increase was higher in DCI compared to the other two primary airway epithelial cultures (Figure 4-3B). DEPE exposure resulted in a significant upregulation of NQO1 mRNA levels in basal AEC and DCI differentiated cells, but not in PneumaCult™-ALI differentiated cells (Figure 4-3B). SLC7A11 mRNA levels were significantly upregulated upon DEPE exposure in all three cell types, but highest in basal AEC. CYP1A1 mRNA, upregulated in all three models upon DEPE exposure, was induced to a highest level in DCI differentiated cells (Figure 4-3B).

Overall, these results indicate that the three different primary airway epithelial models react differently towards airway toxicants, with basal AEC being more sensitive to oxidative stress as shown by NQO1 and SLC7A11 mRNA upregulation and DCI differentiated small airway epithelial cells more sensitive for CSE or DEPE induced increase in CYP1A1 mRNA levels. CSE exposure was previously shown to induce CYP1A1 expression selectively in SCGB1A1 expressing club cells (Chang et al., 2006) and DCI differentiated SAEC showed the highest levels of SCGB1A1 expression within this study (section 3.2). Therefore, although gene expression changes do not necessarily translate to different levels of protein, the results are consistent with what is understood regarding the toxic specificity for these exposures and the relative toxicological responses relevant for small airway regional effects. These SAEC models will be used to evaluate the differentiation specific toxicities and mechanisms of action of amiodarone, busulfan and paraquat.
4.3. Primary airway epithelial cell responses to amiodarone exposure

As described previously, AM is an anti-arrhythmic drug that has been linked to small airway disease via mostly unknown mechanisms. To explore the effect of AM exposure, cells were exposed systemically (on basolateral side of TransWell inserts) to different concentrations of AM for 24 hours. Toxicological impact was explored by determining supernatant LDH and resazurin reduction levels and gene expression differences were explored using qPCR methods as described in chapter 2.

4.3.1. Airway epithelium differentiation dependent responses to Amiodarone

AM exposure did not significantly affect supernatant LDH or resazurin reduction levels in any of the primary airway epithelial models cultured on ALI after 24h exposure at the tested concentrations (3 µM and 15 µM) (Figure 4-4A). Furthermore, qPCR analysis showed no significant upregulation of selected genes commonly involved in toxicity pathways (DDIT3, MDM2, ATF4, HMOX1, SLC7A11, and NQO1) upon AM exposure at the tested concentrations in any model (Figure 4-4B). However, DCI differentiation showed a slight, non-significant upregulation of DDIT3, MDM2 and HMOX1 mRNA levels, that was not observed in Basal, or PneuMaCult\textsuperscript{TM}-ALI differentiated airway epithelium cells (Figure 4-5B). These results indicate that DCI differentiated cells might be more susceptible to AM exposure compared to the other two models. Furthermore, since only six genes were measured using qPCR methods, AM exposure may have resulted in significant upregulation of other genes. Therefore, 15 µM AM exposure was further investigated for DCI differentiated cells using mRNA-Sequencing as described in section 2.4.
Primary small airway epithelial cell responses to chemical exposures

Figure 4-4 Primary airway differentiation effects on responses towards amiodarone. Airway basal cells and PneumaCult™ and DCI differentiated cells were exposed to amiodarone (3 µM and 15 µM) on the basolateral side to mimic systemic exposure for 24 hours and toxicological assessment was carried out using resazurin reduction and LDH release (N=3) (A). Results are shown as mean ± SEM of fold over control (F.O.C) for LDH release and mean ± SEM of percentage (%) of control for resazurin reduction. mRNA expression changes of selected genes upon amiodarone exposure were analysed using RT-qPCR (N=3), corrected for GAPDH expression, and shown as fold change over control (mean ± SEM) (P-value < 0.05) (B).
4.3.2. Global Transcriptome impact of Amiodarone on DCI differentiated ALI airway epithelial cells

Although qPCR methods did not show significant effects in the selected genes, RNA-sequencing was performed to provide a more complete picture on how airway cells respond to this exposure and to gain insight into the molecular mechanisms and pathways of AM toxicity.

Differential expression analysis of mRNA-sequencing data shows significant upregulation of SCD, INSIG1 and GPNMB, and downregulation of IL17C and CSF3 upon 15µM AM exposure (Figure 4-5A). Comparison to BUS and PQ exposures (further discussed in the following sections) shows that GPNMB and SREBF1 are specific for AM exposures, whereas SCD and INSIG1 RPKM levels are also upregulated upon PQ exposure (Figure 4-5B, C). However, as RNA Sequencing was not performed in AM exposed basal AEC or PneumaCult™-ALI differentiated SAEC, no further regional or cellular comparisons can be made.

Figure 4-5 Amiodarone effects in DCI differentiated primary airway epithelium. Amiodarone exposure (24 hours) in DCI differentiated cells was characterized using RNA-sequencing analysis. Significantly differentially expressed genes (fold change > 2 and FDR p-value <0.05) upon 15 µM amiodarone exposure are highlighted in red on a volcano plot (A) and displayed on a heatmap (B) and bar graphs (C) of log2 RPKM values (AM; 15 µM; Bus 50 µM, PQ 100 µM) (mean ± SEM, N=3) to compare to other chemical exposures.
4.4. Primary airway epithelial cell responses to busulfan exposure

To explore the effect of BUS exposure, the three airway epithelial cell cultures were exposed systemically (on basolateral side of TransWell inserts) to different concentrations of BUS for 24 hours. Toxicological impact was explored using supernatant LDH, resazurin reduction, qPCR and mRNA Sequencing as described in chapter 2.

4.4.1. Airway epithelium differentiation specific responses to busulfan

BUS exposure did not significantly affect supernatant LDH or resazurin reduction levels in any of the primary airway epithelial models cultured on ALI after 24h exposure at the tested concentrations (10 µM and 50 µM) (Figure 4-6A). Furthermore, qPCR analysis showed no significant upregulation of mRNA levels of selected genes commonly involved in toxicity pathways (DDIT3, MDM2, ATF4, HMOX1, SLC7A11, and NQO1) upon BUS exposure in any model (Figure 4-6B), indicating a lack of toxicological response. As only six genes were measured using qPCR methods and BUS treatment is known to result in pulmonary toxicity in vivo, BUS exposure may have resulted in significant alterations in other pathways and gene expression. Therefore, 50 µM BUS exposure was explored for global changes in gene expression using RNA-seq. This was carried out using DCI differentiated cells only as a representative of more distal small airway cells.
Figure 4-6 Primary airway differentiation effects on responses towards Busulfan. Airway basal cells and PneumaCult™ and DCI differentiated cells were exposed to busulfan (10 µM and 50 µM) on the basolateral side to mimic systemic exposure for 24 hours and toxicological assessment was carried out using resazurin reduction and LDH release (N=3) (A). Results are shown as mean ± SEM of fold over control (F.O.C) for LDH release and mean ± SEM of percentage (%) of control for resazurin reduction. mRNA expression changes of selected genes upon busulfan exposure were analysed using RT-qPCR (N=3), corrected for GAPDH and shown as fold change over control (mean ± SEM) (B).
4.4.2. Global Transcriptome impact of Busulfan on DCI differentiated ALI airway epithelial cells

RNA-Sequencing was performed to gain insight into the molecular mechanisms and pathways of BUS toxicity. Differential expression analysis showed significant (Fold change >2, FDR P-value <0.05) up- and downregulation of a few uncharacterized protein coding genes with low RPKM values (<1) (Figure 4-7A). Comparison of genes with non-FDR p-value <0.05 and RPKM >1 to AM and PQ chemical exposures indicates no specific BUS effect observed at the tested concentration in DCI differentiated SAEC (Figure 4-7B, C). Overall, these results indicate that DCI differentiated SAEC are unable to detect pharmacological or toxicological responses upon BUS exposure at the tested concentrations.

Figure 4-7 Busulfan effects in DCI differentiated primary airway epithelium. Busulfan exposure (24 hours) in DCI differentiated cells was characterized using RNA-sequencing analysis. Significantly differentially expressed genes (fold change > 2 and FDR p-value <0.05) upon 50 µM busulfan exposure are highlighted in red on a volcano plot (A). Genes with a non-FDR p-value <0.05 and RPKM >1 were displayed on a heatmap (B) and bar graphs (C) of log₂ RPKM values (AM; 15 µM; Bus 50 µM, PQ 100 µM) (mean ± SEM, N=3) to compare to other chemical exposures.
4.5. Primary airway epithelium differentiation and cell type specific responses to paraquat exposure

Airway epithelial models were exposed systemically (on the basolateral side of the TransWell insert) to PQ doses similar to plasma PQ concentrations observed in patients after PQ ingestion (100 µM) (Hong et al., 2014). Toxicological impact was explored by determining supernatant LDH and resazurin reduction levels as well as gene expression levels using qPCR and mRNA-Sequencing methods.

4.5.1. Airway epithelium differentiation specific responses to paraquat

PQ did not alter supernatant LDH or resazurin reduction levels upon 20 or 100 µM exposures (Figure 4-8A). However, 100 µM resulted in a significant upregulation of DDIT3 mRNA in DCI differentiated cells, but not the other two primary SAEC models (Figure 4-8B). Furthermore, SLC7A11 and HMOX1 mRNA levels were upregulated in both DCI and PneumaCultTM-ALI differentiated cells, but not in the undifferentiated basal AEC (Figure 4-8B). SLC7A11 mRNA upregulation was higher in PneumaCultTM-ALI differentiated SAEC, whereas HMOX1 upregulation was higher in DCI differentiated cells. NQO1, ATF4 and MDM2 mRNA levels did not show significant upregulation in the three SAEC models using qPCR methods (Figure 4-8B). Interestingly, PQ exposure of undifferentiated basal AEC did not result in differential expression of any of the six selected genes (Figure 4-8B), indicating differentiation is a fundamental prerequisite of the response of these airway epithelial cells to this compound.
Figure 4-8 Primary airway differentiation effects on responses towards Paraquat. Airway basal cells and PneumaCult™ and DCI differentiated cells were exposed to paraquat (20 µM and 100 µM) on the basolateral side for 24 hours to mimic systemic exposure and toxicological assessment was carried out using resazurin reduction and LDH release (N=3) (A). Results are shown as mean ± SEM of fold over control (F.O.C) for LDH release and mean ± SEM of percentage (%) of control for resazurin reduction. mRNA expression changes of selected genes upon paraquat exposure were analysed using RT-qPCR (N=3), corrected for GAPDH expression, and shown as fold change over control (mean ± SEM), significance shown as * (p-value < 0.05) (B).

RNA-Sequencing was performed in all 3 airway differentiation models to explore cell type and regional differentiation as a determinant of response to PQ exposure. It was also used to gain insights into the molecular mechanisms and pathways of PQ toxicity within the lung. Differential expression analysis showed significantly differentially expressed genes upon 100 µM PQ exposure in all three models. However, the numbers of significantly differentially expressed genes (FDR p-value <0.05 and fold change >2) were different. PQ exposure resulted in 100
differentially expressed genes in basal AEC, 312 differentially expressed genes in DCI differentiated cells and 939 differentially expressed genes in PneumaCult™-ALI differentiated cells (Figure 4-9A).

100 µM PQ exposure resulted in highest upregulation of CRCT1, GPX2 and PPP1R15A in undifferentiated basal AEC, DDIT3, TRIB3 and PSAT1 in DCI differentiated cells, and SLC7A11, CXCL8 and ABCB6 in PneumaCult™-ALI differentiated cells (Figure 4-9B), indicating differentiation dependent responses to PQ. Indeed, comparing Fold Change (FC) values between the different models (Figure 4-9C), shows that several genes, including CSF3, SLC7A11, STC2, IL1A and CXCL2 are preferentially upregulated in PneumaCult™-ALI differentiated cells, whereas other genes such as IL24, TRIB3, PSAT1, DDIT3 and UPP1 are preferentially upregulated in DCI differentiated SAEC. Plotting the RPKM values of these genes in a heatmap (Figure 4-9D) gives more insight in these differences.

Some genes were not significantly upregulated in basal AEC but were upregulated in DCI and PneumaCult cultures (e.g., TRIB3, PSAT1, ASNS, SLC7A11) (Figure 4-9D). Several of these genes demonstrated much higher control RPKM levels of expression in basal AEC compared to the other 2 culture models. It could be argued that these higher control levels may represent an already activated pathway phenotype in basal AEC that may not be able to react to additional toxicant induced pathway activation, and thus result in less differentially regulated genes. In addition, there were genes (GDF15, HMOX1, CXCL8, CCL20), which were activated by PQ in DCI and PneumaCult™-ALI differentiated but not in basal AEC while displaying similarly lower levels of RPKM expression across all control levels. This observation argues that for some pathways, activation level is similar between culture models and that a lack of effect for these genes in basal AEC may be due a reduced sensitivity to the damaging actions of PQ. Whether this involves PQ transport or different levels of PQ redox cycling or other reason is unknown, but we can confidently suggest that differentiation does appear to strongly impact the cells’ ability to respond to PQ.
Figure 4-9 Characterizing primary airway differentiation effects on response to paraquat exposure. Paraquat exposure (24 hours) in airway basal cells and PneumaCult™ and DCI differentiated cells was characterized using RNA-Seq. Significantly differentially expressed genes (fold change > 2 and FDR p-value < 0.05) upon 100 µM paraquat exposure were counted (A) and highlighted in red on a volcano plot (B). Log2 fold change of top 20 differentially expressed genes in each condition is visualised as mean ± SEM (N=3) (C) and log2 RPKM values of these genes are displayed in a heatmap (D).
4.5.2. DCI and PneumaCult™ differentiated cell specific responses to paraquat exposures

From section 3.2, we suggest that DCI differentiation represents a more distal small airway differentiation whereas PneumaCult™-ALI possess properties more consistent with proximal small airway regions. Comparison of DCI to PneumaCult™-ALI differentiated cell responses to PQ, shows that for DCI, control untreated cell levels of inflammatory genes such as CCL20 and CXCL8 are higher when compared to similar untreated PneumaCult™-ALI differentiated cells (Figure 4-9D). These higher levels of inflammatory gene expression in DCI differentiated SAEC may represent an already maximal stimulation for pathways regulating inflammatory gene expression and may possibly contribute to a lack of responsiveness to PQ in these cells. Furthermore, PQ differential responses in individual donors within PneumaCult™-ALI differentiated cells were more uniform than for DCI differentiated cells obtained from the same donors (Figure 4-9D). Therefore, this larger standard deviation (SD) may be another contributory factor for the decreased number of differentially expressed genes in DCI differentiated cells.

The average RPKM values of genes preferentially induced in either DCI or PneumaCult™-ALI differentiated SAEC were plotted to further visualise differentiation dependent effects between cell types (Figure 4-10). Genes including DDIT3, DUSP5, TRIB3, IL1A, IL24 and UPP1 were upregulated to a higher degree in DCI differentiated SAEC compared to other cell types (Figure 4-10A), whereas another cohort of genes including IL36G, ARKR1B10, OSGIN1, ADM2, SLC7A11 and SRXN1 were modestly more upregulated in PneumaCult™-ALI differentiated cells than other cell models (4-10B).
Primary small airway epithelial cell responses to chemical exposures

Figure 4-10 Differentiation specific gene upregulation upon paraquat exposure. Airway basal cells, DCI differentiated cells and PneumaCult™-ALI differentiated cells were exposed to paraquat for 24 hours and analysed using RNA-Sequencing. Genes that were more upregulated in DCI (A) or PneumaCult™-ALI (B) differentiated cells are displayed as mean ± SEM RPKM values (N=3).
4.5.3. Chemical specificity of paraquat responses in DCI differentiated airway epithelial cells

Comparisons of PQ effects between culture models can provide important information on cell differentiation and regional airway susceptibilities as well as providing insight into mechanisms of toxicity. Further comparisons of PQ to other compound effects within the same system can also help to understand PQ mechanisms of toxicity. This was carried out in Figure 4-11 and demonstrates that genes upregulated upon PQ exposure are not upregulated with AM or BUS exposures. This is unsurprising given the minimal changes in transcriptomic responses observed for these compounds (Figures 4-5 and 4-7).

Figure 4-11 Comparison of paraquat effects in DCI differentiated primary airway epithelium to other chemical exposures. Genes differentially expressed upon paraquat (PQ) exposure in DCI differentiated cells were compared to busulfan (BUS) and amiodarone (AM) exposures (AM; 15 µM; Bus 50 µM, PQ 100 µM; 24 hours) and displayed as log$_2$ RPKM values in a heatmap (A) and mean ± SEM RPKM values in bar graphs (N=3) (B).
4.5.4. Differences in pathway activation upon paraquat exposure in DCI and PneumaCultTM-ALI differentiated airway epithelial cells

Differentially expressed genes upon PQ exposure were analysed for pathway activation of biological processes (BP) (GO:BP, Figure 4-12A) and molecular factors (MF) (GO:MF, Figure 4-12B) to identify common and preferentially activated pathways within the SAEC models as described in methods section 2.4. Pathway analysis shows that PneumaCult™-ALI differentiated cells responded most significantly for response to stimulus in BP and oxidoreductase activity and cytokine activity for MF analysis. DCI differentiated SAEC also showed increases in response to stimulus in BP and cytokine activity pathways in MF analysis, but to a lesser extent than PneumaCult™-ALI differentiated cells. Interestingly DCI differentiated cells did not respond significantly for oxidoreductase activity in MF analysis. Both DCI and PneumaCult™-ALI differentiated airway epithelial cell models show a higher level of pathway activation and upregulation compared to undifferentiated basal AEC, indicating differentiation is a key factor in the response to PQ exposure. This preferential activation of oxidoreductase activity pathways within PneumaCult™-ALI differentiated cells is somewhat strengthened when RPKM values of selected pathway genes are plotted (Figure 4-12B), as SRXN1, HMOX1 and NQO1 are preferentially upregulated within these cells compared to basal AEC. However, DCI differentiated cells show increased expression of these genes upon PQ exposure as well, which is not reflected in GO pathway analysis. When looking at genes that are involved in cell death pathways and cytokine activity (Figure 4-12B), these are mostly preferentially upregulated within DCI differentiated cells compared to the other two SAEC models. Stress response pathway genes seem to be upregulated to a similar extend between DCI and PneumaCult™-ALI differentiated SAEC models. However, pathway analysis shows a higher level of pathway activation for PneumaCult™-ALI differentiated cells due to an overall higher number of significantly differentially expressed genes. Although GO pathway analysis might not be particularly useful for comparing pathway activation between cell types, it is still useful to identify broad mechanisms of PQ toxicity.
Primary small airway epithelial cell responses to chemical exposures

Figure 4-12 Pathway activation upon paraquat exposure in basal, DCI and Pneumacult™ differentiated airway epithelial cells. Significantly differentially expressed genes (FDR p-value <0.05, fold change > 2) from RNA-Sequencing analysis of paraquat (100 µM; 24 hours) exposures were analysed for GO biological process (BP) and molecular function (MF) pathway analyses using g:Profiler (A). Selected genes were plotted as mean ± SEM RPKM values (B).
4.5.5. Airway epithelial cell culture model gene expression differences relating to paraquat susceptibility

There are several possible mechanistic explanations as to why differentiation of airway epithelial cells alters the responsiveness to PQ. Some of these can be explored by comparing the mRNA levels of genes, which are known to impact responsiveness to PQ.

The lungs are thought to be particularly sensitive to PQ exposure due to active absorption and accumulation mechanisms, suggested to be mediated through polyamine transporters present on club and alveolar cell types (Dinis-Oliveira et al., 2008). Exploring the mRNA markers of those polyamine transporters implicated in PQ uptake in literature (Hiasa et al., 2014; Uemura et al., 2010) shows that PneumaCult™-ALI differentiated SAEC have higher expression of SLC7A2 and SLC18B1, but not SLC3A2, SLC7A1 and ATP13A2 mRNA (Figure 4-13A). Furthermore, DCI differentiated cells also show higher levels of SLC7A2 and SLC18B1 mRNA compared to basal AEC, indicating presence of these transporters may contribute to PQ induced toxicity.

Increased levels of superoxide dismutase (SOD1/SOD2) have been shown to have a protective effect on PQ induced toxicity (Filograna et al., 2016). Therefore, higher levels of superoxide dismutase would indicate an increased resistance to PQ toxicity. However, RNA-Sequencing shows that SOD1 levels are highest in PneumaCult™-ALI differentiated SAEC whereas SOD2 levels are highest in DCI differentiated SAEC, as lowest levels of PQ toxicity were observed in basal AEC, these differences are unlikely to explain the observed susceptibility to PQ exposures (Figure 4-13B). They may however contribute to differential response between DCI and PneumaCult™-ALI cultures.

As discussed previously, PQ reduction to generate superoxide requires enzymatic activity. Significant oxidoreductase pathway activation was only observed in PneumaCult™-ALI differentiated SAEC. Therefore, the presence of these enzymes and / or co-factors are likely to affect PQ susceptibility. Indeed, RNA-Sequencing shows that POR, TXNDR and CYBA mRNA expression was higher in DCI and PneumaCult™-ALI differentiated cells compared to basal AEC.
Furthermore, UCP2 mRNA levels were higher in PneumaCult\textsuperscript{TM}-ALI differentiated cells compared to the other two cell models (Figure 4-13B). However, UCP2 over-expression within A549 cells was shown to inhibit ROS accumulation and apoptosis (Deng et al., 2012), therefore over-expression of UCP2 within PneumaCult\textsuperscript{TM}-ALI differentiated cells is an unlikely cause of increased levels of gene dysregulation upon PQ exposure within this SAEC model.

A) Polyamine transport

![Graphs of SLC3A2, SLC7A1, SLC7A2, SLC18B1, and ATP13A2 showing basal differences in expression levels of genes relevant to paraquat toxicity.]

B) Superoxide dismutase / oxidoreductases

![Graphs of SOD1, SOD2, CYBA, POR, DUOX2, DUOXA2, DUOXA1, DUOX1, UCP2, and TXNDR1 showing basal differences in expression levels of genes relevant to paraquat toxicity.]

Figure 4-13 Basal differences in expression levels of genes relevant to paraquat toxicity. Genes that are relevant for paraquat toxicity were analysed for their RPKM values in basal, DCI differentiated, and PneumaCult\textsuperscript{TM}-ALI differentiated cells from RNA-sequencing analysis. Genes relevant for polyamine transports (A) superoxide dismutase / antioxidant genes (B) are displayed as mean RPKM value ± SEM (N=6)
4.6. Discussion

The *in vitro* small airway epithelial cell models established in section 3.2 (basal AEC, DCI and PneumaCult™-ALI differentiated cells) have the potential to be valuable models for *in vitro* airway toxicity testing. Airway toxicants show regional airway selectivity that could possibly be linked to regional differences in cellular composition and defence mechanisms such as mucociliary clearance (Okuda et al., 2018; J. Yang et al., 2017). As differences in cell type markers are observed between the three described airway epithelial models, these could be valuable in further investigating mechanisms of cellular susceptibility and regional toxicity of chemicals that cause pulmonary injury.

The models were first tested with CSE and DEPE, pulmonary toxicants that are known to specifically cause airway re-modelling within the small airway epithelium (Chang et al., 2006; J. Yang et al., 2017; L. Zhang et al., 2017). CSE and DEPE have been shown to induce toxicological responses in (bronchial) primary airway epithelial cells, as assessed using CYP1A1/CYP1A2 upregulation (Baxter et al., 2015; Boei et al., 2017). This allowed us to investigate whether the airway epithelial cell models display a differential response in NQO1, SLC7A11 and CYP1A1 mRNA expression to these toxicants. Only basal AEC showed a significantly increased level of NQO1 mRNA upon apical CSE exposure, indicating increased levels of Nrf2 (NFE2L2) pathway activation and oxidative stress within this model. Furthermore, another Nrf2 dependent gene, SLC7A11 was significantly upregulated only within DCI differentiated SAEC upon CSE exposure. These observations might be attributed to a lack of protective mechanisms, such mucociliary clearance within basal AEC and DCI differentiated cultures. Indeed, both cultures showed lower levels of ciliated (FOXJ1) cell markers compared to PneumaCult™-ALI differentiated SAEC (section 3.2). The increased level of NQO1 and SLC7A11 mRNA levels upon DEPE exposure in basal AEC and to a lesser extent in DCI differentiated cells, further indicate that there is a lack of protective mechanism within these models compared to PneumaCult™-ALI. One supporting piece of evidence for more protective mechanisms in PneumaCult™-ALI differentiated cells is a
monolayer with a thicker layer of mucus (data not shown), possibly allowing a higher level of protection against apical exposure of toxicants compared to the other two models. Superoxide dismutase (e.g. SOD2) expression in unexposed cultures was also higher in PneumaCult™-ALI and DCI differentiated cells compared to basal AEC, which might indicate a higher level of protection against ROS within these models, resulting in less increases in NQO1 and SLC7A11 expression.

Within this study, DCI differentiated SAEC are most representative for the distal small airways, as these cultures show the highest level of club cell marker SCGB1A1 expression out of the three tested models (section 3.2). The higher level of increase in expression of CYP1A1 observed in the DCI differentiated cells upon both CSE and DEPE exposures, corresponds with observations of increased CYP1A1 expression in distal club cells upon cigarette smoke exposure in humans in vivo (Chang et al., 2006). The increased level of CYP1A1 implicates increased levels of bioactivation and thus potential toxicological responses within this culture model. Therefore, these SAEC models could be useable to investigate the regional and cellular susceptibility of airway toxicants with unknown mechanisms.

4.6.1. Amiodarone and busulfan pulmonary toxicity responses

AM and BUS are pharmaceuticals that are known to cause pulmonary toxicity and fibrosis. However, AM and BUS exposures did not show significant differences in viability, cytotoxicity or mRNA expression of relevant genes as measured by PCR at the tested concentrations in any primary airway epithelial cell culture model.

RNA-Sequencing analysis was used to get a more complete picture of responses to these chemicals and showed that AM exposure in DCI differentiated SAEC resulted in significant upregulation of 4 genes, SCD, INSIG1, SREBF1 and GPNMB. Upregulation of these lipid metabolism-related genes is a marker for accumulation of phospholipids in lysosomes due to abnormal lipid metabolism, known as phospholipidosis (PL) (Goss et al., 2013). Indeed, SCD and SREBF1 mRNA have been previously shown to be upregulated in BEAS-2B cells exposed to AM
and linked to PL induced by PPARγ signalling pathways (Song et al., 2011). Furthermore, PPARγ signalling pathways regulate INSIG1 (involved in insulin sensitization and glucose homeostasis) and GPNMB (encoding an endogenous glycoprotein involved in inflammation) expression (Kast-Woelbern et al., 2004), indicating PPARγ induced PL may be a mechanism of AM induced pulmonary toxicity. However, as RNA-Seq was not performed in the other two small airway models, no conclusions can be made on regional or cellular susceptibility. Interestingly, INSIG1, SCD and GPNMB are included in a list of blood markers for the detection of AM exposure in humans in vivo (Ryu et al., 2011). From the RNA-sequencing analysis we observed these marker genes, and may therefore infer that AM is displaying a pharmacological effect similar to what it would have in humans. We did not however observe any toxicity pathway activation upon single exposure and may conclude that toxicity for this compound is likely a consequence of repeat dose injury to susceptible tissues. This is supported by observations in clinical pathology, as pulmonary fibrosis side effects are more common after prolonged AM treatments (>2 months) (Jackevicius et al., 2011). Interestingly however, it was previously shown that AM exposure resulted in epithelial-mesenchymal transition in the alveolar cell line A549 at doses of 10 µM for 24 hours, although the effects were more profound upon higher concentrations or exposure times (Weng et al., 2020). Although cell lines do not represent alveolar cells with normal phenotypes, this may be an indication that alveolar cell types can be more susceptible to AM exposures.

RNA-Seq analysis also demonstrated that BUS exposure (50 µM) had limited effects resulting in significantly differential expression of only 5 uncharacterized protein coding genes with low RPKM values. These genes are not associated with any known mechanisms of pharmacological or toxicological mechanisms of BUS activity and are unlikely to represent meaningful observations. It could be argued that they possible fall with the FDR range for false positives. Increasing the dose of BUS could increase validity of the exposure regime as BUS treatment is commonly administrated in doses of 1 mg/kg body weight orally every 6 hours for
a total of 16 doses, with risk of toxicity increased at if plasma concentrations above 3 mM are reached (McCune et al., 2000). However, solubility limits of BUS (approximately 50 mM in organic solvents, e.g., DMSO and insoluble in water) limit the ability to increase dose of 50 µM for in vitro models, as DMSO concentrations exceeding 0.1% are likely to affect cell cultures. Therefore, a lack of effect of BUS in our model systems is likely a consequence that concentrations of BUS typically observed to induce toxicity in humans could not be achieved in vitro due to technical issues around compound solubility.

Another explanation for the lack of observed toxicological responses upon AM or BUS exposure could be a lack of bio-activation within the models. In vivo, these pharmaceuticals will pass the liver in which bioactivation might take place, before reaching the pulmonary tissues. Indeed, there are some indications that AMs major metabolite DEA, catalysed by CYP3A4 and CYP2C8 within the liver might be responsible for AM induced pulmonary toxicity (Ohyama et al., 2000). Both CYP3A4 and CYP2C8 mRNA levels are low in all three airway epithelium models (RPKM levels of <0.1, data not shown). Therefore, exposure to AMs major metabolite or application of an in vitro multi-organ model including liver metabolism, may be a more improved system to test AM susceptibility to pulmonary injury.

4.6.2. Paraquat pulmonary toxicity responses

In contrast to AM and BUS, PQ effects in humans typically manifest as acute toxicity rather than injury after chronic exposure. Indeed, 24h exposure of a single PQ dose resulted in dysregulation of genes in all three airway models at a concentration that did not significantly alter supernatant LDH or resazurin reduction levels. Initial qPCR analysis of selected genes showed that 100 µM PQ exposure resulted in a significant upregulation of DDIT3 in DCI differentiated cells only and a significant upregulation of SCL7A11 and HMOX1 in both DCI and Pneumacult™-ALI differentiated SAEC. Basal AEC did not show upregulation of any of the selected genes upon PQ exposure, indicating differentiation is required to observe pulmonary PQ effects within these models. The PQ effects were then further explored using mRNA-Sequencing analysis, showing
that 100 µM PQ exposure resulted in 100 differentially expressed genes in basal AEC, 312 differentially expressed genes in DCI differentiated cells and 939 differentially expressed genes in PneumaCult™-ALI differentiated cells.

The most significantly differentially expressed genes upon PQ exposure differ between the three SAEC models. CRCT1 and PPP1R15A were the most differentially expressed genes in undifferentiated basal AEC, DDIT3 and TRIB3 in DCI differentiated cells, and SLC7A11, CXCL8 in PneumaCult™-ALI differentiated cells. Comparing the RPKM values of unexposed and PQ exposed cells between the three SAEC models, shows that no genes are preferentially upregulated in basal AEC. Genes including DDIT3, DUSP5, TRIB3, and IL1A, appear to be more upregulated in DCI differentiated SAEC, whereas genes such as IL36G, ARKR1B10, SLC7A11 and SRXN1 appear to be slightly more upregulated in PneumaCult™-ALI differentiated cells. However, comparing GO:MF and GO:BP pathway analyses between indicates preferential pathway induction within PneumaCult™-ALI differentiated SAEC, whereas some genes are more induced in DCI differentiated SAEC. The increased levels of pathway activation within PneumaCult™-ALI differentiated SAEC is likely attributed to increased number of differentially expressed genes upon PQ exposure within this cell model.

Although pathway analysis was not particulary useful for comparisons between cell models, it did give insights into the molecular mechanism of paraquat toxicity. It demonstrated overexpression of pathways such as oxidoreductase activity (AKR1C1, HMOX1, NQO1), cytokotine signalling (CCL20, CXCL8, IL1A) and stress response (DDIT3, DUSP1, GDF15, TRIB3) upon PQ exposures in PneumaCult™-ALI differentiated SAEC. Significance levels of pathway activation were generally lower in DCI differentiated SAEC, however, RPKM levels demonstrated similar gene activation patterns in both cell types, with several stress response genes (DDIT3, GDF15) showing a higher level of expression in DCI differentiated SAEC. Overall, the lack of a profound response in basal AEC models as well as subtle differences between DCI and
PneumaCult\textsuperscript{TM}-ALI differentiated SAEC models upon PQ exposures can be used to explore mechanisms of cellular susceptibility of PQ exposures.

There are clear differences in responses to PQ exposure dependent on cell type differentiation, that cannot be explained by physical differences in the cultures or their exposures. All cultures were cultured at the ALI and the same dose, apical volume and time point of analysis were used. There were more cells in the PneumaCult\textsuperscript{TM}-ALI exposure due to pseudostratified differentiation than basal AEC and DCI (data not shown) but since the greater changes were observed in these cultures compared to basal AEC and DCI, a greater dose per cell or greater mucus layer cannot account for differences between cultures. This leaves us with a more plausible explanation that individual cell differentiation and unique gene and protein expression are at the heart of the differences observed between models. One can explore the molecular mechanisms of such susceptibility by analysis of the RNA-sequencing datasets in this study.

For instance, a difference in the presence of polyamine transporters that mediate active absorption and accumulation of PQ within the tissues could explain differences in PQ toxicity (Dinis-Oliveira et al., 2008). Indeed, PneumaCult\textsuperscript{TM}-ALI differentiated SAEC demonstrated a higher expression of mRNA for the polyamine transporters SLC7A2 and SLC18B1 compared to the other two models. Furthermore, DCI differentiated cells also display higher mRNA levels of these transporters compared to basal AEC, indicating higher levels of these transporters could lead to PQ accumulation within cells and cause susceptibility to PQ exposures (Dinis-Oliveira et al., 2008; Hoet & Nemery, 2000; Silva et al., 2015). However, mRNA levels of these transporters do not necessarily reflect the presence of transporter proteins. However, mRNA levels typical parallel protein levels, whereas protein levels may not be reflected in corresponding mRNA levels. This gives weight to the possibility the differences we see between models at the mRNA level are functional. However, further investigation is thus required to establish differences in transporter proteins and intracellular PQ levels between the models.
A study using a CRISPR library knockdown approach to determine PQ toxicity mechanisms within immortalized human T lymphocyte cells has identified POR (An endoplasmic reticulum membrane-bound enzyme accepting electrons from NADPH and donating these to cytochrome P450s) as the primary gene contributing to PQ induced cell death (Reczek et al., 2017). Interestingly, mRNA Sequencing data in our study showed that POR mRNA levels were higher in DCI and PneumaCult™-ALI differentiated cells compared to basal AEC. Therefore, increased POR mediated PQ reduction resulting in higher levels of superoxide generation could be part of the underlying mechanism of observed increased toxicity within the differentiated SAEC models.

Thioredoxin reductase, encoded by TXNDR, has been identified as a NADPH:paraquat oxidoreductase within mice (J. P. Gray et al., 2007). Although slightly higher in the differentiated SAEC models, RPKM levels of TXNDR are relatively low (RPKM <2) in all three models, indicating that higher TXNDR as unlikely to be involved in increased susceptibility to PQ in the differentiated SAEC models.

CYBA encodes a protein that is critical for superoxide-generating NADPH oxidases (NOXs) (Stasia, 2016). CYBA mRNA levels are shown to be higher in both DCI and PneumaCult™-ALI differentiated cells compared to basal AEC. As NOX enzymes have been implicated in PQ reduction and consequent ROS generation (Cristóvão et al., 2009), higher CYBA levels could be another potential explanation for increased susceptibility within the differentiated cultures.

4.6.3. Conclusions and future directions

This chapter aimed to explore the influence of small airway toxicants on three different primary small airway epithelial cell models to investigate the applicability of these models in examining small airway toxicity. This was first demonstrated using the small airway and club cell specific toxicants CSE and DEPE. The airway model most representative of the distal small airways and containing highest expression of club cell markers (DCI differentiation), showed highest increase of CYP1A1 expression, indicating the SAEC models are able to detect regional or cellular specific toxicity. However, AM and BUS did not show significant levels of toxicity in any model, indicating
limitations of exposure regimes in exploring small airway toxicity (Figure 4-14). Repeated dose exposures, increased dosage for AM and BUS or utilizing a multi organ model with metabolically active liver cells to consider metabolites upon systemic exposures could be used to further explore airway susceptibility of these chemicals.

In contrast, PQ is known for acute toxicity upon ingestion, and indeed, effects were observed after 24h exposure in all three models. Overall, these results indicate that DCI and PneumaCult™-ALI differentiated primary SAEC are more capable in detecting PQ induced acute toxicity within a 24h exposure period than undifferentiated basal AEC, showing that differentiation of primary basal AEC is required to observe PQ toxicity responses. Pathway analysis indicated involvement of oxidoreductase activity, cytokine signalling and stress response pathways in PQ toxicity. However, the exact mechanism of cellular and regional susceptibility to PQ exposure is still unknown. Protein analysis or single cell sequencing analysis could give more insight in different levels of polyamine transport systems between the cell cultures and their relation to PQ accumulation and toxicity. Furthermore, the extent of the involvement of ROS in PQ toxicity is currently unknown and functional studies may be required to fully explore this. New insights into PQ airway toxicity are however observed and will be further explored in chapter 6 to establish cellular specificity and toxicity mechanisms.

Figure 4-14 Schematic overview of conclusions of Chapter 4
CHAPTER 5: COMPARISON OF CHEMICAL INDUCED TOXICITY RESPONSES IN CD34+ HSC AND IPSC DERIVED MACROPHAGES AND DENDRITIC CELLS
5.1. Introduction

Myeloid cells such as macrophages and dendritic cells play an important role in innate defence mechanisms in most human tissues, including the small airways, and are therefore potential targets for small airway toxicants (Janeway et al., 2001; Kelly & O’Neill, 2015; Kopf et al., 2015). This chapter aims to explore the toxicity responses of the CD34+ hematopoietic stem cell derived myeloid cultures, DC$^{cd34}$ and MC$^{cd34}$, and the iPSC derived myeloid cultures, DC$^{iPSC}$ and MC$^{iPSC}$, established in chapter 3. Primary CD34 derived immune cells were characterised for cell type specific markers indicative of macrophage and dendritic cell function. While these cells may not directly reflect the precise profile of myeloid cell types within the small airways, we believe they are still an important model system representative of how such cells would respond to airway toxicant exposures. As the iPSC myeloid models demonstrated some similarities and differences to primary CD34+ counterparts, comparing the toxicological response of chemical exposures will allow for a more detailed assessment of these cells suitability for toxicity testing. The chemicals AM, BUS and PQ have been discussed in detail in chapter 4 with regards to small airway epithelium susceptibility. Here, we will further discuss the possible impact of these chemicals on the immune cells residing in the small airways.

5.1.1. Amiodarone, busulfan and paraquat immunotoxicity

As discussed previously in chapter 4, AM can interfere with the phospholipid movements across intracellular membranes, which can lead to accumulation of high concentrations of phospholipid-bound AM in membrane-rich structures such as lysosomes within macrophages (Lapinsky et al., 1993). This lipid accumulation causes morphological abnormalities within alveolar macrophages, also referred to as “foamy macrophages”, typically observed in BAL of AM exposed patients (Papiris et al., 2010). This “foamy” macrophage phenotype is rarely found in unexposed subjects, and can then be used as a clinical indicator of AM exposure within patients (Mermolja et al., 1994). Thus, alveolar macrophages might be susceptible to phospholipidosis caused by AM exposure. Furthermore, AM exposure can cause an altered
Comparison of chemical induced toxicity responses in CD34+ HSC and iPSC derived macrophages and dendritic cells

production of cytokines within macrophages, as shown by TNFα and TGFβ release by alveolar macrophages in a rodent model of AM toxicity (Reinhart & Gairola, 1997). Whereas AM, or its toxic metabolites, are known human macrophage toxicants, their impact on dendritic cells has not yet been explored.

BUS, a potent alkylating agent currently used exclusively in preparation for hemopoietic stem cell transplantation, is known to cause DNA damage and thus induce cellular senescence (Iwamoto et al., 2004; Probin et al., 2007). Interestingly, interstitial pulmonary fibrosis has also been reported as a complication of BUS administration in several cases (Oliner et al., 1961; Pearl, 1977), speculated to be caused by initial inflammation of alveoli followed by fibrous thickening of alveolar walls (Littler et al., 1969). The exact mechanisms by which BUS, or its metabolites, can result in pulmonary fibrosis are still largely unknown but may involve airway resident immune cells such as macrophages and dendritic cells (A. Myers et al., 2017).

Small airway responses to PQ exposure involve a prominent inflammatory component, as shown by recruitment of innate immune cells such as polymorphonuclear leukocytes, NK-cells and macrophages within the lung upon PQ exposure (Dinis-Oliveira et al., 2008; Wu et al., 2020). While PQ induced airway epithelial tissue injury can induce inflammatory processes, PQ may also have a direct effect on airway resident immune cells further propagating inflammatory events alongside airway epithelial injury. This may occur through redox sensitive signalling pathways such as NF-κB and MAPK activation via toll-like receptors (TLR4 and 9) (Subbiah & Tiwari, 2021). Macrophages are central regulators of tissue homeostasis and play a role in the response to PQ. For instance, freshly isolated rat primary alveolar macrophages and type II pneumocytes exposed to PQ were more sensitive to DNA strand breakage compared to other cell types (Dušinská et al., 1998; Petrovská & Dušinská, 1999). Furthermore, murine alveolar macrophages were observed to respond to systemic PQ exposure by increasing expression of the transporter SLC7A11 that regulates levels of the anti-oxidant glutathione (Kobayashi et al., 2012). Interestingly, murine bone marrow derived macrophages showed a dose-dependent
Comparison of chemical induced toxicity responses in CD34+ HSC and iPSC derived macrophages and dendritic cells increase in inflammatory cytokine IL6 mRNA and protein expression upon PQ exposure, whereas fibroblasts and bronchial airway epithelial cells (16HBE) did not respond (Hu et al., 2017). These altered inflammatory signalling pathways might contribute to the development of pulmonary fibrosis upon PQ exposure (Hu et al., 2017).

5.1.2. Overall aims

The broad aims of this chapter are to investigate how small airway toxicants may target myeloid MC and DC cells as a mechanism of pulmonary injury, through analysis of cell type specific effects. It is also a main aim to investigate how iPSC derived myeloid cells compare to primary cell responses to toxicant exposure.

More specifically the chemicals AM, BUS and PQ used within the in3-MSCA-ITN project and in chapter 4 of this thesis, will be used to investigate if CD34+ and iPSC derived macrophage and dendritic cells are comparable and to further explore the mechanisms of small airway toxicity. Despite some evidence for the role of macrophages in the response to AM, BUS and PQ induced tissue injury and inflammatory cascades, there has been no comprehensive investigation of the intracellular mechanisms involved or whether these cells form a major target for injury upon exposure. Even less information is available on the possible impact of these chemicals on dendritic cells. Here we explore the effects of different concentrations of AM, BUS and PQ exposure on DC^{cd34} and MC^{cd34} cultures to determine cellular specificity and possible mechanisms of toxicity, using targeted RNA-sequencing; TempO-seq. Furthermore, DC^{ipSC} and MC^{ipSC} cultures were exposed to the same chemicals and their toxicological responses were compared to CD34+ counterparts to investigate their applicability to in vitro toxicity testing (Figure 5-1).
Comparison of chemical induced toxicity responses in CD34+ HSC and iPSC derived macrophages and dendritic cells

**In vitro model**
- MC\textsuperscript{CD34}
- DC\textsuperscript{CD34}
- MC\textsuperscript{iPSC}
- DC\textsuperscript{iPSC}

**Toxicity testing**

**Chemical toxicity**
- Amiodarone
- Busulfan
- Paraquat

**Objectives**
1) Identify cellular specificity and toxicity of chemicals
2) Compare toxicological responses of cells differentiated from iPSC to cells differentiated from CD34+ hemopoietic stem cells

Figure 5-1 Overview of Chapter 5
Comparison of chemical induced toxicity responses in CD34+ HSC and iPSC derived macrophages and dendritic cells

Whereas chapter 4 has utilized bulk whole transcriptome mRNA Sequencing methods to investigate the responses of primary airway epithelial cells upon PQ exposure, this chapter will utilize targeted TempO-Seq transcriptomic analysis instead. The TempO-Seq platform allows for high throughput gene expression analysis and requires low input amounts. TempO-Seq technology is typically used to identify differential expression of a selected number of genes with particular interest to toxicology and has previously shown similar outcomes compared to RNA-Sequencing or Microarray methods (Bushel et al., 2018). The panel of genes used for TempO-Seq was designed to include commonly altered toxicity pathways.

Aims:

- Identify DC\textsuperscript{cd34} and MC\textsuperscript{cd34} specific responses to understudied airway toxicants (AM, BUS and PQ)
- Compare DC\textsuperscript{iPSC} and MC\textsuperscript{iPSC} responses to the airway toxicants AM, BUS and PQ with primary CD34+ counterparts to identify applicability of this iPSC model to chemical toxicity assessment.
- Understand differences in cell culture models underlying differential responses to small airway toxicants and establish cellular specificity of response.
Comparison of chemical induced toxicity responses in CD34+ HSC and iPSC derived macrophages and dendritic cells

5.2. Myeloid cell responses to amiodarone

To explore the effect of AM exposure, DC^{cd34}, MC^{cd34}, DC^{iPSC}, and MC^{iPSC}, were exposed to different concentrations of AM for 24 hours. Toxicological impact was determined using LDH, resazurin reduction and gene expression differences. Gene expression levels were explored using TempO-Seq methods as further described in chapter 2.

5.2.1. CD34+ primary cell differentiation dependent responses to amiodarone

AM exposure significantly increased supernatant LDH levels at concentrations higher than 25 µM in both DC^{cd34} or MC^{cd34} cultures after 24h exposure (Figure 5-2A), indicating a cytotoxic effect upon AM exposure. This effect was more profound in DC^{cd34} cultures. Furthermore, AM exposure resulted in a significant decrease in viability levels as measured with resazurin reduction at concentrations higher than 15 µM in DC^{cd34} and 45 µM in MC^{cd34} (Figure 5-2A). Therefore, 15 µM AM exposure was further explored using TempO-Seq methods. Unfortunately, one replicate of 15 µM AM exposure in MC^{cd34} did not meet sample quality standards as shown in a Poisson distance heatmap (Supplemental figure 9-7). Therefore, only two replicates were included in the analysis for the MC^{cd34} cultures and no significantly differentially expressed genes were detected upon 15 µM AM exposure (Figure 5-2B). In DC^{cd34} cultures however, AM exposure resulted in a significant increase in CYP51A1 and decrease in PHOSPHO1 expression (Figure 5-2B). However, as N=2 was used for the MC^{cd34} cultures, no reliable comparisons can be made. To explore the data sets further, the normalized counts of the genes with the highest fold change and a non-adjusted p-value of <0.05 were plotted on a heatmap (Figure 5-2C) and showed a non-significant increase in genes such as CXCL8 and PSAT1 in both DC^{cd34} and MC^{cd34} cultures upon AM exposure (Figure 5-2D). Furthermore, when comparing genes that are non-significantly increased upon AM exposure to BUS and PQ exposures, genes such as INSIG1, ALAS1 and TNFSF12 were more increased upon AM exposure compared to the other chemicals.
Comparison of chemical induced toxicity responses in CD34+ HSC and iPSC derived macrophages and dendritic cells

Figure 5-2 Amiodarone effects in primary CD34 macrophage and dendritic cells. CD34+ derived macrophages (MC\textsuperscript{cd34}) and dendritic cells (DC\textsuperscript{cd34}) were exposed to amiodarone for 24 hours and toxicological assessment was carried out using resazurin reduction and LDH release (A). Results are shown as mean ± SEM fold over control (F.O.C) (LDH) or mean ± SEM % of negative control (resazurin) (N=3). Statistical differences compared to control are shown as * (p-value <0.05). Analysis of mRNA transcripts was carried out using TemO-Seq after 15 µM amiodarone exposure and significant genes (adjusted p-value <0.05 and fold change > 2) are highlighted in red in volcano plots (DC\textsuperscript{cd34}: N=3, MC\textsuperscript{cd34}: N=2) (B). Log2 normalized count of genes with the highest fold change and non-adjusted p-value <0.05 are visualized in a heatmap (C) and mean ± SEM (N=3) normalized count of selected non-significant genes was plotted (D) and compared to other chemical exposures (E).
5.2.2. iPSC macrophage and dendritic cell responses to amiodarone

DC\textsuperscript{iPSC} and MC\textsuperscript{iPSC} did not show a significant difference in supernatant LDH or resazurin reduction levels upon 0-15 µM AM exposure, as also observed for the CD34+ cultures (Figure 5-3A). Furthermore, TempO-Seq analysis of 15 µM AM exposure did not show significant differentially expressed genes (Figure 5-3B). Exploration of the genes that showed a significant difference in the CD34+ cultures (CYP51A1, PHOSPHO1) did not show an up- or downregulation in either iPSC culture (5-3C). However, the limited observed effects in CD34+ cultures upon AM exposure hamper evaluation of applicability of the iPSC models for AM toxicity assessment.

Figure 5-3 Comparing Amiodarone effects in iPSC to CD34+ macrophages and dendritic cells. iPSC derived macrophages (MC\textsuperscript{iPSC}) and dendritic cells (DC\textsuperscript{iPSC}) were exposed to amiodarone and toxicological assessment was carried out using LDH release (A). Results are shown as mean ± SEM fold over control (F.O.C) (N=3) and compared to responses in CD34+ derived macrophages (MC\textsuperscript{cd34}) and dendritic cells (DC\textsuperscript{cd34}). Analysis of mRNA transcripts was carried out using TemO-Seq after 15 µM amiodarone exposure and visualized in volcano plots (B). Normalized counts of genes with the highest fold change (non-adjusted P-value <0.05) in CD34 (C) were visualized as Mean ± SEM (N=3).
5.3. Myeloid cell responses to busulfan

To explore the effect of BUS exposure on myeloid cells, DC^{cd34}, MC^{cd34}, DC^{iPSC} and MC^{iPSC} cultures were exposed to different concentrations of BUS for 24 hours. Toxicological impact was explored using supernatant LDH, resazurin reduction and TempO-Seq as described in chapter 2.

5.3.1. CD34 differentiation dependent responses to busulfan

BUS exposure did not significantly affect supernatant LDH or resazurin reduction levels in DC^{cd34} or MC^{cd34} cultures after 24h exposure at the tested concentrations (0 – 150 µM) (Figure 5-4A). A slight, non-significant increase in supernatant LDH levels was observed at 100 µM BUS exposure in DC^{cd34} cultures. Furthermore, a non-significant decrease in resazurin reduction levels was observed at 150 µM BUS exposure in both cultures. Therefore, 50 µM BUS exposure was further explored using TempO-Seq analysis. No significantly altered genes were observed after 50 µM BUS exposure in DC^{cd34} and MC^{cd34} cultures (Figure 5-4B). The normalized counts of the genes with the highest fold change and a non-adjusted p-value of <0.05 were further explored (Figure 5-4C) and showed a non-significant increase in genes such as SGK2 and ZNF586 in both DC^{cd34} and MC^{cd34} cultures upon BUS exposure. Overall, no significant effects were observed upon BUS exposure in either culture at the tested concentrations.
Comparison of chemical induced toxicity responses in CD34+ HSC and iPSC derived macrophages and dendritic cells

Figure 5-4 Busulfan effects in CD34+ macrophages and dendritic cells. CD34+ derived macrophages (MC\textsuperscript{cd34}) and dendritic cells (DC\textsuperscript{cd34}) were exposed to busulfan for 24 hours and toxicological assessment was carried out using resazurin reduction and LDH release (A). Results are shown as mean ± SEM fold over control (F.O.C) (LDH) or mean ± SEM % of negative control (resazurin) (N=3). Analysis of mRNA transcripts was carried out using TemO-Seq after 50 µM busulfan exposure and visualized volcano plots (B). Log\textsubscript{2} normalized count of genes with the highest fold change and non-adjusted p-value <0.05 are visualized in a heatmap (C) and mean ± SEM (N=3) normalized count of selected genes was plotted (D).
5.3.2. iPSC macrophage and dendritic cell responses to busulfan

Similar to the primary CD34+ cultures, DC\textsuperscript{iPSC} and MC\textsuperscript{iPSC} did not show a significant difference in supernatant LDH or resazurin reduction levels upon 0-50 µM BUS exposure (Figure 5-5A). Furthermore, TempO-Seq analysis of 50 µM BUS exposure did not show significant differentially expressed genes (Figure 5-5B). Exploration of the genes that showed a non-significant increase in the CD34+ cultures (SGK2, ZNF586) did not show an upregulation upon BUS exposure in either iPSC culture (5-5C). However, as no significantly differentially expressed genes were detected upon BUS exposure within the CD34+ cultures, the applicability of the iPSC cultures for BUS toxicity assessment cannot be fully determined.

Figure 5-5 Comparing Busulfan effects in iPSC to CD34+ macrophages and dendritic cells. iPSC derived macrophages (MC\textsuperscript{iPSC}) and dendritic cells (DC\textsuperscript{iPSC}) were exposed to busulfan and toxicological assessment was carried out using LDH release (A). Results are shown as mean ± SEM fold over control (F.O.C) (N=3) and compared to responses in CD34+ derived macrophages (MC\textsuperscript{cd34}) and dendritic cells (DC\textsuperscript{cd34}). Analysis of mRNA transcripts was carried out using TemO-Seq after 50 µM busulfan exposure and visualized in volcano plots (B). Normalized counts of genes with the highest fold change (non-adjusted P-value <0.05) in iPSC (C) were visualized as Mean ± SEM (N=3).
5.4. Myeloid cell responses to paraquat

Paraquat has been shown to induce small airway toxicity upon ingestion in humans. To explore how this toxin may impact immune cells resident within the small airway as a mechanism of toxicity \textit{in vitro} models of macrophages and dendritic cells were used. iPSC derived myeloid cells were also tested for PQ toxicity responses and compared to primary CD34+ derived cells to evaluate their suitability for toxicity testing. DC$^{cd34}$, MC$^{cd34}$, DC$^{iPSC}$ and MC$^{iPSC}$ models were exposed to a range of PQ doses, for 24 hours, similar to those used for primary small airway epithelial cell cultures.

5.4.1. CD34 differentiation dependent responses to paraquat

PQ induced a dose dependent decrease in resazurin reduction and increase in supernatant LDH levels, which was more pronounced in DC$^{cd34}$ than MC$^{cd34}$ cultures, indicating an increased sensitivity within the dendritic lineage (Figure 5-6A). PQ responses were further explored at doses that precede toxicological effects (20 and 100µM) to be able to detect changes in gene expression in the absence of active cytotoxic processes. Transcriptomic changes in response to PQ exposure was assessed using TempO-Seq methods (Figure 5-6B). PQ at 20 and 100µM resulted in differential expression of 19 and 192 genes in DC$^{cd34}$, and 16 and 123 genes within MC$^{cd34}$ cultures (Figure 5-6C). Fold change differences between DC$^{cd34}$ and MC$^{cd34}$ were further explored (Figure 5-6D) and showed that CXCL8, GDF15 and TRIB3 were among the genes preferentially induced in DC$^{cd34}$ while CHAC1, RRAD, and SLC7A11 were preferentially induced in MC$^{cd34}$. Normalized count levels were displayed on a heatmap to further explore these differences (Figure 5-6E). This shows that some genes, including GDF15 and CXCL8 that showed a higher fold change in one culture, displayed similar count values of gene expression after PQ exposure in both DC$^{cd34}$ and MC$^{cd34}$ cultures. The observed preferential fold change values were due to differences in control gene expression between the two cultures, as also shown for genes such as CXCL1 and CXCL2. However, other genes that showed preferential fold changes upon PQ exposure, showed similar control expression levels, for example DDIT3 and TRIB3 in the DC$^{cd34}$ population, indicating preferential induction of these genes within this population (Figure 5-6E).
Comparison of chemical induced toxicity responses in CD34+ HSC and iPSC derived macrophages and dendritic cells

**Figure 5-6** Paraquat effects in CD34 macrophages and dendritic cells. CD34+ derived macrophages (MC\textsuperscript{CD34}) and dendritic cells (DC\textsuperscript{CD34}) were exposed to paraquat for 24 hours and toxicological assessment was carried out using resazurin reduction and LDH release (A). Results are shown as mean ± SEM fold over control (F.O.C) (LDH) or mean ± SEM % of negative control (resazurin) (N=3). Statistical differences compared to control are shown as * (p-value <0.05). Analysis of mRNA transcripts was carried out using TemO-Seq after 100 µM paraquat exposure and significant genes (adjusted p-value <0.05 and fold change > 2) are highlighted in red in volcano plots (B). Significantly differentially expressed genes (fold change > 2 and adjusted p-value <0.05) upon 100 µM paraquat exposure were counted (D) and Log\textsubscript{2} fold change of top 20 differentially expressed genes in each condition is visualised as mean ± SEM (N=3) (E) and log\textsubscript{2} RPKM values of these genes are displayed in a heatmap (F).
5.4.2. DCCD34 and MCcd34 cultures display differential responses to paraquat

Pathway analysis of biological processes (GO:BP) and molecular factors (GO:MF) was carried out on the significantly differentially expressed genes upon PQ exposure in DC\textsuperscript{cd34} and MC\textsuperscript{cd34} cultures (Figure 5-7A) to identify common and preferentially activated pathways within these myeloid cultures. This showed that DC\textsuperscript{cd34} cultures responded most significantly for stress response in GO:BP and cytokine activity in GO:MF pathway analysis in response to PQ exposure, which were also upregulated to a lesser extent in MC\textsuperscript{cd34} cultures. However, normalized count plots (Figure 5-7B) show upregulation of several stress response and cytokine activity genes, such as GDF15, DUSP1 and IL1A to a similar extent upon PQ exposure within both cultures. However, other stress response pathway genes such as NR1D1 and BBC3 and chemical stimulus response genes such as TRIB3, and ASNS were preferentially induced in DC\textsuperscript{cd34} cultures.

Furthermore, MC\textsuperscript{cd34} showed a slightly more significant response for pathways such as cell death (BP), apoptotic processes (BP) and signalling receptor binding (MF) (Figure 5-7A). Exploring normalized count values within selected pathway genes (Figure 5-7B), however, showed that several cell death pathway related genes such as CHAC1 and DDIT3 are upregulated to a higher normalized count within the DC\textsuperscript{cd34} cultures, indicating increased PQ susceptibility in this culture.

Oxidoreductase activity related genes, such as HMOX1 and NQO1 were slightly upregulated within both cultures (Figure 5-7B), indicating increased levels of oxidative stress might be indeed a mechanism of the PQ induced toxicity.
Comparison of chemical induced toxicity responses in CD34+ HSC and iPSC derived macrophages and dendritic cells

A) GO:BP

- Response to stress
- Response to biotic stimulus
- Response to organic substance
- Response to chemical stimulus
- Cellular response to organic substance
- Cellular response to chemical stimulus
- Response to cytokine
- Cellular response to cytokine stimulus
- Cell population proliferation
- Apoptotic process
- Regulation of apoptotic process
- Regulation of programmed cell death
- Cell cycle
- Cell death
- Mitotic cell cycle

A) GO:MF

- Cytokine activity
- Receptor activity
- Signaling receptor activity
- Molecular function regulator
- Protein binding
- Cytokine receptor binding
- Arachidonic acid binding
- Interleukin-7 receptor activity
- Receptor regulator activity
- Tubulin binding
- DNA replication origin binding
- Microtubule binding
- Identical protein binding
- Enzyme binding

Figure 5-7 Pathway activation upon paraquat exposure in CD34+ derived macrophages and dendritic cells. Significantly differentially expressed genes (FDR p-value < 0.05, fold change > 2) from RNA-Sequencing analysis of paraquat (100 µM; 24 hours) exposures were analysed for GO biological process (BP) and molecular function (MF) pathway analyses using g:Profiler (A). Selected genes were plotted as mean ± SEM RPKM values (B).
5.4.3. Chemical specificity of PQ responses

Comparison of significantly differentially expressed genes observed upon PQ exposure to the other chemical exposures (AM and BUS) may allow for some level of chemical specificity pathway responses. Figure 5-8 shows that the SLC7A11 was also non-significantly upregulated upon AM exposure in DC<sup>cd34</sup>. However, TRIB3 and DDIT3 were uniquely upregulated upon PQ exposure in DC<sup>cd34</sup> and CXCL8 and MDM2 are upregulated upon PQ exposure only in both DC<sup>cd34</sup> and MC<sup>cd34</sup> cultures. In addition SERPINE1 was uniquely upregulated upon PQ exposure in MC<sup>cd34</sup>, indicating both chemical and differentiation specific effects.

5.4.4. iPSC macrophage and dendritic cell responses to paraquat exposure

As CD34+ derived cultures displayed a strong transcriptional response to PQ exposure, it forms a strong baseline response to compare iPSC derived myeloid cell responses to the same toxicant.
Comparison of chemical induced toxicity responses in CD34+ HSC and iPSC derived macrophages and dendritic cells

Both DC\textsuperscript{iPSC} and MC\textsuperscript{iPSC} cultures did not show a significant difference in cytotoxicity or cellular viability levels, as measured by supernatant LDH and resazurin reduction, upon 0-100 µM PQ exposure, similar to that observed for CD34+ derived cells (Figure 5-9A). One replicate of DC\textsuperscript{iPSC} was excluded from TempO-Seq analysis due to relatively low count values (Supplemental figure 9-9).

TempO-Seq analysis of 100 µM PQ exposure demonstrated a significant down regulation of DUSP1 and TNC in DC\textsuperscript{iPSC} cultures but no significantly differentially expressed genes within the MC\textsuperscript{iPSC} cultures (Figure 5-9B). Comparing the responses of DUSP1 and TNC upon PQ exposure to those observed in CD34+ derived cultures, shows a significant increase in DUSP1 in both DC\textsuperscript{cd34} and MC\textsuperscript{cd34} cultures and no significant difference for TNC (Figure 5-9C). Furthermore, genes that were significantly upregulated in both primary CD34+ derived cell types (GDF15, MDM2, CXCL8 and DDIT3), did not follow the same pattern in iPSC cultures, except perhaps for GDF15 in DC\textsuperscript{iPSC} cultures (5-9D). It should be noted that the use of N=2 replicates for DC\textsuperscript{iPSC} culture may be considered a weakness for our ability to perform a confident comparison of effects between DC cell types.

TempO-Seq may not be a sensitive enough method to detect changes upon 24-hour PQ exposure within the iPSC cultures, if the levels of expression are relatively low. qPCR allows for the detection of low levels of individual mRNAs (Bustin, 2000), and was therefore used to further explore the PQ induced response in iPSC and CD34+ macrophage cultures. qPCR analysis also did not show upregulation of CXCL8 and MDM2 mRNA in MC\textsuperscript{iPSC}, whereas these genes were significantly induced in MC\textsuperscript{cd34} (Figure 5-9E). Overall, these results indicate the iPSC macrophage and dendritic cell models may need to be improved to be adequate models for PQ toxicity detection \textit{in vitro} using TempO-Seq or qPCR methods.
Comparison of chemical induced toxicity responses in CD34+ HSC and iPSC derived macrophages and dendritic cells

**Figure 5-9** Comparing Paraquat effects in iPSC to CD34+ macrophages and dendritic cells. iPSC derived macrophages (MC\textsuperscript{iPSC}) and dendritic cells (DC\textsuperscript{iPSC}) were exposed to paraquat and toxicological assessment was carried out using LDH release (A). Results are shown as mean ± SEM fold over control (F.O.C) and compared to responses in CD34+ derived macrophages (MC\textsuperscript{cd34}) and dendritic cells (DC\textsuperscript{cd34}). Analysis of mRNA transcripts was carried out using TemO-Seq after 100 µM paraquat exposure and visualized in volcano plots (N=2 for DC\textsuperscript{iPSC}, N=3 for MC\textsuperscript{iPSC}) (B). Normalized counts of genes significantly differentially expressed in iPSC (C) and CD34 (D) were visualized as Mean ± SEM (N=3). RT-qPCR of selected genes was carried out upon 100 µM paraquat exposure in MC\textsuperscript{iPSC} (blue) MC\textsuperscript{cd34} (green) and visualized as mean ± SEM fold change over control (N=3), corrected for GAPDH (E). Significance is shown as * (p-value <0.05).
Comparison of chemical induced toxicity responses in CD34+ HSC and iPSC derived macrophages and dendritic cells

5.4.5. Gene expression differences underlying cell type specific paraquat susceptibility

There are several possible physiological explanations as to why differences in PQ exposure susceptibility between the DC\textsuperscript{cd34} and MC\textsuperscript{cd34} models occur and why a lack of PQ susceptibility in iPSC derived models were observed. Some of the reasons can be explored by examining the mRNA levels of relevant genes present in the TempO-Seq gene set in the unexposed cultures. Normalized count values of a control gene GAPDH were similar (Figure 5-10A), whereas the mitochondrial marker gene MT-ND6 was higher in iPSC cultures, especially in DC\textsuperscript{iPSC}, which may indicate a higher level of mitochondria within these cultures.

As discussed in chapter 4, SOD1 and SOD2 levels have a protective effect on PQ induced toxicity (Filograna et al., 2016), thus higher levels of expression of these genes would indicate an increase in resistance to PQ induced toxicity. SOD2 levels were higher in MC\textsuperscript{cd34} cultures compared to DC\textsuperscript{cd34} cultures, which may explain increased susceptibility of the DC\textsuperscript{cd34} cultures to PQ through a lack of antioxidant protection (Figure 5-10B). However, SOD2 levels in iPSC cultures were similar to those observed in DC\textsuperscript{cd34} cultures, indicating antioxidant protection is unlikely to result in a lack of response upon PQ exposure in iPSC cultures.

As discussed previously, PQ requires enzymatic activity for its reduction to regenerate ROS. A lack of these enzymes could thus possibly prevent PQ induced toxicity. Indeed, POR, CYBB, NCF2 and NCF4 mRNA levels were shown to be lower within the iPSC cultures, indicating a lack of enzymatic activity may be preventing PQ reduction within the iPSC cultures. Furthermore, PQ susceptibility may also be mediated by PQ absorption and accumulation due to polyamine transporters (Dinis-Oliveira et al., 2008). SLC3A2 mRNA was shown to be higher in MC\textsuperscript{iPSC} cultures, whereas SCL47A1 was higher in DC\textsuperscript{cd34} cultures. A higher level of polyamine transport in DC\textsuperscript{cd34} cultures could possibly explain the increased susceptibility to PQ within this culture. Unfortunately, TempO-Seq probe set did not include a wider range of genes related to polyamine transport, hampering further comparisons.
Comparison of chemical induced toxicity responses in CD34+ HSC and iPSC derived macrophages and dendritic cells

Figure 5-10 Basal differences in expression levels of genes relevant to paraquat toxicity in iPSC and CD34+ derived macrophages and dendritic cells. Genes that are relevant for paraquat toxicity were analysed for their normalized count values in iPSC derived macrophages (MCiPSC) and dendritic cells (DCiPSC) and CD34+ derived macrophages (MCcd34) and dendritic cells (DCcd34) from TempO-seq analysis. Marker genes for cellular RNA and mitochondrial RNA (A), superoxide dismutase (B), oxidoreductases (C), and polyamine transport (D) are visualized as mean normalized count value ± SEM (N=3).
5.5. Discussion

As airway resident immune cells play an important role in toxicological responses within the small airways, the primary in vitro myeloid models established in section 3.4 (DC\text{cd34} and MC\text{cd34} cultures) and iPSC myeloid models established in section 3.5 (DC\text{iPSC} and MC\text{iPSC} cultures) are potentially valuable models for in vitro small airway toxicity testing. Here we have explored the toxicological responses of these models to AM, BUS and PQ; chemicals which were also tested in primary SAEC models in chapter 4, allowing for further comparisons between cell types within chapter 6.

5.5.1. Amiodarone and busulfan responses in primary CD34+ derived macrophages and dendritic cells

AM and BUS are commonly used pharmaceuticals known to induce pulmonary toxicity upon high dose or prolonged exposure in several cases. The involvement of immunotoxicity in pulmonary disease development upon AM or BUS exposure is currently unknown but a likely mode of action, especially for AM, as AM exposure results in foamy macrophages development within the lungs of exposed patients (Papiris et al., 2010). Indeed, AM exposure resulted in significant increase in supernatant LDH levels at concentrations surpassing 25 μM in both DC\text{cd34} and MC\text{cd34}, indicating cytotoxicity occurs upon higher concentrations of AM exposure. Furthermore, a reduction in cellular viability as measured by resazurin reduction levels was observed at AM concentrations higher than 15 μM in DC\text{cd34} and 45 μM in MC\text{cd34} cultures. This indicates an increased susceptibility of DC\text{cd34} culture for AM induced toxicity. TempO-Seq analysis showed a significant dysregulation of 2 genes in of DC\text{cd34} cultures upon 15 μM AM exposure, while MC\text{cd34} cultures did not show significantly differentially expressed genes. AM exposure resulted in an increase in CYP51A1 and a decrease in PHOSPHO1 expression in DC\text{cd34} cultures. CYP51A1 is a cholesterol biosynthesis enzyme and thus the observed overexpression could be an indication of lipid accumulation within the dendritic cell populations (Horvat et al., 2011). PHOSPHO1 gene has been suggested to be a regulator of insulin resistance in mice.
Comparison of chemical induced toxicity responses in CD34+ HSC and iPSC derived macrophages and dendritic cells (Suchacki et al., 2020) and to be associated with type 2 diabetes risk in humans (Dayeh et al., 2016). These observed gene expression changes may thus indicate that insulin resistance and lipid accumulation are indeed possible mechanisms of AM toxicity, as also suggested in chapter 4 (Lapinsky et al., 1993). However, due to an outlier as observed by sample distance, N=2 was used for the MC\textsuperscript{cd34} cultures, hampering direct comparisons and thus conclusions with regards to cellular specificity of AM exposures. However, non-significant increases in expression of genes such as CXCL8 and PSAT1 were observed in both DC\textsuperscript{cd34} and MC\textsuperscript{cd34} cultures upon AM exposure (Figure 5-2D), indicating AM exposure may also impact inflammatory pathways in both cell types (Vroling et al., 2008). As discussed previously, AM metabolites may also be at least partially responsible for AM induced pulmonary toxicity. Indeed, studies using rat or rabbit alveolar macrophages showed a larger effect of AM’s major metabolites, mono-N-desethylamiodarone (MDEA) and di-N-desethylamiodarone (DDEA) on viability and cytotoxicity markers compared to their parent compound (Ogle & Reasor, 1990; Quaglino et al., 2004). However, the effect of AM and its metabolites on human alveolar macrophages and dendritic cells is currently unknown and requires further investigation. As AM exposure was shown to have a significant effect on cytotoxicity and viability markers in this study but resulted in a low amount of significantly differentially expressed genes, a detection method that is more sensitive than TempO-Seq analysis may be required to detect small changes in gene expression upon AM exposure. Indeed, qPCR analysis of the same samples as used for TempO-Seq analysis showed a significant increase in CXCL8 expression in DC\textsuperscript{cd34} cultures only and a significant increase in DDIT3 expression in both DC\textsuperscript{cd34} and MC\textsuperscript{cd34} cultures (Supplemental figure 9-8).

In contrast, BUS exposure did not significantly alter cytotoxicity, viability, or gene expression levels both DC\textsuperscript{cd34} and MC\textsuperscript{cd34} cultures. The presence of the GM-CSF in both culture media supplements, maybe acting as a protective factor against BUS induced effects, as this growth factor was previously shown to protect an erythro-myeloid cell line against BUS induced toxicity (Ertan et al., 2007). Furthermore, granulocyte-macrophages were shown to be more resistant to BUS exposure compared to erythroid-progenitor cells isolated from the same individual
Comparison of chemical induced toxicity responses in CD34+ HSC and iPSC derived macrophages and dendritic cells (Kubota et al., 1983). Although our study did not show a profound effect of BUS exposure, additional studies are needed to determine macrophage and dendritic cell susceptibility to BUS toxicity. As discussed before in chapter 4, a higher BUS dose, a repeated dose exposure regime, or appropriate levels of bio-activation within the model could increase ability of our CD34+ models to detect BUS toxicity (Younis et al., 2008). Overall, whereas AM resulted in altered toxicity, which was more profound in DC^{cd34} cultures. No major toxicity was found upon BUS exposure in either culture.

5.5.2. Primary CD34+ derived macrophage and dendritic cells display differential responses to paraquat

Exposure to the herbicide PQ commonly results in acute pulmonary toxicity and delayed pulmonary fibrosis. As shown in section 4.5, PQ exposure resulted in a large toxicological response in primary airway epithelial cells. However, the possible contribution of airway resident immune cells to PQ induced pulmonary toxicity has not been adequately investigated. Primary CD34+ cultures were exposed to the same concentrations of PQ as used for the primary airway epithelial cells to allow for more in-depth comparisons to be made in chapter 6. PQ exposure did not significantly alter toxicity or viability markers in DC^{cd34} and MC^{cd34} cultures in concentrations below 1 mM. However, the decrease in resazurin reductase activity and increase in supernatant LDH levels shown at 1 mM PQ exposure in both cultures was more pronounced in DC^{cd34}. To understand the mechanism by which PQ might differentially impact these two myeloid cultures, gene expression patterns were explored using TempO-Seq analysis. Whereas PQ exposure resulted in a similar increase in several stress response genes such as GDF15 and DUSP1 in DC^{cd34} and MC^{cd34} cultures, other genes such as DDIT3 and TRIB3 were preferentially induced in DC^{cd34} cultures. Overall, these results indicate that whereas both DC^{cd34} and MC^{cd34} cultures show responsiveness to PQ exposure, increased responsiveness of DC^{cd34} cultures of several stress response and cell death pathway genes may underly differences in cytotoxicity and viability markers upon PQ exposure in both cultures.
There are several possible explanations for the differences in PQ responses observed between both cell types. One of these explanations are possible inherent biological functions within these cell types resulting in differential susceptibility. As an increased production of ROS is a common defence mechanism against pathogens for macrophages, these cells have self-protective mechanisms against the self-generated oxidative stress (Virág et al., 2019; P. Wang et al., 2019). Within inflammatory macrophages, NRF2 pathway activation was shown to be the main signalling cascade that alleviates oxidative stress by upregulating anti-oxidant genes such as HMOX and NQO1 (Tonelli et al., 2018; P. Wang et al., 2019). However, no differences in upregulation of NRF2 pathway activation or HMOX1 and NQO1 was observed between MC<sub>cd34</sub> and DC<sub>cd34</sub> cultures, indicating NRF2 mediated protective mechanisms are unlikely to be the underlying mechanism of the observed differential responses to PQ exposures. In dendritic cells, mitochondrial ROS production is required for their ability to induce CD8+ T cell responses (Matsue et al., 2003; Oberkampf et al., 2018), indicating that specific ROS mediated pathway activations observed within DC<sub>cd34</sub> cultures upon PQ exposure might be related to innate biological functions of dendritic cells.

Examination of inherent differences in gene expression patterns between cell types can give further insight in differential susceptibilities to PQ exposure. Although this does not consider possible contribution of post translational modifications or epigenetic changes, it might give useful insight into possible mechanism of PQ susceptibility. One possible explanation for difference in susceptibility could be a different level of intracellular PQ exposure. PQ has been suggested to be taken up by cells through polyamine transporters (Dinis-Oliveira et al., 2008). TempO-Seq probe set includes SCL3A2 and SLC47A1 genes which encode proteins that may play a role in PQ uptake. Whereas no difference was observed in SCL3A2 mRNA levels between DC<sub>cd34</sub> and MC<sub>cd34</sub> cultures, SLC47A1 mRNA levels were shown to be higher in DC<sub>cd34</sub> cultures. Indeed, PQ accumulation and toxicity was shown to be larger in human embryonic kidney cells expressing hMATE1 which is encoded by SLC47A1 (Y. Chen et al., 2007), indicating increased PQ susceptibility observed in DC<sub>cd34</sub> cultures might indeed be due to increased levels of PQ uptake.
Comparison of chemical induced toxicity responses in CD34+ HSC and iPSC derived macrophages and dendritic cells

Although mRNA levels might not accurately reflect protein levels of polyamine transporters, they may give an indication of their levels. Other relevant genes related to polyamine transport as measured by RNA-Sequencing methods will be further discussed in chapter 6.

SOD activity plays an important role in reducing PQ induced toxicity (Filograna et al., 2016), as shown by a study that demonstrated CRISPR knockout of SOD1 in immortalized human T lymphocyte cells resulted in increased sensitivity to PQ injury (Reczek et al., 2017). Although no difference in SOD1 mRNA levels was observed between DC\textsuperscript{cd34} and MC\textsuperscript{cd34} cultures within this study, DC\textsuperscript{cd34} cultures showed lower levels of SOD2 gene levels compared to MC\textsuperscript{cd34} cultures, which may indicate a reduced capability to detoxify superoxide molecules in DC\textsuperscript{cd34} cultures. As SOD2 is located within mitochondria (Flynn & Melov, 2013), it may be that increased mitochondrial levels of superoxide within DC resulted in selective activation of signalling pathways originating in mitochondria.

Mitochondrial stress response pathways include oxidative phosphorylation, inflammatory signalling, apoptosis, and induction of mitochondrial unfolded protein responses (UPR) (Münch, 2018; Patergnani et al., 2020). Selective induction of genes which are related to the UPR pathway such as DDIT3, ATF4 and TRIB3 were observed in DC\textsuperscript{cd34} cultures. To verify this specific UPR activation as a mechanism of PQ toxicity within DC\textsuperscript{cd34} cultures requires further studies as other stress response or inflammatory pathways might be causing the difference in gene expression patterns (West et al., 2015). Further insights in PQ effects on DC\textsuperscript{cd34} and MC\textsuperscript{cd34} cultures and comparison to primary airway epithelial cell responses to PQ will be explored in chapter 6.

5.5.3. iPSC derived macrophage and dendritic cell responses to amiodarone, busulfan and paraquat

To explore the applicability of iPSC derived myeloid cultures to \textit{in vitro} toxicity testing, DC\textsuperscript{iPSC} and MC\textsuperscript{iPSC} cultures were exposed to AM, BUS and PQ to be able to compare to CD34+ counterpart responses and determine similarity. Both DC\textsuperscript{iPSC} and MC\textsuperscript{iPSC} cultures did not show a significant cytotoxic response or gene expression alteration with AM or BUS treatment. Primary CD34+
Comparison of chemical induced toxicity responses in CD34+ HSC and iPSC derived macrophages and dendritic cells counterparts also did not show changes in supernatant LDH levels upon AM or BUS exposure at concentrations up to 15 µM. Resazurin reduction levels were not measured in the iPSC cultures. TempO-Seq analysis showed that 15 µM AM exposure resulted in dysregulation of 2 genes in DC_{cd34} cultures, CYP51A1 and PHOSPHO1, which was not observed in iPSC cultures. As CD34+ counterparts also did not show a profound effect upon AM and BUS exposures either, comparisons are limited. As discussed in section 3.5, the iPSC macrophage and dendritic cell cultures are not pure populations and consist partly of mesenchymal cell lineages. However, human fibroblasts cell lines have been shown to be susceptible to AM, BUS, and PQ induced oxidative stress and fibrosis, albeit at slightly higher concentrations as used in our experiments (Probin et al., 2007; Silva Santos et al., 2017; R. Yao et al., 2015). It is therefore unlikely that the presence of fibroblasts in the iPSC cultures completely masked toxicological effects.

iPSC derived macrophages have been previously used to assess cytokine and calcium release upon drug administration (Gutbier et al., 2020), however their ability to detect redox toxicity has not been determined. Unlike their CD34+ counterparts, both DC_{iPSC} and MC_{iPSC} cultures did not show a profound effect on cytotoxicity, viability, or gene expression patterns upon PQ exposure. There could be technical issues with sensitivity of the TempO-Seq method to detect gene expression changes, as cell density and count values were generally lower in iPSC compared to CD34+ models (Supplemental figure 9-9). However, a lower cell density would generally result in an increased susceptibility as the PQ dose per cell would be higher. Therefore, this is an unlikely cause of the lack of PQ toxicity observed in iPSC cultures. As previously discussed, higher SOD2 levels may have a protective effect on PQ induced toxicity. However, both DC_{iPSC} and MC_{iPSC} cultures showed low levels of SOD2, comparable to DC_{cd34} cultures, indicating this protective mechanism is not the cause of a lack of PQ induced toxicity observed in iPSC cultures. As PQ toxicity is likely mediated by enzymatic redox cycling and superoxide production (Karnati et al., 2013), gene expression differences in possible targets of PQ induced toxicity were examined (Dinis-Oliveira et al., 2008). POR and NOX have been identified as possible enzymes that play a role in reduction of PQ to produce superoxide anions (Cristóvão et
Comparison of chemical induced toxicity responses in CD34+ HSC and iPSC derived macrophages and dendritic cells (al., 2009; Han et al., 2006), with POR being the primary mediator in immortalized human T lymphocyte cell (Reczek et al., 2017). Both iPSC cultures showed lower levels of POR and CYBB (NOX2) mRNA. Furthermore, the iPSC cultures showed much lower mRNA levels of several NOX enzyme co-factors, including NCF2 and NCF4 compared to their CD34+ counterparts. Lower mRNA levels of primary enzymes involved in PQ reduction and toxicity indicate that a lack of PQ redox cycling activity might be a reason for lack of observed toxicity in the iPSC cultures. Increasing POR and NOX enzyme capacity of these cultures might therefore increase their ability to identify redox cycling toxicants.

5.5.4. Conclusions and future directions

This chapter aimed to explore the effect of small airway toxicants on CD34+ and iPSC derived macrophage and dendritic cell models to investigate the applicability of these models to toxicity testing. Although the immune cell models used within this chapter are more representative for inflammatory phenotypes, their responses to toxicants are presumably similar to small airway resident immune cells. AM and BUS did not show profound transcriptomic changes on CD34+ cultures, as was also observed for primary airway epithelial cell cultures. As these compounds are known to induce pulmonary toxicity, the models used within this thesis may not be adequate to detect this, possibly due to limitations in detecting small transcriptomic changes using TempO-Seq methods, relatively low exposure doses or lack of bio-activation capacity. Therefore, these compounds will not be further discussed in chapter 6 (Figure 5-11). PQ exposure did result in altered transcriptomic responses in both DC$^{cd34}$ and MC$^{cd34}$ cultures, with several genes selectively upregulated in DC$^{cd34}$ cultures. However, the exact mechanism of action of PQ toxicity is still unknown. Examining protein levels of polyamine transporters or levels of intracellular ROS production could provide future insights into PQ toxicity. However, this chapter provides new insights into PQ toxicity that will be further explored in chapter 6. The iPSC cultures did not show a similar response upon PQ exposure as observed for the CD34+ cultures and will therefore not be further explored within the next chapter as additional efforts are needed to optimize these cultures for toxicity testing purposes.
Comparison of chemical induced toxicity responses in CD34+ HSC and iPSC derived macrophages and dendritic cells

Figure 5-11 Conclusions of Chapter 5
CHAPTER 6: UTILISATION OF IN VITRO MODELS TO EXPLORE CELL SPECIFICITY AND MECHANISMS OF ACTIONS OF RESPIRATORY TOXINS
6.1. Introduction

The previous chapters have focussed on the development, optimisation, and characterisation of *in vitro* small airway models, inclusive of immune cell types and their application to *in vitro* toxicity testing. This chapter builds on these initial observations and to use the power of comparative transcriptomics to interrogate cellular specificity and mechanisms of action of respiratory chemical and environmental exposure toxicity.

Different cell types exist by virtue of different gene expression landscapes that underpin cell specific differentiation. Indeed, one of the major goals of *in vitro* toxicology is to ensure there is a cell type and organ specific competency that can recapitulate whole body responses. A considerable component of organ and cell type specific toxicity to chemical exposure can be attributed to unique properties of particular cell types independent of external dose exposure they may experience. Such intrinsic properties include expression of transporters and metabolising enzymes (Dinis-Oliveira et al., 2008), which can be detected at the mRNA level using techniques such as RNA-seq. Initial observations from paraquat exposure in small airway epithelial and immune cell *in vitro* experiments in chapters 4 and 5, demonstrate differential responses depending on the cell type used. This included increased stress response pathway activation (e.g UPR activation) detected using transcriptomic analysis in DC<sup>CD34</sup> cells. In this chapter we will directly compare toxicity pathway activation within these datasets to reveal cell type and pathway specific responses. We will then interrogate basal levels of gene expression of each cell type in order to understand how unique features of a cell type may underly susceptibility to toxicity.

In addition to investigations involving paraquat for which we have introduced previously (section 4.1.3), this chapter will also use the same methodological approach described above to explore how other respiratory toxins (nanoparticles and dust mite allergen) respond in a cell type specific manner and how this may have implications for our understanding of allergic airway disease and asthma and how in vitro models may be used to assess these effects.
6.1.1. Allergic airway disease and nanoparticle exposure

As a means to understand relevant mechanisms and endpoints assessed in vitro assays, this section will focus on introducing nanoparticle and allergen exposure as it impacts pulmonary disease and asthma.

Nanoparticle exposure has been suggested to increase responses to allergens within the lung and to result in asthma symptoms, specifically within the small airways (Meldrum et al., 2017). Asthma is described as a heterogeneous chronic inflammatory lung disease that results in respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough (Reddel et al., 2015). It is no longer considered as a single disease, but as a syndrome comprising different endotypes that involve distinct underlying molecular mechanisms (Fehrenbach et al., 2017), small airway disease might be a distinct phenotype of asthma (Lipworth et al., 2014). Indeed, several clinical studies suggest that the small airways form the major site of obstruction in asthma (Bommart et al., 2017; Gonem et al., 2014; J. Huang et al., 2015; Kjellberg et al., 2016; Perez et al., 2013; Skylogianni et al., 2018; Stenberg et al., 2017; Uppala et al., 2019; van der Wiel et al., 2014). The most common small airway abnormalities in asthma include goblet cell metaplasia, subepithelial fibrosis, increased airway smooth muscle mass, angiogenesis, chronic inflammatory infiltrates and alterations in the extracellular matrix components (Halwani et al., 2010; Mauad et al., 2007). Furthermore, small airways of asthma patients show a decreased number of club cells and an increased number of T cells, activated eosinophils and mast cells, indicating a decreased control of inflammation in asthma (Shijubo et al., 1999).

Several nanoparticle (TiO$_2$, SiO$_2$, CeO$_2$, ZnO) and toxicant exposures can exacerbate allergen induced type II inflammation through mechanisms involving airway epithelial and/or immune cells (De Haar et al., 2006; Meldrum et al., 2017, 2020). So far, it is unclear which (combination of) cell types are the most likely initial target for the cascade of signalling pathways induced by chemical, nanoparticle and allergen co-exposures. Dendritic cells are typically associated with
allergen sensitization. For instance, dendritic cells have been shown to be essential for ultrafine carbon black particles induced allergen sensitization in mice (de Haar et al., 2008). Environmental particle exposures were shown to induce dendritic cell antigen uptake and processing, cytokine production, upregulation of maturation markers and migration to lymph nodes, ultimately resulting in a type II immune response (de Haar et al., 2008; Pfeffer et al., 2018). Although dendritic cells play an essential role in airway sensitization, they are not necessarily the primary target for nanoparticle induced airway sensitization. Macrophages form a first line of defence within the human small airway lumen, as these cells contain receptors for allergens and the ability to crosstalk with other immune cell types (Currie et al., 2000; Lee et al., 2015), they form another potential target for type II airway inflammation and sensitization (Hussell & Bell, 2014). Indeed, an in vitro study has shown that HDM extracts can activate toll-like receptors TLR2 and TL4 on mouse alveolar macrophages playing a key role in its polarization to Th2 type immune responses (C. Liu et al., 2013).

Furthermore, airway epithelial cells express cell-surface and intracellular pattern recognition receptors that allow these cells to detect signals such as microbial infection, particulate matter, oxidant gasses and cellular damage in order to synthesize pro-inflammatory cytokines activating or recruiting other immune cells (Poynter, 2012). For instance, several studies showed that metal nanoparticles can induce cytotoxicity and production of cytokines in alveolar macrophages and airway epithelial cells resulting in recruitment and activation of dendritic cells (De Haar et al., 2006; Rabolli et al., 2014; Val et al., 2009).

6.1.2. Specific aims

As both Airway epithelial cell and CD34+ models were exposed to 100 µM PQ, comparison of transcriptomic responses of these models gives further insight into cellular specificity of PQ exposures (Figure 6-1) and mechanisms of action. Additional concentrations of PQ were only tested in DCI differentiated SAEC and CD34+ models, therefore comparison of classical cytotoxicity assays was limited to these models. Additionally, nanoparticle (15 µg/cm²) and
Utilisation of in vitro models to explore cell specificity and mechanisms of actions of respiratory toxins

allergen (25 µg/cm²) co-exposures effects will be explored in DCI differentiated SAEC and CD34+ models as well, to determine the applicability of the in vitro models developed within this thesis to detect nanoparticle exacerbation effects.

Figure 6-1 Overview of Chapter 6. Classical toxicological and mRNA sequencing methods used to investigate respiratory exposure cell type specific responses for PQ exposure. Particle and HDM exposure responses were explored using LDH cytotoxicity and qPCR methods in all models and TempO-Seq in MC<sub>cd34</sub> cultures only.

Aims:

- Compare PQ induced toxicological and transcriptomic changes as described in chapters 4 and 5 to further insights into PQ mechanism of toxicity
- Generate additional data using nanoparticle and HDM exposure to further investigate how in vitro models may be useful for pulmonary toxicity assessment and to investigate the mechanisms involved.
6.2. **Cellular specificity of paraquat toxicity**

PQ has been shown to induce pulmonary toxicity upon ingestion, likely to be mediated by multiple cellular mechanisms, including redox cycling and inflammatory pathways (Subbiah & Tiwari, 2021). SAEC cultured on ALI and CD34+ models were exposed to PQ as described in the previous chapters 4 and 5. The toxicological impact of such exposure was explored by determining supernatant LDH and resazurin reduction levels and transcriptomic changes were determined using qPCR, mRNA Sequencing and TempO-Seq methods as described in chapter 2. Responses are generally provided as fold over control for each cell type to allow for comparisons to be made between cell type.

6.2.1. **Cell type specific impact of paraquat cytotoxicity levels**

Figure 6-2 shows a significant increase in supernatant LDH levels in DC⁺cd34 cultures upon 1000 μM PQ exposure. In contrast, MC⁺cd34 and DCI differentiated SAEC cultures did not show a significant impact on supernatant LDH levels at the same concentration range. 1000 μM PQ exposure also resulted in a significantly decreased resazurin reduction level in all three tested cultures, which was most profound in DC⁺cd34 cultures. However, DCI differentiated SAEC cultures showed a significant concentration dependent decrease in resazurin reduction levels upon lower PQ exposure within the 50 – 1000 μM range, which was not observed in CD34+ cultures. Overall, it may be suggested that DC⁺cd34 cells displayed greater general toxicity to paraquat than SAEC and CD34 derived macrophages.
Figure 6-2 **Paraquat toxicity curves in in vitro models.** CD34+ derived macrophages (MC\(^{cd34}\)) and dendritic cells (DC\(^{cd34}\)), and DCI differentiated SAEC were exposed with paraquat for 24 hours and toxicological assessment was carried out using resazurin reduction and LDH release assays. Results are shown as mean ± SEM fold over control (F.O.C) (LDH) or mean ± SEM % of negative control (resazurin) (N=3) and significance over control is shown as * (P<0.05).

6.2.2. Cell type specific transcriptomic responses to paraquat

In order to further delineate differences between cell type exposures to PQ, transcriptomic analysis was next examined. As different methods were used to determine the transcriptomic responses in CD34+ and SAEC cultures, read count metrics from the different platforms (TempO-seq and mRNA Sequencing) cannot be directly compared. We therefore used the approach to compare fold change (FC) values over untreated samples for each transcriptomic analysis. Using this approach allowed both CD34+ derived macrophage and dendritic cell cultures as well as SAEC Basal, DCI and PneumCult\(^{TM}\)-ALI differentiated cultures to be compared. Since DC\(^{cd34}\) cultures arguably displayed the greatest gross toxicity response to PQ, the top significantly differentially expressed genes upon 100 \(\mu\)M PQ exposure within these cells (DDIT3, GDF15, MDM2 and TRIB3) was further examined and compared to the other cell types (Figure 6-3). DDIT3, GDF15 and TRIB3 stress response genes were shown to be upregulated in both CD34+ and differentiated SAEC cultures. In contrast, MDM2, a negative regulator of p53 pathway, was shown to be significantly upregulated upon PQ exposure in CD34+, but not SAEC models. Within CD34+ cultures, DDIT3, GDF15, MDM2 and TRIB3 mRNA were preferentially induced within the DC\(^{cd34}\) cell type.
Utilisation of in vitro models to explore cell specificity and mechanisms of actions of respiratory toxins

Figure 6-3 Top significantly differentially expressed genes upon paraquat exposure in DC^{cd34} cultures. CD34+ derived macrophages (MC^{cd34}) and dendritic cells (DC^{cd34}), airway basal cells, DCI differentiated cells and PneumaCult^{TM}-ALI differentiated cells were exposed with paraquat for 24 hours. Log2 fold ov (FC) values (mean ± SEM) upon 100 µM paraquat exposure were compared between RNA-Seqencing (SAEC cultures) and TempO-Seq (CD34 cultures) analysis (N=3).

Overall, basal SAEC showed a less profound response to PQ exposure compared to the other models as shown in a heatmap of FC values of top differentially expressed genes in both SAEC and CD34+ cultures (Figure 6-4). DCI differentiated SAEC showed a similar upregulation of DDIT3, GDF15 and TRIB3 stress response genes upon PQ exposure compared to DC^{cd34} cultures but lack responsiveness for genes such as MDM2 and CXCL8 (Figure 6-4). PneumaCult^{TM}-ALI differentiated SAEC showed upregulation of a large number of genes upon PQ exposure, including inflammatory genes such as CXCL8 and IL1A. Interestingly, oxidoreductase activity genes such as SRXN1, HMOX1 and AKR1B10 are upregulated in differentiated SAEC, in particular within the PneumaCult^{TM}-ALI differentiated model. In contrast, genes such as CXCL8 and MDM2 show a higher FC value in CD34+ cultures, especially within the DC^{cd34} model. Whereas genes such as GDF15 and TRIB3, are top significantly differentially expressed genes in both DC^{cd34} and MC^{cd34} cultures, genes such as CHAC1 and SLC7A11 specifically might be more specifically upregulated in MC^{cd34} cultures.
Figure 6-4 Comparing fold change value of top significantly differentially expressed genes upon paraquat exposure in different in vitro cultures. CD34+ derived macrophages (MC\textsuperscript{cd34}) and dendritic cells (DC\textsuperscript{cd34}), airway basal cells, DCI differentiated cells and PneumaCult\textsuperscript{TM}-ALI differentiated cells were exposed with paraquat for 24 hours. Log2 fold change (FC) values for the top differentially expressed genes upon 100 µM paraquat exposure were compared between RNA-sequencing (SAEC cultures) and TempO-Seq (CD34 cultures) analysis.

As different platforms were used in Figures 6-3 and 6-4 to compare cell type responses, qPCR analysis was also performed to confirm these responses within the same assay (Figure 6-5). Indeed, qPCR analysis showed a significant upregulation of DDIT3 mRNA in DCI differentiated SAEC and DC\textsuperscript{cd34} cultures only, as also observed with sequencing methods. SLC7A11 mRNA was upregulated in DCI and PneumaCult\textsuperscript{TM}-ALI differentiated SAEC as well as MC\textsuperscript{cd34} cultures upon PQ exposure. HMOX1 mRNA showed upregulation in DCI and PneumaCult\textsuperscript{TM}-ALI differentiated SAEC and DC\textsuperscript{cd34} cultures upon PQ exposure. Furthermore, qPCR analysis showed that MDM2 and ATF4 mRNA were only significantly upregulated in DC\textsuperscript{cd34} cultures. Highest upregulation of these genes within DC\textsuperscript{cd34} was also observed using sequencing methods.
Overall, basal SAEC showed no significant upregulations of the selected genes using qPCR methods, further demonstrating differentiation of SAEC is required to detect PQ responses in vitro. Furthermore, DC\(^{\text{cd34}}\) cultures showed the highest number of dysregulated genes (DDIT3, HMOX1, MDM2, ATF4) in response to 100 µM PQ exposure. Interestingly, both DDIT3 and ATF4 are key markers for UPR pathway activation (Fusakio et al., 2016), indicating a unique role for dendritic cells in responses to PQ exposure.
6.2.3. Gene expression differences related to paraquat susceptibility

There are several possible mechanistic explanations as to why cells respond differentially to PQ exposure. Some of these can be explored by comparing the mRNA levels of genes which are likely to impact responsiveness to PQ as discussed before in chapters 4 and 5. As RNA-Sequencing methods were used to characterise the cultures, a comparison of RPKM values can be used to give insights into mechanisms of differential PQ susceptibility beyond what was discussed in the previous chapters.

Figure 6-6 displays RPKM values of genes previously related to PQ susceptibility from RNA-Seq data of all cell types used in this chapter. This figure shows that polyamine transporters (SLC3A2, SLC7A1 and ATP13A2), copper transporters (SCL13A1 and SLC13A2) and superoxide dismutase (SOD1 and SOD2) genes are expressed to a higher degree in CD34+ derived cultures, with copper transport and SOD2 being notably higher in MC<sup>cd34</sup> cultures. As SOD1 activity is dependent on intracellular copper levels, copper transporters may impact cellular toxicity through modulation of superoxide levels. Indeed, a CRISPR screen has previously shown SLC31A1 knock-out sensitised cells to PQ injury (Reczek et al., 2017). Therefore, higher levels of SOD2 and copper transporters with MC<sup>cd34</sup> cultures compared to DC<sup>cd34</sup> cultures might underly their increased resistance to PQ exposure.

The oxidoreductase gene DUOXA1 was more highly expressed in basal SAEC cultures, whereas UCP2 mRNA was higher in PneumaCult™-ALI differentiated SAEC and CD34+ cultures compared to the other two cell models. Overall, oxidoreductase related genes were notably more highly expressed in CD34+ cultures compared to SAEC. Mitochondrial RNA (MT-ND1) was more comparable between cultures, indicating this higher level of oxidoreductase genes may not be caused by higher levels of mitochondria in CD34+ cultures.

Additional research is required to fully explore the involvement of mechanisms such as PQ accumulation due to polyamine transporters, and ROS production due to oxidoreductase activity in the cellular susceptibility to PQ exposures.
Utilisation of in vitro models to explore cell specificity and mechanisms of actions of respiratory toxins

Figure 6-6 Differences in control RPKM values in airway and CD34 cultures. Genes that are relevant for paraquat toxicity were analysed for their RPKM value in CD34+ derived macrophages (MC\textsuperscript{cd34}) and dendritic cells (DC\textsuperscript{cd34}) and airway basal, DCI differentiated, and PneumaCult\textsuperscript{TM}-ALI differentiated cells from RNA sequencing analysis. Genes that are relevant for polyamine transport (A), copper transport (B), superoxide dismutase (C) and oxidoreductase activity (D) are visualized as mean normalized count value ± SEM (N=6).
6.3. **Nanoparticle and allergen effects on *in vitro* airway models**

Cell type specific responses for additional respiratory toxins and exposures were next examined. Nanoparticle and dust mite exposures were carried out apically for airway epithelial *in vitro* models to represent airway apical exposure as would occur in inhalation exposure in humans. This route of exposure is in contrast to that for paraquat exposure, where exposure in poisoning is basolateral (section 6.2). Immune cells were exposed in suspension. Calculations of dosing were carried out such that the same dose/cm² exposure was achieved.

As macrophages are one the primary responders to allergens within the human airways (Puttur et al., 2019), MC^cd34^ cultures were used to determine which particles to analyse across other cell types. Responses to nanoparticle and house dust mite (HDM) exposures alone or in combination were investigated in MC^cd34^ cultures by qPCR (Supplemental figure 9-10). A panel of different common nanoparticles were initially selected to investigate inflammatory responses, from which two would be chosen for more in-depth analysis and cell type specific response assessment.

Upregulation of genes such as metallothioneins (e.g., MT1G) can be controlled by metal transcription factors which require Zinc metal ions. Indeed ZnO nanoparticles have been previously shown to induce MT1G gene expression in several human cell lines (Moos et al., 2011). Using ZnO nanoparticles as a positive control, we show increased mRNA levels of CXCL5, MT1G and CCL5 upon co-exposure with HDM within MC^cd34^ cultures (Supplemental figure 9-10). SiO₂ and CeO₂ nanoparticles were chosen to be further explored within other *in vitro* small airway models, as these nanoparticles demonstrated increased inflammatory responses (including increased MT1G expression) upon co-exposures with HDM as compared to the other test particles (Supplemental figure 9-10).

SiO₂ and CeO₂ nanoparticles were analysed for size (Figure 6-7A) using DLS methods. Addition of the particles to DMEM or DMEM + HDM increased particle size when compared to water dispersion alone, indicating agglomeration of particles, likely a consequence of salt ions present
within the culture media. Addition of HDM to particle solutions further increased particle
diameter and may represent further agglomeration or protein corona formation (Maiorano et
al., 2010).

Small airway models are most appropriate for nanoparticle toxicity assessment as these airway
regions are most impacted in asthma and allergic airway disease (Lipworth et al., 2014).
Therefore, the DCI differentiated SAEC which show a small airway-like phenotype (section 3.2)
were exposed to nanoparticles and HDM. Furthermore, MC\textsuperscript{cd34} and DC\textsuperscript{cd34} cultures were also
exposed to particles and HDM to explore the airway immune responses and to determine
cellular specific responses.

Cells were treated with HDM (25 \(\mu g/cm^2\)), SiO\textsubscript{2} nanoparticles (15 \(\mu g/cm^2\)), CeO\textsubscript{2} nanoparticles
(15 \(\mu g/cm^2\)) alone, or combinations of HDM and nanoparticles at these same doses were also
included. No significant changes in supernatant LDH levels were observed upon HDM,
nanoparticle or co-exposure in any cell type (Figure 6-7B), indicating exposures did not cause
significant levels of gross cytotoxicity. As gene expression changes often precede toxicological
changes, qPCR was also performed (Figure 6-7C). DCI differentiated SAEC showed no significant
changes in gene expression upon HDM, SiO\textsubscript{2}, CeO\textsubscript{2} or co-exposure treatment. DC\textsuperscript{cd34} cultures did
however demonstrate a significant increase of CCL15 and MT1G upon HDM and CeO\textsubscript{2} + HDM co-
exposures compared to controls. Interestingly, MC\textsuperscript{cd34} cultures showed the most pronounced
responses, with significant increases in SLC7A11, MT1G, CCL15 and SLC2A6 mRNA expression
upon HDM exposure. Interestingly while CCL15 expression was not altered by SiO\textsubscript{2} alone, it was
significantly increased in SiO\textsubscript{2} + HDM co-exposures compared to HDM exposure alone. Broadly
speaking, macrophage cells responded to a greater extent to HDM exposures than other cell
types.
Utilisation of in vitro models to explore cell specificity and mechanisms of actions of respiratory toxins

A)

<table>
<thead>
<tr>
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<th>H$_2$O</th>
<th>DMEM</th>
<th>DMEM + HDM</th>
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<tbody>
<tr>
<td>CeO$_2$ NPs</td>
<td>137.5 ± 7.1 nm</td>
<td>228.3 ± 61.5 nm</td>
<td>386.9 ± 71.0 nm</td>
</tr>
<tr>
<td>SiO$_2$ NPs</td>
<td>230.6 ± 63.3 nm</td>
<td>419.8 ± 145.4 nm</td>
<td>554.5 ± 171.2 nm</td>
</tr>
</tbody>
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B)

Figure 6-7 Cellular specificity of nanoparticle exposure. CD34+ derived macrophages (MC$^{cd34}$) and dendritic cells (DC$^{cd34}$) and DCI differentiated airway epithelium were exposed to house dust mite (HDM, 25 µg/cm$^2$), cerium dioxide nanoparticles (CeO$_2$ NPs, 15 µg/cm$^2$), silica nanoparticles (SiO$_2$ NPs 15 µg/cm$^2$) and co-exposures. Nanoparticles were characterized for their size using dynamic light scattering (DLS) in different media (A). Values are shown as mean ± SD size (nm). Toxicological assessment was carried out using LDH release assay (N=4) displayed as mean ± SEM fold over control (F.O.C.) (B). RT-qPCR analysis of selected genes was carried out upon single and co-exposures (24 hours), N=4, shown as mean ± SEM fold change over control, corrected for GAPDH expression (C). Significance is shown as * (compared to control) or # (compared to HDM exposure), p-value <0.05.
As these initial pPCR observations demonstrate that MC\textsuperscript{cd34} cells had the greatest response to nanoparticle and dust mite exposure, it was decided to focus in on these cells to explore potential further immune responses and mechanisms underlying any potential adjuvant or enhanced responses that may underly allergic airway disease. TempO-Seq analysis was thus used to interrogate responses to HDM, CeO\textsubscript{2}, SiO\textsubscript{2} alone and in combination after 24hr exposure. HDM exposure resulted in upregulation of 1 gene, MT1M (Figure 6-8A). Whereas neither nanoparticle resulted in a significant dysregulation of genes within the TempO-Seq gene panel, co-exposure of HDM with CeO\textsubscript{2} nanoparticles showed upregulation of genes such as CCL15, MT1M and SLC2A6 and co-exposure of HDM with SiO\textsubscript{2} nanoparticles revealed upregulation of genes such as CCL15, CXCL8 and SOD2 (Figure 6-8A,B). Interestingly CCL15 was upregulated beyond HDM exposure alone when combined with nanoparticle (CeO\textsubscript{2} + HDM and SiO\textsubscript{2} + HDM) exposures (Figure 6-8B,C). IL-4+IL-13 exposure was used to represent responses of these cells to type II inflammation as would occur within an allergic airway environment (Gour & Wills-Karp, 2015). This treatment produced a large change in gene expression typified by an increased CCL22 and decreased CD14 gene expression not observed upon HDM, nanoparticle or HDM + nanoparticle co-exposures.
Figure 6-8 Characterizing effects of nanoparticle and co-exposure with HDM in CD34+ macrophages. CD34+ derived macrophages (MC\textsuperscript{cd34}) were exposed to house dust mite (HDM, 25 µg/cm\textsuperscript{2}), cerium dioxide nanoparticles (CeO\textsubscript{2} NPs, 15 µg/cm\textsuperscript{2}), silica nanoparticles (SiO\textsubscript{2} NPs 15 µg/cm\textsuperscript{2}) and co-exposures for 24 hours and analysis of mRNA transcripts was carried out using TempO-Seq. Significantly differentially expressed genes (adjusted p-value <0.05 and fold change > 2) are highlighted in red in volcano plots (A). Log\textsubscript{2} RPKM values of highest differentially expressed genes are displayed a heatmap (B) and normalized counts (mean ± SEM) of selected genes are plotted in bar graphs (C).
A comparison of control RPKM levels obtained from mRNA Sequencing analysis of all primary cell types examined in this thesis, showed that genes for HDM receptors or co-receptors have higher RPKM values in CD34+ cultures as compared to airway epithelial cell cultures (C. Liu et al., 2005; Lundell et al., 2005). CD14, CLEC6A and CLEC4D displayed particularly high levels of gene expression in MC<sup>cd34</sup> cultures (Figure 6-9) and may underly the increased responsiveness of MC<sup>cd34</sup> cultures to HDM and co-exposure effects, when compared to other cell types. It does not however explain why combination exposures have greater effects than HDM alone.

Figure 6-9 Differences in control RPKM values of house dust mite receptors in airway and CD34+ cultures. RNA-Sequencing RPKM values of genes related to house dust mite receptors in CD34+ derived macrophages (MC<sup>cd34</sup>) and dendritic cells (DC<sup>cd34</sup>) and airway basal, DCI differentiated, and PneumaCult<sup>TM</sup>-ALI differentiated cells are visualized as RPKM ± SEM (N=6).
6.4. Discussion

As *in vitro* models can be valuable tools in gaining understanding of mechanisms of toxicity as well as in developing regulatory tools such as AOPs, this chapter has utilised the previously described primary airway epithelial and immune cell models optimised in this thesis, to gain insights into airway toxin mechanism of action and how cell type dependent susceptibilities may be key drivers of responses. These results also reinforce some fundamental principles of *in vitro* toxicology, that cellular differentiation reflective of how specific cell types exist in the human body is a critical parameter to recapitulate, such that information gained from *in vitro* toxicity assessment is relevant for human responses and not an artifact of the model. It also reinforces the importance of choosing the correct cell type(s) in *in vitro* toxicity testing exercises to identify toxicity of toxicant exposure.

6.4.1. Mechanisms of paraquat susceptibility

Comparing the toxicological responses upon PQ exposure between different cell types *in vitro*, allows for further insights in the mechanisms of action of PQ relevant for multi-cellular response and tissue injury. Our results show that DC<sup>cd34</sup> show a larger toxicological response to PQ exposure compared to MC<sup>cd34</sup>. Furthermore, whereas undifferentiated SAEC did not show a large toxicological response to PQ exposure, differentiated SAEC did, with partially overlapping and different gene expression profiles compared to the immune cell cultures. All *in vitro* models, apart from basal AEC, showed activation of stress response pathway genes (GDF15, TRIB3) indicating involvement of stress response pathways in the PQ general mechanism of toxicity. Interestingly, the PneumaCult<sup>TM</sup>-ALI and to a lesser extent the DCI differentiated SAEC cultures showed upregulation of oxidative stress related gene expression (HMOX1, SRXN1, and AKR1B10) upon PQ exposure, whereas the CD34+ cultures did not show a significant difference. In contrast, MDM2, a negative regulator of p53 pathway, was exclusively upregulated in CD34+ cell cultures, especially DC<sup>cd34</sup>. ATF4, one of the main transcription factors for UPR pathways, was
shown to be upregulated in DC\textsuperscript{cd34} only. Overall, these results indicate that there are overlapping mechanisms and differences in the toxicological response to PQ exposure between cell types.

Previous work has suggested that airway epithelial and immune cells are particularly susceptible to PQ toxicity as discussed in sections 4.1.3 and 5.1.1 respectively (Dinis-Oliveira et al., 2008; Hoet & Nemery, 2000). Taking together, these findings and other findings within literature allows us to describe a potential mechanism of action (MoA) for PQ induced toxicity within the lung (Figure 6-10). Increased levels of polyamine transport mechanism has been suggested to be causing increased airway susceptibility to PQ (Dinis-Oliveira et al., 2008; Hoet & Nemery, 2000; Silva et al., 2015), although other mechanisms may be involved (Dunbar et al., 1988). Immune cultures, especially DC\textsuperscript{cd34}, showed a higher level of polyamine transporter mRNA (SLC3A2 and SCL7A1) compared to airway epithelial cell cultures, possibly explaining higher levels of supernatant LDH within DC\textsuperscript{cd34} cultures upon PQ exposure. However, PQ exposure also resulted in a large number of differentially expressed genes in PneumaCult\textsuperscript{TM}-ALI differentiated SAEC, which showed low mRNA levels of these polyamine transporter genes. Indeed, mRNA levels do not necessarily represent protein levels within the cells, therefore, analysis of differences in protein levels of polyamine transporters between the cell models is needed to further insights in mechanisms of PQ uptake and susceptibility.

After uptake, PQ exposure results in an upregulation of stress response and inflammatory pathway related genes in immune and differentiated SAEC cultures. These stress response pathways are likely mediated via oxidative stress (Petrovská & Dušinská, 1999). Enzymatic reduction of PQ is a suggested to result in both NAD(P)H enzyme depletion and ROS generation (Cristóvão et al., 2009). Superoxide anion generation is dependent on both enzymatic activity and the presence of oxygen molecules (Dinis-Oliveira et al., 2008). CRISPR studies have increased the knowledge of possible mechanisms underlying cellular specificity of PQ toxicity, including the involvement of POR in PQ induced ROS production (Reczek et al., 2017). POR mRNA levels were shown to be higher in CD34+ cell cultures, indicating a possible increased susceptibility to
PQ exposure within these cultures. However, as SAEC are cultured on ALI, oxygen concentrations within these cultures are likely higher than the submerged CD34+ cell cultures, possibly adding to increased susceptibility to PQ induced oxidative stress. Indeed, genes indicative of oxidative stress levels, such as HMOX1, were upregulated to a larger extent in the differentiated SAEC cultures.

Detoxification of reactive superoxide is mediated by enzymes such as SOD (Filograna et al., 2016). It has been suggested that airway epithelial cells show relatively low levels of SOD activity (Montgomery, 1977), adding to their susceptibility to PQ induced oxidative stress (Pollak et al., 1990). Indeed, our study also showed that CD34+ cultures contained a higher mRNA level of SOD genes compared to SAEC cultures. Especially the gene SOD2 showed high mRNA levels within MC\textsubscript{cd34} cultures. This is most likely related to the primary functions of macrophages. Since an increased production of ROS is a defence mechanism against pathogens for macrophages, there are self-protective mechanisms (SOD2) against the potential oxidative stress (Virág et al., 2019; P. Wang et al., 2019). As SOD1 activity is dependent on intracellular copper levels, copper transporters might impact cellular toxicity through regulation of superoxide levels (Reczek et al., 2017). mRNA levels of several genes related to copper transporter (SLC31A1 and SLC31A2) are also elevated in CD34+ cultures, specifically in MC\textsubscript{cd34}. Lower levels of these antioxidant enzymes and copper transporters could be another explanation for to the more profound oxidative stress responses observed in SAEC cultures. Ultimately, at higher concentrations, PQ induced upregulation of stress response genes can result in cell death (Figure 6-10). Interestingly, within this study only DC\textsubscript{cd34} cultures responded to PQ exposure with ATF4 upregulation. ATF4 is a key regulator of mitochondrial stress and UPR pathways (Quirós et al., 2017). ASNS and DDIT3, genes that are also regulated by the UPR pathway, were also upregulated in DC\textsubscript{cd34} cultures upon PQ exposure, indicating a potential unique role for dendritic cells in airway responses to PQ exposure if such effects were to be also found in humans.
Inflammatory responses upon PQ have been previously observed in rat studies (Toygar et al., 2015) and hypothesized to be mediated by oxidative stress induced activation of NF-κB inducing increased transcription of inflammatory cytokines and chemokines (Schoonbroodt & Piette, 2000). In this study, PQ exposure resulted, either directly or indirectly via oxidative stress mediated pathways, in increased expression cytokine signalling genes (CXCL8, IL1A) possibly resulting in inflammatory state in CD34+ cultures, especially DC<sup>CD34</sup>, and to a lesser extent in PneumaCult<sup>TM</sup>-ALI differentiated SAEC. However, as both DCI differentiated SAEC and MC<sup>CD34</sup> cultures have high basal expression levels of CXCL8 and IL1A genes, the impact of PQ exposure might be masked in these cultures, hampering conclusions on cellular specificity of PQ induced inflammatory responses.

**Figure 6-10 Proposed mechanism of action (MoA) of paraquat exposure.** Paraquat uptake is connected to outcomes observed in this thesis via several possible mechanisms of action. Connections are facilitatory (●) or inhibitory (⊥) and dashed lines indicate speculative interactions.

To mimic systemic PQ exposure, CD34+ cultures were exposed under submerged conditions whereas ALI cultured SAEC cultures were exposed on the basolateral side to the same concentrations of PQ. However, as differentiated SAEC cultures form a pseudostratified layer of cells, the dose of PQ per cell could differ depending on uptake mechanisms whereas the concentration may be more uniform within CD34+ cultures. Single cell sequencing efforts could further indicate the cell types within this culture that are most susceptible to systemic PQ exposure, especially when using co-cultures. Furthermore, using the same method for transcriptomic analysis between cultures could give further insights into levels of gene activation.
Utilisation of in vitro models to explore cell specificity and mechanisms of actions of respiratory toxins

beyond F.O.C. comparisons by qPCR in this study. Nevertheless, comparisons of F.O.C of mRNA levels do give an indication of relative PQ toxicity between cells and further insights in its mechanism of action.

In summary, the comparisons between cell types for their responses to PQ exposure revealed obvious differences. Interrogation of basal mRNA levels for pathway specific genes as a means to understand inherent susceptibility across immune and epithelial cell types did not reveal any obvious over-arching mechanism. This may be due to the targeted nature of the analysis and other pathways yet to be identified may contribute to responses to PQ. It also may be the case that a combination of different factors independent of baseline pathway mRNA levels may contribute including cellular oxygen levels and protein levels, which may differ. Comparisons within closely related types (e.g. macrophages vs dendritic cells) did reveal some potential mechanisms (e.g. SOD2 expression) but how these mechanisms of susceptibility may translate to other cell type susceptibilities is more difficult to assess and highlights the complex nature of toxicological assessment \textit{in vitro}.

6.4.2. Mechanisms of nanoparticle and allergen effects on airway models

Previous work has shown that several nanoparticles have the ability to exacerbate allergen induced type II airway inflammation (Meldrum et al., 2017, 2018). However, the molecular initiating events (MIE) of nanoparticle induced allergen exacerbation are currently unknown.

To gain insights into the MIE of nanoparticle and allergen co-exposure, DCI differentiated SAEC and CD34+ derived macrophage and dendritic cell cultures were exposed to cerium and silica nanoparticles in absence or presence of the common allergen HDM. qPCR results showed a possible nanoparticle induced exacerbation of allergen effects in MC\textsubscript{cd34} but not DC\textsubscript{cd34} or DCI differentiated SAEC cultures. This exacerbation effect was further demonstrated using TempO-Seq techniques and was observed as an upregulation of genes such as CCL15, CXCL8 and IL1A. SiO\textsubscript{2} nanoparticles have previously been shown to have proinflammatory and immunomodulatory effects on ovalbumin sensitized mice \textit{in vivo} (Marzaioli et al., 2014). CeO\textsubscript{2}
nanoparticles have also been shown to modulate the murine pulmonary response to HDM towards a type II immune response (Meldrum et al., 2018). Interestingly, this study also showed an increased expression of genes such as SLC26A4 and CLCA1 in primary human bronchial epithelial cells after one week repeat exposure treatments of CeO$_2$ nanoparticles and HDM co-exposure. Longer term and repeat treatments, therefore may reveal epithelial changes not observed after a single short-term exposure of 24hrs in our model.

The primary cell in vitro models used in this analysis can be characterised as having an elevated level of inflammatory activation as would occur in diseased tissue and therefore represent an appropriate system to test for exacerbation effects, that may occur with respiratory exposure to allergens and particles. To understand why MC$_{cd34}$ cultures respond to these stimuli, driven mainly by HDM, baseline levels of gene expression for HDM component ligands were compared. This showed that MC$_{cd34}$ cells had higher levels of genes related to known cellular receptor components of HDM mixtures (CLEC4D and CLEC6A) (C. Liu et al., 2005; Lundell et al., 2005). A major bioactive property of HDM is through LPS activation of TLR4 (Hammad et al., 2009). The TLR4 co-factor CD14 was also shown to be highly expressed in MC$_{cd34}$ cultures, further indicating that enhanced responses in MC$_{cd34}$ may be a consequence of differences in HDM receptor profiles between cell cultures.

While receptor expression may explain HDM effects, how nanoparticles exert their impacts on cells remain more elusive. It has been suggested that exposure to inhaled particles can cause an inhibition of alveolar macrophage phagocytosis and thereby an increased susceptibility to allergen exposure and exacerbation of diseases such as asthma (Lundborg et al., 2006). It has also been suggested that M2 polarization as would occur in type II inflammation enhances the uptake of nanoparticles by alveolar macrophages (Hoppstädter et al., 2015). Furthermore, activation of NLRP3 inflammasome due to accumulation of particles in phagocytes and subsequent release of inflammatory cytokines might be other mechanisms underlying adjuvant activity (Marrack et al., 2009). More research is required to fully define these mechanisms of
nanoparticle action. This information can in the future be integrated in an AOP, for instance the AOP that was previously developed for low molecular weight chemical induced airway sensitisation (Sullivan et al., 2017). However, although sensitisation effects were explored within this study, no relevant outcomes were found. The results would better fit within an AOP related to exacerbation effects rather than sensitization, which is yet to be developed.

6.4.3. Conclusions and future directions

This chapter aimed to utilize the in vitro models characterized within this thesis as powerful tools to gain understanding of mechanisms of action and explore how they may be applied to in silico tools such as AOPs. PQ exposure demonstrated differences and similarities in oxidative stress and inflammation signalling pathway activations between cell types. The insights gained in this chapter further highlight the importance of differentiation of basal SAEC cells to mimic airway regions relevant for human toxicity testing. CD34+ cells differentiated towards macrophages and dendritic cells also demonstrated differences in responses to PQ exposure, with dendritic cells being more susceptible, highlighting the relevance of in vitro models to gain knowledge on mechanisms of actions of chemical exposures.

Furthermore, a nanoparticle induced exacerbation of HDM effects were observed in MC<sup>cd34</sup> cultures but not in DC<sup>cd34</sup> or DCI differentiated SAEC cultures. Functional studies are however required to gain more insights in the mechanism of action of nanoparticle and allergen co-exposures and thus fully define the MIE, which can then integrated within an AOP (Sullivan et al., 2017). Ultimately, increased applicability of in vitro and in silico approaches to chemical and particle toxicity testing could allow for a reduction of animal testing required for research and legal purposes as well as better prediction of human toxicity.

Overall, the results demonstrate appropriate organotypic differentiation is essential for in vitro small airway models to detect relevant toxicant responses. Future work could focus on utilising co-cultures to gain a more complete overview of small airway responses and interactions between cell types.
CHAPTER 7: GENERAL DISCUSSION
7.1. Summary

The aim of this thesis was to characterize in vitro small airway models for the purpose of regional and cell type specific small airway toxicity testing. These efforts are required to ensure safe exposure levels of chemicals and nanomaterials. Currently, animal models are often used as a gold standard for regulatory purposes (Movia et al., 2020). However, species differences in function and morphology of the small airways hamper extrapolation of toxicity data in many cases (Kolanjiyil et al., 2019). Also, the advancement of 3R efforts further demonstrates the need for in vitro alternatives to animal testing (Krewski et al., 2020).

As discussed in chapter 1, human small airways and especially club cells are susceptible to chemical and nanoparticle induced toxicity (Zuo et al., 2018). However, in vitro small airway models that are currently used are sub-optimal as they often lack regional organotypic specific differentiation and thus require further optimization. Although relatively challenging to culture, primary cells show normal cell morphology and maintain markers and functions as seen in vivo (Pan et al., 2009). However, their limited life span and availability hampers their use for large scale in vitro toxicity assessments (Lauenstein et al., 2014). iPSC cultures on the other hand, form an in theory, unlimited supply of donor specific cells generated from easily accessible somatic cells, making them an attractive alternative to primary cell cultures for in vitro toxicity testing.

For these reasons, this thesis initially focused on the differentiation and characterization of primary cells and iPSC towards SAEC and airway resident macrophages and dendritic cell cultures that may subsequently be used for toxicity testing. Compounds with known small airway toxicity were used to further evaluate the in vitro small airway models and to determine cellular specificity of these compounds.

7.1.1. Characterization of in vitro small airway models

Initially, primary basal SAEC were differentiated using DCI and PneumaCult™-ALI differentiation media to establish region specific airway cultures. Modification of cell differentiation in vitro is highly condition dependent. Culturing basal SAEC using ROCK signalling inhibition increased the
proliferative capacity of these primary cells substantially while maintaining airway basal cell characteristics and morphology, facilitating their use in toxicity studies (Mou et al., 2016). The relevance of culture medium in primary nasal AEC differentiation has been previously demonstrated and showed importance of RA, VEGF, EGF and FGFβ concentrations balance for the formation of cilia in the cell cultures (Luengen et al., 2020). DCI compounds have been used to achieve small airway differentiation from iPSC derived lung progenitor cells (McCauley, Alysandratos, et al., 2018), further illustrating the importance of medium components for regional specific airway differentiation. In this study, RNA-Sequencing analysis showed that differentiation of primary basal SAEC on ALI using DCI differentiation medium indeed resulted in a small airway-like phenotype containing club cells (SCGB1A1+), whereas PneumaCult™-ALI differentiation medium showed a more proximal ciliated (FOXJ1+) airway-like phenotype. As these cell cultures show similarity to different airway regions, differences in toxicological responses between these cell states have the potential to identify regional airway susceptibility. However, the DCI differentiated SAEC showed relatively high levels of inflammatory genes such as CXCL8, limiting their potential somewhat, arguably being more relevant to examination of toxicological effects in disease and inflammatory conditions such as asthma. Nevertheless, these results demonstrate that medium components indeed play an important role in organotypic differentiation.

Differentiation of iPSC towards a mature airway epithelium was attempted using several published protocols (Firth et al., 2014; S. Huang et al., 2015; Mitchell et al., 2017; Roelandt et al., 2013). Although iPSC were successfully differentiated towards lung progenitor cells (NKX2.1+, TP63+), differentiation was not adequately achieved within this thesis, as these cultures lacked typical airway markers (FOXJ1, MUC5AC, SCGB1A1) using PneumaCult™-ALI differentiation medium and the ability to form a monolayer on ALI using DCI differentiation medium. Therefore, these cultures were not used for chemical toxicity testing.
Macrophages and dendritic cells play an important role in innate immune responses in the small airways, existing as primary innate immune responders to environmental stimuli including allergens, pollutants and pathogens (Iwasaki et al., 2017). Both CD34+ HSC and iPSC were differentiated into macrophage and dendritic cell-like cultures. Compounds that were previously shown to favour differentiation to an alveolar macrophage-like phenotype (GM-CSF, TGFβ) were added to the culture medium to recreate a macrophage model relevant for small airway toxicity (Schneider et al., 2014). CD34+ cultures were guided through alveolar macrophage-like (IL-6, M-CSF, GM-CSF, and TGFβ) and dendritic cell-like (FLT3L, SCF, IL-4, and GM-CSF) differentiation to establish MCcd34 and DCcd34 cultures. mRNA-sequencing showed successful myeloid differentiation as observed by high expression of macrophage (e.g. MARCO, CD163, ORM1) and dendritic cell (e.g. CD1B, CD1E) markers within MCcd34 and DCcd34 cultures respectively. Since gene expression does not necessarily reflect the levels of proteins in immune cells, single cell AbSeq analysis was also performed to further characterize these cultures. There was a complete separation of both cell types further confirming our cultures as macrophage and dendritic cell lineages by macrophage marker (CD14 and CD163) and dendritic marker (CD1A and CD1C) antibody staining respectively. Further optimization of the differentiation protocol might be required, as the presence of immature (iMC and iMC) and activated inflammatory cells were also observed within the MCcd34 and DCcd34 cultures. Furthermore, to be relevant for small airway toxicity testing, these myeloid cells need to resemble the populations present within the small airways in vivo. The mature MC-like population within the MCcd34 cultures showed relatively low levels of some human alveolar macrophage markers (HLA-DR, CD206) (Nayak et al., 2018). No in vitro model for small airway specific dendritic cell populations has been developed so far. However, as a mixture of airway resident and inflammatory monocyte derived macrophages and dendritic cells are also likely to be present the human small airways in vivo (Collin & Bigley, 2018), we consider the cell types developed in this study at the forefront of in vitro models used to assess immune cell responses to toxic exposures as may occur within the small airways.
iPSC (SBAD3) were successfully differentiated towards a monocyte-like population based on an established protocol (van Wilgenburg et al., 2013). This protocol allows for the generation of a large number of monocytes over a prolonged period of time resulting in applicability of these cells to toxicity testing efforts. After monocyte-differentiation, the iPSC cultures were guided through alveolar macrophage-like (IL-6, M-CSF, GM-CSF, and TGFβ) and dendritic cell-like (FLT3L, SCF, IL-4, and GM-CSF) differentiation, similar to CD34+ cultures. Although TempO-Seq analysis showed high levels of mesenchymal cell markers (COL12A1, CCDC80) within both differentiated iPSC cultures, qPCR analysis confirmed increased levels of macrophage (MARCO, MRC1) and dendritic cell (CCR7) markers in MC<sub>iPSC</sub> and DC<sub>iPSC</sub> cultures as well, indicating mixed cell populations arose from the iPSC derived monocytes. Single cell Abseq analysis further confirmed a fibroblast-like population in both MC<sub>iPSC</sub> and DC<sub>iPSC</sub> cultures. However, MC<sub>iPSC</sub> and DC<sub>iPSC</sub> cultures could be clearly separated and MC<sub>iPSC</sub> grouped closer to CD34+ iMC populations whereas DC<sub>iPSC</sub> grouped closer to CD34+ iDC populations, indicating that differentiation trajectories were appropriate, but additional efforts are needed to achieve both purification and maturation of the cell cultures. Currently, no protocol has been established to efficiently differentiate iPSC towards alveolar specific macrophages. However, iPSC derived macrophages have been previously established using a similar protocol and demonstrated their applicability to drug screening (Gutbier et al., 2020). Although resembling immature phenotypes and containing mesenchymal cell lineages, the MC<sub>iPSC</sub> and DC<sub>iPSC</sub> cultures characterized in this study may be considered for in vitro toxicity testing.

7.1.2. Chemical toxicity assessment

As primary SAEC cultures were differentiated towards airway epithelia representing different regions of the human airways, these could be used to investigate the mechanism of action of known small airway toxicants. AM and BUS, commonly used pharmaceuticals with reported small airway toxicity side effects (A. Myers et al., 2017; Papiris et al., 2010), did not show significant upregulation of toxicity genes upon 24 hour exposure using qPCR in any SAEC culture. As DCI differentiated SAEC most closely resemble the small airways, RNA Sequencing analysis
was performed in this culture only. This showed upregulation of pharmacologically relevant
genes (SCD, INSIG1, GPNMB, SREBF1) upon AM, but not BUS exposure. This indicates that the
established primary SAEC culture and exposure regime was adequate to detect pharmacological,
but not toxicological responses upon AM exposure. Further repeat and higher dose regimens
are likely needed to further explore mechanisms of toxicity in these cells for both compounds.

PQ, a herbicide with reported acute small airway toxicity, resulted in increased levels of
toxicological relevant genes as measured by qPCR in all three primary epithelial cell cultures.
The magnitude of these effects however differed between cell types, with basal SAEC showing
the least amount of differentially expressed genes highlighting the importance of differentiation
for small airway toxicity testing. Partially due to the higher level of inflammatory genes in DCI
differentiated SAEC cultures, RNA sequencing analysis showed a lower number of dysregulated
genes within this culture compared to PneumaCult™-ALI differentiation upon PQ exposure.
However, the RPKM and fold change values of several relevant toxicological genes (DDIT3,
TRIB3) were significantly higher in DCI differentiated SAEC cultures, indicating susceptibility to
PQ toxicity within this culture. As DCI differentiation showed a higher level of club cell markers
(SCGB1A1), this observation corresponds to previous theories that club cells are especially
susceptible to PQ exposure (Dinis-Oliveira et al., 2008).

To further investigate the mechanisms of small airway toxicity, CD34+ and iPSC derived
macrophages and dendritic cells were also exposed to AM, BUS, and PQ. Although LDH and
resazurin assays showed a cytotoxic effect of AM which was more profound in DC<sup>Cd34</sup>
cultures and not observed in SAEC models, both AM and BUS exposure showed no significant
toxicological responses in any cell culture as measured by qPCR and TempO-Seq methods. iPSC
derived macrophages and dendritic cell cultures also did not show significant differentially
expressed genes upon PQ exposure. However, PQ exposure showed a profound effect on gene
expression in DC<sup>Cd34</sup> and MC<sup>cd34</sup> cultures. Interestingly, iPSC cultures showed a lower level of
expression of genes related to redox enzymes (POR, NCF2), indicating the redox capacity of
these cells might not be adequate to allow for PQ toxicity effects. Indeed, the presence of POR has been shown to be important for PQ induced oxidative stress (Reczek et al., 2017). Within CD34+ cultures, DC<sup>cd34</sup> showed a higher response to PQ compared to MC<sup>cd34</sup> cultures as shown by classical toxicity assays (LDH and resazurin) as well as the level of significantly differentially expressed genes (ATF4, DDIT3, TRIB3) typically involved in UPR pathways. Comparing gene expression patterns of unexposed DC<sup>cd34</sup> and MC<sup>cd34</sup> cultures reveals that SOD2 gene expression levels were higher in MC<sup>cd34</sup> cultures, possibly protecting these cultures from PQ induced ROS damage (Filograna et al., 2016).

Even though different methods were used for exposure and sequencing in SAEC and CD34+ cultures, the fold change values can be compared (Figure 7-1) and integrated to further the knowledge of the PQ mechanism of action. Within this study, CD34+ showed higher levels of POR gene expression as measured by RNA Sequencing analysis compared to SAEC cultures, indicating CD34+ cultures could be more susceptible to PQ exposure. Indeed, several genes, such as MDM2 and CXCL8 were preferentially induced in CD34+ cultures. However, other stress response genes such as DDIT3 and were shown to be significantly upregulated exclusively in DCI differentiated SAEC and DC<sup>cd34</sup> cultures, indicating other mechanisms of PQ susceptibility might be involved. Furthermore, SAEC cultures showed a higher amount of overexpression of oxidative stress related genes (SRXN1, HMOX1, ARK1B10) upon PQ exposure, which could be speculated to be due to presence of O<sub>2</sub> as these cultures were on ALI in contrast to the submerged CD34+ cultures. Overall, these results gave insights in the mechanisms of PQ toxicity but also demonstrate that additional research is required to further unravel mechanisms of PQ susceptibility.
Figure 7.1 Overview of chemical toxicity assessment. iPSC derived airway epithelium were not used for chemical toxicity testing. Amiodarone and busulfan did not show significant effects in any culture and were not further used to determine cellular susceptibility. iPSC derived macrophages and dendritic cells did not show significant effects for paraquat toxicity and were therefore not further investigated. The Paraquat effects in primary SAEC cultures and CD34+ macrophages and dendritic cells were further explored.

7.1.3. Nanoparticle and allergen toxicity assessment

The *in vitro* models developed within this thesis were used to determine cellular susceptibility to nanoparticle and allergen exposures as previous work has shown that nanoparticles might have the ability to exacerbate allergen induced type II inflammation (Meldrum et al., 2017). DCI differentiated SAEC, DC<sup>cd34</sup> and MC<sup>cd34</sup> models were exposed to nanoparticles, allergens, and co-exposures to gain insight in which cell types play an important role in allergen exacerbation. This study found that MC<sup>cd34</sup> cultures showed upregulation of inflammatory genes (CCL15, CXCL8) upon exposure of CeO<sub>2</sub> and SiO<sub>2</sub> nanoparticles in co-exposure with the common allergen HDM. This is speculated to be due to either phagocytosis or higher levels of HDM receptors within this culture. Overall, this shows that macrophages may be prioritized for nanoparticle and allergen co-exposure toxicity testing for regulation purposes and demonstrates an example of the relevance of the *in vitro* small airway models characterized in this study.
Overall, it can be concluded that *in vitro* primary SAEC and CD34+ immune cell cultures may be optimized and applied to small airway toxicity testing, whereas iPSC cultures require further research. Furthermore, cell types are critically important for biological responses and therefore the choice of toxicity testing model to assess a chemical with unknown effects is crucially important.

### 7.2. Limitations

Cell culture medium components impact SAEC differentiation towards different cell types when cultured on ALI. However, some of the culture media components used in this thesis are not fully defined. The dexamethasone, cAMP and IBMX compounds added to the DCI differentiation medium likely affect small airway differentiation, however, other undefined compounds could also play a role. For instance, Ultroser G, added to the DCI differentiation medium, is a serum substitute containing mostly unknown compounds and has previously been shown to have a large effect on tracheal AEC differentiation (Sachs et al., 2003). Furthermore, PneumaCult™-ALI is a commercially available differentiation medium of which the compounds are undefined, however, have higher concentrations of EGF and RA compared to other cell culture media (Luengen et al., 2020), but the complete repertoire of growth factors and small molecules within this media supplement is unknown. This limits the conclusions that can be made regarding specific cell culture medium components that may guide differentiation towards small airway epithelium and thus can be reasonably be modified for future culture optimisation. The lack of a defined media formulation hampers the ability to adjust conditions to improve for instance the inflammation state of DCI differentiated cultures. Using fully defined medium would help in exploring compounds required for optimal differentiation.

The use of iPSC within this thesis also proved to have limitations. iPSC from different origins (SBAD2 and SBAD3) showed discrepancies in their ability to differentiate to specific cell types. Myeloid differentiation was only established for SBAD3 whereas the airway differentiation showed a slightly better ability to form a monolayer on ALI for SBAD2 (data not shown),
indicating not all iPSC are equally able to differentiate towards specific cell types. Furthermore, as no iPSC derived mature airway epithelium could be achieved and iPSC derived macrophages and dendritic cells show a more immature phenotype compared to CD34+ derived counterparts, it is clear iPSC derived cell cultures still represent a more underdeveloped and embryonic phenotype when compared to primary counterparts.

Characterization of in vitro small airway models was mainly performed using methods to detect mRNA levels within the cell cultures (qPCR, RNA-Sequencing and TempO-Seq). Gene expression levels give an indication of the presence of specific proteins within the cell cultures (e.g. enzymes or transporters), however, might not accurately reflect the levels protein expression, limiting comparisons between the in vitro SAEC models. Further characterization using immunostaining methods would give more insights into the presence of specific cell types within the SAEC models. However, the culturing of these cells on TransWell inserts hampered immunocytochemistry efforts within this thesis. ScSeq showed a good correlation between antibody and mRNA levels in subpopulations of CD34+ cell cultures, indicating that mRNA levels might accurately reflect the presence of proteins within the cell cultures.

The toxicological impacts of compounds within this thesis have been mainly described using alterations in gene expression levels (qPCR, RNA-Sequencing and TempO-Seq). As gene expression differences often precede physical changes within the cell, it represents a good starting point for determining the biological and toxicological effects of compounds. However, not all compounds strongly affect gene expression, thus using additional methods such as proteomics or functional studies, could aid in determining the toxicological effect of compounds. For instance, demonstrating the presence of polyamine transporters or measuring intracellular PQ levels would allow examination of differences in cellular uptake of PQ between cell types as a possible contributor to the differential effects upon treatment.

SAEC cultures cultured on ALI were exposed to chemicals on the basolateral side to mimic systemic exposure, whereas for the submerged CD34+ chemicals had to be exposed in
suspension as these cells were not cultured on TransWell inserts. Differences in these exposure methods may explain in part some of the differences in toxicological responses, the exact nature of which needs further investigation. Furthermore, SAEC cultures were exposed to nanoparticles and HDM apically in a minimum volume (50 µL), whereas the nanoparticles and allergens were added directly to the culture medium for the CD34+ cultures. Interaction with proteins and electrolytes present in the cell culture medium can impact the charge, protein corona, agglomeration and aggregation levels of the nanoparticles and thereby affect the toxicity levels (Maiorano et al., 2010). Differences in exposure methods could hamper comparisons between the SAEC and CD34+ models. However, care has been taken that the concentrations of compounds were the same between the cell types.

Although the initial seeding density of iPSC derived monocytes and primary CD34+ cells was similar, differences in proliferative capacity caused a lower cell number in the fully differentiated iPSC cultures compared to the CD34+ cultures. Since cells were exposed to the same concentration of chemicals, the amount of chemical per cell was considerably higher within the iPSC cultures. However, this would more likely result in a larger toxic effect and therefore is unlikely to be the cause of the lack of response to PQ exposure in the iPSC cultures. The lower amount of iPSC cells also resulted in lower raw count values within the TempO-Seq dataset, which may have resulted in a higher threshold for differentially expressed genes. qPCR measurements also did not detect an increase in relevant toxicity genes upon PQ exposures within the MC<sup>iPSC</sup> cell cultures. As this method for mRNA quantification has a higher sensitivity compared to RNA-Sequencing methods (Costa et al., 2013), it is more likely the iPSC derived cultures were indeed unresponsive to PQ treatment as compared to primary cell types.

Different methods of sequencing were used to detect gene expression changes upon chemical exposures in primary SAEC (RNA-Sequencing) and in iPSC and CD34+ immune cell cultures (TempO-Seq). TempO-Seq was used to allow future comparisons of chemical responses to other organ models derived from the same iPSC lines within the in3 project. For this reason, no direct
comparisons could be made regarding differences in gene expression levels of toxicologically relevant genes between SAEC and CD34+ cultures. Nevertheless, fold over control values were determined for both methods and directly compared, giving an indication of cellular specificity of the compound toxicological effects.

Only one time point (24 hours) after chemicals and particle and allergen exposures was analysed within this thesis, due to both time and study size restrictions. Including multiple time points would give, a) the opportunity to create temporal data relevant for quantitative AOP development, and b) increased validity for compounds that humans are commonly exposed to for prolonged periods of time such as AM and BUS within this study. Furthermore, repeated dose toxicity could enhance the ability of the in vitro models to detect toxicity upon exposure of pharmaceutical compounds for which acute toxicity is less likely. Indeed, SAEC cultures were able to detect specific pharmacological effects of AM after 24-hour exposure, indicating limiting factors for detecting AM induced toxicity are more likely to be the exposure regime used rather than the in vitro small airway models developed within this thesis.

7.3. Future work

The development of suitable in vitro models for airway toxicity testing is of high importance for efforts such as drug screening and toxicity assessment of environmental toxicants. Additional research is needed for current in vitro small airway models to be up to standards required for legislative efforts. This thesis has shown feasibility for the use of iPSC and primary cells in toxicological investigations, but also highlighted the need of further optimization and standardization of in vitro small airway models.

A recently developed commercially available cell culture medium PneumaCult™-ALI-S optimized for small airway differentiation showed higher expression of SCGB1A1 and SCGB3A2 compared to PneumaCult™-ALI medium (Bluhmki et al., 2020) and could be useful for future investigations of small airway differentiation. However, using fully defined cell culture medium would allow
further optimization of differentiation media, as the effect of specific compounds could be investigated.

Small airway epithelium cells are tightly regulated by signals from adjacent mesenchyme and immune cells. Therefore, co-culture of airway epithelial cells with fibroblasts, endothelial cells, smooth muscle cells and immune cells, such as macrophages and dendritic cells would increase validity of in vitro models (Hiemstra et al., 2018). Most small airway co-culture studies have focused on tumour or immortalized cell lines, as these are easier to control and require less cell specific culture media (Hiemstra et al., 2018). For instance, a co-culture of human alveolar epithelial (hAELVi) and macrophage (THP-1) cell lines has been described to model the air-blood barrier (Kletting et al., 2018). Primary cells, although more closely resembling the in vivo physiology, require specific media and have variable growth rates making the establishment of co-cultures more challenging (Hiemstra et al., 2018). Culturing primary cells using ROCK and TGFβ/BMP inhibition might facilitate the development of co-cultures (Yonker et al., 2017).

Establishing a co-culture of primary differentiated SAEC and CD34+ macrophages and dendritic cells as established in this thesis would be an interesting future direction highly relevant for human toxicity assessment. Although the CD34+ differentiation towards macrophages and dendritic cells was shown to be successful within this thesis, alveolar specific macrophage differentiation was not adequately achieved. A recent study has shown that addition of the PPARγ agonist rosiglitazone to differentiation medium containing GM-CSF and TGFβ is effective in differentiating mouse bonne marrow cells to alveolar macrophage phenotype (Luo et al., 2021). Furthermore, using co-cultures with SAEC cells could also help guide differentiation of CD34+ cells towards a more airway resident cell phenotype.

Furthermore, the development of iPSC derived small airway models could advance the development of co-cultures, as material from the same donor could be used for all cell types within the culture, creating a donor specific model (Mertens et al., 2017). iPSC models are a promising alternative to primary cell models as these cells are easily accessible with an in theory
unlimited supply, however, more studies are required to optimize iPSC models. Both SAEC and myeloid cultures showed an immature phenotype within this thesis. As the DC\textsuperscript{iPSC} and MC\textsuperscript{iPSC} cultures showed the presence of a mesenchymal lineage, cell sorting (e.g. CD14) could be useful to achieve a higher level of purity within the cell cultures. However, cell sorting using CD14+ microbeads also showed a presence of collagen expression in iPSC derived macrophages and dendritic cell cultures (Monkley et al., 2020). Optimization of cell culture environment such as the plastic, coating or culture medium could be explored to prevent mesenchymal differentiation within the iPSC cultures.

\textit{In vitro} small airway models could also be applied to investigate disease specific responses to compounds in future studies, using cells obtained from specific patient populations. Limited availability of patient specific primary cells however hampers their use in large scale toxicity studies. Gene editing of disease specific genes would be an interesting alternative, as edited and unedited cells could be directly compared. iPSC and iPSC derived cells (e.g. macrophages) have been shown to be especially suitable for gene editing using CRISPR-Cas9 (Navarro-Guerrero et al., 2021). CRISPR-Cas9 gene editing requires stable clonal cells and is thus often not applicable to primary cell cultures, due to limited life span and proliferative capacity. However, the addition of dual SMAD inhibition has been shown to significantly increase the life span of primary SAEC and could thus facilitate applicability of CRISPR-Cas9 gene editing to these cultures (Everman et al., 2018). Furthermore, using a guide-swap method to introduce targeted guide RNAs in a complex with nontargeting guide RNA has been proven successful in applying CRISPR-Cas9 methods to primary cultures such as CD34+ HSC (Ting et al., 2018).

Microphysiological systems (MPS), or “Organ-on-a-chip” models aim to recapitulate \textit{in vivo} physiology in a microenvironment and could be highly relevant for human \textit{in vitro} toxicity assessments in the future (B. Zhang et al., 2018). These systems contain multiple cell types as well as microfluidics for continuous delivery of nutrients and disposal of waste products (B. Zhang et al., 2018). The first “lung-on-a-chip” model included an alveolar cell line and
microvascular endothelial cells with shear stress mimicking breathing motions (Huh et al., 2010). The use of cell lines, however, limits physiological relevance of this model. A bronchiolar model including primary bronchial epithelium cells, lung microvascular endothelium, fibroblasts, extracellular matrix and neutrophils did not show adequate mucociliary differentiation (Barkal et al., 2017). A human bronchial epithelial and endothelial “airway-on-a-chip” model with a central epithelial channel exposed to air surrounded by vascular cells was also described, enabling functional imaging of the interior of the lumen, e.g. mucociliary transport mimicking in vivo situation (Z. Liu et al., 2019). Advances in 3D bioprinting could facilitate the development of in vitro “lung-on-a-chip” models. The plausibility of a 3D bio printed air-blood-tissue barrier was shown using A549 alveolar cell line combined with endothelial cells in a layer-by-layer printing approach (Horvath et al., 2015). Recently, a 3D cell printed vascular platform was utilized to develop an airway-on-a-chip model with primary tracheal epithelial cells, able to induce asthmatic symptoms upon exposure with IL-13 (Park et al., 2019). A “Small airway-on-a-chip” model with relevant function and morphology, metabolic enzymes and club cells requires further research. Especially the development of iPSC derived “Small Airway-on-a-chip”, or integration with other models to create “body-on-a-chip” could be highly relevant for toxicity testing or drug development. However, the costs and labour intensity of this model still limit its suitability for high-throughput screening and more research is needed for these models to be adequate for human toxicity assessment (Probst et al., 2018).

The use of in vitro and in silico integration tools would also be beneficial in advancing predictive toxicology. In3 efforts for in vitro and in silico integration have showed feasibility of a multi-disciplinary approach to integrate data obtained from in vitro studies with models such as QSAR and AOP (Nunes et al., 2022; Spinu et al., 2020; Vukovic et al., 2019). AOP models could be used to identify the sequences of molecular and cellular events that are required to produce an effect upon toxicant exposure. The use of in vitro models, such as the ones developed in this thesis, could aid the development of in silico tools and thereby play an important role in replacing animal testing for regulatory purposes.
8. REFERENCES


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https://doi.org/10.1038/nbt.1788


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Differentiation of Club Cells to Alveolar Epithelial Cells in Vitro. *Scientific Reports*, 7(1), 1–9. https://doi.org/10.1038/srep41661


9. APPENDIX

9.1. Supplemental tables and figures

**Supplemental table 9-1 GAPDH values.** Airway epithelial cell models (N=4) and immune cell models (N=3) were differentiated as described in sections 2.1.4 and 2.1.5 and exposed for 24 hours to amiodarone, busulfan or paraquat. GAPDH values were determined using PCR and displayed as average CT values (±SD). No significant differences (P<0.1) between chemical treatment and control samples were observed.

<table>
<thead>
<tr>
<th>In vitro model</th>
<th>Control</th>
<th>Amiodarone 3 µM</th>
<th>Amiodarone 15 µM</th>
<th>Amiodarone 50 µM</th>
<th>Busulfan 10 µM</th>
<th>Busulfan 20 µM</th>
<th>Busulfan 100 µM</th>
<th>Paraquat 10 µM</th>
<th>Paraquat 20 µM</th>
<th>Paraquat 100 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Airway epithelial cell models</td>
<td>Basal</td>
<td>15.5 (±1.1)</td>
<td>15.1 (±1.6)</td>
<td>15.0 (±1.3)</td>
<td>15.3 (±1.0)</td>
<td>15.8 (±0.9)</td>
<td>15.5 (±0.9)</td>
<td>15.8 (±1.0)</td>
<td>15.8 (±1.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PneumaCult™</td>
<td>17.8 (±1.1)</td>
<td>17.3 (±1.6)</td>
<td>18.2 (±1.4)</td>
<td>17.4 (±1.3)</td>
<td>17.6 (±1.2)</td>
<td>17.9 (±0.8)</td>
<td>17.6 (±1.3)</td>
<td>17.6 (±1.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DCI</td>
<td>17.1 (±1.6)</td>
<td>17.7 (±2.5)</td>
<td>18.6 (±1.4)</td>
<td>16.8 (±1.3)</td>
<td>17.7 (±1.2)</td>
<td>16.7 (±1.2)</td>
<td>18.6 (±3.7)</td>
<td>18.6 (±3.7)</td>
<td></td>
</tr>
<tr>
<td>Immune cell models</td>
<td>MC&lt;sup&gt;cd34&lt;/sup&gt;</td>
<td>18.0 (±0.6)</td>
<td>18.2 (±0.7)</td>
<td>18.8 (±1.2)</td>
<td>17.2 (±0.7)</td>
<td>18.2 (±1.6)</td>
<td>16.9 (±0.6)</td>
<td>16.5 (±1.4)</td>
<td>16.5 (±1.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DC&lt;sup&gt;cd34&lt;/sup&gt;</td>
<td>18.7 (±1.4)</td>
<td>17.5 (±0.4)</td>
<td>19.1 (±2.0)</td>
<td>20.4 (±1.6)</td>
<td>19.3 (±1.4)</td>
<td>19.4 (±2.3)</td>
<td>19.1 (±0.3)</td>
<td>19.1 (±0.3)</td>
<td></td>
</tr>
</tbody>
</table>
Supplemental figure 9-1 Chemical cytotoxicity assessment in submerged primary airway epithelial cell cultures. SAEC were differentiated for 7 days using DCI differentiation medium in submerged conditions and were exposed to concentration ranges of amiodarone, busulfan and paraquat. Toxicological assessment was carried out using resazurin reduction and LDH release. Results are shown as % of negative control for Resazurin (●, left y-axis) and % of positive control (10x RLT buffer) for LDH (■, right y-axis), significant differences compared to control shown as # (LDH, p<0.05) or * (Resazurin, p<0.05).
Supplemental figure 9-2 Effect of differentiation media on primary small airway epithelium differentiation. Primary SAEC were differentiated on ALI using several differentiation medium components for 21 days. A) Differentiation medium components, B) TEER values (mean ± SEM), C) Heatmap of fold change (rt-qPCR, corrected for GAPDH expression), D) Fold change of rt-qPCR selected genes in different media, fold change over control (mean ± SEM).
Appendix

Supplemental figure 9-3 PCR analysis of primary airway differentiation using DCI and PneumaCult™-ALI differentiation medium. SAEC (day 0, basal cells) were differentiated for 21 days on ALI using DCI and PneumaCult™-ALI differentiation medium. qPCR values were corrected for GAPDH expression and shown as mean fold over control (basal cells) ± SEM. Significance is shown compared to basal cells (* P<0.01, ** P<0.001, *** P<0.0001) and compared to DCI differentiation (# P<0.01, ## <0.001, ### <0.0001)
Supplemental figure 9-4 2D iPSC airway differentiation protocol 1. iPSC (SBAD2 and SBAD3) were differentiated as described in iPSC airway protocol 1 (section 2.1.3.1). A) protocol overview, B) microscopy images (x100), C) qPCR values were corrected for GAPDH expression and shown as mean fold over control (undifferentiated iPSC) ± SEM (N=3). Significance is shown compared to control (* P<0.05).
Supplemental figure 9-5 iPSC airway differentiation protocol 2. iPSC (SBAD2 and SBAD3) were differentiated as described in iPSC airway protocol 2 (section 2.1.3.2). A) protocol overview, B) microscopy images (x100), C) qPCR values were corrected for GAPDH expression and shown as mean fold over control (undifferentiated iPSC) ± SEM (N=3). Significance is shown compared to control (* P<0.05).
Supplemental figure 9-6 CD34+ derived macrophage differentiation over time. CD34+ hematopoietic stem cells were differentiated as described in section 2.1.5 and analysed using qPCR at multiple time points. qPCR values were corrected for GAPDH expression and shown as mean fold over control (undifferentiated CD34+ cells) ± SEM (N=3). Significance is shown compared to control (* P<0.05).
Supplemental figure 9-7 Poisson sample distance of chemical exposures in CD34+ macrophages. CD34+ hematopoietic stem cells were differentiated towards macrophages as described in section 2.1.5. Data from TempO-Seq data was analysed for sample distribution using Poisson distribution within DESeq2 package in R. Data shows that amiodarone (15 µM) exposed donor 6 can be considered as an outlier.

Supplemental figure 9-8 qPCR analysis of amiodarone exposures in CD34+ macrophages and dendritic cells. CD34+ hematopoietic stem cells were differentiated as described in section 2.1.5, exposed to 15 µM amiodarone for 24 hours and analysed using qPCR. qPCR values were corrected for GAPDH expression and shown as mean fold over control ± SEM (N=3). Significance is shown compared to control (* P<0.05).
Supplemental figure 9-9 Cell number differences between CD34+ and iPSC derived macrophages and dendritic cells. Differences in cell numbers exposed to chemicals were analysed using microscopy images (A) and TempO-Seq raw count values (B). DC\textsuperscript{iPSC} replicate 3 was excluded from further analysis due to low count value. Normalized counts were similar between samples (C). Raw counts upon PQ exposure showed low counts throughout DC\textsuperscript{iPSC} rep 3 and for low dose PQ exposure in the other DC\textsuperscript{iPSC} replicates (D).

Supplemental table 9-2 Nanoparticle characterization. Nanoparticles were suspended in DMEM or DMEM + HDM and analysing using dynamic light scattering (DLS), data shown as mean (N=3) ± SD of size (nm).

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>DMEM</th>
<th>DMEM + HDM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al\textsubscript{2}O\textsubscript{3} NPs</td>
<td>275.2 ± 28.5</td>
<td>350.1 ± 59.5</td>
</tr>
<tr>
<td>C NPs</td>
<td>352.4 ± 15.3</td>
<td>447.8 ± 67.9</td>
</tr>
<tr>
<td>TiO\textsubscript{2} NPs</td>
<td>374.3 ± 17.1</td>
<td>462.4 ± 58.1</td>
</tr>
<tr>
<td>ZnO NPs</td>
<td>299.5 ± 62.3</td>
<td>434.3 ± 18.7</td>
</tr>
</tbody>
</table>
Appendix

Supplemental figure 9-10 PCR analysis of nanoparticle exposures in CD34+ macrophages. CD34+ hematopoietic stem cells were differentiated as described in section 2.1.5, exposed to 15 µg/cm² nanoparticles and 25 µg/cm² HDM for 24 hours and analysed using qPCR. qPCR values were corrected for GAPDH expression and shown as mean fold over control ± SEM (N=3). Significance is shown compared to control (* P<0.05) or HDM exposure (# P<0.05).
9.2. Published papers

Small Airway Susceptibility to Chemical and Particle Injury

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Keywords
Lung · Airway disease · Toxicity · Cellular differentiation

Abstract
Small airways (SA) in humans are commonly defined as those conducting airways <2 mm in diameter. They are susceptible to particle- and chemical-induced injury and play a major role in the development of airway disease such as COPD and asthma. Susceptibility to injury can be attributed in part to structural features including airflow dynamics and tissue architecture, but recent evidence may indicate a more prominent role for cellular composition in directing toxicological responses. Animal studies support the hypothesis that inherent cellular differences across the tracheobronchial tree, including metabolic CYP450 expression in the distal conducting airways, can influence SA susceptibility to injury. Currently, there is insufficient information in humans to make similar conclusions, prompting further necessary work in this area. An understanding of why the SA are more susceptible to certain chemical and particle exposures than other airway regions is fundamental to our ability to identify hazardous materials, their properties, and accompanying exposure scenarios that compromise lung function. It is also important for the ability to develop appropriate models for toxicity testing. Moreover, it is central to our understanding of SA disease aetiology and how interventional strategies for treatment may be developed. In this review, we will document the structural and cellular airway regional differences that are likely to influence airway susceptibility to injury, including the role of secretory club cells. We will also describe recent advances in single-cell sequencing of human airways, which have provided unprecedented details of cell phenotype, likely to impact airway chemical and particle injury.

Introduction
Human small airways (SA) are those non-cartilaginous airways within the lung with an internal diameter <2 mm and typically range from the 6–8th up to the 19–22nd airway generations [1]. They include proximal, distal, and terminal bronchioles (TBs) [2–4], with some descriptions also including respiratory bronchioles (Fig. 1). They comprise a luminal surface epithelium supported by a collagen-rich basement membrane and in more proximal regions, a smooth muscle layer. They can be innervated and are populated by immune cells predominantly at the mucosal surface [5–7]. The main function of the SA are as pathways of low pulmonary resistance, conducting air for respiratory function. Importantly, they are increasingly recognized as primary targets for pulmonary injury and disease development [8] and as the major site of airflow limitation in most airways diseases [8].

Full text available: https://www.karger.com/Article/FullText/519344
CD34+ derived macrophage and dendritic cells display differential responses to paraquat

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1 Introduction

Paraquat (PQ) is a broad-spectrum herbicide known to be highly toxic to humans (Dias-Oliveira et al., 2009; Subhah and Tiwari, 2021). While it is currently banned within the European Union, in much of the rest of the world it is still commonly used. Upon ingestion, it results in systemic toxicity with acute mortality due to multiple organ failure and delayed mortality due to pulmonary fibrosis (Subhah and Tiwari, 2021). Higher levels of PQ within the lung have been attributed to distal alveolar epithelial cell accumulation due to active absorption, suggested to be mediated through polyamine transporter expression (Dias-Oliveira et al., 2008). While studies have demonstrated competitive inhibition of PQ uptake by polyamines (Hoch and Nunney, 2005; Silva et al., 2013), some in vivo work suggest additional mechanisms may be involved (Dusbar et al., 1986). The intermolecular molecular mechanism of toxicity of PQ involves the reduction of PQ2+ seen as the initial step in the ability of this compound to act as a redox cycle, generating superoxide and subsequent H2O2. These reactive chemical species cause cellular injury due to oxidation of lipids, proteins and DNA (Dias-Oliveira et al., 2009). The reduction of PQ to generate superoxide within