Development of a Primary Cell-Based Model of the Human Alveolar Epithelium

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Background

While complex organotypic *in vitro* models of the nasal, tracheal and bronchial (and oral) region of the human respiratory tract are available today, models of the alveolar epithelium still mainly rely on cell lines or on cellular monocultures. The alveolar epithelium provides a surface area that is about 140-fold that of the airways *(1)*, is a highly vascularized surface at which (diffusion driven, hence non-specific) gas exchange between the inhaled air and the bloodstream takes place and possesses unique and highly balanced immunological defense mechanisms with high relevance to secondary pulmonary and systemic toxicity *(2, 3)*. Evidently, the development of primary cell- based organotypic models of the human alveolar epithelium is of paramount importance for further promoting the scientific relevance of *in vitro* inhalation toxicology and accordingly the implementation of the 3R principles (replacement, reduction, and refinement of animal testing) in this field.

The most prevalent cell types in the normal human alveolar region are epithelial and endothelial cells, fibroblasts, and macrophages *(1, 4)*. Using primary human alveolar epithelial cells and primary CD14+ human peripheral blood monocytes, we are currently establishing a triple cell co-culture of the human alveolar epithelium, consisting of type I and II alveolar epithelial cells (ATI, ATII) and monocyte-derived macrophages. The inclusion of primary human endothelial cells and primary human fibroblasts will be considered once stable triple cell co-cultures can be established and successfully used for toxicological testing of aerosols. Being placed on transwell cell culture inserts, the cultures will be feasible for exposure at the air-liquid interface (ALI) and will allow studying effects of aerosol exposure towards the alveolar epithelium itself as well as the transfer of aerosol constituents across the epithelium, hence potential systemic effects of aerosol exposure.

Project Description and Current State

The current work aims at *i)* developing protocols for the differentiation of primary peripheral blood cell-derived monocytes (PBMC) to macrophages with high phenotypic resemblance to *in vivo* alveolar macrophages (AMs) and *ii)* developing cultivation protocols for primary human alveolar epithelial cells that result in the formation of structurally and functionally stable cultures containing ATI and ATII cells in the required ratio of ~1:2 . Once established, co-cultures as shown schematically in Figure 1 will be composed. The workflow is schematically shown in Figure 2. Table 1 lists the criteria to be used for the characterization of mono-and co-cultures.

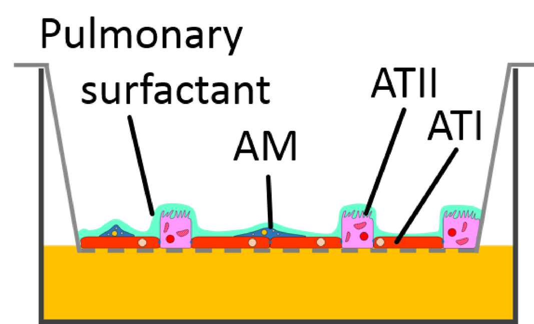
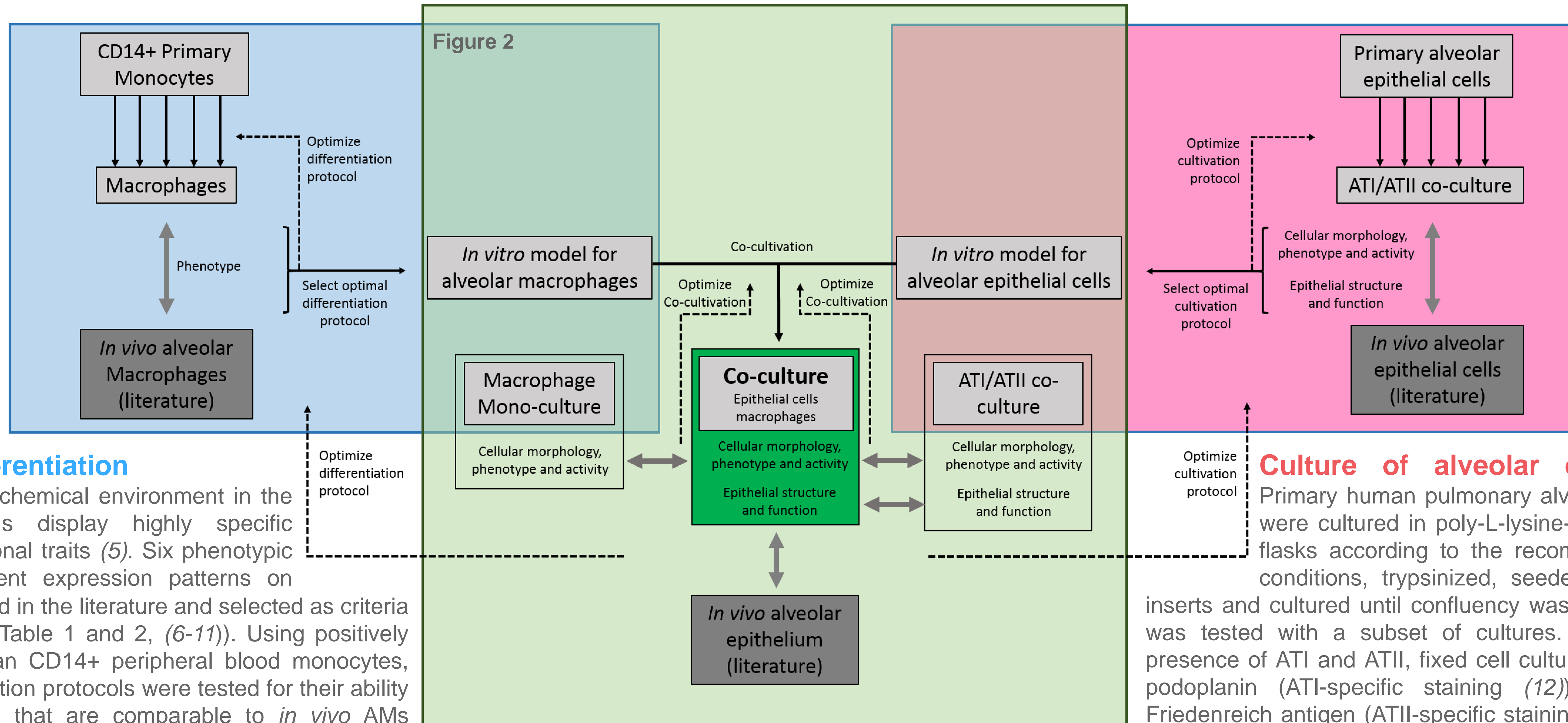


Figure 1: Schematic representation of the anticipated co-culture model of the human alveolar epithelium (ATI/II: Type I/II alveolar epithelial cell, AM: alveolar macrophages).

Table 1: Markers to be applied for characterization of mono- and co-cultures

	AM	ATI/ATII	Co-cultures
Cellular morphology	Immunohistochemistry		
Phenotype (surface markers) *(6-13)*	CD11b, CD11c, CD14, CD71, CD86, HLA-DQ	Thomsen-Friedenreich antigen, Podoplanin	all
Viability	PI/Trypan blue exclusion		
Epithelial integrity	N/A	TEER, adherens/tight junction formation	
Surfactant production	N/A	Immunoassay	
Catalase activity *(15)*	Intra- and extracellular	Extracellular	
Resistance to H_2O_2 *(15)*	Viability upon H_2O_2 treatment		
Fibronectin secretion upon LPS treatment	Immunoassay		
Phagocytic activity	Uptake of fluorescent particles		
Cytokine secretion (LPS stimulation) *(6, 16-21)*	GM-CSF, IL1b, IL6, IL8, IL10, MCP-1, MIP-1α, TGFβ, TNFα		
Relative Cell numbers *(1, 4)*	N/A	ATI:ATII ~1:2	AM:AT ~1:7



Macrophage differentiation

Due to the unique biochemical environment in the alveolar region, AMs display highly specific phenotypic and functional traits *(5)*. Six phenotypic markers with consistent expression patterns on human AMs were found in the literature and selected as criteria for AM resemblance (Table 1 and 2, *(6-11)*). Using positively selected normal human CD14+ peripheral blood monocytes, eight *in vitro* differentiation protocols were tested for their ability to yield macrophages that are comparable to *in vivo* AMs (Table 3). Best results were obtained by differentiation in supplemented macrophage basal medium containing 50 ng/ml recombinant human GM-CSF, during 6 days. Flow cytometry results for a representative experiment are shown in Figure 3. Further optimization of the differentiation protocol will be conducted only if changes in the macrophage phenotype occur in response to co-culture with the epithelial cells.

Table 2: Selected surface markers for macrophage characterization

Marker	Expected expression on AMs
CD11b	+
CD11c	+
CD14	(-)
CD71	+
CD86	(-)
HLA-DQ	+

Table 3: Macrophage differentiation protocols

Differentiation (6 days)	Polarization (24 hours)
GM-CSF	-
GM-CSF	LPS/IFNγ
GM-CSF	IL4/IL10/TGFβ
M-CSF	-
M-CSF	LPS/IFNγ
M-CSF	IL4/IL10/TGFβ
M-CSF	GM-CSF
M-CSF/IL4	-

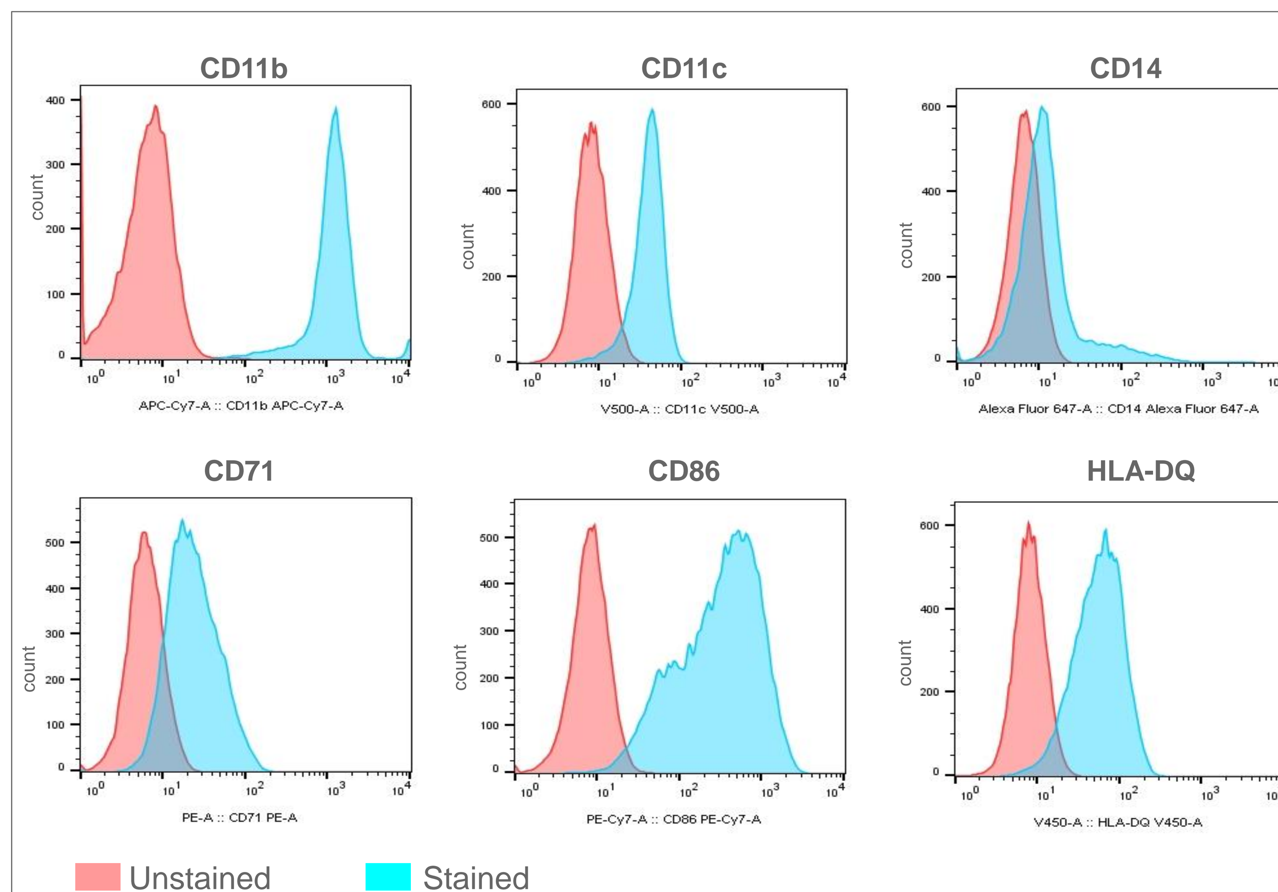


Figure 3: Flow cytometry analysis of PBMC-derived macrophages. CD14+ monocytes were differentiated *in vitro* for 6 six days in the presence of 50 ng/ml GM-CSF. A comparison with literature on the the six selected phenotypic markers *(6-11)* shows an overall good agreement with the *in vivo* expression patterns (see Table 2)

Culture of alveolar epithelial cells

Primary human pulmonary alveolar epithelial cells were cultured in poly-L-lysine-coated cell cultured flasks according to the recommended cultivation conditions, trypsinized, seeded onto cell culture inserts and cultured until confluency was reached. ALI lifting was tested with a subset of cultures. For assessing the presence of ATI and ATII, fixed cell cultures were stained for podoplanin (ATI-specific staining *(12)*) and Thomsen-Friedenreich antigen (ATII-specific staining *(13)*), but ATI/ATII specific labelling could not be obtained (Figure 4). Due to the occurence of a large number of spindle-shaped cells, additional stainings for E-cadherin (an epithelial cell-specific marker) and vimentin (a mesenchymal marker) were performed (Figure 5). The observed expression of vimentin in absence of E-cadherin is indicative for fibroblasts, but has also been reported for alveolar epithelial progenitor cells (14) further investigations on the nature of the vimentin-positive cells is therefore ongoing.

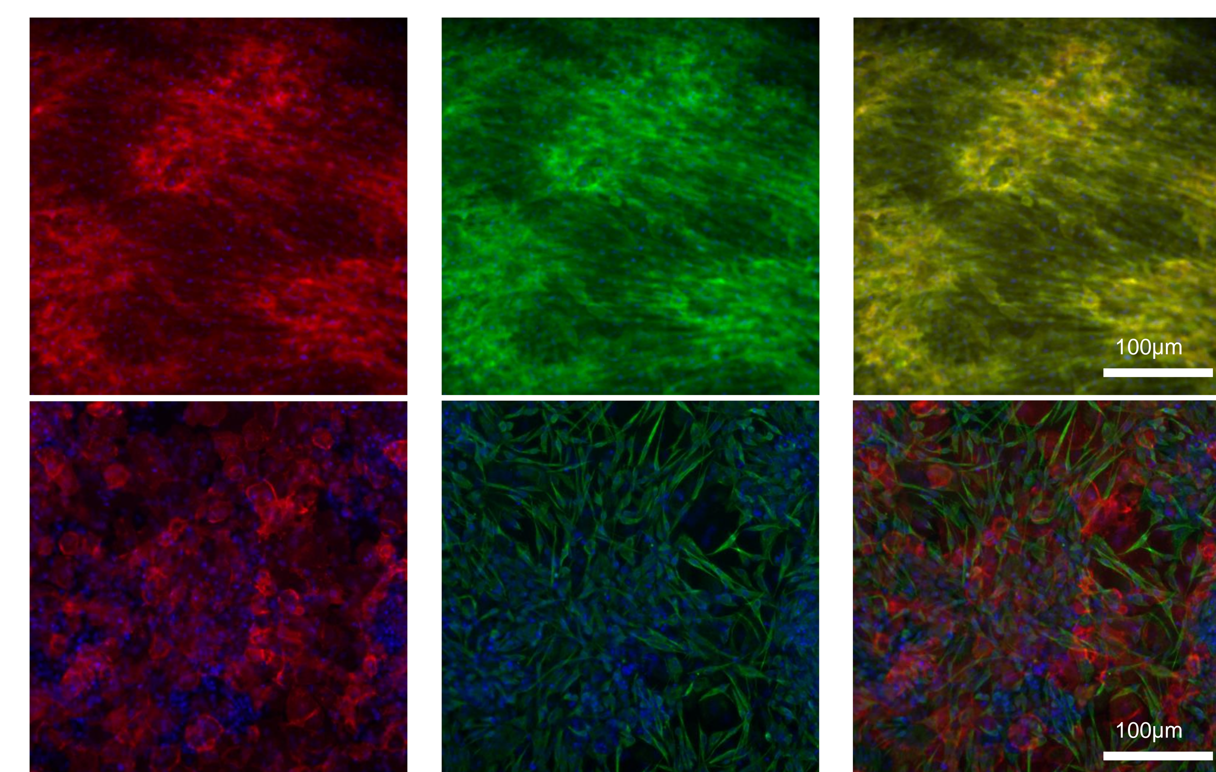


Figure 4: Epithelial cell cultures after 24h at the ALI, stained for podoplanin (ATI marker, red and Thomsen-Friedenreich antigen (ATII marker, green).

Figure 5: Epithelial cell cultures after 24h at the ALI, stained for E-cadherin (red,) and vimentin (green).

Future Developments

Further steps will include the adaptation of the cultivation and characterization of epithelial cells. Protocols for co-culturing the epithelial cells with *in vitro* differentiated macrophages and the procedures for the detailed structural and functional characterization of mono- and co-cultures will be developed.

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