

Characterization and validation of the Arlo cell line as an *in vitro* model of the air-blood barrier

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“All models are wrong, but some are useful.”

- George E. P. Box

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Summary

Animal models remain widely used in pulmonary research despite limited translational relevance and ethical concerns. Therefore, physiologically relevant human *in vitro* lung models are increasingly needed. However, the complexity of alveolar biology makes representative *in vitro* models challenging, and only a few relevant deep lung models exist.

This thesis aimed to characterize the recently established human alveolar epithelial cell line Arlo. Comparative analyses with primary human alveolar epithelial cells (hAEPc) and established epithelial cell lines showed that Arlo forms a tight epithelial barrier with high transepithelial electrical resistance (TEER), pronounced transepithelial potential difference (PD), and low permeability. The high PD was driven by strong epithelial sodium channel (ENaC) activity. Inhibition experiments revealed additional contributions of the actin cytoskeleton and tight junctions to the barrier properties. Ultrastructural and histological analyses indicated features of an alveolar type II-like phenotype.

These properties enabled the establishment of an *in vitro* pulmonary edema model reproducing altered electrophysiology and impaired fluid clearance that could be pharmacologically modulated using ENaC-targeting drugs.

Arlo was also applied in co-culture with differentiated THP-1 macrophage-like cells to investigate the effects of the ACOD1 inhibitor Citra01 during pulmonary inflammation, although epithelial-macrophage crosstalk appeared limited.

Zusammenfassung

Tiermodelle werden in der Lungenforschung weiterhin häufig eingesetzt, obwohl ihre translationale Relevanz begrenzt ist und ethische Bedenken bestehen. Daher besteht ein wachsender Bedarf an physiologisch relevanten *in vitro* Modellen der humanen Lunge. Die komplexe Zellbiologie der Alveolen erschwert jedoch die Entwicklung repräsentativer Modelle, sodass bislang nur wenige Modelle existieren.

In dieser Arbeit wurde die kürzlich etablierte humane alveoläre Epithelzelllinie Arlo charakterisiert. Vergleichende Analysen mit primären alveolären Epithelzellen und etablierten Zelllinien zeigten, dass Arlo eine ausgeprägte epitheliale Barriere mit hoher transepithelialer Resistenz, starkem transepitheliale Potentialunterschied und geringer Permeabilität bildet. Der hohe Potentialunterschied wird maßgeblich durch eine starke Aktivität des epithelialen Natriumkanals (ENaC) verursacht. Inhibitionsversuche zeigten Beiträge des Aktinskeletts und der Tight Junctions zur Barrierefunktion, während ultrastrukturelle und histologische Analysen auf einen AT2-ähnlichen Phänotyp hinweisen.

Darauf basierend wurde ein *in vitro* Modell pulmonaler Ödeme etabliert, das veränderte Elektrophysiologie und beeinträchtigte Flüssigkeitsclearance abbildet und über ENaC pharmakologisch beeinflusst werden kann. Zudem wurde Arlo in Co-Kultur mit dTHP-1-Makrophagen-ähnlichen Zellen zur Untersuchung des ACOD1-Inhibitors Citra01 bei Lungenentzündung eingesetzt, wobei die Interaktion beider Zelltypen begrenzt erschien.

List of abbreviations

3D	Three-dimensional
ACE2	Angiotensin-converting enzyme 2
ACOD1	Aconitate decarboxylase 1
ACTB	Beta-actin
AFC	Alveolar fluid clearance
ALI	Air-liquid interface
ARDS	Acute respiratory distress syndrome
AT1	Alveolar type I epithelial cell
AT2	Alveolar type II epithelial cell
BCS	Biopharmaceutics classification system
BSA	Bovine serum albumin
BSSB	Balanced salt solution buffer
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
COPD	Chronic obstructive pulmonary disease
CRDs	Chronic respiratory diseases
DCFDA	2,7-Dichlorofluorescein Diacetate
DPH	Diphenhydramine
dTHP-1	Differentiated THP-1
ECHA	European Chemicals Agency
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EMA	European Medicines Agency
ENaC	Epithelial sodium channels
EpCAM	Epithelial cell adhesion molecule
EURL ECVAM	European Union Reference Laboratory for alternatives to animal testing
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FDA	U.S. Food and Drug Administration
GCCP	Good Cell Culture Practice
GIVIMP	Guidance Document on Good In Vitro Method Practices
GSH	Glutathione
hAEPc	Human alveolar epithelial cells
hAM	Human alveolar macrophages
hTERT	Human Telomerase Reverse Transcriptase

List of abbreviations

IFN- γ	Interferon- γ
IPL	Isolated perfused lung
iPSC	Induced pluripotent stem cell
IRG1	Immunoresponsive gene 1
LCC	Liquid-covered conditions
LLOQ	Lower limit of quantification
LoC	Lung-on-chip
LPS	Lipopolysaccharide
MDA	Malondialdehyde
MDM	Monocyte-derived macrophages
MPS	Microphysiological systems
NA	No annotation
Na ⁺ /K ⁺ -ATPase	Sodium-potassium ATPases
NAMs	New approach methodologies
NHBE	Normal human bronchial epithelial
NHP	Non-human primates
NSCLC	Non-small cell lung cancer
OECD	Organization for Economic Co-operation and Development
OoC	Organ-on-chip
P/S	Penicillin/Streptomycin
P _{app}	Apparent permeability coefficient
PARC	Partnership for the Assessment of Risks from Chemicals
PBS	Phosphate-buffered saline
PCD	Primary ciliary dyskinesia
PCLS	Precision-cut lung slices
PD	Potential difference
P-gp	P-glycoprotein
PK/PD	Pharmacokinetic and pharmacodynamic
PMA	Phorbol-12-myristate-13-acetate
PMDA	Pharmaceuticals and Medical Devices Agency
PNECs	Pulmonary neuroendocrine cells
REACH	Registration, Evaluation, Authorization, and Restriction of Chemicals
RIN	RNA integrity number
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SAGM	Small Airway Epithelial Cell Growth Medium
SCLC	Small cell lung cancer

List of abbreviations

SOD.....	<i>Superoxide dismutase</i>
TBP	TATA-box binding protein
TEER.....	<i>Transepithelial electrical resistance</i>
WHO.....	<i>World Health Organization</i>

1 General introduction

1.1 Drug discovery and development process

The drug development process is typically initiated by an unmet medical need for a new or improved therapy for a specific disease [1]. In addition to *de novo* drug discovery, development efforts may also arise from drug repurposing, where new therapeutic indications are identified for existing compounds, from traditional medicine with subsequent mechanistic exploration, or from serendipitous observations [1], [2], [3]. Early drug discovery generally begins with target identification and validation, aiming to establish a clear biological rationale for therapeutic intervention [1]. Once a suitable target has been defined, potential drug candidates are identified through various approaches, including high-throughput screening, structure-based drug design, phenotypic screening, omics-based strategies such as proteomics or transcriptomics, and increasingly, artificial intelligence-driven methods [1], [2], [4], [5]. Identified hit compounds undergo further optimization during lead discovery and lead optimization, where chemical modifications are introduced to improve potency, selectivity, stability, and other physicochemical and biopharmaceutical properties. These optimization steps are guided by iterative *in vitro* and *in vivo* experiments that assess pharmacological activity and early safety profiles. Following successful lead optimization, compounds enter preclinical development, which comprises comprehensive *in vitro* and *in vivo* studies to evaluate efficacy, toxicity, and pharmacokinetic and pharmacodynamic (PK/PD) properties [1], [6], [7]. Importantly, insufficient efficacy, unexpected toxicity, and unfavorable PK/PD characteristics represent the primary causes of failure during later stages of drug development, underscoring the critical importance of robust early testing strategies [4], [8], [9]. If preclinical data sufficiently support safety and potential efficacy, the candidate may be advanced to clinical development [1], [4]. Clinical trials are conducted in sequential phases (Phase 0–IV), with an increasing number of participants [10], [11]. Phase 0 (also known as pre-phase I) studies, also referred to as exploratory or first-in-human studies, involve limited human exposure and are primarily designed to assess early pharmacokinetics and target engagement without therapeutic intent [10]. Phase I trials primarily evaluate safety, tolerability, and dosing. Phase II trials focus on preliminary efficacy and dose optimization. In contrast, Phase III trials aim to confirm efficacy and safety in larger patient populations, and Phase IV involves post-marketing surveillance. Clinical trials require prior authorization by regulatory authorities and are conducted under continuous regulatory oversight throughout all clinical phases [11]. Following successful completion of the clinical program, the drug candidate may be submitted for marketing authorization to agencies such as the European Medicines Agency (EMA), the U.S. Food and Drug Administration (FDA), or the Pharmaceuticals and Medical Devices Agency (PMDA) in Japan [11], [12], [13], [14]. After approval and market entry, the development process continues through post-market surveillance, during which adverse events, rare side effects, and real-world effectiveness are

continuously monitored. This pharmacovigilance process is ongoing and serves to continuously reassess the benefit-risk ratio of the drug throughout its lifecycle (Phase IV) [11]. The major stages of the drug development process from discovery to post-market surveillance are summarized in Figure 1.

Overview of the Drug Development Process

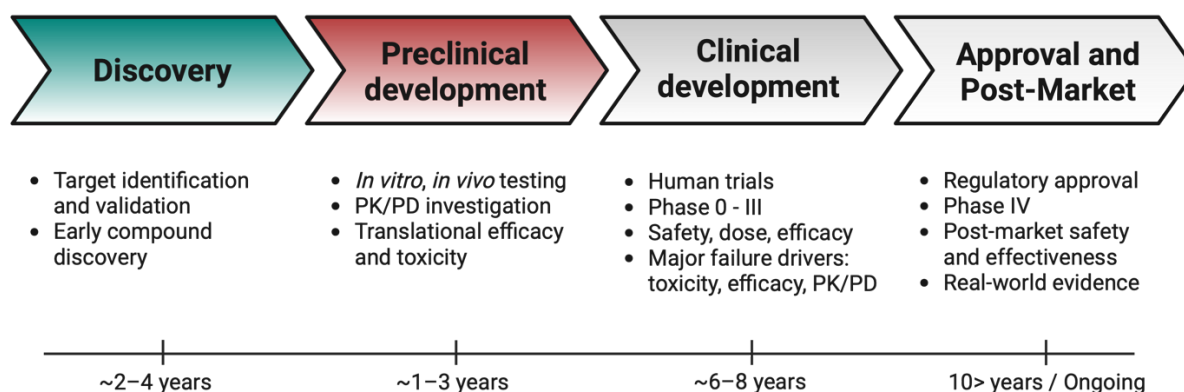


Figure 1: Schematic overview of the drug development process. Overview of the major stages from discovery through preclinical and clinical development to regulatory approval and post-market surveillance. Discovery comprises target identification, validation, and early compound discovery. Preclinical development includes *in vitro* and *in vivo* testing to assess efficacy, toxicity, and pharmacokinetic and pharmacodynamic properties. Clinical development encompasses Phase 0–III trials evaluating safety, dosing, and efficacy in humans, followed by regulatory approval and post-market monitoring (phase IV) to assess long-term safety and real-world effectiveness. The figure was created based on information from Hughes et al., van Norman et al., and Sun et al. [1], [4], [11]. Image created with Biorender.com.

Overall, drug discovery and development is a lengthy, costly, and high-risk process, typically requiring 12–15 years and investments amounting up to more than one billion U.S. dollars [1], [11], [15], [16]. Consequently, improving the currently high failure rates is of major importance. In particular, the transition from Phase I clinical trials to successful market approval remains highly inefficient, with an overall probability of launch of only approximately 7–11 %. This indicates that nearly nine out of ten drug candidates fail despite having already entered first-in-human studies [1], [4], [8], [17]. Beyond the substantial economic and temporal burden, such late-stage failure also raises ethical considerations, as it entails exposing human participants to potential risks without sufficient likelihood of clinical benefit.

The predominant reasons for clinical failure are a lack of sufficient efficacy, accounting for approximately 40–50 % of failed drug candidates, followed by unmanageable toxicity of around 30 % and unfavorable pharmacokinetic or other drug-like properties of approximately 10–15 % [4]. Importantly, the likelihood of successful progression also strongly depends on the therapeutic area. Especially during Phase I and Phase II development, anti-infective drugs show success probabilities of approximately 16 % in Phase I and 27 % in Phase II, while anticancer agents reach about 9 % and 23 %, respectively. These values are approximately twofold higher compared with respiratory drugs, which show success probabilities of around

7 % in Phase I and 15 % in Phase II. Upon entry into Phase III clinical trials, these differences become less pronounced, with on average two out of three candidates ultimately receiving regulatory approval [17].

Taken together, these high failure rates, which are particularly pronounced for respiratory drug candidates, indicate that clinical attrition is largely driven by inadequate preclinical predictivity with respect to toxicity, pharmacological properties, and therapeutic efficacy [1], [6], [7], [18]. This underscores an urgent need for improved and predictive *in vitro* and *in vivo* disease models during preclinical development. Such models are essential to identify and eliminate non-promising drug candidates as early as possible, as current systems often fail to reliably translate into clinical outcomes [6]. In this context, human-relevant *in vitro* models represent a promising opportunity to improve translational predictivity, especially for complex disease areas such as pulmonary disorders [19], [20], [21]. The lung is a highly specialized and heterogeneous organ with distinct structural and functional characteristics, including a complex epithelial barrier and close interaction with immune and vascular compartments, which critically influence disease mechanisms and therapeutic responses and provide unique opportunities for therapeutic intervention. A detailed understanding of lung anatomy and biology is therefore essential to guide the development of physiologically relevant experimental models.

1.2 Anatomy and Cellular Organization of the Human Respiratory Tract

The human respiratory tract is a highly specialized organ system optimized for efficient gas exchange while simultaneously providing a robust barrier against environmental insults [22]. Structurally, it is organized into a conducting zone and a respiratory zone (Figure 2) [23], [24]. The conducting airways extend from the nasal cavity through the trachea, bronchi, and bronchioles and terminate at the level of the terminal bronchioles, which represent the distal boundary. In contrast, the respiratory zone begins at the respiratory bronchioles and comprises respiratory bronchioles, alveolar ducts, and alveoli, which collectively constitute the primary site of oxygen and carbon dioxide exchange [25], [26], [27]. This hierarchical organization enables the lung to maximize surface area while minimizing diffusion distance, thereby ensuring efficient respiratory function [28].

The conducting airways are lined by a pseudostratified epithelium composed of multiple specialized cell types, including ciliated cells, mucus-secreting goblet cells, basal cells, club cells, and pulmonary neuroendocrine cells (PNECs). Together, these cells form a dynamic barrier that facilitates mucociliary clearance, humidifies inhaled air, and provides a first line of defense against inhaled pathogens and particulate matter. Ciliated cells drive mucociliary clearance by transporting mucus secreted by goblet cells from the bronchi toward the pharynx, thereby enabling the removal of trapped particles and microorganisms. Basal cells serve as structural support cells and function as progenitor and stem cells that maintain epithelial integrity and contribute to airway repair following injury. Club cells, predominantly located in

the bronchiolar epithelium, possess secretory, metabolic, and detoxifying functions and act as progenitor cells for bronchiolar epithelial renewal; additionally, they contribute to airway homeostasis through the secretion of surfactant-like components. Pulmonary neuroendocrine cells represent a rare but functionally important epithelial population involved in airway sensing and regulation, secreting neuropeptides and bioactive mediators that modulate epithelial differentiation, immune responses, and airway tone [29]. Beyond cellular defense mechanisms, airway protection is mediated by complementary physicochemical and biochemical barriers. These include the mucus layer that traps inhaled particles and pathogens, pulmonary surfactant with antimicrobial and surface-stabilizing functions, lung-specific metabolic and detoxification pathways, epithelial efflux transporters limiting intracellular xenobiotic accumulation, and continuous exhalation facilitating mechanical clearance of inhaled substances. Regional differences along the airway tree reflect functional specialization, with progressive thinning of the epithelium and shifts in cellular composition toward the distal lung [29], [30].

The distal lung culminates in the alveoli, which constitute the functional gas-exchange units of the respiratory system. Human lungs contain approximately 300–400 million alveoli, forming an enormous surface area that enables rapid diffusion of respiratory gases across the alveolar-capillary barrier [25], [31]. While the total internal surface area of the conducting airways amounts to only a few square meters, the alveolar compartment expands this interface dramatically, providing an estimated 130–140 m² of gas-exchange surface area [32]. This barrier is exceptionally thin, with a thickness of approximately 2 µm, and is composed primarily of alveolar epithelial cells, a shared basement membrane, and the pulmonary capillary endothelium, reflecting the stringent structural requirements for efficient gas exchange [31], [32]. The alveolar epithelium consists predominantly of two highly specialized cell types: alveolar type I (AT1) and alveolar type II (AT2) epithelial cells. AT1 cells are large, flattened epithelial cells that are structurally optimized to minimize diffusion distance and therefore cover the vast majority of the alveolar surface. While AT1 cells account for approximately 95 % of the alveolar surface area, they represent only around 40 % of the alveolar epithelial cell population, reflecting their extreme morphological specialization. Despite their numerical minority, AT1 cells play a central role in maintaining barrier integrity and regulating transepithelial transport. In contrast, AT2 cells constitute the remaining majority of alveolar epithelial cells, accounting for roughly 60 % of the epithelial cell population, while occupying only a comparatively small fraction of the alveolar surface. AT2 cells are cuboidal epithelial cells responsible for the synthesis and secretion of pulmonary surfactant, a lipid-protein complex that reduces surface tension and prevents alveolar collapse during respiration [31], [32]. In addition, AT2 cells serve as progenitor cells within the adult lung, supporting epithelial maintenance and regeneration following injury through their capacity to self-renew and

differentiate into AT1 cells [33]. However, under specific conditions, mature AT1 cells have also been shown to exhibit phenotypic plasticity and can revert to an AT2-like state, thereby contributing to alveolar epithelial regeneration [34], [35]. In addition to their structural and functional specialization, alveolar epithelial cells operate within a dynamic mechanical environment imposed by breathing. Cyclic stretch and airflow-associated shear forces continuously act on the alveolar epithelium and have been shown to modulate epithelial differentiation, barrier integrity, and cellular signaling [36], [37].

Beyond epithelial specialization, the alveolar compartment is characterized by a distinct immune environment dominated by alveolar macrophages, which reside on the luminal surface of the alveolar epithelium. These macrophages act as first-line immune sentinels, clearing inhaled particles and microorganisms while maintaining immune tolerance within the delicate gas-exchange region. Through close bidirectional interactions with alveolar epithelial cells, particularly AT2 cells, alveolar macrophages contribute to surfactant homeostasis, epithelial repair, and the regulation of inflammatory responses, thereby preserving alveolar structure and function under homeostatic conditions [38], [39].

Together, alveolar epithelial and immune cells exemplify one level of the complex cellular organization present throughout the human lung. More broadly, the lung is one of the most cellularly diverse organs in the human body, comprising not only epithelial cells but also endothelial cells, fibroblasts, smooth muscle cells, and a broad spectrum of resident and recruited immune populations. Large-scale single-cell atlasing efforts have demonstrated that the adult human lung consists of more than 50 distinct cell types and cellular states, reflecting pronounced regional specialization and functional heterogeneity across the respiratory tract [40], [41].

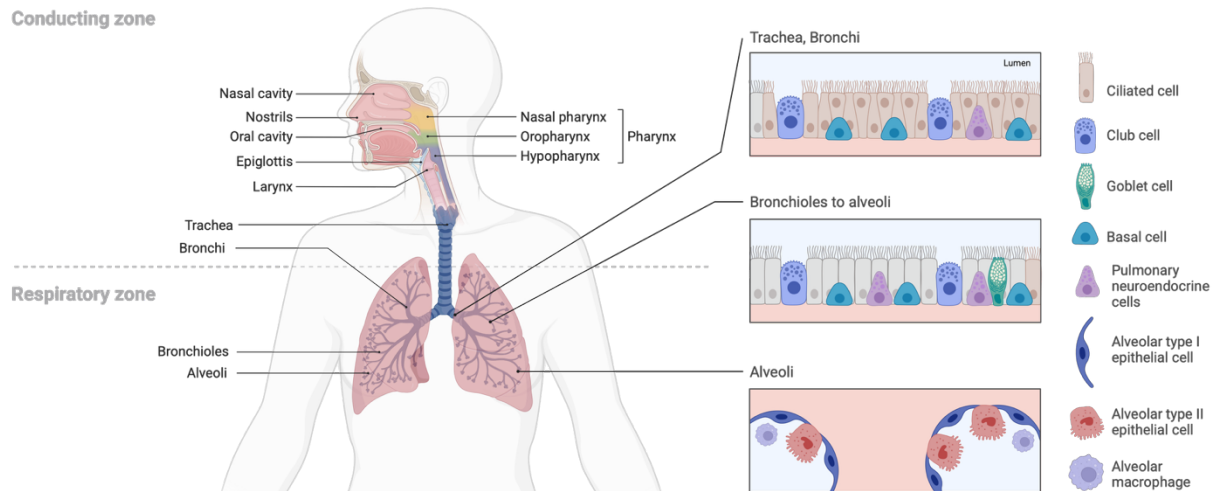


Figure 2: Structural and cellular organization of the human respiratory tract. Schematic overview of the conducting and respiratory zones of the human lung. The conducting airways extend from the upper airways to the terminal bronchioles and are lined by a pseudostratified epithelium containing ciliated, goblet, basal, and club cells. The respiratory zone begins at the level of the respiratory bronchioles and extends into alveolar ducts and alveoli, which are formed by alveolar type I and type II epithelial cells in close association with the pulmonary capillary endothelium. Alveolar macrophages are located on the luminal surface of the alveoli and contribute to immune surveillance and tissue homeostasis. Figure adapted from Debnath et al. [23]. Image created with Biorender.com.

While this extensive cellular diversity enables the lung to integrate gas exchange, barrier function, and immune surveillance, it also poses a major challenge for modeling of pulmonary biology in research. No single experimental system can fully recapitulate the structural, cellular, and functional complexity of the human lung. Consequently, a wide spectrum of experimental model systems has been established over the past decades, ranging from *in silico* approaches to *in vivo*, *ex vivo*, and *in vitro* models. Each of these systems is associated with inherent advantages and limitations. A thorough understanding of their respective strengths and constraints is therefore essential to enable the careful selection or rational design of appropriate models tailored to the specific biological question under investigation. Notably, different model systems capture distinct aspects of lung biology, including epithelial function, immune interactions, barrier integrity, and regenerative processes [42], [43].

1.3 Pathophysiology of human lung diseases

Disruption of the highly specialized cellular and structural organization of the lung gives rise to a wide spectrum of pulmonary diseases. An understanding of these disease mechanisms is essential for the rational selection and development of experimental disease models that aim to recapitulate clinically relevant aspects of lung pathology.

1.3.1 Overview of major pulmonary disease classes

According to the World Health Organization (WHO), lung-related diseases account for a substantial proportion of global mortality. Five of the leading causes of death worldwide are directly linked to pulmonary pathology, including infectious diseases such as COVID-19 and tuberculosis, which primarily affect the lung despite potential systemic involvement, other lower

respiratory infections, chronic obstructive pulmonary disease (COPD), and lung cancer. Together, these conditions accounted for approximately 17 million deaths in 2021. Given a total of around 69 million deaths worldwide, this corresponds to roughly one in four deaths being attributable to lung-related diseases. COVID-19 alone ranked as the second leading cause of death globally in 2021, with approximately 8.8 million deaths, closely following ischemic heart disease. Marked regional differences exist in the distribution of pulmonary mortality. In low-income countries, lower respiratory infections represent the leading cause of death, surpassing stroke and ischemic heart disease. In contrast, middle- and high-income countries exhibit a shift toward non-communicable diseases such as metabolic disorders and cancer; however, pulmonary diseases including COVID-19 and COPD remain among the leading causes of mortality [44].

Pulmonary diseases comprise a heterogeneous group of disorders that impair normal lung function and gas exchange. They can be categorized according to different criteria, including disease etiology, underlying pathophysiological mechanisms, or characteristic symptoms. A commonly used classification distinguishes between obstructive and restrictive lung diseases, as well as chronic respiratory diseases, infectious lung diseases, lung cancer, and genetic pulmonary disorders [45]. Obstructive lung diseases are characterized by airflow limitation resulting from partial or complete obstruction of the airways, thereby reducing the amount of air that reaches the alveoli for gas exchange. Representative examples include asthma, chronic obstructive pulmonary disease (COPD), chronic bronchitis, and bronchiectasis [46]. In contrast, restrictive lung diseases are defined by reduced lung compliance and impaired lung expansion. These disorders may arise from intrinsic pulmonary causes, such as exposure to toxic substances including tobacco smoke or asbestos, infections, or fibrosis, as well as from extrinsic factors, including irregular chest movement or neuromuscular dysfunction [47]. Chronic respiratory diseases (CRDs) represent long-term, often progressive conditions, with asthma and COPD being the most prevalent examples. Major risk factors for CRDs include tobacco smoke, air pollution, occupational exposure to chemicals or dusts, and environmental pollutants. Although these diseases are generally not curable, a variety of therapeutic strategies are available to alleviate symptoms, slow disease progression, and improve quality of life [48], [49]. Infectious diseases of the respiratory tract are commonly divided into upper and lower respiratory tract infections. Upper respiratory tract infections include conditions such as the common cold, sinusitis, and pharyngitis, and may be caused by viral (e.g., Rhinoviruses, Adenoviruses), bacterial (e.g., *Mycoplasma* and *Streptococcus* species), or fungal (e.g., *Candida albicans*) pathogens [50]. Lower respiratory tract infections primarily comprise pneumonia, which can be triggered by viruses, including influenza A viruses and SARS-CoV-2, bacteria such as *Streptococcus pneumoniae*, or fungi such as *Aspergillus* species [51], [52]. Severe pneumonia may progress to acute respiratory distress syndrome (ARDS), a life-

threatening form of acute lung injury characterized by diffuse alveolar damage, non-cardiogenic pulmonary edema, and severe impairment of gas exchange, as discussed in detail in section 1.3.2 [53]. Inherited pulmonary diseases constitute another important category and include disorders such as cystic fibrosis (CF), in which mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel result in viscous mucus accumulation and impaired mucociliary clearance in the lung, although CF affects multiple organs beyond the lung. Primary ciliary dyskinesia (PCD), another genetic pulmonary disorder, is characterized by dysfunctional ciliary motility and ineffective mucociliary transport [54], [55]. Lung cancer is one of the most common and lethal malignancies worldwide and remains a leading cause of cancer-related mortality. It is commonly classified into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) based on histological characteristics, with these two major subtypes differing markedly in clinical behavior and therapeutic management. Its development is primarily driven by tobacco smoke-induced genetic alterations, which promote disease initiation and progression and ultimately lead to progressive loss of pulmonary function and systemic homeostasis [56], [57].

Despite their diverse etiologies and clinical presentations, many pulmonary diseases share common pathophysiological features, including epithelial barrier dysfunction, dysregulated fluid transport, inflammatory signaling, and tissue remodeling. These shared mechanisms play central roles in disease progression and clinical outcome, representing key targets for experimental disease modeling.

1.3.2 Acute respiratory distress syndrome and pulmonary edema

Among acute pulmonary diseases, acute respiratory distress syndrome (ARDS) represents a prototypical condition in which epithelial barrier dysfunction, dysregulated fluid transport, and inflammation converge. It is characterized by hypoxaemic respiratory failure resulting from diffuse alveolar damage accompanied by a pronounced inflammatory response, which subsequently leads to pulmonary edema due to increased alveolocapillary permeability. Importantly, ARDS does not constitute a single, uniform disease but rather a heterogeneous syndrome with diverse underlying pathological causes, clinical manifestations, and therapeutic responses. This heterogeneity arises in part from the involvement of multiple cell types, including alveolar epithelial cells, endothelial cells, macrophages, and other immune cells, each contributing distinct pathological mechanisms. Together, these processes result in key disease features such as endothelial barrier dysfunction and impaired resolution of pulmonary edema, both of which are commonly observed in ARDS patients and are associated with disease progression and increased mortality. Beyond alveolar epithelial cell death, dysregulation of sodium transport across the air-blood barrier plays a critical role in ARDS pathophysiology. As illustrated in Figure 3, under healthy conditions sodium enters AT1 and AT2 cells through epithelial sodium channels (ENaC) located at the apical membrane [58],

[59], [60]. ENaC is a heterotrimer composed of three subunits, although four ENaC subunits (α , β , γ , and δ) exist, functional channels incorporate only three at a time, with distinct subunit combinations causing different channel conductance properties [61], [62]. Sodium is subsequently transported into the lung interstitium by basolaterally localized sodium-potassium ATPases (Na^+/K^+ -ATPase). This vectorial sodium transport establishes an osmotic gradient that drives water movement from the alveolar space into the interstitium, thereby maintaining relatively dry alveoli, apart from the thin alveolar lining fluid, and enabling efficient gas exchange. The absorbed fluid is then removed from the interstitium either by reabsorption into the pulmonary vasculature or via lymphatic drainage. This process is referred to as alveolar fluid clearance (AFC) [63]. In ARDS, AFC is frequently impaired and can be reduced by up to 50 %, particularly under hypoxaemic conditions. In addition, pro-inflammatory cytokines further compromise AFC, which is consistent with the strong inflammatory nature of ARDS. Viral infections such as influenza A have also been shown to directly impair ENaC channel function, thereby exacerbating alveolar fluid accumulation [58].

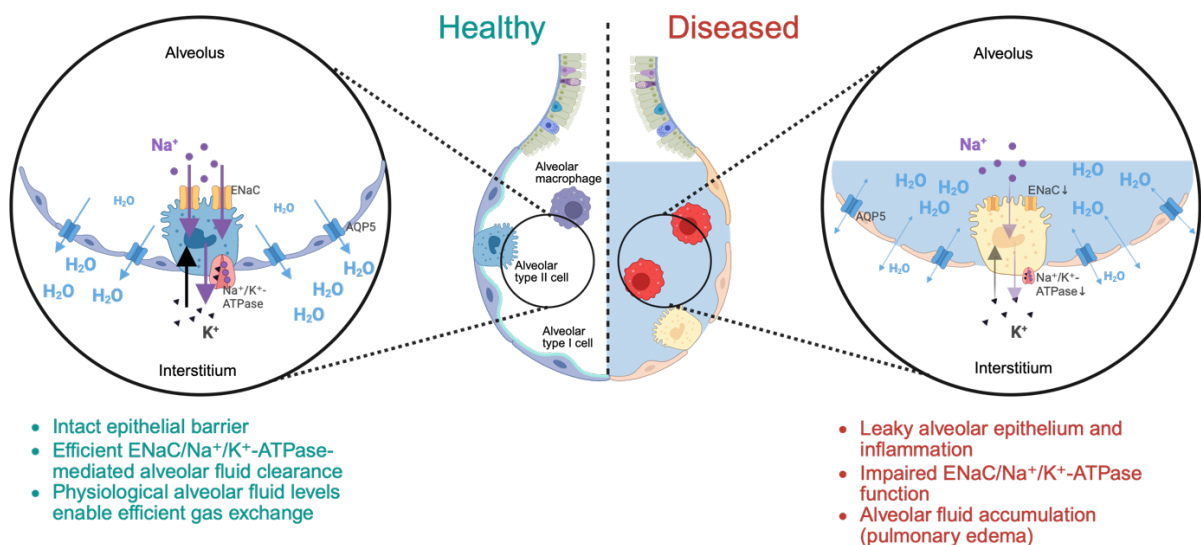


Figure 3: Alveolar fluid clearance under healthy conditions and its impairment in acute respiratory distress syndrome. Under physiological conditions (left), alveolar fluid clearance is mediated by epithelial sodium transport across alveolar epithelial cells. Sodium enters the cells via apical epithelial sodium channels and is transported into the interstitium by basolateral sodium-potassium ATPases, generating an osmotic gradient that drives water movement from the alveolar space into the interstitium. The fluid is subsequently cleared from the interstitium via reabsorption into the pulmonary vasculature or lymphatic drainage, thereby maintaining a thin alveolar lining fluid enabling efficient gas exchange. In ARDS (right), epithelial barrier disruption and inflammation impair epithelial sodium transport, resulting in reduced alveolar fluid clearance, accumulation of fluid within the alveolar space, and compromised gas exchange. Figure adapted from Huppert et al. [63]. Image created with Biorender.com.

Because this sodium-dependent fluid clearance mechanism is predominantly mediated by alveolar epithelial cells, cellular *in vitro* models represent a powerful platform to study AFC dysfunction and to evaluate therapeutic strategies aimed at restoring epithelial ion transport and enhancing edema resolution in ARDS [60].

1.3.3 The role of Aconitate decarboxylase 1 in pulmonary diseases

Aconitate decarboxylase 1 (ACOD1), also known as immunoresponsive gene 1 (IRG1), has emerged as a central regulator of immunometabolism during inflammation and infection. Immunometabolism describes the dynamic interaction between immune signaling pathways and cellular metabolic processes in response to stress. Upon infectious or inflammatory stimulation, ACOD1 expression is rapidly induced by microbial components such as lipopolysaccharide and by pro-inflammatory cytokines including tumor necrosis factor- α and interferon- γ , through tightly regulated signal transduction networks. ACOD1 is predominantly expressed in mitochondria of macrophages, where it catalyzes the conversion of the tricarboxylic acid cycle intermediate cis-aconitate into itaconate. Itaconate functions as a pleiotropic immune metabolite with broadly anti-inflammatory and antimicrobial properties. In addition to limiting bacterial and viral replication, ACOD1 activity is associated with the generation of mitochondrial reactive oxygen species, contributing to host defense but also bearing the potential for tissue injury. Dysregulation of ACOD1-itaconate signaling has been implicated in a wide range of pathological conditions, including inflammatory and infectious diseases, cancer, and neurodegenerative disorders [64]. In pulmonary fibrosis and asthma, experimental evidence supports a protective role of ACOD1 activity, as intact itaconate signaling attenuates disease progression, whereas ACOD1 knockout exacerbates pathological outcomes, corresponding data in humans remain limited [65], [66]. In respiratory tract infections, ACOD1 signaling exerts a dual function by combining antimicrobial activity with strong anti-inflammatory effects, which may in some contexts impair efficient pathogen clearance [64]. In contrast, in cancer, these anti-inflammatory properties can be harmful, as ACOD1 knockout suppresses tumor growth and enhances the efficacy of immune checkpoint inhibition, including anti-PD-(L)1 therapy [67], [68].

All in all, ACOD1 represents a highly promising and context-dependent therapeutic target in pulmonary disease. Given the complex interplay between immune cells, epithelial barriers, and metabolic signaling pathways, multicellular and co-culture *in vitro* models are particularly well suited to investigate ACOD1-mediated mechanisms and to support translational drug development [64], [69].

1.4 Modeling the human lung in health and disease

To investigate the complex biology of the human lung, a broad range of experimental model systems has been developed across different levels of biological organization. These include *in vivo* models, *ex vivo* tissue-based approaches, and *in vitro* cell-based platforms, each designed to address specific scientific and translational questions. *In vivo* models range from rodents to larger species and non-human primates, offering increasing physiological similarity to the human lung, while *ex vivo* systems such as precision-cut lung slices and isolated

perfused lungs preserve native tissue architecture under controlled experimental conditions [70], [71].

At the cellular level, *in vitro* lung models comprise immortalized cell lines and primary human cells, which differ in their regional origin, differentiation status, and mode of immortalization. Primary cells provide higher physiological relevance but are limited by donor variability and restricted lifespan. These cellular systems can be cultured in a variety of experimental formats, including conventional monolayers, permeable support systems, microphysiological lung-on-chip devices, and three-dimensional models such as spheroids and organoids. In addition, many platforms enable co-culture of epithelial, endothelial, immune, and mesenchymal cells, allowing key aspects of multicellular lung biology to be recapitulated in a controlled manner. Together, this diversity of available models underscores the necessity of selecting experimental systems that are appropriately matched to the specific biological question under investigation [71], [43].

1.4.1 Animal models

Animal models continue to play a central role in pulmonary research and drug development, as they enable investigation of respiratory biology within the context of an intact organism. In contrast to simple experimental systems, animal models allow the study of complex physiological processes, systemic effects, multiorgan interactions, disease progression, and pharmacokinetics [72]. As direct experimentation in humans is not feasible during early stages of drug development, animal species with varying degrees of anatomical and physiological similarity to humans are used as surrogate systems [73]. A wide spectrum of animal models is available, ranging from small rodents to non-human primates (NHP), each associated with distinct advantages and limitations. Consequently, the selection of an appropriate animal model and experimental assay must be carefully aligned with the specific research question. Critical evaluation criteria include anatomical and physiological comparability to the human lung, metabolic and immunological differences, and the relevance of key molecular pathways [74], [75]. A prominent example highlighting the importance of model selection is SARS-CoV-2 research, where wild-type mice were initially unsuitable due to differences in the amino acid sequence of the angiotensin-converting enzyme 2 (ACE2) receptor, rendering them naturally resistant to infection. In contrast, golden hamsters, ferrets, and non-human primates exhibit natural susceptibility to SARS-CoV-2 and therefore served as more appropriate models for studying viral infection and pathogenesis [76]. Within pulmonary research, mice represent the most widely used animal species, primarily due to their small size, cost-efficient breeding, short generation times, and extensive availability of genetic tools. Mice share approximately 95 % of their genes with humans and can be easily genetically modified, enabling precise investigation of molecular mechanisms underlying lung development, disease, and repair. Furthermore, decades of use have resulted in well-established experimental protocols,

analytical methods, and broad regulatory acceptance. Accordingly, mice and other rodents are frequently employed in mechanistic studies of pulmonary diseases, including chronic obstructive pulmonary disease, asthma, pulmonary fibrosis, acute lung injury, as well as infectious and inflammatory models [72], [77], [78]. Large-animal models, such as pigs and sheep, are used to address limitations associated with small-animal systems. Porcine lungs closely resemble human lungs in airway architecture, alveolar structure, and respiratory mechanics, making pigs particularly suitable for inhalation studies, ventilation-associated lung injury, and cystic fibrosis research [79]. Ovine models, by contrast, are commonly applied to investigate alveolar fluid clearance, surfactant biology, and neonatal lung disease [80], [81]. Non-human primates are used when a high degree of anatomical, physiological, and immunological similarity to the human lung is required. Due to their close resemblance to humans, NHP models are primarily used in translational research, including respiratory infections, vaccine development, and safety evaluation of therapeutics. However, their use is severely constrained by ethical considerations, high costs, and stringent regulatory requirements, and is therefore typically limited to specific late-stage preclinical applications for which alternative models are inadequate [82], [83], [84].

Despite these clear advantages, animal experimentation is accompanied by substantial limitations, encompassing not only ethical concerns but also scientific and practical constraints. Ethical considerations have long been addressed through the 3R principle (Replacement, Reduction, and Refinement), introduced by Russell and Burch in 1959, which aim to minimize animal use and suffering [85]. In addition, animal models face scientific limitations, including interspecies variability and limited translational predictivity, as well as high costs and lengthy study durations [20]. Humans differ fundamentally from animal species, with substantial differences, for example, in enzyme expression and activity, anatomical size and organ dimensions, immune system function, and metabolic pathways, which can markedly affect the translation of animal data to humans. In the context of toxicity testing, for example, toxicological findings obtained in rodents, dogs, or non-human primates show agreement with human outcomes in only approximately 40–60 % of cases, which represents a major contributor to the high attrition rates observed during clinical development. In addition, the reproducibility of toxicological studies, estimated at around 70 %, remains limited and further undermines the reliability of animal-based safety predictions [86], [21]. In pulmonary research specifically, pronounced anatomical differences exist between the lungs of humans and commonly used animal models. According to Weibel's morphometric analysis, the human lung exhibits a highly ordered branching architecture comprising approximately 23 airway generations from the trachea to the alveolar sacs [87]. In contrast, rodents such as mice and rats display substantially fewer generations, typically in the range of 13–18, which contributes to differences in airflow dynamics and aerosol deposition compared with humans [80]. Pigs, by

comparison, exhibit a similar number of airway generations to humans; however, their branching architecture differs fundamentally. The human lung is predominantly characterized by bipodial branching, in which an airway divides into two daughter branches of comparable size, whereas pigs and rodents mainly show a monopodial branching pattern, consisting of a dominant main airway with multiple smaller side branches [79]. Beyond airway branching, numerous additional interspecies differences exist, including variations in the number of lung lobes, epithelial thickness, cellular composition, and the thickness of the air-blood barrier, as well as further structural and physiological characteristics. Together, these differences substantially alter pulmonary function and restrict the direct transferability of findings from animal models to the human lung [80].

Despite their central role in pulmonary research, the limitations of animal models highlight the need for complementary experimental approaches. Consequently, increasing emphasis has been placed on cell-based *in vitro* lung models, which enable the investigation of human-specific pulmonary mechanisms under controlled conditions.

1.4.2 Primary cells and induced pluripotent stem cells (IPSC)

One of the most physiologically relevant approaches for *in vitro* lung research is the use of primary human cells, including primary human alveolar epithelial cells (hAEpC), normal human bronchial epithelial cells (NHBE), and primary human alveolar macrophages (hAM) [88]. These cells are isolated directly from human lung tissue, which is typically obtained during surgical procedures, and therefore retain functional, and molecular characteristics of the cells *in vivo* [22]. Following isolation, primary cells are commonly purified based on the expression of specific surface markers using magnetic sorting or fluorescence-activated cell sorting (FACS), for example epithelial cell adhesion molecule (EpCAM) or cytokeratins [89], [90], [91]. Bronchial epithelial cells are generally isolated by enzymatic digestion or bronchial brushing and, depending on the isolation method, may allow limited subcultivation [92], [93]. Upon differentiation, primary bronchial epithelial cells form a pseudostratified epithelial layer characterized by mucus production and functional cilia, a combination of features that is not simultaneously achieved by many immortalized cell lines. In contrast, primary alveolar epithelial cells are most commonly obtained through enzymatic isolation of alveolar type II cells, which subsequently transdifferentiate into non-proliferative alveolar type I-like cells during *in vitro* culture, thereby precluding subcultivation [22]. Primary alveolar epithelial cells are characterized by the formation of a tight epithelial barrier, reflected by high transepithelial electrical resistance (TEER) [43], [94]. Disease-specific *in vitro* models can be generated by isolating primary cells from donors with defined pathological conditions. Moreover, autologous co-culture systems combining different cell types from the same donor, such as alveolar epithelial cells and alveolar macrophages, provide valuable opportunities for disease modeling [95], [96].

Despite their high physiological relevance, the use of primary cells is associated with several major limitations. The availability of human lung tissue is inherently restricted, as it depends on surgical material, which limits scalability and makes primary cells unsuitable for high-throughput screening approaches [22]. In addition, tissue samples are predominantly derived from patients undergoing surgery for severe conditions such as lung cancer. Although the isolated tissue is often obtained from non-tumorous regions, many donors have a history of smoking or underlying lung disease, which may already affect gene and protein expression profiles and thereby influence experimental outcomes. Furthermore, primary cells are subject to pronounced inter-donor variability resulting from differences in age, sex, genetic background, disease status, medication, and environmental exposures (e.g. smoking), leading to limited reproducibility [71], [97], [98]. The isolation process itself is time-consuming, costly, and technically demanding and carries a substantial risk of low yields or complete failure of cell outgrowth, particularly when tissue quality is compromised. Importantly, although robust and well-established protocols exist for the isolation and culture of primary human lung cells, these procedures are labor-intensive and require specialized expertise and infrastructure, which limits their routine application and scalability [91]. In addition, the maintenance and differentiation of primary cells require highly defined, cell type-specific culture media supplemented with selective growth factors and appropriate extracellular matrix components, further increasing experimental complexity and costs [88], [99]. Even when successfully established, primary cells display a limited proliferative capacity, can only be passaged a few times, and frequently undergo rapid phenotypic changes under *in vitro* conditions, restricting their suitability for long-term experiments [93]. Accordingly, primary cells are widely regarded as reference systems for mechanistic and translational studies rather than as routine platforms for standardized, long-term, or large-scale experimentation [71].

In addition to in-house isolation, primary human cells are commercially available from specialized providers as frozen aliquots or ready-to-use cultures, including material from healthy donors and patients with pulmonary diseases such as fibrosis, asthma, COPD, or cystic fibrosis [94], [100], [22].

As an alternative approach, induced pluripotent stem cell (iPSC)-derived lung models have emerged as a promising strategy to address several limitations associated with primary cells, including limited availability, reproducibility, and scalability. iPSCs can be generated from adult somatic cells by reprogramming, allow theoretically unlimited expansion, and enable patient-specific disease modeling. They can be differentiated at scale into a broad range of lung-relevant cell types, including epithelial cells, innate immune cells, endothelial cells, and fibroblasts, while providing genetic tractability and a consistent genetic background that reduces donor-dependent variability. Importantly, iPSC-based approaches enable the

generation of distinct alveolar type I- and type II-like cell populations, which are often difficult to maintain separately in primary cultures due to transdifferentiation processes [101].

Differentiation is achieved through defined protocols employing small molecules, growth factors and transcription factors, with increasing attention being directed toward non-soluble cues such as extracellular matrix composition, air-liquid interface (ALI) culture, and mechanical forces to enhance lung-like maturation [102], [103], [104], [105]. While many iPSC-derived lung cell types recapitulate key functional aspects of their *in vivo* counterparts and are amenable to genetic manipulation, differentiation toward mature alveolar epithelial phenotypes remains complex, time-consuming, and frequently results in heterogeneous or incompletely matured, fetal-like cell populations. This can limit the faithful modeling of adult lung physiology [106], [107]. In addition, differentiation efficiency and cellular identity vary substantially between protocols and laboratories, and iPSC-based systems require specialized expertise, infrastructure, and high costs, which constrain their routine use in mechanistic and screening-based studies [101].

Taken together, although primary cells and iPSC-derived models provide high physiological relevance, their practical limitations underscore the need for complementary *in vitro* systems that combine biological relevance with reproducibility, scalability, and experimental robustness. One such approach is the use of immortalized epithelial cell lines as standardized and controllable model systems.

1.4.3 Cell lines

Continuous cell lines are widely used because of their distinct advantages over other *in vitro* models. These include ease of handling, long-term availability, simple and cost-effective culture using well-established protocols, and their suitability for high-throughput screening applications. In addition, continuous cell lines do not exhibit inter-donor variability, as they originate from a single donor, which represents a clear advantage compared to primary cells. However, this lack of donor heterogeneity also limits the generalizability of findings derived from a single cell line [22]. In general, most established cell lines are either directly derived from tumor tissue or generated by immortalization of primary cells. Immortalization is commonly achieved via viral or non-viral gene transfer approaches that target key regulators of the cell cycle or cell division, for example SV40 LT-Antigen or human Telomerase Reverse Transcriptase (hTERT), thereby enabling indefinite proliferation [108], [109]. Traditionally, such strategies rely on the introduction of a single dominant immortalizing gene, which frequently interferes with fundamental regulatory pathways and is therefore associated with pronounced phenotypic and functional alterations [109], [110]. However, alternative approaches have been developed that aim to minimize these undesired effects by targeting multiple genes simultaneously, each exerting only mild effects on proliferation-associated pathways and thereby reducing the risk of substantial phenotypic change [111], [112], [113]. However, due

to the process of immortalization, artificial culture conditions, and their simplicity, cell lines are limited in their ability to faithfully recapitulate the physiological *in vivo* situation and are therefore best suited for the investigation of specific, isolated aspects of complex biological processes [107]. Consequently, the selection of an appropriate cell line must be carefully considered and aligned with the specific research question being addressed.

Several criteria should be taken into account when selecting an appropriate cell line. For epithelial cell models in particular, barrier properties represent a key aspect, reflecting the degree to which cells form tight intercellular junctions or, conversely, a leaky monolayer. These properties can be assessed using various complementary approaches, including transepithelial electrical resistance (TEER), transepithelial potential difference (PD), and the apparent permeability coefficient (P_{app}), which will be briefly introduced in the following sections.

TEER is a non-invasive, easy, low-cost, and rapid measure of epithelial barrier integrity, reflecting resistance to ionic flow and tight junction integrity across a cell layer. Due to its non-destructive nature, it is well suited for continuous monitoring of barrier formation rather than endpoint analysis. However, TEER measurements are highly sensitive to experimental conditions such as temperature, medium composition, and electrode handling. Although TEER values are commonly normalized to the growth area to improve comparability, they provide only limited information on functional permeability and must therefore be interpreted with caution [114].

The apparent permeability coefficient describes a normalized measure of the translocation of defined marker molecules across an epithelial cell layer and provides a functional assessment of barrier permeability. Typically, high-permeability marker compounds that preferentially cross the epithelium via the transcellular route and show high P_{app} values and low-permeability markers that mainly traverse the paracellular pathway are used to distinguish between different transport mechanisms. In addition, substrates of specific transport proteins can be applied to assess transporter-mediated flux, which can substantially influence the resulting P_{app} values. Depending on the polarized localization of these transport proteins, both apical-to-basolateral and basolateral-to-apical transport experiments can be performed, with the apical side representing the air-exposed surface and the basolateral side corresponding to the blood-facing compartment in the lung [115], [116], [117].

Transepithelial potential difference (PD), also referred to as the transepithelial potential difference, reflects the electrical potential difference across an epithelial cell layer and arises from enzyme-mediated active ion transport processes [118]. In the lung, PD is of particular relevance, as vectorial ion transport establishes electrochemical gradients that drive alveolar fluid clearance [119]. As with other electrophysiological readouts, PD is dependent on experimental conditions such as temperature, medium composition, and ionic strength and

therefore requires careful standardization and interpretation. Beyond functional barrier measurements, combined transcriptomic and proteomic profiling can provide complementary molecular context by revealing the expression of relevant genes, pathways, and transport proteins. The integration of RNA- and protein-level data enables an informed assessment of model suitability for specific research questions based on publicly accessible databases, thereby reducing the need for extensive preliminary experimental screening. However, the interpretation of these data requires caution, as low-abundance transcripts or proteins, post-transcriptional regulation, and technical detection limits may influence the apparent representation of functional pathways [120], [121], [122].

Another important criterion in the selection of an appropriate cell line is its anatomical origin, particularly in the lung, which comprises more than 50 distinct cell types as discussed in section 1.2, as it determines key epithelial properties relevant to the respective experimental application [43]. Table 1 provides an overview of commonly used epithelial cell lines representing the upper and lower lung, including their tissue origin, immortalization strategy, characteristic features, and typical fields of application.

Table 1: Overview of commonly used human lung epithelial cell lines, their origin, immortalization strategies, characteristics, and application fields.

Cell line	Lung region	Origin	Immortalization	Functional characteristics	Application field	Ref.
BEAS-2B	Upper	NHBE	Viral transduction (SV40 LT-Antigen)	- No functional barrier	- Cell biology - Toxicology - Metabolism	[71], [123], [124]
Calu-3	Upper	Bronchial adenocarcinoma	Cancer	- Functional barrier with TJ - Transport protein expression - Mucus production at ALI	- Permeability - Mucus - Drug delivery	[71], [125], [126], [127]
CFBE41o-	Upper	NHBE (CF patient)	Transfection (SV40 LT-Antigen)	- Functional barrier with TJ - $\Delta F508$ CFTR	- CF models - CFTR studies	[128], [129], [130], [131]
16HBE14o-	Upper	NHBE	Transfection (SV40 LT-Antigen)	- Functional barrier with TJ - Functional CFTR - Cilia expression at ALI	- CF studies - Cell biology - Permeability - Ion transport	[99], [128], [129], [132]
NCI-H441	Upper & lower	Lung adenocarcinoma	Cancer	- Features of club cells - Features of AT1 & AT2 cells - Functional barrier with TJ - Transport protein expression	- Permeability - Ion transport - Nanoparticle deposition - Co-cultures	[71], [133], [134]
A549	Lower	Lung adenocarcinoma	Cancer	- AT2-like - Poor barrier properties	- Toxicology - Inflammation	[71], [135], [136]

General introduction

Cell line	Lung region	Origin	Immortalization	Functional characteristics	Application field	Ref.
				- Transport protein expression	- Cell uptake - Transfection	
TT1	Lower	hAEpC	Viral transduction (hTERT & U19tsA58 LT)	- AT1-like - Poor barrier properties - Flat morphology	- AT1 physiology - Inflammation - Toxicology	[108], [137], [138]
hAELVi	Lower	hAEpC	Viral transduction (multi-gene approach)	- AT1-like - Tight barrier with high TEER	- Toxicology - Nanoparticle testing - Co-cultures - Permeability	[71], [111], [139]
Arlo	Lower	hAEpC	Viral transduction (multi-gene approach)	- Proliferating AT2-like - Tight barrier with extreme TEER - High transepithelial PD - Highly active ENaC	- Permeability - Alveolar physiology - Nanoparticle testing - Fluid clearance - Co-culture - Inflammation - Electrophysiology	[140]

While many lung epithelial cell lines can be broadly assigned to either the upper or lower airways, this classification is not always clear. Nonetheless, several cell lines are well established as representative models of distinct airway compartments, with BEAS-2B, Calu-3, CFBE41o-, and 16HBE14o- commonly used to model the upper airways, and A549, TT1, hAELVi, and Arlo the distal lung. In contrast, NCI-H441 occupies an intermediate position, as it exhibits features of bronchial club cells while simultaneously expressing markers associated with both AT1 and AT2 cells [71].

Epithelial cell models of the bronchial region are characterized by a broad diversity and are available for a wide range of research questions. Most of these cell lines form functional epithelial barriers with high TEER values when cultured as monolayers, rendering them suitable for permeability and transport studies [71]. An exception is BEAS-2B, which does not establish a tight barrier and is therefore primarily applied in cell biology and metabolic studies [88]. Beyond barrier formation, bronchial epithelial cell lines differ in additional functional characteristics that render them suitable for specific applications. For instance, Calu-3 cells are capable of forming a mucus layer on top of the epithelial monolayer, which is of particular relevance for studies investigating mucus as an additional barrier, such as aerosol or nanoparticle deposition in the airways. However, mucus production by Calu-3 cells is dependent on specific culture conditions and is only observed under air-liquid interface (ALI) conditions, in which cells are cultured on permeable membranes with basolateral medium supply while being exposed to air at the apical surface. In contrast, cultivation with medium on both sides of the insert is referred to as liquid-covered conditions (LCC), where secreted mucus is continuously diluted and washed away. Such differences in culture configuration can induce pronounced phenotypic changes, including mucus production in Calu-3 cells under ALI conditions or cilia expression in 16HBE14o- cells, which is likewise restricted to ALI culture [127], [129]. CFBE41o- cells originate from a cystic fibrosis (CF) patient and harbor the most common CF-causing mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) channel, namely the deletion of phenylalanine at position 508 ($\Delta F508$). As a result, CFBE41o- cells represent a well-established *in vitro* model for CF-related research questions [128]. In this context, 16HBE14o- cells are frequently employed as a reference model, as they express a functional CFTR channel [128]. Together with additional features such as the club cell-like phenotype and the expression of relevant transport proteins, including P-glycoprotein (P-gp), NCI-H441 cells complement the spectrum of commonly used bronchial epithelial cell lines [71], [134]. While these examples do not represent an exhaustive list, they illustrate the diversity of available bronchial epithelial models. Overall, the bronchial region is represented by a wide array of established *in vitro* models, enabling the investigation of diverse aspects ranging from epithelial permeability to disease-specific mechanisms.

In contrast, the availability of suitable epithelial cell models for the alveolar region has long been limited. For many years, research in this area relied predominantly on the A549 cell line and primary alveolar epithelial cells, with TT1 cells being used less frequently. Although A549 and TT1 cells exhibit AT2-like and AT1-like features, respectively, neither cell line forms a tight epithelial barrier with functional tight junctions [71]. However, the formation of an intact epithelial barrier is a key characteristic of the alveolar epithelium, as it is essential for efficient gas exchange while simultaneously preventing alveolar flooding through controlled fluid clearance [141]. Consequently, A549 and TT1 cells are mainly applied in toxicological, inflammatory, and other non-barrier-related studies, where they nevertheless represent valuable experimental tools. The NCI-H441 cell line exhibits improved barrier properties with moderate to high TEER values and expresses several markers associated with AT1 and AT2 cells. However, the simultaneous expression of club cell-related features positions NCI-H441 at the interface between bronchial and alveolar epithelial models, limiting its suitability as a faithful representation of the alveolar epithelium [71]. A major advance in the field was achieved in 2016 with the introduction of the hAELVi cell line by Kuehn et al. This cell line was generated from hAEPc using a mild immortalization strategy and displays pronounced barrier properties with reported TEER values exceeding $1500 \Omega \times \text{cm}^2$ [111]. hAELVi cells are commonly described as exhibiting an AT1-like phenotype, although some studies have suggested AT2-associated characteristics [71]. Despite these advantages, hAELVi cells were generated through immortalization of a heterogeneous population of primary cells, resulting in a polyclonal cell line. This polyclonality has been associated with batch-to-batch variability, including inconsistent barrier formation and, in some cases, the development of multilayered structures instead of homogeneous monolayers, thereby complicating data interpretation and reproducibility. To address these limitations, Carius et al. applied single-cell printing to generate monoclonal derivatives of hAELVi cells. This approach enabled the isolation of stable clones with defined growth characteristics. One such clone, termed Arlo, exhibited strict monolayer formation, a phenotype of proliferating AT2-like cells, and exceptionally tight barrier properties. Compared to both parental hAELVi cells and primary hAEPc, Arlo demonstrated superior epithelial integrity, characterized by TEER values exceeding $3000 \Omega \times \text{cm}^2$ and high transepithelial potential difference, accompanied by very low P_{app} [140]. These properties render Arlo a particularly promising *in vitro* model for studying alveolar barrier formation and function, alveolar disease mechanisms, and vectorial ion transport processes underlying alveolar fluid clearance. Furthermore, Arlo has been shown to be permissive to viral infection, including SARS-CoV-2, supporting its applicability in infectious disease research [140].

While continuous epithelial cell lines provide robust and reproducible systems for studying defined aspects of lung epithelial biology, their inherent simplicity limits the representation of

higher-order tissue complexity. To address these limitations, a range of advanced *in vitro* and *ex vivo* lung models has been developed, which are discussed below.

1.4.4 Advanced *in vitro* and *ex vivo* models

The described cell lines and primary cell types constitute the biological basis of *in vitro* lung models, while the applied culture platforms and devices define the experimental environment in which these cells are maintained and studied. Conventional approaches typically rely on standard well plates, in which cells are grown under liquid covered conditions (LCC). More advanced systems include permeable filter membrane inserts, such as Transwell® systems, allowing cells to be cultured on porous membranes with independent access to the apical and basolateral compartments. This configuration enables the application of distinct culture conditions, including air-liquid interface (ALI) or submerged LCC [142]. Beyond monocultures, co-culture and multicellular models involving two or more cell types have been developed to better reflect the cellular interplay present *in vivo*. In pulmonary research, epithelial cells are frequently combined with endothelial cells, macrophages, fibroblasts, or dendritic cells to study intercellular communication, inflammatory responses and host-pathogen interactions [71], [143]. While such approaches increase physiological relevance, their implementation is technically demanding, as culture media and supplementation must be carefully optimized to support all participating cell types [71]. Co- and multicellular models can be implemented across a range of platforms, from conventional well plates to advanced microphysiological systems [144], [145].

An important extension of two-dimensional monolayer cultures is three-dimensional (3D) cell culture, which includes spheroids and organoids. These models enable enhanced cell-cell and cell-extracellular matrix interactions, improved tissue architecture, and the formation of nutrition gradients that more closely resemble *in vivo* conditions [146]. Spheroids are relatively simple 3D aggregates derived from a broad range of cell types, including cell lines, and are used to model tumor microenvironments and assess drug penetration and efficacy [147]. Organoids, in contrast, are complex 3D structures derived from embryonic, adult, or induced pluripotent stem cells that self-organize into organ-specific architectures and recapitulate key structural and functional features of the desired organ [148]. Lung organoids are applied in studies of lung biology, disease mechanisms, host-pathogen interactions, and drug responses and can be generated from patient-derived material, enabling investigation of interindividual variability and offering promising perspectives for personalized medicine [72]. Despite their physiological relevance, spheroids and organoids are still challenged by limited standardization, batch variability, and the restricted availability of non-destructive, high-resolution analytical methods suitable for long-term studies [148].

Another increase in model complexity is achieved with microphysiological systems (MPS), commonly referred to as organ-on-chip (OoC) platforms and, in the context of respiratory

research, lung-on-chip (LoC) models. LoC models are microengineered *in vitro* devices in which lung-relevant cells are cultured in microfluidic compartments, allowing controlled perfusion and, in many designs, integration of dynamic cues such as cyclic stretch to mimic breathing [94]. Such features have been shown to modulate epithelial barrier function, transport processes, inflammatory responses, and nanoparticle translocation and can be complemented by aerosol exposure and on-chip measurements of barrier integrity or permeability [71]. Owing to their increased physiological relevance, LoC systems are increasingly applied in inhalation toxicology, disease modeling, and preclinical drug development. However, their broader application is currently limited by higher technical complexity, increased costs, and challenges related to standardization and reproducibility [36], [71].

Ex vivo lung tissue models provide an intermediate level of complexity between *in vitro* systems and *in vivo* studies by preserving large parts of the native lung architecture while allowing experimental control under isolated conditions. The isolated perfused lung (IPL) is an *ex vivo* whole-organ model in which an isolated lung is ventilated and perfused under controlled conditions, thereby maintaining intact epithelial and endothelial barriers, ventilation, and perfusion for investigation of pulmonary drug deposition, absorption, and clearance. However, its applicability is limited by short tissue viability, high technical complexity, and restricted throughput [71], [149].

Precision-cut lung slices (PCLS) are thin, reproducible sections of lung tissue that retain the native three-dimensional microarchitecture and cellular diversity of the lung, enabling parallel pharmacological, toxicological, and disease-modeling studies *ex vivo*. Their use is constrained by limited culture duration, partial loss of tissue polarization, and variability between slices, necessitating careful optimization and interpretation [71].

Taken together, advanced *in vitro* and *ex vivo* lung models provide increasing levels of physiological relevance at the cost of growing experimental complexity, underscoring the need for standardized validation, reproducibility, and clearly defined performance criteria when such models are considered for regulatory decision-making in preclinical drug development.

1.5 Regulatory perspectives on *in vitro* lung models and non-animal approaches

In recent years, the regulatory landscape governing preclinical drug development has undergone a substantial paradigm shift, driven by ethical considerations, scientific limitations of animal models, and the increasing availability of advanced non-animal methodologies. This development is closely aligned with the long-standing 3R principle (Replacement, Reduction, and Refinement), which aims to minimize animal use and suffering in biomedical research [85]. In 2022, the U.S. Food and Drug Administration (FDA) responded to this development by enacting the FDA Modernization Act 2.0, which removed the long-standing requirement for

mandatory animal testing in preclinical studies prior to first-in-human trials, marking the first substantial regulatory change in this area in more than 80 years. Under this framework, animal studies are no longer the default, and alternative non-clinical approaches such as cell-based assays, microphysiological systems, bioprinted, and computational models may be used when scientifically justified [150], [151]. Building on this legislative change, the FDA published the Roadmap to Reducing Animal Testing in Preclinical Safety Studies in 2025, outlining a strategic plan to progressively reduce animal use through the implementation of scientifically validated new approach methodologies (NAMs). The roadmap emphasizes interagency collaboration to improve the predictive value of these methods, reduce drug development costs, and strengthen regulatory innovation. In the longer term, the FDA envisions a transition toward a NAM-based default for preclinical safety assessment, with animal studies becoming the exception rather than the norm [152], [153], [154]. While the Roadmap defines the agency's strategic direction, a related legislative proposal, the FDA Modernization Act 3.0, has been introduced but has not yet been enacted and is therefore not legally binding at the time of writing this thesis [155]. At the European level, the validation and regulatory evaluation of new approach methodologies is coordinated by the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) [156]. In parallel, the European Parliament has called for a clear phase-out plan for animal testing across research sectors (2021 Resolution), and agencies such as ECHA (European Chemicals Agency) and EFSA (European Food Safety Authority) are actively integrating non-animal approaches into regulatory risk assessment workflows under the evolving REACH (Registration, Evaluation, Authorization, and Restriction of Chemicals) framework [157], [158], [159]. In addition, the Partnership for the Assessment of Risks from Chemicals (PARC) promotes cross-agency harmonization and shared validation infrastructure to support the regulatory implementation of new approach methodologies [160]. For regulatory acceptance, *in vitro* models must demonstrate reliability, robustness, reproducibility, relevance, and a clearly defined fit for their intended purpose [161]. Central to this process is rigorous validation, which aims to establish confidence in a method by demonstrating consistent performance in a clearly defined application and regulatory relevance [162], [163]. Validation typically involves systematic optimization and standardization of new methods as well as the demonstration of reproducibility across laboratories [163]. Importantly, validation of new approach methodologies should be guided by a clearly defined context of use, emphasizing fit-for-purpose qualification for specific mechanistic, functional, or safety-relevant endpoints rather than one-to-one replacement of whole-animal experiments [163].

To support this process, several international frameworks and guidance documents have been established that define quality standards for cell-based *in vitro* models. The principles summarized in the Good Cell Culture Practice (GCCP) 2.0 guidelines emphasize core

requirements such as cell identity control, contamination prevention, traceable documentation, and standardized culture and handling procedures across increasingly complex *in vitro* systems [164]. Complementing these general standards, the Organization for Economic Co-operation and Development (OECD) Guidance Document on Good *In Vitro* Method Practices (GIVIMP) provides a regulatory-oriented framework for the development and implementation of *in vitro* methods, with a particular focus on transparency, quality assurance, and fit-for-purpose application in regulatory contexts [165].

Despite the availability of such frameworks, the validation and broader implementation of advanced *in vitro* models remain challenging [161]. This is particularly true for *in vitro* lung models, where the physiological complexity of the air-liquid interface, mechanical stretch, high blood flow, aerosol exposure, presence of surfactant, and a thin air-blood barrier introduces additional layers of biological and technical variability [166]. This can lead to pronounced challenges in standardization, reproducibility, and regulatory qualification [167]. A prominent example illustrating how these principles can be successfully implemented is the Caco-2 cell line, which has become an established reference model for intestinal permeability assessment in the context of the biopharmaceutics classification system (BCS) and regulatory biowaiver decisions [168], [169]. Despite its tumor origin and acknowledged biological limitations, Caco-2 has achieved broad scientific and regulatory acceptance through extensive characterization, standardized culture conditions, clearly defined functional readouts, and a well-delimited domain of applicability [170]. This example highlights that regulatory confidence in cell-based models is primarily driven by thorough characterization and fit-for-purpose validation, rather than by complete biological recapitulation of the native tissue.

For *in vitro* systems more broadly, this underscores the necessity of comprehensive biological and functional characterization. The importance of such multiparametric evaluation becomes particularly evident for alveolar epithelial models, where suitable *in vitro* systems have historically been scarce. Although recent developments, such as the Arlo and hAELVi cell lines, represent promising advances, limited characterization has so far constrained their broader application in drug development, toxicology, and disease modeling. Consequently, scientific – and ultimately regulatory – acceptance of advanced alveolar epithelial models requires systematic validation strategies that integrate comprehensive biological characterization with reproducible functional performance and clearly defined translational relevance

2 Aim of the thesis

The aim of this thesis was to comprehensively characterize and validate the recently established Arlo cell line as an *in vitro* model of the human alveolar epithelium. Following the successful generation of this novel cell line, a systematic evaluation of its functional properties and biological characteristics was required in order to assess its strengths and limitations and to determine its potential for physiologically relevant research applications.

To achieve this objective, this thesis was designed to apply a multiparametric and comparative characterization strategy. A central goal was to compare Arlo with established *in vitro* models of the pulmonary epithelium, including primary human alveolar epithelial cells as well as widely used epithelial cell lines. This comparative analysis was intended to evaluate key properties relevant for modeling the alveolar barrier, including epithelial barrier development, electrophysiological properties such as transepithelial electrical resistance and potential difference, drug permeability, as well as ultrastructural organization and histological features. Through this systematic comparison, the work aimed not only to identify the specific strengths and limitations of Arlo and to position the cell line within the existing landscape of pulmonary epithelial models, but also to support a more rational selection of *in vitro* lung models by linking defined cellular properties with the requirements of different research questions.

Another focus of this thesis was to investigate the mechanisms underlying the pronounced barrier properties observed in Arlo cells. In particular, the work aimed to identify factors contributing to the high transepithelial resistance and electrophysiological behavior of Arlo in order to gain further insights into the processes governing alveolar epithelial barrier formation. Furthermore, the thesis aimed to further define the cellular phenotype of Arlo. As the cell line originates from hAELVi cells, which have been described as exhibiting an alveolar type I-like phenotype, while Arlo itself has been reported to display characteristics of proliferating alveolar type II cells, the work sought to clarify the phenotypic characteristics of Arlo and to better define its position within the spectrum of alveolar epithelial cell types.

Finally, this thesis aimed to explore potential translational applications of the Arlo cell line in pulmonary research. Based on the characterization of the cell line, the work intended to evaluate the suitability of Arlo-based systems for modeling disease-relevant processes of the deep lung and for investigating epithelial responses under physiologically relevant conditions. In this context, the thesis aimed to assess whether Arlo-based *in vitro* models could serve as a useful platform for studying pulmonary disease mechanisms and for supporting drug discovery and pharmacological research in the respiratory system.

3 Outcome of the thesis

3.1 Cell-based in vitro models for pulmonary permeability studies

Main Contributors:

Tobias Neu, Clémentine Richter, Lorenz Latta, Nicole Schneider-Daum, Claus-Michael Lehr

The contributions were as follows:

T. Neu	Equally contributing first author, writing of the original manuscript, reviewing, and editing
C. Richter	Equally contributing first author, writing of the original manuscript, reviewing, and editing
L. Latta	Writing of the original manuscript, reviewing, and editing,
N. Schneider-Daum	Supervision, conceptualization, language refinement and alignment, reviewing, and editing
C.-M. Lehr	Supervision, conceptualization, reviewing, editing

Chapter 3.5

Cell-based in vitro models for pulmonary permeability studies

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3.5.1 Introduction

3.5.1.1 Pharmaceutical relevance of lung barrier models

The lung, the primary respiratory organ in humans, is a highly complex organ built from over 50 cell types that differ in morphology, function, and architectural assembly (Travaglini et al., 2020).

When looking at lung permeability, there are two main regions of interest to discern. The first region is comprised of the conducting airways (bronchi), where permeating drugs and toxins end up in the surrounding sub-epithelial tissue. The tubular conducting airways are characterized by a pseudostratified epithelial barrier expressing ciliae and producing mucus (Hiemstra, Grootaers, van der Does, Krul, & Kooter, 2018).

While the conducting airways have only an approximate surface of 1–2 m², most of the lung surface is comprised of the respiratory area (alveoli), adding up to 100–140 m² in the deep lung. The relevance of the respiratory area for drug delivery and permeation is enhanced by the fact that permeating drugs and toxins may directly reach the bloodstream through the air–blood barrier. The respiratory area is characterized by a large surface area, high vascularization, and a very thin air–blood barrier. The air–blood barrier itself is formed by endothelial cells, a basal membrane, alveolar epithelial type 1 cells (AT1), and lining fluid containing surfactant. In total, the air–blood barrier measures 1–2 µm and is highly permeable to gases, as well as highly

a. Equally contributing first authors.

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impermeable to water (Gehr, Bachofen, & Weibel, 1978; Hiemstra et al., 2018; Stone, Mercer, Gehr, Stockstill, & Crapo, 1992).

Which region a drug or particle will reach depends mainly on its size and the correct breathing technique of the patient. Particles around 5–10 μm size can impact and sediment in the conducting airways, whereas particles smaller around 2 μm can reach the respiratory area (Costa et al., 2016). Drug administration via the lung can be used for efficient local treatment while avoiding systemic adverse effects, but on the other hand, it could also be used for systemic treatment to avoid the hepatic first-pass effect (Schneider-Daum, Carius, Horstmann, & Lehr, 2019). Inhalation has been used for both therapeutic aerosols and drugs of abuse for over 3000 years. Today, inhalation drugs for local treatment are widely used against inflammatory diseases such as asthma and chronic obstructive pulmonary disease (COPD). These drugs are being administered since the 1950s with, for example, a metered dose inhaler or a dry powder inhaler (Stein & Thiel, 2017). Drugs for local treatment of lung diseases should have a very low permeability to the surrounding tissue and bloodstream to avoid systemic adverse effects. On the other hand, there are also inhaled drugs for systemic use, such as inhaled insulin for diabetes patients (Fleming, Fleming, & Davis, 2015) or inhaled levodopa for patients with Parkinson's disease (Paik, 2020). These drugs need to have a high permeability through lung epithelia to reach the blood stream and their intended site of action (Schneider-Daum et al., 2019). The other way around, drugs for pulmonary diseases that are given orally need to permeate into the lung and accumulate there. Therefore, there is an increasing interest in investigating the distribution of systemically administered drugs into the lungs.

The main defense mechanisms of the lung are exhalation, mucus, mucociliary clearance, patrolling immune cells, surfactant, lung-specific metabolism, and efflux transporters (Ibrahim & Garcia-Contreras, 2013; Rothen-Rutishauser, Gibb, He, Petri-Fink, & Sayes, 2023). In general, the lung is assumed to have lower drug metabolizing enzymatic activity compared to the liver and inhaled drugs avoid the hepatic first-pass effect (Rubin et al., 2020). However, the lung metabolism of inhaled drugs should always be considered regarding local drug activation, systemic bioavailability, and systemic adverse effects (Enlo-Scott, Bäckström, Mudway, & Forbes, 2021).

3.5.1.2 Relevance of air–liquid interface and aerosolization

One of the most important parameters when reproducing the lung barrier in vitro, regardless of which region, is the air–liquid interface (ALI) culture. It is not only a differentiation trigger for primary bronchial epithelial cells, but it allows for the nebulization of substances or particles onto the epithelial barrier model. Since inhalation is the most common route of entering the lung, it is crucial to be able to simulate it in vitro (Hiemstra et al., 2018). Transport properties and cellular uptake of nebulized drugs are highly dependent on the

liquid volume in the apical donor compartment and the nebulized concentration (Brandenberger et al., 2010; Bur, Huwer, Muys, & Lehr, 2010; Lenz et al., 2013). The biological response is also highly dependent on the experiment setup and the presence of ALI (Friesen et al., 2022). The interaction with bronchial or alveolar lining fluid (mucus and/or surfactant) should also be taken into consideration (Xu et al., 2023). Considering the lack of measurable amounts of liquid in the apical compartment of an ALI model, some barrier readouts must be adapted. The most commonly used ways to characterize the tightness of the epithelial barrier is the measurement of transepithelial electrical resistance (TEER) in $\Omega \cdot \text{cm}^2$ (Srinivasan et al., 2015) and the apparent permeability of tracer molecules. The tightness is correlated to the presence of intercellular junctions, mainly tight junctions (TJs). As a noninvasive way to determine the barrier integrity, the TEER measurement can be performed in between other assays as in-process control. Therefore, two electrodes, one in the basal and one in the apical compartment, are used to measure the resistance across the cell layer. It should be considered that the results depend on many different parameters which influence the measurement (e.g., the type of electrode, the temperature, the membrane of the insert, the TEER device, and the ion concentration) (Doryab & Schmid, 2022). In the case of ALI conditions, liquid-covered conditions (LCCs) must be re-established for a fixed amount of time before TEER measurement. Also, apparent permeability cannot be calculated and should be reported in the percent transported over time (Bur et al., 2010). Furthermore, specifying particle or substance concentrations as dose per volume proves challenging at ALI and should be replaced by dose per surface (Schmid & Stoeger, 2016). Besides TEER, another bio-electrical parameter of interest for characterizing epithelial barriers is the potential difference (PD). Whereas in relatively leaky epithelia like the gut, PD only reaches barely measurable values of below 1 mV (Grasset, Pinto, Dussaulx, Zweibaum, & Desjeux, 1984), but the PD in the airways reaches values of up to -30 mV or even more in cystic fibrosis patients (Mac Sweeney, Fischer, & McAuley, 2011). Therefore, while not being relevant for orally administered drugs, PD is an important parameter of the lung's ion transport and other active transport mechanisms.

There are many ways to make particles or substances airborne, the most common being nebulizing of a liquid (Horstmann et al., 2021; Lenz et al., 2009), dispersion of a dry powder (Hein, Bur, Schaefer, & Lehr, 2011) or exposure to gas or smoke (Mastalerz et al., 2022). Some setups can easily be built in research laboratories, while others can be bought from specialized companies (Hiemstra et al., 2018). However, regardless of which aerosolization technique is used, the deposition should be high enough to reach pharmacologically relevant dosing, evenly distributed and reproducible, for example, by monitoring the deposition with an incorporated quartz crystal microbalance (Ehrmann et al., 2020; Lenz et al., 2009).

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In conclusion, lung permeability is dependent on the deposition site (cell types, defense mechanisms, TJs, presence of drug-specific transporters, or efflux transporters), the physicochemical drug properties (size and hydrophobicity (Ibrahim & Garcia-Contreras, 2013), and the disease state (Hiemstra et al., 2018; Rothen-Rutishauser et al., 2023). Up to date, commonly used animal models for inhalation studies comprise mice and rats. While these models show high anatomical and physiological complexity, unfortunately, the lung anatomy, physiology, cellular structure, and size ratios are highly dissimilar to humans, implicating poor predictability for the clinical situation in patients. Therefore, this chapter will focus on how to emulate and investigate lung permeability in vitro. While there are many approaches to in vitro lung models in research, an important premise for the application in drug development is their validation, so that they may be used to set new regulatory standards and shall be accepted by the relevant authorities. This appears to be particularly important in light of the recent change in US law, where the Food and Drug Administration (FDA) has recently made it possible to approve drugs that have not gone through animal testing. Should appropriate in vitro methods be validated and used, animal models are no longer mandatory in the process of preclinical drug development (Wadman, 2023).

International guidelines and strict standard operating procedures (SOPs) need to be established and followed to demonstrate the scientific suitability, robustness, and quality of an in vitro model that might be used in regulatory decision-making (Barosova et al., 2021; OECD, 2018). In general, an in vitro lung permeability model should mimic the specific properties of the biological barrier of interest in the closest possible way, while keeping in mind that some functions may be more relevant to a particular question of research than others (Hiemstra et al., 2018; Rothen-Rutishauser et al., 2023).

3.5.1.3 Relevant cell types for lung barrier models and their biological identity

The barrier-forming entities of the conducting airways and respiratory area are the epithelia. Since the 1960s, the knowledge about these epithelia has increased, starting with morphology descriptions (Crapo, Barry, Gehr, Bachofen, & Weibel, 1982; Gehr et al., 1978), identification of cell-specific markers (Dobbs, Gonzalez, Allen, & Froh, 1999; Gonzalez, Allen, Gonzales, Ballard, & Dobbs, 2010; You et al., 2004), and physiological functions (Johnson et al., 2006; Yamaya, Finkbeiner, Chun, & Widdicombe, 1992).

For permeability studies, not all cell types occurring in the epithelium are of the same interest. The conducting airways consist of ciliated cells, mucus-secreting goblet cells, secretory (club) cells, basal cells, tuft (brush) cells, pulmonary neuroendocrine cells, and bronchioalveolar stem cells (Sun et al., 2022). Pulmonary neuroendocrine and Tuft cells act as sensors in the airways, are rare cells (Sun et al., 2022), and are not considered in in vitro permeability

models. The deeper lung epithelium is composed of alveolar type 1 (AT1) and alveolar type 2 (AT2) cells (Crapo et al., 1982). In addition to classical histological studies, lung cells are extensively characterized by lineage tracing and single cell-sequencing. Analysis of single cells from whole lung identified or proposed new cell types (Sikkema et al., 2022; Sun et al., 2022; Travaglini et al., 2020). New and rare cell types are of interest in tissue regeneration, cell lineage, or immune response and not for barrier function. Cell lineage studies and spatiotemporal expression studies in mice (Frank et al., 2019; Gokey et al., 2021) are particularly of interest in generating lung epithelial cells from induced pluripotent stem cell (iPSCs) lines. These lineage studies create knowledge critical to discover cell fate-defining signals and transcription factors that are essential for the correct differentiation of iPSCs into airway epithelial cells (McCauley et al., 2017), AT1 (Burgess et al., 2023), or AT2 (Jacob et al., 2017) pneumocytes (Calvert & Ryan, 2020). Figure 3.5.1 shows the barrier-relevant cell types, ignoring some rare and more specialized cells.

Description of barrier-forming lung epithelial cells, including their markers, channels, transporters, and anatomical and histological features is essential to successfully bridge these features to the applied scientific question of drug transport across pulmonary epithelia. Mimicking a functional biological barrier adds another layer of complexity: Transferring qualitative descriptions of junctional complexes (Overgaard, Mitchell, & Koval, 2012; Wittekindt, 2017), ion channels (Hollenhorst, Richter, & Fronius, 2011), or further relevant properties like cell polarization and associated signaling cascades from numerous individual unrelated basic-research papers into defined and concise in vitro models to allow a quantitative prediction of drug transport is a big challenge.

3.5.1.3.1 Conducting airways: Ciliated cells, goblet cells, and basal cells

A rational approach is to directly compare drug transport of in vitro models to ex vivo tissue properties. Such comparison has been used frequently in in vitro models of conduction airways (see numbers in orange in Figure 3.5.2). Specific transporter expression, activity, or expression of junctional components can link drug transport with its respective in vitro models (Selo, Sake, Kim, & Ehrhardt, 2021; Silva, Bicker, Falcão, & Fortuna, 2023; Wittekindt, 2017). Primary bronchial-derived cells from human donors form tight cellular layers in vitro. In the early 1980s, researchers adopted protocols to differentiate primary cells into ciliated and goblet cells (Figure 3.5.2). Implementing techniques like Ussing chambers and microperfusion allowed a comparative view between cellular models and in vivo/ex vivo properties regarding ion and drug transport processes (Blouquit et al., 2002; Yamaya et al., 1992) without knowing the exact and quantitative composition of transporters and junctions. After the correlation of primary human bronchial epithelial cells (hBECs) and

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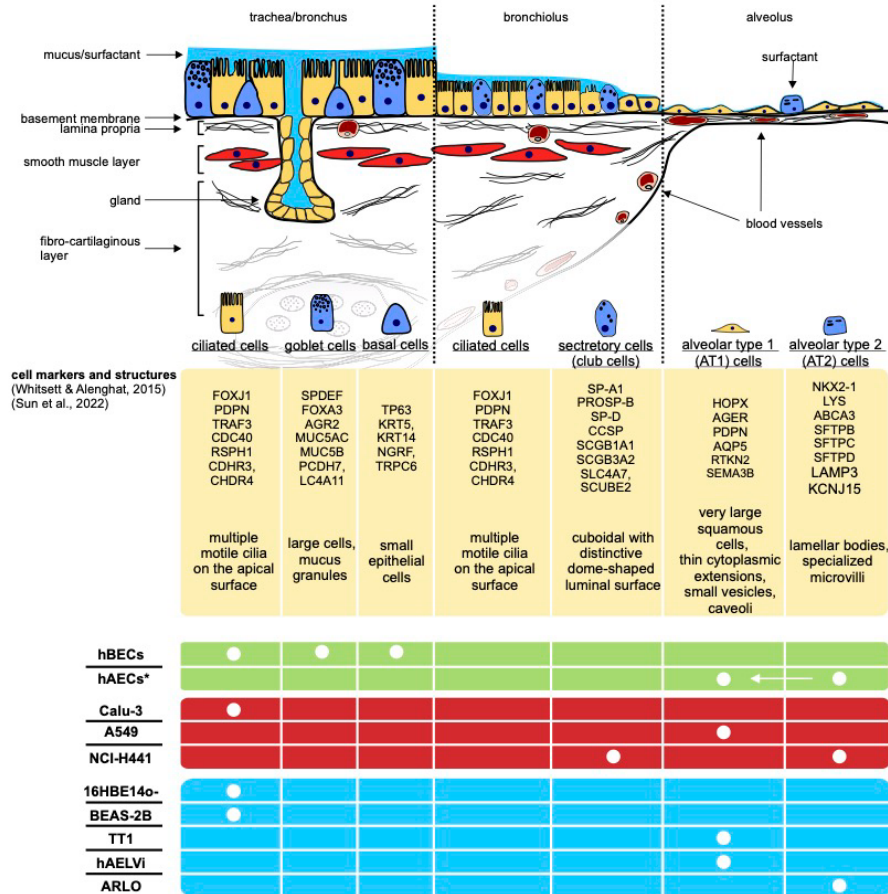


FIGURE 3.5.1 Anatomical structure with localization of cell types related to barrier functions. Cell lines and primary cells are assigned to their in vivo corresponding counterparts (white circles). In addition to cell type-specific markers, morphologic parameters and ultrastructure can be used for classification. Further established classical markers are predominantly used in in vitro models (see Tables 3.5.1, 3.5.2, and 3.5.3). New omics approaches can suggest additional marker genes complementing established markers. The figure is adapted from Klein, Hennen, Serchi, Blömeke, and Gutleb (2011).

ex vivo tissue had been established, further in vitro models could reliably be compared to primary hBECs. In contrast to the brain, kidney, liver, placenta, or small intestine, no database for the lung and its in vitro models exists (<https://transportal.compbio.ucsf.edu/index/> Morrissey et al., 2012) so far.

The advantage of lung/airway-specific models for drug transport is still under debate. Some reports claim a good correlation between airway in vitro models and IPF (isolated perfused lung models), but also to non-airway derived in vitro models like Caco-2 (Manford et al., 2005).

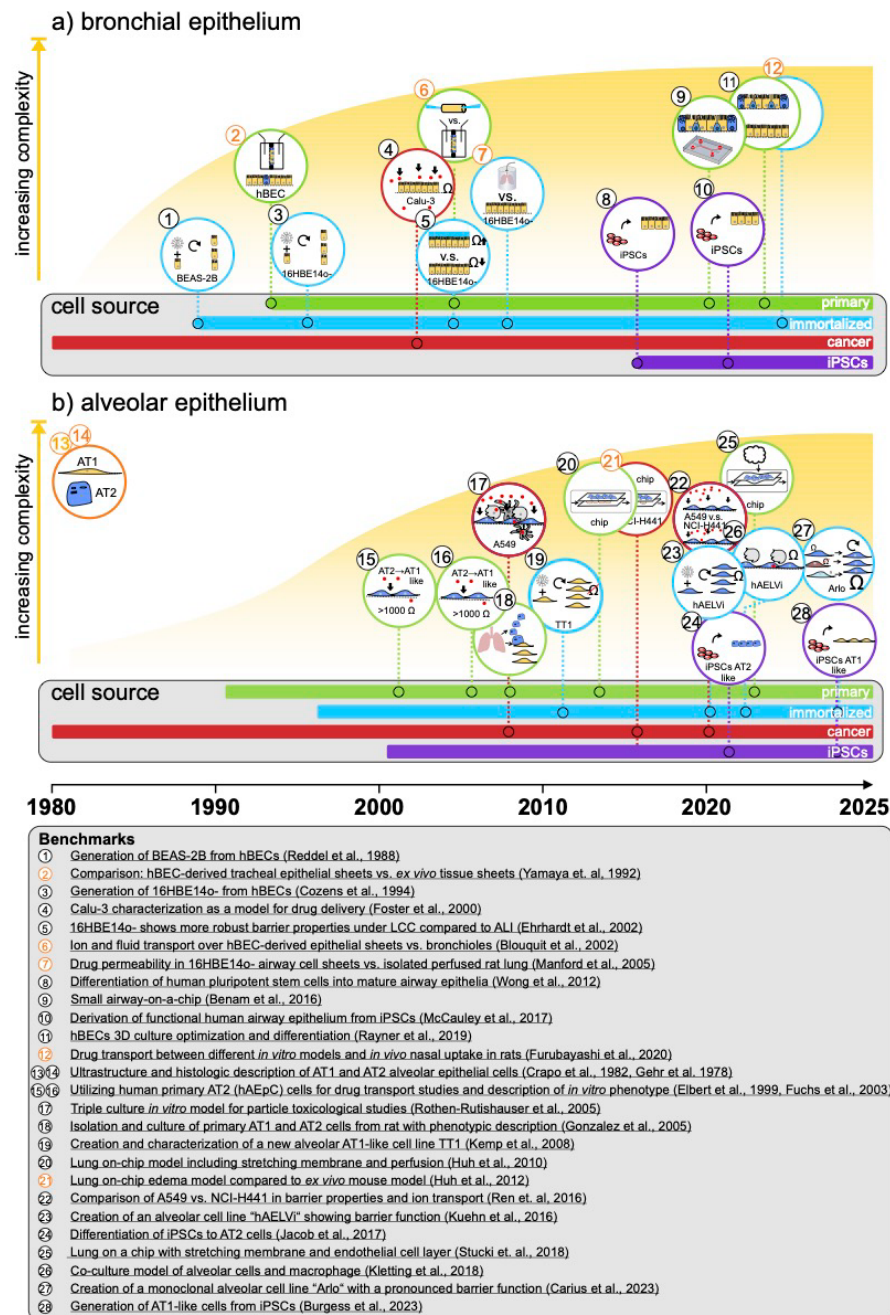


FIGURE 3.5.2 Comparison of *in vitro* model development benchmarks between conducting airways (a) and alveolar epithelium (b). Different cellular systems are displayed in different colors (primary cells: green, immortalized cells: light blue, cancer cell lines: red, and induced pluripotent cells in magenta). The numbers beside the pictograms refer to the model's description, respectively. Numbers for manuscripts, including *in vitro/ex vivo* comparisons, are highlighted in orange. The y-axis (yellow) reflects the increasing complexity of the models; the x-axis shows the development over time (Ehrhardt et al., 2002; Foster, Avery, Yazdani, & Audus, 2000; Furubayashi et al., 2020; Rayner, Makena, Prasad, & Cormet-Boyaka, 2019; Wong et al., 2012).

3.5.1.3.2 Alveolar region: AT1 and AT2

Compared to the conducting airways, it is more challenging for the alveolar region to measure or correlate cellular parameters influencing drug transport. At the same time, it appears to be even more important to study transport across the so-called “air–blood barrier” of the deep/peripheral lung, as it represents a much larger surface area (100–140 m²), compared to the conducting airways of the upper/central lung (1–2 m²) (Gehr et al., 1978; Stone et al., 1992).

In the alveolar epithelium, AT1 cells cover most of the surface area. The AT2 cells can function as the progenitor of AT1. Their surfactant production acts on surface tension and is crucial for immunomodulatory tasks. Since for the in vitro models, predominantly few classical markers are used for phenotypic characterization, it is still challenging to assign an in vitro model either to AT1 (flat, caveolae, AQP5 expression) or AT2 (cuboidal, lamellar bodies, surfactant expression) phenotype. Furthermore, Gonzales et al. observed general transcriptional changes if primary rat AT1 and AT2 cells were taken into culture (Gonzalez et al., 2005). Intensive characterization of marker gene or protein expression could be an option to compare the in vitro alveolar cell type with ex vivo-based data and hopefully correlate with drug transporting properties.

AT1 cells are likely terminally differentiated. Human AT1 cells have predominantly been characterized in transcriptional studies but not in functional assays. Since AT2 cells can differentiate to AT1 in vivo (Barkauskas et al., 2017; Desai, Brownfield, & Krasnow, 2014), this concept was transferred to cell culture early. In cell culture, AT2-isolated cells develop an AT1-like phenotype based on biochemical markers (Cheek, Evans, & Crandall, 1989) or cellular ultrastructure (Fuchs et al., 2003). In contrast to conducting airways, the physiological parameters and linkage to AT1 and AT2 cellular phenotypes are not well established, since drug transport studies cannot be conducted on complex alveolus tissue due to the complex alveolar architecture. Isolating both cell types AT1 and AT2 from rats allowed to link cell properties like water and sodium transport (Dobbs et al., 1998; Eaton, Helms, Koval, Bao, & Jain, 2009; Johnson, Widdicombe, Allen, Barbry, & Dobbs, 2002). To the authors' knowledge, only human-derived transdifferentiated AT1 cells have been described so far. Also, in vivo/ex vivo data of human alveoli regarding ion flux and transport processes is sparse.

3.5.1.3.3 Progress and further cell types

The knowledge about the epithelial cell properties has also been used whenever possible to develop or improve in vitro permeability models. The progress in this field is linked to the successful development of protocols for isolating cancer cell lines, primary epithelial cells, iPSCs, or the immortalization of primary cells (Figure 3.5.2). The better availability of barrier-forming cell

lines might also explain why conducting airway models appear to be further developed, and standardized ex vivo methods exist to evaluate the models. In addition, technology-linked innovations such as organ-on-chips have also contributed to the dynamics in the field. Although not considered in barrier models described here, mucus formed by the goblet cells of conducting airways, alveolar lining fluid, and macrophages are essential for nonabsorptive clearance and critical parameters for drug delivery and permeation studies. These aspects have been recently reviewed elsewhere (Donnelley, Gardner, Morgan, & Parsons, 2021).

3.5.2 Primary cells

Regarding the validity of the in vitro cell characteristics and functions, primary cells are considered one of the closest approximations to in vivo models. Primary cells are isolated from native tissue, in contrast to those differentiated from stem cells or immortalized cell lines, which will be discussed in the next sections. Unfortunately, primary cells show high patient-to-patient variance and have the drawbacks of a short life cycle in cell culture and limited access (Schneider-Daum et al., 2019). But they can be grown on permeable supports and at ALI and can demonstrate disease-specific characteristics (Hiemstra et al., 2018). In addition, transport properties and drug responses are particular for the region of interest, that is, either the bronchi in the conducting airways or the alveoli in the respiratory area (Haghi et al., 2015). An overview of primary lung epithelial cells used in vitro is given in Table 3.5.1.

3.5.2.1 Primary bronchial epithelial cells

Primary bronchial epithelial cells can be isolated from human bronchial tissue or bought from specialized companies either in frozen suspension or in ready-to-use ALI culture (Hiemstra et al., 2018). Isolation of primary bronchial epithelial cells can be performed via enzymatic digestion and/or bronchial brushing (Clifford et al., 2019). Depending on the specific protocol used, freshly isolated primary bronchial cells may be sub-cultured by passaging a few times in a cell culture flask and grown on permeable support for several weeks (Leung et al., 2020). After seeding on a permeable support, primary bronchial cells should attach, grow into a confluent layer and differentiate into a tight pseudostratified epithelial barrier with ciliated and mucus-producing cells (Bals et al., 2004). Important parameters to achieve a differentiated bronchial epithelial barrier are proper surface coating with matrix proteins, use of selective media with essential growth factors, and culture at ALI on permeable supports (Hiemstra, Tetley, & Janes, 2019; Leung et al., 2020; Min, Rosania, & Shin, 2016). Furthermore, a co-culture with immune cells, for example, macrophages, can be considered if the research question demands it (Haghi et al., 2015; Rothen-Rutishauser et al., 2023). Culture at ALI also

TABLE 3.5.1 Primary lung epithelial cells in vitro (comparison of different isolation methods and respective in vitro characterization)

Cell types	Origin	Markers used to determine the phenotype	Characteristics	References
Bronchial epithelial cells (large airways)	Resected lung tissue—scraping or enzymatic digestion of large airways	Ciliae Muc5AC MUC2 ZO-1 E-cadherin	Growth at ALI Mucociliary differentiation Mucus production Tight barrier formation Mixed culture of ciliated, goblet, and basal cells	Bals, Beisswenger, Blouquit, and Chinet (2004) Leung, Wadsworth, Yang, and Dorscheid (2020)
Alveolar epithelial cells type 2 (AT2)	Induced from progenitor cells (isolated from resected lung tissue)	pro-SP-C/SP-C SP-B CD90 Vimentin α -SMA AQP5	Surfactant production	Fujino et al. (2011)
	Isolated from resected lung tissue (short-time cultivation)	EpCAM pro-SP-C/SP-C pro-SP-B/SP-B	Surfactant production	Kosmider, Mason, and Bahmed (2018)
Alveolar epithelial cells type 1 (AT1)	Differentiated from AT2 cells (isolated from resected lung tissue)	EpCAM ZO-1	Tight barrier formation Growth at ALI Mixed culture of decreasing amount of AT2 and increasing amount of AT1	Daum et al. (2012) Elbert et al. (1999) Hittinger et al. (2016)

enables the investigation of the effects of inhaled particles on the bronchial epithelial barrier ([Hiemstra et al., 2019](#)). Inhaled particles in the lower micron size will impact and sediment in the bronchi, before having to cross the mucus layer, resist mucociliary clearance, and avoid phagocytosis by patrolling immune cells. Only after overcoming these first barriers, the inhaled particles can reach the epithelial cell barrier with tight junctions and efflux transporters, protecting the underlying tissue. All these different barrier types may be affected by the patient's disease state, which should be considered when choosing the patient cohort from which to isolate primary bronchial epithelial cells ([He et al., 2019](#)).

Companies selling cryopreserved human primary cells include Promocell ([PromoCell GmbH](#)), Cell biologics ([Cell Biologics](#)), InnoProt ([Innoprot](#)), Epithelix ([Epithelix](#)), ScienCell Research Laboratories ([ScienCell Research Laboratories](#)), and Creative Bioarray ([Creative Bioarray](#)). Some companies offer distinct disease-specific human primary cells, like AcceGen ([AcceGen](#)) and Lifeline Cell Technology ([Lifeline Cell Technology](#)). Ready-to-use differentiated models at ALI include the EpiAirway ([MatTek](#)) model from MatTek and the MucilAir ([Epithelix](#)) and SmallAir ([Epithelix](#)) models from Epithelix.

3.5.2.2 Primary alveolar epithelial cells

Alveolar epithelial cells can also be bought from specialized companies or isolated from human lung tissue ([Rothen-Rutishauser et al., 2023](#)). Isolation and culture of alveolar epithelial cells differ slightly from the isolation of bronchial epithelial cells. In general, protocols describe the isolation of AT2 cells, which will differentiate in a matter of days into a physiological co-culture of AT2 and AT1 cells ([Daum et al., 2012](#); [Elbert et al., 1999](#)). Separation from other cell types during isolation can be achieved via magnetic beads or fluorescence-activated cell sorting (FACS) ([Hiemstra et al., 2018](#)). However, as AT1 cells do not proliferate, long-term cultivation and sub-cultivation like for bronchial epithelial cells are not possible. The relevant parameters for physiological differentiation are surface coating with matrix proteins, the use of selective media with essential growth factors, and culture at ALI on permeable supports ([Ehrhardt, Kim, & Lehr, 2005](#)). To mimic the human alveolar barrier, the primary cell culture model at ALI should be comprised of a mixture of AT2 and AT1 cells and show a mono-layered phenotype with strong barrier properties. Such conditions are prerequisites to investigating cellular transport and uptake of airborne particles ([Hiemstra et al., 2018](#)). If relevant, co-cultures with macrophages, ideally from the same donor, can be generated ([Hittinger et al., 2016](#)). However, as with all primary cell models, availability, high costs, and donor differences limit their use in pharmaceutical research.

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Companies selling cryopreserved human primary alveolar cells include Cell biologics ([Cell Biologics](#)), InnoProt ([Innoprot](#)), Epithelix ([Epithelix](#)), ScienCell Research Laboratories ([ScienCell Research Laboratories](#)), and Creative Bioarray ([Creative Bioarray](#)).

3.5.3 Cell lines

Besides primary cells, cell lines are an essential and intensively used tool in in vitro cell culture models. These cancer-derived or immortalized cells show advantages in terms of easy access, relatively low costs, reproducibility, and well-established protocols. However, due to immortalization and culture conditions, cell lines may be limited to represent the physiological in vivo situation in a relevant way and can only be used to investigate some particular aspect ([Miller & Spence, 2017](#)).

The composition and therefore the characteristics of the lung epithelium change along the pulmonary tract ([Rothen-Rutishauser et al., 2023](#)). To resemble the different areas with their unique morphologies and characteristics, different cell lines are used and chosen depending on the suitability of the cell line for the specific experiment. The most commonly used cell lines for bronchial and alveolar permeability studies are shown in [Tables 3.5.2 and 3.5.3](#), respectively. Most cell lines are isolated from cancer tissues or derived from primary cells. Their immortalization usually occurs by viral or nonviral transduction, targeting crucial genes of the cell cycle regulation or cell division (e.g., telomerase), which then allows indefinite dividing of the cells ([Kemp et al., 2008](#); [Maqsood, Matin, Bahrami, & Ghasroldasht, 2013](#)). Both cancer-derived and immortalized cell lines exhibit advantages and disadvantages depending on the scientific question, the cell type, and the characteristics of the cell line. Another ongoing approach is to use iPSCs for cell culture models ([Burgess et al., 2023](#); [Calvert & Ryan, 2020](#)).

To perform meaningful transport studies, especially of small drug-like molecules, the most crucial attribute of any such cell culture model is the tightness of the epithelial barrier. Epithelial tightness is correlated to the presence of functional TJ and is measurable as TEER as mentioned previously ([Srinivasan et al., 2015](#)). While some epithelia may also display a significant PD, the ability to form functional TJs remains the most essential property for permeability assays.

3.5.3.1 Bronchial epithelial cell lines

The most prominent cell lines of the bronchial epithelium are Calu-3, 16HBE14o-, NCI-H441, and CFBE41o. All these cell lines form a functional barrier with tight junctions, which is essential for pulmonary drug delivery investigations and exhibit cell-specific characteristics. NCI-H441 expresses some key drug transporters like P-glycoprotein (P-gp) ([Salomon et al., 2014](#)).

TABLE 3.5.2 Bronchial cell lines, their origin and characteristics matching in vivo/ex vivo phenotype described

Cell line	Origin ^a	Characteristics ^b	Cell identity markers/features ^c	Markers/features compared to	References
NCI-H441	Adenocarcinoma (bronchial or alveolar epithelial cells)	Functional barrier with TJ Expression of key drug transporters	P-gp, OCT1–3, OCTN1, and OCTN2 SPRR1B	Freshly isolated human alveolar epithelial cells BEAS-2B clone S6	Kulaksiz, Schmid, Hönscheid, Ramaswamy, and Cetin (2002) ^c Salomon et al. (2014) ^{b,c} Vuong et al. (2002) ^c Zhang, Whitsett, and Stripp (1997) ^a
BEAS-2B	Immortalized (bronchial epithelial cells)	No functional barrier	E-cadherin		Reddel et al. (1988) ^a Zhao and Klimecki (2015) ^b
CFBE41o- (cystic fibrosis bronchial epithelial cell line)	Immortalized (bronchial epithelial cells)	Functional barrier with TJ P-gp expression ΔF508 CFTR	ZO-1 E-cadherin LRP Caveolin-1		No citation for first description ^a Ehrhardt et al. (2006) ^{b,c} Murgia et al. (2017) ^a
Calu-3 (cultured human airway epithelial cells)	Adenocarcinoma (bronchial epithelial cells)	Functional barrier with TJ Mucus production at ALI	Microvilli Immature cilia		Fogh, Fogh, and Orfeo (1977) and Grainger, Greenwell, Lockley, Martin, and Forbes (2006) ^{b,c}
16HBE14o- (human bronchial epithelial cell line)	Immortalized (bronchial epithelial cells)	Functional barrier with TJ Cilia expression	CFTR Ion transport		Cozens et al. (1994) ^{a,c} Forbes, Shah, Martin, and Lansley (2003) ^b ; Kozlova, Nilsson, Henriksnäs, and Roomans (2006) ^{b,c}

TABLE 3.5.3 Alveolar cell lines, their origin and characteristics matching in vivo/ex vivo phenotype described

Cell line	Origin ^a	Characteristics ^b	Cell identity markers/features ^c	Markers/features *compared to	References
A549	Adenocarcinoma (AT2-like cells)	No functional TJ Tool for inflammation and toxicology studies	Cytoplasmic multilamellar bodies Phospholipid biosynthesis	Microscopic images of AT2 cells	Foster, Oster, Mayer, Avery, and Audus (1998) ^c Giard et al. (1973) ^a Lieber, Todaro, Smith, Szakal, and Nelson-Rees (1976) ^c Winton et al. (1998) ^b
			Absorptive endocytosis Lamellar bodies Tannic acid CYP1A1 CYP2B6	Direct comparison to isolated AT2 cells	
NCI-H441	Adenocarcinoma (AT2-like cells)	Functional barrier	ZO-1, CDH1, CLD5, ATP1A1	A549	Kulaksiz et al. (2002) ^c Rehan et al. (2002) ^c Ren, Birch, and Suresh (2016) ^c Rehan et al. (2002) ^c Salomon et al. (2014) ^{b,c} Vuong et al. (2002) ^c Zhang et al. (1997) ^c
			Vitamin D ₃ metabolism, SFTPB production	Concluded from literature (D ₃ receptor expressed in AT2 in vivo (NGUYEN et al., 1990))	
			CTFR activation (usually not expressed in alveolar cells)	Human and rat lung	
			NKX2-1 binding affects CSSP expression in NCI-H441 cell extracts	Compared to HELA cells	
			Properties of surfactant regulation (club cell-like phenotype)		

TT1 (Transformed type 1)	Immortalized (AT1-like cells)	No functional TJ	Flat morphology, alkaline phosphatase expression lower* SFTPC Lower* NKX2-1 (TTF1) Higher* CAV1 Higher* AGER	Primary AT2 (cultured)	Kemp et al. (2008)^{a,b,c}
hAELVi (human alveolar epithelial lentivirus immortalized)	Immortalized (AT1-like cells)	Functional barrier with TJ Polyclonal	Lower* CAV1 Lower* SFTPC Lower* AQP5 ZO-1 OCLDN	Primary fresh AT2	Kuehn et al. (2016)^{a,b,c}
Arlo	Immortalized ("proliferating AT2 cells")	Functional barrier with TJ Monoclonal	Extensive characterization at transcript level including junctional components and transporters	Bulk seq AT2 compared to single-cell data	Carius et al. (2023)^{a,b,c}

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CFBE41o-can express P-gp as well, but more importantly, they show the $\Delta F508$ CFTR (cystic fibrosis transmembrane conductance regulator) mutation, which is the most common CF (cystic fibrosis) mutation (Murgia et al., 2017). Therefore, this cell line is often used for CF studies.

The culture conditions can also influence the characteristics of cell lines. Calu-3, for example, shows higher TEER values and secretes mucus when cultured at ALI compared to LCC (Grainger et al., 2006; Haghi et al., 2010). 16HBE14o-expresses ciliae when cultured at ALI but not when cultured in LCC (Kozlova et al. 2006).

3.5.3.2 Alveolar epithelial cell lines

The alveolar epithelial cell lines A549 and TT1 do not express tight junctions and do not form a tight barrier (Kemp et al., 2008; Winton et al., 1998). Therefore, they are of limited use for permeability studies, at least for (small) drug molecules. Until the implementation of the hAELVi cell line, primary cells were the only suitable model for drug transport across the alveolar epithelium of the deep lung. hAELVi was generated by immortalization of human alveolar epithelial cells (hAEpC), displays an AT1-like phenotype with functional TJs, and shows high TEER values (Kuehn et al., 2016). To improve long-term cultivation in monolayers, Arlo was created via single-cell printing of hAELVi cells. The resulting monoclonal Arlo has been described as “proliferating AT2 cell” and shows a very tight barrier with TEER values of approximately $3000 \Omega \cdot \text{cm}^2$ (Carius et al., 2023).

Even when grown as 2D (two-dimensional) monolayers, which are most useful for permeability studies, any single cell line cannot mimic the high complexity of native tissue. As a next step to increase complexity, two or more cell lines can be cultured simultaneously.

3.5.4 Complex in vitro models

Many parameters influence the relevance and complexity of lung barrier models. Although some have already been briefly mentioned (culture at ALI, co-culturing with different cell types), they will be discussed in detail in this section. Compared to a mono-culture, co-cultures provide increased intercellular cell-cell communication and thus a more realistic extracellular environment (Miki et al., 2012). The cells which are cultured together are selected according to the lung region and disease state they should represent. Cells to mimic the immune response like macrophages and dendritic cells or supportive cells like fibroblasts and endothelial cells are commonly used for co-cultures (Kletting et al., 2018; Miki et al., 2012; Osei, Booth, & Hackett, 2020; Rothen-Rutishauser, Kiama, & Gehr, 2005; Viola et al., 2021).

All these features lead to a closer simulation of the physiological state, though not all of them are always necessary to implement, depending on the

scientific question. It takes careful consideration to decide which parameters influence the results and are therefore needed in a specific model (Ehrmann et al., 2020). In addition, standardization of in vitro models and readouts, as well as correlation to clinical (i.e., human) in vivo data are essential to get regulatory acceptance of such in vitro models as a better alternative to animal models (Doryab & Schmid, 2022; Hittinger, Schneider-Daum, & Lehr, 2017; Metz et al., 2020).

3.5.4.1 Dynamic microfluidic systems

Microphysiological systems (MPSs), also known as organ-on-chips (OoCs), or in this case lung-on-chips (LoCs), aim to mimic the architecture and dynamics of the lung more closely than traditional 2D submerged cell culture, to create models that might surpass animal models in clinical relevance (Ehrmann et al., 2020). If successfully accepted by regulatory authorities, MPSs have the potential to reduce costs and improve the success rate of preclinical drug research and development (Franzen et al., 2019). Various MPSs have already been described in the literature, so the choice of MPSs should always be dictated by relevant parameters to assist hypothesis-driven research (Ainslie et al., 2019). An LoC can reflect the architecture of specific lung regions, for example, the acini, to model airflow in the most realistic way (Artzy-Schnirman et al., 2019). There is also the possibility of including a flexible membrane support in a LoC to emulate the mechanical forces on the cell in an expanding alveolus (Guenat & Berthiaume, 2018; Nossa, Costa, Cacopardo, & Ahluwalia, 2021). This can also be combined with aerosol deposition of particles and substances (Cei et al., 2021; Lenz et al., 2014; Stucki et al., 2015, 2018). One of the first and game-changing LoCs includes not only a flexible growth support but also allows for fluids to flow in the basal compartment (Huh, 2015; Huh et al., 2010). Flow can also be simulated in an easy-to-built LoC (Carius et al., 2021). While flow enables continuous sampling in sink conditions in permeability experiments, the stretch itself seems to have a barrier-modulating effect and should be taken into consideration when modeling the human air–blood barrier (Huh, 2015; Stucki et al., 2018). Many MPSs can measure barrier integrity and permeability directly on chip, mostly via resistance or impedance measurement (Arik et al., 2018; Guenat, Geiser, & Berthiaume, 2020; Henry et al., 2017; Nicolas et al., 2021; van der Helm et al., 2017, 2019). All these MPS aim to create a more realistic in vitro lung setup, from gas flow to the structure and relation of cells. Compared to 2D submerged cell culture or animal models, they have the potential for higher throughput and higher relevance, as well as the advantage of optical transparency and miniaturization (Ainslie et al., 2019). However, the handling is sometimes more complex, the costs are higher, and the reproducibility is yet to be investigated (Ainslie et al., 2019; Franzen et al., 2019).

3.5.4.2 3D cell culture models

Further development of the conventional 2D monolayer models in cell culture is 3D (three-dimensional) models. In 3D cell culture, two kinds of cell aggregates are distinguished, spheroids and organoids. An overview of the differences between spheroids and organoids is displayed in [Figure 3.5.3b](#). Organoids are derived from either embryonic or adult stem cells or induced pluripotent stem cells and show a complex self-organized structure of the organ-specific cell types ([Clevers, 2016](#); [Corrò, Novellasdemunt, & Li, 2020](#)). Spheroids, on the other hand, are derived from a broad range of cells and do not self-organize. They are simple aggregates of cells and are not as advanced as organoids ([Gunti, Hoke, Vu, & London, 2021](#)). Unlike monolayers, these models can resemble the in vivo situation better because of advanced 3D cell–cell and cell–extracellular matrix (ECM) contacts, tissue architecture, more in vivo-like gene and protein expression, and nutrition gradients inside the 3D structures ([Archer, Bobet-Erny, & Gomes, 2021](#); [Jensen & Teng, 2020](#); [Torrás, García-Díaz, Fernández-Majada, & Martínez, 2018](#)). Despite these benefits, the analysis, interpretation, and reproducibility of 3D cell culture models remain challenging ([Kapałczyńska et al., 2018](#); [Torrás et al., 2018](#)). Two approaches can be considered for evaluating the permeability of 3D cell culture models. Firstly, micro-injection can be used to apply the drug into the lumen of the organoid and thus to investigate the transport from the lumen out of the organoid. Secondly, the polarity of the organoids can be modified to achieve an apical-out model, allowing to investigate transport of drugs from the surrounding medium into the lumen of the organoids ([Kastlmeier, Guenther, Stoeger, & Voss, 2022](#)).

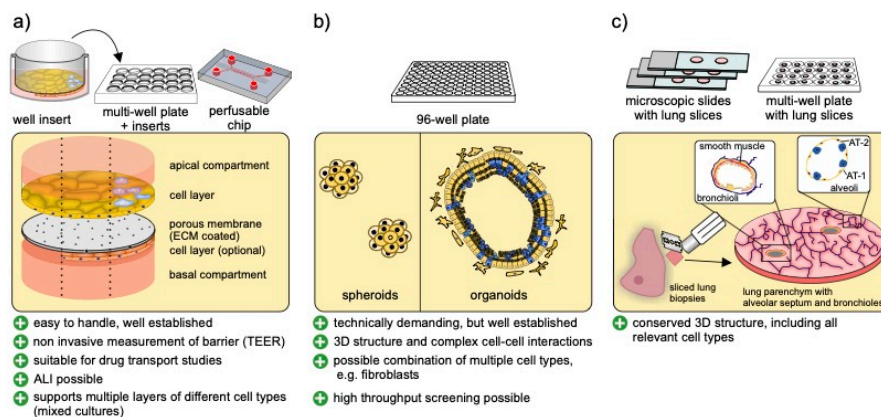


FIGURE 3.5.3 Comparison of the advantages of different in vitro models used in drug transport studies: (a) filter-grown cell (mono-) layers, with or without perfusion or membrane stretching (b) organoid and spheroid models (c) precision-cut lung slices.

3D cell culture models show great potential for modeling cancer. Individual patient-derived organoids can thus be used for personalized medicine and biobanking of different cancer types. Furthermore, these models hold great potential for regenerative medicine including gene therapy if combined with gene editing of the cells (Clevers, 2016; Corrò et al., 2020).

3.5.4.3 Complex co-cultures mimicking specific lung diseases

The commonly used animal models often fail in their prediction of human toxicology and drug response due to dissimilar lung anatomy, physiology, and cellular structure. To better simulate the state of a given disease, many complex in vitro models have been proposed. In this last section, all discussed parameters (i.e., cell types, growth support, and inhalation techniques) will come together to form disease-relevant models for human lung pathophysiology. These models might help understand lung disease mechanisms or be used to measure the efficacy rather than just the safety of candidate drugs (Rothen-Rutishauser et al., 2023). Some complex models can be bought ready-to-use like the EpiAlveolar model (MatTek) or the MucilAir, SmallAir, and AveolAir model co-cultured with macrophages (Epithelix).

Several models have been proposed in the literature to study the interaction of inhaled substances and particles with the air–blood barrier, showing the effects of co-culturing alveolar epithelial cells with endothelial cells and/or macrophages on cellular crosstalk and barrier formation (Barosova et al., 2020; Costa, Souza Carvalho-Wodarz, Seabra, Sarmiento, & Lehr, 2019; Hittinger et al., 2016; Kletting et al., 2018; Stucki et al., 2018). To study how some diseases might affect the permeability of drugs, barrier disruption and pulmonary edema can be simulated on chip (Huh et al., 2012), as well as alveolar inflammation and its effect on air–blood barrier permeability (Drasler et al., 2020). A LoC with human primary bronchial cells was able to emulate inflamed states like asthma and COPD (Benam et al., 2016). And most recently, co-cultures of human cells with bacteria and/or viruses were achieved in vitro to measure the inflammatory response of host cells as well as the therapeutic efficacy of inhaled anti-infectives (Deinhardt-Emmer, Böttcher, et al., 2020; Deinhardt-Emmer, Rennert, et al., 2020; Horstmann et al., 2022; Montefusco-Pereira et al., 2020; Schicke et al., 2020). Current SARS-CoV-2 variants have been tested in alveolar epithelial cell line hAELVi, showing promising results for establishing alternative fast and relevant in vitro models (Mache et al., 2022).

3.5.5 Conclusion

In contrast to in vitro skin and gut models, lung models are not yet as developed and accepted by regulatory authorities. Cells for skin models have been described since the 1970s (Barrandon & Green, 1987; Boukamp et al.,

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1988; Niimura, Pass, Wooley, & Soutor, 1975), and these findings have quickly led to the clinical use of cultured epithelial grafts for burn patients (Blight, Mountford, Cheshire, Clancy, & Levick, 1991). Another driving factor for innovation was the ban of animal use for toxicology testing of cosmetics in 1993, which was finally effective in 2004 (European Animal Research Association), after one of the first correlations of in vitro to in vivo skin toxicology data was reported in 1999 (Perkins, Osborne, Rana, Ghassemi, & Robinson, 1999).

Cells for gut models have been described in the 1990s (Fogh et al., 1977; Perreault & Beaulieu, 1998). Even though primary intestine cells were difficult to maintain in culture, the Caco-2 cell line soon proved to be a good in vitro approximation for clinical permeability data (Yee, 1997). In 2016, the EMA issued a biowaiver based on the biopharmaceutical classification system (BCS), replacing some previously mandatory human trials with in vitro methods for highly soluble and highly permeating drugs (GMP Navigator).

With a timely delay, bronchial cells were established and correlated to physiological parameters (Blouquit et al., 2002; Manford et al., 2005; Yamaya et al., 1992). The culture of alveolar cells is feasible, albeit more challenging, and lacks physiological correlation due to nonexistent appropriate methods. Hence, while there are in vitro models of skin and gut that are accepted by regulatory authorities and used by the pharmaceutical and cosmetic industry, no in vitro model of the lung has so far been validated. Nevertheless, in vitro lung models are needed and relevant for early inhaled drug development to reduce the particularly high attrition rate during clinical studies (Barnes et al., 2015). To achieve that, we need a better understanding of the mechanistic of drug transport and metabolism across the pulmonary epithelium, making in vitro models of the lung, at least for the time being, still more of a target than a tool of research.

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3.2 Multiparametric characterization of the human alveolar epithelial cell line Arlo for modeling pulmonary edema

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The contributions were as follows:

T. Neu	Conceptualization, experimental design, execution of experiments, data analysis, visualization, and manuscript writing
L. Latta	Conceptual input and methodological guidance
P. Donate, T. Risch	Technical assistance with Proteomics
C. Richter	Contribution to study design and initial pilot experiments of pulmonary edema model
M. Jurisic	Technical assistance with histology
S. El-Garawany	Assistance with experimental work about mechanistic insights on Arlo's barrier
A. Aljohmani	Technical assistance with ECIS measurements
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A. Wahid, H. Metwaly, D. Yildiz, R. Müller, M. Schneider	Manuscript review
N. Schneider-Daum, C.-M. Lehr	Initiated and supervised the project and contributed to the conceptual development and finalization of the manuscript.

Multiparametric characterization of the human alveolar epithelial cell line Arlo for modeling pulmonary edema

Short title: Profiling the human alveolar epithelial cell line Arlo as pulmonary edema model

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Keywords:

Alveolar barrier integrity; ENaC-mediated ion transport; Alveolar fluid homeostasis; Deep lung *in vitro* model; Transepithelial electrophysiology; Human lung epithelial barrier

1 Abstract

Introduction:

The human lung comprises more than 50 specialized cell types with distinct functions across the airways and alveoli. Diseases of the distal lung, including pulmonary edema, require *in vitro* models that reflect relevant physiological properties of the alveolar epithelium. However, many widely used lung epithelial cell lines, such as Calu-3 or A549, fall short in this respect and provide limited relevance for deep-lung pathophysiology.

Objectives:

Aim of this study was a multiparametric comparative characterization of the human alveolar epithelial monoclonal cell line Arlo in relation to commonly used epithelial models regarding alveolar properties and to assess its suitability for investigating diseases of the distal lung, like pulmonary edema.

Methods:

Arlo was systematically compared with commonly used epithelial models, such as Calu-3, A549, NCI-H441, primary human alveolar epithelial cells, and intestinal Caco-2 cells as reference. Barrier integrity, electrophysiological properties, permeability, morphology, and ENaC channel expression were assessed by transepithelial electrical resistance (TEER) and potential difference (PD), permeability assays, histological and ultrastructural analyses, and label-free quantitative proteomics. Based on these characteristics, Arlo was chosen as potential pulmonary edema model. After induction by cytarabine and bleomycin, respectively, changes of PD and fluid clearance were evaluated for treatment with the epithelial sodium channel (ENaC) modulating drugs rosiglitazone, salbutamol, forskolin, and aldosterone.

Results:

Arlo's pronounced epithelial tightness and PD go along with expression of the three relevant subunits of the ENaC, otherwise only found in primary cells, supported by proteomic profiling. ENaC-dependent PD and fluid clearance qualified Arlo as an *in vitro* pulmonary edema model for drug screening, where rosiglitazone reduced, salbutamol did not alter, and forskolin and aldosterone increased PD and fluid clearance.

Conclusion:

Based on this multiparametric characterization Arlo displayed relevant physiological features to model severe pulmonary diseases, like ARDS or Covid-19, going along with an impaired alveolar barrier.

2 Introduction

The human lung is a structurally and functionally complex organ that supports efficient gas exchange through a highly branched airway system terminating in the alveoli. With a surface area of up to 140 m², the lung is continuously exposed to the external environment, requiring a tightly regulated epithelial barrier to maintain fluid homeostasis and protect against inhaled pathogens and pollutants [1]. The alveolar region contains an ultrathin air-blood interface formed by closely apposed epithelial cells, enabling rapid gas exchange while restricting fluid leakage and microbial invasion. This anatomical specialization of the lung is supported by substantial cellular diversity, as different lung epithelial cell types exhibit distinct structural and functional properties, limiting the transferability of findings between *in vitro* models [2,3]. Single-cell transcriptomic studies have identified more than 50 distinct lung cell types, including multiple epithelial, endothelial, stromal, and immune subsets [3]. Among these, alveolar epithelial type I (AT1) and type II (AT2) cells are essential for gas exchange, surfactant production, and fluid homeostasis.

Alveolar ion and fluid transport is particularly relevant for deep-lung pathologies such as pulmonary edema and acute respiratory distress syndrome (ARDS). The epithelial sodium channel (ENaC), in the lung predominantly expressed in AT2 cells, drives apical sodium uptake and generates the osmotic gradients necessary for alveolar fluid clearance. Impaired ENaC activity contributes to alveolar flooding and respiratory failure, underscoring the need for model systems that faithfully reproduce alveolar physiology [4–6]. However, *in vitro* modeling of the alveolar epithelium remains challenging. Primary human alveolar epithelial cells (hAEPc) are considered the most physiologically accurate system, but are limited by donor variability, restricted availability, and short culture lifespan. Conversely, widely used immortalized cell lines such as the bronchial Calu-3 line do not recapitulate essential alveolar features, including appropriate barrier properties, ion transport mechanisms, or protein expression [7]. More generally, cancer-derived epithelial cell lines may exhibit altered signaling, limited differentiation capacity, and culture-dependent phenotypes that differ from native tissue [8]. These limitations constrain their utility for modeling diseases and therapeutic interventions targeting the distal lung. Recent reviews emphasize that lung epithelial *in vitro* models differ substantially in regional identity, structural complexity, and functional relevance, highlighting the importance of application-driven model selection [9].

To address the need for physiologically relevant alveolar models, we recently established Arlo, a human immortalized alveolar epithelial cell line derived from hAEPc [10]. In this study, we systematically compare Arlo with primary human alveolar epithelial cells and widely used lung-derived models, including A549, Calu-3, and NCI-H441, as well as Caco-2 as reference. Using a multiparametric approach that integrates electrophysiology, permeability, histology, and proteomics, we define characteristic features and functional capacities across models. This analysis defines strengths and limitations that guide appropriate model selection. Within this context, Arlo emerges as a promising model of the alveolar epithelium, exhibiting key features that align with those of primary alveolar cells. Based on these properties, we explored Arlo as pulmonary edema model by demonstrating pathophysiological changes of alveolar electrophysiology and fluid clearance under such conditions as well as the effects of different ENaC modulating drugs.

3 Material and Methods

Detailed protocols are provided in the supplementary information.

3.1 Cell culture

For this comparative study, various lung epithelial cell lines were analyzed alongside primary human alveolar epithelial cells and Caco-2 cells. hAEPc, isolated directly from human lung tissue, served as the *in vitro* gold standard for alveolar epithelial cells. Caco-2 cells were included as an example for regulatory acceptance.

hAEPc were isolated from tissue samples provided by SHG Clinics Völklingen and Clinic Saarbrücken, using the isolation and cultivation protocol described in Daum et al [11,12]. The use of human material for this research was approved by the Ethics Committee of the State of Saarland, Germany. Approval was granted for SHG Clinics Völklingen on May 21, 2019 (Reference No. 113/19) and on May 10, 2021 (Reference No. 97/21) for Clinic Saarbrücken.

Arlo cells were cultured as described in Carius et al [10]. A549, NCI-H441, Calu-3, Caco-2 were cultured under standard conditions as previously reported with minor changes [13–16].

3.2 Transepithelial electrical resistance (TEER) and potential difference (PD)

TEER and PD were measured under liquid-covered conditions using an STX2 electrode and volt-ohm-meter (World Precision Instruments) to assess barrier integrity and electrophysiology. Measurements were performed under sterile, temperature-controlled conditions; blank values were subtracted and TEER normalized to insert area ($\Omega \times \text{cm}^2$). Electrodes were equilibrated in KCl prior to PD recording, which was performed before TEER when both were measured. ENaC dependence of PD was assessed by amiloride treatment.

3.3 Electric cell-substrate impedance sensing (ECIS)

ECIS was used to monitor barrier development and electrophysiological properties using an ECIS Z θ with 96W10idf plates (both Applied BioPhysics). Impedance was continuously recorded over 14 days at 4,000, 16,000, and 64,000 Hz to resolve resistance, capacitance, and total impedance. Wells were calibrated with L-cysteine prior to seeding.

3.4 qPCR of α -, β -, and γ -subunits of ENaC

Total RNA was isolated using the RNeasy Micro Kit with the RNase-Free DNase Set (Qiagen, 74004 and 79254), reverse-transcribed using the qScriber™ cDNA Synthesis Kit (highQu, RTK0104), and ENaC α -, β -, and γ -subunit expression was quantified by qPCR using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, A25742) and gene-specific primers.

3.5 Transcriptomic data mining and gene expression analysis

To investigate the mechanisms underlying the pronounced barrier properties of Arlo and to assess the involvement of alveolar-specific barrier-forming processes, RNA sequencing data were analyzed. Gene expression data from previous work and public datasets were used to compare barrier-forming and non-barrier-forming lung epithelial cells and to identify genes for downstream analysis [10,17].

3.6 PrestobluTM metabolic activity assay

Metabolic activity was assessed using the PrestoBlue™ Cell Viability Reagent (Invitrogen™, A13262). Cells were treated with increasing concentrations of EDTA (Sigma-Aldrich, E9884), amiloride (Sigma-Aldrich, A7410), cytochalasin D (Sigma-Aldrich, C8273), ouabain (Sigma-Aldrich, O3125), and Y-27632 (Sigma-Aldrich, Y0503), and fluorescence was measured to define non-toxic dose ranges.

3.7 Label-free quantitative proteomics

Label-free quantitative proteomics was performed to compare epithelial cell models under different culture conditions. Whole-cell lysates (T75 flasks), Transwell® (Corning, 3460) cultures, and membrane-enriched fractions were analyzed to support rational model selection. Cells were lysed, proteins were reduced, alkylated, acetone-precipitated, and digested with trypsin (Promega; VA9000). Peptides were desalted, dried, and analyzed by nanoLC-MS/MS using a DIA-PASEF method on a timsTOF Pro. Data were processed using DIA-NN against the UniProt human reference proteome and analyzed in Perseus. Differential protein abundance was assessed by Student's t-test and results were visualized using volcano plots. For comparative analyses, proteins detected in ≥ 3 of 4 replicates were considered abundant.

3.8 Apparent permeability (P_{app})

Apparent permeability was determined in an apical-to-basolateral Transwell® transport setup. The test compounds included fluorescein (quantified via fluorescence), and budesonide (TargetMol, T0094), ciprofloxacin (Merck, PHR1044), carbamazepine (Sigma-Aldrich, C4024), and atenolol (Sigma-Aldrich, A7655) (quantified by LC-MS/MS). Samples were collected from the basolateral compartment at defined time points with or without prior treatment.

3.9 Ultrastructural & histological analysis

Cells cultured on Transwell® inserts were fixed at peak TEER and processed for ultrastructural and histological analyses. Samples were dehydrated through graded ethanol series. For scanning electron microscopy, membranes were dried using hexamethyldisilazane (Sigma-Aldrich, 440191), sputter-coated with gold, and imaged at 1,000 – 30,000 $\times$ magnification. For histology, samples were cleared, paraffin-embedded, sectioned (6–8 μ m), stained with hematoxylin and eosin (Carl Roth, T865.2 and X883.2), and imaged by light microscopy.

3.10 *In vitro* pulmonary edema model

To model pulmonary edema *in vitro*, Arlo cells were cultured on 1.12 cm² Transwell® inserts and treated on day 5 post-seeding with cytarabine (10 μ M; Sigma-Aldrich, 251010) or bleomycin (5 μ g/mL; Sigma-Aldrich, BP971). Potential differences were measured on day 11 to assess epithelial electrophysiological alterations, followed by treatment with salbutamol (10 μ M; Sigma-Aldrich S0150000), forskolin (25 μ M; Sigma-Aldrich, F6886), rosiglitazone (100 μ M, Sigma-Aldrich, R2408), or aldosterone (1 μ M; Sigma-Aldrich, A9477). After 72 h, PD was re-measured and cells were lysed. In separate experiments, apical fluid clearance was assessed as a functional readout by adding 20 μ L medium to the apical compartment on day 14 and quantifying the remaining fluid volume after 72 h.

4 Results and Discussion

4.1 Electrophysiological assessment of epithelial barrier function

To identify epithelial models that best reflect alveolar physiology, we assessed electrophysiological properties defining barrier integrity and ion transport in the distal lung. These parameters are particularly relevant for pulmonary edema, where impaired barrier function and dysregulated ENaC activity drive fluid accumulation. TEER is a widely accepted readout of epithelial barrier integrity, enabling quantitative comparison of barrier properties [18]. Comparative electrophysiological characterization of Arlo and primary human alveolar epithelial cells (hAEPc), Calu-3, NCI-H441, A549, and Caco-2 revealed superior barrier tightness and transepithelial potential difference in Arlo.

As shown in Figure 1A, Arlo and hAEPc reached peak TEER values between 3300 and 4200 $\Omega \times \text{cm}^2$. Unlike hAEPc, Arlo maintained these high values over 14 days, while hAEPc TEER already declined earlier. In contrast, other epithelial cell lines

reached only 300–700 $\Omega \times \text{cm}^2$, indicating substantially weaker barrier properties. These differences became more pronounced for potential difference (PD; Figure 1B), an indicator for active ion transport across cell layers. Arlo reached approximately 43 mV, hAEPc 7 mV, whereas the remaining cell lines showed no measurable potential difference. A likely explanation for the elevated PD in alveolar cells is increased ENaC activity, which is critical for alveolar fluid clearance [19]. To investigate ENaC contribution to elevated PD in Arlo, cells were treated with the ENaC inhibitor amiloride. As shown in Figure 1C, amiloride induced a concentration-dependent reduction of PD with near-complete inhibition at 25 μM . Consistently, qPCR analysis revealed expression of all three ENaC subunits (α , β , γ) in Arlo at a ratio compatible with functional heterotrimeric channel formation (Figure 1D) [20]. In contrast, Calu-3, NCI-H441, and A549 lacked at least one ENaC subunit, and hAEPc displayed a distinct subunit expression ratio. These findings suggest that Arlo's elevated PD is driven by high ENaC activity resulting from balanced expression of all three subunits. To further support differences in TEER and PD, electric cell-substrate impedance sensing (ECIS; Figure 1E–F) was performed. Arlo showed a rapid and steep increase in resistance at 4,000 Hz, reaching a stable maximum after 4–5 days. Medium exchange induced sharp transient spikes in resistance in Arlo, likely reflecting higher electrosensitivity and rapid response to ion concentration changes. In parallel, capacitance at 64,000 Hz decreased quickly, indicating fast cell spreading, full coverage of the electrode surface, and minimal paracellular leakiness [21,22]. Calu-3 showed a slower increase in resistance, but ultimately reached similar values, suggesting slower barrier and monolayer formation compared to Arlo. In contrast, Caco-2, NCI-H441, and A549 exhibited only modest resistance increases and relatively high residual capacitance, consistent with weaker barrier integrity and incomplete or slower surface coverage. Together, these results confirm the distinct and superior electrophysiological phenotype of Arlo relative to commonly used pulmonary cell lines and highlight its suitability as a physiologically relevant disease model of ENaC associated pathologies. Additional ECIS data at 4,000, 16,000, and 64,000 Hz are shown in Supplementary Figure S1.

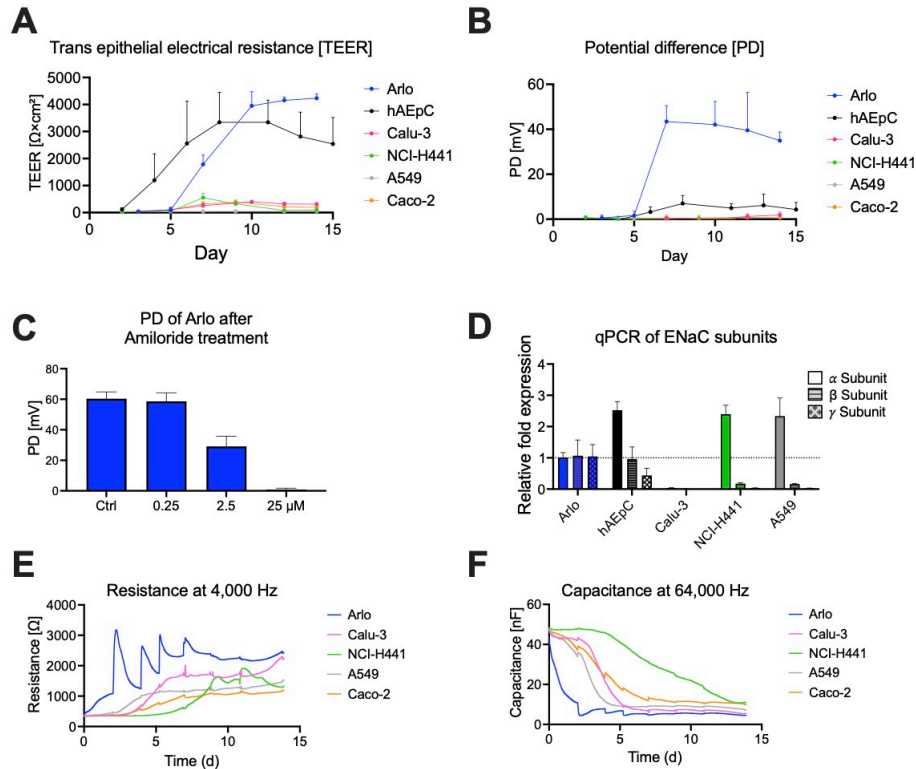


Figure 1: Comparative electrophysiological analysis of Arlo compared to primary human alveolar epithelial cells (hAEPc), Calu-3, Caco-2, NCI-H441, and A549. (A) TEER development over 15 days. (B) Potential difference (PD) development over 15 days. (C) Effect of amiloride on PD of Arlo. (D) qPCR of α , β , and γ ENaC subunits (E-F) Impedance analysis, N=3-4, n=9-20

4.2 Permeability profiling across epithelial models via P_{app} assays

Tight regulation of paracellular and transcellular permeability is essential in the distal lung to prevent penetration of inhaled pollutants, while restricting excessive water flux into the lung. We examined both pathways using fluorescein, ciprofloxacin, and budesonide as reference compounds and determined apical-to-basolateral apparent permeability coefficients (P_{app}) for Arlo, hAEPc, Calu-3, and Caco-2 monolayers. A549 and NCI-H441 were excluded due to insufficient barrier formation, as NCI-H441 did not develop adequate tight junction integrity under our conditions and A549 inherently lack functional tight junctions required to restrict paracellular flux [23].

Fluorescein and ciprofloxacin are hydrophilic, low-permeability markers that predominantly traverse epithelial layers via the paracellular route. Fluorescein shows lower permeability than ciprofloxacin and is therefore widely used as a sensitive indicator of tight junction integrity [24,25]. In contrast, the lipophilic compound budesonide favors transcellular diffusion (Figure 2A).

As shown in Figure 2B, Arlo exhibited the lowest P_{app} values for all three markers and reflected the expected permeability order: fluorescein < ciprofloxacin < budesonide. A similar ability to discriminate permeability was observed in Calu-3, which showed the second-lowest P_{app} values. In contrast, hAEPc and Caco-2 identified budesonide as

the most permeable compound but failed to distinguish between fluorescein and ciprofloxacin, potentially due to transporter-mediated ciprofloxacin uptake [26,27]. Although proteomics data show, as discussed later in the manuscript, comparable expression of transporters such as ABCG2, OCT, and OATP across all cell lines, subtle differences in transporter abundance affecting ciprofloxacin disposition as well as unidentified transport mechanisms, underline these discrepancies. Notably, hAEPc showed nearly twofold higher P_{app} values for budesonide ($\sim 18 \times 10^{-6}$ cm/s) compared to the other cell lines, which ranged between approximately $5\text{--}12 \times 10^{-6}$ cm/s. This may be attributed to the flat morphology of hAEPc monolayers, as discussed later. Additionally, hAEPc displayed substantially higher standard deviations, likely reflecting donor-to-donor variations. Following 1 hour pre-treatment with 16 mM EDTA, all cell lines exhibited increased P_{app} values due to EDTA-mediated chelation of divalent cations required for tight junction stability, resulting in paracellular integrity disruption [28]. Consequently, the low-permeability compounds fluorescein and ciprofloxacin exhibited marked P_{app} increases. In contrast, the transcellularly transported budesonide showed a significant permeability increase only in Arlo and Calu-3. Together, these results demonstrate that Arlo most effectively restricts solute flux and discriminates between permeability pathways, reflecting key features of the alveolar barrier. This robust and reproducible permeability profile supports Arlo as the most suitable model for investigating epithelial integrity and drug transport in the deep lung.

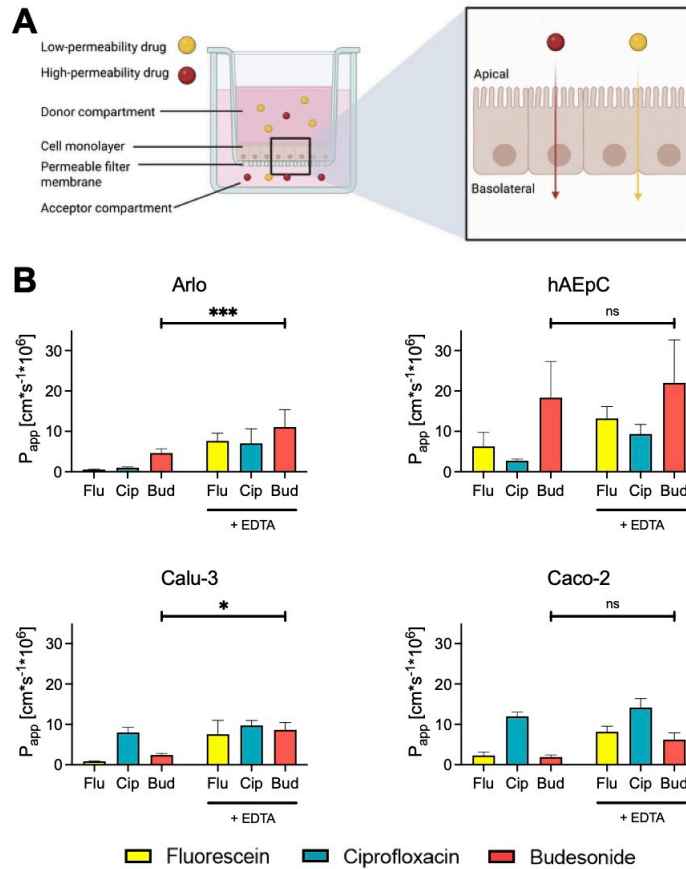


Figure 2: Permeability profiling of Arlo compared to hAEpC, Calu-3, and Caco-2. (A) Schematic representation of the Transwell®-based apical-to-basolateral permeability assay. (B) Apparent permeability coefficients (P_{app}) of fluorescein (Flu; yellow), ciprofloxacin (Cip; cyan), and budesonide (Bud; red) with and without EDTA pre-treatment. Data were analyzed using unpaired two-tailed t-tests, with $p < 0.05$ considered statistically significant. $N=3$, $n=9-11$

4.3 Mechanistic insights into Arlo's barrier function

Following the identification of Arlo's remarkable barrier properties, we next investigated the underlying mechanisms. Therefore, gene expression data from epithelial cells forming tight *in vitro* barriers (Arlo, hAEpC, Calu-3, and NCI-H441) were analyzed in comparison to leaky cell types (A549 and BEAS-2B) to identify genes consistently upregulated in barrier-forming cells using data sets from Carius et al., and GEO data sets [10,17]. Key findings were ENaC subunits, Na^+/K^+ -ATPase, α - and β -actin, and various claudins, which are closely associated with epithelial ion transport and junctional integrity. ENaC and the Na^+/K^+ -ATPase drive vectorial sodium transport across polarized epithelia, thereby maintaining ion gradients and fluid homeostasis [19]. Claudins and the actin cytoskeleton are essential for tight junction integrity and the regulation of paracellular permeability [29,30]. The Rho kinase (ROCK) pathway was additionally included due to its known role in cytoskeletal regulation and AT2-to-AT1 transdifferentiation [31]. The modes of action of these compounds are illustrated in Figure 3A. To assess their functional relevance, established inhibitors were applied,

including amiloride, ouabain, cytochalasin D, ethylenediaminetetraacetic acid (EDTA), and Y-27632 as ROCK inhibitor [32–35].

Figure 3B shows the metabolic activity of Arlo cells after treatment with increasing concentrations of the selected inhibitors to determine the highest non-cytotoxic concentration, defined as >80% metabolic activity. Based on these results, the experimental settings shown in Table 1 were selected. Values exceeding 100% may reflect mild hormetic or survival response at low concentrations, temporarily increasing cellular metabolic activity or proliferation.

Table 1: Experimental settings derived from Figure 3B results

Inhibitor	Effect or target	Concentration	Incubation time
Amiloride	ENaC	100 μ M	24 h
Ouabain	Na ⁺ /K ⁺ -ATPase	10 μ M	24 h
Cytochalasin D	Actin skeleton	100 μ M	3 h
EDTA	calcium-dependent junctions	16 mM	1 h
Y-27632	ROCK	100 μ M	24 h

Figure 3C displays the effect of the selected inhibitors on barrier integrity, as measured by TEER. EDTA treatment caused a complete loss of barrier function in all tested cell lines, likely due to its non-specific chelation of divalent cations such as Ca²⁺, which are essential for tight junction integrity [28]. In contrast, amiloride had no measurable impact on TEER across any cell line, indicating that ENaC activity does not contribute to paracellular barrier tightness in this setup [36]. Cytochalasin D caused a moderate decrease in TEER for most cell lines, but led to a pronounced reduction of approximately 70% in hAEPc, likely reflecting a stronger dependence of primary alveolar cells on their peri-junctional actin ring for junctional stability compared to immortal cells, e.g. Calu-3 [37,38]. Ouabain treatment reduced TEER in all cell lines except Arlo, although literature reports TEER-lowering effects in alveolar cells [39]. This resistance of Arlo may arise from differences in tight junction regulation, cellular localization, ouabain sensitivity, or intrinsically high RhoA activity [40–42]. Finally, both Arlo and hAEPc responded strongly to Y-27632, a ROCK inhibitor affecting actin cytoskeletal dynamics. This effect may reflect a Y-27632-induced reorganization of the peri-junctional actin ring, which could be particularly consequential in alveolar cells due to their morphology and distinct tight junction organization [43,44]. The parallel response suggests a shared cytoskeletal architecture with high sensitivity to ROCK inhibition, further supporting their similarity.

Lastly, the effects of the different inhibitors on the P_{app} of the cell lines were evaluated using atenolol, ciprofloxacin, and carbamazepine [45,46]. Figure 3D shows the relative P_{app} increase for atenolol and ciprofloxacin, while data for carbamazepine and the other inhibitors are provided in the Supplement Figure S2, as no substantial changes were observed for this highly permeable compound. This supports the hypothesis that the inhibitors primarily affect the paracellular route without significantly altering transcellular transport. For the lower-permeability drugs atenolol and ciprofloxacin, significant P_{app} changes were observed following certain treatments, whereas other inhibitors showed no measurable effects. Amiloride had no impact on P_{app} , consistent with unchanged TEER values. Notably, Cytochalasin D markedly reduced TEER in hAEPc but did not alter P_{app} in any cell line, while Y-27632 caused only a mild permeability increase in Arlo and minimal effect on Calu-3 and Caco-2 but not on hAEPc. This suggests that cytoskeletal manipulation predominantly influences TEER

rather than paracellular permeability. Ouabain modestly increased P_{app} in Arlo and hAEPc, but had a strong effect in Calu-3, with up to 15-fold increase for atenolol, while Caco-2 showed a 4-fold increase. These results partially mirror the TEER data, where Arlo was resistant to ouabain-induced barrier disruption. Notably, despite reduced TEER in hAEPc, permeability remained unchanged, indicating that TEER loss does not necessarily correlate with increased solute permeability. EDTA increased permeability in all cell lines, consistent with its unspecific mode of action, but effects were more pronounced in Calu-3 and Caco-2 than in Arlo and hAEPc. This suggests a greater reliance of Arlo and hAEPc on cytoskeletal support for barrier maintenance, supported by their sensitivity to cytochalasin D and Y-27632. In contrast, Calu-3 and Caco-2 may depend more strongly on calcium-mediated adhesion, rendering them more susceptible to EDTA.

Overall, treatments such as amiloride and EDTA, which act via general mechanisms, produced comparable effects across all cell lines, indicating that some barrier-regulating pathways are shared among epithelial cells. In contrast, the alveolar-derived models Arlo and hAEPc consistently responded in a similar yet distinct manner compared with Calu-3 and Caco-2, suggesting a stronger contribution of alveolar-specific mechanisms. This indicates that certain epithelial cell lines, despite originating from the same organ, may not adequately capture these processes. Notably, the pronounced TEER sensitivity to cytoskeleton-targeting compounds and the distinct P_{app} response to ouabain underscore fundamental differences in barrier maintenance between alveolar and non-alveolar epithelia. Together, these findings further support Arlo as a physiologically relevant model for mechanistic studies of alveolar barrier regulation in the deep lung.

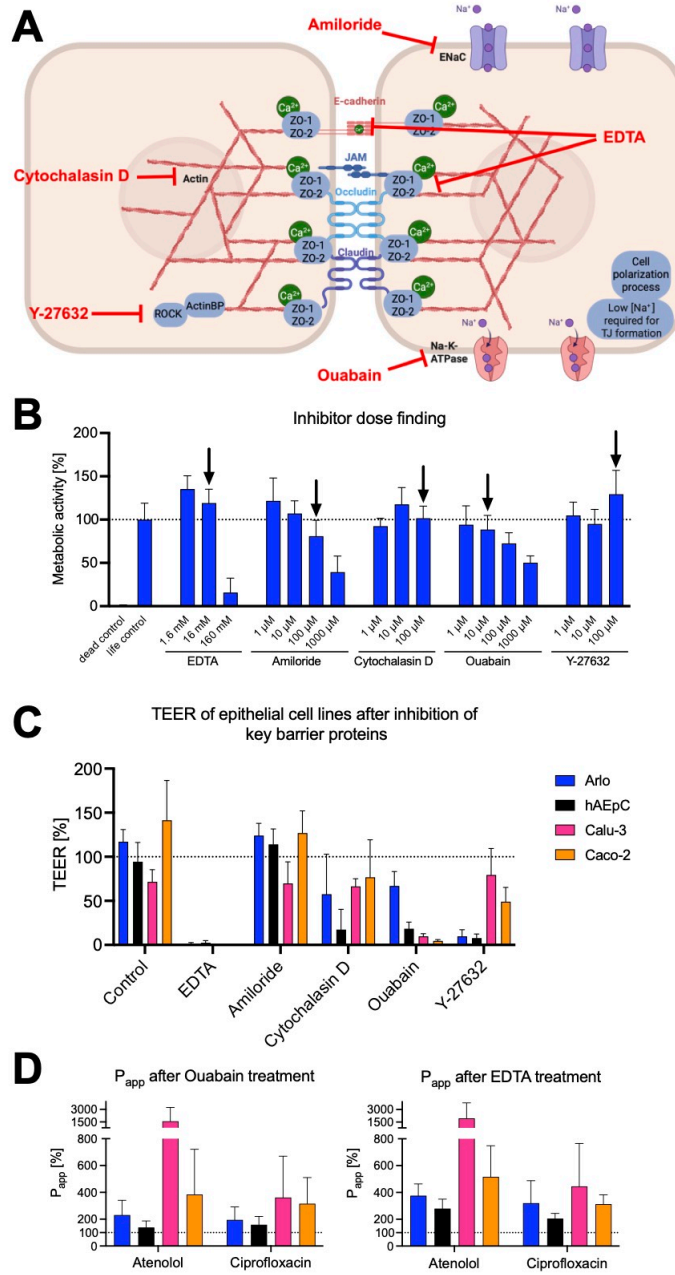


Figure 3: Mechanistic dissection of barrier regulation in Arlo and hAEPc, Calu-3, and Caco-2 epithelial cell lines. (A) Schematic overview of pathways and targets modulated by inhibitors: amiloride (ENaC), ouabain (Na^+/K^+ -ATPase), cytochalasin D (actin), EDTA (calcium-dependent junctions), and Y-27632 (ROCK). (B) Metabolic activity of Arlo cells after treatment with increasing concentrations of EDTA, amiloride, cytochalasin D, ouabain, and Y-27632. Arrows indicate which concentration was chosen for downstream experiments. (C) TEER response after inhibitor treatment. (D) Effects of inhibitors on apparent permeability (P_{app}) for atenolol and ciprofloxacin after indicated treatment normalized to untreated control. N=3, n=8–9, except hAEPc N=2, n=6.

4.4 Proteomic characterization

To assess Arlo's suitability as an alveolar epithelial model, its proteome was compared with that of established epithelial cell lines, focusing on proteins involved in infectious disease susceptibility and epithelial permeability. Given the central role of membrane proteins in both processes, the membrane proteome (MP) was analyzed in parallel with the total proteome. As membrane proteins are low in abundance, an enrichment step was applied to enable robust quantitative analysis (Figure 4A). Additionally, proteomes of cells grown on Transwell® culture (TC) were compared with those grown in T75 flask culture (FC), since epithelial cells alter their proteome upon polarization on permeable supports [47].

Figure 4B shows a volcano plot used to validate the successful enrichment of membrane proteins, comparing the purified membrane proteome fraction to the flask culture total protein fraction of Arlo. The pronounced enrichment of canonical membrane protein families, including SLC (solute carriers; e.g. SLC3A2), ABC (ATP-binding cassette transporters; e.g. ABCC1), TLR (Toll-like receptors; e.g. TLR2), integrins (e.g. ITGA3), claudins (e.g. CLDN7), and cadherins (e.g. CDH4), confirms the effectiveness of the enrichment of membrane proteins in this fraction. Figure 4C shows PCA (principal component analysis) plots of the total proteome of cells grown in flask culture (FC) or on Transwell® culture (TC), used to assess data quality and to visualize global proteomic differences between cell lines [48]. The flask culture proteome PCA revealed clear separation between cell lines, indicating pronounced differences in global protein expression profiles, with tight clustering of the replicates reflecting high reproducibility. Arlo was positioned between the alveolar cell lines NCI-H441/A549 and hAEPc, reflecting both, shared alveolar-specific features but also distinct protein expression patterns. PCA of Transwell®-cultured inserts similarly revealed clear separation with close replicate clustering, whereas hAEPc at day 2 and 7 showed pronounced donor-to-donor variability, resulting in less consistent clustering. Accordingly, Arlo clustered closely with hAEPc cultured for 14 days, while earlier hAEPc stages were more dispersed, indicating greater similarity of Arlo to later-stage hAEPc. Other lung-derived cell lines and intestinal Caco-2 cells formed distinct clusters, reflecting substantial proteomic differences.

Based on the differences observed in the PCA, volcano plot analyses were performed for selected pairwise comparisons (Figure 4D). Comparison of hAEPc cultured on Transwell® inserts at day 2 versus day 14 revealed approximately 9% significantly differentially expressed proteins, indicating that about one in ten proteins changes in abundance during culture and AT2-to-AT1-like transdifferentiation. Comparison of the flask cultured total proteomes of hAEPc and Arlo showed approximately 31% differentially expressed proteins, indicating greater similarity than observed between Arlo and either Caco-2 (40%) or Calu-3 (37%). Notably, despite their shared lung origin, Arlo and Calu-3 were not more similar to each other than Arlo was to the intestinal Caco-2 cell line. Comparisons between cells cultured on Transwell® inserts and in T75 flasks revealed differences ranging from 12 to 44%, depending on the cell type (Supplementary Figure S3), highlighting the strong impact of culture format on proteomic profiles.

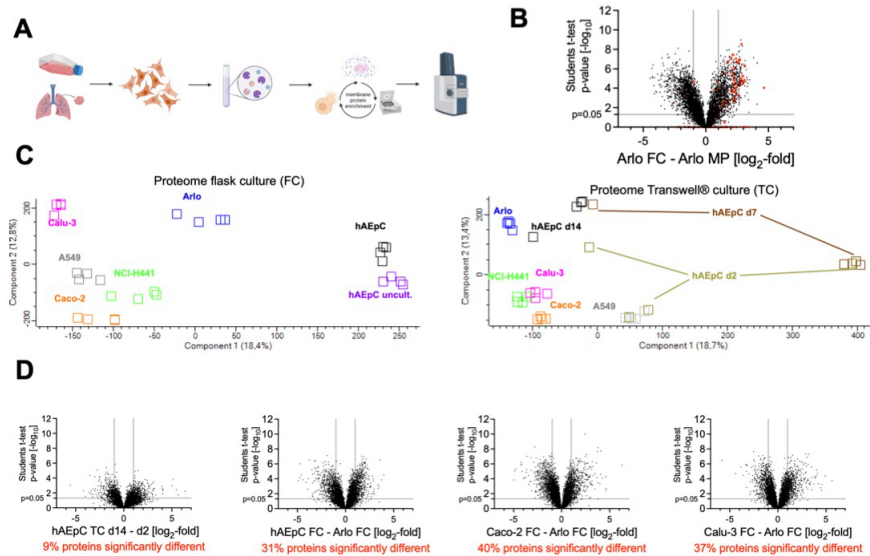


Figure 4: Proteomic characterization of epithelial cell models relevant for barrier and infection studies. (A) Workflow of label-free quantitative proteomics, including enrichment of membrane protein fraction. (B) Volcano plot demonstrating enrichment of typical membrane protein families in the membrane proteome (MP) in red (e.g., solute carriers, ABC transporters, Toll-like receptors, integrins, claudins, and cadherins). (C) Principal component analysis of flask culture (FC) total proteomes (all cultured for 7 days) and Transwell® culture (TC) total proteomes (all cultured 10–12 days except hAEPc 2, 7, or 14 days as indicated). (D) Volcano plots of pairwise comparisons. The complete list of quantified proteins, including gene names, fold changes, and adjusted p-values, is provided in the Supplementary Material Table S2. N=4

Because the most relevant differences often concern the presence or absence of specific proteins rather than expression magnitude, we examined key proteins relevant to permeability studies and lung infection modeling (Table 2 and Supplementary Table S1). In this context, Arlo appears well suited for modeling viral lung infections and associated disease manifestations such as pulmonary edema, as well as passive permeability, but less suitable for transporter-mediated transport studies. More generally, each cell type showed advantages for specific applications: hAEPc are appropriate for studying pulmonary peptide transport via PEPT2, whereas Caco-2 cells are useful for investigations involving organic anion transporters. These findings underscore the importance of proteomic profiling prior to experiments targeting defined pathways, as functional suitability depends more on protein presence than on tissue origin alone [49].

Table 2: Overview of differential expression of selected drug transporters across lung epithelial cell lines with Caco-2 as reference. Lung epithelial cell lines (Arlo, hAEPc, A549, NCI-H441, and Calu-3) are shown alongside Caco-2 cells as intestinal reference model. Check marks (✓) indicate protein expression detected in at least 3 out of 4 biological replicates, whereas crosses (X) indicate detection in fewer than 3 out of 4 replicates. FC (flask cultured) refers the whole lysates from T75 flasks, TC (Transwell® cultured) to total protein from cells cultured on Transwell® inserts, and MP (membrane protein fraction) to the enriched membrane protein fraction from T75 cultured cells. N=4

Protein	Arlo			hAEPc			A549			NCI-H441			Calu-3			Caco-2		
	FC	TC	MP	FC	TC	MP	FC	TC	MP	FC	TC	MP	FC	TC	MP	FC	TC	MP
ABCB1 (PGP)	✓	X	X	X	X	X	✓	X	X	X	X	X	✓	✓	✓	✓	✓	✓
ABCC1 (MRP1)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
ABCC2 (MRP2)	✓	X	✓	X	✓	✓	✓	✓	X	X	✓	X	X	✓	✓	✓	✓	✓
ABCG2 (BCRP)	✓	X	✓	X	✓	✓	✓	✓	X	X	✓	X	X	✓	✓	✓	✓	✓
OATP1B1/3	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
OCT1-2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
PEPT1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
PEPT2	X	X	X	✓	X	X	X	X	X	X	X	X	X	X	X	X	X	X

4.5 Morphological and histological characterization

To understand how structural features contribute to the functional differences observed between epithelial models, we examined morphology and apical surface architecture using scanning electron microscopy (SEM) and histological analyses. SEM at magnifications up to 30,000× enabled detailed visualization of apical features, including microvilli organization, while hematoxylin and eosin (H&E) staining provided complementary information on epithelial architecture, such as mono- versus multilayer, cell height, and shape. These analyses revealed marked differences in epithelial organization and surface specialization among the models. SEM imaging (Figure 5A) showed a generally regular cell arrangement, whereas H&E cross-sections (Figure 5B) highlighted differences in layer thickness, morphology, and packing density. Together, these approaches enabled direct comparison of structural and functional characteristics.

Arlo formed a densely packed monolayer of uniformly cuboidal cells, consistent with an alveolar type II-like phenotype (Figure 5B). The homogeneous morphology and tight cell-cell contacts create a large intercellular contact area, likely contributing to the pronounced junctional tightness observed in this model. *In vivo*, about 95% of the alveolar surface is covered by type I cells; therefore, a layer composed exclusively of type II-like cells, as seen in Arlo, may even form a tighter barrier than the mixed AT1/AT2 layers formed by hAEPc [50]. SEM analysis (Figure 5A) revealed a few short microvilli, supporting its AT2-like character [51]. hAEPc displayed a large, flat morphology and formed a thin yet continuous epithelial layer characteristic of alveolar type I cells (Figure 5B). The extremely flattened shape reduces intercellular contact area and may account for their slightly lower TEER values compared with Arlo. SEM analysis (Figure 5A) confirmed smooth apical surfaces without prominent microvilli,

consistent with a gas-exchange rather than absorptive phenotype [52]. Occasional short microvilli described in the literature may reflect donor variability or culture duration. Calu-3 cells formed a compact, well-organized epithelial layer characteristic of airway secretory epithelia (Figure 5B). SEM analysis (Figure 5A) showed numerous small microvilli covering most of the apical surface [53]. This cohesive architecture promotes extensive cell-cell junctions and supports tight barrier formation, although not to the same extent as observed in Arlo. NCI-H441 displayed a heterogeneous morphology with variable cell height and occasional multilayered areas, indicating partial pseudostratification and less uniform organization (Figure 5B). This irregular architecture likely disrupts continuous intercellular contacts and may contribute to the reduced barrier tightness observed in this model. SEM analysis (Figure 5A) revealed long, dense microvilli suggestive of secretory activity, although previous reports describe generally lower microvilli abundance [54]. Caco-2 cells formed a densely packed, columnar epithelium characteristic of intestinal absorptive cells (Figure 5B), although an even more elongated morphology might be expected given their intestinal origin. SEM imaging (Figure 5A) revealed abundant, prominent microvilli that increase apical surface area by approximately 5–15 ×, consistent with their absorptive function and the relatively high permeability reported for this model [55,56]. A549 cultures appeared loosely organized and irregular, occasionally forming small multilayered clusters instead of a uniform monolayer, consistent with their poorly differentiated, tumor-derived origin (Figure 5B). SEM analysis (Figure 5A) showed smooth apical surfaces without prominent microvilli, in line with their limited differentiation and AT2 phenotype.

Taken together, these structural findings demonstrate how cellular origin and differentiation state determine epithelial architecture and apical specialization. Such model-dependent morphological differences are well recognized in lung epithelial *in vitro* systems and reflect region-specific functional specialization of the respiratory epithelium [9]. The observed structural diversity corresponds to the previously described differences in barrier tightness and permeability, further underscoring the suitability of Arlo's alveolar-like phenotype for modeling distal lung epithelial function.

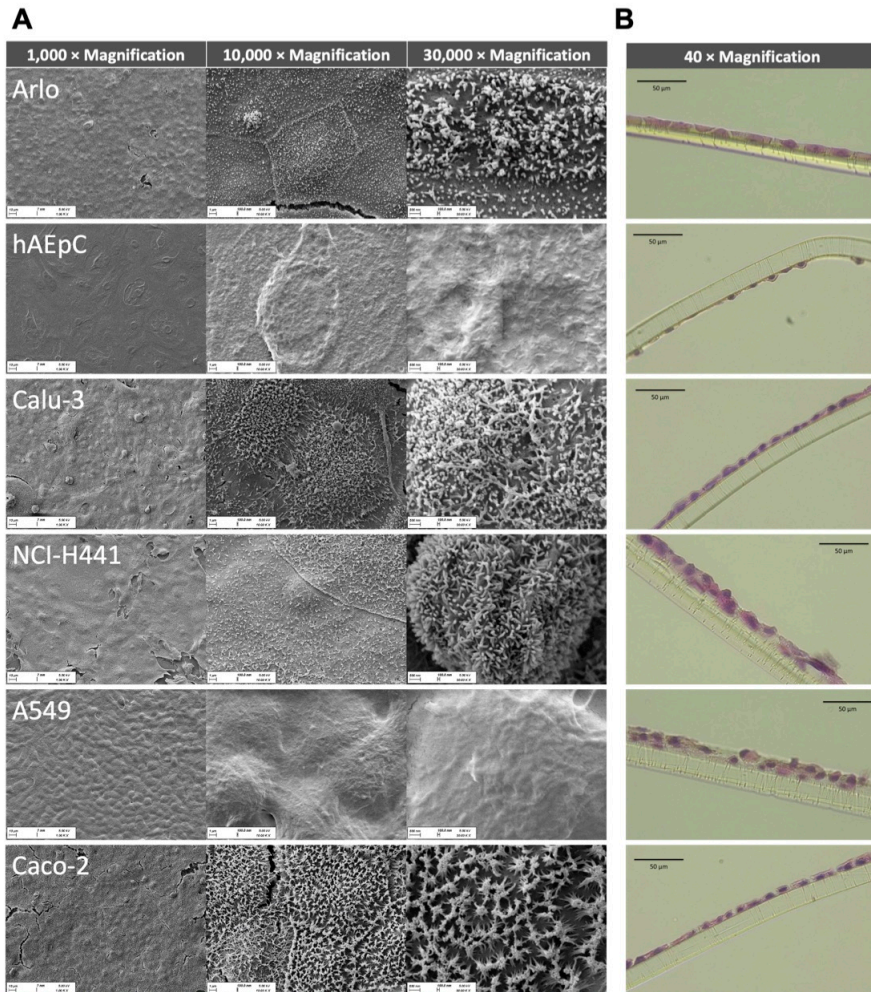


Figure 5: Morphological and histological characterization of epithelial cell models. (A) SEM imaging of cells cultured on Transwell® inserts for 10–12 days (hAEPc 7 days) at magnifications of up to 30,000x. (B) Hematoxylin and eosin (H&E) staining of cross-sections from Transwell® cultures (10–12 days, except hAEPc 7 days) at 40x magnification.

4.6 Application of Arlo as pulmonary edema model

Following the comparative characterization of Arlo, which revealed elevated PD and functional ENaC activity, we evaluated its suitability as a pulmonary edema model, as both parameters are central to alveolar sodium transport and fluid clearance *in vivo*. Pulmonary edema is a key feature of acute lung injury, including viral pneumonias such as COVID-19, where impaired ENaC function and disrupted epithelial transport promote alveolar flooding. To investigate this, we established a simplified *in vitro* system recapitulating core aspects of alveolar fluid imbalance.

Figure 6A schematically depicts alveolar fluid clearance, where sodium enters via apical ENaC channels and is actively transported by basolateral Na^+/K^+ -ATPases, with water following osmotically to the basolateral side [19]. This mechanism maintains alveolar dryness and is often impaired during inflammatory or edematous states [4].

To functionally assess ENaC-mediated fluid transport, we used two complementary assays. PD served as a surrogate for ENaC activity and allowed detection of ion transport changes after edema induction with cytarabine, bleomycin, or after pharmacological treatments. In parallel, a fluid clearance assay was established, in which a defined apical fluid volume was applied and the remaining volume quantified after 72 hours, providing a functional measure of epithelial fluid removal capacity. Together, these assays enabled assessment of ion transport and fluid clearance under healthy and edema-like conditions, as well as therapeutic responses to rosiglitazone, salbutamol, forskolin, and aldosterone. Rosiglitazone activates PPAR γ to enhance ENaC expression and anti-inflammatory effects; salbutamol stimulates ENaC via β_2 -adrenergic cAMP/PKA signaling; forskolin increases intracellular cAMP through adenylyl cyclase activation; and aldosterone upregulates ENaC and Na $^+$ /K $^+$ -ATPase via the mineralocorticoid receptor [57–62].

In Figure 6B, control conditions (without edema induction) showed that rosiglitazone completely reduced PD, whereas salbutamol had no measurable effect. In contrast, forskolin increased PD approximately five-fold and aldosterone approximately three-fold. Based on their mechanisms, a stronger response to aldosterone would have been expected. However, hydrocortisone present in the SAGM medium, which also binds the mineralocorticoid receptor, likely attenuated aldosterone's effect through competitive receptor binding and limited transcriptional activation. This trend was mirrored in the qPCR results: rosiglitazone reduced ENaC mRNA levels, salbutamol had no significant effect, forskolin induced a modest increase, and aldosterone produced the strongest upregulation. This aligns with aldosterone's role as a transcriptional activator of ENaC via the mineralocorticoid receptor. In contrast, forskolin likely enhanced ENaC activity through increased intracellular cAMP, primarily modulating channel function rather than gene expression. In contrast, cytarabine-induced edema elicited a markedly different response. As a nucleoside analog, cytarabine incorporates into DNA during replication, inhibits DNA polymerase, and thereby suppresses DNA synthesis and transcriptional activity. Accordingly, ENaC mRNA levels were reduced upon treatment with rosiglitazone, salbutamol, and forskolin, whereas only aldosterone induced a slight upregulation as a transcription factor of ENaC. Despite the reduced baseline PD following cytarabine treatment, the untreated control, likely due to hydrocortisone in the medium, and salbutamol restored PD back to non-edematous baseline levels, while forskolin increased PD two-fold. Since aldosterone primarily acts via transcriptional upregulation of ENaC, its effect was likely limited by cytarabine-mediated inhibition of mRNA synthesis. In contrast, treatments acting predominantly through cAMP-dependent ENaC activation increased PD. Following bleomycin-induced edema, the response resembled that observed under non-edematous conditions. Bleomycin generates reactive oxygen species that cause DNA strand breaks, cell cycle arrest, and apoptosis, explaining the reduced baseline PD compared to healthy controls. After 72 hours, PD in the untreated control exceeded baseline levels, again possibly due to hydrocortisone-mediated ENaC activation. Under edematous conditions, salbutamol increased PD, unlike in non-edema controls. Forskolin and aldosterone exerted the strongest stimulatory effects, increasing PD by up to five-fold and three-fold, respectively, whereas rosiglitazone markedly reduced PD. qPCR analyses mirrored this trend, showing ENaC downregulation with rosiglitazone, minimal regulation with forskolin, and slight upregulation with aldosterone, supporting predominant cAMP-mediated ENaC activation. The overall lower values compared to non-edema controls likely reflect bleomycin-induced cellular damage.

In Figure 6C, the impact of the treatments on apical fluid clearance is shown as the percentage of fluid remaining 72 hours after application of a defined volume, with untreated controls (no edema) set to 100%. Rosiglitazone increased residual fluid, consistent with its reduction of PD and ENaC mRNA, supporting the central role of ENaC in alveolar fluid clearance. Salbutamol had no significant effect, in line with the PD and expression data. Although forskolin markedly increased PD, it produced only a moderate reduction in apical fluid. In contrast, aldosterone led to a more pronounced decrease in apical fluid, suggesting a sustained enhancement of clearance capacity via transcriptional upregulation of ENaC rather than solely cAMP-mediated channel activation. Edema induction with cytarabine and bleomycin increased baseline values to approximately 150% of untreated controls. Under cytarabine-induced edema, rosiglitazone further impaired fluid clearance, consistent with PD and qPCR data, whereas the other treatments showed no significant improvement despite effects observed at the PD, potentially due to the influence of cytarabine on the ENaC expression. In bleomycin-induced edema, forskolin and aldosterone enhanced clearance by ~30%, while rosiglitazone again reduced it, in line with PD and qPCR findings.

Overall, increases in PD correlated with enhanced alveolar fluid clearance, indicating that this dual-readout approach reliably models both impaired and pharmacologically improved fluid removal, possibly exploitable as platform for evaluating therapeutic strategies in pulmonary edema.

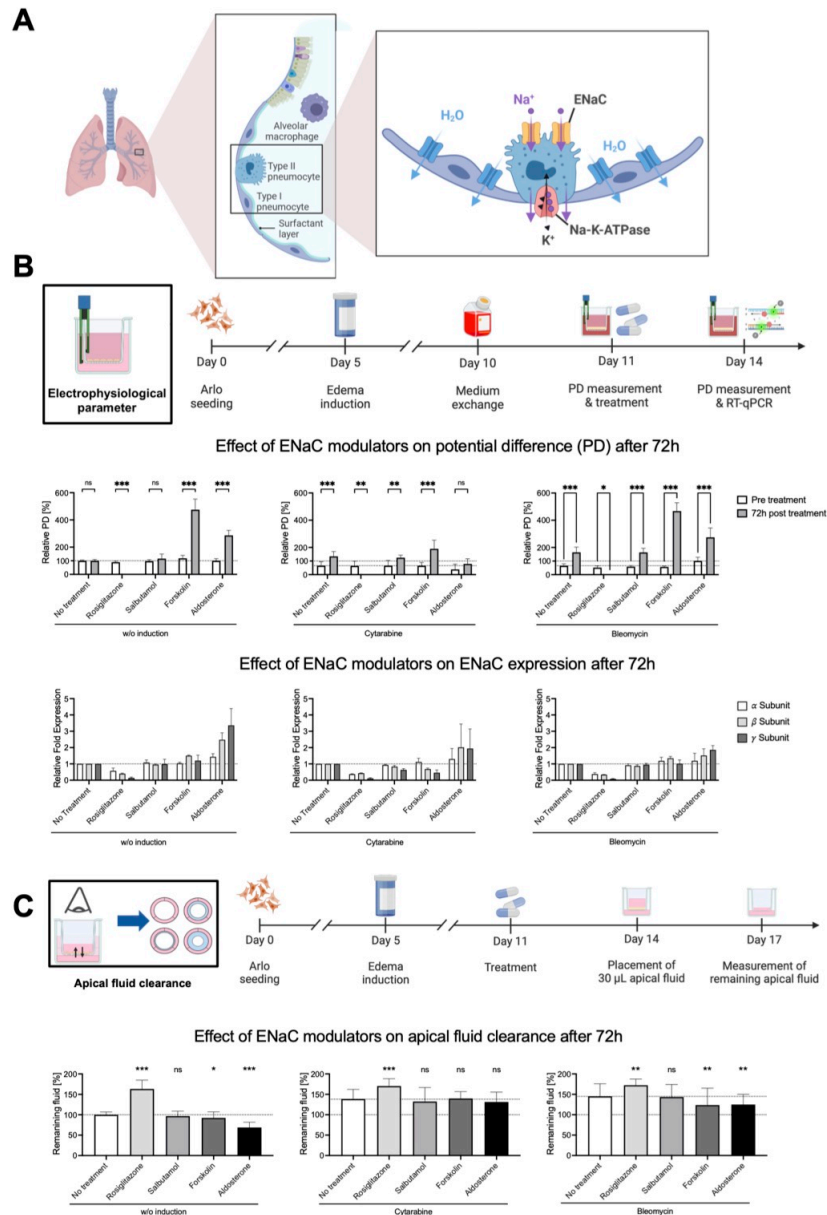


Figure 6: Arlo-based in vitro model of alveolar fluid clearance and pulmonary edema. (A) Scheme of sodium-dependent alveolar fluid clearance. (B) Experimental setup and results of manipulation of transepithelial potential difference (PD) and corresponding ENaC mRNA expression under control conditions and after edema induction with cytarabine or bleomycin and treatment with rosiglitazone, salbutamol, forskolin, or aldosterone. Data were analyzed by two-way repeated-measures ANOVA, followed by Šidák's post hoc multiple comparisons test for pairwise comparisons between 0 h and 72 h within each treatment group. Statistical significance was defined at $p < 0.05$. (C) Experimental setup and fluid clearance assay under control conditions and after edema induction with cytarabine or bleomycin and treatment with rosiglitazone, salbutamol, forskolin, or aldosterone. Data were analyzed using unpaired two-tailed t-tests, with $p < 0.05$ considered statistically significant. Treatments were compared with untreated control. N=3–6, n=6–15

5 Conclusion

In this study, we demonstrate that the immortalized human alveolar epithelial cell line Arlo combines robust barrier integrity with functional ENaC activity, distinguishing it from commonly used lung-derived cell lines such as A549, Calu-3, or NCI-H441. Comparative analyses revealed that Arlo resembles primary human alveolar epithelial cells while offering greater stability and reproducibility. Proteomic profiling further underscored its suitability for mechanistic and application-oriented studies. In a translational context, Arlo enabled us to establish a bleomycin-induced *in vitro* pulmonary edema model that recapitulates impaired fluid clearance as well as the effects of different ENaC modulating drugs. Together, these findings position Arlo as a versatile and physiologically relevant tool for drug transport, disease modeling, and infection research of the distal lung.

6 Data availability

Datasets and further information of the studies are available upon request from the corresponding author. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD060822.

Reviewers access details:

Project accession: PXD060822

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7 Conflict of interests

The authors declare no competing interests.

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10 Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work the authors used ChatGPT-5 in order to support language refinement. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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3.3 Application of the Arlo cell line in an epithelial-macrophage alveolar co-culture model to study ACOD1 inhibition during pulmonary inflammation

As outlined in section 1.3.3, ACOD1 represents a central regulator of immunometabolism in the lung during inflammation, modulating itaconate production, mitochondrial ROS generation, PD-(L)1 activity, and host-pathogen interactions [64], [67]. Its context-dependent role spans both protective and potentially detrimental effects, depending on the disease setting [64]. Given the complex interplay between immune cells and the epithelial barrier, appropriate *in vitro* models are required to investigate these mechanisms [69]. Therefore, a co-culture model of Arlo epithelial cells and dTHP-1 macrophages was established to study ACOD1-mediated responses under inflammatory conditions. In addition, the effects of pharmacological ACOD1 inhibition using Citra01 were evaluated.

3.3.1 Material and methods

3.3.1.1 Isolation of primary human alveolar epithelial cells (hAEpC)

Human alveolar epithelial cells were isolated from lung tissue obtained from SHG Clinics Völklingen and Clinic Saarbrücken, following the procedure described by Daum et al [91]. The use of human material was approved by the Ethics Committee of the State of Saarland, Germany (approval no. 113/19, issued May 21, 2019, for SHG Clinics Völklingen; and approval no. 97/21, issued May 10, 2021, for Clinic Saarbrücken). Lung tissue was cut into small cubes (approximately 1 × 1 × 1 cm) using sterile scissors and subsequently sliced into 15 µm sections with a McIlwain Tissue Chopper (Plano GmbH, TC752). The tissue fragments were transferred into 50 mL falcon tubes containing 30 mL of balanced salt solution buffer (BSSB; 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 5.5 mM glucose; all Sigma-Aldrich, and 10 mM HEPES; Carl Roth; pH 7.4). Samples were washed by filtration through a 100 µm cell strainer (Greiner Bio-One, 542000). For enzymatic digestion, the tissue suspension was incubated in 30 mL BSSB containing 1.5 mL trypsin (100 mg/mL; Sigma-Aldrich, T8003) and 0.3 mL elastase (10 mg/mL; Worthington Biochemical, LS002279). The digested material was subsequently filtered through a 40 µm strainer (Greiner Bio-One, 542040). The resulting cell suspension was plated on Petri dishes (Corning, 430167) to facilitate adhesion of immune cells and erythrocytes, thereby enriching the epithelial fraction. Further purification was performed by Percoll density gradient centrifugation (Sigma-Aldrich, P1644) followed by magnetic separation using CD326 (EpCAM) MicroBeads (Miltenyi Biotec, 130-061-101). The EpCAM-positive epithelial cells were finally resuspended in 5 mL Small Airway Epithelial Cell Growth Medium (SAGM BulletKit™, Lonza Bioscience, CC-3118).

3.3.1.2 Primary human alveolar macrophages (hAM)

Primary human alveolar macrophages were obtained as part of the isolation process hAEpC [95]. All buffer fractions collected during the tissue chopping and washing steps prior to

enzymatic digestion were pooled in 50 mL falcon tubes and centrifuged. The resulting cell pellet was resuspended in 40 mL RPMI 1640 medium supplemented with 10 % fetal calf serum (FCS; Capricorn Scientific, FBS-12A) and 1 % penicillin/streptomycin (P/S; Gibco, 15140-122), and the suspension was distributed onto three Petri dishes. After incubation for 90 min at 37 °C and 5 % CO₂, the medium was carefully aspirated, and each petri dish was washed three times with 13 mL phosphate-buffered saline (PBS; Sigma-Aldrich, D8537). Subsequently, 13 mL of fresh RPMI 1640 medium containing 10 % FCS and 1 % P/S was added to each petri dish. Cells were maintained under standard culture conditions and fed every other day until day 6. On day 6, cultures were washed three times with 13 mL PBS per dish, followed by the addition of 3 mL Accutase® (Sigma-Aldrich; A6964) to detach adherent cells. After 30 min incubation at 37 °C, cells were gently scraped off using a cell scraper, and the enzymatic reaction was quenched by adding 9 mL RPMI medium per petri dish. The resulting cell suspensions were collected, centrifuged for 4 min at 300 × g, and resuspended in mixed culture medium composed of 50 % RPMI 1640 (supplemented with 10 % FCS) and 50 % Small Airway Epithelial Cell Growth Medium (SAGM; without hydrocortisone).

3.3.1.3 Arlo

Arlo is a monoclonal human alveolar epithelial cell line generated via single-cell printing, developed to serve as a stable and reproducible *in vitro* model of the alveolar epithelium for biopharmaceutical and pulmonary research applications [140]. Cells were maintained in Small Airway Growth Medium supplemented with 1 % (v/v) fetal calf serum and 1 % (v/v) penicillin-streptomycin, and cultured in a humidified incubator at 37 °C with 5 % CO₂. For routine maintenance in T25 cm² flasks, 7 mL of medium was replaced every other day, and cells were subcultured approximately once per week. For passaging, cells were rinsed twice with 5 mL PBS, detached using 1.8 mL trypsin-EDTA (Gibco, 25300-054) for 6 min at 37 °C/5 % CO₂, and collected by centrifugation at 300 × g for 4 min. The resulting cell pellet was resuspended in 5 mL fresh SAGM medium, and 0.7×10^6 cells were seeded into new T25 flasks (Greiner, 690160). Prior to cell seeding, culture flasks were coated with 2 mL of a coating solution containing 1 % (v/v) fibronectin (1 mg/mL; Corning, 356008) and 1 % (v/v) collagen (3 mg/mL; Sigma-Aldrich, C4243) in sterile distilled water and incubated for 1 h at 37 °C/5 % CO₂.

3.3.1.4 THP-1

The human monocytic cell line THP-1 (DSMZ ACC 16) was used as a surrogate for alveolar macrophages to establish a cell line-based co-culture with Arlo cells representing the alveolar epithelium. THP-1 cells were cultured in T75 flasks containing 12 mL RPMI 1640 medium (Gibco, 21875-034) supplemented with 10 % fetal calf serum as published previously [144]. Cells were subcultured twice weekly by transferring either 2×10^6 cells on Mondays or 3×10^6 cells on Fridays into new T75 flasks containing 12 mL fresh medium. For differentiation into macrophage-like cells (dTHP-1; differentiated THP-1), 3×10^6 cells were seeded in 10 mL

RPMI-1640 supplemented with 7.5 ng/mL phorbol-12-myristate-13-acetate (PMA; Sigma, P1585) and incubated for 48 h at 37 °C in a humidified atmosphere with 5 % CO₂ in a T75 flask. Following incubation, cells were washed twice with 10 mL PBS and detached by adding 3 mL Accutase® (Sigma, A6964) per flask. After 30 min at 37 °C/5 % CO₂, cells were gently released using a cell scraper (Greiner Bio-One, 541070). The enzymatic reaction was quenched by adding 7 mL RPMI medium, after which the suspension was centrifuged for 4 min at 300 × g and the resulting pellet resuspended in 1.5 mL mixed medium.

3.3.1.5 Co-culture, inflammation, and treatment with ACOD1 inhibitor Citra01

Epithelial cells, either primary human alveolar epithelial cells (hAEPc) or Arlo cells, were seeded apically in 500 µL SAGM medium onto collagen/fibronectin-coated Transwell® inserts (Corning, CLS3460; 1.12 cm² growth area) at seeding densities of 300,000 cells/cm² or 100,000 cells/cm², respectively. The basolateral compartment was filled with 1500 µL SAGM medium. Cells were maintained under standard culture conditions (37 °C, 5 % CO₂) with medium exchange every other day. hAEPc were cultivated for 6 days and Arlo cells for 9 days to allow barrier formation. On day 6 (hAEPc) or day 9 (Arlo), the transepithelial electrical resistance (TEER) was measured to confirm epithelial integrity. Subsequently, both apical and basolateral compartments were washed with phosphate-buffered saline (PBS) to remove residual hydrocortisone from the SAGM medium. For co-culture establishment, differentiated THP-1 or human alveolar primary macrophages were seeded onto the apical side in 500 µL mixed medium (50 % RPMI 1640 supplemented with 10 % FCS and 50 % SAGM without hydrocortisone) at densities of 240,000 dTHP-1 cells or 100,000 hAM per insert. The basolateral compartment was filled with 1500 µL of the same mixed medium. Co-cultures were allowed to equilibrate for 24 h before further treatment. After 24 h, TEER was measured again, and cells were stimulated with lipopolysaccharide (LPS; Sigma-Aldrich, L2762) and interferon-gamma (IFN-γ; Miltenyi Biotec, 130-096-484) to induce inflammation, reaching final concentrations of 100 ng/mL LPS and 30 ng/mL IFN-γ. For ACOD1 inhibition, cells were treated with Citra01 (final concentration 300 µM). The following experimental conditions were investigated: control (Ctrl) – no inflammation, no treatment; inflammation (Infl) – LPS/IFN-γ stimulation, no treatment; treatment (Treat) – Citra01 treatment, no inflammation; and inflammation + treatment (Infl+Treat) – combined LPS/IFN-γ stimulation and Citra01 treatment. All conditions were tested in both co-culture and corresponding monoculture systems. For dTHP-1 monocultures, cells were seeded into 48-well plates instead of Transwell inserts to achieve same growth areas. After an additional 24 h incubation, TEER was measured once more to assess the impact of inflammatory stimulation and treatment on epithelial barrier integrity.

3.3.1.6 Transepithelial electrical resistance (TEER) measurement

TEER measurements were performed under liquid-covered conditions with 500 µL apical and 1500 µL basolateral medium. A STX2 chopstick electrode (World Precision Instruments, STX2) in combination with a volt-ohm meter (World Precision Instruments, EVOM2) was used to assess the resistance of the cell layer. Measurements were carried out under sterile conditions on a heating plate inside a fume hood. Blank values from cell-free inserts were subtracted, and TEER values were normalized to the insert surface area ($\Omega \times \text{cm}^2$) according to Equation 1.

$$(Resistance_{Cell\ layer}(\Omega) - Resistance_{Blank}(\Omega)) \times Growth\ area\ (cm^2) = TEER\ (\Omega \times cm^2)$$

Equation 1: Calculation of the transepithelial electrical resistance

3.3.1.7 Magnetic bead-based separation of Arlo and dTHP-1

For co-cultures of Arlo and dTHP-1 cells, separation of both cell types was performed after the experiment to enable independent analysis of epithelial and macrophage responses to inflammation and drug exposure. To ensure comparability between co-culture and monoculture conditions, all separation steps described below were identically applied to the corresponding monocultures, thereby minimizing potential bias in subsequent RNA sequencing and downstream analyses. Initially, the dTHP-1 cells were gently detached from the Arlo monolayer by repeatedly pipetting the 500 µL apical medium up and down. The resulting suspension containing detached dTHP-1 cells was transferred into 1.5 mL Eppendorf tubes and centrifuged for 4 min at $300 \times g$. The supernatant was stored at $-80\text{ }^{\circ}\text{C}$ for downstream analyses. To ensure complete removal of residual dTHP-1 cells from the epithelial layer, magnetic bead-based cell separation was subsequently performed. For preparation of a single-cell suspension, Arlo cells remaining on the Transwell inserts were first treated with Dispase II (0.5 U/mL; Sigma-Aldrich, D4693) applied to both apical and basolateral compartments for 45 min at $37\text{ }^{\circ}\text{C}$, until the epithelial sheets detached from the membrane and rolled up. The detached sheets were transferred into Eppendorf tubes, pooling material from two Transwells per tube to obtain sufficient cell numbers, and centrifuged at $300 \times g$ for 4 min. Pellets were washed once with 1 mL PBS, after which the supernatant was removed.

Each pellet was resuspended in 500 µL Accutase® and transferred into a 24-well plate. Cells were incubated for three consecutive 10 min intervals at $37\text{ }^{\circ}\text{C}$, with gentle mixing by pipetting up and down after each interval to facilitate dissociation into single cells. The enzymatic reaction was stopped by adding 500 µL mixed medium (50 % RPMI 1640 with 10 % FCS and 50 % SAGM without hydrocortisone). The cell suspension was then transferred back into Eppendorf tubes and passed through Flowmi® cell strainers (Bel Art, BAH136800040) attached to pipette tips to remove residual aggregates.

Cells were centrifuged again (4 min, 300 × g) and washed once with 1 mL ice-cold autoMACS rinsing solution buffer (Miltenyi, 130-091-222) supplemented with 0.5 % bovine serum albumin (BSA; Sigma-Aldrich, A9647). For magnetic labeling, cells were incubated with 500 µL autoMACS rinsing solution buffer containing 15 µL anti-EpCAM (CD326) MicroBeads per tube for 30 min on a rotator at 4 °C.

After incubation, magnetic separation was performed using MS Columns (Miltenyi Biotec, 130-042-201), which were pre-equilibrated with 500 µL autoMACS rinsing solution buffer before sample loading. Columns were washed four times with 500 µL autoMACS rinsing solution after sample application. Two MS Columns were used per sample to ensure complete separation of dTHP-1 from Arlo cells. The EpCAM-positive Arlo cell fraction was finally eluted in 1 mL PBS and collected by centrifugation. The supernatant was discarded, and the resulting cell pellets were used for subsequent analyses.

A separation of hAEpC and hAM was not possible, likely due to intensive cell-cell-attachment between these cell types.

3.3.1.8 RNA isolation

Cell pellets obtained after separation of dTHP-1 and Arlo cells were lysed in 350 µL RLT buffer, and total RNA was isolated using the RNeasy Micro Kit (Qiagen, 74004) in combination with the RNase-Free DNase Set (Qiagen, 79254) according to the manufacturer's instructions, with minor modifications. Centrifugation steps were extended to 1 min (instead of 15 s), and RNA was eluted in 20 µL RNase-free water (instead of 14 µL). To minimize RNase activity, all steps were carried out on ice whenever feasible, except for the on-column DNase I digestion, which was performed at room temperature. RNA concentration (ng/µL) and purity were determined using a Tecan Spark® Cyto microplate reader (Tecan) equipped with a NanoQuant Plate™ (Tecan, 30033939). Absorbance was measured at 230, 260, 280, and 310 nm, and full spectral scans ranging from 200 to 400 nm were performed to evaluate RNA concentration and purity. RNA was considered of sufficient quality when the 260/230 ratio was ≥ 1.8 , the 260/280 ratio ranged between 1.8 and 2.2, and the RNA integrity number (RIN) was ≥ 8 . RIN were determined using the 4150 TapeStation System (Agilent Technologies) with RNA ScreenTape (5067-5576), RNA ScreenTape Sample Buffer (5067-5577), and RNA ScreenTape Ladder (5067-5578) according to the manufacturer's instructions. Data acquisition and analysis were performed using TapeStation Analysis Software version 5.1 (Agilent Technologies). Sample identifiers were structured as follows: A = Arlo, D = dTHP-1; M = monoculture, C = co-culture; C = control, I = inflammation, IT = inflammation + treatment, and T = treatment; the final number indicates the biological replicate (e.g., A-M-I-2).

3.3.1.9 Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR) for separation evaluation of Arlo and dTHP-1

RT-qPCR was conducted to verify the efficiency of Arlo and dTHP-1 cell separation by assessing cell type-specific marker expression for epithelial cells (EpCAM) and macrophages (CD45). Based on the measured RNA concentrations, 1,000 ng total RNA were reverse-transcribed into cDNA using the qScriber™ cDNA Synthesis Kit (highQu, RTK0104) according to the manufacturer's instructions on a Veriti™ 96-Well Thermal Cycler (Applied Biosystems). The resulting 20 µL cDNA was diluted with 80 µL RNase-free water to obtain a working concentration of 10 ng/µL. Quantitative PCR of target genes (Table 1) was performed using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, A25742) on a Bio-Rad real-time PCR system (C1000™ Thermal Cycler, CFX96™ Real-Time System). Each 10 µL reaction contained 5.0 µL 2× SYBR mix, 0.3 µL primer-pair mix (10 µM each; final concentration 300 nM per primer), 2.7 µL nuclease-free water, and 2.0 µL cDNA (~ 20 ng). Amplification was carried out for 40 cycles following the manufacturer's recommended cycling parameters. Primers were designed using Primer-BLAST (NCBI) and verified for specificity by BLAST alignment against the human transcriptome. Lyophilized primers were briefly centrifuged (≤ 1 min) to collect the material and dissolved in TE buffer (10 mM Tris-HCl, Carl Roth, 9090.2; 0.1 mM EDTA, Sigma-Aldrich, E9884) to yield 100 µM stock solutions. Primer-pair working solutions (10 µM each) were prepared by mixing 100 µL forward primer (100 µM), 100 µL reverse primer (100 µM), and 800 µL nuclease-free water. Graphs were created with GraphPad Prism (10.5.0).

Table 2: Target and reference genes for RT-qPCR and corresponding primer sequences

Gene	Target or reference gene	Forward primer sequence (5' → 3')	Reverse primer sequence (5' → 3')
EpCAM	Epithelial cell target gene	TGCTGGGGTCAGAAGAACAG	TCCTTCTGAAGTGCAGTCCG
CD45	Macrophage cell target gene	CAACAGTGGAGAAAGGACGC	GGCAAAGCCAAATGCCAAGA
β -Actin	Reference gene	CCCCGCGAGCACAGAG	ATCATCCATGGTGAGCTGGC
TATA-Binding Protein	Reference gene	TGTGCTACCCACCAACAAT	TGCTCTGACTTTAGCACCTGTT

3.3.1.10 Cis-aconitate decarboxylase (ACOD1) activity measurement

Arlo and dTHP-1 cells separated from co-cultures and monocultures were analyzed for ACOD1 enzymatic activity by LC-MS/MS measurement itaconate. Itaconate served as a surrogate marker for ACOD1 activity, as the enzyme cis-aconitate decarboxylase catalyzes the conversion of cis-aconitate to itaconate during inflammatory activation. For itaconate

extraction, 200 µL extraction buffer consisting of 12.5 nM diphenhydramine (internal standard) in acetonitrile:methanol:water (2:2:1, v/v/v) was added to the cell pellets received after the separation and incubated overnight at -80 °C. Samples were subsequently centrifuged at $20,800 \times g$ for 10 min at 4 °C to separate cell from the extraction buffer. The resulting supernatant, containing the intracellular metabolites including itaconate, was collected for LC-MS/MS analysis.

Quantification of itaconate was performed according to Winterhoff et al. with minor modifications [171]. Analyses were carried out using a Vanquish Flex UHPLC system (Thermo Fisher Scientific, Germany) coupled to a TSQ Altis Plus triple quadrupole mass spectrometer (Thermo Fisher Scientific, Germany). Chromatographic separation was achieved on an EC 150/2 Nucleodur C18 Pyramid column (5 µm, 150 × 2 mm; Macherey-Nagel, Germany) maintained at 40 °C with a flow rate of 0.6 mL/min. The mobile phases consisted of solvent A (water with 0.2 % formic acid) and solvent B (methanol with 0.2 % formic acid). The gradient profile was as follows: 0-0.5 min, 1 % B; 0.5-2.2 min, 1-90 % B; 2.2-3.2 min, 90 % B; 3.2-4.0 min, 1 % B. Mass spectrometry parameters were: vaporizer temperature 350 °C, ion transfer tube temperature 380 °C, sheath gas 30, auxiliary gas 10, and sweep gas 2. Diphenhydramine (DPH) was used as internal standard. Quantification of itaconate concentrations was based on a calibration curve ranging from 62.5 nM to 2000 nM prepared in 2-fold serial dilutions. Graphs were created with GraphPad Prism (10.5.0).

Table 3: LC-MS/MS settings for Itaconate quantification

Analogue	Parent (m/z)	Fragment (m/z)	Collision energy [V]	Polarity	Tube lens [V]	Spray Voltage [V]	RT [min]
Itaconate	128.883	84.88	9.4	-	30	5000	2.20
Itaconate	128.883	98.83	9.7	-	30	5000	2.20
DPH	256.050	164.97	41.8	+	30	1560	2.66
DPH	256.050	166.97	13.0	+	30	1560	2.66

3.3.1.11 RNA Sequencing

mRNA libraries were generated using the NEBNext® Ultra™ II Directional RNA Library Prep Kit (NEB #E7765) and sequenced on an Illumina NovaSeq X system employing paired-end 150 bp reads (PE150, 2 × 150 bp). This resulted in approximately 30 million reads per sample, with an overall output of around 800 million clusters per flow cell.

Raw FASTQ data were analyzed using a standardized pipeline implemented in a Docker environment running RNADetector [172]. Sequence alignment was carried out with STAR, followed by gene-level quantification using featureCounts. Normalization of read counts to

account for library size and composition was performed using the Bioconductor package edgeR.

Gene expression data were subsequently visualized using volcano plots and heatmaps created with GraphPad Prism (version 10.5.0).

3.3.1.12 Reactive oxygen species (ROS) evaluation

Reactive oxygen species were analyzed using the Oxidative Stress Combo Assay Cell Kit (Stagnosis, EA-7006) following the manufacturer's instructions with minor adaptations. The kit enables the quantification of four distinct oxidative stress markers: general ROS via DCFDA (2,7-Dichlorofluoroscine Diacetate) assay, lipid peroxidation via malondialdehyde (MDA) assay, glutathione (GSH) levels, and superoxide dismutase (SOD) activity.

To this end, Arlo monocultures, dTHP-1 monocultures, and Arlo-dTHP-1 co-cultures were established in clear 96-well plates (Corning, 3596). Wells designated for Arlo mono- and co-cultures were pre-coated with 100 μ L of collagen-fibronectin solution and incubated for 1 h at room temperature prior to seeding. Cells were seeded in 200 μ L of hydrocortisone-free mixed medium at a density of 33,000 Arlo and/or 71,000 dTHP-1 cells per well, depending on the culture condition. After 24 h of resting, cells were stimulated with 100 ng/mL lipopolysaccharide (LPS), 30 ng/mL interferon- γ (IFN- γ), and 300 μ M Citra01. In addition to control (Ctrl), inflammation (Infl), treatment (Treat), and inflammation + treatment (Infl+Treat), a positive control with 500 μ M hydrogen peroxide (H_2O_2) was included.

After 24 h of incubation, cells were washed with KRPG buffer, and 100 μ L of 10 μ M DCFDA solution was added per well and incubated for 45 min at 37 °C and 5 % CO_2 . Following DCFDA incubation, the dye was removed and replaced with KRPG buffer. For the H_2O_2 control, this buffer was supplemented with 500 μ M H_2O_2 . Plates were incubated for an additional 90 min under identical conditions, and fluorescence was measured at 485/535 nm (excitation/emission). Cells were subsequently lysed and stored at -80 °C. MDA, GSH, and SOD assays were carried out according to the manufacturer's instructions. Graphs were created with GraphPad Prism (10.5.0).

3.3.1.13 Citra01 effect on PD-L1

The concentration of soluble PD-L1 in cell culture supernatants was determined using the Human PD-L1 ELISA Kit (Invitrogen, BMS2327) according to the manufacturer's protocol. Supernatants of control (Ctrl), inflammation (Infl), and inflammation + treatment (Infl+Treat) samples collected during the separation process (see Section 3.3.1.7) were analyzed in duplicate. After an initial incubation of standards and samples in human PD-L1 antibody-precoated 96-well plates, wells were sequentially incubated with a biotin-conjugated anti-PD-L1 detection antibody and horseradish peroxidase (HRP)-conjugated streptavidin. Color development was performed using 3,3',5,5'-tetramethylbenzidine (TMB) as chromogenic substrate. The enzymatic reaction was stopped by addition of stop solution, and absorbance

was measured at 450 nm with 620 nm as reference wavelength. Sample concentrations were calculated based on a standard curve and corrected for the dilution factor. Graphs were created with GraphPad Prism (10.5.0).

3.3.2 Results and Discussion

3.3.2.1 Barrier integrity of alveolar monocultures and co-cultures with macrophages after inflammation with LPS/IFN- γ and treatment with Citra01

The integrity of the alveolar epithelial barrier is essential for maintaining efficient gas exchange by preventing fluid accumulation within the airspace. In pulmonary inflammatory diseases, disruption of this barrier contributes to severe complications such as acute respiratory distress syndrome (ARDS), where increased permeability and impaired epithelial function promote alveolar flooding, as discussed in section 1.3.2 [58], [173]. To assess the barrier function *in vitro*, barrier integrity can be quantitatively measured by transepithelial electrical resistance (TEER), which reflects the tightness of intercellular junctions and overall epithelial cohesion [114]. Figure 4 summarizes the TEER development of alveolar epithelial monocultures and corresponding epithelial-macrophage co-cultures. Arlo-dTHP-1 co-cultures were used as a cell line-based model system and directly compared to an autologous primary co-culture of human alveolar epithelial cells (hAEPc) and human alveolar macrophages (hAM). This comparative approach was designed to assess whether the Arlo-based system recapitulates barrier responses observed in primary human cells. All models were investigated under four experimental conditions: untreated control (Ctrl), inflammatory stimulation with LPS and IFN- γ (Infl), inflammation combined with Citra01 treatment (Infl+Treat), and Citra01 treatment alone (Treat) as an additional control. Citra01 acts as an inhibitor of ACOD1, a key enzyme involved in immunometabolic reprogramming during pulmonary inflammation and infection [174], [175]. TEER measurements were performed to evaluate how pharmacological inhibition of ACOD1 by Citra01 affects alveolar barrier integrity under inflammatory conditions.

Figure 4A depicts TEER development in Arlo monocultures and in co-cultures with PMA-differentiated THP-1 (dTHP-1) cells, which were used to model macrophage-like cells and thereby approximate the cellular microenvironment of the alveoli [144]. TEER values were expressed relative to the initial resistance measured at the start of the experiment (T0), with each condition normalized to its own baseline. Following baseline assessment, epithelial-macrophage co-cultures were established and maintained for 24 h to allow stabilization and cellular adaptation (t24). Subsequently, cultures were subjected to control conditions, Citra01 treatment, inflammatory stimulation (LPS + IFN- γ), or a combination of inflammation and Citra01. Final TEER measurements were obtained 24 h after stimulation or treatment (t48). In control Arlo monocultures, TEER increased to approximately 240 % between T0 and t24, likely reflecting ongoing barrier maturation at day 10 of culture, at which point TEER development may not yet be fully completed. Between t24 and t48, TEER declined to approximately 150 %,

which may be attributable to the use of mixed medium lacking hydrocortisone, a factor known to support epithelial barrier stability [176]. Previous studies with hAELVi cells and dTHP-1 cells also showed a similar increase in TEER in mono and co-cultures even without stimulation, especially for ALI cultures [139]. Upon stimulation with LPS and IFN- γ , Arlo monocultures exhibited a further increase in TEER, reaching approximately 300 % at t48, suggesting enhanced barrier tightening in response to inflammatory cues. Inflammatory stimulation in pulmonary epithelial models is typically associated with reduced TEER values reflecting barrier disruption [140]. However, temporary TEER increases have been reported in other epithelial systems, such as Caco-2 cells, where exposure to *E. coli* induced a temporary elevation in TEER prior to subsequent decline [190]. A comparable increase of TEER was observed in inflamed cultures treated with Citra01. In contrast, Citra01 treatment alone did not alter TEER development relative to the control condition. The TEER development pattern in Arlo-dTHP-1 co-cultures largely mirrored that of monocultures. Control co-cultures showed an increase to approximately 150 % at t24, followed by only a modest decline (~15 %) until t48, in contrast to the more pronounced decrease observed in monocultures. Both inflammatory conditions (with or without Citra01) resulted in a similar TEER increase as seen in monocultures, reaching approximately 300 % of baseline at t48. As in monocultures, Citra01 treatment in the absence of inflammation did not significantly affect TEER compared to the control condition. Overall, the presence of dTHP-1 macrophage-like cells in co-culture showed only minor effects on barrier development under basal conditions, primarily reflected by a slightly attenuated TEER decline between t24 and t48. Under inflammatory conditions, TEER increased in both mono- and co-cultures, and this response was not modified by Citra01 treatment. Thus, neither the co-culture configuration nor ACOD1 inhibition by Citra01 substantially influenced TEER-based barrier integrity in this experimental setting.

Figure 4B shows the corresponding experiment performed with primary human alveolar epithelial cells (hAEPc) and autologous human alveolar macrophages (hAM). In hAEPc monocultures under control conditions, TEER steadily declined from baseline to approximately 40 % at t48. In contrast, inflammatory stimulation led to stabilization of TEER at approximately 100 % at t48 following a moderate decrease to around 80 % at t24, consistent with the inflammation-associated barrier tightening observed in Arlo cells. Treatment with Citra01 under inflammatory conditions did not alter TEER compared to inflammation alone. Citra01 treatment without inflammatory stimulation was not performed due to limited primary cell yield.

In autologous hAEPc-hAM co-cultures, TEER declined markedly from approximately 100 % to around 20 % across all conditions, indicating substantial barrier disruption. This effect was not observed in Arlo-dTHP-1 co-cultures, where the TEER kept increasing. The pronounced TEER reduction observed with primary hAM likely reflects differences in inflammatory responsiveness between primary macrophages and PMA-differentiated THP-1 cells. While

THP-1 cells are a convenient simplified model, they do not entirely reproduce the complex activation profiles and responses of primary human macrophages under pro-inflammatory conditions [177]. Notably, even in the absence of exogenous LPS and IFN- γ stimulation, under control conditions, hAM appeared to compromise epithelial barrier integrity, potentially due to activation during isolation procedures and cultivation.

Overall, Arlo monocultures and Arlo-dTHP-1 co-cultures exhibited a relative barrier-tightening response, particularly under inflammatory conditions, whereas primary hAEpC monocultures did not show TEER values exceeding baseline levels. In hAEpC monocultures, barrier integrity could be maintained under inflammatory stimulation but declined under control conditions. The presence of primary hAM, however, led to pronounced barrier disruption independent of additional inflammatory stimulation. Thus, under the present experimental conditions, the Arlo-based system does not fully recapitulate the barrier behavior observed in primary hAEpC-hAM co-cultures. One possible explanation may lie in the cellular phenotype, Arlo cells exhibit an AT2-like profile, whereas the primary hAEpC population represents a mixed AT2/AT1 population with ongoing differentiation toward an AT1-like phenotype. Given that AT1 cells are extremely thin and hardly proliferate, they may be more susceptible to inflammatory or mechanical stress compared to a stable AT2-like monolayer which also have a higher regenerative capacity [178]. Importantly, ACOD1 inhibition by Citra01 did not substantially influence barrier integrity in any experimental condition.

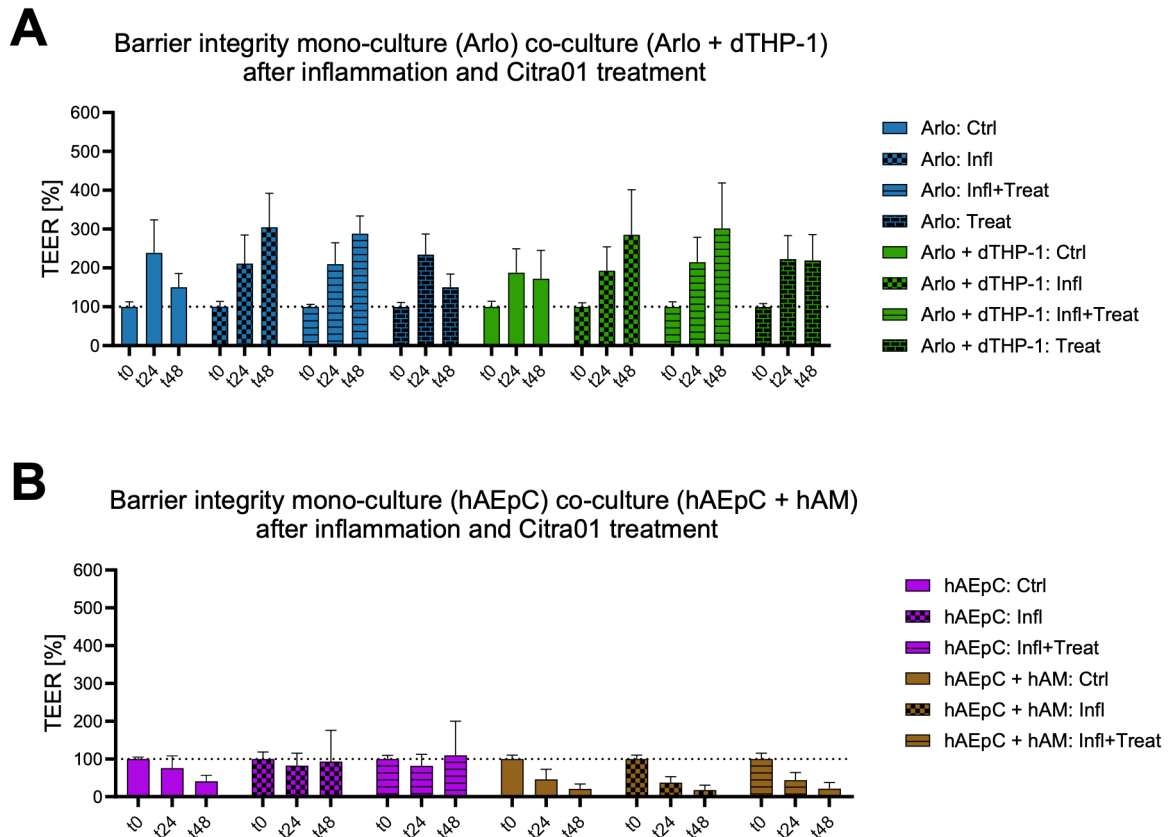


Figure 4: Transepithelial electrical resistance (TEER) development of Arlo and dTHP-1 monocultures and co-cultures under control, inflammatory, and Citra01-treated conditions. A) Normalized TEER values of Arlo monocultures (blue) and Arlo-dTHP-1 co-cultures (green). All values were normalized to the respective baseline TEER of each condition at T0. T0 represents baseline TEER prior to initiation of the experiment. Subsequently, co-cultures were assembled and allowed to equilibrate for 24 h (t24). After equilibration, cultures were exposed to control conditions (Ctrl), inflammation with 100 ng/mL LPS and 30 ng/mL IFN- γ (Infl), inflammation + 300 μ M Citra01 (Infl+Treat), or only Citra01 treatment (Treat). t48 represents TEER values measured 24 h after stimulation or treatment. (B) Corresponding TEER measurements of primary human alveolar epithelial cells (hAEpC; magenta) and autologous hAEpC-hAM co-cultures (brown) at T0 (baseline), t24 (after 24 h equilibration), and t48 (24 h post-treatment) under identical experimental conditions. All values were normalized to the respective baseline TEER of each condition at T0. Data represent 3–4 independent biological replicates (N = 3–4) with 6–12 technical replicates (n = 6–12).

3.3.2.2 Validation of Arlo and dTHP-1 cell separation following magnetic bead sorting

To independently evaluate the effects of inflammatory stimulation, Citra01 treatment, and co-culture conditions on epithelial cells and macrophages, magnetic bead-based cell separation was performed using EpCAM (epithelial cell adhesion molecule) as a positive selection marker. EpCAM is expressed on epithelial cells but absent on macrophages, enabling selective isolation of the epithelial fraction. The efficiency of cell separation was evaluated by RT-qPCR analysis of EpCAM (epithelial marker), CD45 (macrophage marker), and the reference genes β -actin (ACTB) and TATA-box binding protein (TBP) (Figure 5). CD45 was undetectable or detected only at very high Ct values (≥ 38) in Arlo cells obtained from both mono- and co-cultures, confirming the absence of CD45 expression in the epithelial cell line and indicating highly efficient separation of the epithelial fraction (Figure 5A) [179], [180]. As expected,

dTHP-1 cells from mono- and co-cultures showed strong CD45 expression (Ct 21–23), with no apparent differences between experimental conditions. EpCAM (Figure 5B) was strongly expressed in all Arlo samples (Ct 19–20) and was absent in dTHP-1 monocultures (Ct 34–35). In contrast, dTHP-1 cells isolated from co-cultures displayed intermediate EpCAM signals (Ct 24–25), suggesting minor epithelial carryover. However, based on the $2^{-\Delta Ct}$ method, a ΔCt of approximately 5 translates into an estimated ~32-fold difference in transcript abundance, indicating that separation was largely effective, although not entirely complete for the macrophage fraction. The reference genes ACTB and TBP (Figure 5C+D) were consistently expressed across all samples and conditions, supporting the technical reliability and comparability of the qPCR measurements.

The demonstrated separation efficiency ensured adequate cellular purity of the isolated fractions, thus enabling reliable downstream analyses, including ACOD1 activity measurements (Section 3.3.2.3) and transcriptomic profiling by RNA sequencing (Section 3.3.2.6 and 3.3.2.7). Separation of hAEpC and hAM could not be accomplished under the applied conditions, possibly reflecting tight intercellular interactions between epithelial cells and macrophages.

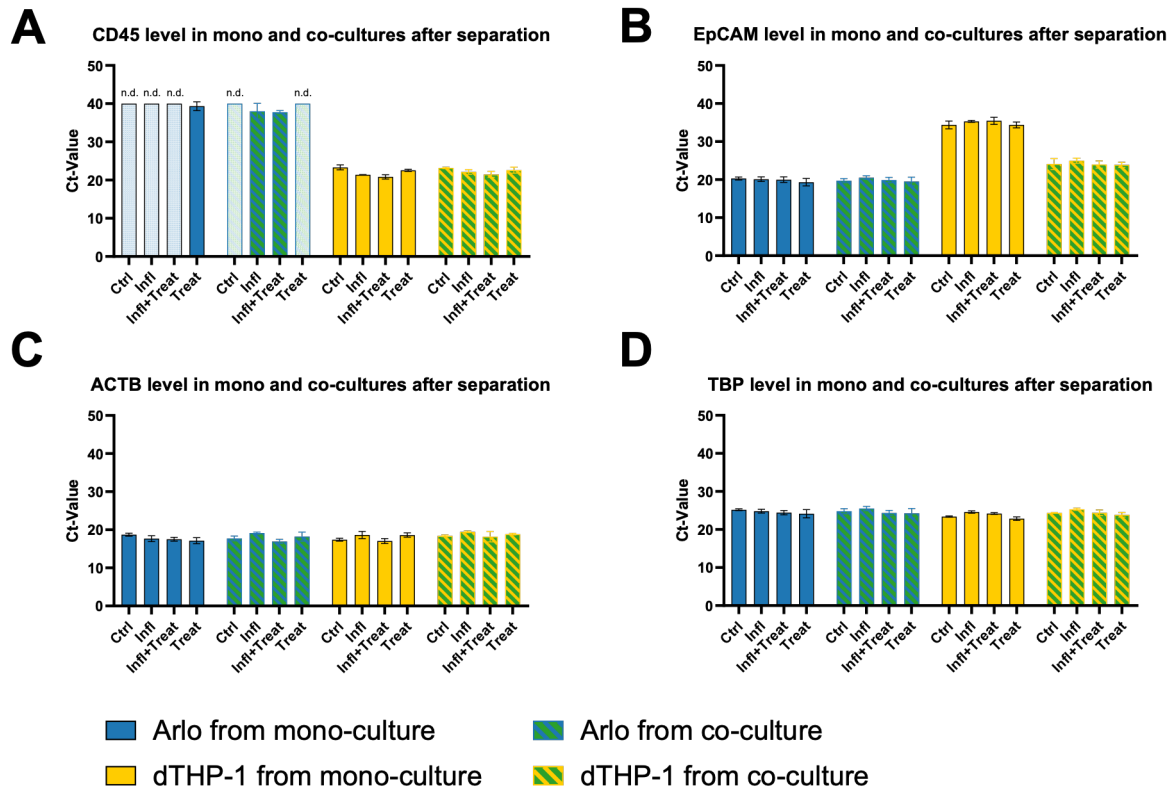


Figure 5: Gene expression analysis of Arlo epithelial cells and dTHP-1 macrophage-like cells following EpCAM-based magnetic bead sorting from mono- and co-cultures. (A) CD45, (B) EpCAM, (C) ACTB, and (D) TBP qPCR measurements in Arlo epithelial cells and dTHP-1 macrophage-like cells separated from mono- and co-cultures under control conditions (Ctrl), inflammatory stimulation with 100 ng/mL LPS and 30 ng/mL IFN- γ (Infl), inflammation combined with 300 μ M Citra01 treatment (Infl+Treat), and Citra01 treatment alone (Treat). Solid bars represent cells obtained from monoculture samples (Arlo, blue; dTHP-1, yellow), whereas striped bars indicate cells obtained from co-culture samples (Arlo, blue-green; dTHP-1, yellow-green). Less intense colors indicate that gene expression was not detectable (n.d.) within 40 PCR cycles ($Ct \geq 40$). Data represent three independent biological replicates ($N=3$), each measured in duplicate technical replicates ($n=6$).

3.3.2.3 Indirect ACOD1 activity assessment via Itaconate as primary metabolite

ACOD1 activity was assessed in mono- and co-cultures of Arlo, dTHP-1, hAEPc, and hAM to evaluate the effectiveness of Citra01 as an ACOD1 inhibitor. Activity was measured indirectly by quantifying itaconate, the product of the ACOD1-catalyzed reaction, which serves as a surrogate marker for ACOD1 enzymatic activity (Figure 6). ACOD1-mediated itaconate production is a key regulator of immunometabolic reprogramming during inflammation. In a situation-dependent manner, ACOD1 can exert both pro- and anti-inflammatory effects, influencing cytokine signaling, reactive oxygen species (ROS) generation, and antimicrobial host defense [181].

Figure 6A presents relative itaconate levels in Arlo and dTHP-1 monocultures as well as in Arlo-dTHP-1 co-cultures, normalized to the internal standard diphenhydramine.

In Arlo monocultures, no itaconate was detectable under any condition, indicating absence of measurable ACOD1 activity. In dTHP-1 monocultures, itaconate was likewise undetectable under control conditions, suggesting low basal ACOD1 activity. Following inflammatory

stimulation with LPS and IFN- γ , dTHP-1 cells exhibited marked ACOD1 activation, reflected by increased itaconate levels reaching approximately 0.3 (relative to diphenhydramine). This induction was effectively abrogated by Citra01 treatment, reducing itaconate levels to below the detection limit and thereby confirming the suitability and inhibitory efficacy of Citra01 toward ACOD1. Treatment with Citra01 alone did not induce detectable itaconate production, consistent with the absence of ACOD1 activation in non-inflamed conditions.

In Arlo-dTHP-1 co-cultures, itaconate was similarly detectable under inflammatory conditions and was substantially reduced upon combined inflammation and Citra01 treatment. Control and treatment-only conditions did not show measurable ACOD1 activity. Notably, inflammatory itaconate levels in co-cultures were lower than in dTHP-1 monocultures, despite identical cell numbers and experimental conditions. This observation may indicate epithelial-macrophage interactions that attenuate macrophage inflammatory activation within the co-culture system.

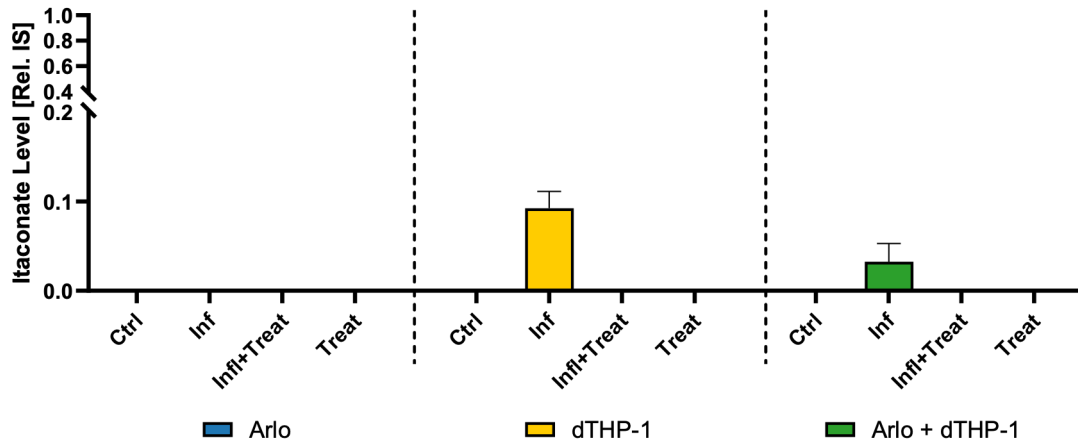
Figure 6B shows the corresponding data for primary hAEPc and hAM cultures. The Citra01-only condition was not included due to limited primary cell yield. Similar to Arlo cells, hAEPc monocultures showed no detectable itaconate under control conditions. Interestingly, inflamed hAEPc monocultures exhibited a small but detectable itaconate signal, which was abolished by Citra01 treatment. This finding was unexpected, as ACOD1 expression is typically associated with myeloid cells such as macrophages. However, recent reports have described inducible ACOD1 expression and itaconate production in epithelial cells, suggesting that epithelial ACOD1 expression may occur under specific inflammatory contexts [182], [183].

In hAM monocultures and hAEPc-hAM co-cultures, no itaconate was detected under control conditions. Upon inflammatory stimulation, both systems exhibited markedly elevated itaconate levels (~ 0.6), approximately twofold higher than those observed in dTHP-1 monocultures. This strong ACOD1 activation was almost completely reversed by Citra01 treatment, reducing itaconate levels to approximately 0.02 relative to diphenhydramine. In contrast to the Arlo-dTHP-1 model, no attenuation of macrophage-derived itaconate production by epithelial cells was observed in the primary hAEPc-hAM co-culture, as inflammatory levels were comparable to hAM monocultures.

Overall, these results confirm the mode of action and inhibitory efficacy of Citra01 in both model systems. While Citra01 activity in dTHP-1 monocultures had been demonstrated previously, the present data extend these findings to co-culture conditions. The observed attenuation of macrophage activation in the Arlo-based system, but not in the primary cell model, suggests model-specific epithelial-macrophage interactions that warrant further investigation, particularly at the transcriptomic level. Differences in signal intensity between Arlo/dTHP-1 and primary systems are likely attributable to the comparatively lower inflammatory responsiveness of differentiated THP-1 cells relative to primary human alveolar macrophages [177].

A

ACOD1 activity in Arlo & dTHP-1 mono-cultures and co-cultures after 24h

**B**

ACOD1 activity in hAEpC & hAM mono-cultures and co-cultures after 24h

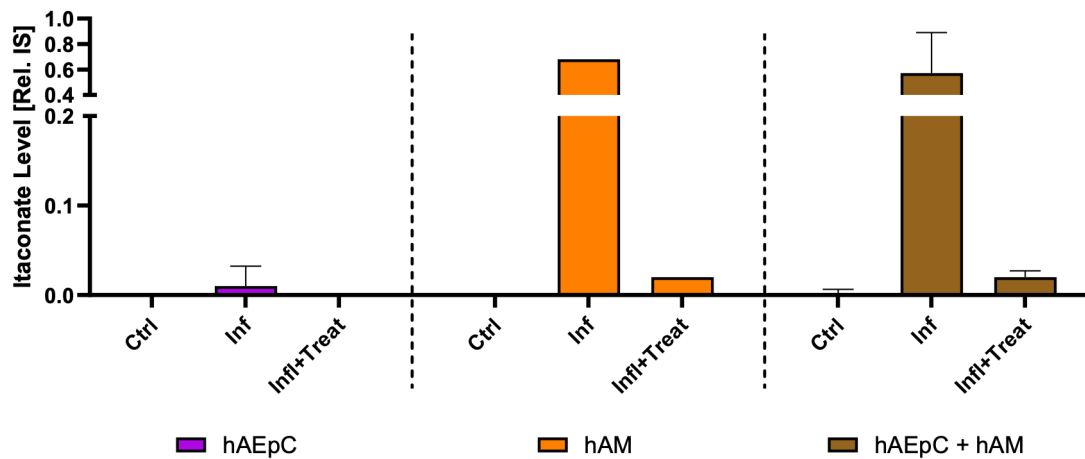


Figure 6: Indirect assessment of ACOD1 activity by quantification of itaconate levels in mono- and co-culture systems of Arlo and dTHP-1, as well as hAEpC and hAM. (A) Relative itaconate levels in Arlo (blue) and dTHP-1 (yellow) monocultures as well as Arlo-dTHP-1 co-cultures (green) under control (Ctrl), inflammatory stimulation with 100 ng/mL LPS and 30 ng/mL IFN- γ (Inf), inflammation combined with 300 μ M Citra01 treatment (Inf+Treat), and Citra01 treatment alone (Treat) conditions. Itaconate levels were normalized to the internal standard diphenhydramine. (B) Relative itaconate levels in primary human alveolar epithelial cells (hAEpC; purple), human alveolar macrophages (hAM; orange), and hAEpC-hAM co-cultures (brown) under control, inflammatory, and inflammation combined with Citra01 treatment conditions. The Citra01-only condition was not included due to limited primary cell yield. Absence of a visible bar indicates that itaconate levels were below the detection limit. Data represent three independent biological replicates (N=3), except hAM with N=1.

3.3.2.4 Role of ACOD1 in the regulation of oxidative stress during Inflammation in mono- and co-cultures of Arlo and dTHP-1

Itaconate, generated by ACOD1, inhibits succinate dehydrogenase and thereby limits mitochondrial production of reactive oxygen species (ROS) [181], [184]. While this mechanism protects against excessive oxidative stress during inflammation, it may also restrict effective pathogen clearance [64]. In addition, itaconate signaling promotes activation of the antioxidant

transcription factor NRF2, enhancing the expression of antioxidant genes while suppressing pro-inflammatory gene expression [181], [185]. To investigate the contribution of this pathway to redox regulation under inflammatory conditions, we quantified total ROS levels, lipid peroxidation, glutathione content, and superoxide dismutase activity in Arlo and dTHP-1 monocultures as well as in the corresponding co-culture systems under control (Ctrl; non-inflammatory control) conditions, inflammation with LPS + IFN- γ (Infl), inflammation plus treatment with Citra01 (Infl+Treat), only Citra01 treatment (Treat), and H₂O₂ control conditions (H₂O₂; Inflammatory control) (Figure 7).

Figure 7A shows the assessment of general ROS levels using the DCFDA (2,7-dichlorofluorescein diacetate) assay. DCFDA enters cells, is deacetylated by intracellular esterases, and subsequently oxidized by ROS to fluorescent DCF, enabling quantitative measurement of total ROS. However, the assay does not distinguish between specific ROS species [186]. Arlo monocultures exhibited the highest overall ROS levels; however, no significant differences were observed between Ctrl, Infl, Infl+Treat, or Treat conditions, except for the H₂O₂ positive control, which showed the expected increase in fluorescence. dTHP-1 monocultures displayed a comparable pattern, with no condition-dependent differences apart from the positive control, but overall signal intensities were approximately one third of those detected in Arlo cells. This reduced ROS signal in dTHP-1 cells may reflect several factors, including their non-proliferative state following PMA differentiation, in contrast to the highly proliferative Arlo cells, which likely exhibit higher metabolic activity and mitochondrial ROS production [187]. Additionally, differences in intracellular esterase activity or efflux pump expression may have influenced DCFDA signal intensity [186], [188]. Co-cultures of Arlo and dTHP-1 demonstrated increased ROS levels compared to dTHP-1 monocultures, reaching approximately 80–85 % of the signal observed in Arlo monocultures, while maintaining the same overall pattern with no condition-dependent differences except for H₂O₂ stimulation. The absence of an inflammation-induced ROS increase was unexpected but may be attributable to the LPS concentration used (100 ng/mL), which, although sufficient for previous inflammatory readouts, may not have been high enough to robustly induce ROS production via TLR4 signaling, particularly as other studies have employed substantially higher LPS concentrations ranging from 1–40 μ g/mL to elicit measurable ROS responses [189].

Figure 7B depicts intracellular glutathione (GSH) levels in Arlo and dTHP-1 mono- and co-cultures under the same experimental conditions. As ACOD1-derived itaconate can modulate GSH homeostasis via NRF2 activation, total GSH levels were assessed. Arlo monocultures displayed stable GSH concentrations of approximately 0.6 mM across Ctrl, Infl, Infl+Treat, and Treat conditions, with a slight reduction to ~0.5 mM in the H₂O₂ group, consistent with oxidative consumption of GSH. A comparable pattern was observed in dTHP-1 monocultures and Arlo-dTHP-1 co-cultures. The overall stability of GSH levels suggests that no pronounced oxidative

imbalance was induced under the applied inflammatory conditions after 24 h. This is in agreement with literature reporting a transient early decline in GSH within the first 6 h after LPS exposure (10 ng/mL), followed by recovery to baseline levels at 24 h [190]. As the inflammatory incubation time in the present study was 24 h, the absence of significant differences is consistent with these observations. To detect potential early redox alterations, shorter exposure times should be further investigated.

Figure 7C shows malondialdehyde (MDA) levels as a marker of lipid peroxidation in Arlo and dTHP-1 monocultures as well as in the corresponding co-culture model under Ctrl, Infl, Infl+Treat, Treat, and H₂O₂ conditions. In Arlo monocultures, MDA levels were highest under control conditions and showed a tendency to decrease under inflammatory and H₂O₂ treatment conditions. Pharmacological inhibition of ACOD1 using Citra01 did not alter the inflammation-associated decrease in MDA levels. A similar pattern was observed in dTHP-1 monocultures, where overall MDA levels were lower compared to Arlo, likely reflecting reduced cell numbers due to the non-proliferative state of PMA-differentiated dTHP-1 cells [187]. Again, no marked increase in lipid peroxidation was detected upon inflammatory stimulation. In the Arlo-dTHP-1 co-culture model, MDA levels were comparable to Arlo monocultures and likewise did not exhibit a pronounced increase in response to inflammation or oxidative challenge, but rather a slight decrease. The absence of an inflammation-induced rise in MDA suggests that the applied inflammatory conditions did not result in sustained lipid peroxidation at the 24 h post-stimulation time point. The low MDA levels are consistent with the stable GSH and total ROS measurements at 24 h. Although literature reports increased lipid peroxidation in PMA-differentiated THP-1 macrophages after stimulation with 200 ng/mL LPS, the lower concentration used in this study (100 ng/mL) may have been sufficient to induce inflammatory signaling but not strong enough to cause sustained membrane oxidation [191]. Higher LPS doses or earlier time points may therefore be required to detect transient lipid peroxidation in this model.

Figure 7D presents superoxide dismutase (SOD) activity in Arlo and dTHP-1 monocultures as well as in the corresponding co-culture model under Ctrl, Infl, Infl+Treat, Treat, and H₂O₂ conditions. SOD catalyzes the dismutation of superoxide anions (O₂^{•-}) into hydrogen peroxide (H₂O₂) thereby constituting a central enzymatic defense against oxidative stress. Across all culture systems, no pronounced differences between experimental conditions were observed. However, dTHP-1 monocultures exhibited overall lower SOD activity compared to Arlo, suggesting greater superoxide availability. This difference may again be influenced by variations in cell number and proliferative status. The Arlo-dTHP-1 co-culture displayed SOD activity comparable to Arlo monocultures, further supporting the notion that the epithelial component predominates in shaping the overall antioxidative capacity of the system. In line with the previous experiments, literature reports that stimulation of PMA-differentiated THP-1

macrophages with 200 ng/mL LPS results in reduced SOD activity and consequently increased superoxide levels after 24 h, indicating that higher inflammatory strength may be required to induce measurable impairment of antioxidative defenses [191].

Collectively, the combined ROS, GSH, MDA, and SOD analyses indicate that the applied inflammatory setting did not induce substantial oxidative stress at the 24 h time point. Specifically, stimulation with 30 ng/mL IFN- γ and 100 ng/mL LPS was sufficient to induce inflammatory responses in prior analyses of barrier integrity (Section 3.3.2.1) and ACOD1 activity (Section 3.3.2.3) but not strong enough to provoke sustained redox imbalance or lipid peroxidation. Higher LPS concentrations and/or earlier time points may therefore be required to reveal more pronounced oxidative alterations in the Arlo-dTHP-1 model.

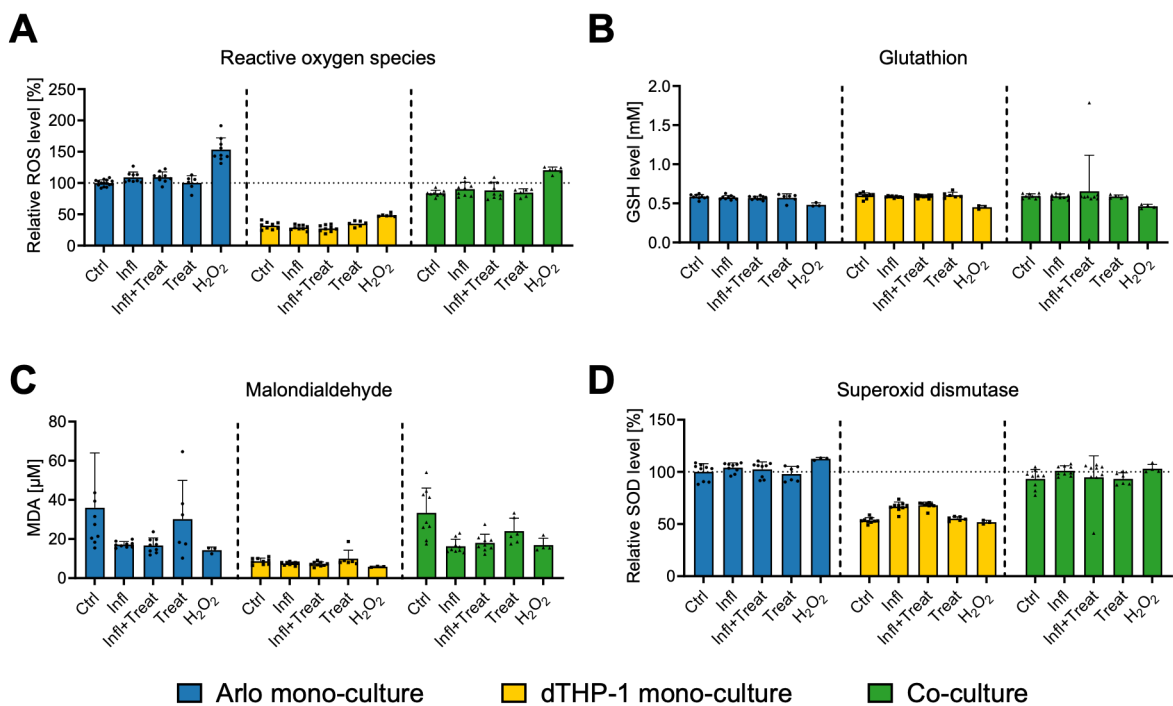


Figure 7: Multiparametric assessment of oxidative stress in mono- and co-culture systems of Arlo and dTHP-1. (A) Relative total ROS levels in Arlo (blue) and dTHP-1 (yellow) monocultures as well as Arlo-dTHP-1 co-cultures (green) under control (Ctrl), inflammatory stimulation with 30 ng/mL IFN- γ and 100 ng/mL LPS (Infl), inflammation combined with 300 μ M Citra01 treatment (Infl+Treat), Citra01 treatment alone (Treat), and 500 μ M H₂O₂ positive control conditions. (B) Intracellular glutathione (GSH) levels in Arlo and dTHP-1 monocultures as well as in Arlo-dTHP-1 co-cultures under the same experimental conditions. (C) Malondialdehyde (MDA) levels as a marker of lipid peroxidation in Arlo and dTHP-1 monocultures as well as in Arlo-dTHP-1 co-cultures under the indicated conditions. (D) Relative superoxide dismutase (SOD) activity in Arlo and dTHP-1 monocultures as well as in Arlo-dTHP-1 co-cultures under the respective conditions. Data represent independent biological replicates (N) with corresponding technical replicates (n): Ctrl, Infl, and Infl+Treat (N=3, n=9); Treat (N=2, n=6); H₂O₂ (N=1, n=4).

3.3.2.5 Modulation of PD-L1 under inflammatory conditions and ACOD1 inhibition

In addition to its role in inflammatory and infectious diseases, itaconate has been implicated in tumor immune escape via modulation of programmed death-ligand 1 (PD-L1). PD-L1 mediates immune evasion by binding to PD-1 on activated T cells and suppressing their immune function. Beyond cytokine-driven transcriptional regulation, PD-L1 stability is controlled by

ubiquitination and subsequent proteasomal degradation. ACOD1-derived itaconate has been reported to stabilize PD-L1 through cysteine alkylation, thereby limiting its degradation and promoting immune escape of tumor cells [67]. We therefore investigated whether ACOD1 activity modulates PD-L1 expression in Arlo and dTHP-1 mono- and co-cultures under control (Ctrl), inflammatory stimulation with IFN- γ and LPS (Infl), and inflammation combined with Citra01 treatment (Infl+Treat) (Figure 8).

Figure 8 presents PD-L1 levels measured in supernatants collected from Arlo and dTHP-1 monocultures as well as from Arlo-dTHP-1 co-cultures. The calibration standards yielded the expected values; however, none of the experimental conditions produced signals above the lower limit of quantification (LLOQ), limiting the interpretability of the data as all sample values required extrapolation beyond the reliable range of the calibration curve.

Despite this limitation, a trend toward increased PD-L1 levels under inflammatory stimulation was observed across culture systems, with the most pronounced effect in dTHP-1 monocultures. This increase may be attributable to ACOD1 induction and subsequent itaconate production following LPS and IFN- γ stimulation. Treatment with Citra01 appeared to attenuate this effect. However, given that all values were below the LLOQ, these observations must be interpreted with considerable caution.

Overall, the data suggest a potential modulation of PD-L1 under inflammatory conditions, but definitive conclusions cannot be drawn due to the insufficient signal intensity. Future experiments using cell lysates rather than supernatants may provide greater sensitivity and enable more reliable quantification, as PD-L1 is primarily membrane-bound and may not be robustly detectable in the supernatant of the cells.

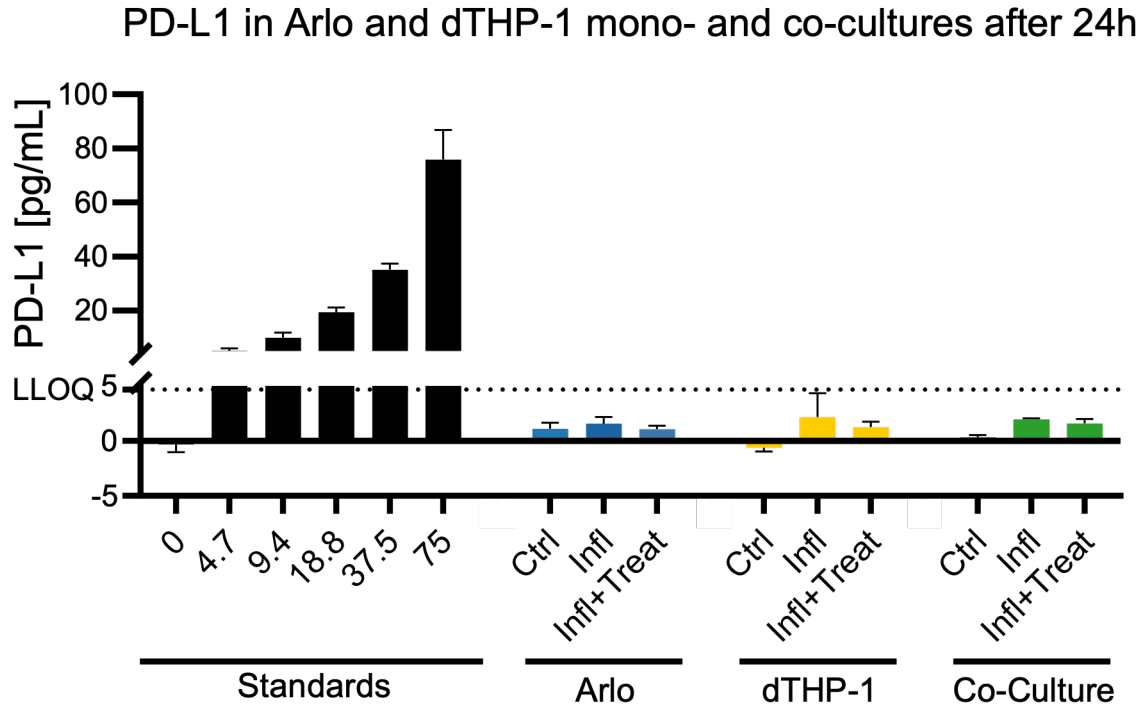


Figure 8: PD-L1 levels in mono- and co-culture systems of Arlo and dTHP-1. Relative PD-L1 concentrations measured in supernatants of Arlo (blue) and dTHP-1 (yellow) monocultures as well as Arlo-dTHP-1 co-cultures (green) under control (Ctrl), inflammatory stimulation with 30 ng/mL IFN- γ and 100 ng/mL LPS (Infl), and inflammation combined with 300 μ M Citra01 treatment (Infl+Treat). Calibration standards are shown in black. The lower limit of quantification (LLOQ) was 4.7 pg/mL. Data represent three independent biological replicates (N=3).

3.3.2.6 Transcriptomic analysis of intercellular crosstalk between Arlo and dTHP-1 cells in co-cultures compared to mono-cultures

To elucidate the mechanisms underlying the observed effects and to characterize intercellular crosstalk between Arlo and dTHP-1 cells in co-culture, we performed RNA sequencing. To enable direct comparison, Arlo-dTHP-1 co-cultures as well as Arlo and dTHP-1 monocultures were analyzed under control (Ctrl) conditions, inflammatory stimulation with LPS + IFN- γ (Infl), inflammation combined with Citra01 treatment (Infl+Treat), and Citra01 treatment alone (Treat).

Prior to RNA sequencing, quality control was performed to assess RNA purity and integrity. Absorbance was measured at 230, 260, and 280 nm to determine purity ratios, and RNA integrity numbers (RIN) were obtained to evaluate RNA degradation. All samples met the predefined quality criteria, with 260/230 ratios ≥ 1.8 , indicating minimal contamination by residual salts or organic compounds from the isolation process, and 260/280 ratios between 1.8 and 2.2, consistent with low protein contamination. RIN values were ≥ 8 for all samples, demonstrating high RNA integrity and suitability for downstream sequencing. Detailed values for each individual sample are provided in the Supplementary Information of this thesis. (Supplementary Table S1)

Figure 9 presents volcano plots comparing the transcriptomic profiles of Arlo cells cultured in monoculture with those cultured in co-culture with dTHP-1 macrophages under the different experimental conditions. This analysis was performed to investigate whether the presence of macrophages induces transcriptional changes in epithelial Arlo cells, potentially indicating cellular crosstalk within the co-culture system. Under control conditions (Figure 9A), only a limited number of genes were differentially expressed between mono- and co-cultured Arlo cells, with 12 genes upregulated and 14 genes downregulated in the co-culture. Considering that more than 11,000 genes were detected in total, this comparatively small number of regulated genes suggests that the presence of dTHP-1 cells has only a minor effect on the transcriptional profile of Arlo cells. A slightly higher number of differentially expressed genes was observed under inflammatory stimulation (Figure 9B), where 39 genes were upregulated and 25 downregulated in co-cultured Arlo cells compared to monoculture. Similar trends were observed for the inflammation combined with Citra01 treatment condition (Figure 9C), with 26 genes upregulated and 35 downregulated, and for the Citra01-only condition (Figure 9D), where 39 genes were upregulated and 21 genes downregulated. Despite these modest increases in regulated genes under stimulated conditions, the overall number of differentially expressed genes remained small relative to the total number of detected transcripts. Moreover, several of the identified differentially expressed entries corresponded to pseudogenes or genes without clear annotation (NA), further reducing the number of biologically meaningful candidates. Given the relatively small sample size ($N = 3$), some of the detected differences may therefore also reflect stochastic variation rather than consistent biological effects. Taken together, these findings indicate that co-culture with dTHP-1 macrophages induces only limited transcriptional alterations in Arlo cells across the tested conditions, suggesting that the epithelial transcriptome is largely preserved in the presence of macrophages in this experimental system.

Previous studies using hAELVi cells in co-culture with dTHP-1 macrophages likewise reported only minor effects of the co-culture on epithelial barrier integrity compared to epithelial monocultures [139]. At the same time, macrophage-epithelial interactions in *legionella pneumophila* infections in similar systems have been described to occur via paracrine signaling mediated by macrophage-derived cytokines that modulate epithelial responses [192]. However, direct cell-cell communication between macrophages and epithelial cells has also been reported, for example through surface receptor interactions, immune checkpoint molecules, or gap junction-mediated signaling [69]. Nevertheless, the limited number of differentially expressed genes observed in Arlo cells in the present RNA-seq analysis suggests that, within the experimental setup used here, macrophage-epithelial communication does not lead to extensive transcriptional reprogramming of epithelial cells. This may partly reflect the experimental configuration, in which THP-1 cells were differentiated with PMA prior to co-

culture, during which they strongly adhere to the plastic surface of the cell culture flask. For the co-culture experiments, these differentiated macrophages were subsequently detached and transferred onto the epithelial monolayer, where they remained only loosely associated with the epithelial layer, potentially limiting the formation of stable cell-cell contacts.

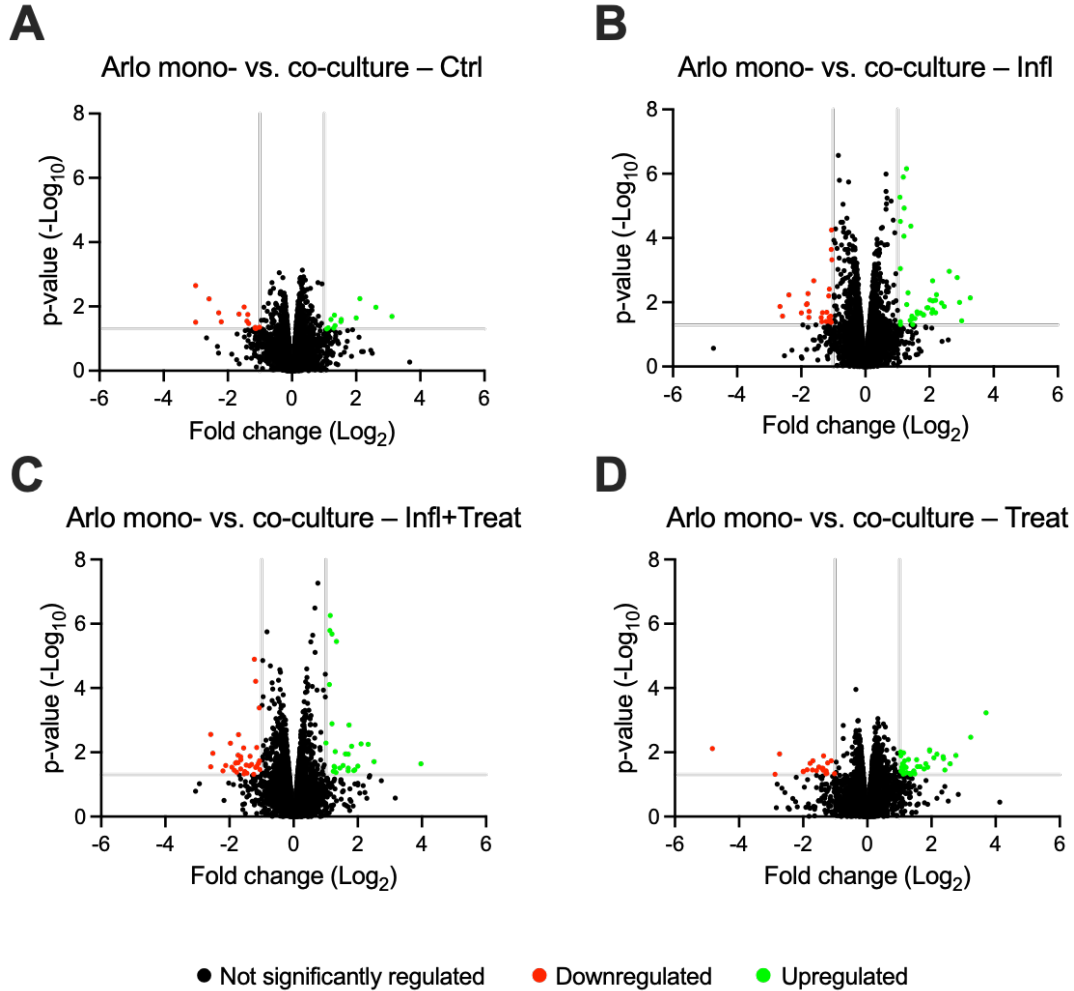


Figure 9: Transcriptomic impact of co-culture with dTHP-1 on Arlo cells. Volcano plots showing differential gene expression in Arlo cells cultured in monoculture compared to Arlo cells cultured together with dTHP-1 in co-culture under (A) control (Ctrl), (B) inflammatory stimulation (Infl), (C) inflammation combined with Citra01 treatment (Infl+Treat), and (D) Citra01 treatment alone (Treat) conditions. Inflammatory stimulation was performed using 30 ng/mL IFN- γ and 100 ng/mL LPS, and Citra01 was applied at 300 μ M. The x-axis represents $\log_2$ fold change (co-culture vs monoculture), and the y-axis shows $-\log_{10}$ adjusted p-values. Genes meeting the significance criteria of $|\log_2\text{FC}| \geq 1$, corresponding to at least a two-fold change in expression, and an adjusted p-value ≤ 0.05 ($-\log_{10} p \geq 1.3$) are highlighted in green (upregulated in co-culture) and red (downregulated in co-culture), while non-significant genes are shown in black. Vertical and horizontal lines indicate the respective fold change and significance thresholds. Data represent three independent biological replicates (N=3)

In contrast to the epithelial cells, a markedly higher number of genes appeared to be differentially expressed in dTHP-1 cells when comparing monoculture and co-culture conditions. Figure 10 shows the corresponding volcano plots for dTHP-1 cells. Under control conditions (Figure 10A), approximately 1,800 genes were upregulated and around 900 genes were downregulated in co-cultured macrophages compared to monoculture. Similarly large numbers of regulated genes were observed under inflammatory stimulation (Figure 10B),

inflammation combined with Citra01 treatment (Figure 10C), and Citra01 treatment alone (Figure 10D). At first glance, these results suggest that the presence of epithelial cells strongly influences the transcriptional profile of macrophages in the co-culture system.

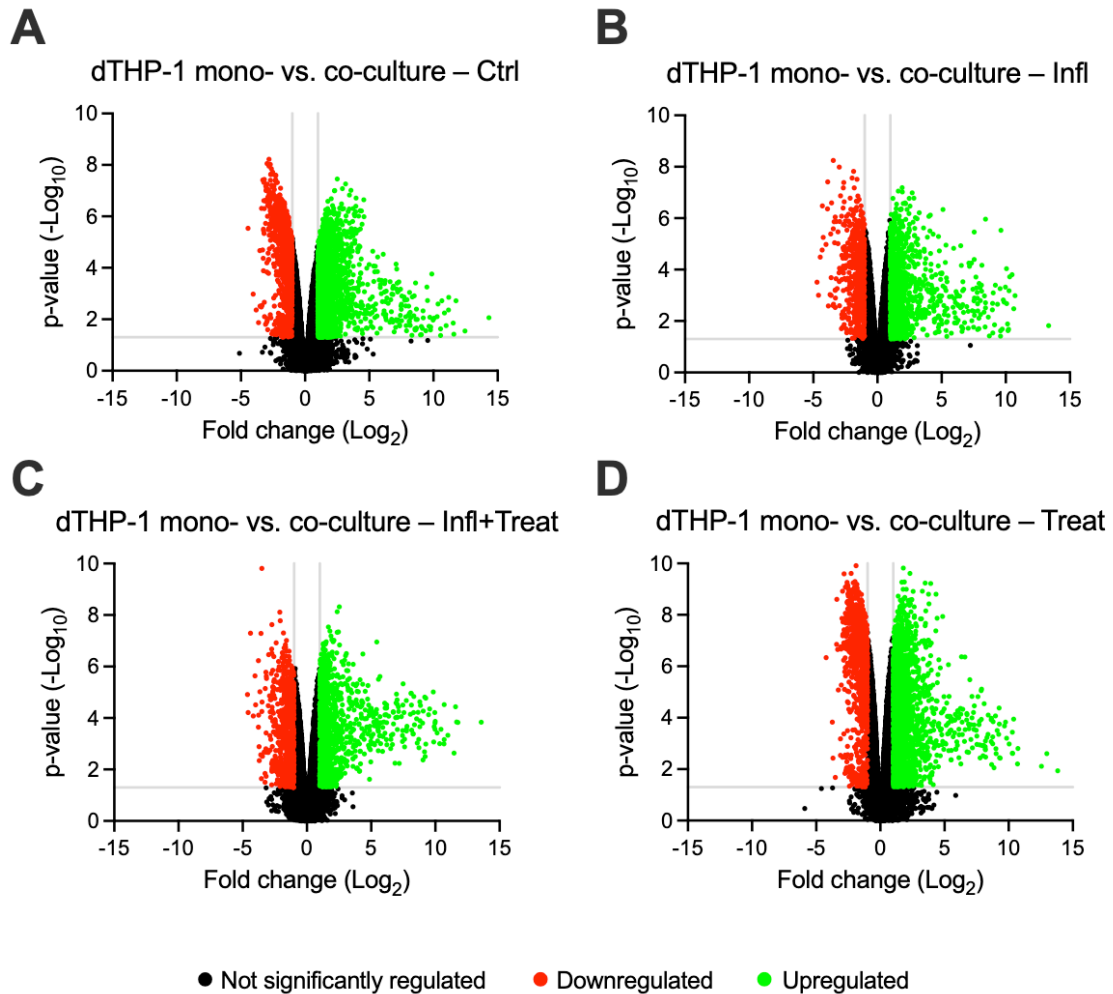


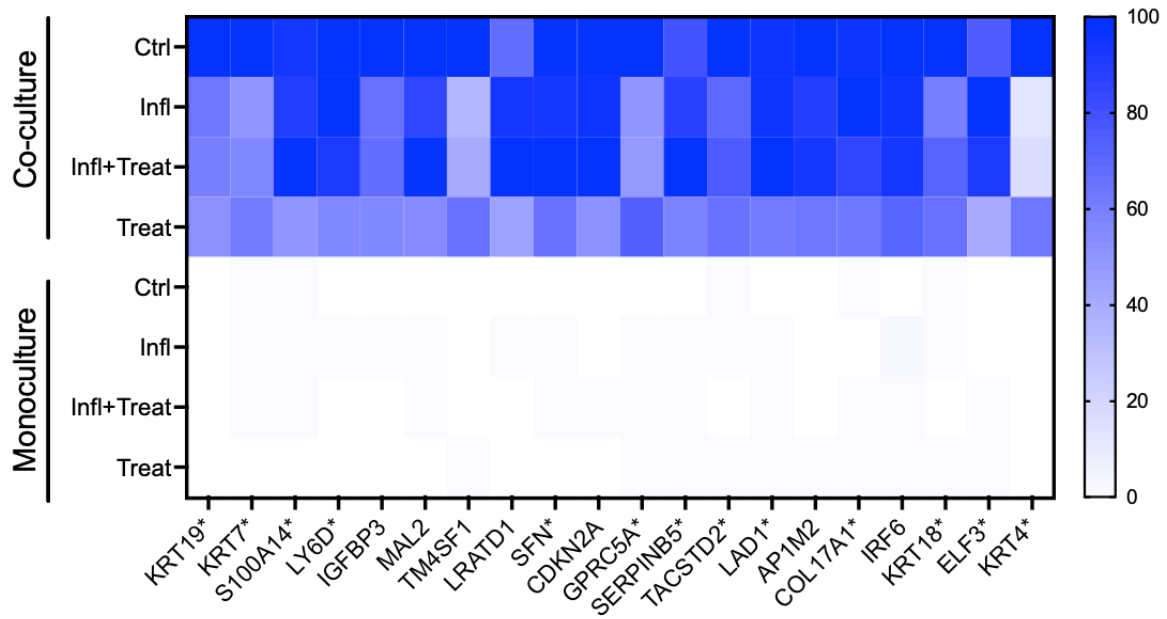
Figure 10: Transcriptomic impact of co-culture with Arlo on dTHP-1 cells. Volcano plots showing differential gene expression in dTHP-1 cells cultured in monoculture compared to dTHP-1 cells cultured together with Arlo in co-culture under (A) control (Ctrl), (B) inflammatory stimulation (Infl), (C) inflammation combined with Citra01 treatment (Infl+Treat), and (D) Citra01 treatment alone (Treat) conditions. Inflammatory stimulation was performed using 30 ng/mL IFN- γ and 100 ng/mL LPS, and Citra01 was applied at 300 μ M. The x-axis represents $\log_2$ fold change (co-culture vs monoculture), and the y-axis shows $-\log_{10}$ adjusted p-values. Genes meeting the significance criteria of $|\log_2FC| \geq 1$, corresponding to at least a two-fold change in expression, and an adjusted p-value ≤ 0.05 ($-\log_{10} p \geq 1.3$) are highlighted in green (upregulated in co-culture) and red (downregulated in co-culture), while non-significant genes are shown in black. Vertical and horizontal lines indicate the respective fold change and significance thresholds. Data represent three independent biological replicates (N=3)

However, this initially observed strong transcriptional effect must be interpreted with caution. Following co-culture, separation of macrophages and epithelial cells was performed prior to RNA extraction. Although this procedure removed the majority of epithelial cells, complete separation could not be achieved. Quality control of the separation by qPCR analysis of the epithelial marker EpCAM (Section 3.3.2.2) revealed an approximately 32-fold difference between the fractions, indicating efficient but not complete separation. While this level of enrichment suggests a largely successful isolation of the macrophage fraction, it may still be

insufficient for highly sensitive methods such as RNA sequencing. Consequently, residual epithelial cells in the macrophage samples may have contributed epithelial transcripts to the RNA-seq dataset. To further investigate whether the observed transcriptional differences reflect true macrophage responses or are influenced by epithelial contamination, the most strongly regulated genes were examined in more detail.

A

Top 20 upregulated genes in dTHP-1 co-culture vs monoculture (Ctrl)

**B**

Top 20 downregulated genes in dTHP-1 co-culture vs monoculture (Ctrl)

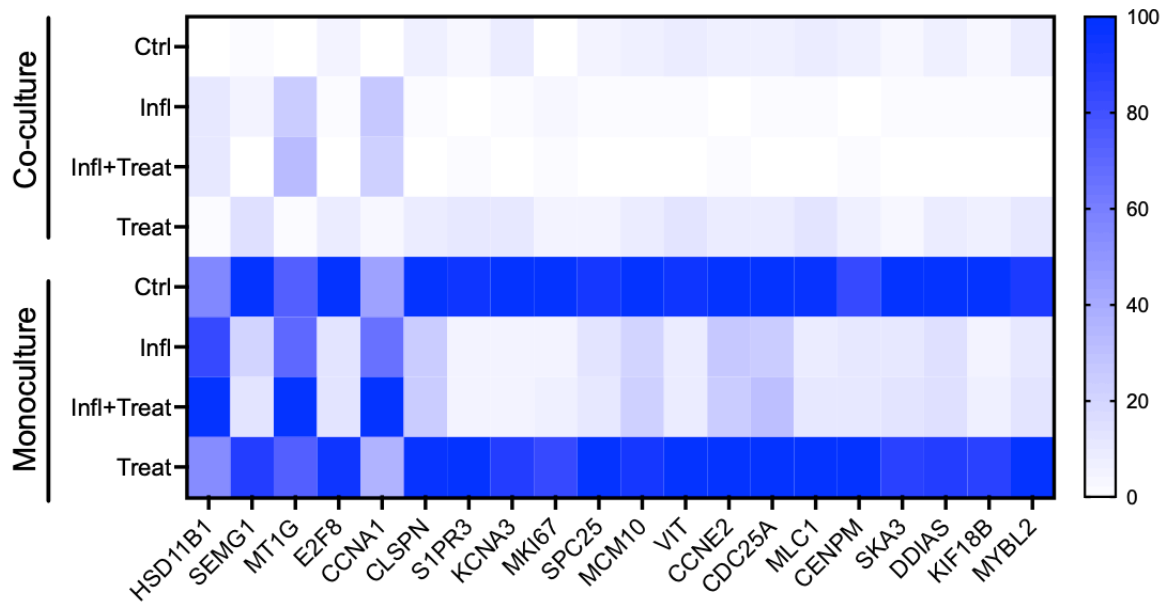


Figure 11: Heatmap of the top genes up- and downregulated in dTHP-1 cells in Arlo-dTHP-1 co-culture compared to monoculture under control (Ctrl) conditions (A) Heatmap showing the top 20 genes upregulated in dTHP-1 cells in Arlo-dTHP-1 co-culture relative to dTHP-1 monoculture under control conditions. (B) Heatmap showing the top 20 genes downregulated in dTHP-1 cells in Arlo-dTHP-1 co-culture relative to monoculture under control conditions. The experimental conditions comprised control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M Citra01). Gene expression values represent mean expression levels of biological replicates and were column-wise min-max normalized for visualization. Color intensity reflects relative expression levels, with high expression shown in blue and low expression in white. Genes are displayed along the x-axis, and the respective mono- and co-culture conditions are shown along the y-axis. Genes annotated as epithelial according to the Human Protein Atlas are marked with an asterisk (*) in panel A [193]. Data represent three independent biological replicates (N=3)

Figure 11 shows a heatmap of the 20 most upregulated (Figure 11A) and downregulated (Figure 11B) genes in dTHP-1 cells in co-culture compared to monoculture. Notably, several of the most strongly upregulated genes correspond to epithelial-associated transcripts. Genes annotated as epithelial according to the Human Protein Atlas are marked with an asterisk in Figure 11A [193]. In total, 13 out of the 20 most upregulated genes can be clearly assigned to epithelial cells, excluding genes that may also be expressed in macrophages. This observation indicates that residual epithelial cells remained in the macrophage fraction after separation and contributed epithelial transcripts to the RNA-seq dataset. Consequently, the large number of differentially expressed genes observed in Figure 10 likely overestimates the true transcriptional changes occurring in macrophages upon co-culture. These findings highlight the technical challenge of achieving complete cell-type separation in co-culture systems and underline the need for careful interpretation of cell-type-specific transcriptomic data derived from such experimental setups. Additional heatmaps of regulated genes across the different conditions are provided in the Supplementary Figures Supplementary Figure S1–S7.

In summary, transcriptomic analysis revealed only limited evidence for direct macrophage-epithelial crosstalk in the Arlo-dTHP-1 co-culture system. Across all tested conditions, the presence of dTHP-1 macrophages induced only minor transcriptional changes in Arlo cells, suggesting that the epithelial transcriptome remains largely stable in the presence of macrophages. Macrophage-epithelial interactions in similar systems have been described to occur through multiple mechanisms, including paracrine signaling mediated by soluble factors as well as direct cell-cell communication [69]. Nevertheless, the limited number of differentially expressed genes observed in Arlo cells in the present RNA-seq analysis indicates that such interactions may not necessarily result in pronounced transcriptional changes in epithelial cells or reflect only minimal crosstalk between both cell types under the experimental conditions applied. While initial analyses suggested pronounced transcriptional changes in dTHP-1 cells upon co-culture, further investigation indicated that these observations were largely influenced by residual epithelial transcripts remaining after cell separation. The limited transcriptional interaction observed here may additionally be influenced by the experimental configuration of the model system, which may restrict stable cell-cell contacts between both cell types. Future studies could therefore explore alternative approaches, such as differentiating THP-1 cells directly within the co-culture system or employing primary monocyte-derived macrophages (MDM) or human alveolar macrophages (hAMs), to promote stronger and potentially more physiologically relevant macrophage-epithelial interactions.

3.3.2.7 Transcriptomic responses of Arlo and dTHP-1 monocultures to inflammatory stimulation and Citra01 treatment

After investigating the effects of co-culture on the individual cell types, the impact of inflammatory stimulation and, more importantly, Citra01 treatment on the transcriptomic

profiles of Arlo and dTHP-1 cells was analyzed. As the culture conditions (mono- vs. co-culture) appeared to have only a minor influence on Arlo cells and the analysis of dTHP-1 cells is clearer in monoculture due to the absence of epithelial contamination, subsequent transcriptomic analyses were performed using Arlo and dTHP-1 monocultures.

Figure 12 shows volcano plots comparing the different experimental conditions in Arlo cells. Panel A shows Infl vs Ctrl, B shows Treat vs Ctrl, C shows Treat vs Infl, D shows Infl+Treat vs Ctrl, E shows Infl+Treat vs Infl, and F shows Treat vs Infl+Treat. In panel A, it is clearly visible that inflammatory stimulation with LPS and IFN- γ significantly alters the transcriptome of Arlo cells, resulting in both up- and downregulation of numerous genes. A heatmap of the 20 most up- and downregulated genes is provided in the Supplementary Material (Supplementary Figure S8). The corresponding heatmaps showing the 20 most up- and downregulated genes for each comparison are provided in Supplementary Figure S8–S13. The upregulated genes include several chemokines (e.g., CXCL9, CXCL10, CXCL11), genes related to antigen presentation (e.g., HLA-DPA1, HLA-DQA1 and others), as well as genes involved in immune regulation (e.g., IDO1, SERPING1), indicating a successful inflammatory response [193]. ACOD1 was not among the upregulated genes, which is consistent with its low expression in epithelial cells as discussed previously. Comparison of the volcano plots in panels A (Ctrl vs Infl) and D (Ctrl vs Infl+Treat) suggests that treatment with Citra01 has only minor effects on the transcriptome. Similarly, panel B (Ctrl vs Treat) shows very few regulated genes, indicating that Citra01 treatment alone does not substantially alter gene expression in Arlo cells. Consistent with this observation, the comparison between Infl and Infl+Treat in panel E also reveals only minimal transcriptional differences. After excluding pseudogenes and genes without annotation (NA), only one upregulated gene in the Infl+Treat samples (LTB; lymphotoxin- β , involved in immune response regulation) and two downregulated genes (KCNF1; potassium voltage-gated channel modifier subfamily F member 1, a potassium channel regulator, and CCN5; cellular communication network factor 5, involved in bone turnover) were identified, as shown in the heatmap in Supplementary Figure S12 [193]. Considering that approximately 11,000 genes were detected in the RNA-seq dataset, the lack of a clear relationship of these genes with ACOD1 signaling, and the limited number of biological replicates (N=3), it is likely that these differences represent stochastic variation rather than biologically meaningful effects.

Overall, the transcriptomic response of Arlo monocultures is strongly driven by LPS and IFN- γ -induced inflammation, which is clearly visible in panels A, C, D, and F. These patterns appear highly similar regardless of whether Citra01 treatment was applied, suggesting that inhibition of ACOD1 has little impact on the global transcriptional response of Arlo cells under the tested conditions.

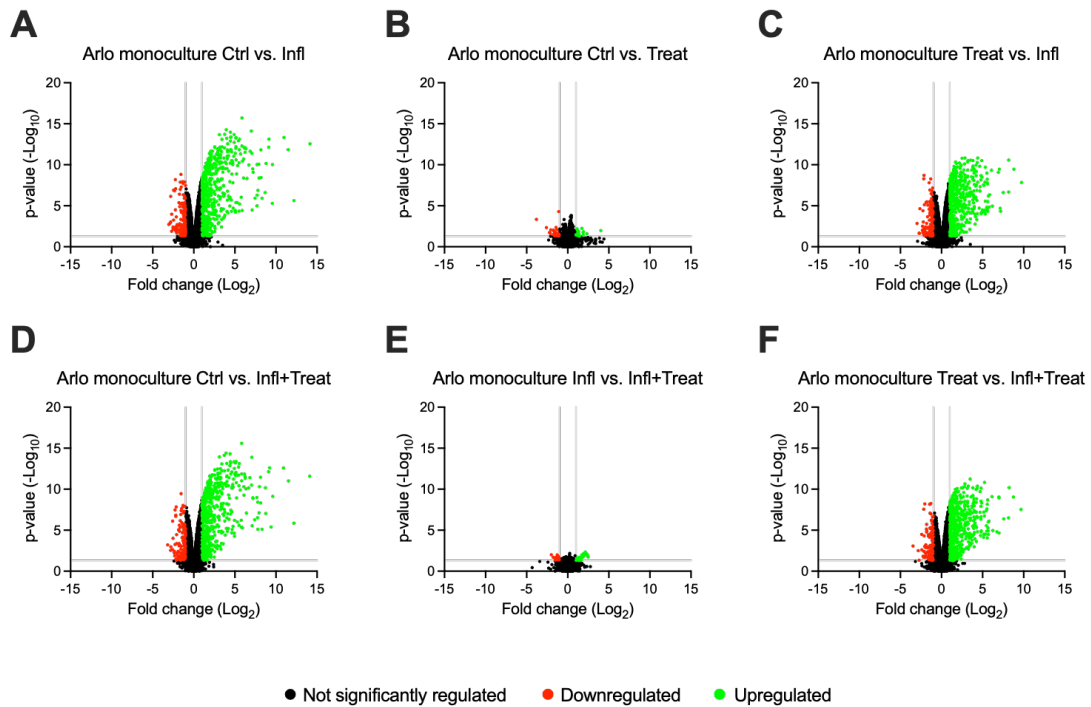


Figure 12: Transcriptional effects of inflammatory stimulation and Citra01 treatment in Arlo monocultures. Volcano plots showing differential gene expression in Arlo cells cultured in monoculture under control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M) conditions. Comparisons are shown as follows: (A) Ctrl vs. Infl; (B) Ctrl vs. Treat; (C) Treat vs. Infl; (D) Ctrl vs. Infl+Treat; (E) Infl vs. Infl+Treat; and (F) Treat vs. Infl+Treat. Each plot displays log₂ fold change on the x-axis and -log₁₀(p-value) on the y-axis. Vertical lines indicate the fold change (FC) threshold ($|\log_2FC| \geq 1$), and the horizontal line represents the statistical significance threshold ($p < 0.05$). Genes meeting both criteria are highlighted, with upregulated genes shown in green and downregulated genes in red, while non-significant genes are depicted in black. Data represent three independent biological replicates (N=3)

For the dTHP-1 monoculture samples shown in Figure 13, a very similar trend is observed. The figure is organized in the same way as the Arlo dataset: panel A shows Infl vs Ctrl, panel B Treat vs Ctrl, panel C Treat vs Infl, panel D Infl+Treat vs Ctrl, panel E Infl+Treat vs Infl, and panel F Treat vs Infl+Treat. Again, the predominant transcriptional changes are driven by inflammatory stimulation, which is clearly visible in panels A, C, D, and F. In addition to several chemokines (e.g., CXCL9, CXCL10, CXCL11, CCL2, and CCL4) and interferon-induced genes (e.g., IFI27, IFI44L, and GBP1), ACOD1 was also found to be strongly upregulated among the top 20 regulated genes (Supplementary Figure S14) [193]. The corresponding heatmaps showing the 20 most up- and downregulated genes for each comparison are provided in Supplementary Figure S14–S19. Upregulation of ACOD1 was expected, as elevated itaconate levels had already been observed in previous experiments (Section 3.3.2.3). The strong induction of ACOD1 in inflamed dTHP-1 cells highlights the importance of macrophages as the primary source of ACOD1 activity within the co-culture system and underlines their relevance for studying ACOD1-dependent immunometabolic processes and the effects of ACOD1 inhibition. Apart from this difference, the overall transcriptional patterns are highly comparable to those observed in Arlo cells. In dTHP-1 monocultures, treatment with Citra01 again appears

to have only minor effects on the transcriptome. This is particularly evident in panel B, where the comparison between Ctrl and Citra01-treated samples results in only 7 upregulated and 7 downregulated genes (Supplementary Figure S15). Similarly, the comparison between Infl and Infl+Treat in panel E reveals only 4 upregulated and 8 downregulated genes (Supplementary Figure S18).

For completeness, volcano plots of Arlo and dTHP-1 cells derived from co-cultures are provided in Supplementary Figure S20–S21. These plots show patterns comparable to those observed in the respective monocultures, further indicating that Citra01 treatment does not substantially alter the global transcriptomic response under the tested conditions.

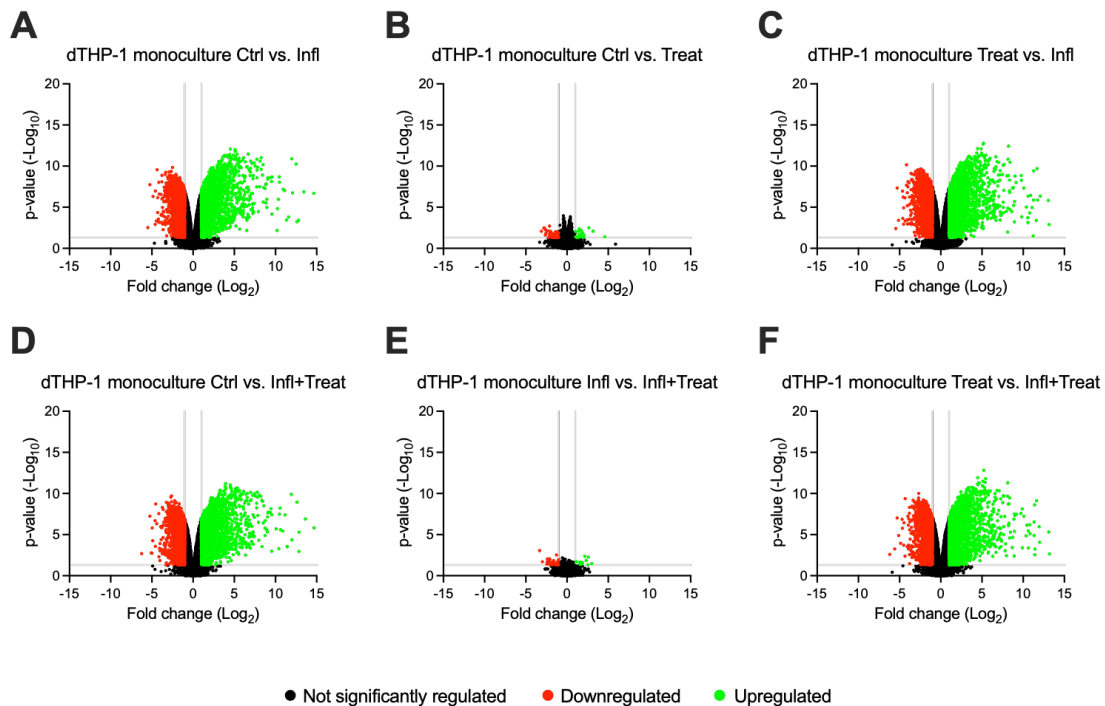


Figure 13: Transcriptional effects of inflammatory stimulation and Citra01 treatment in dTHP-1 monocultures. Volcano plots showing differential gene expression in dTHP-1 cells cultured in monoculture under control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M) conditions. Comparisons are shown as follows: (A) Ctrl vs. Infl; (B) Ctrl vs. Treat; (C) Treat vs. Infl; (D) Ctrl vs. Infl+Treat; (E) Infl vs. Infl+Treat; and (F) Treat vs. Infl+Treat. Each plot displays $\log_2$ fold change on the x-axis and $-\log_{10}(\text{p-value})$ on the y-axis. Vertical lines indicate the fold change (FC) threshold ($|\log_2\text{FC}| \geq 1$), and the horizontal line represents the statistical significance threshold ($p < 0.05$). Genes meeting both criteria are highlighted, with upregulated genes shown in green and downregulated genes in red, while non-significant genes are depicted in black. Data represent three independent biological replicates (N=3)

In summary, transcriptomic analysis revealed that inflammatory stimulation with LPS and IFN- γ is the primary driver of gene expression changes in both Arlo and dTHP-1 monocultures. While Arlo cells showed a pronounced inflammatory response, ACOD1 expression remained low, consistent with their limited capacity for itaconate production. In contrast, dTHP-1 macrophages displayed strong induction of ACOD1 upon inflammatory stimulation, highlighting their role as the main ACOD1-expressing component within the system. Despite effective inhibition of ACOD1 activity as indicated by reduced itaconate levels, treatment with

Citra01 had only minor effects on the global transcriptomic profiles of both cell types. Together, these findings suggest that ACOD1 inhibition in this model primarily affects immunometabolic pathways rather than causing broad transcriptional changes.

4 Conclusion and outlook

This thesis aimed to comprehensively characterize and validate the recently established human alveolar epithelial cell line Arlo as an *in vitro* model of the human alveolar epithelium. To achieve this goal, a comprehensive, multiparametric, and comparative characterization strategy was applied in order to define the biological properties of the cell line, position it within the landscape of existing epithelial models such as primary human alveolar epithelial cells and established cell lines, and evaluate its strengths and limitations.

Through systematic comparison with primary human alveolar epithelial cells and widely used epithelial cell lines, Arlo was shown to possess pronounced epithelial barrier properties and electrophysiological characteristics that resemble those of primary human alveolar epithelial cells (hAEPc). In particular, Arlo exhibited high transepithelial electrical resistance, a strong transepithelial potential difference, and low permeability for paracellular and transcellular marker compounds in a highly reproducible manner. Notably, the transepithelial potential difference was even more pronounced than in hAEPc. A major contributor to this strong electrophysiological phenotype was identified as the high activity of the epithelial sodium channel (ENaC), further supporting the alveolar character of the Arlo cell line. Structural analyses confirmed the formation of a tightly organized epithelial monolayer with morphological characteristics consistent with an alveolar type II-like phenotype. Beyond descriptive characterization, this work also investigated the mechanisms underlying the pronounced barrier properties of Arlo. Functional inhibition experiments targeting cytoskeletal organization, tight junction stability, and ion transport demonstrated that the barrier phenotype of Arlo is supported by contributions of the actin cytoskeleton and tight junctions.

To further complement the functional characterization, a comparative proteomic analysis was performed to assess the abundance of proteins in Arlo relative to hAEPc and other commonly used epithelial cell lines. Particular focus was placed on proteins relevant for epithelial transport processes and for host-pathogen interactions. This analysis provides an additional layer of information that may support the rational selection of appropriate *in vitro* lung models depending on the specific research question. Based on these functional and molecular characteristics, Arlo was subsequently applied in a disease-relevant *in vitro* model. Taking advantage of its pronounced ENaC-mediated ion transport activity, a pulmonary edema model was established that enabled the investigation of altered epithelial electrophysiology and impaired alveolar fluid clearance. The model further allowed the evaluation of pharmacological modulation of epithelial sodium transport using several ENaC-modulating drugs, demonstrating its potential as a platform for drug screening and pharmacological studies targeting epithelial ion transport.

Finally, Arlo was integrated into a more complex *in vitro* system through co-culture with differentiated THP-1 macrophage-like cells (dTHP-1). This co-culture approach enabled the

investigation of epithelial-immune cell interactions and inflammatory signaling within the alveolar environment. The results demonstrate that Arlo can be incorporated into more physiologically relevant multicellular lung models, thereby expanding its applicability beyond epithelial monoculture systems. At the same time, the findings also indicate certain limitations regarding the crosstalk between Arlo and dTHP-1 cells, which should be further investigated in future studies.

Taken together, this thesis provides a comprehensive multiparametric characterization of the Arlo cell line and demonstrates its functional capabilities for modeling key aspects of distal lung physiology and pathology. By combining proteomic profiling of different lung epithelial models with defined functional readouts and experimental applications, this work contributes to a more rational selection of *in vitro* lung models for specific research applications. Importantly, while Arlo represents a powerful tool for modeling the alveolar epithelium, it should not be considered a universal solution for all research questions related to the distal lung. Rather, the choice of an appropriate *in vitro* model should always be guided by the specific biological processes and experimental questions under investigation.

Future work should focus on the systematic validation of Arlo-based models for clearly defined contexts of use. In this regard, fit-for-purpose validation strategies, standardized experimental protocols, and interlaboratory reproducibility studies will be essential to assess the robustness and transferability of the model across laboratories, as required for widely used *in vitro* models in general. Establishing well-defined culture conditions, ideally based on serum-free and animal component-free media, could further improve reproducibility and reduce variability between experiments. In parallel, the development of guidelines and reference testing strategies, including benchmarking with defined compound panels, will be important steps toward broader adoption of such systems. Ultimately, these efforts may support the integration of Arlo-based models into emerging New Approach Methodologies (NAMs) and contribute to their long-term regulatory acceptance as physiologically relevant alternatives to animal experiments.

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6 Appendix

6.1 Supporting information to “Application of the Arlo cell line in an epithelial-macrophage alveolar co-culture model to study ACOD1 inhibition during pulmonary inflammation”

Supplementary Table S1: RNA quality metrics of samples used for RNA sequencing. RNA purity was assessed by spectrophotometric absorbance ratios at 260/280 nm and 260/230 nm, and RNA integrity was determined by RNA integrity number (RIN). Sample identifiers are structured as follows: A = Arlo, D = dTHP-1; M = monoculture, C = co-culture; C = control, I = inflammatory stimulation (30 ng/mL IFN- γ + 100 ng/mL LPS), IT = inflammation + Citra01 treatment, and T = Citra01 treatment alone. The final number indicates the biological replicate. For example, A-M-IT-2 denotes Arlo cells cultured as a monoculture under inflammatory stimulation with Citra01 treatment, replicate 2.

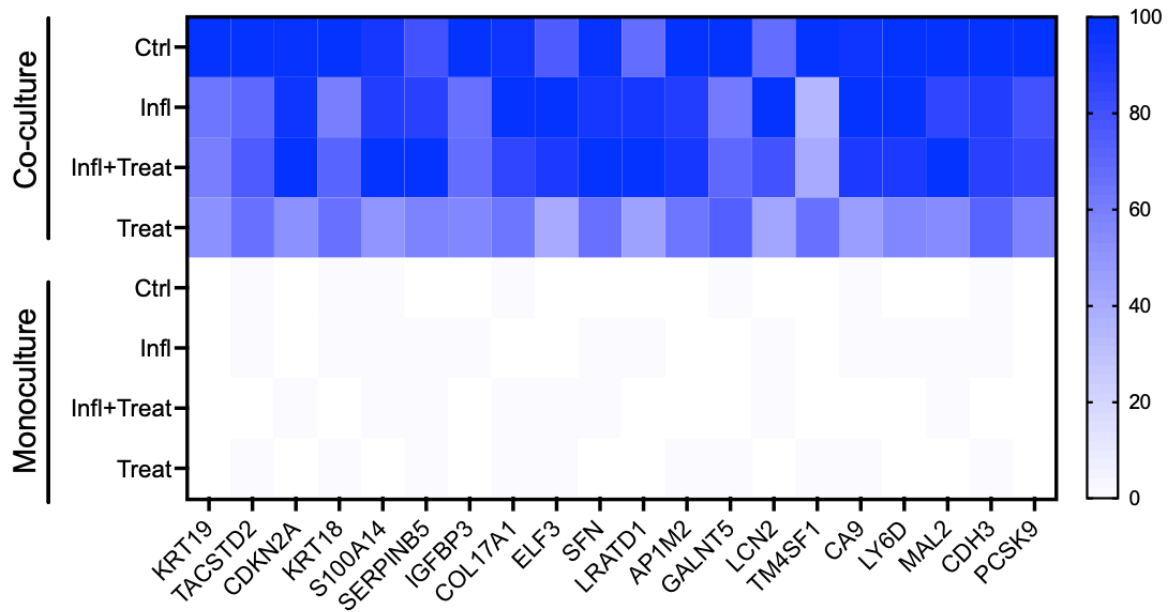
Sample	260/280 nm	260/230 nm	RIN
Arlo samples obtained from monoculture			
A-M-C-1	2.15	2.14	9.2
A-M-C-2	2.14	2.21	9.8
A-M-C-3	2.17	2.23	9.8
A-M-I-1	2.16	2.18	9.5
A-M-I-2	2.15	2.21	9.7
A-M-I-3	2.16	2.23	9.7
A-M-IT-1	2.17	1.94	9.7
A-M-IT-2	2.15	2.10	9.8
A-M-IT-3	2.16	2.17	9.7
A-M-T-1	2.17	1.96	9.6
A-M-T-2	2.16	2.22	9.6
A-M-T-3	2.16	2.22	9.9
Arlo samples obtained from co-culture			
A-C-C-1	2.15	2.16	9.4
A-C-C-2	2.16	2.17	9.8
A-C-C-3	2.18	1.86	9.8
A-C-I-1	2.15	2.13	9.3
A-C-I-2	2.14	2.18	9.7
A-C-I-3	2.17	1.88	9.7
A-C-IT-1	2.15	2.05	9.7
A-C-IT-2	2.14	2.18	9.7
A-C-IT-3	2.17	2.14	9.5
A-C-T-1	2.15	2.16	9.4
A-C-T-2	2.16	2.08	9.7
A-C-T-3	2.17	2.21	9.8

Appendix

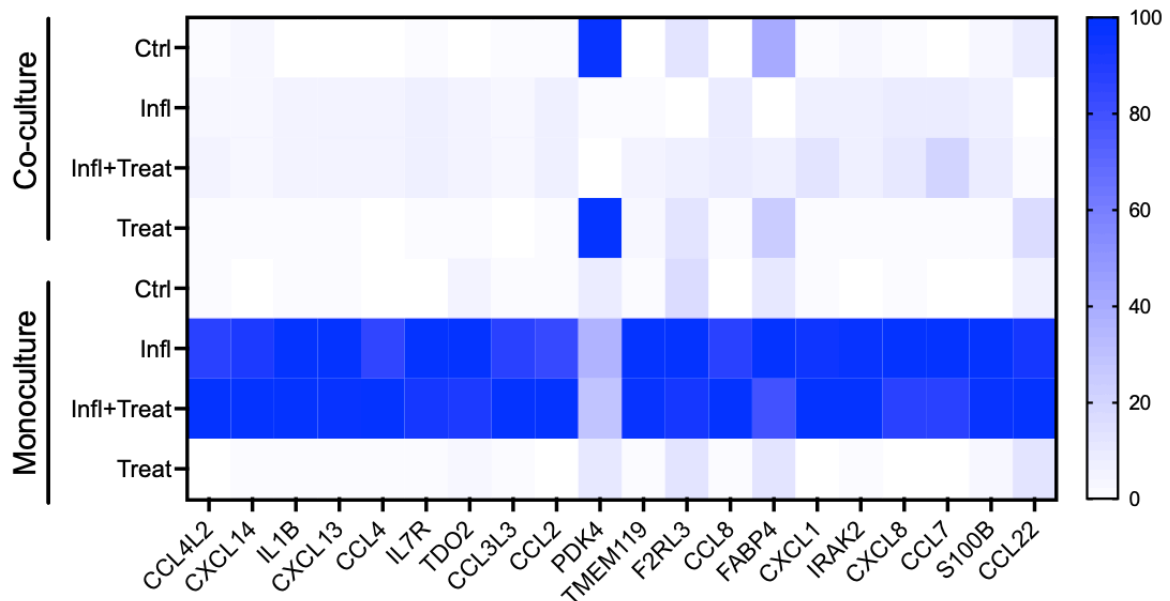
Sample	260/280 nm	260/230 nm	RIN
dTHP-1 samples obtained from monoculture			
D-M-C-1	2.13	2.10	10
D-M-C-2	2.13	2.01	9.9
D-M-C-3	2.13	2.13	10
D-M-I-1	2.12	2.06	9.7
D-M-I-2	2.14	2.14	9.5
D-M-I-3	2.13	2.19	9.4
D-M-IT-1	2.13	1.91	9.1
D-M-IT-2	2.11	2.06	9.8
D-M-IT-3	2.14	1.87	9.4
D-M-T-1	2.13	2.11	10
D-M-T-2	2.13	2.19	9.9
D-M-T-3	2.10	2.02	10
dTHP-1 samples obtained from co-culture			
D-C-C-1	2.12	2.03	10
D-C-C-2	2.13	1.97	10
D-C-C-3	2.14	2.21	10
D-C-I-1	2.15	1.99	9.7
D-C-I-2	2.14	2.13	10
D-C-I-3	2.13	1.96	10
D-C-IT-1	2.15	2.00	10
D-C-IT-2	2.10	1.84	10
D-C-IT-3	2.13	1.98	10
D-C-T-1	2.14	1.88	10
D-C-T-2	2.13	1.86	10
D-C-T-3	2.17	2.19	10

A

Top 20 upregulated genes in dTHP-1 co-culture vs monoculture (Infl)

**B**

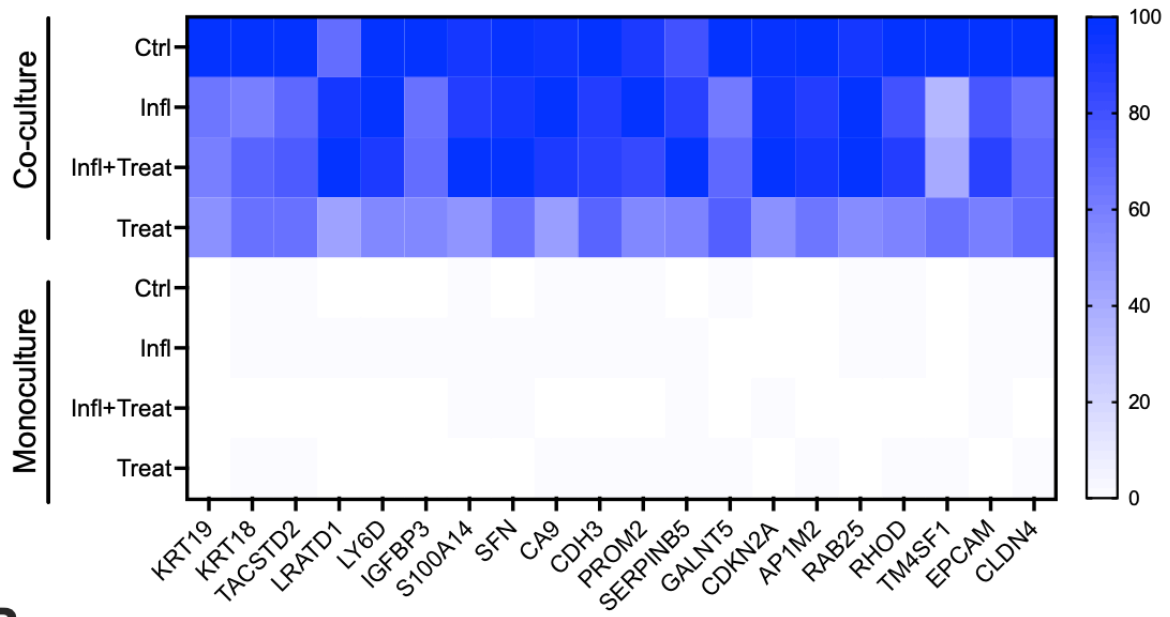
Top 20 downregulated genes in dTHP-1 co-culture vs monoculture (Infl)



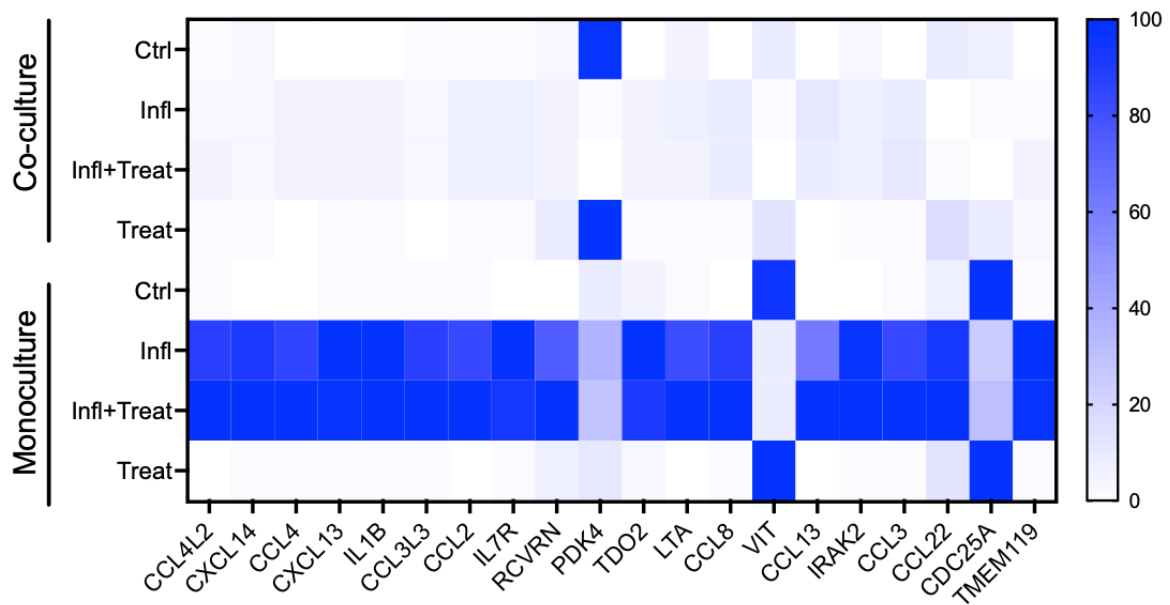
Supplementary Figure S1: Heatmap of the top genes up- and downregulated in dTHP-1 cells in Arlo-dTHP-1 co-culture compared to monoculture under inflamed (Infl) conditions (A) Heatmap showing the top 20 genes upregulated in dTHP-1 cells in Arlo-dTHP-1 co-culture relative to dTHP-1 monoculture under inflamed conditions. (B) Heatmap showing the top 20 genes downregulated in dTHP-1 cells in Arlo-dTHP-1 co-culture relative to dTHP-1 monoculture under inflamed conditions. The experimental conditions comprised control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M Citra01). Gene expression values represent mean expression levels of biological replicates and were column-wise min-max normalized for visualization. Color intensity reflects relative expression levels, with high expression shown in blue and low expression in white. Genes are displayed along the x-axis, and the respective mono- and co-culture conditions are shown along the y-axis. Data represent three independent biological replicates (N=3)

A

Top 20 upregulated genes in dTHP-1 co-culture vs monoculture (Infl+Treat)

**B**

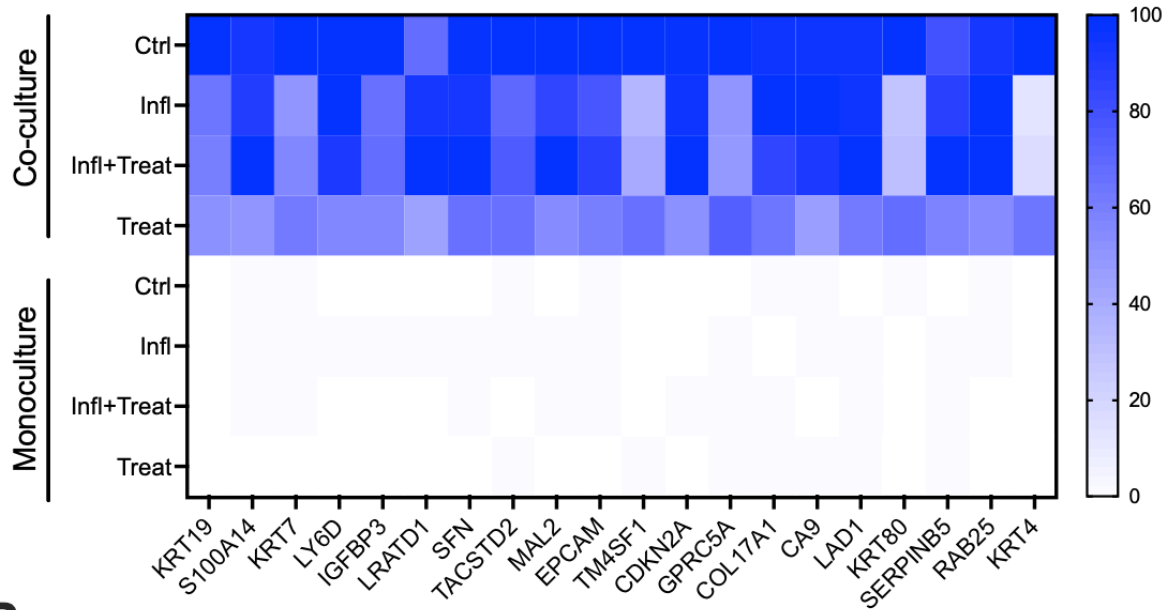
Top 20 downregulated genes in dTHP-1 co-culture vs monoculture (Infl+Treat)



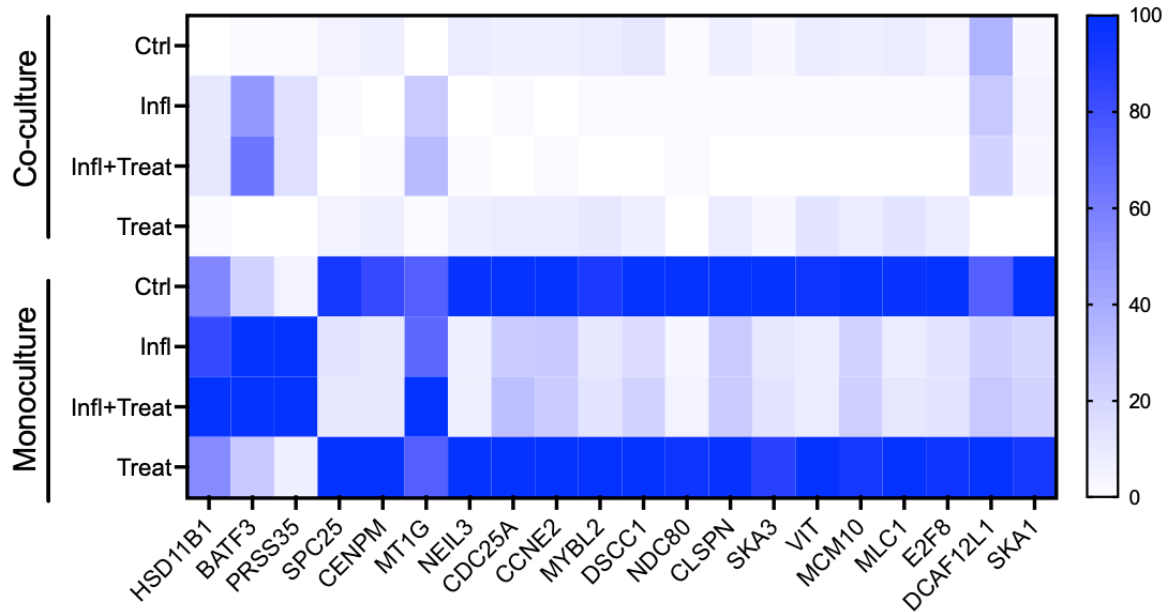
Supplementary Figure S2: Heatmap of the top genes up- and downregulated in dTHP-1 cells in Arlo-dTHP-1 co-culture compared to monoculture under inflamed and treated (Infl+Treat) conditions (A) Heatmap showing the top 20 genes upregulated in dTHP-1 cells in Arlo-dTHP-1 co-culture relative to dTHP-1 monoculture under inflamed and treated conditions. (B) Heatmap showing the top 20 genes downregulated in dTHP-1 cells in Arlo-dTHP-1 co-culture relative to dTHP-1 monoculture under inflamed and treated conditions. The experimental conditions comprised control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M Citra01). Gene expression values represent mean expression levels of biological replicates and were column-wise min-max normalized for visualization. Color intensity reflects relative expression levels, with high expression shown in blue and low expression in white. Genes are displayed along the x-axis, and the respective mono- and co-culture conditions are shown along the y-axis. Data represent three independent biological replicates (N=3)

A

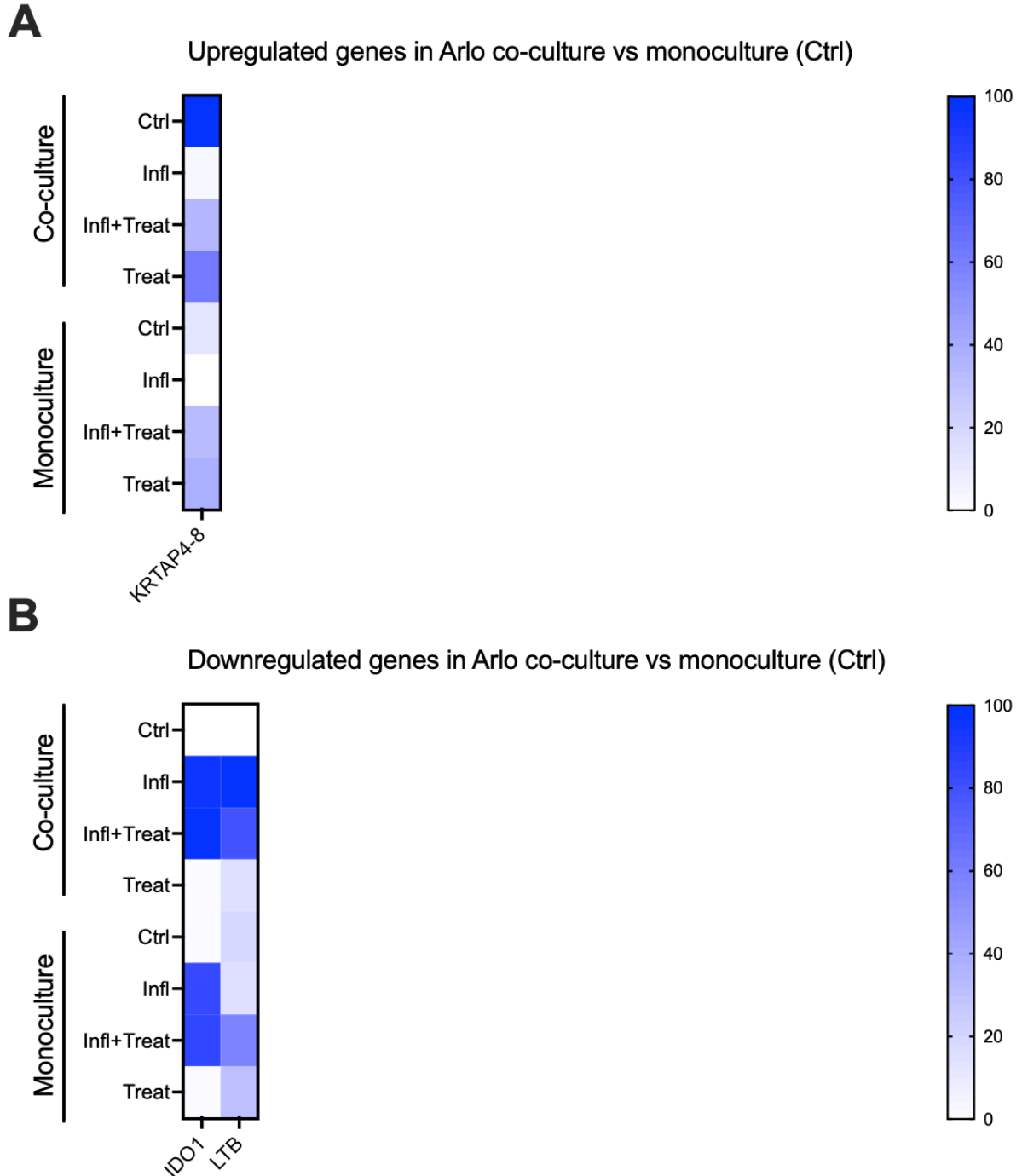
Top 20 upregulated genes in dTHP-1 co-culture vs monoculture (Treat)

**B**

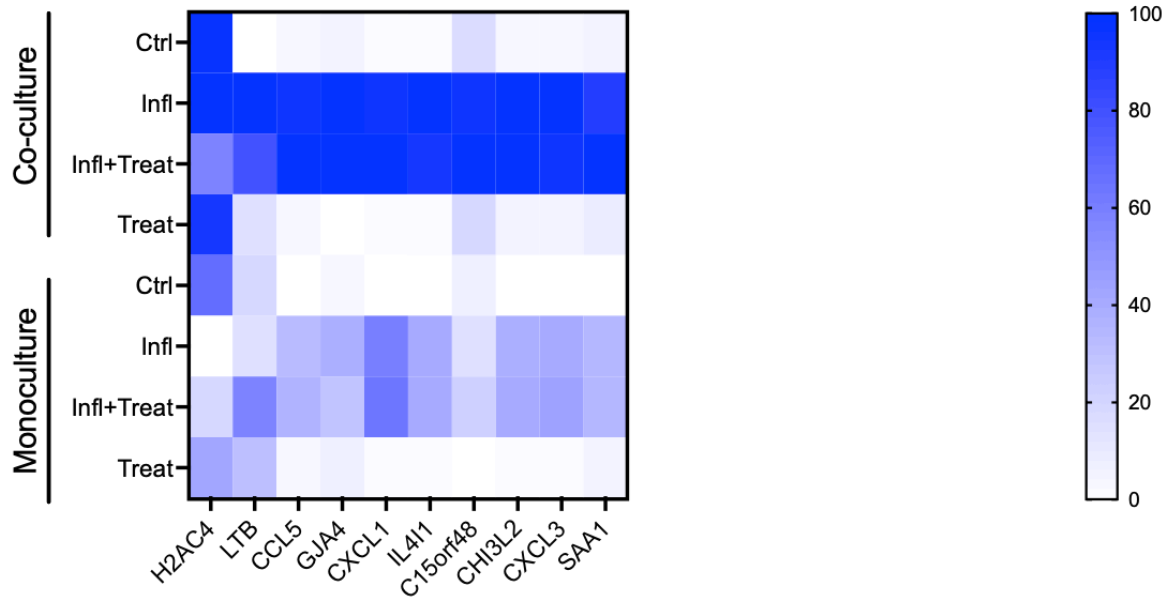
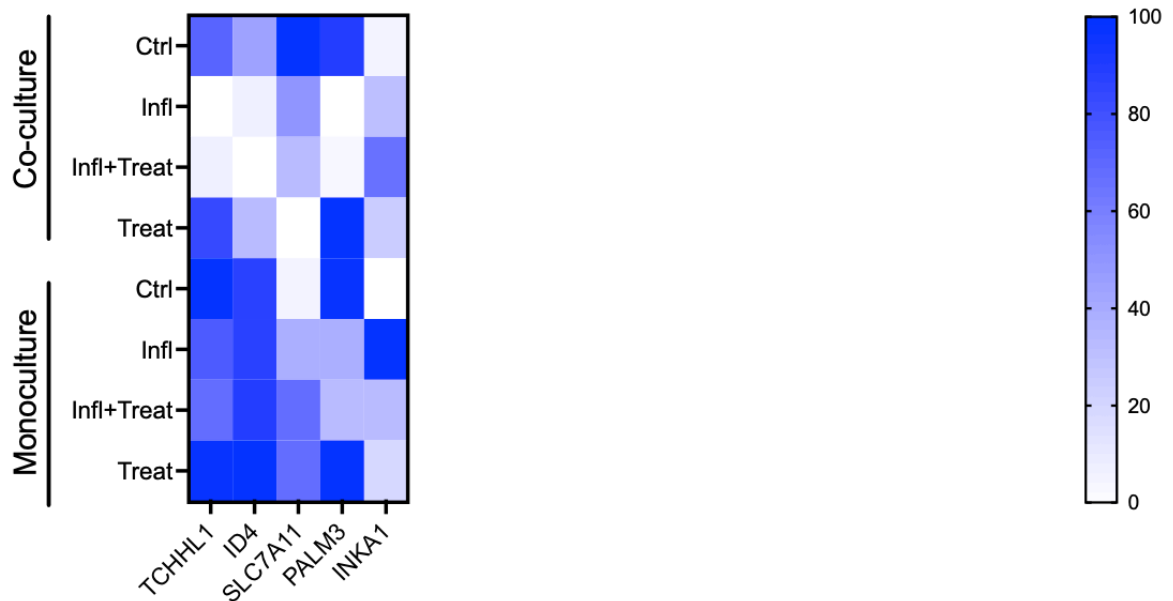
Top 20 downregulated genes in dTHP-1 co-culture vs monoculture (Treat)



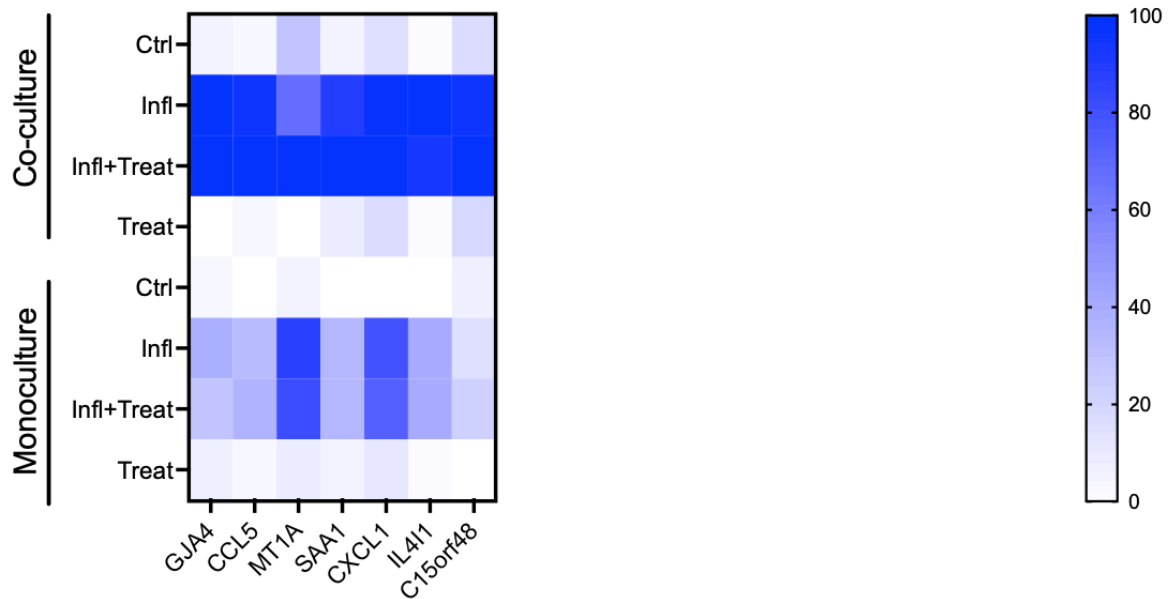
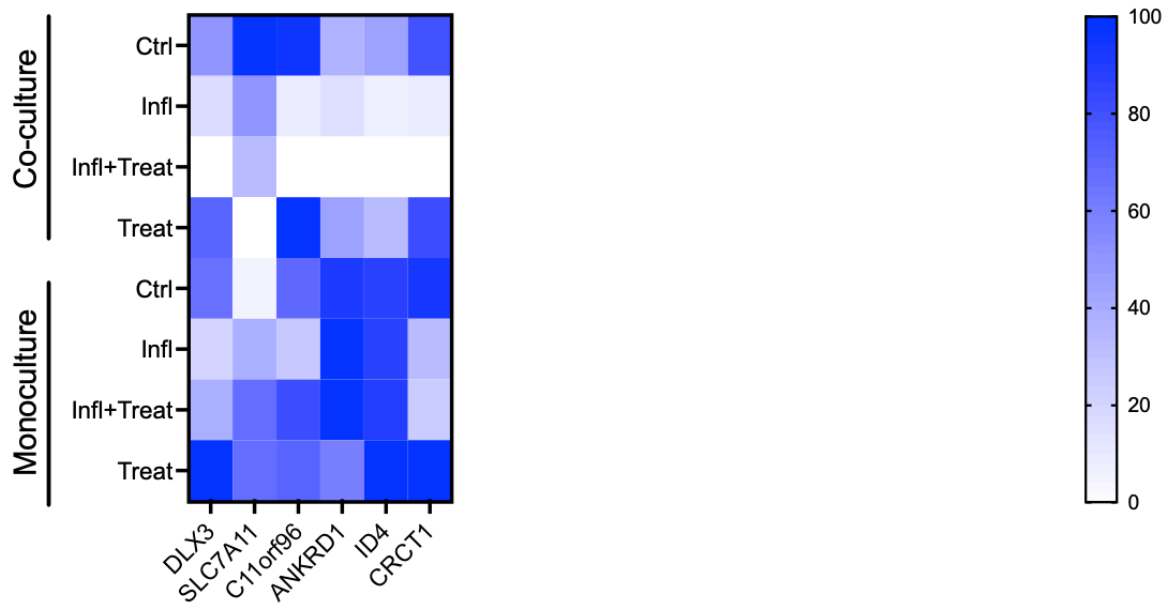
Supplementary Figure S3: Heatmap of the top genes up- and downregulated in dTHP-1 cells in Arlo-dTHP-1 co-culture compared to monoculture under treated (Treat) conditions (A) Heatmap showing the top 20 genes upregulated in dTHP-1 cells in Arlo-dTHP-1 co-culture relative to dTHP-1 monoculture under treated conditions. (B) Heatmap showing the top 20 genes downregulated in dTHP-1 cells in Arlo-dTHP-1 co-culture relative to dTHP-1 monoculture under treated conditions. The experimental conditions comprised control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M Citra01). Gene expression values represent mean expression levels of biological replicates and were column-wise min-max normalized for visualization. Color intensity reflects relative expression levels, with high expression shown in blue and low expression in white. Genes are displayed along the x-axis, and the respective mono- and co-culture conditions are shown along the y-axis. Data represent three independent biological replicates (N=3)



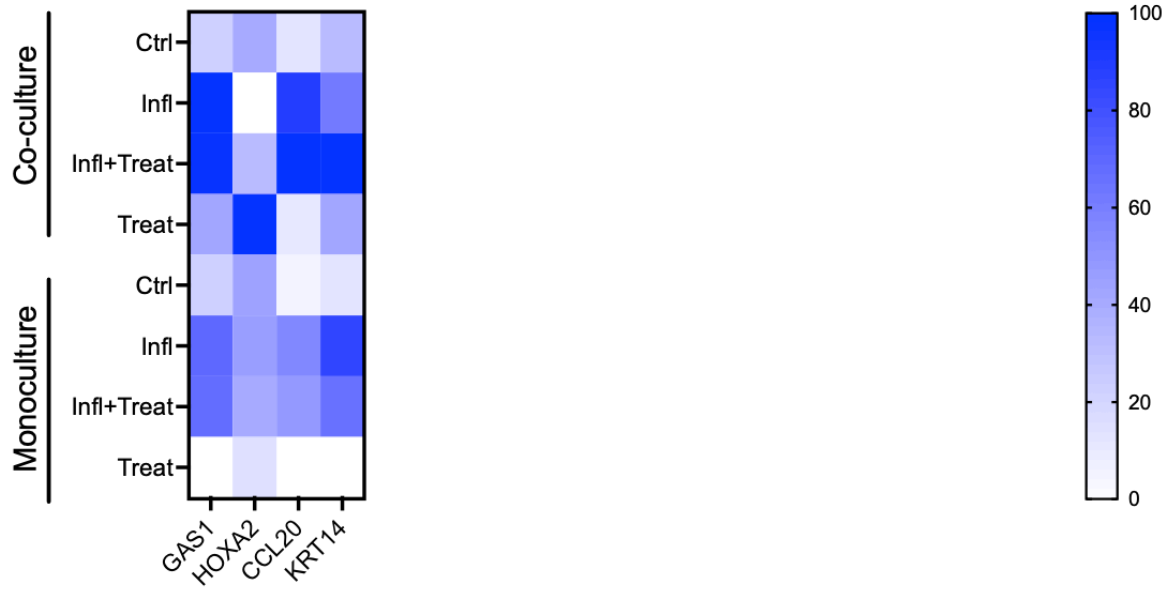
Supplementary Figure S4: Heatmap of the top genes up- and downregulated in Arlo cells in Arlo-dTHP-1 co-culture compared to monoculture under control (Ctrl) conditions (A) Heatmap showing the top 20 genes upregulated in Arlo cells in Arlo-dTHP-1 co-culture relative to Arlo monoculture under control conditions. (B) Heatmap showing the top 20 genes downregulated in Arlo cells in Arlo-dTHP-1 co-culture relative to Arlo monoculture under control conditions. The experimental conditions comprised control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M Citra01). Gene expression values represent mean expression levels of biological replicates and were column-wise min-max normalized for visualization. Color intensity reflects relative expression levels, with high expression shown in blue and low expression in white. Genes are displayed along the x-axis, and the respective mono- and co-culture conditions are shown along the y-axis. Data represent three independent biological replicates (N=3)

A**Upregulated genes in Arlo co-culture vs monoculture (Infl)****B****Downregulated genes in Arlo co-culture vs monoculture (Infl)**

Supplementary Figure S5: Heatmap of the top genes up- and downregulated in Arlo cells in Arlo-dTHP-1 co-culture compared to monoculture under inflamed (Infl) conditions (A) Heatmap showing the top 20 genes upregulated in Arlo cells in Arlo-dTHP-1 co-culture relative to Arlo monoculture under inflamed conditions. (B) Heatmap showing the top 20 genes downregulated in Arlo cells in Arlo-dTHP-1 co-culture relative to Arlo monoculture under inflamed conditions. The experimental conditions comprised control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M Citra01). Gene expression values represent mean expression levels of biological replicates and were column-wise min-max normalized for visualization. Color intensity reflects relative expression levels, with high expression shown in blue and low expression in white. Genes are displayed along the x-axis, and the respective mono- and co-culture conditions are shown along the y-axis. Data represent three independent biological replicates (N=3)

A**Upregulated genes in Arlo co-culture vs monoculture (Infl+Treat)****B****Downregulated genes in Arlo co-culture vs monoculture (Infl+Treat)**

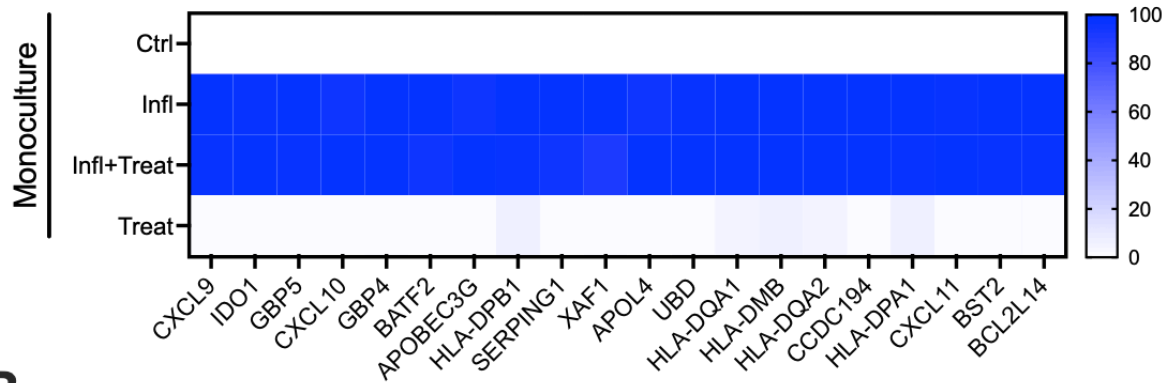
Supplementary Figure S6: Heatmap of the top genes up- and downregulated in Arlo cells in Arlo-dTHP-1 co-culture compared to monoculture under inflamed and treated (Infl+Treat) conditions
 (A) Heatmap showing the top 20 genes upregulated in Arlo cells in Arlo-dTHP-1 co-culture relative to Arlo monoculture under inflamed and treated conditions. (B) Heatmap showing the top 20 genes downregulated in Arlo cells in Arlo-dTHP-1 co-culture relative to Arlo monoculture under inflamed and treated conditions. The experimental conditions comprised control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M Citra01). Gene expression values represent mean expression levels of biological replicates and were column-wise min-max normalized for visualization. Color intensity reflects relative expression levels, with high expression shown in blue and low expression in white. Genes are displayed along the x-axis, and the respective mono- and co-culture conditions are shown along the y-axis. Data represent three independent biological replicates (N=3)

A**Upregulated genes in Arlo co-culture vs monoculture (Treat)****B****Downregulated genes in Arlo co-culture vs monoculture (Treat)**

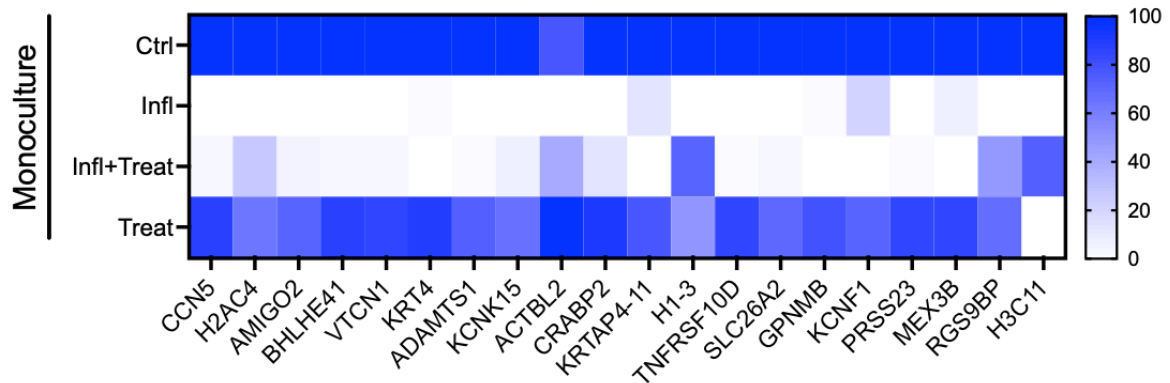
Supplementary Figure S7: Heatmap of the top genes up- and downregulated in Arlo cells in Arlo-dTHP-1 co-culture compared to monoculture under treated (Treat) conditions (A) Heatmap showing the top 20 genes upregulated in Arlo cells in Arlo-dTHP-1 co-culture relative to Arlo monoculture under treated conditions. (B) Heatmap showing the top 20 genes downregulated in Arlo cells in Arlo-dTHP-1 co-culture relative to Arlo monoculture under treated conditions. The experimental conditions comprised control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M Citra01). Gene expression values represent mean expression levels of biological replicates and were column-wise min-max normalized for visualization. Color intensity reflects relative expression levels, with high expression shown in blue and low expression in white. Genes are displayed along the x-axis, and the respective mono- and co-culture conditions are shown along the y-axis. Data represent three independent biological replicates (N=3)

A

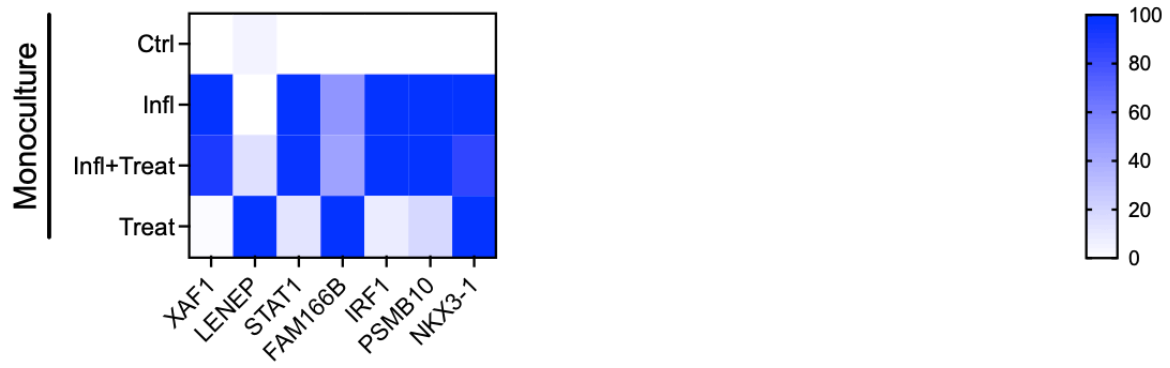
Arlo monoculture upregulated genes in Infl compared to Ctrl

**B**

Arlo monoculture downregulated genes in Infl compared to Ctrl



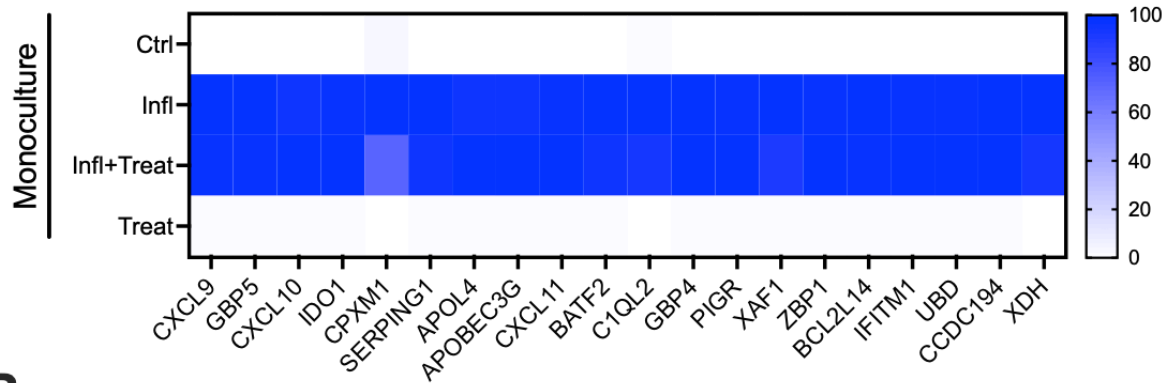
Supplementary Figure S8: Heatmap of the top genes up- and downregulated in Arlo monoculture cells in inflamed conditions compared to control conditions (A) Heatmap showing the top 20 upregulated genes (B) Heatmap showing the top 20 downregulated genes. The experimental conditions comprised control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M Citra01). Gene expression values represent mean expression levels of biological replicates and were column-wise min-max normalized for visualization. Color intensity reflects relative expression levels, with high expression shown in blue and low expression in white. Genes are displayed along the x-axis, and the experimental conditions are shown along the y-axis. Data represent three independent biological replicates (N=3)

A**Arlo monoculture upregulated genes in Treat compared to Ctrl****B****Arlo monoculture downregulated genes in Treat compared to Ctrl**

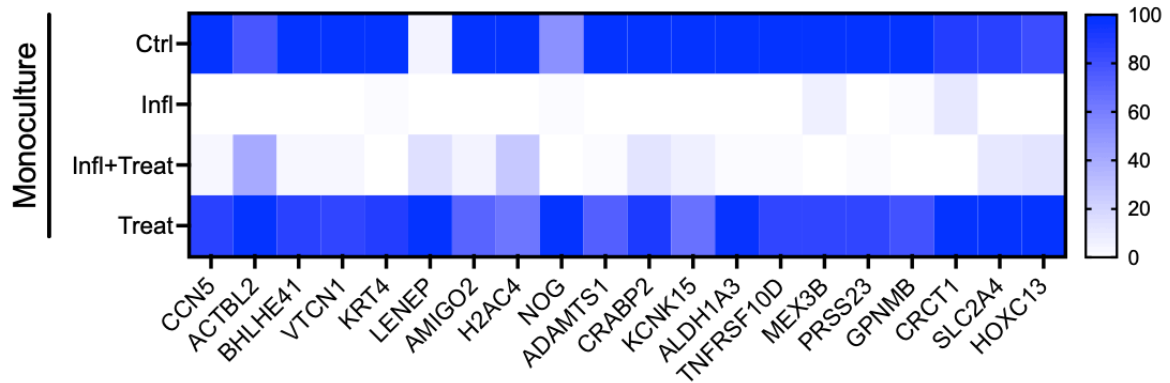
Supplementary Figure S9: Heatmap of the top genes up- and downregulated in Arlo monoculture cells in Citra01-treated conditions compared to control conditions (A) Heatmap showing the top 20 upregulated genes (B) Heatmap showing the top 20 downregulated genes. The experimental conditions comprised control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M Citra01). Gene expression values represent mean expression levels of biological replicates and were column-wise min-max normalized for visualization. Color intensity reflects relative expression levels, with high expression shown in blue and low expression in white. Genes are displayed along the x-axis, and the experimental conditions are shown along the y-axis. Data represent three independent biological replicates (N=3)

A

Arlo monoculture upregulated genes in Infl compared to Treat

**B**

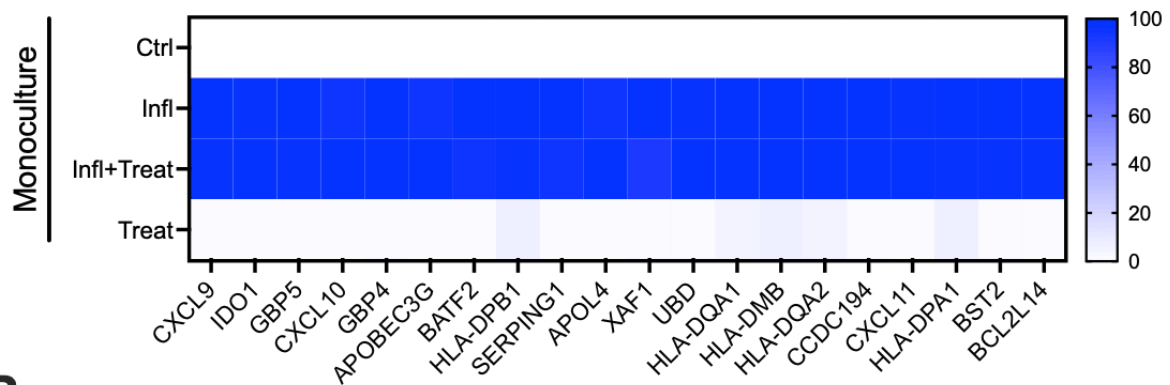
Arlo monoculture downregulated genes in Infl compared to Treat



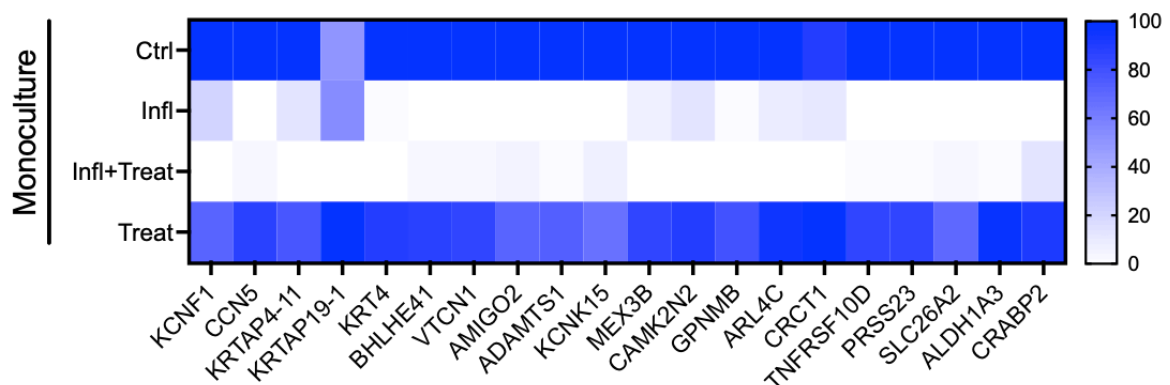
Supplementary Figure S10: Heatmap of the top genes up- and downregulated in Arlo monoculture cells in inflamed conditions compared to Citra01-treated conditions (A) Heatmap showing the top 20 upregulated genes (B) Heatmap showing the top 20 downregulated genes. The experimental conditions comprised control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M Citra01). Gene expression values represent mean expression levels of biological replicates and were column-wise min-max normalized for visualization. Color intensity reflects relative expression levels, with high expression shown in blue and low expression in white. Genes are displayed along the x-axis, and the experimental conditions are shown along the y-axis. Data represent three independent biological replicates (N=3)

A

Arlo monoculture upregulated genes in Infl+Treat compared to Ctrl

**B**

Arlo monoculture downregulated genes in Infl+Treat compared to Ctrl



Supplementary Figure S11: Heatmap of the top genes up- and downregulated in Arlo monoculture cells in inflamed and treated conditions compared to control conditions (A) Heatmap showing the top 20 upregulated genes (B) Heatmap showing the top 20 downregulated genes. The experimental conditions comprised control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M Citra01). Gene expression values represent mean expression levels of biological replicates and were column-wise min-max normalized for visualization. Color intensity reflects relative expression levels, with high expression shown in blue and low expression in white. Genes are displayed along the x-axis, and the experimental conditions are shown along the y-axis. Data represent three independent biological replicates (N=3)

A

Arlo monoculture upregulated genes in Infl+Treat compared to Infl

**B**

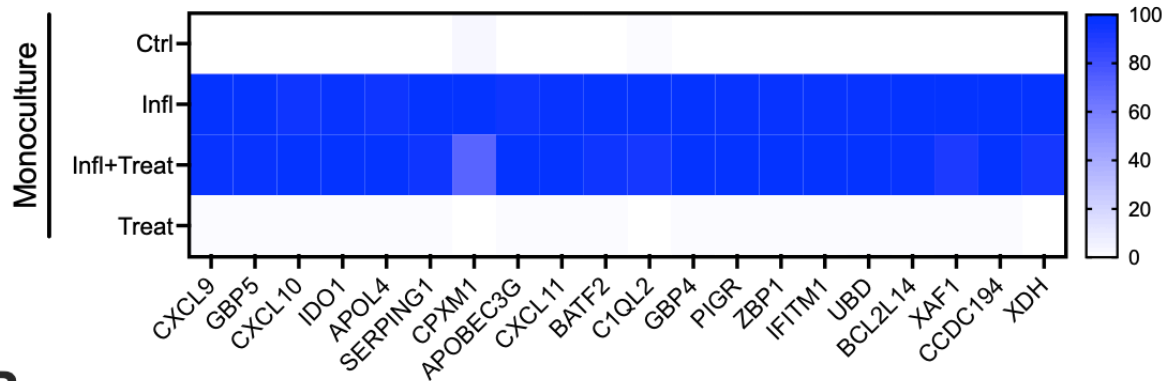
Arlo monoculture downregulated genes in Infl+Treat compared to Infl



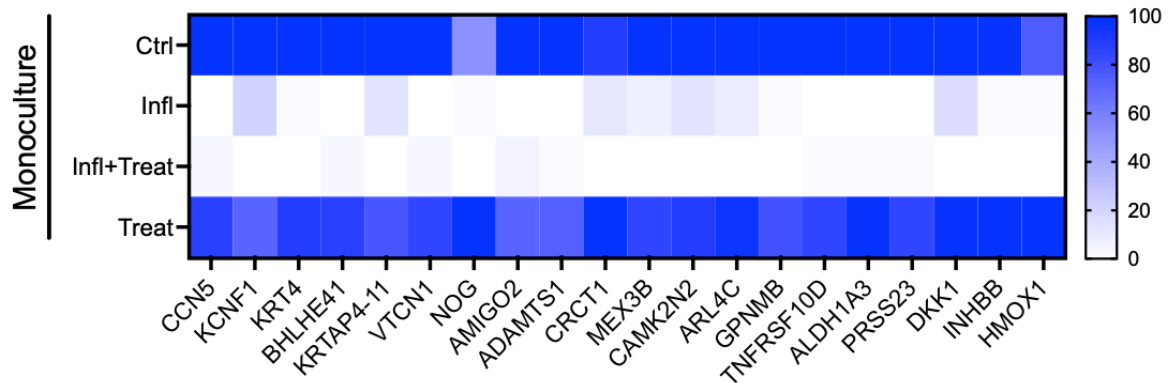
Supplementary Figure S12: Heatmap of the top genes up- and downregulated in Arlo monoculture cells in inflamed and treated conditions compared to inflamed conditions (A) Heatmap showing the top 20 upregulated genes (B) Heatmap showing the top 20 downregulated genes. The experimental conditions comprised control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M Citra01). Gene expression values represent mean expression levels of biological replicates and were column-wise min-max normalized for visualization. Color intensity reflects relative expression levels, with high expression shown in blue and low expression in white. Genes are displayed along the x-axis, and the experimental conditions are shown along the y-axis. Data represent three independent biological replicates (N=3)

A

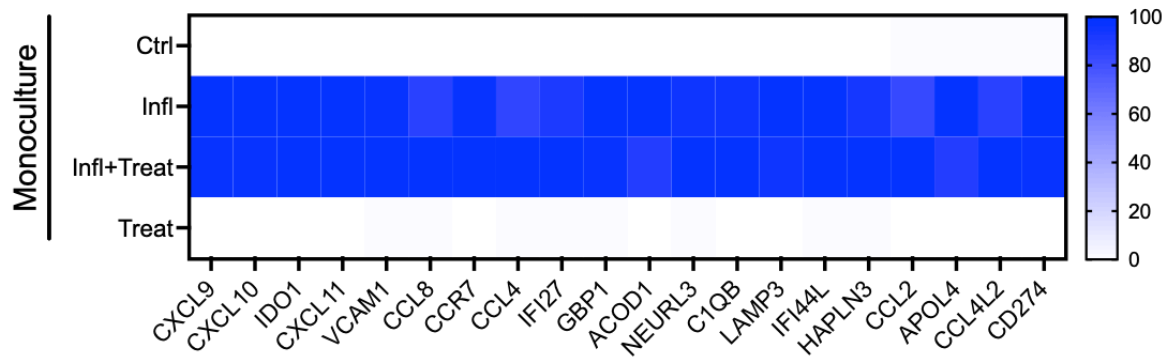
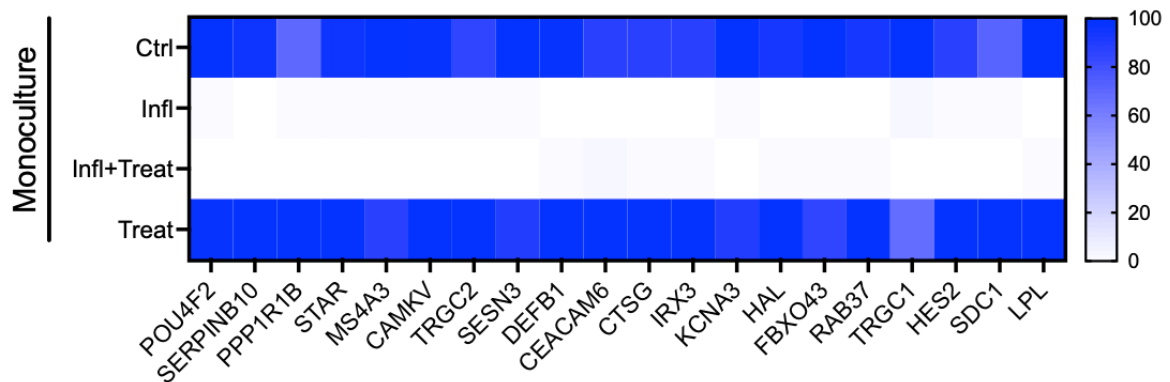
Arlo monoculture upregulated genes in Infl+Treat compared to Treat

**B**

Arlo monoculture downregulated genes in Infl+Treat compared to Treat



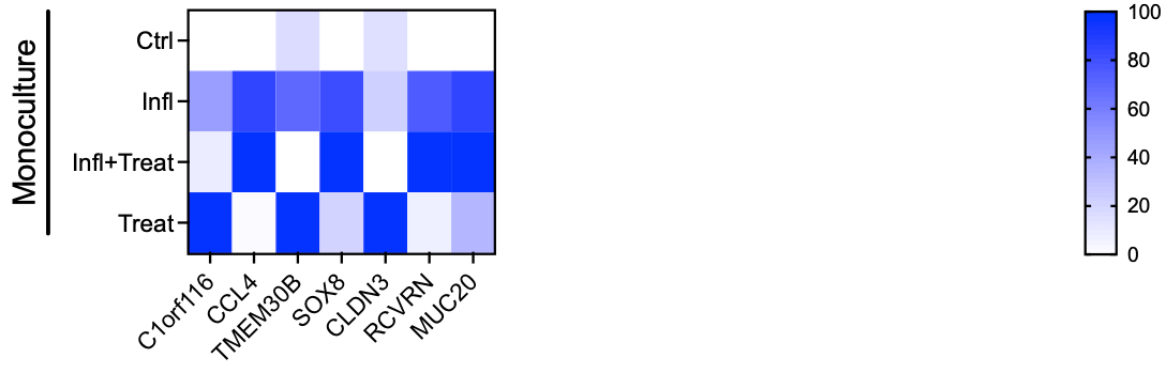
Supplementary Figure S13: Heatmap of the top genes up- and downregulated in Arlo monoculture cells in inflamed and treated conditions compared to Citra01-treated conditions (A) Heatmap showing the top 20 upregulated genes (B) Heatmap showing the top 20 downregulated genes. The experimental conditions comprised control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M Citra01). Gene expression values represent mean expression levels of biological replicates and were column-wise min-max normalized for visualization. Color intensity reflects relative expression levels, with high expression shown in blue and low expression in white. Genes are displayed along the x-axis, and the experimental conditions are shown along the y-axis. Data represent three independent biological replicates (N=3)

A**dTHP-1 monoculture upregulated genes in Infl compared to Ctrl****B****dTHP-1 monoculture downregulated genes in Infl compared to Ctrl**

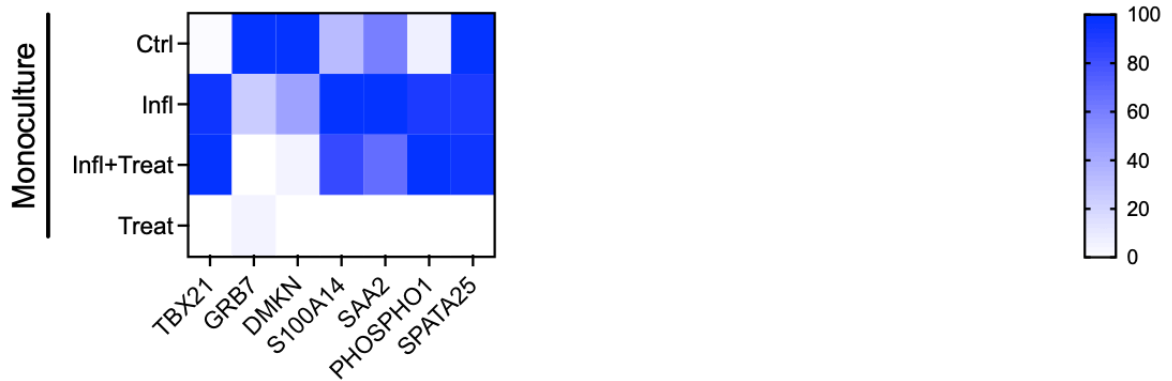
Supplementary Figure S14: Heatmap of the top genes up- and downregulated in dTHP-1 monoculture cells in inflamed conditions compared to control conditions (A) Heatmap showing the top 20 upregulated genes (B) Heatmap showing the top 20 downregulated genes. The experimental conditions comprised control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M Citra01). Gene expression values represent mean expression levels of biological replicates and were column-wise min-max normalized for visualization. Color intensity reflects relative expression levels, with high expression shown in blue and low expression in white. Genes are displayed along the x-axis, and the experimental conditions are shown along the y-axis. Data represent three independent biological replicates (N=3)

A

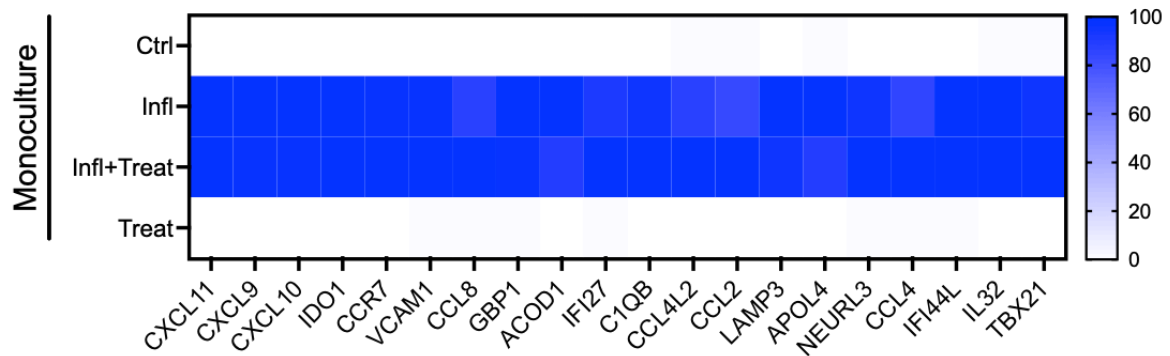
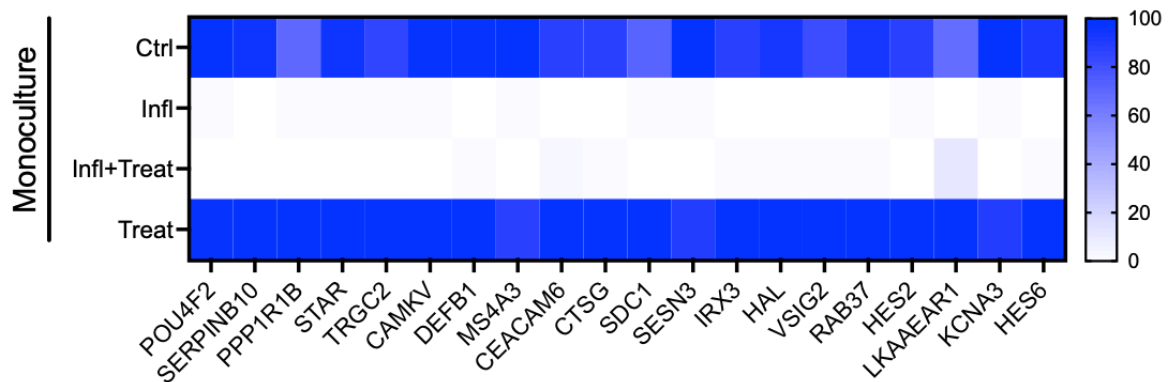
dTHP-1 monoculture upregulated genes in Treat compared to Ctrl

**B**

dTHP-1 monoculture downregulated genes in Treat compared to Ctrl



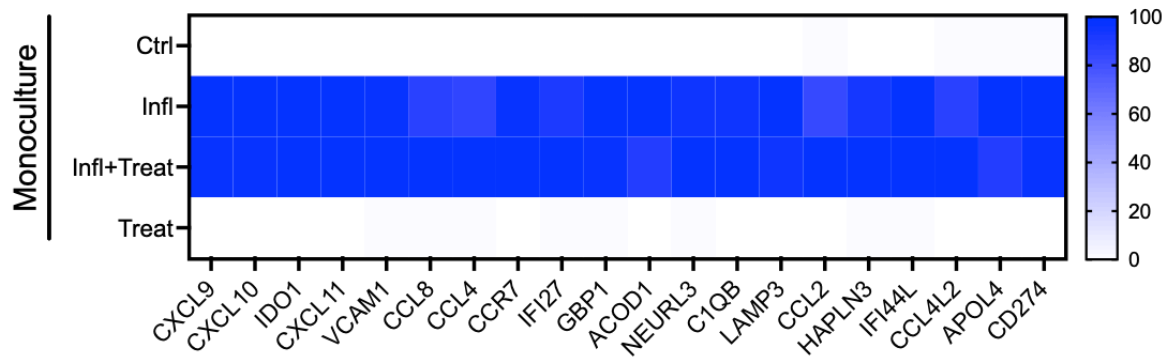
Supplementary Figure S15: Heatmap of the top genes up- and downregulated in dTHP-1 monoculture cells in Citra01-treated conditions compared to control conditions (A) Heatmap showing the top 20 upregulated genes (B) Heatmap showing the top 20 downregulated genes. The experimental conditions comprised control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M Citra01). Gene expression values represent mean expression levels of biological replicates and were column-wise min-max normalized for visualization. Color intensity reflects relative expression levels, with high expression shown in blue and low expression in white. Genes are displayed along the x-axis, and the experimental conditions are shown along the y-axis. Data represent three independent biological replicates (N=3)

A**dTHP-1 monoculture upregulated genes in Infl compared to Treat****B****dTHP-1 monoculture downregulated genes in Infl compared to Treat**

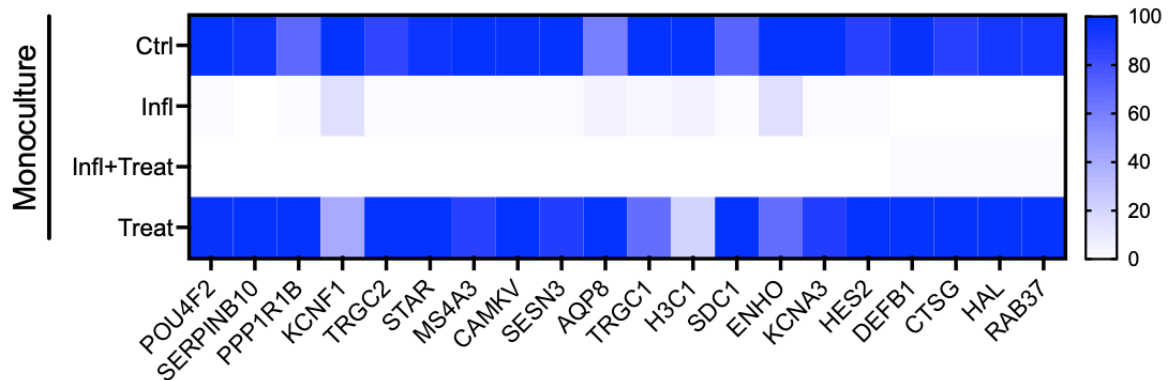
Supplementary Figure S16: Heatmap of the top genes up- and downregulated in dTHP-1 monoculture cells in inflamed conditions compared to Citra01-treated conditions (A) Heatmap showing the top 20 upregulated genes (B) Heatmap showing the top 20 downregulated genes. The experimental conditions comprised control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M Citra01). Gene expression values represent mean expression levels of biological replicates and were column-wise min-max normalized for visualization. Color intensity reflects relative expression levels, with high expression shown in blue and low expression in white. Genes are displayed along the x-axis, and the experimental conditions are shown along the y-axis. Data represent three independent biological replicates (N=3)

A

dTHP-1 monoculture upregulated genes in Infl+Treat compared to Ctrl

**B**

dTHP-1 monoculture downregulated genes in Infl+Treat compared to Ctrl



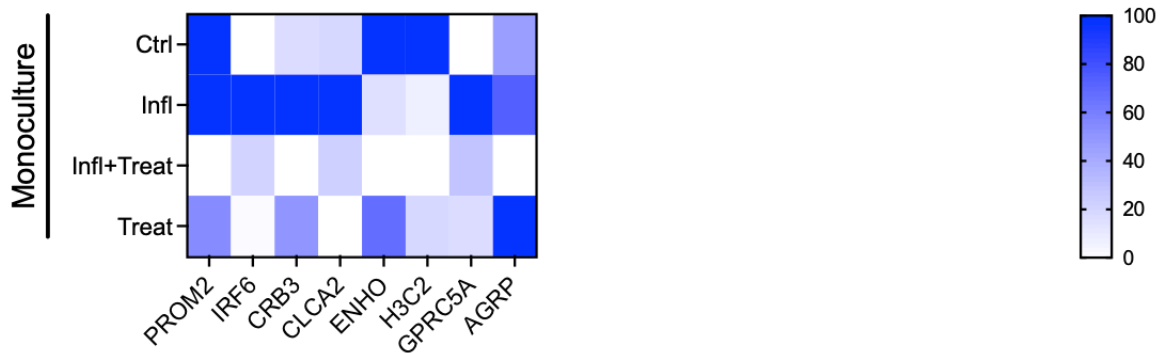
Supplementary Figure S17: Heatmap of the top genes up- and downregulated in dTHP-1 monoculture cells in inflamed and treated conditions compared to control conditions (A) Heatmap showing the top 20 upregulated genes (B) Heatmap showing the top 20 downregulated genes. The experimental conditions comprised control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M Citra01). Gene expression values represent mean expression levels of biological replicates and were column-wise min-max normalized for visualization. Color intensity reflects relative expression levels, with high expression shown in blue and low expression in white. Genes are displayed along the x-axis, and the experimental conditions are shown along the y-axis. Data represent three independent biological replicates (N=3)

A

dTHP-1 monoculture upregulated genes in Infl+Treat compared to Infl

**B**

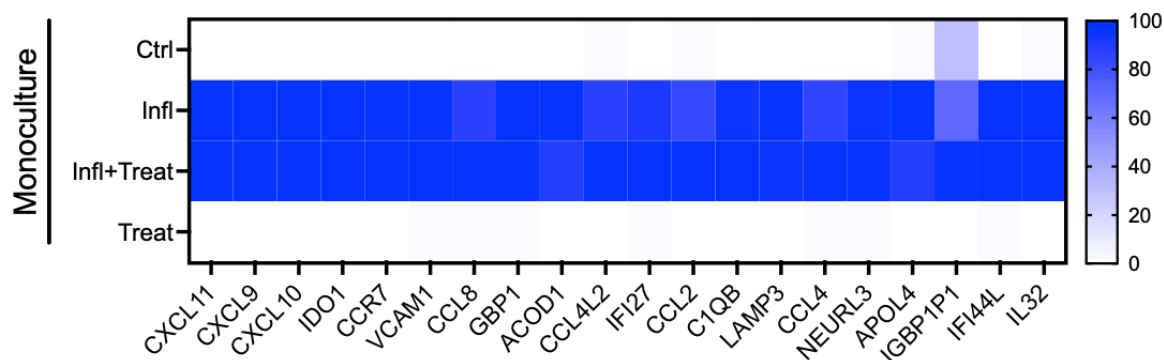
dTHP-1 monoculture downregulated genes in Infl+Treat compared to Infl



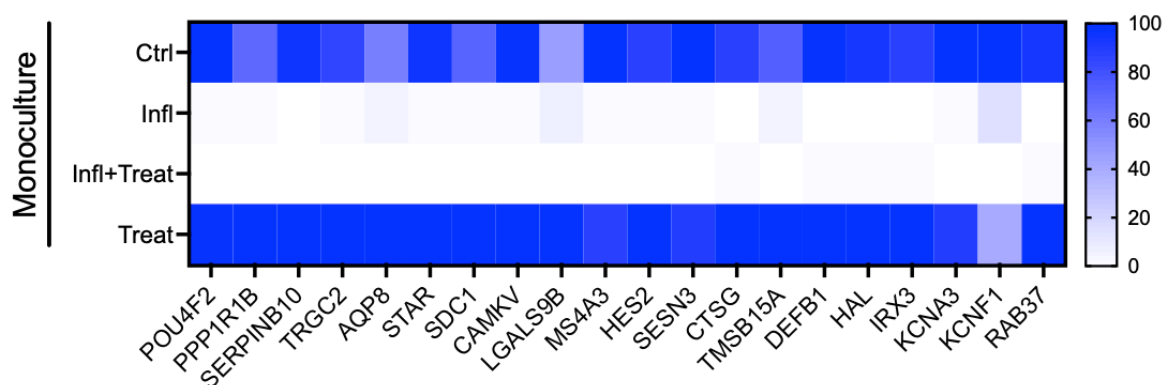
Supplementary Figure S18: Heatmap of the top genes up- and downregulated in dTHP-1 monoculture cells in inflamed and treated conditions compared to inflamed conditions (A) Heatmap showing the top 20 upregulated genes (B) Heatmap showing the top 20 downregulated genes. The experimental conditions comprised control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M Citra01). Gene expression values represent mean expression levels of biological replicates and were column-wise min-max normalized for visualization. Color intensity reflects relative expression levels, with high expression shown in blue and low expression in white. Genes are displayed along the x-axis, and the experimental conditions are shown along the y-axis. Data represent three independent biological replicates (N=3)

A

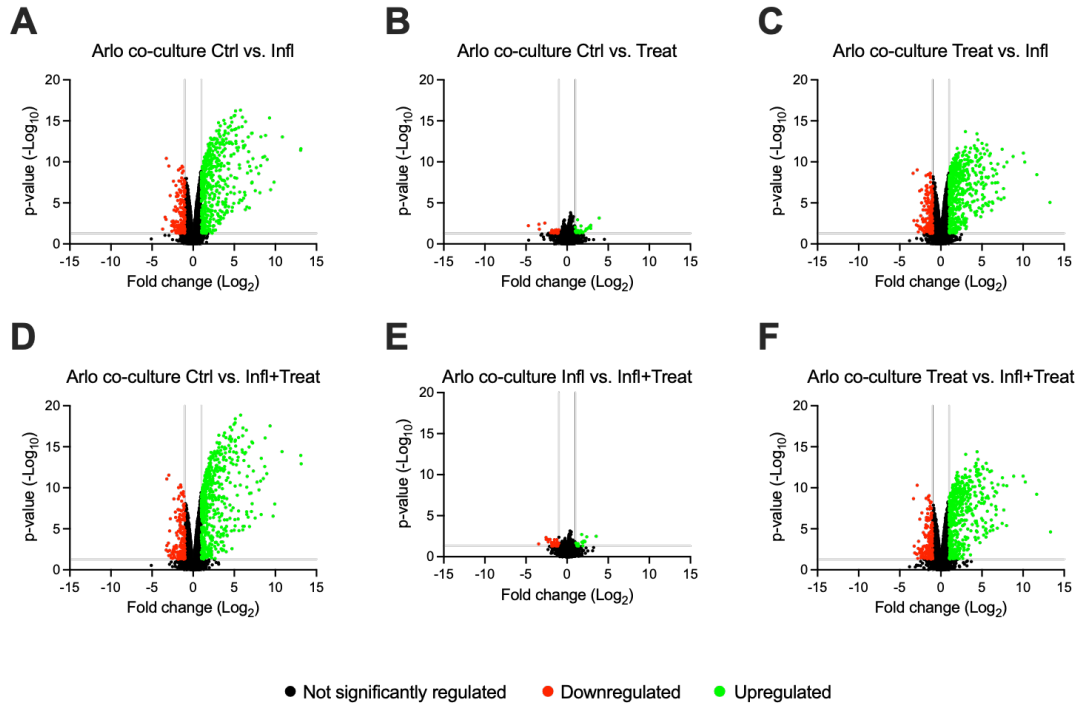
dTHP-1 monoculture upregulated genes in Infl+Treat compared to Treat

**B**

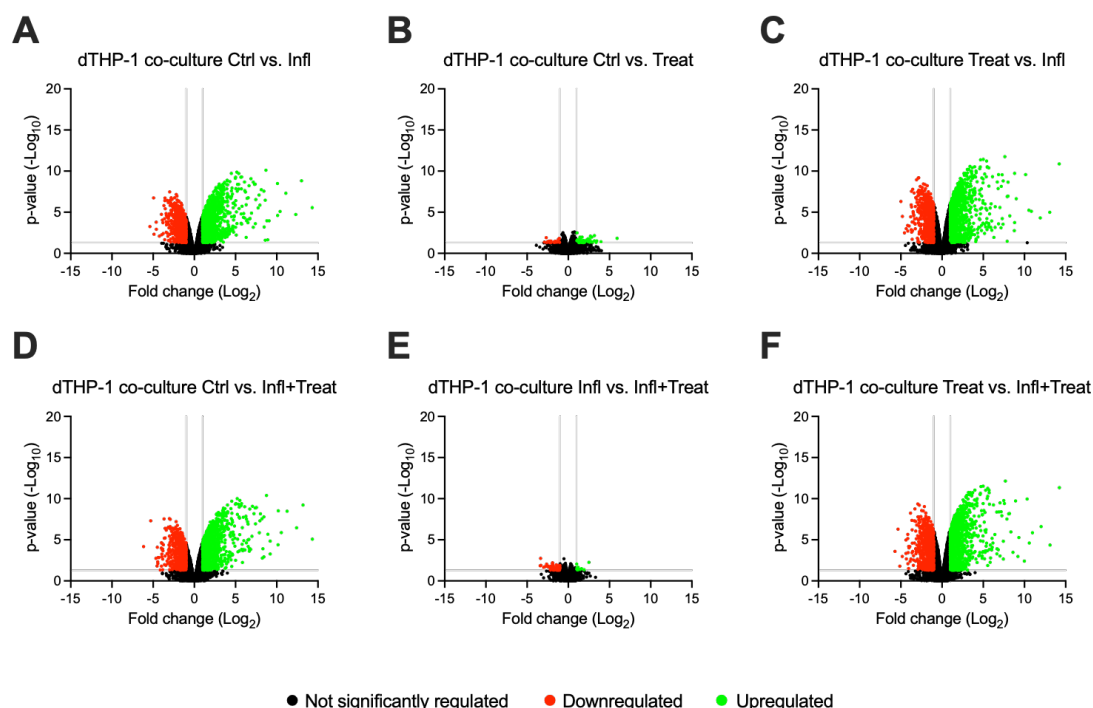
dTHP-1 monoculture downregulated genes in Infl+Treat compared to Treat



Supplementary Figure S19: Heatmap of the top genes up- and downregulated in dTHP-1 monoculture cells in inflamed and treated conditions compared to Citra01-treated conditions (A) Heatmap showing the top 20 upregulated genes (B) Heatmap showing the top 20 downregulated genes. The experimental conditions comprised control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M Citra01). Gene expression values represent mean expression levels of biological replicates and were column-wise min-max normalized for visualization. Color intensity reflects relative expression levels, with high expression shown in blue and low expression in white. Genes are displayed along the x-axis, and the experimental conditions are shown along the y-axis. Data represent three independent biological replicates (N=3)



Supplementary Figure S20: Transcriptional effects of inflammatory stimulation and Citra01 treatment in Arlo cells cultured in co-culture with dTHP-1 cells. Volcano plots showing differential gene expression in Arlo cells cultured in co-culture with dTHP-1 cells under control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M Citra01) conditions. Comparisons are shown as follows: (A) Ctrl vs. Infl; (B) Ctrl vs. Treat; (C) Treat vs. Infl; (D) Ctrl vs. Infl+Treat; (E) Infl vs. Infl+Treat; and (F) Treat vs. Infl+Treat. Each plot displays $\log_2$ fold change on the x-axis and $-\log_{10}(\text{p-value})$ on the y-axis. Vertical lines indicate the fold change (FC) threshold ($|\log_2\text{FC}| \geq 1$), and the horizontal line represents the statistical significance threshold ($p < 0.05$). Genes meeting both criteria are highlighted, with upregulated genes shown in green and downregulated genes in red, while non-significant genes are depicted in black. Data represent three independent biological replicates (N=3)



Supplementary Figure S21: Transcriptional effects of inflammatory stimulation and Citra01 treatment in dTHP-1 cells cultured in co-culture with Arlo cells. Volcano plots showing differential gene expression in dTHP-1 cells cultured in co-culture with Arlo cells under control (Ctrl), inflammatory stimulation (Infl; 30 ng/mL IFN- γ and 100 ng/mL LPS), inflammation combined with Citra01 treatment (Infl+Treat; 30 ng/mL IFN- γ , 100 ng/mL LPS, and 300 μ M Citra01), and Citra01 treatment alone (Treat; 300 μ M Citra01) conditions. Comparisons are shown as follows: (A) Ctrl vs. Infl; (B) Ctrl vs. Treat; (C) Treat vs. Infl; (D) Ctrl vs. Infl+Treat; (E) Infl vs. Infl+Treat; and (F) Treat vs. Infl+Treat. Each plot displays log₂ fold change on the x-axis and -log₁₀(p-value) on the y-axis. Vertical lines indicate the fold change (FC) threshold ($|\log_2FC| \geq 1$), and the horizontal line represents the statistical significance threshold ($p < 0.05$). Genes meeting both criteria are highlighted, with upregulated genes shown in green and downregulated genes in red, while non-significant genes are depicted in black. Data represent three independent biological replicates (N=3)

6.2 Scientific output

6.2.1 Publications in peer-reviewed journals

- 1) **Tobias Neu***, Clémentine Richter*, Lorenz Latta, Nicole Schneider-Daum, Claus-Michael Lehr (2024): **Cell-based *in vitro* models for pulmonary permeability studies**. In Concepts and Models for Drug Permeability Studies (Second Edition) Cell and Tissue Based *in Vitro* Culture Models. 2024, Pages 137-168, Elsevier, DOI: 10.1016/B978-0-443-15510-9.00009-8
- 2) **Tobias Neu**, Lorenz Latta, Philipp Donate, Timo Risch, Clémentine Richter, Marijas Jurisic, Sara El-Garawany, Ahmad Aljohmani, Alexander Gamrekeli, Felix Stöckle, Klaus Urbschat, Ahmed Wahid, Heba Metwaly, Daniela Yildiz, Rolf Müller, Marc Schneider, Nicole Schneider-Daum, Claus-Michael Lehr (2026): **Multiparametric characterization of the human alveolar epithelial cell line Arlo for modeling pulmonary edema** Submitted to Journal of Advanced Research (JAR).

- 3) Petra Obioma Nnamani, Chinekwu Nwagwu, Edith Obioma Diovu, Obiora Emmanuel Abonyi, Mariia Nesterkina, **Tobias Neu**, Clémentine Richter, Brigitta Loretz, Claus-Michael Lehr (2024): **Design and evaluation of nanostructured lipid carrier of bergenin isolated from *Pentaclethra macrophylla* for anti-inflammatory effect on lipopolysaccharide-induced inflammatory responses in macrophages.** In European Journal of Pharmaceutics and Biopharmaceutics Volume 200, July 2024, 114307. DOI: 10.1016/j.ejpb.2024.114307
 - 4) Camilla Passi, **Tobias Neu**, Nicole Schneider-Daum, Claus-Michael Lehr, Marc Schneider, Sangeun Lee (2025): **Nanoparticles Formed by Isoprenoid-Antitumoral Polymer Conjugates via Schiff-base for Superior Drug Loading and Enhanced Efficacy in Endosomes.** In ChemMedChem. DOI: 10.1002/cmdc.202500810
 - 5) Sina Simon, Thanusa Shanmugalingam, **Tobias Neu**, Nicole Schneider-Daum, Claus-Michael Lehr, Carina Canthrell (2025): **Mapping the ABC transporter landscape in the human lung: A comprehensive characterisation of P-gp, MRP1 and BCRP expression and functional activity in human lung epithelial cells.** In European Journal of Pharmaceutical Sciences, Volume 215, 1 December 2025, 107333. DOI: 10.1016/j.ejps.2025.107333
 - 6) Samy Aliyazdi, Sarah Frisch, **Tobias Neu**, Barbara Veldung, Pankaj Karande, Ulrich F. Schaefer, Brigitta Loretz, Thomas Vogt, Claus-Michael Lehr (2024): **3D-Printed Human Hair Follicle Model to Investigate Topically Administered Nano-Antibiotics.** In ACS Biomaterials Science & Engineering, Vol 10/Issue 8. DOI: 10.1021/acsbiomaterials.4c00570
 - 7) Roya Shafiei, Alaa Alhayek, Lorenz Latta, **Tobias Neu**, Ahmed Aljohmani, Lukas Hiller, Sahar Abdollahibiroun, Eva-Maria Schönborn, Nicole Schneider-Daum, Jörg Haupenthal, Daniela Yildiz, Claus-Michael Lehr, Anna K.H. Hirsch (2025): **Exploring the role of *Pseudomonas aeruginosa* Elastase B in Lung Epithelial Barrier Dysfunction: Advancing Towards Anti-Virulence Therapies.** In Revision at ACS Infectious diseases
 - 8) Chutao Chen, Mingming Zhao, Fakhar Waqas, Eleonora Campus, **Tobias Neu**, Andreas Kany, Fangfang Chen, Ulrike Beutling, Bosede Owolabi, Callum O’Kane, Taosan Li, Jenny Worthington, Nicole Schneider-Daum, Claus-Michael Lehr, Anna KH Hirsch, Mark Brönstrup, Walid AM Elgaher, Nikolaos Chalkias, Wulf Blankenfeldt, Alessandro Michelucci, Frank Pessler, Konrad Büssow (2026): **A small molecule ACOD1 inhibitor reveals enzyme-dependent vs. –independent effects of ACOD1 on cell metabolism and inflammation and restricts mammary tumor growth *in vivo*.** Submitted to Nature Metabolism
- 6.2.2 Oral presentations
- 1) “The novel monoclonal human alveolar epithelial cell line “Arlo” for modeling the pulmonary air-blood-barrier *in vitro*” at EUSAAT in Linz (Austria), 2024.

- 2) **“Multiparametric characterization of the human alveolar epithelial cell line Arlo: From electrophysiological properties to pulmonary edema model”** at CRS DeChAt meets BioBarriers in Saarbrücken (Germany), 2026

6.2.3 Poster presentations

- 1) **“*In vitro* applications of Arlo, a novel monoclonal human alveolar epithelial cell line”** at HIPS Symposium in Saarbrücken (Germany), 2023
- 2) **“The novel monoclonal human alveolar epithelial cell line “Arlo” as *in vitro* model of the air-blood barrier”** at ISAM Congress in Saarbrücken (Germany), 2023
- 3) **“The novel monoclonal human alveolar epithelial cell line “Arlo” as *in vitro* model of the air-blood barrier”** at CRS Local Chapter Germany in Bad Dürkheim (Germany), 2024
- 4) **“The novel monoclonal human alveolar epithelial cell line “Arlo” as *in vitro* model of the air-blood barrier”** at HIPS Symposium in Saarbrücken (Germany), 2024
- 5) **“The novel monoclonal human alveolar epithelial cell line “Arlo” as *in vitro* model of the air-blood barrier”** at 3R Symposium Saarland in Saarbrücken (Germany), 2024
- 6) **“The novel monoclonal human alveolar epithelial cell line “Arlo” as *in vitro* model of the air-blood barrier”** at CRS DeChAt in Bern (Switzerland), 2025
- 7) **“The novel monoclonal human alveolar epithelial cell line “Arlo” as *in vitro* model of the air-blood barrier”** at International Conference of Anti-infectives in Saarbrücken (Germany), 2025
- 8) **“The novel monoclonal human alveolar epithelial cell line “Arlo” as *in vitro* model of the air-blood barrier”** at PhD Students Day 2025 in Saarbrücken (Germany), 2025
- 9) **“The novel monoclonal human alveolar epithelial cell line “Arlo” as *in vitro* model of the air-blood barrier”** at ISAM Congress in Washington D.C. (USA), 2025

6.2.4 Awards

- 1) **Saarland Research Award for Alternatives to Animal Testing.** In Homburg, 2024
- 2) **Young Scientist Travel Award,** at EUSAAT in Linz, 2024

6.3 Acknowledgements

At the beginning of this 3.5-year journey, I was driven by curiosity about how to model the human lung, an honestly very complex organ, *in vitro*. The motivation was not only to help reduce the number of animals sacrificed for experiments that often provide limited translational benefit, but also to contribute to better science and, ultimately, to the faster development of new drugs and medical advances. However, this journey would not have been possible without a number of remarkable people.

First and foremost, I would like to thank my Doktorvater, Prof. Claus-Michael Lehr, for giving me the opportunity to pursue my PhD in his group and for creating such a great scientific environment with outstanding experts in the field. Without his expertise and steady hand at the helm, this project would hardly have reached a safe harbor.

I am also very grateful to my two supervisors, Dr. Nicole Schneider-Daum and Dr. Lorenz Latta. Their complementary perspectives greatly shaped this project: Lorenz continuously brought creative ideas and enthusiasm, while Nicole balanced them with her experience and strategic perspective, helping to guide the project safely to completion. Beyond their scientific guidance, I feel very fortunate to have worked with both of them on a personal level and could hardly have wished for a better supervisory team.

A big thank you also goes to Clémentine for giving me an easy start and for generously sharing her experience with me. Special thanks also go to Achim and Josch. We shared nearly the entire duration of our PhDs together and experienced many moments both in and outside the lab, forming a great friendship along the way.

I am also very grateful for my amazing office mates over the years and for the many great discussions as well as the moments of procrastination. In particular, I would like to mention Aghiad, Mohamed, Jesús, and Ana. Beyond office borders, I am equally thankful for many wonderful colleagues who made this time memorable. Cheers to Mélina, Mariia, Vesa, Anne, Fabiana, Jo, Sven, Rafael, and Steffen.

Another group of people without whom this PhD would not have been possible is our fantastic technician team, who keep the lab running. Pascal, Petra, Tabea, and Clara, you are the hidden but essential gears that keep this group moving.

Many thanks also go to Jan and Philipp for the countless philosophical discussions about life choices and the inevitable ups and downs of the PhD journey.

My sincere gratitude also goes to all co-authors, collaborators, and colleagues who contributed to this work but could not be mentioned individually here.

Finally, and most importantly, I want to thank my family and friends, who formed the opposite pole to my scientific life. With them I could step away from science for a while and forget about the struggles of academic life, whether at festivals, concerts, on vacations, or simply by

spending quality time together. Mina, our dog, was also an important part of this, reliably getting me away from my desk and out into the fresh air.

In particular, I would like to thank my parents, my sister, the family of my partner, and of course Vera. Our anniversary falls on the first of October, which also happens to be the day I officially started my PhD. She has been by my side throughout this entire journey and has been an incredible support in every regard, from planning vacations to recover from stressful phases, to helping me prepare for conferences, or simply by always having an open ear for my complaints. Thank you.

6.4 Declaration of the use of generative AI

As part of the preparation of this thesis, ChatGPT powered by GPT-5 was utilized to support language refinement. All content was subsequently reviewed and edited by the author, who retains full responsibility for the final manuscript.