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# Polarized Airway Epithelial Models for Immunological Co-Culture Studies

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## Key Words

Co-culture models · Airway epithelium · Dendritic cells · T cells · Immune homeostasis · Tolerance

## Abstract

Epithelial cells line all cavities and surfaces throughout the body and play a substantial role in maintaining tissue homeostasis. Asthma and other atopic diseases are increasing worldwide and allergic disorders are hypothesized to be a consequence of a combination of dysregulation of the epithelial response towards environmental antigens and genetic susceptibility, resulting in inflammation and T cell-derived immune responses. In vivo animal models have long been used to study immune homeostasis of the airways but are limited by species restriction and lack of exposure to a natural environment of both potential allergens and microflora. Limitations of these models prompt a need to develop new human cell-based in vitro models. A variety of co-culture systems for modelling the respiratory epithelium exist and are available to the scientific community. The models have become increasingly sophisticated and specific care needs to be taken with regard to cell types, culture medium and culture models, depending on the aim of the study. Although great strides have been made, there is still a need for further optimization, and optimally also for standardization, in order for in vitro co-culture models to become powerful tools in

the discovery of key molecules dictating immunity and/or tolerance, and for understanding the complex interplay that takes place between mucosa, airway epithelium and resident or infiltrating immune cells. This review focuses on current knowledge and the advantages and limitations of the different cell types and culture methods used in co-culture models of the human airways.

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## Introduction

All cavities and surfaces throughout the body contain a layer of epithelial cells and are thus often the first cells to interact with external antigens. The skin is the most straightforward example but the lumen of the intestines and airways also include an epithelial cell layer to protect the physiological 'interior' against harmful external pathogens or noxious substances. Due to its role as an interface, many diseases involve the epithelium in various ways, even with pathogens that are not primarily aimed at the epithelia. In order for pathogens to gain entry to their target organs, they need to pass and interact with the epithelium, and this may involve cell damage and necrosis. With as much as 15,000 litres of air passing through our lungs every day, we are constantly exposed to a multitude of pollutants, environmental allergens and patho-

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gens of different kinds. Not all of these exposures are potentially harmful. One of the airway epithelium's main functions, besides providing a structural barrier and allowing respiration to proceed, is to help maintain homeostasis by assisting our immune cells to distinguish between harmful and harmless exposure. When this mechanism fails we experience inflammatory reactions against antigens that are not inherently dangerous, for example environmental allergens, and repeated exposures together with the memory of our adaptive immunity lead to deleterious reactions that may develop into a chronic state of disease.

Asthma and other atopic diseases are increasing worldwide. It has been hypothesized that allergic disorders may be a consequence of complex interactions between environmental allergens and epithelial tissues combined with genetic susceptibility resulting in T cell-derived immune responses [1]. Resident and circulating antigen-presenting cells, primarily dendritic cells (DCs), continuously sample their environment at epithelial surfaces. These sentinel cells express a variety of pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), recognizing pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). When exposed to PAMPs or DAMPs, DCs are able to orchestrate immune responses by migrating to local draining lymph nodes and presenting antigens to T cells. Through cell-to-cell contact and the production of cytokines and chemokines, DCs play an important part in the polarization of T cell responses, and hence for the activation and recruitment of other inflammatory cells to the epithelial tissue [2]. The maturation and polarization of DCs in response to many pathogens have both been well studied, but interactions between the epithelium and DCs while they sample antigens are likely to also instruct and modulate DC responses. Other, more newly identified cell types, such as type 2 innate lymphoid cells (ICL2), may also play important roles in epithelial type 2 responses. ICL2s produce type 2 cytokines in response to epithelial-derived IL-25 and IL-33, and furthermore appear to represent the majority of IL-5- and IL-13-producing lung cells in murine asthma models [3–6]. The epithelial coordination of immune responses has become a field of increasing interest with emerging theories viewing allergy and asthma at least partially as a result of epithelial disorders. The theory is that persistent type 2 responses are a symptom of erroneous-instructed antigen-presenting cells combined with a Th2-biased microenvironment in both the local environment and the lymphatic tissues of the airways. Whether homeostasis in non-allergic in-

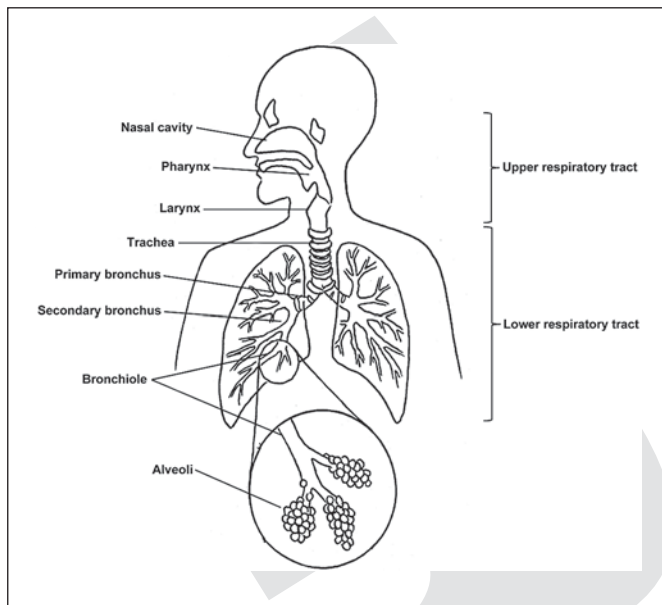
dividuals is maintained due to the triggering of mechanisms that are not present in certain individuals genetically susceptible to allergy, or if allergic individuals have an exaggerated response towards antigens, is still debated [7, 8]. There are now emerging data supporting the belief that epithelial cell barrier dysfunction leads to an overzealous immune activation [9–13].

In vivo animal models have long been used to study homeostasis in the lungs but are often hampered by a lack of functional homology between animal and human molecules, a lack of lengthy exposure to environmental factors and a lack of unknown predisposing genetic polymorphisms. Dissecting how tissue-specific non-immune cells and DCs co-operate in co-ordinating tissue homeostasis and inflammation in humans, for example, has multiple practical problems and there is a need to develop new cell-based in vitro human tissue models to overcome many of the limitations with animal models. Many of the existing in vitro models were initially used to study mechanisms of drug uptake across the epithelium but have become increasingly sophisticated, and can now be used to study cell-cell and cell-matrix interactions during homeostasis and inflammation [14–19]. This review focuses on the advantages and limitations of the different cell types and culture conditions applied in co-culture models of the airways, aiming to study cell-cell interaction between the airway epithelium and their neighbouring innate immune cells.

## The Airway Epithelium

### *Epithelial Morphology of the Airways and Danger Signalling*

'The airways' is a collective term encompassing all tissues involved in the transportation and exchange of gases. It is normally divided into the upper airways, comprising the nose, the nasopharynx and the larynx, and the lower airways, comprising the trachea, the bronchi, the bronchioles and finally the alveoli, where the gas exchange takes place (fig. 1) [20]. There are several structural and cellular mechanisms within the airways that help to protect them against harmful material and potential pathogens. A surfactant film is present along the lower airways [21, 22] and, together with the mucociliary escalator of the upper airways, matter can be transported and removed back up the airways where it can subsequently be swallowed into the esophagus [23]. Innate cell populations such as macrophages (professional phagocytes) are also constantly present in the airways and in the alveoli,



**Fig. 1.** Schematic illustration of the human airways. The human airways encompass all organs involved in the process of respiration from the site of inhalation at the nasal cavity to the lungs and the alveoli, where the exchange of oxygen and carbon dioxide takes place. Adapted from Castro and Kraft [204].

and a population of DCs resides inside and underneath the airway epithelium [24, 25]. Lastly, there are specific tight junction (TJ) complexes maintaining the structural integrity of the epithelial layer between the cells [26, 27].

The upper airway epithelium is lined by predominantly ciliated columnar cells together with goblet cells, whereas the epithelium in the lower airways is dominated by cuboidal ciliated cells together with Clara cells [28]. Apart from these tissue-specific cells within the epithelium there are also a variety of non-epithelial migratory cells, such as lymphocytes, leukocytes and mast cells [29]. Moving further down the respiratory system to the alveoli there are two main epithelial cell types. The squamous alveolar type I pneumocytes are responsible for the gas exchange and cover roughly 96% of the alveolar surface area [30], whereas the smaller cuboidal alveolar type II (ATII) pneumocytes, which cover about 3% of the alveolar surface, are responsible for synthesizing and secreting antimicrobial and surface-active components [31]. Alveolar type I pneumocyte cells lack the ability to renew themselves but are generated from type II pneumocytes.

In parallel with DCs, epithelial cells are also equipped to recognize PAMPs through specialized PRRs such as TLRs [32]. However, as TLRs and other PRRs in essence are unable to discriminate between pathogenic and non-

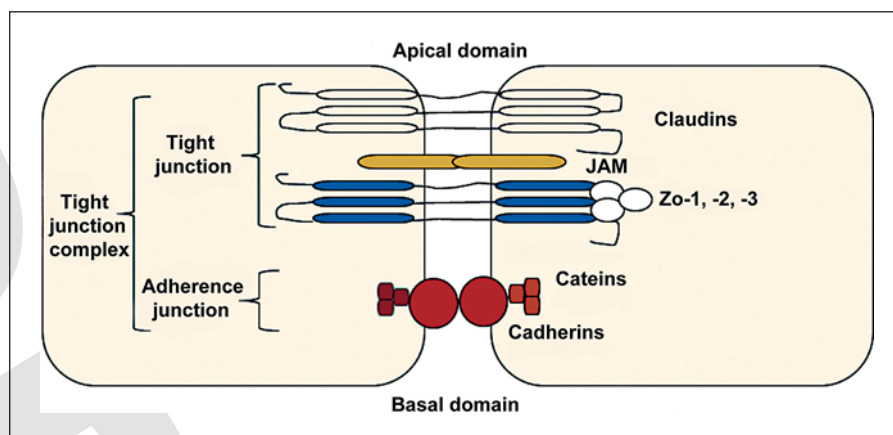
pathogenic microorganisms, other mechanisms are involved to maintain a correct balance between immune homeostasis and activation in a non-sterile environment such as the airways. These are likely to involve the recognition of DAMPs, and several concepts of organ-specific and graded immune responses have been proposed in the last couple of years to answer some of these questions [33]. According to some of these, the different organs sense infectious danger in different ways depending on their natural morphology and physiology, and act upon danger to modulate and instruct local immune responses thereafter [33]. For example, in sterile environments such as in the spleen, maximum sensitivity and immune activation towards any pathogen recognition is expected. In the opposite case where there is constant microbial exposure, as in the colon, the natural response is instead tolerance where recognition by PAMPs needs to be constantly suppressed. As the airways are not sterile but not as heavily exposed to microbes as the colon, a more graded response would be necessary to uphold homeostasis [34].

There have been several studies showing that the sensitivity and the activation threshold of TLRs in bronchial epithelial cells are indeed regulated [35], supporting the concept that alveolar epithelial cells (AECs) actively discriminate between potential danger signals. One such mechanism is the bronchial epithelium's hyporesponsiveness towards Gram-positive bacteria, due to low expression of TLR2 and a lack of expression of CD36, which mediates the phagocytosis of Gram-positive bacteria [35, 36]. Others have shown that certain micro-organisms or aeroallergens may function as adjuvants for epithelial activation in both protease-dependent and independent manners [37, 38]. The airway epithelium also maintains a steady-state condition by producing anti-inflammatory cytokines, such as TGF- $\beta$  and indoleamine 2,3-dioxygenase, which suppress T cell-mediated immunity, as well as the activation of antigen-presenting cells [34, 39].

In an inflammatory setting, the activation of PRRs on epithelial cells leads to the release of cytokines, chemokines and antimicrobial peptides that are both able to attract and activate innate and adaptive immune cells. It is plausible that prior exposure to DAMPs and PAMPs alters the activation threshold of epithelial cells, which could either increase the expression of PRRs themselves or other signalling proteins downstream of PRRs. Epithelial exposure to cigarette smoke and some respiratory viruses have been found to upregulate the expression of TLR4 and promotes its localization to the cell membrane, thereby increasing epithelial responsiveness to subsequent endotoxin exposure [40, 41]. Most PRR signalling



**Fig. 2.** The TJ complexes between neighbouring epithelial cells may actually be divided into bona fide TJs and adherence junctions. The TJ consists of claudins, JAM and occludin, on which ZO-1, 2 and 3 molecules attach and connect the TJ to the actin cytoskeleton. The adherence junction consists of cadherins associated with different catenins that also attach to the actin cytoskeleton of the cells.



pathways activate the transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B), responsible for the expression of multiple genes of inflammatory cytokines [42]. These signalling pathways have primarily been studied in mice, taking advantage of the model allergen ovalbumin, and have revealed that constitutive activation of NF- $\kappa$ B in AECs was sufficient to activate DCs and break inhalational tolerance towards ovalbumin [43]. Inhibition of the expression of NF- $\kappa$ B in AECs reversely decreased recruitment of type 2 effector cells and reduced airway remodelling [44]. Taken together, it is now clear that AECs not only play a crucial role in upholding homeostasis, but are also important in the recognition and initiation of immune responses towards pathogens.

#### *Barrier Function and Epithelial Polarization*

In order for the heterogeneous composition of airway epithelial cells to act as a barrier between the air we breathe and the body, there is a large variety of TJ protein complexes between neighbouring cells, resulting in variable tightness of the epithelium [26, 27, 45]. TJ formation between epithelial cells happens during a process known as polarization [46]. Polarization includes the sorting and distribution of certain membrane proteins and lipids into the three surface domains: apical, basal and lateral. Studies have shown that proteins move more or less freely between the lateral and basal domains, and the localization of proteins hereto is often referred to as being basolateral. The apical domain is faced towards the luminal compartment, the physiological exterior, and contains proteins that are important, for example, in absorption and for PRRs to recognize any inhaled pathogens.

The lateral domains include proteins involved in cell-to-cell interaction between neighbouring cells, with pro-

teins responsible for epithelial cell-to-cell contact and with TJs located the most apically, representing a boundary between the apical and basolateral domains [47]. The TJs are made up of three distinct types of transmembrane proteins comprising: occludin, claudins and junctional adhesion molecules (JAM; fig. 2) [48–50]. The TJ proteins are the basis for AEC barrier function and enable control of paracellular transport and cellular communication [46, 51, 52]. Besides the proteins that are part of the TJ complex there are other cell-to-cell associated proteins, such as gap junction proteins (connexins) and transmembrane proteins (cadherins), which are linked to both the actin cytoskeleton and cell adhesion molecules through intracellular proteins such as catenins [53–55]. The basal domain faces the extracellular compartment, the physiological interior, allowing contact with the extracellular matrix as well as with resident and migratory immune cells responsible for homeostasis or the triggering of the immune system. Although the intricate machinery responsible for the intracellular changes and the trafficking and sorting of membrane proteins in the polarization process have been fairly well described [56, 57], the organization of immuno-related receptors on epithelial cells have yet to be fully elucidated.

E-cadherin is a major component of the TJ complex, and its involvement has been associated with inhalational tolerance towards allergens [58]. E-cadherin expression is reduced in biopsies from asthmatics and inhibitory signals are transmitted to DCs via E-cadherin interactions between DCs and epithelial cells [59, 60]. Loss of E-cadherin expression leads to decreased epithelial polarization and hampers inhibitory signalling, facilitating allergic sensitization by the activation of DCs [59]. Loss of E-cadherin in cultured epithelial cells has also been found

to enhance the production of inflammatory Th2 cytokines such as thymic stromal lymphopoietin (TSLP), and could potentially be an important factor in breaching tolerance towards inhaled allergens [61]. Besides the secretion of TSLP, AECs have also been found to recruit immature DCs by producing CCL20 and  $\beta$ -defensins [56], as well as several pro-inflammatory cytokines responsible for the activation effector cells in type 2 responses, such as IL-6, IL-33, IL-25 and GM-CSF [12, 62–67].

Little is known about how airway epithelial integrity and the degree of polarization in terms of how receptor diversity of the epithelial basolateral and apical domains influence immune homeostasis and activation. In the digestive system, unstimulated, polarized epithelial cells of the gastric and intestinal mucosa modulate the function of local DCs, mainly via the release of soluble mediators, resulting in a non-inflammatory phenotype crucial for maintaining homeostasis [68–71]. The conditioning either by direct contact or through soluble factors, or both, instructing the DCs on how to polarize ensuing T cell responses, has been referred to as imprinting [71]. In the airways we have recently shown, using a bronchial epithelial cell line, that a polarized, intact epithelial layer dampens immune responses and maintains homeostasis, even when exposed to allergens. DCs in direct contact with polarized AECs developed a tolerogenic phenotype when exposed to bacterial components, and AEC-imprinted DCs derived from allergic individuals had a decreased ability to stimulate autologous T cells after allergen exposure [72].

The continuous exposure to both harmful and harmless antigens and the vast surface area of the airways makes these defence mechanisms and barrier functions crucial [73]. However, despite the protective properties of the airways, respiratory diseases are common and increasing [74], resulting in more attention being directed towards examining how allergens and certain antigens manage to overcome the epithelial barrier and how the epithelium responds towards the challenge.

#### *Epithelial Uptake and Antigen Presentation*

Besides its role as a protective barrier, the polarized epithelium most likely also plays an important part in facilitating the uptake of antigens and allergens. Proteins, which are normally unable to penetrate and move through the epithelium, are normally deposited on the luminal surface to the apical side. DCs residing underneath the epithelium capture these antigens and allergen proteins without affecting the epithelial integrity [75]. The exact mechanism by which the DC dendrites cross the TJs and sample these molecules is still not clear [76]. In gut mu-

cosa, sub-epithelial DCs capture antigens across the epithelial barrier by extending fine cytoplasmic dendrites between the epithelial cells and through their TJs [77, 78]. The DCs express certain TJ proteins that fuse with the epithelial TJs to reach across the epithelial cells, without disturbing the structural integrity. This mechanism has also been reported in situ for the human nasal mucosa, where dendrite extension was shown to penetrate beyond well-developed epithelial TJs [73, 79]. However, the epithelium itself may also be involved in the transport and uptake of allergens. Recent observations indicated that the timothy grass allergen Phl p 1 is rapidly transported through the epithelium by an active process, but this was only observed through the epithelium in allergic patients, and not in healthy subjects [80]. Studies done with the birch allergen Bet v 1 on nasal epithelium showed similar results, with the allergen only taken up in patients allergic to birch and not in healthy controls [81, 82]. These findings support new emerging theories that allergy and asthma may be driven by epithelial dysfunction. This emphasizes further the need to establish robust AEC models to better elucidate the mechanisms behind the antigen sampling across the epithelium. Lastly, AECs, and specifically ATII pneumocytes, express MHC class II molecules and can act as antigen-presenting cells upon microbial activation [83]. The type II cells have subsequently been shown to directly present antigens to T cells and may play a role in both the priming and re-stimulation of adaptive T cell responses [84]. Although the type II pneumocytes express MHC class II molecules, they lack expression of co-stimulatory molecules, e.g. CD80 and CD86 [85]. Studies have shown that epithelial antigen presentation to T cells in the absence of co-stimulatory molecules in combination with the secretion of non-inflammatory cytokines, such as TGF- $\beta$ , leads to the induction of T cell anergy and/or regulatory T cell responses [84, 86]. T cell activation and commitment is normally initiated in the primary draining lymph node but during inflammation bronchoalveolar lymphatic tissue may develop. Under such circumstances the direct interaction between epithelial cells and T cells may be of utmost importance for regulating the activation of both effector and naïve T cells. One would assume that for AECs to be able to present antigens and to induce regulatory T cell responses in these settings, it is crucial that the epithelium maintains its barrier properties and polarization, expressing PRRs at the apical domain, while the MHC molecules are expressed at the basolateral domain. It is therefore important for co-culture models investigating AEC interactions with other cell types to ensure AEC polarization.

## Analysis of Epithelial Cell Polarization

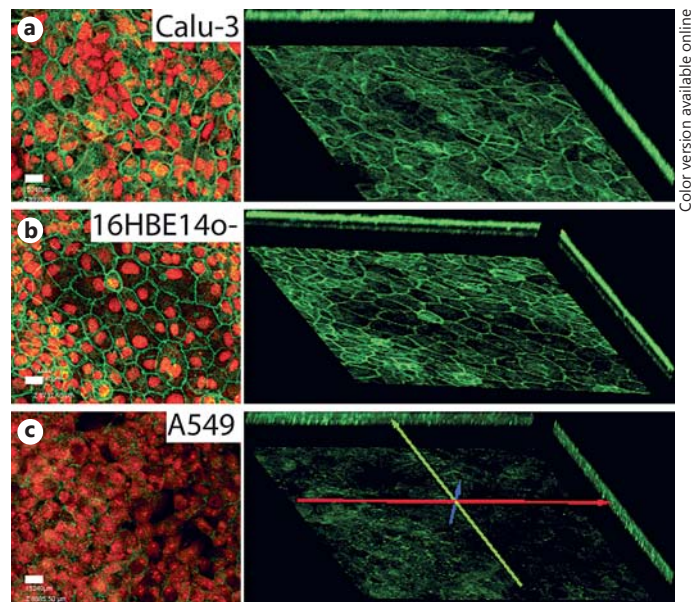
As discussed, polarization is a key process for establishing a proper barrier function, and the degree of polarization can be analyzed by measuring different aspects of the polarization mechanism. Essentially, there are three strategies to ascertain polarization: (a) expression and localization of TJ proteins by fluorescence or confocal microscopy, (b) assessment of barrier function by means of diffusion of small tracer molecules or (c) by measurement of transepithelial electrical resistance (TEER) across the epithelial layer. Several studies have compared these strategies and found that they correspond well with each other [87–94]. For practical reasons the measurement of TEER appears to be the easiest accessible of the three methods, and TEER measurement can be applied routinely without disturbing the integrity of the AECs and thus allowing the same cells to be examined in various setups.

### *Fluorescence and Confocal Microscopy-Assisted Localization of TJ Proteins*

Fluorescence and confocal microscopy is another widely used method to detect cytoplasmic and membrane proteins of the TJs. Illumination in a confocal microscope is achieved by scanning one or more focused beams of light, usually from a laser, across the specimen. The light excites fluorophores conjugated to antibodies, and the emitted light is subsequently captured by a confocal microscope.

Immunofluorescent staining and fluorescence microscopy was traditionally limited in its sensitivity, but with improvements in fluorescent conjugates as well as better microscopes has made this less of an issue today. To verify TJ formation in AECs, the most commonly analyzed proteins are occludin, E-cadherin or zonula occludens (ZO)-1, 2 or 3. In fully differentiated and polarized epithelial cells immunofluorescent staining of TJs produces an image with a honeycomb-like pattern when viewed in the x-y plane, vertical to the plane of the monolayers (fig. 3). Some adherence proteins, such as E-cadherin, are not located at the upmost apical portion of the membrane, and confocal microscopy also allows for staining to be viewed in the x-z plane when visualizing 3D structures.

Protocols for preparing samples differ in some details between different laboratories but the steps are generally similar. Samples need to be washed, fixated and permeabilized, and subsequently blocked to minimize background fluorescence, after which specific antibodies spe-



Color version available online

**Fig. 3.** 2D and 3D confocal microscopy images of the airway epithelial cell lines Calu-3 (a), 16HBE14o- (b) and A549 (c), visualizing the expression and localization of the TJ protein occludin. Cell nuclei were visualized by DAPI (red) and occludin was visualized by Alexa 488-conjugated anti-occludin antibody (green; colors refer to the online version only) [72].

cific to the desired protein to stain are added. There are a multitude of commercially available fluorescently labeled secondary antibodies that emit light in different spectrums, facilitating the use of several colors simultaneously. In order to clarify cell placement, the cell nuclei are traditionally also stained, most commonly with DAPI (4',6-diamidino-2-phenylindole) that stains the nucleus red.

During preparation, epithelial cell samples are most likely cultured on cell culture inserts. In order to avoid disruption of the cells prior to confocal analysis the AECs can be stained directly on culture inserts. Inserts with the AECs can be cut out of their plastic frame and mounted on object glass prior to the staining protocol. As with any protocol utilizing fluorescent labelling, many of these steps need to be done in dim light with the inclusion of an anti-fading reagent in the mounting media to prevent the loss of fluorescence.

### *Diffusion Studies to Assess Barrier Function*

One of the main functions of the AECs is to prevent the free diffusion of particles across the epithelial barrier. This can also be utilized to assess epithelial polarization and the establishment of TJs. In vitro models with im-



mortalized epithelial cells have been used for absorption and diffusion studies since the late 1980s, initially with intestinal epithelial cells, and later with airway epithelium [19, 95].

Different compounds cross the epithelial barrier by different mechanisms. Most exogenous macromolecules with lower molecular weights are most likely absorbed by passive diffusion across the airway epithelium via the TJ pathways [96]. Large hydrophilic compounds, such as mannitol and dextran, are absorbed through similar mechanisms across the epithelium and are therefore often used as model compounds to analyze the diffusion properties of polarized AECs *in vitro*. Comparative studies of solute permeability between *in vivo* and *in vitro* epithelium are limited, but available data for the two most commonly used bronchial epithelial cell lines, Calu-3 and 16HBE14o–, correlate well with primary epithelial cell cultures [97]. Permeability studies with *in vitro* epithelial cultures are more common and several studies have shown a very clear correlation between TEER values and diffusion rates of mannitol [91, 98–101] as well as dextran [92, 102–104]. Further studies by Man et al. [91] illustrated this relationship by the disruption of E-cadherin function, resulting in the loss of TEER and increased permeability to mannitol and dextran paracellular flux. AEC permeability is often presented by calculating a permeability co-efficient ( $P_{app}$ ) for a specific compound. This is done by the following equation:

$$P_{app} = (dc/dt)/(A \times C_0), \quad (1)$$

where  $dc/dt$  is the transport rate in mol/s,  $A$  is the surface area of the membrane in  $cm^2$  and  $C_0$  is the initial concentration of the compound added to the apical chamber of the AECs in  $mol/cm^3$ . The compound of interest can be either radiolabelled or fluorescently labelled. In order to calculate the transport rate (mol/s), samples are consecutively obtained from the basolateral medium and the measurement of fluorescence or radioactivity allows for an estimation of the concentration, and hence of the diffusion across the epithelial barrier.

#### Measurement of TEER

When establishing *in vitro* models for co-culture studies, two chamber models are commonly used. This is done with cell culture inserts composed of a porous membrane upon which the AECs are cultured. After the AECs polarize, the basal and apical surfaces are easily accessed on either side of the cell inserts. TEER can then be assessed by measuring the difference in electric potential – movements of ions – across the epithelium, which is a

measure of the tightness of the cell-cell contacts within the epithelium. Specialized epithelial volt-ohmmeters are commercially available (catalogue No. MERS00002, Millipore, Billerica, Mass., USA, and catalogue No. EVOM2, World Precision Instruments, Sarasota, Fla., USA) and used to measure TEER across cell inserts using ‘chopstick’ electrodes (catalogue No. MERSSTX03, Millipore, or catalogue No. STX3, World Precision Instruments) that are adjustable to enable placement of the electrodes into either side of filter inserts in cell culture wells (fig. 4) [105]. By inducing an alternating current, the specialized epithelial volt-ohmmeter produces a zero net charge, thereby eliminating any adverse effects on the AEC layer that a static direct current would create. A more detailed schematic of the current and blueprint of a homemade epithelial volt-ohmmeter is provided in online supplementary figure 1 (see [www.karger.com/doi/10.1159/000445833](http://www.karger.com/doi/10.1159/000445833) for all online suppl. material). The volt-ohmmeter is also unaffected by any inherent membrane capacitance and membrane voltage when measuring TEER across the cultured AECs.

The TEER value is calculated by subtracting the electrical resistance of the filter insert in the absence of epithelial cells from the value measured with cells, and then by multiplying the result with the surface area of the insert. As a measure of TEER, the resulting unit is given in  $\Omega \cdot cm^2$ :

$$\Delta R_{TEER} = A_{Insert} \times (\overline{R_{Cell+insert}} - \overline{R_{Insert}}). \quad (2)$$

where  $TEER$  ( $R_{TEER}$ ) is obtained by measuring the difference in resistance across culture inserts with AEC cultures

$$(\overline{R_{Cell+insert}})$$

and culture insert without cells

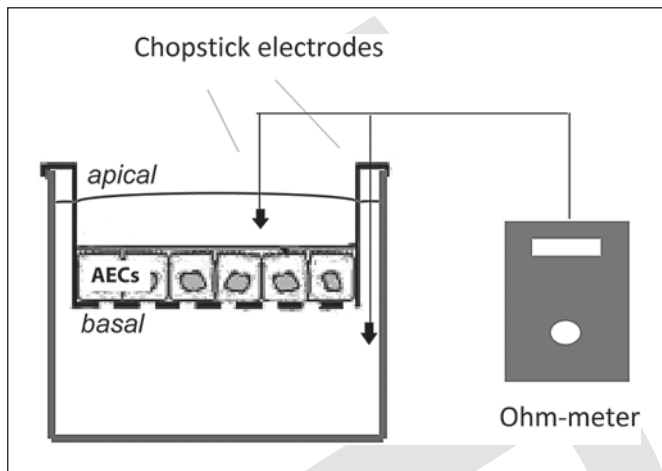
$$(\overline{R_{Insert}}),$$

and then multiplied with the insert surface area  $A_{Insert}$  (in  $cm^2$ ).

This process requires that the epithelial cell layer is in contact with culture medium on both sides, meaning that culture models designed to have the epithelial cells at an air-liquid interface (ALI), which is discussed later, would require the addition of culture medium to the apical side for the duration of the measurement.

The precise definition of what is a relevant degree of polarization and what are adequate TEER values differs quite a lot among different groups. TEER values are reported within a variable span, even when using the same cell lines. These variations are most likely due to differ-





**Fig. 4.** Measuring TEER across a polarized epithelial cell layer is performed in a two-chamber system using a specialized ohm-meter and two chopstick electrodes.

ences in culture conditions, such as seeding number, use of matrix proteins or culture medium, but differences in the handling technique and protocol for measurement could also play an important role. Working with the bronchial cell line 16HBE14o–, our group noticed that TEER values could differ by up to 5% depending on how long after the addition of culture medium measurements were made [unpubl. data]. For primary alveolar epithelial cultures, TEER values of  $>1,000 \Omega \cdot \text{cm}^2$  have generally been obtained [102], whereas bronchial primary cells generally lead to TEER values of  $400\text{--}4,000 \Omega \cdot \text{cm}^2$  [106]. Although, some groups report ‘polarized’ epithelium with TEER values as low as  $150 \Omega \cdot \text{cm}^2$  [107], we propose that it is rational to set the bar at the lowest TEER observed in cultures with primary airway epithelial cells, at approximately  $400 \Omega \cdot \text{cm}^2$ . Due to its easiness, the measurement of TEER is the most common way to ascertain polarization, but it is highly recommended that it be supplemented by other techniques when characterizing new model systems.

### Epithelial Cell Types in Airway Co-Culture Models

In short, there are three main approaches to the study of cell-cell interactions: in vivo animal experiments, ex vivo studies of cells from isolated lung tissue or biopsies and, lastly, in vitro cell culture systems using cell lines or primary cells in combination with accompanying cells, such as DCs. Such in vitro cell culture systems create sim-

plified biological systems in basic science that offer more controllable, versatile and reproducible setups compared with in vivo and ex vivo systems.

The characterization and use of epithelial barriers in vitro started in the 1980s with the aim of finding an ideal cell culture model to study the transcellular transport of drugs which would also be robust and reproducible to simplify large-scale studies and minimize animal testing [108]. The co-culture models in use today are all based on the pioneer work done by Hidalgo et al. [95] on the intestinal cell line Caco-2, which have been adapted to co-culture models of the airway epithelium and comprise a large span of different setups. In the more basic models, other cell types are only exposed to AEC supernatants either by the transfer of supernatants from cultured AECs or by separating the cell cultures with culture inserts in transwells. This has been done, for example, by Bleck et al. [109, 110] to investigate the impact of diesel exhaust particles on AEC cytokine production and, with no direct contact between the cell types investigated or uptake across the epithelium, these models do not necessarily utilize polarized epithelium. As airway co-culture models become more sophisticated they often include polarizing epithelial cells with or without cell culture inserts [72, 87, 111–113]. These models can be further utilized to investigate the additional influence of direct epithelial contact on neighbouring cells as well as the effect on uptake across the epithelium by innate cells such as macrophages and DCs.

The source of the AECs in co-culture models is also of importance and epithelial cells from freshly isolated tissue (primary cultures) or from a continuous cell line can be used. Depending on the aim of the co-culture study, different epithelial cells can be utilized for optimal results. Here we will provide an overview of the most commonly used cell types in human airway co-culture studies. Different cell types and characteristics are also summarized in table 1.

#### Primary Cells

Primary cultures isolated from tissue will most often represent a heterogeneous population of several different (epithelial) cell types and each isolate will be unique and impossible to fully reproduce. The number of cells will also often be a limiting factor when isolating from normal human airway tissue [114, 115], making it difficult to co-ordinate large and/or repetitive studies using primary cells. However, it is also important to emphasize that primary epithelial cells represent the native and original cells of the microenvironment, which is intend-

**Table 1.** Physiological features and culture conditions for commonly used airway epithelial cells

Cell type/ name	Origin	Phenotype	ALI culture	TEER, $\Omega \cdot \text{cm}^2$	TEER ALI, $\Omega \cdot \text{cm}^2$	TJ-protein expression	Reference No.
RPMI 2650	Nasal squamous cell carcinoma	Small monolayers	Yes	100	200	Yes (at ALI)	109, 113, 114, 117, 119
Primary bronchial ECs	Healthy tissue	Mixed, ciliated cells, goblet cells, serous cells, basal cells	Yes	400–4,000	~750	High (localized)	101, 104, 113
16HBE14o–	Healthy tissue, immortalized	Rounded, cuboidal, non-ciliated	No	700–2,500	n.a.	High (localized)	66, 82, 84, 104, 118
Calu-3	Adenocarcinoma	Non-ciliated (some report ciliated), columnar	Yes	~1,200	~600	High (localized)	66, 83, 101, 106, 107, 111, 118
BEAS-2B	Healthy bronchial tissue, immortalized	Squamous	No	0–150	n.a.	Low/medium (part-localized)	101, 118
Primary AECs	Healthy tissue	Mixed alveolar type I and II, lamellar bodies, squamous cells	Yes	>1,000	>1,000	High (localized)	84, 96, 116, 118
A549	Alveolar adenocarcinoma	Membrane-bound inclusions, alveolar type II-like	No	0–150	n.a.	Low (non-localized)	66, 84, 105, 112, 115, 120
NH441	Alveolar adenocarcinoma	Mixed alveolar type II cells and Clara cells	No	300	n.a.	n.a.	108, 110, 115

The ranking of cell types are given according to their location in the airways. ECs = Epithelial cells; n.a. = not applied.

ed for study, and in theory the optimal candidate for establishing epithelial co-culture models. Similarly, primary bronchial epithelial cells from laboratory animals have been in use over the last 30 years [116–121]. Human primary AECs can be obtained in several different ways: (a) autopsy of non-cancerous individuals, (b) atraumatic methods or (c) traumatic methods [121]. Traumatic methods also include the surgical removal of nasal polyps or biopsies and, together with post-mortem autopsy methods, their main advantage is ample cells or tissue that can be harvested and used for culture. One disadvantage of obtaining cells from surgical procedures is that they can rarely be repeated and post-mortem samples are obviously unique. Epithelial cells obtained from polyp surgery are the most frequently reported source of excised human nasal tissue and are widely used for primary cell cultures [122, 123]. However, polyp epithelium has been found to exhibit higher levels of  $\text{Na}^+$  absorption, increased  $\text{Cl}^-$  permeability and increased numbers of ILC2s in patients with chronic rhinosinusitis compared to healthy epithelium [124], indicating that AECs from nasal polyps may be functionally different than primary cells from other parts of the nasal mucosa [125]. Other techniques to obtain primary cells are through atraumatic techniques such as cellular scrapings or brushings. These samples can be easier to obtain from the nasal mu-

cosa in comparison with samples from the lower airways. In contrast to the more traumatic methods, these techniques can often be done more than once from the same donor, albeit cell counts are often lower in comparison with traumatic techniques.

Model systems using primary tracheobronchial epithelial cells from humans were first published over 20 years ago [126]. Most of the protocols have been used for drug absorption studies, and primary AECs function well for a few generations but lose their ability to form TJs after a couple of generations, and TEER measurements indicate that polarization is lost [127]. AECs isolated from bronchoscopies are terminally differentiated and short-lived cells that have been shown to have lower metabolic capacities than actively growing cells in vivo or in vitro [128]. One study showed that the primary cells failed to generate any active ion transport, in addition to losing their ability to polarize, after only 4 generations [127]. Although primary bronchial epithelial cells mimic a functional mucosal barrier by depositing extracellular matrix proteins and developing TJs and adherence proteins, such as MUC5AC,  $\beta$ -tubulin IV, ZO-1 and E-cadherin, studies have shown that these abilities may heavily depend on the individual donor [107, 129].

Human alveolar epithelial type II pneumocytes that are isolated from normal human lung tissue undergo

morphological and histochemical changes, causing them to change from type II to type I-like cells [115]. Several studies have also shown that cultured type II pneumocytes differentiate into heterocellular compositions of type I and type II pneumocytes. These heterocultures form monolayers with well-established TJs and TEER above  $1,000 \Omega \cdot \text{cm}^2$  [115, 130]. Although this method makes it possible to obtain polarized alveolar epithelium with a desirable phenotype and intact TJs, it still has some limitations. Primary cells from other tissues can often be used for at least a couple of generations, but primary AECs generally exhibit very limited proliferation in culture [106].

Even though biopsy samples can lead to higher cell numbers than brushings, for example, it is difficult to obtain cell yields adequate for large scale studies. This makes primary cells an inconvenient alternative for large-scale experiments such as drug screening, and primary cells have thus not been widely utilized for biopharmaceutical purposes [131]. Other factors to consider are the complicated maintenance and high degree of variability between donors, which decreases the reproducibility between experiments, particularly with respect to the development of TEER, as the primary cells also have a finite lifespan and may be difficult to manipulate. In a publication by Lehmann et al. [90] it is concluded that working with primary AECs is much more time consuming than the culturing of epithelial cell lines and, as cultivation time can vary for primary cells, for co-culture studies it can be challenging to work with other cells in parallel. It should also be mentioned that a ready-to-use culture system based on primary human tracheobronchial cells has been made commercially available by the Mattek Corporation (EpiAirway™, Mattek Corp., Ashland, Mass., USA) [132]. EpiAirway™ has been marketed for use in drug delivery studies, but has recently been used in co-culture studies [133]. Additionally, Lonza (Basel, Switzerland) has recently made a panel of human primary bronchial epithelial cells commercially available, offering the choice of healthy primary cells with guaranteed cilia formation, mucin production and high TEER levels, or primary cells from a variety of donors diagnosed with asthma, COPD or cystic fibrosis. Such standardized cultured primary epithelial cells could potentially prove to be great tools in future co-culture studies. Despite the obstacles mentioned above, and depending on the situation and due to the current lack of strongly polarizing alveolar-derived cell lines (TEER  $>4,000 \Omega \cdot \text{cm}^2$ ), primary AECs may be the best choice for studies of airway epithelium.

### Primary Cells in ex vivo Models

Conducting experiments under ex vivo conditions allows for more controlled conditions than is possible in vivo experiments, but also suffers from similar disadvantages, such as donor and specimen variability and lowered reproducibility. As with culture models using primary cells, one of the main advantages of using ex vivo tissues is the ability to perform tests or measurements that would otherwise not be possible or ethically acceptable in living subjects and still maintain a mixed phenotype. Ex vivo models of the airway epithelium have mainly been used to study characteristics of the epithelium in patients with asthma or allergy, and not to study cellular interactions between immune cells and epithelial cells. One such study, published by Allam et al. [134], investigated epithelial immune responses in tissues from the oral mucosa from allergic donors. Excised tissue samples were placed in Petri dishes and exposed to FITC-conjugated allergen after which migrating tissue antigen-presenting cells, defined as oral Langerhans cells, could be collected and their uptake, phenotype, cytokine production and ability to prime T cells analyzed [134]. This is an elegant and simple model that to a large extent manages to retain the complex interplay between different cell types within a tissue whilst retaining many of the practical issues stated earlier with regard to donor-specific variability and availability.

### Primary Cells from Asthmatic Donors

The comparison of primary bronchial epithelial cells from asthma and non-asthma subjects has been made in a number of studies to investigate intrinsic differences in the asthmatic epithelium. The AECs from asthmatic patients exhibit an increased proliferation and secretion of inflammatory cytokines in comparison with AECs from non-atopics [135], and also possess a reduced ability to repair mechanical wounds [136, 137]. Several groups have cultured asthmatic epithelial cells, showing that they obtain a less differentiated phenotype with increased numbers of basal cells and decreased TJ formation [138, 139], and it has even been reported that primary epithelial cells from asthmatics are not able to polarize at all in vitro [51].

### Epithelial Cell Lines

Epithelial cell lines are more homogenous with little phenotypic differentiation compared to primary cells. This makes them more stable during culturing and relatively easy to work with. However, due to the transformation process and clonality, they are at risk of lacking important molecules that are otherwise present in vivo.



Using cell lines instead of primary cells are also often associated with significantly low costs and reduce the variability between different experiments. Several detailed protocols for how to culture and maintain different airway epithelial cell lines have been published during the last decade, spanning from the nasal epithelium down to the alveolar epithelium [131, 140].

#### RPMI 2650

There is presently only one nasal epithelial cell line in use for *in vitro* studies. The human nasal cell line RPMI 2650 (ATCC CCL 30) originates from an anaplastic squamous cell carcinoma of the human nasal septum [141]. The karyotype and cytokeratin polypeptide pattern of RPMI 2650 has been found to closely resemble that of normal human nasal epithelium [142, 143]. During culturing, RPMI 2650 cells grow in sheets of non-ciliated cells instead of polarized monolayers and only obtain TEER values of around  $100 \Omega \cdot \text{cm}^2$  [121]. Compared with TEER values obtained using primary cells from the lower airways (table 1), the TEER values of RPMI 2650 cells are very low and would normally not be considered polarized. However, primary nasal epithelial cells from excised human nasal epithelium also obtain very low TEER values when cultured *in vitro* with groups reporting TEER values below  $100 \Omega \cdot \text{cm}^2$  and other groups reporting values between 360 and  $640 \Omega \cdot \text{cm}^2$ , indicating that *in vitro* culture of nasal epithelial cells have yet to be fully optimized [144, 145]. The expression of TJ proteins was initially reported to be absent, but recent studies with RPMI 2650 cells at an ALI revealed the expression of several TJ proteins as well as a doubling of TEER [98, 145, 146]. This suggests that improving culture conditions could possibly make this cell line more viable for use in future *in vitro* co-culture models.

#### Calu-3

One of the most widely used bronchial cell lines is the Calu-3 cell line (ATCC HTB-55) derived from a bronchial epithelial adenocarcinoma of a 25-year-old Caucasian male [147]. The cell line shows excellent polarization with high levels of the TJ proteins occludin and E-cadherin localized at the junctions [72, 89] (fig. 3a). The Calu-3 cell line often reaches full polarization after 8–10 days in culture and obtains TEER values often exceeding  $1,000 \Omega \cdot \text{cm}^2$ , depending on the culture conditions [106, 107]. Interestingly, a study comparing Calu-3 cells and primary human bronchial epithelial cells found that TEER was higher and more robust in the Calu-3 cell line than in the primary bronchial epithelial cells. However, the expression of ZO-1 was higher in human bronchial

epithelial cells than in the Calu-3 cells at both the mRNA and protein levels, indicating that other TJ proteins play a more important role in maintaining a functionally polarized barrier [107].

The Calu-3 cell line exhibits serous cell properties and forms confluent monolayers of mixed cell phenotypes of ciliated and secretory properties and mucus production. The ability of the Calu-3 cell line to express cilia appears to be highly inconsistent and has only been reported by some investigators [148, 149], and not by others [92, 150–152]. As some groups have reported that the expression of cilia seems to disappear with an increasing passage number, the discrepancy could be related to the number of cell passages [89, 152]. Calu-3 cells have also been found to differentiate and polarize under both liquid-covered conditions as well as under ALI conditions, as discussed below.

#### 16HBE14o–

Another widely used bronchial epithelial cell line, 16HBE14o–, was developed by the transformation and immortalization of normal bronchial epithelial cells obtained from a 1-year-old male heart and lung transplant patient. The cells express high levels of the TJ proteins occludin and E-cadherin and form TJs (fig. 3b) [19, 72, 153]. 16HBE14o– cells are smaller and generally rounder or more cuboidal in shape compared to the Calu-3 cells, which appear to possess a columnar phenotype. The 16HBE14o– cells retain an epithelial phenotype, as demonstrated for the Calu-3 cells, with TJs and a proper transepithelial resistance [88, 154, 155]. In parallel with Calu-3 cells, there are conflicting reports of whether 16HBE14o– cells are ciliated or not, but they have been shown to express several transport proteins [88, 155].

When cultured under ALI conditions, 16HBE14o– cells fail to polarize and do not develop normal epithelial phenotypical and morphological traits, unlike the Calu-3 cell line [88]. In general, bronchial cell lines are expected to function and polarize when cultured at the ALI *in vitro* because epithelial cells are normally located at an air interface *in vivo*. The inability of the 16HBE14o– cell line to polarize under ALI conditions is an exception for which the exact reason or mechanism is currently unknown. Future optimization of culture conditions, such as changes in culture medium, and the inclusion of matrix proteins for the coating or implementation of other culture techniques, such as the use of rotating wall vessels (RWV, discussed below), could possibly enable the 16HBE14o– cell line to polarize when cultured at ALI, as described for the nasal RPMI 2650 cell line.



## BEAS-2B

The BEAS-2B cell line (ATCC CRL-9609) is available from the American Type Culture Collection and derives from normal human epithelial cells immortalized using the adenovirus 12-simian virus 40 hybrid virus [156]. The cell line has been used to study bronchial epithelial structure and the expression and activity of drug-metabolizing enzymes [157, 158]. The BEAS-2B cell line does not appear to polarize and form TJs and only obtains TEER values below  $100 \Omega \cdot \text{cm}^2$  in vitro [107]. Even though the cell line fails to polarize, it has still been used in some co-culture studies to evaluate the epithelial influence on different accompanying immune cells after challenges with tobacco smoke, diesel particles or endotoxin [110, 159, 160]. These studies have used models where accompanying cells were either mixed together or separated by filter inserts, with the accompanying cells being exposed to only soluble factors secreted by the BEAS-2B cells [110, 159, 160].

## A549

The most frequently used alveolar epithelial cell line is the A549 cell line (ATCC CL-185), which is derived from a human pulmonary adenocarcinoma [161]. The A549 cells contain multilamellar cytoplasmic inclusion bodies, which are typically seen on human lung ATII cells, although the inclusion bodies disappear with increasing culture time [162]. A549 cells are functionally deficient in TJs, and most research groups have concluded that the A549 cell line is unable to polarize and incapable of forming functional TJs [72, 102, 131, 163–165]. However, A549 cells do express certain TJ and adherence proteins, such as desmosomes and zonulae adherents, to an even greater extent than the 16HBE14o– cells that otherwise obtain high TEER values [165]. The inability of A549 cells to polarize also affects protein and dextran permeability, which has been reported to be over 2 orders of magnitude higher in A549 cells compared with other cell lines [131]. One group has reported TEER of around  $600 \Omega \cdot \text{cm}^2$  with the A549 cell line, although they also showed that large hydrophilic molecules translocated across the A549 cell layer at rates very close to free diffusion limits [103]. As this is the only report suggesting that the A549 cell line can polarize, the reported TEER measurements may be misleading. We and other groups have shown that both occludin and E-cadherin are expressed by A549 cells [72, 166]. However, this expression was relatively low and not localized to the same extent as seen for the Calu-3 and 16HBE14o– cell lines (fig. 3c). Despite its limitations, the A549 cell line is still used in co-culture

studies, often with the addition of other epithelial cell types in order to facilitate a polarized cell layer [87, 90, 167].

## NCI-H441

Besides A549, another human alveolar cell line called NCI-H441 (ATCC HTB-174) has been used in some published studies. The NCI-H441 cell line originates from a human lung adenocarcinoma and has been described to have characteristics of both ATII [168, 169] and bronchiolar Clara cells [170, 171]. Ion transport characteristics of the H441 cells suggest that it forms polarized monolayers, although the polarization appears to be highly variable with the majority of cultures only reaching TEER values of around  $300 \Omega \cdot \text{cm}^2$  [172, 173]. The NCI-H441 cell line has been used in co-culture models together with endothelial cells in studies aiming to characterize the air-blood barrier, but remains to be tested in co-cultures with immune cells [174, 175].

## The Accompanying Co-Cultured Cells

The two main applications of airway co-culture models have been either to investigate the effects on AECs and neighbouring cell types in response to antigens, or to develop competent uptake models for drug delivery. One of the main reasons for the increased interest in co-culture models with airway epithelium together with antigen-presenting cells is the finding that AECs secrete a variety of pro-inflammatory cytokines, especially TSLP, but also IL-25, IL-33 and IL-1b, that all seem to play important roles in the cross-talk between antigen-presenting cells and the epithelium in type 2-mediated immune responses [64]. Co-culture models provide a powerful tool to understand the crosstalk between the epithelium and its neighbouring cells. However, it is important to carefully characterize the phenotype of the accompanying cell type with the purpose of ensuring that it has not been activated due to handling.

From the onset of developing in vitro models of the epithelial surface there has been constant progress in optimizing culture conditions for the functional use of AECs. This work is still ongoing for many of the epithelial cell lines in use today. In contrast, many of the accompanying cell types used in these co-culture models, e.g. DCs, have established protocols detailing refined and specific culture conditions, but in co-culture setups the inclusion of additional cell types in the form of AECs is not always trivial in order to preserve the integrity of both

cell types. Antigen-presenting cells, such as macrophages and DCs, are in nature especially sensitive to changes in their surroundings. In suboptimal culture conditions these cells may become stressed or damaged, or even react towards an unknown component in a culture medium, something that can activate DCs and consequently affect the results [176]. Nonetheless, the most commonly used accompanying cell types in these co-culture models have been the DCs and macrophages, investigating how their responses towards particulate matter, pathogens and their subsequent effects on T-cell responses are influenced by AECs [67, 109, 110, 112, 113]. Both DCs and macrophages are very relevant cell types to investigate with AECs and both can be generated from monocytes, which are readily obtained in high quantities from buffy coats or full-blood samples [177].

In the airways, AECs have been found to affect and interact with cell types, such as mast cells, eosinophils, ILC2 and basophils, and co-culture models with AECs could become a powerful tool in further elucidating their roles in airway inflammation and homeostasis [67]. Peripheral blood eosinophils have been used in some co-culture studies with primary nasal epithelial cells, mainly to evaluate the effect of AEC-soluble factors on eosinophil migration and activation, but also how eosinophil soluble factors affect nasal epithelium [178–180]. Similar studies with AEC-conditioned medium have also been used to investigate mast cell proliferation and one group has investigated mast cell adhesion to tracheal epithelial cells in a canine system [181, 182]. However, to the authors' knowledge, ILC2s or basophils have yet to be used in any AEC co-culture model.

Airway co-culture models with one accompanying cell type have proven to be a great tool for investigating specific cell-cell interactions with AECs, and also to investigate interactions between several cell types. Some groups have taken this even further, incorporating macrophages or fibroblasts in addition to DCs [87, 90, 113, 159, 183]. This is a very interesting development for airway co-culture models, which in the future could potentially lead to complex multicell co-culture models, bringing us close to recreating airway tissues in vitro and becoming a rational supplement – or possible substitution – for in vivo and ex vivo studies.

## Culture Conditions

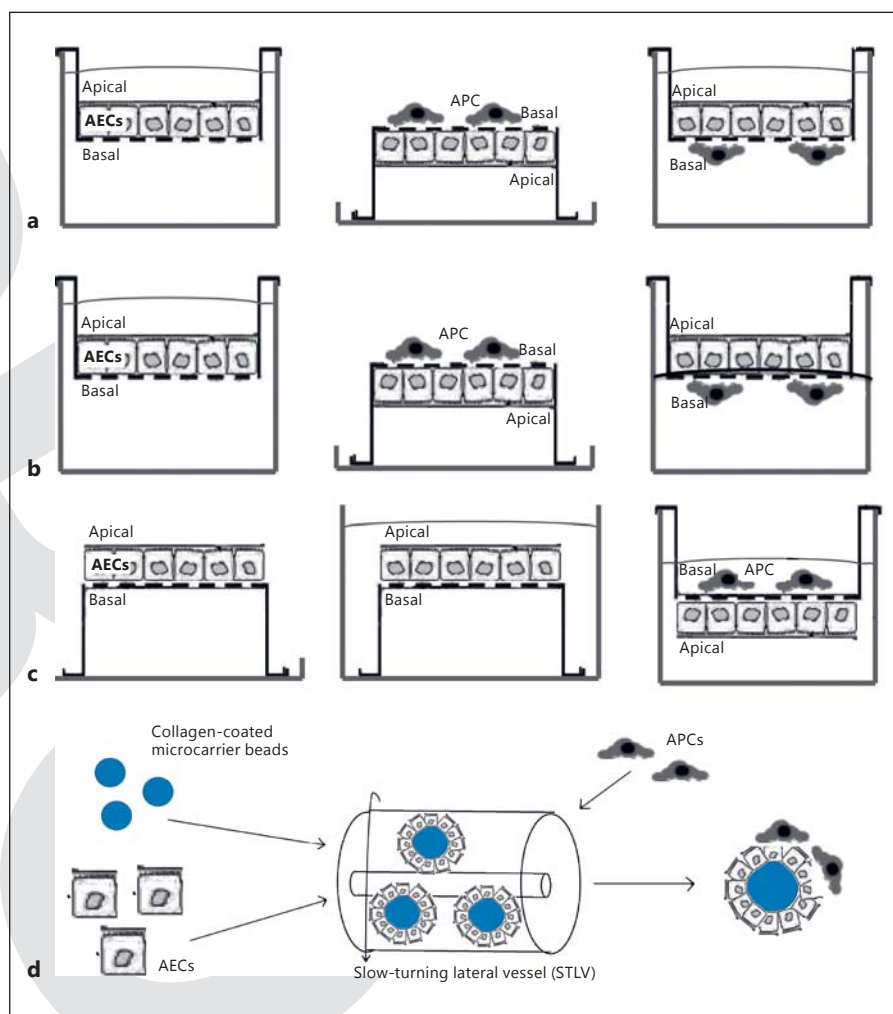
The culture conditions employed are critical for the characteristics of the resulting cell layer for both primary cells and cell lines [184, 185]. As seen in table 1, different

groups obtain different levels of polarization and TEER using the same cell lines. Besides inevitable variations in cell handling between laboratories and experimental set-ups, other factors might also affect the properties of epithelial cell layers. Early studies with intestinal epithelial cell culture models showed that differences in culture conditions, such as the coating of matrix proteins and culture medium, influenced the permeability and expression of transporter proteins [186, 187]. Similar differences in culture conditions have also been reported to greatly affect the growth and differentiation of primary airway epithelial cell cultures [94, 188]. Growing a confluent polarized epithelial layer also depends on the number of AECs seeded onto culture inserts. Most of the polarizing airway epithelial cell lines are most commonly used between day 7 and 10 [72, 89, 98, 99, 107, 183], similar to the use of primary bronchial and alveolar cells [116, 127, 189], although some have reported using primary cells at later time points, such as on day 14 [90]. The seeding densities of AECs are generally between  $10^5$  and  $10^6$  cells per square centimetre. Although it has been reported to not directly affect their ability to form confluent and polarized monolayers, seeding densities appear to mostly influence how many days of culture are needed before confluence and polarization are reached [98, 99, 190].

The inclusion of additional cell types in co-culture set-ups can also influence culture conditions for the AECs in several ways. In a triple cell co-culture study by Lehmann et al. [90] it was found that both the A549 and the 16HBE14o– cell lines obtained significantly lower TEER values after the addition of macrophages and DCs. Furthermore, the specific design and protocol for co-culturing also influences the ability of AECs to proliferate. A schematic representation of the most common co-culture models is provided in figure 5. Some co-culture protocols entail adding accompanying cells to the basal side of AECs by letting them attach to upended culture inserts (fig. 5) [72, 115, 167]. This step unfortunately leads to the full exposure of the AECs to air (high humidity, 37°C, 5% CO<sub>2</sub>) in the absence of culture medium, and we have found that such exposure severely damages epithelial polarization and decreases TEER by over 60% within 24 h [72].

### *ALI or Liquid-Covered Conditions*

One central decision when establishing an airway in vitro model is whether to culture the AECs at an ALI or under liquid-covered conditions. With AECs cultured on filter inserts it provides the opportunity to only supply medium to the basal side of the cells. This restriction in nutrients brings the AECs to an environment close to the



**Fig. 5.** Schematics of four different co-culture techniques. AECs are traditionally cultured in filter inserts submerged in media until polarized. These filter inserts can then be removed and upended to allow the attachment of accompanying cell types before the attachment filters are placed back in the well with culture media either fully covering all cells (**a**) or placing the AECs at an ALI (**b**). AECs can also be cultured on the opposite side of filter inserts, allowing for the addition of accompanying cells without air exposure (**c**). RWV technology allows for AECs to grow on coated microcarrier beads in suspension in low fluid-shear conditions inside cylindrical bioreactors, termed slow-turning lateral vessels (**d**). APCs = Antigen-presenting cells.

physiological situation. These ALI cultures can be used with both primary epithelial cells and certain epithelial cell lines. The precise culture techniques for ALI conditions have been described for several airway epithelial cell lines [92, 111, 191, 192].

Primary cells obtained during bronchoscopy of normal human volunteers are normally cultured on tissue culture plates coated with human collagen and used in studies after at least one passage [106]. These primary human bronchial epithelial cells can be cultured in ALI conditions using a defined medium to drive a differentiation into ciliated and goblet cells with a high degree of polarization [185, 193]. As most airway in vitro models build upon the work done with the intestinal epithelial cell line Caco-2 [95, 104], which was cultured fully suspended in medium, initial work with airway epithelial cell lines were tested in similar conditions. Such liquid-covered condi-

tions also offer some advantages, as particle uptake and transfer studies are more straightforward with TEER being easily monitored. However, for models designed to represent the airways, liquid-covered conditions partly fail to reflect the physiological conditions under which the airway epithelium normally operates. In vivo, the apical surface of AECs is exposed to air with only a thin layer of liquid covered by a surfactant film [22]. Several groups have suggested that culturing cells at an air-liquid interface plays an important role in the differentiation of respiratory epithelial cells and that AECs grown under ALI conditions undergo enhanced differentiation and attain better bioelectrical properties due to the increased aerobic respiration compared with AECs immersed in medium [89, 99, 131].

Although the bronchial cell lines Calu-3 and 16HBE14o- are generally considered to develop an ade-



quate and favourable phenotype under liquid-covered conditions, a few studies have suggested that the ALI condition may also be critical for the growth differentiation of these two cell lines [100, 101]. A study by Grainger et al. [89] showed that Calu-3 cells in ALI conditions produced a more columnar epithelium with a more rugged apical topography and greater glycoprotein secretion compared with cells cultured under liquid-covered conditions. In their study, the authors observed secretory vesicles in cells grown under both conditions, but extracellular glycoproteins were only detected at the cell surface when ALI conditions were applied. However, they recognized that any secreted mucus consisting of glycoproteins would be dispersed and removed with the media at the apical membrane under liquid-covered conditions [89]. Concurrent with other studies, the authors concluded that the Calu-3 cells at the ALI represent a more morphologically correct model of the bronchial epithelium in comparison with Calu-3 cells cultured under liquid-covered conditions [89, 151, 189]. However, Calu-3 cells obtain substantially lower TEER values under ALI conditions compared to culturing in liquid-covered conditions. During liquid-covered conditions TEER values are often over  $1000 \Omega \cdot \text{cm}^2$  but have been reported to reach as high as  $2,500 \Omega \cdot \text{cm}^2$  [72, 106, 194], whereas TEER values when cultured under ALI are typically  $300\text{--}600 \Omega \cdot \text{cm}^2$  [89, 106]. Thus, it is possible that even with a better morphology Calu-3 TJ formation is not as complete at the ALI and might not be optimal for all investigations of the bronchial epithelium.

It remains unknown why the Calu-3 cell line obtains higher TEER values during liquid-covered conditions compared to ALI conditions. It could be a matter of culture conditions not yet being refined enough at ALI conditions and that future optimization of culture conditions in terms of the use of matrix proteins or RVWs (discussed below) could possibly enable the Calu-3 cell line to better polarize at an ALI, as described for the nasal RPMI 2650 cell line or A549 cells. In the same context, 16HBE14o- cells remain to be successfully cultured under ALI conditions, and fully submerged cultures are commonly favoured [88]. There are a few examples of studies culturing 16HBE14o- in ALI conditions with positive staining for the TJ proteins ZO-1, occludin and the intercellular protein E-cadherin, but TEER values did not exceed  $250 \Omega \cdot \text{cm}^2$  [93, 99].

#### *RWV 3D Culture*

Over the last decade increasing effort has been made to utilize technology for creating 3D cell culture models to study cellular responses to external stimuli [195–197].

Through a bioreactor technology known as rotating wall vessel, or RWV, a few studies have been published in which the A549 cell line was used to create a 3D model of the alveolar epithelium [198, 199]. In brief, the RWV technology is an optimized suspension culture method in which cells are grown on coated microcarrier beads at low fluid-shear conditions inside cylindrical bioreactors, called slow-turning lateral vessels, STLV, or high aspect ratio vessels, HARV [200]. Through improved 3D cell-matrix and cell-cell interactions, the model showed significant improvements on morphological and phenotypical characteristics compared with the traditional 2D models using the A549 cell line. These improvements included the establishment of apical and basolateral polarity, enhanced expression of TJs, extracellular matrix proteins and extramembrane mucin localization [198]. The RWV modelling of the epithelium does not allow for traditional TEER measurements, but the fact that the 3D model showed a clear increased expression of TJ proteins compared to traditional 2D models suggests an improved polarization and makes the A549 cell line suitable for future co-culture studies using RWV technology.

#### *Optimization of Medium*

As different media result in different cell phenotypes regardless of whether AECs are cultured at an ALI or in liquid-covered conditions, AEC culturing requires specialized medium in order to obtain morphologically representative epithelial cells [107, 184, 201]. Many of the airway epithelial cell lines, such as the RPMI 2650, Calu-3 and 16HBE14o- cells, are cultured in MEM-based medium [72, 90, 98, 113, 167, 183], whereas the A549 and H441 cell lines are cultured in RPMI-based medium [72, 90, 167]. The majority of co-culture models utilize AECs cultured on transwell inserts, where they are allowed to form monolayers and polarize for several days before use. As it is important to maintain the epithelial properties, the most common practice has been to simply add whichever additional cell type is to be tested to the transwell system using the specialized AEC culture medium. With good use of controls, these studies can still yield important results, but if the aim is to characterize and study in vivo interactions, such a change of recommended medium for the accompanying cells without adaptation and/or optimization could affect their responses and hence phenotypes. In our co-culture experiments with the 16HBE14o- bronchial cell line and monocyte-derived DCs, we investigated how the two cell types responded to each other's medium. The 16HBE14o- cell line was found not to proliferate as readily in the culture medium recom-



mended for culturing monocyte-derived DCs, whereas monocyte-derived DCs failed to preserve their immature phenotype in the AEC medium. The DCs upregulated co-stimulatory receptors and produced pro-inflammatory cytokines, indicating a matured profile when cultured in the recommended medium for the AECs [72]. These results indicate that non-optimized culture medium for co-cultures affects results, making it incredibly important to determine the effect of a change in culture medium on each individual cell type. Optimizing culture mediums for more than one cell type can be done in several ways. By blending certain components of both recommended media it may be possible to find a medium which supports both or all cell types without affecting phenotypes [72]. Another approach is to mix the different recommended media at different ratios and find a composition suitable for both cell types. Although it might seem trivial, this is an important parameter to consider when designing a co-culture model, especially in order to study the parameters of cells involved in activating or repressing the immune response.

#### *Matrix Proteins*

Primary cells from the airways are normally cultured on tissue culture plates coated with human collagen and fibronectin [90, 106]. With the exception of a few alveolar cell lines, the AEC lines discussed in this review are also recommended to grow on surfaces coated with matrix proteins. Extracellular matrix proteins, including fibronectin and collagen, enhance cell adherence. The basement membrane in the airways is composed mainly of collagen fibers and, among the extracellular matrix proteins, type 1 collagen results in the best differentiation of airway epithelium [202, 203]. Coating of the culture surface affects the polarization of primary cultured airway cells; however, both Calu-3 and 16HBE14o- cells have

been reported to be able to form adequate barriers in the absence of any coating [131, 185]. Probably in line with other findings but as yet unpublished, we found that coated inserts resulted in the most consistent TEER values when growing these two cell lines to confluency. For the nasal epithelial-derived cell line RPMI 2650, collagen coating is a prerequisite for monolayer formation and also leads to improved differentiation, functional polarization and a significant TEER increase [98].

#### **Concluding Remarks**

The mechanisms regulating airway responses to allergens and other antigens are extremely complex and are influenced by the interaction of different cell types and cytokines in the airway microenvironment. Recreating such a complex environment in vitro using epithelial cell lines or primary cells as a field of research is still progressing. Choosing how to design a co-culture model depends on various factors, including the experimental question being addressed together with the cost, training and expertise required to successfully establish, optimize and apply the model. Depending on which part of the airways one aims to investigate, there are multiple options on how to design a suitable model. This review has illustrated the diverse set of options available today in order to recreate certain aspects of the morphological, biochemical and immunological features of the airways. The most commonly used cell lines for different areas of the airways have been discussed together with important considerations of the culture conditions. Recreating – or mimicking – organs and tissues as complex as the airways stresses the importance of careful validation and a clear aim of the immunological mechanism of study before designing and utilizing any airway co-culture models.

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