

Review

Potential of coculture *in vitro* models to study inflammatory and sensitizing effects of particles on the lung

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ABSTRACT

Exposure to particulate matter (PM) like nanoparticles (NPs) has increased in the last century due to increased combustion processes, road traffic, etc. In addition, the progress in chemical and cosmetic industry led to many new compounds, e.g. fragrances, which humans are exposed to every day. Many chemicals are known to act as contact and some as respiratory sensitizers, causing allergic reactions. Exposure to small particles of less than 100 nm in diameter is linked with an increased risk of respiratory diseases, such as asthma or rhinitis. To date already more than 1000 consumer products contain eNPs without knowing much about the health effects. In comparison to chemicals, the mechanisms by which PM and eNPs can cause sensitization are still not fully understood. Validated and regulatory accepted *in vitro* models to assess this hazard in its full range are still missing. While a huge number of animal studies contributed to our knowledge about sensitization processes, knowledge on involved cellular mechanisms is still limited. In this review relevant *in vitro* models to study and elucidate these mechanisms in more detail are presented and their potential to serve as part of a tiered testing strategy is discussed.

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1. Introduction

For a long time, *in vivo* experiments dominated the field of toxicological research. Already Greek philosophers reported about

experiments on living animals during the third and fourth century BC (Cohen and Loew, 1984). Nowadays, ethical discussions about the broad use of animals arose in the public opinion, spreading them to politics. Since the formulation of the 3R strategy (Replacement, Reduction and Refinement) in 1959 (Russel and Burch, 1959), there are increasing efforts to reduce the numbers of animals which are routinely used for toxicological tests. Organisations

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like FRAME (Fund for the Replacement of Animals in Medical Experiments) or CAAT (Johns Hopkins Center for Alternatives for Animal Testing) are committed to reduce and replace animal tests to a minimum by use of alternative methods. According to the 7th amendment of the 1976 European cosmetics directive (The European Parliament and the council of the European union, 2003), the use of animals for the testing of cosmetic formulations as well as cosmetic ingredients is forbidden in Europe since March, 11th 2009. Furthermore, a marketing ban on cosmetics, which concerns products containing animal tested compounds, has been decided by the European Union (Commission of the European Community, 2004). For drugs and their components there is no actual ban or amendment to change the testing strategies for these chemicals. The active principle of a drug must be tested *in vivo* to proof the impact and to fully understand the systemic effects. Regarding this legal background, the demand for alternative methods increased, but the replacement of well established *in vivo* assays by *in vitro* methods is a challenging task, and some investigators suspect that the current cut-off-dates for the testing and marketing bans are questionable (Hartung, 2008). Currently it seems as if this deadline has to be extended, since validated *in vivo* replacements for several fields of toxicology, like toxicokinetics, repeated dose or skin sensitization, will not be ready for regulatory use until 2013 (Adler et al., 2011).

To produce reliable and comparable results, it is necessary to have standardised and validated *in vitro* systems. In Europe, ECVAM (European Centre for the Validation of Alternative Methods) is responsible for this task. To date, validated alternative methods already exist for testing skin corrosivity or eye irritation (OECD, 2004b, 2009a,b). The biggest part of the currently available alternative methods contributes to the reduction or refinement of existing *in vivo* assays, but does not serve as full replacements. For example, the commonly used Draize test for eye irritation (Draize et al., 1944) can be partially replaced by *ex vivo* assays like “The Bovine Corneal Opacity and Permeability test methods [BCOP]” and the “Isolated Chicken Eye [ICE] test method” (Prinsen and Koëter, 1990, 1992, 1993; Southee, 1998; Southee and Curren, 1996). In these *ex vivo* assays, slaughterhouse waste derived eyes from cows and chicken are used, so that no living animals will be harmed. These two assays have a relatively low sensitivity, but they are able to predict the irritant potential of compounds accurately under defined conditions; however, negative results have to be verified by *in vivo* experiments (ICCVAM, 2006). Most of the alternative methods which are currently available in the ECVAM database (<http://ecvam.jrc.ec.europa.eu/>) show similar problems and still need *in vivo* data to finally verify the negative results in order to avoid the risk of false negatives. However, all positive results following the *ex vivo* assay are considered as true positive and do not need any further validation, thus as a consequence reducing the number of experimental animals.

Many chemicals are known to be able to act as contact sensitizers (Gerberick et al., 2005), but only few chemicals are known to cause sensitization of the respiratory tract (Commission of the European Community, 2004). Nevertheless, the exposure to inhalable sensitizers with either low molecular weight (LMW) (chemicals and some drugs) or high molecular weight (HMW) (mostly proteins) plays an important role in occupational lung diseases such as occupational asthma (reviewed in Chan-Yeung, 1990; Jeebhay and Quirce, 2007). It is estimated that up to 20% of adult asthma is induced by occupational factors and that approximately 90% of these involve immunological mechanisms (Mapp, 2005). A relatively new class of compounds that is suspected to have the potential to induce respiratory sensitization are nanoparticles (NPs). These can be of rather different origins such as combustion processes, erosion, volcanoes, human industrial engineering (eNPs), etc. (Biswas and Chang-Yu, 2005; Buzea et al., 2007; Shi et al.,

2001). In respect to their different sources, NPs can be dramatically different in their properties, such as solubility, net charge, hydrophobicity and size. Furthermore, eNPs may have a special surface coating or contain several different compounds, influencing e.g. the potential to induce oxidative stress (Limbach et al., 2007). As an example, polycationic particle surfaces can lead to a strong interaction with cell membranes *in vitro* and a considerable toxicity for some kinds of particles (Hoet et al., 2004).

Nanotechnology has attracted great attention during recent years due to their technologically interesting properties, e.g. small size but large surface area, reactivity, etc. (Adlakha-Hutcheon et al., 2009). It is expected that nanotechnology applications will contribute positively to the quality of life through the production of light and durable materials, cleaner energy and inexpensive clean water production, etc. (Adlakha-Hutcheon et al., 2009; Maynard et al., 2006; Savolainen et al., 2010). To date more than 1000 consumer products contain eNPs (Woodrow Wilson International Centre for Scholars, <http://nanotechproject.org>). However, some of the properties that make eNPs so unique and beneficial may also be dangerous for human health through the potential induction of cyto- and genotoxic effects, inflammation and allergy (Borm et al., 2006; Donaldson et al., 2005; Savolainen et al., 2010; Yang et al., 2009). There is strong evidence that the inhalation of particles like PM (less than 10 µm in size) and also of NPs (less than 100 nm in size) can cause and exacerbate lung diseases (Pietropaoli et al., 2004; Inoue et al., 2006). Due to their small sizes, NPs can cross cellular membranes and reach the bloodstream where they can have adverse effects on the cardiovascular system (Geiser et al., 2005; Schulz et al., 2005; Donaldson et al., 2005). Furthermore PM and some kinds of NPs such as carbon black or titanium dioxide can cause and worsen inflammatory reactions due to the generation of reactive oxygen species (ROS) either in a direct or indirect manner (Li et al., 2008). Both *in vitro* and *in vivo* assays show that intracellular ROS production after the inhalation and uptake of high doses of PM or NPs can lead to genotoxicity, and to an increased cancer risk as a consequence (Schins and Knaapen, 2007). Especially the large surface area of NPs in comparison to coarser particles (between 2.5 and 10 µm) seems to have an impact on the toxic potential of certain NPs (Carlsson et al., 2008; Karlsson et al., 2009). Due to the increasing number of PM and NPs in the environment, it becomes more and more relevant to have fast, easy and reliable methodology to forecast the toxic potential of small particles (Nel et al., 2006).

In contrast to the lung, some progress has been made regarding method development for the *in vitro* characterization of the interactions of chemicals with the skin. With the introduction of the “EPISKIN™” and “The Rat Skin Transcutaneous Electrical Resistance (TER) Test”, two full replacements for *in vivo* skin corrosivity evaluation are available (ECVAM, 1998a,b). For other endpoints like skin sensitization, it is generally accepted that a battery of assays, each representing one important step of the immunological process, are needed for hazard identification (Basketter and Kimber, 2009; Divkovic et al., 2005; Jowsey et al., 2006; Natsch et al., 2009). One *in chemico* assay (direct peptide reactivity assay, DPRA) and three *in vitro* assays (myeloid U937 skin sensitization test, MUSST; human cell line activation test, h-CLAT; and the Keratino-Sens assay) are currently being pre-validated by ECVAM (Aeby et al., 2010; Natsch et al., 2011; Sakaguchi et al., 2010).

Much less is available as alternative to animal testing in respect to respiratory sensitizers. Methods like the Local Lymph Node Assay (LLNA) or the reduced LLNA (rLLNA), which are monitoring the induced proliferative response of lymphocytes in the draining lymph nodes of mice (reviewed in Kimber et al., 2007), are partially able to recognize respiratory sensitizers but can not distinguish them from contact sensitizers (Boverhof et al., 2008; Kimber et al., 2010). However, a proper hazard assessment of sensitization is needed to improve occupational safety.

The aim of this review is to give an overview on the state-of-the-art of relevant lung cell-based *in vitro* assays, which are currently under development for the evaluation of particles and chemicals. Furthermore, the available approaches for contact and respiratory sensitization will be compared and in the discussion we will attempt to describe the advantages as well as the gaps of the current systems.

2. About the organization of the lung – a short overview

The respiratory system is highly specialized and it is designed to perform gas exchange between the blood stream and the inhaled air at a high yield. It can be subdivided into the upper and the lower respiratory tract. The upper respiratory tract comprises the nasal cavity, the pharynx and the larynx. The lower respiratory tract is composed of the trachea, which is fused to the larynx; the primary bronchi, which lead to the bronchioles; and the alveolar region at the end.

The bronchial tree in the lower respiratory tract can be roughly described as an accumulation of branching tubes, stemming from the main trachea (Mann-Jong et al., 2008). The diameter of these tubes, the bronchi, decreases progressively until the terminal bronchioles with the alveolar sacs at the end, which is also referred to as the distal respiratory tract (Magno and Fishman, 1982). The main function of the bronchi and bronchioli is to conduct inhaled air to the lower respiratory tract where the actual gas exchange takes place in more than 300 million alveoli. The conducting zone itself does not undergo gas exchange (BéruBé et al., 2010). The total surface area for gas exchange in an adult body is between 55 m² and 80 m² (Leonhardt, 1990) and at the end of a normal breath, the lung consists of about 80% air, 10% blood, and only 10% tissue by volume (Mühlfeld and Ochs, 2010).

The surface of the respiratory tree is formed by a continuous layer of epithelial cells, which play an important role in maintaining the functionality of the lung (e.g. forming a barrier to pathogens and other harmful compounds, facilitating mucociliary clearance,

secreting protective substances, etc.) (Proud, 2008). Furthermore, it has been found that the epithelium can influence the modulation of allergic immune responses (Toppila-Salmi et al., 2011). The epithelium shows a complex and different composition regarding e.g. the bronchioli and the alveoli (Fig. 1). In total, more than 40 cell types have been discovered within the epithelia of the respiratory system (Sorokin, 1970). Many of these are intermediates or differentiating cells, leaving more or less a dozen morphologically and functionally unique epithelial cell types that can be identified (Fig. 1) (Breeze and Wheeldon, 1977). The epithelium in the lower respiratory tract (trachea and bronchi) is pseudostratified and columnar, composed of secretory goblet cells which produce mucus and basal cells and ciliated cells, which are necessary for mucociliary clearance (Fig. 1). In the bronchioles, the epithelium consists of more cuboidal-shaped cells with shorter cilia and secretory clara cells. The distal respiratory tract with the alveoli mainly consists of alveolar type I and II (AT-I, AT-II) cells, which cover the alveolar epithelium (Fig. 1) (Mann-Jong et al., 2008). The alveolar epithelium is highly vascularised by capillaries underneath the basal membrane to improve the gas exchange between air and blood. AT-II cells serve many different functions, including surfactant production or as a progenitor of AT-I cells. A resident population of alveolar macrophages resides in the interstitial spaces to perform alveolar clearance of inhaled particles and pathogens (BéruBé et al., 2010). The role of the cells present on alveolar spaces will be discussed in detail at a later stage of this review (see Section 4.3).

3. Comparison between mono- and coculture systems

A monoculture system, which represents the classical *in vitro* system, can be used for many toxicological questions, ranging from studying the uptake or the cell death after the exposure to a compound or particle to modern -omic approaches (Lasserre et al., 2009). However, in order to gain more insight and enhanced understanding of the relevant interactions of inflammatory processes involving the interplay of several different cell types and

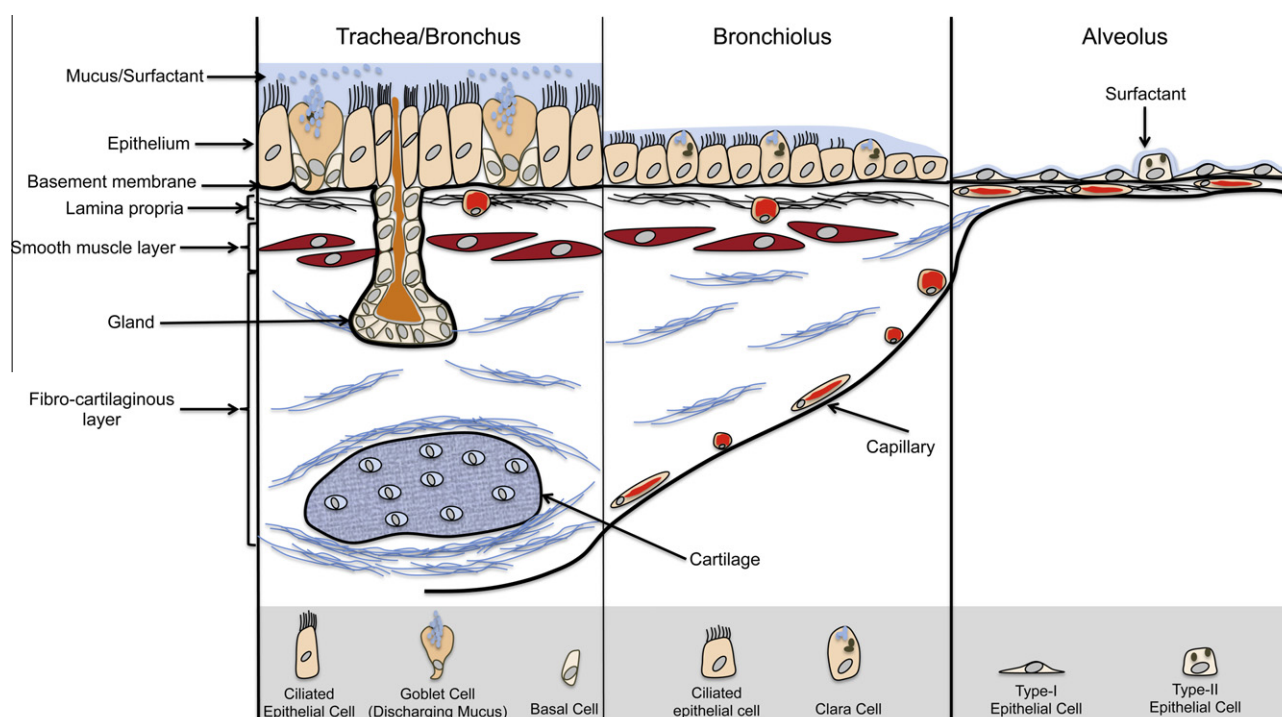


Fig. 1. Simplified scheme of the structure of the airway wall at the three principal levels. The epithelial layer gradually becomes reduced from pseudostratified to cuboidal and then to squamous. The organization of the airway wall is mainly a mosaic of epithelial and secretory cells. In the alveoli, the smooth muscle layer is not present. The fibrous layer contains cartilage only in bronchi and gradually becomes thinner as the alveolus is approached. Adapted and modified from Ochs and Weibel (2008).

mechanisms, coculture systems may be valuable tools allowing better prediction of effects to be expected.

For monoculture as well as for coculture systems it is possible either to use primary cells derived from biopsies, surplus biological material from surgeries or cell lines as a continuous source. The isolation of primary cells is often experimentally challenging and labour intensive, due to the problems to find adequate donors to assure the constant and homogenous supply with cell material. Freshly isolated primary cells are highly differentiated, but will start dedifferentiation soon after isolation when cultured *in vitro* (Hartung et al., 2002). In addition, isolates of primary cells represent a highly heterogeneous population in respect to state of differentiation and cell types present. The latter aspect is mainly a question of good isolation procedures and could be partially overcome. Cell lines are more homogenous, but show only few characteristics of differentiated cells, wherefore they often only poorly represent the *in vivo* situation (Hartung et al., 2002). In addition they may be less uniform than previously thought depending on their passage and culture conditions used (Bonifas et al., 2010). Nevertheless, for establishing a new assay it is often better to use cell lines instead of primary cells to avoid possible donor variations, otherwise experiments could be hard to standardise (BéruBé et al., 2009; Hartung et al., 2002).

In a coculture system at least two cell types are being cultivated together and the system can mimic more closely the *in vivo* situation. Although coculture systems are still lacking many details and characteristics of a differentiated tissue, such systems allow cell-cell communications. The development of such systems is challenging and time consuming due to e.g. difficulties in establishing the right cell ratios, the proper medium composition and the need to characterise the system for many properties, e.g. the formation of tight junctions, development of Trans Epithelial Electrical Resistance (TEER), etc. The presence of tight junctions and the development of a high TEER, showing the integrity of the epithelium, are of particular interest for studies concerning the trans-epithelial transport of compounds or their cellular uptake (Agu et al., 2001; Geys et al., 2006).

Frequently it may not be possible to meet the ideal requirements for each cell type, so that the best compromise concerning e.g. different amounts of serum or percentage of CO₂ has to be found. It has to be considered that some cell lines still hold the potential to differentiate into different cell types during the experimental course depending on culture conditions and factors present in the medium (Brackman et al., 1995; Schwende et al., 1996). HL-60 cells are e.g. able to differentiate into four different phenotypes (granulocytes, monocytes, macrophages and eosinophiles), depending on the stimulating agent (Collins, 1987) (Table 4). Therefore, many systems are quite versatile and the coculture system needs to be carefully characterised.

Complex models consisting of various epithelial cells with or without immune cells may be established to serve as models for inhalation toxicology studies. Furthermore this may include *in vitro* aerosol exposure at the air-liquid-interface (ALI). Such coculture systems can be especially useful for studying the interplay of epithelial cells with immune cells, which is an important event during inflammation.

4. Lung – *in vitro* models

4.1. Sensitization processes in skin and lung: what is already feasible *in vitro*?

While a large number of *in vivo* studies contributed much to our mechanistic understanding of complex processes such as contact or respiratory sensitization, it is still not possible to assess the risk of a new chemical or NP *in vitro*. The aim of this review is to briefly

summarize what is possible *in vitro* for contact sensitization and describe available and potential models for pulmonary exposure with particles (PM, NPs, etc.) as the main topic.

Regarding the mechanistic ladder of the skin sensitization process (Table 1), haptenation is a crucial step for many chemical sensitizers (Divkovic et al., 2005). This process is dependent on the intrinsic potential of a chemical to react with proteins and generate an immunogen. This protein-binding process can be predicted by *in silico* and *in chemico* methods, although it cannot be determined in these assays whether the generated hapten-protein-complexes could then activate the immune system. The *in silico* methods are comparing structural aspects of new compounds to chemicals which are already known as sensitizers. Other approaches evaluate the peptide reactivity in a cell-free *in chemico* system based on the evaluation of the reactivity of chemicals with glutathione or synthetic model peptides (Aleksic et al., 2009; Gerberick et al., 2009, 2004, 2007; Natsch et al., 2007).

Although peptide reactivity can be considered as a complex process, a protocol for measurement has already successfully been established. In contrast to that, much more research still needs to be done for the prediction of other steps leading to sensitization. *In vitro* assays representing the activation of dendritic cells (MUSST and h-CLAT) (Ade et al., 2006; Python et al., 2007; Sakaguchi et al., 2006; Sakaguchi et al., 2010) and the response of keratinocytes (KeratinSens) (Natsch et al., 2011) are already in pre-validation. However, to simulate steps such as dendritic cell (DC) migration or finally naïve T-cell priming, the interplay of epithelial and immune cells is needed. To date, only a few approaches are able to assess some of these steps in skin relevant models (Table 1).

In contrast to that, methodological approaches for the lung are much more limited. Except for the *in silico* and *in chemico* peptide reactivity, there are hardly any approaches available for the remaining steps (Table 1). *In silico* and *in chemico* approaches are also in development to forecast the reactivity of respiratory sensitizers (Vandebriel and van Loveren, 2010), but up to now no regulatory accepted or validated methods are available. In addition, an interesting approach to assess the epidermal involvement and the stimulation of antigen presenting cells in response to respiratory (non)sensitizers, by studying the gene profiles of BEAS-2B, A549 but also THP-1 has been described (Verstraelen et al., 2009a,b,c; Verstraelen et al., 2008). A more detailed summary about the advances in assessing respiratory inflammation and irritation has been published recently (Vandebriel et al., 2011).

4.2. Monoculture models of cells relevant for the respiratory system

Monoculture systems are commonly used for studying the uptake and the toxic effects of particles, e.g. particulate matter (PM) or NPs, or chemicals on epithelial or immune cells (AshaRani et al., 2009; Becker et al., 2005; Geys et al., 2006; Hussain et al., 2009, 2010; Jang et al., 2006; Penn et al., 2005; Richard, 2010; Sanchez-Perez et al., 2009). The two main ways to cultivate lung cells and to expose them to particles are submerged culture and air-liquid-interface (ALI). These exposure systems are also used for studying chemicals. However, it has to be considered that applying chemicals to cells via an aerosol chamber could lead to an unintended oxidation of the compound due to the enhanced contact with air. This air oxidation could alter the sensitizing potential (Karlberg et al., 1985, 1992; Sköld et al., 2002, 2004, 2006). Nevertheless, no system has yet been validated or pre-validated and all concepts still need further improvements.

4.2.1. Submerged cultivation vs. cultivation at the air-liquid-interface (ALI)

The submerged cultivation is the classical method to grow cells and to study effects of different agents. Depending on the assay,

Table 1Comparison of approaches to evaluate the hazard of sensitization in skin and lung *in vitro*.

Mechanistic step	Allergic contact dermatitis – skin		Respiratory sensitization – lung		
	Assay/approach	Status	Assay/approach	Status	Remarks
Absorption	(Q)SAR <i>in silico</i> approaches to predict the reactivity potential (Chaudhry et al., 2010; Dimitrov et al., 2005; Fedorowicz et al., 2005)	In development	(Q)SAR <i>in silico</i> approaches to predict the reactivity potential (Enoch et al., 2010; Seed and Agius, 2010; Seed et al., 2008)	In development	
	In vitro skin absorption studies using OECD guideline 427 and 428 (OECD, 2004a, 2008)	Regulatory accepted	Not applicable		
Haptentation	Direct peptide reactivity assay (DPRA) (Aleksic et al., 2009; Gerberick et al., 2009, 2004, 2007; Natsch et al., 2007)	In pre-validation	Direct peptide reactivity assay (DRPA) (Gerberick et al., 2004, 2007), also to distinguish between contact and respiratory sensitizer (Gauggel et al., 1993; Hopkins et al., 2005; Reichardt et al., 2003; Wass and Belin, 1990)	In development	1
Epidermal involvement	Epidermal equivalents such as RHE or epiderm (www.skinethic.com) Assessing the activation of keratinocytes (KeratinoSens) (Natsch et al., 2011)	In development/in pre-validation (KeratinoSens)	Based on 3D-reconstituted human tissue cytokine profiling (http://www.skinethic.com ; http://www.mattek.com) (Huang et al., 2009; Roggen et al., 2006) Gene profiles of epidermal cells (Verstraelen et al., 2009a, b)	In development	2
Antigen presenting cell stimulation	Human cell line activation test (h-CLAT) (Ashikaga et al., 2006; Sakaguchi et al., 2006); Myeloid U937 skin sensitization test (MUSST) (Ade et al., 2006; Python et al., 2007) Gene profiles of THP1 macrophages and monocytes (Lambrechts et al., 2009) Cross talk between keratinocytes and dendritic cells (Hennen et al., 2011)	h-CLAT and MUSST in pre-validation	Role of p38 MAPK in the selective release of IL-8 induced by chemical allergen in naïve THP-1 cells (Mitjans et al., 2008) Gene profiles of THP-1 cells (Verstraelen et al., 2009c)	In development	
Dendritic cell migration	Not applicable		Not applicable		
T cell proliferation	Human T cell priming assay (hTCPA) (Aeby et al., 2010)	In development	Not applicable		

Remarks: (1) Some studies were conducted including contact and respiratory sensitizers. It will also be necessary to consider the special conditions in the lung, such as the mucus or surfactant film. (2) Only limited datasets available, which suggest the use for *in vitro* sensitization assessment. Model systems for respiratory sensitization based on reconstituted human tissue are designed in the style of skin model systems.

this model is relatively simple to use, shows high reproducibility and is rather cost efficient. It is particularly relevant to study the toxicity of chemicals and particles that are water soluble, i.e. pneumotoxic heavy metals. These can be dissolved in cell culture medium and then be used to expose the cells of interest to study, i.e. effects on the viability of a particular cell type. For the submerged cultivation, the cells are studied in culture wells, covered by the medium (Fig. 2a). The surface of the cells has no contact with the ambient air and therefore PM or NPs have to cross the medium be-

fore they can reach the cell surface. Along this way, modification of particles due to the proteins and supplements of the medium may take place, which can enhance or totally quench potential toxic effects. It is further assumed that it is the outer layer of proteins around a particle that is recognized by the cells, especially of the immune system, and leads to their responses (Lynch et al., 2007). Contact with the culture medium can also be considered as a critical step for other chemicals, especially because chemical sensitizers hold the potential to react with proteins *in vitro* too and exhibit

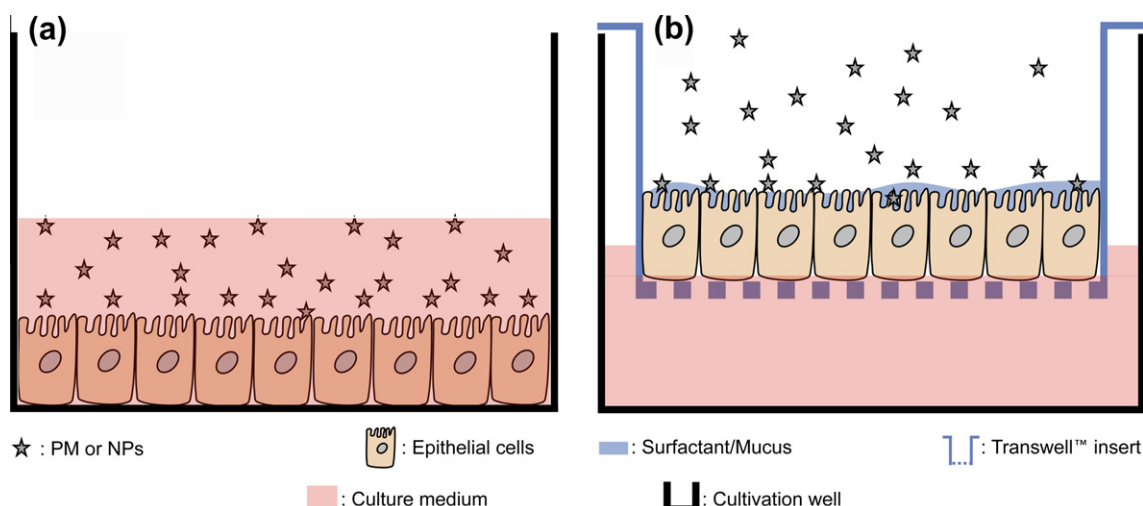


Fig. 2. (a) Scheme of the submerged cultivation of a monoculture system. (b) Scheme of the ALI cultivation of a mono-culture system for aerosol exposure.

their sensitizing potential after the haptentation step (Landsteiner and Jacobs, 1936).

The administered mass of a particle in a submerged *in vitro* system will never reflect properly the deposited mass on the cell surface *in vivo* because of effects like the Brownian diffusion or agglomeration (Mühlfeld et al., 2008; Teeguarden et al., 2007). Due to these conditions for particulate compounds, dose determination in submerged cultures and thus evaluation of toxicity can lead to huge errors, meaning that, at this stage, submerged cultures are far behind of being considered as a valid substitution for *in vivo* assays. To circumvent all these issues, cells can be cultivated at the ALI (Voisin et al., 1977) and be exposed in a special chamber (Aufderheide et al., 2003), which provides the possibility of creating an aerosol and using a microbalance for dose determination (Paur et al., 2008) (Table 2).

Cells cultivated in this way are seeded in a Transwell™ insert and are fed from the medium below (Fig. 2b). During the ALI cultivation the cells in the apical part will have no contact with the medium, but will be in direct contact with the airphase. Although no medium is added into the upper compartment, depending on the applied cell type a very thin layer of liquid, which is similar to the surfactant film for alveolar cells or to the secreted mucus for bronchial cells, will be on top of the cells (Aufderheide, 2008; Paur et al., 2008). The surface structure of cells in ALI culture is different from the surface observed in a submerged culture. A549 lung epithelial cells cultivated at the ALI begin to secrete surfactant, which is forming a protection layer against drying and agents (Ross et al., 2007; Rothen-Rutishauser et al., 2008). The surfactant is also important for particle retention and displacement under *in vivo* conditions (Gehr et al., 1996; Schürch et al., 1990) and its presence in the *in vitro* system increases similarity of the model. The pulmonary surfactant system serves three major functions in the alveolar system. First, it lowers surface tension at the ALI, thus keeping alveoli open and preventing them from collapsing. Second, the surfactant prevents fluid fluxes into the alveolar lumen by lowering alveolar surface tension, thus keeping alveoli relatively dry. Third, it contributes to several important components of the lung's innate immune system, thus keeping alveoli clean. These biophysical and immunomodulatory functions are dependent on a complex system of biochemical and morphological factors (reviewed in Mühlfeld and Ochs, 2010).

Primary tracheal epithelial cells have been shown to contain considerably more ciliated surface cells when cultivated at the ALI compared to submerged cultures. Extensive ciliogenesis is dependent on growth at the ALI and also on the presence of retinoic acid in the culture medium (Kaartinen et al., 1993). These morphological differences were also attributed to the greater exposure to oxygen under ALI conditions (BéruBé et al., 2010).

A lot of efforts were put into standardisation of aerosol exposure chambers for reproducible exposure of cells cultivated at the ALI (Aufderheide et al., 2003; Aufderheide, 2006, 2009; Aufderheide and Mohr, 2000). A crucial benefit of the ALI cultivation is

the fact that the particle properties should not be changed, as there is no contact with cell culture medium components prior to exposure. However, it may change due to the interaction with surfactant or mucus, although this is likely to happen under *in vivo* conditions, too. However, the interaction between proteins and particles can also be limited in a submerged system by using serum-free exposure conditions to study the toxicity (Bihari et al., 2008; Kroll et al., 2011). The administered dose can be continuously determined via microbalance-measurements, thereby allowing exact dosimetry (Paur et al., 2008). Nevertheless, there is still space for improvements of the cell culture systems which are used together with the aerosol chamber, and none of these models has yet entered a pre-validation phase. Altogether the ALI exposure system is a promising approach to study and simulate the toxicity of aerosols (Table 2).

4.3. Coculture models of cells relevant for the pulmonary system

Since the *in vivo* testing of cosmetic ingredients will be strictly forbidden in the future (Adler et al., 2011), cell-based systems like cocultures of epithelial cells and immune cells may gain attention for the use as alternative *in vitro* methods for hazard assessment.

The respiratory system with its vast surface area serves as a major portal for ambient materials such as PM and eNPs. There is an ongoing effort to develop and improve *in vitro* models which should be suitable for studying inflammatory and toxic effects of chemicals and particles on the respiratory tract (BéruBé et al., 2009; Kimber et al., 2007). A challenging aspect for the development of coculture *in vitro* model systems is the question regarding the cell types to be used and if it is better to choose primary cells or cell lines. The fact that the lung is composed of at least 40 different cell types (Sorokin, 1970) complicates the development of such models even further. The lung is functionally divided into two main areas, conducting zone and respiratory zone, which are again subdivided into several more specialised areas with differences in cell composition (Fig. 1) (Germann and Stanfield, 2004). Thus, a single *in vitro* model system using just a few different cell types will not be able to represent such a complex system as the respiratory tract. Therefore it is more reasonable to develop specialised models for certain anatomical parts of interest, e.g. the alveoli or bronchioli.

Small particles and also pollen can penetrate deep into the lung and even reach the alveoli in contrast to coarser particles (10–100 µm) (Pietropaoli et al., 2004). In addition, ultrafine particles (UFPs), such as eNPs and NPs with an aerodynamic diameter of less than 100 nm, are mainly deposited in the alveolar region (International Commission on Radiological Protection, 1994). UFPs can even reach the blood stream by crossing the alveolar epithelium (Möller et al., 2010). In this part of the lung, two types of epithelial cells are present: the squamous AT-I cells that form the actual lining and the cuboidal AT-II cells that have secretory functions. AT-I cells cover a large area per cell, but are flat and squamous in shape. Their main function is to serve as a thin and gas-permeable epithelial barrier (Mann-Jong et al., 2008). Although AT-II cells are bigger in number than AT-I cells, about 95% of the alveolar surface is covered by AT-I cells (Crapo et al., 1982, 1983; Mühlfeld and Ochs, 2010). Furthermore, the epithelium is sealed by tight junctions between the cells against the entrance of foreign material and pathogens into the interstitium (Bartels, 1979). Since AT-I cells are considered to be terminally differentiated and unable to perform further divisions they must depend on the mitosis and differentiation of AT-II cells (Evans et al., 1975). AT-II cells have therefore two main functions: they produce and secrete the protective surfactant layer and they are the progenitors for both types of alveolar cells. Because of their importance for the alveolar system and the defence of the alveoli against pathogens, they have been referred to

Table 2
Submerged cultivation vs. ALI cultivation, advantages and disadvantages in summary.

Submerged cultivation	Air–liquid–interface cultivation
+ Technically simple and cost efficient	+ No alteration of particles properties
+ High reproducibility	+ Microbalance-based dose determination
	+ Better simulation of inhalation exposure
	+ High reproducibility
– Dose determination is difficult	– Complex and cost extensive system
– Medium can alter particles properties	

as “defender of the alveolus” (Fehrenbach, 2001; Mason and Williams, 1977). In addition to the secretion of the surfactant, these cells are also capable of secreting a spectrum of pro-inflammatory mediators, including a broad profile of eicosanoid metabolites, cytokines and chemokines, having a primary role in inflammatory response (Fehrenbach, 2001; Mühlfeld and Ochs, 2010). The removal of particles and pathogens in the alveoli is predominantly done by macrophage phagocytosis because of the lack of a mucociliary escalator (Möller et al., 2010). These alveolar macrophages (AMs) are the principal phagocytic and scavenger cells on alveolar surfaces and form up to 90% of cells found in the alveolar spaces (Little et al., 2008). AMs are the first line of protection against inhaled antigens and pathogens (Sibille and Reynolds, 1990) and show a strong phagocytic activity that is even enhanced by the contact with opsonised particulates or following inflammatory signals such as IFN- γ (Brain, 1988; Geiser et al., 1994). Non soluble particles and microbes that reach the alveolar surface are efficiently removed by AMs, as long as they do not interfere with the phagocytic activity but can also be transported into the interstitium (Hoet et al., 2004). However, particles which are insoluble in mucus or in surfactant, are generally not easily engulfed by macrophages and particles with a size below 0.26 μm can elude the macrophage defence system (Chono et al., 2006). It seems that the size of a particle plays a crucial role in the clearance process. A subchronic inhalation exposure of rats to ultrafine (around 20 nm) and fine (around 200 nm) titanium dioxide (TiO_2) showed a significantly slower clearance for the small particles due to extended translocation to interstitial spaces and to regional lymph nodes in comparison to the fine TiO_2 particles (Oberdörster et al., 1994). These particles can get easily into contact with the epithelial cells and be translocated through the epithelium and reach interstitial spaces or the blood stream (Nel et al., 2006). Therefore, for NPs a coculture with macrophages and epithelial cells alone could not be adequate to mimic an *in vivo* situation without considering

a possible translocation and the effect on the underlying cells *in vitro*. Furthermore it should also be considered that the applied particle doses *in vitro* are often far beyond the range of what humans are exposed to *in vivo* (Oberdörster and Finkelstein, 2006).

The phagocytic cells are playing a dual role in the respiratory tract: first as a phagocyte to engulf particulates, to process foreign antigens and to kill ingested microorganisms and second as an effector cell in the course of an immune response in order to initiate immune and inflammatory state responses (Nelson and Summer, 1998; Reynolds, 1989). The phagocytosis of deposited particles in the alveoli by macrophages can result in the activation of AMs and the secretion of chemokines, cytokines, ROS, etc. By the secretion of pro-inflammatory chemotactic factors like the chemokine family cytokines, alveolar macrophages can induce the migration of polymorphonuclear cells (PMNs) and other cells to the lung and an inflammation emerges (Ishii et al., 2005; Ozaki et al., 1989; Sibille and Reynolds, 1990; Toews, 1986). Airway epithelial cells can also generate pro-inflammatory cytokines to assist with PMNs attraction (Becker et al., 2005; Fehrenbach, 2001). Therefore, the different cells of the pulmonary defence system are not functionally distinct players, but they communicate with each other by a great amount of cytokines and receptors (Holt et al., 1993, 1988; Kuipers and Lambrecht, 2004; Mantovani et al., 2001; Reynolds, 2000) (Fig. 3).

Since macrophage activation is one of the most important events in the vast array of pulmonary responses to inhaled substances (Finkelstein and Barrett, 2000), it seems reasonable to develop alveolar *in vitro* model systems consistent of the predominant epithelial cell type supplemented with macrophage cells to evaluate the hazard of small particles. Although the surface of the alveoli is mainly composed of AT-II cells, many proposed *in vitro* systems (Alfaro-Moreno et al., 2008; Crabbé et al., 2010; Liu et al., 2010; Rothen-Rutishauser et al., 2005; Rubovitch et al., 2007; Wottrich et al., 2004) are using the AT-II-like cell line

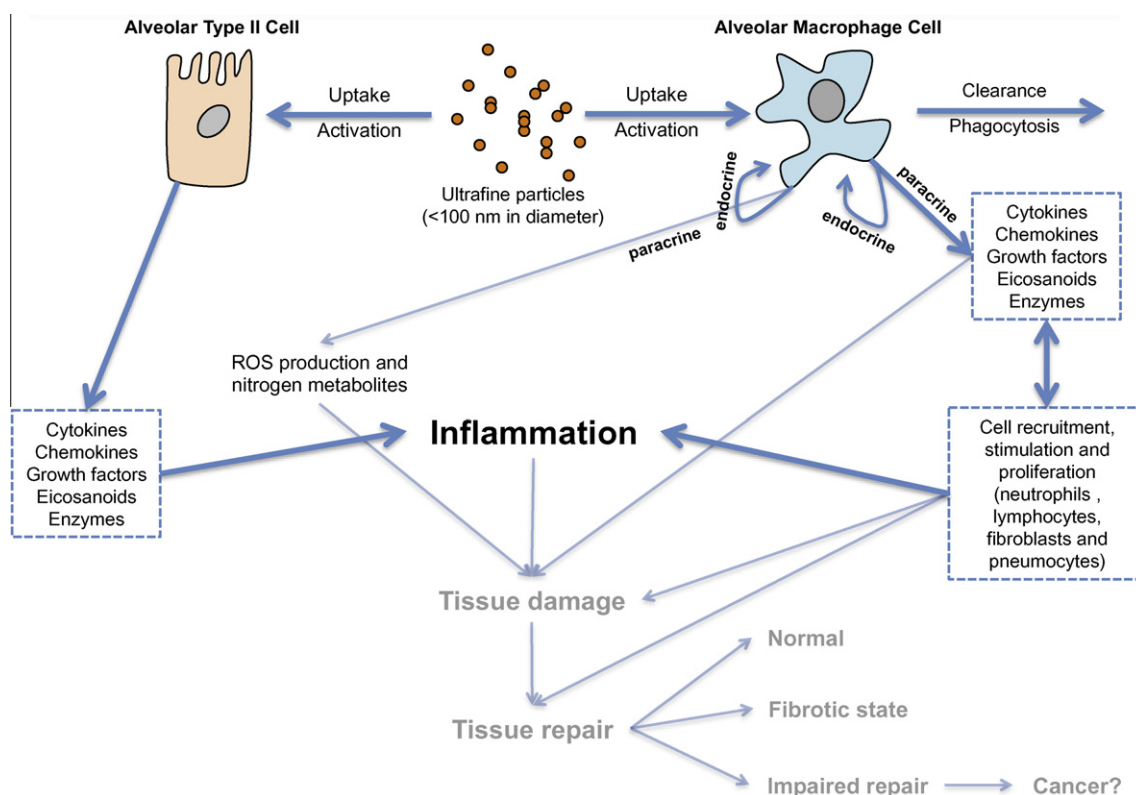


Fig. 3. Suggested role of the alveolar type II cells and macrophages in particle-induced inflammation. Adapted and modified from Finkelstein and Barrett (2000).

A549, which is derived from a human adenocarcinoma (Lieber et al., 1976) (Table 3). The morphology and functionality of A549 cells, e.g. surfactant synthesis, oxidative metabolism and transport properties, are consistent with that of AT-II pulmonary epithelial cells *in vivo* (Balis et al., 1984; Foster et al., 1998). This cell type provides certain benefits such as the protective surfactant film and the secretion of pro-inflammatory mediators. In addition, these cells are able to perform phase I xenobiotic biotransformation, although the capacity does not fully reflect that of the human lung, while phase II activity levels found in A549 cells are in the same range of the lung tissue. A549 cells are appropriate to investigate the mechanism of active toxins, but compounds that have to be biotransformed in order to elicit toxicity are less accurately detected (Castell et al., 2005).

4.3.1. Coculture *in vitro* models to study respiratory inflammation

An interesting *in vitro* approach combining A549 AT-II cells, THP-1 cells differentiated to macrophages and a human mast cell line (HMC-1; Butterfield et al., 1988) in a coculture to assess the hazard of PM₁₀ on the alveolar epithelium has been introduced (Alfaro-Moreno et al., 2008). Due to the fact that the procedure of isolating and purifying human AMs is not trivial and volunteers are not always readily available, the monocytic cell line THP-1 (Tsuchiya et al., 1980), differentiated to macrophages, has been used. Generally, primary AM cells can be isolated by performing bronchoalveolar lavage (BAL), where alveoli are washed out repeatedly with a sterile solution that finally contains the cells present on top of the alveolar epithelium (Blaschke et al., 1985; Henderson, 1994).

Mast cells are also present within the alveolar wall and even particularly on the alveolar surface (Fox et al., 1981), but their role in aggravating alveolar inflammation has not been studied intensively in relation to PM or eNPs. It has been demonstrated that histamine plays an important role in the systemic effects after

exposure to PM *in vivo* (Nemmar et al., 2003, 2004; Salvi et al., 1999). Histamine is mainly secreted by mast cells and therefore it seems that mast cells may be crucial for the understanding of systemic effects of PM and also for eNPs (Alfaro-Moreno et al., 2008). Since PM is capable of inducing an endothelial dysfunction phenotype (Alfaro-Moreno et al., 2007; Montiel-Davalos et al., 2007; Nurkiewicz et al., 2006) and pro-thrombotic effects (Mutlu et al., 2007; Nemmar et al., 2004), in addition to the above-mentioned cell lines, a fourth cell line (EA.hy 926, endothelial cells; Suggs et al., 1986) was used. With the addition of the endothelial cells on top of the system (Fig. 4a), it is possible to study second messenger-induced effects after PM₁₀ exposure. The communication between endothelial cells and the cells that have primary contact with particles may play an important role in the systemic effects of PM secondary after an inflammatory reaction. AT-II cells, macrophage cells and mast cells were seeded together in the wells (Alfaro-Moreno et al., 2008) and the endothelial cells were seeded in the Transwell™ inserts in the same well (Fig. 4a). The system should also offer possibilities to evaluate the inflammatory effects of NPs and eNPs via aerosol exposure at the ALI. However, we consider that for the ALI exposure, the epithelial cells as well as the immune cells should be seeded in the Transwell™ insert and not on the bottom of the well (Fig. 4b). After a certain time in culture, the medium will be removed from the Transwell™ insert, while the endothelial cells will grow to confluency in the well (Fig. 4b). This change is necessary to avoid an unintended diffusion of particles or chemicals from the upper to the lower compartment across the microporous membrane. A similar system, but without ALI cultivation, has already been used for a coculture system testing crystalline silica particles (Herseeth et al., 2008).

The NPs or chemicals could be added to the upper compartment comprising the triculture. Eventually secreted second messengers can cross the microporous membrane and potentially affect the endothelial cells in the lower compartment. Although the epithe-

Table 3
Common types of lung and endothelial cells for mono- and coculture *in vitro* systems for inhalation toxicology.

Name	Origin	Qualities	Described for	Endpoints
<i>Relevant cell types for the pulmonary system</i>				
16HBE14o-	Human bronchial epithelium	Immortalised; formation of tight junctions and cytokine expression ¹ ; retains differentiated epithelial morphology and functions, e.g. cilia; no mucus production ²	Monoculture	Oxidative stress and inflammation ^{3,4}
A549	Human adeno-carcinoma	Alveolar type-II (ATII)-like characteristics ² ; potential to form monolayer with tight junctions ⁵ ; mucin gene and mucus expression ⁶	Mono- and coculture	Inflammation and cytotoxicity ^{7,8,9} ; oxidative stress and inflammation ¹⁰
BEAS-2B	Normal human bronchial epithelial cells	Human bronchial epithelium transformed by an Adenovirus12-SV40 hybrid virus ¹¹ ; retain electron microscopic features of epithelial cells; show positive staining with antibodies to cytokeratin; no tight junctions ¹² ; secrete cytokines; express anti oxidants; no mucus secretion ² ; can differentiate into a squamous phenotype ¹³	Monoculture	Cellular interaction, uptake of ultrafine particles ¹⁴
BET-1A	Normal human bronchial epithelial cells	Immortalised simian virus 40-transformed human bronchial epithelial cell line; remain most of the characteristics of untransformed NHBE, e.g. tight junctions and differentiation ¹¹	Monoculture	Inflammation ¹⁵
Calu-3	Carcinoma of the human bronchus	Immortalised; formation of tight junctions ¹⁶ ; mucus production ⁶	Monoculture	Drug transport studies ¹⁷
NCI-H292	Human mucoepidermoid pulmonary carcinoma	Remain mucoepidermoid characteristics in culture; evidence for squamous differentiation ¹⁸ ; mucus gene and protein expression ⁶	Monoculture	Cytotoxicity ¹⁹
NCI-H441	Human papillary adenocarcinoma	Presumably of Clara-cell origin; grow as an attached monolayer with epithelial cell characteristics; expression of SP-A and SP-B ²⁰	Monoculture	Cytotoxicity ⁸
NHBE	Normal human bronchial epithelial cells	Primary cells, not immortalised; differentiated; form cilia and tight-junctions ²¹ ; mucin secretion ²² ; express cytokines ²³	Coculture	Apoptosis ^{4,24}
<i>Relevant endothelial cell types</i>				
EA.hy 926	Human somatic cell hybrid between umbilical vein cells and A549 cells	Show characteristics of differentiated endothelial cells, e.g. prostacyclin production ²⁵	Mono- and coculture	Inflammation ⁷ ; Cytotoxicity ²⁶

References: ¹ Wan et al. (2000b); ² Bérubé et al. (2009); ³ Hussain et al. (2009); ⁴ Hussain et al. (2010); ⁵ Rothen-Rutishauser et al. (2005); ⁶ Berger et al. (1999); ⁷ Alfaro-Moreno et al. (2008); ⁸ Uboldi et al. (2009); ⁹ Wottrich et al. (2004); ¹⁰ Singh et al. (2007); ¹¹ Reddel et al. (1988); ¹² Atsuta et al. (1997); ¹³ Ke et al. (1988); ¹⁴ Penn et al. (2005); ¹⁵ Abe et al. (2000); ¹⁶ Wan et al. (2000b); ¹⁷ Grainger et al. (2006); ¹⁸ Banks-Schlegel et al. (1985); ¹⁹ Phillips et al. (2005); ²⁰ O'Reilly et al. (1988); ²¹ Lin et al. (2007); ²² Park et al. (2005); ²³ Wang et al. (1999); ²⁴ Gray et al. (2007); ²⁵ Suggs et al. (1986); ²⁶ Lison et al. (2008).

Table 4Common types of immune cells for mono- and coculture *in vitro* systems for inhalation toxicology.

Relevant cell types for the immune system				
Name	Origin	Qualities	Described for	Endpoints
Airway macrophages	Human peripheral blood derived	Primary cells, not immortalised ¹	Coculture	Particle transport and inflammation ^{1,2}
Dendritic cells	Human peripheral blood derived	Primary cells, not immortalised ¹	Coculture	Particle transport and inflammation ^{1,2}
HL-60	Human acute promyelocytic leukemia	Spontaneous and also directed differentiation because of different stimuli; phagocytic activity and response to chemotactic stimuli ³	Monoculture	Cytotoxicity ⁴
HMC-1	Human mast cell leukaemia	They do not respond in all way like mature mast cells; lack of T and B lymphocyte, as well as myeloid cell markers; no functional IgE receptors ⁵	Mono- and coculture	Inflammation ⁶
Mono Mac 6	Human acute monocytic leukemia	Show characteristics of mature macrophages, e.g. ROS production or phagocytosis; expression of specific markers for mature monocytes ⁷	Mono- and coculture	Inflammation ^{8,9}
THP-1	Human acute monocytic leukemia	Phagocytotic activity; lack of surface and cytoplasmic immunoglobulin ¹⁰ ; potential to differentiate because of different stimuli ^{10,11}	Mono- and coculture	Inflammation ⁶ ; Cytotoxicity ⁴

References: ¹ Rothen-Rutishauser et al. (2005); ² Rothen-Rutishauser et al. (2007); ³ Collins (1987); ⁴ Bregoli et al. (2009); ⁵ Butterfield et al. (1988); ⁶ Alfaro-Moreno et al. (2008); ⁷ Ziegler-Heitbrock et al. (1988); ⁸ Brown et al. (2000); ⁹ Wottrich et al. (2004); ¹⁰ Tsuchiya et al. (1982); ¹¹ Schwende et al. (1996).

lial integrity in a system consisting of A549 cells is, due to the possible lack of sealing tight junctions, controversially discussed (Bitterle et al., 2006; Hermanns et al., 2004; Rothen-Rutishauser et al., 2005), it seems as if not all NPs are able to cross microporous membranes with a 0.4 µm pore size in a significant amount even without a cell layer on top (Geys et al., 2006). For the skin it has been shown that an impairment of the presence of tight junctions can contribute to allergic reactions, e.g. allergic contact dermatitis (ACD), due to an increased permeability of the epithelium and thereby causing an enhanced uptake of the allergen, underlining the importance of tight junctions for the integrity of an epithelial barrier *in vivo* (Brandner et al., 2010; De Benedetto et al., 2010).

Applying the above-mentioned changes in orientation of the cell layers, it should also be possible to study second messenger mediated effects on the endothelial cells after NPs exposure without the risk that NPs could reach the cells at the bottom by crossing the microporous membrane. Nevertheless, several *in vivo* and *in vitro* studies have demonstrated a possible translocation of NPs through the epithelium (Möller et al., 2010). Therefore it is rea-

sonable to design and develop *in vitro* model systems suitable for also studying the direct effects due to the transport of particles through the epithelial barrier. However, the setup of the model system is dependent on the scientific question. The results, obtained by both kinds of model systems, could then be integrated to draft a more detailed model about the potential toxic effects and mechanisms of particles. In order to evaluate the effects of chemicals and a possible translocation of particles, other lung epithelial cell lines, like Calu-3 or 16HBE14o- (Wan et al., 2000b) should be chosen (Table 3). These express tight junctions to avoid a translocation of chemicals and particles through the intercellular spaces and then through the microporous membrane. Furthermore, this system should also be capable for ALI cultivation.

The combination of a complex triculture *in vitro* system, cultivated at the ALI and exposed to an aerosol of a particle or chemical with a state-of-the-art exposure system (Aufderheide, 2005; Aufderheide and Mohr, 2000) offers the possibility of studying the interaction of aerosol particles of native atmospheres without altering the particle properties, in a non-physiological way.

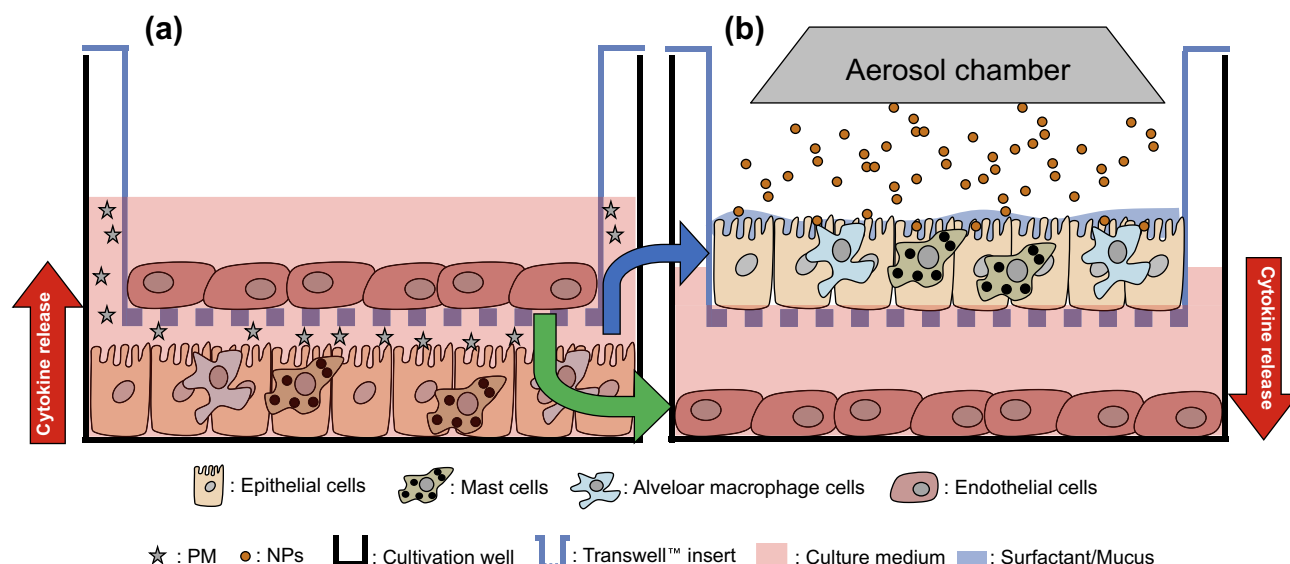


Fig. 4. (a) Triculture system with endothelial cells to study the inflammatory effects and pulmonary cell communication *in vitro* after exposure to PM₁₀ (Alfaro-Moreno et al., 2008). (b) Variant of the system to study the potential inflammatory effects of NPs at the ALI with native aerosol exposure.

4.3.2. Coculture *in vitro* models to study respiratory sensitization

An allergy can be described in general as an adverse condition, which occurs after an immune response to an otherwise harmless antigen, such as pollen or house dust. The allergic reaction, as the clinical manifestation of the sensitized status, is classified as a member of effects termed hypersensitivity reactions, which are defined as harmful immune responses that result in tissue injury (Murphy et al., 2008). The development of hypersensitivity resulting in respiratory allergy or ACD can be separated into two different stages: during the first step of sensitization, the compound is recognized as foreign by DCs, a distinct group of leukocytes characterized by a unique morphology and ability to initiate immune responses (Ryan et al., 2005; Steinman and Cohn, 1973). This recognition induces the maturation of these DCs into mature DCs, which are professional antigen presenting cells (APCs). The antigen will then be presented to T cells in the draining lymph nodes, and a specific primary immune response is provoked which results in sensitization (summarized in Fig. 5). The elicitation due to the subsequent contact to the compound is mediated through the activation of an immune response and the corresponding cellular signals, which result in an inflammatory reaction and symptoms of the allergy (Arts and Frieke Kuper, 2003; Arts et al., 2006). Studies with contact and respiratory sensitizers have shown that contact sensitizers tend to induce a T_H1 -response, whereas respiratory sensitizer tend to induce a T_H2 -response, depending on the local cytokine composition (Dearman and Kimber, 1991, 1992; Dearman et al., 1992) (Fig. 5).

Chemicals causing respiratory allergy, typically associated with rhinitis and/or asthma, are considered to cause an adverse health effect of high concern (Seed et al., 2008; United Nations, 2007). Compared to skin sensitizers, fewer chemicals are known to cause sensitization of the respiratory tract. One of the classes of chemicals most commonly associated with respiratory sensitization and occupational asthma are the acid-anhydrides, some diisocyanates, some reactive dyes, and platinum salts (Bariatico-Vangosa et al., 1994; Seed et al., 2008). Chemical allergy, including chemical-induced sensitization of the respiratory tract, is by definition dependent on the elicitation of an adaptive immune response, but uncertainty exists on the relevant exposure route. While it was initially assumed that chemical-induced sensitization of the respiratory tract is dependent on exposure via inhalation, there is some evidence that skin exposure to chemical allergens such as diisocyanates and acid-anhydrides can induce sensitization of the respiratory tract as well (Klink and Meade, 2003; Sailstad et al., 2003). Although animal models have offered significant insight into the identification of both contact and respiratory sensitizers, the elicitation of an allergic reaction in the respiratory tract is just poorly understood and no generally accepted *in vivo* models are available. As a consequence, we will focus in this part of the review on *in vitro* model systems which could be suitable for studying single steps of the development of respiratory sensitization, but not elicitation *in vitro*.

Allergic reactions can be assorted according to the classification proposed by Gell and Coombs (1963). This classification comprises four main classes of allergic reactions: Type I describes immediate reactions after a subsequent contact with an allergen (mostly proteins). A hallmark of this type is the production of antigen specific antibodies, which can lead to mast cell activation and to anaphylaxis as a consequence. A classic example for a Type I reaction is asthma. Type II is characterized by a cytotoxic reaction mediated by specific antibodies (IgM and IgG), which can bind to the cell surface and opsonize the cell. The antigen for a Type II reaction can be either extrinsic or intrinsic derived. In a Type III reaction an immune complex is formed due to extensive crosslinking of IgM and IgG antibodies with free antigens in the blood stream, which can accumulate in tissues and attract phagocytes.

The delayed-type hypersensitivity (Type IV) shows in contrast to a Type I reaction no immediate phenotype. The symptoms, like pain, reddening or heating need several days to appear.

Common allergic reactions are mainly due to the contact with sensitizing chemicals, which can be present in cosmetics or other products of our daily life. Protein sensitizers in almost all circumstances induce the production of antigen specific IgE antibodies, while this is just true for a small fraction of chemical sensitizers (Lalko et al., 2011). Among the environmental high molecular weight (HMW) allergens, pollens are the most prevalent source of airborne allergens (Wuthrich et al., 1995) and it has been shown that air pollution contributes to the release of allergic determinants by pollen (Behrendt and Becker, 2001). Under normal conditions, pollen do not only release antigens but rather a combination of allergens together with starch grains and other non-protein substances (Gilles et al., 2009) for instance lipids, which are defined as pollen-associated lipid mediators (PALMs). These lipids share structural and also functional aspects with eicosanoids, namely leucotriene- B_4 -like and prostaglandin- E_2 -like substances (Behrendt et al., 2001). These leucotriene-like PALMs attract and activate innate cells like neutrophils and eosinophils. Prostaglandin-like PALMs, probably in combination with other unknown factors, can modulate the migratory and T_H cell polarizing capacities of resident dendritic cells, which favour a T_H2 response (Gilles et al., 2009). The mechanisms, by which the epithelium (lung cells and keratinocytes) interact with pollen allergens are different (Blume et al., 2009). *In vitro*, A549 lung cells showed endocytotic as well as exocytotic activity for pollen allergens. This gives evidence that they may cross the epithelial barrier without proteolytic modifications via transcytotic transport and afterwards get into contact with resident immune cells. In contrast, keratinocytes did not show transcytotic activities, but rather take them up by endocytosis and storage in lysosomal compartments. In addition, the endocytotic activity of keratinocytes could even be enhanced by adding inflammatory mediators. This higher engulfment of allergens by inflammatory-type keratinocytes suggests a higher susceptibility of inflamed skin for the uptake of allergens and consequently a potentially higher risk for sensitization (Blume et al., 2009). Independently, some protein sensitizers, like the excrements of the house dust mite, from fungi but also from pollen, are known to act as proteolytic enzymes (Arruda et al., 1995; Chou et al., 2008; Chua et al., 1988; Grobe et al., 2002; Ibrahim et al., 2010; Poll et al., 2009; Robinson et al., 1997; Shen et al., 2007; Stewart et al., 1991; Tai et al., 2006; Wunschmann et al., 2005). By acting as cysteine, serine or aspartic proteases, these allergens can directly affect the permeability of the epithelium by cleaving tight junction proteins, such as occludin and zonula occludens-1 (Tai et al., 2006; Wan et al., 2000a, 2001). This activity is a possible mechanism for facilitating allergen delivery across epithelia (Runswick et al., 2007) and offering facilitated contact between allergens and DCs in subepithelial tissue (Jacquet, 2011). Furthermore, the contact of airway epithelial cells with protease allergens induces the secretion of large amounts of pro-inflammatory cytokines, favoring a T_H2 mediated response.

Up to now, data assessing the sensitization potential of NPs are limited and these are mainly dealing with the aggravating effects of particles after sensitization to another compound (Inoue and Takano, 2010). To the best of our knowledge, no data regarding the ability of NPs to cause sensitization without adding an adjuvant have been published. Since NPs can interact with proteins (Lynch et al., 2007), which is an essential step for chemical sensitizers (Landsteiner and Jacobs, 1936), particles might induce sensitization by using similar pathways as chemical sensitizers. Within the scientific community the possibility to test NPs *in vitro* is questioned and some investigators assume that it is not possible to translate *in vitro* results to the *in vivo* situation without further

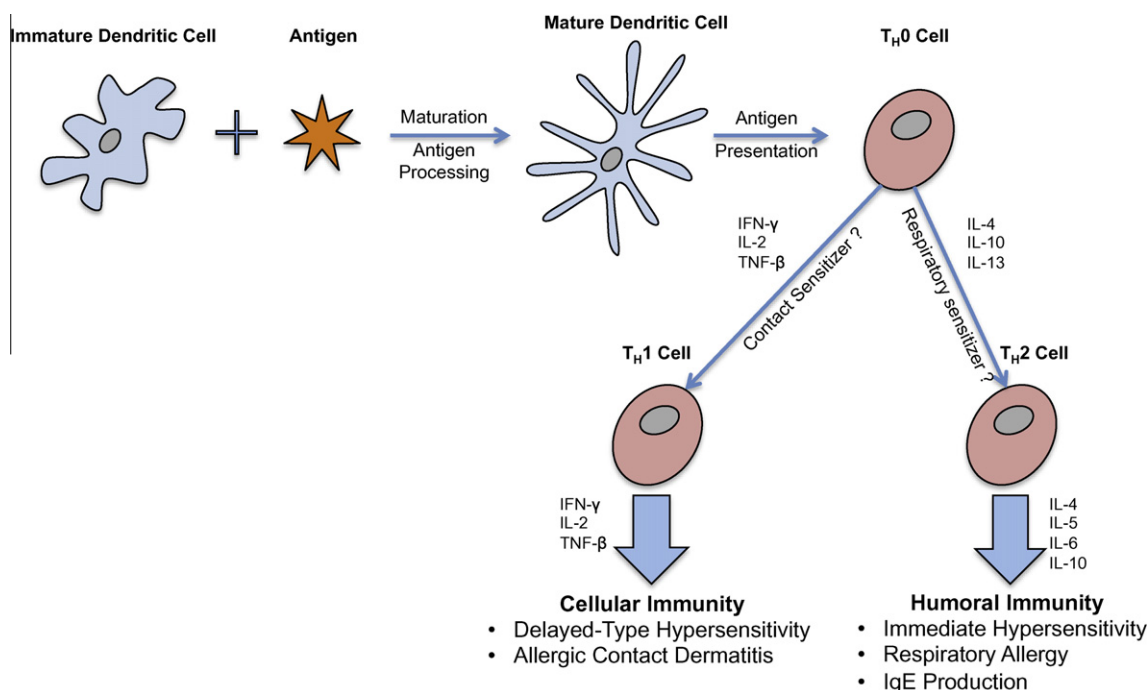


Fig. 5. Simplified scheme of the main events after exposure of dendritic cells to a sensitizing antigen, not considering the interplay of other T_H subsets, such as T_{reg} or T_H17 . After antigen challenge, the compound will be internalized and processed by dendritic cells, followed by a migration to the draining lymph node and the activation of T lymphocytes due to antigen presentation. Interaction between antigen presenting cells and naïve T lymphocytes can result in a proliferative stimulus and differentiation of T_H0 cells into a T_H1 or T_H2 phenotype. T_H1 phenotype is driven and characterised by secretion of cytokines, such as IL-2, IFN- γ and TNF- β , leading to the development of cellular immunity associated with delayed-type hypersensitivity and allergic contact dermatitis. Differentiation into T_H2 cells is driven by another composition of cytokines, such as IL-4, IL-10 and IL-13, and is associated with the development of humoral immunity, immediate hypersensitivity and respiratory allergy (Blackwell, 2006; Boverhof et al., 2008).

development and standardization (Clift et al., 2010; Sayes et al., 2007, 2009; Warheit et al., 2009). However, for different particles (NP and PM), inflammatory effects were tested *in vitro* (Alfaro-Moreno et al., 2008; Brandenberger et al., 2010; Hussain et al., 2009; Müller et al., 2010; Waldman et al., 2007), suggesting that it is possible to exploit these systems to evaluate the sensitization potential of NPs and PM *in vitro*.

A system, which was originally developed to study the interaction of particles deposited at the airway wall, has been described (Rothen-Rutishauser et al., 2005). The model is using cells of the human airway barrier and it comprises monocyte-derived dendritic cells instead of mast cells. The macrophages in this model were also generated *in vitro* from blood monocytes. Although there are resident dendritic cells as well in the skin as in the lung airway mucosa, peripheral blood monocyte-derived dendritic cells were used, due to their relative ease of isolation and the ability to obtain larger quantities of cells (Casati et al., 2005). A549 cells were used in this system as model for epithelial cells.

The seeding process of the cells needs to be done in different steps (Fig. 6). In a first step, A549 cells are seeded in a Transwell™ insert (3 μ m pore size) and cultivated for several days (Fig. 6; step 1). Then, the insert is turned upside-down and placed in a sterile petri dish for the seeding of the human blood-derived dendritic cells on the top of the bottom of the Transwell™ insert (Fig. 6; step 2). In a final step, the Transwell™ insert is turned again to seed the human blood monocyte-derived macrophages on the top of A549 cells (Fig. 6; step 3). By seeding the cells in this way, the system mimics the *in vivo* arrangement of cells in the epithelial barrier of the alveoli (Blank et al., 2007; McWilliam et al., 2000). This system has been well characterised for the formation of tight junctions or TEER (Rothen-Rutishauser et al., 2005). In their experimental design, A549 cells were able to produce tight junctions and related proteins (Rothen-Rutishauser et al., 2005). How-

ever, others found that A549 are not able to produce functional tight junctions (Godfrey, 1997; Hermanns et al., 2004; Winton et al., 1998). Although such models may lack functional tight junctions, they are still suitable to study effects independently from the selective transepithelial transport of compounds. Nevertheless, the importance of functional tight junctions in an *in vitro* model of respiratory sensitization has not yet been evaluated. This system has been used to study different kinds of UFPs (Brandenberger et al., 2010; Gehr et al., 2006; Müller et al., 2010; Rothen-Rutishauser et al., 2007).

By removing the liquid from the upper compartment of the insert, it is also suitable for ALI cultivation, as the utilised A549 cells have the potential to produce the protective surfactant layer (Brandenberger et al., 2010) (Fig. 6; step 4). Assuming that mechanisms by which particles are causing sensitization are similar to chemicals and in the light of the importance of DCs linking innate and adaptive immunity (Novak et al., 2010; Palucka and Banchereau, 1999), the above described system could serve as one part of a tiered strategy to evaluate the hazard of respiratory sensitization. Such a system would in addition offer the possibility of interaction between macrophages, dendritic cells and epithelial cells by forming a trans-epithelial network (Blank et al., 2007).

To assess the full range of the sensitization potential of a compound, even a complex triculture system will not be fully suitable as many other key players of the immune system are still missing. While in the described system epithelial cells and DCs, as well as macrophages can interact with each other, *in vivo* DCs must engulf and process antigens to further prime naïve T cells in the draining lymph nodes, a step that is missing in all these systems.

Another disadvantage of all the presented model systems is that these are not considering the physical forces that are applied to the alveolar epithelium during a breath. *In vivo*, an active tissue–tissue

interface between the epithelial cells and the underlying microvascular endothelium exists, which is necessary to permit the transport of nutrients, immune cells, second messengers, etc. The behavior of this interface is influenced by and dependent on the applied physical forces (Ingber, 2006). To solve this methodic gap, a multifunctional biomimetic microsystem of the alveolar–capillary interface, the fundamental functional unit of the living lung, has been developed. With this system, it is possible to study the effects of particles with and without applying physical forces to the alveolar–capillary interface. With the microsystem, it has been shown that the response after the exposure to silica NPs (12 nm) was different with and without applying physical force (Huh et al., 2010).

5. Commercially available *in vitro* systems relevant for the pulmonary system

In contrast to these coculture systems, there are also commercial cell systems available (MatTek, Epithelix). These are based on normal human bronchial epithelial cells (NHBE), primary cells, either derived from surplus biological material from hospitals, e.g. lung resections, or patient donations (includes post-mortem samples) (BéruBé et al., 2010). NHBE cells need several weeks to differentiate, before they start showing beating cilia on their surface and in addition the requirements on the culture medium are very high. The cells need a serum-free medium supplemented with growth factors and hormones such as retinoic acid, epinephrine, triiodothyronine, human recombinant epidermal growth factor, etc. (Lechner and LaVeck, 1985). The great advantage of this system

compared to others is the well-differentiated surface which is similar to the bronchial surface *in vivo* (BéruBé et al., 2009, 2010).

In order to facilitate the cultivation of NHBE cells and to assure a constant quality, the EpiAirway™ (human tracheal or bronchial epithelium) from MatTek as well as the MucilAir™ (human respiratory tract) from Epithelix were developed. These systems are ready to use prefilled multiwell plates or Transwell™ inserts with the pre-differentiated cells. Both models have a long lifespan, but are not immortalized. Therefore they cannot be used as a constant supply of material due to the limited passage numbers. The system may remain in a usable condition for more than one year, without dedifferentiation (www.epithelix.com). Another advantage for the field of respiratory sensitization is the metabolic activity and detoxification ability still present (Hayden et al., 2007). This metabolic activity is needed for some chemical sensitizers (prohaptens) with very low chemical reactivity. They need a conversion by metabolic enzymes into more reactive molecules, which can finally react with proteins (Dupuis and Benezra, 1982; Lepoittevin, 2006; Smith and Hotchkiss, 2001). Furthermore, the systems are suitable for uptake studies, as well as for transepithelial transport because of the presence of tight junctions. The cultivation at the ALI and therefore the exposure to an aerosol is possible (Balharry et al., 2008; Hayden et al., 2007). Furthermore tissues from diseased persons such as smokers or patients suffering from cystic fibrosis, etc. are also available (www.mattek.com; www.epithelix.com). This offers the possibility to study the potential of chemicals and particles to worsen or interfere with a pre-existing disease.

However, these primary cell models seem to tolerate some compounds better than common lung cell lines, probably reflecting

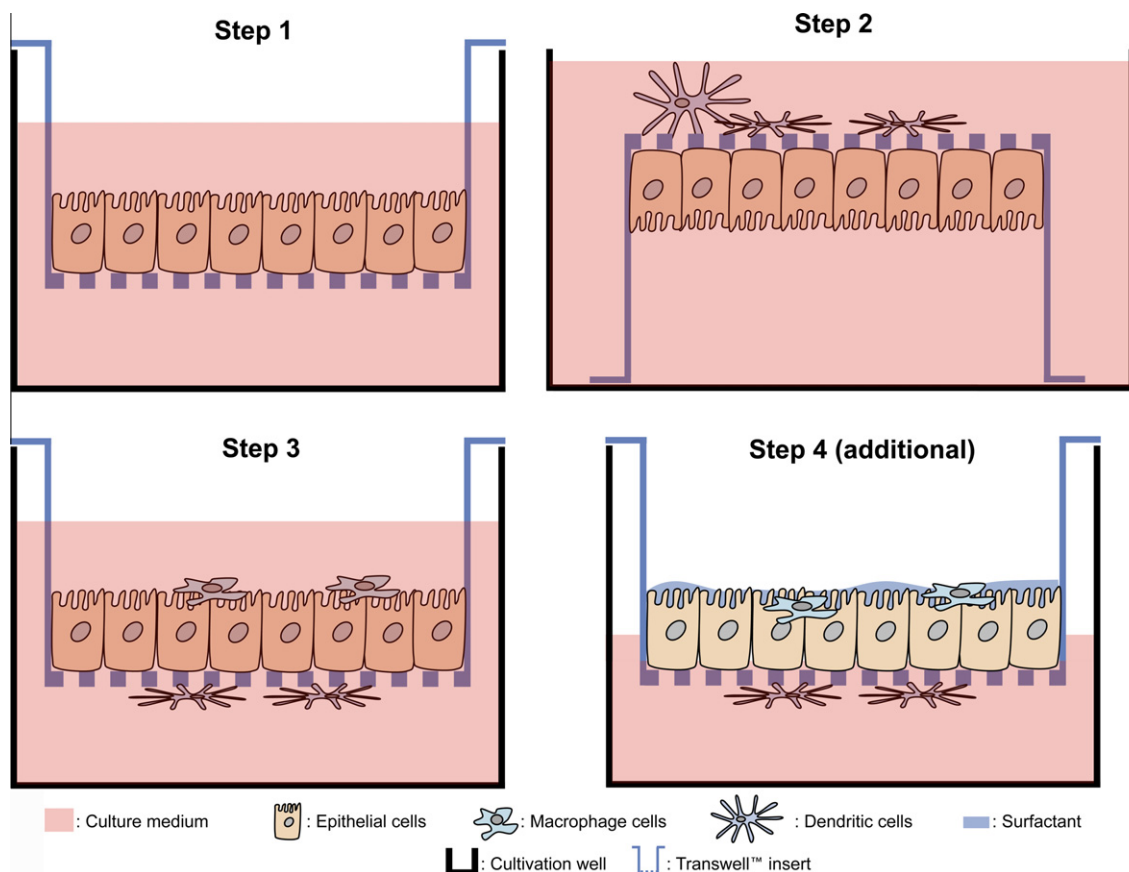


Fig. 6. Scheme of the seeding procedure of a triculture model system originally developed by Rothen-Rutishauser and coworkers (2005). Step 1: Seeding of epithelial cells in a Transwell™ insert; step 2: inverting the Transwell™ insert to seed dendritic cells on the bottom of the insert; step 3: seeding of macrophage cells on top of the epithelial cells; step 4 (additional): removing the medium from the Transwell™ insert to cultivate and expose the system at the ALI.

more the *in vivo* conditions than cell lines (Balharry et al., 2008). This robust, *in vivo*-like nature of NHBE cells was considered to be due to interactions between its mixed-cell phenotype consisting of ciliated, mucus-secreting goblet and basal cells (BéruBé et al., 2010).

6. Discussion

Up to now, *in vivo* models dominated toxicological risk assessment, but nowadays cell based *in vitro* models have become indispensable tools in toxicology. *In vivo* data from many thousands of animal studies were used to steadily improve and advance cell culture systems. But even current state-of-the-art coculture systems are not yet able to fully assess mechanistically complex hazards like sensitization.

Sensitization can be caused by chemicals, present in products of the daily life, like personal care products or chemicals at work, but also by certain proteins. The mechanisms by which the various compound classes cause sensitization are different, at least for chemical sensitizers and proteins, regarding f. e. the lack of IgE antibody production in response to many chemicals sensitizers (Lalko et al., 2011). The exposure to some chemicals and proteins may lead to a sensitization and possibly result in ACD of the skin or allergic asthma or rhinitis of the respiratory tract. While proteins mostly trigger the production of IgE antibodies it is not the case for many chemicals. Studies conducted with pollen have shown that the epithelium plays an important role in the sensitization process, in the skin as well as in the respiratory system (Blume et al., 2009; Toppila-Salmi et al., 2011). Additionally to the differences between LMW and HMW sensitizers, the skin and the lung seem to react different in response to HMW sensitizer. Epithelial cells in the lung are translocating HMW sensitizers, such as pollen proteins, beyond the epithelial barrier but in the skin they are stored and processed in compartments inside the cell (Blume et al., 2009). This points out one more time the complexity of the sensitization process. In addition to the adverse effects, mediated by chemicals and proteins, it has been shown that particles can aggravate allergic asthma *in vivo* (Hussain et al., 2011). eNP can react and interact with proteins (Lynch et al., 2007), wherefore it might be assumed that they could also cause a sensitization similar to the chemically mediated one, but no data on this topic are yet available.

Though much is already known about the processes involved in sensitization, our mechanistic understanding still needs to be extended to facilitate the design and further improvement of existing model systems. Coculture systems, as the ones described in this review could potentially contribute to this task by serving as model systems for single steps of the sensitization process. In contrast to the available approaches for assessing contact sensitization in skin *in vitro*, less is available for the respiratory system (Table 1). Achievements have been made *in silico* and *in chemico* to predict the reactivity of potential respiratory sensitizers, but suitable models to assess absorption of chemicals in the respiratory tract or epidermal inflammation are not in sight.

Future assays should consider the special conditions in the respiratory system, such as the presence of the surfactant film in the alveoli (Gehr et al., 1996; Schürch et al., 1990) or a mucus layer in the bronchioli (Lippmann et al., 1980), since these are also additional barriers *in vivo* and could affect the toxicity of a compound due to an interaction (Mühlfeld et al., 2008). To realize this, the ALI exposure technique has been developed. After removing the liquid from top of the epithelial cells (Fig. 2b, 4b and 6), they are exposed to an aerosol, including the potential hazardous material, which simulates the *in vivo* deposition *in vitro*. Due to the possibility of including a microbalance into the aerosol exposure system (Paur et al., 2008), dose determination becomes easier and more traceable. However, the switch from a submerged hypoxic state to an ALI cultivation

could result in an enhanced ROS production (Kameyama et al., 2003; Kondo et al., 1997) because of the greater availability of oxygen, whereby slight effects on the oxidative stress level, caused by particulates, could remain unnoticed. Nevertheless, results like the observation of significant differences of cellular response after ZnO NPs exposure under submerged and ALI conditions suggest that pharmaceutical and toxicological studies with inhaled NPs should be performed under the more realistic ALI cultivation, rather than submerged cell conditions (Lenz et al., 2009).

As of yet, no attempts have been made to assess the ability of the presented *in vitro* systems, to simulate important steps, such as epithelial inflammation of the alveoli or DC activation. Regarding the *in vivo* role of the involved cell types in the *in vitro* systems (Figs. 3 and 5), one might suggest that a coculture system could serve as one step in a tiered approach to assess potential hazards of particles. However, only little is known about the mechanisms by which eNPs and PM affect the immune system and exhibit their adverse potential. It can be that eNPs are sharing mechanistic similarities with both, LMW and HMW or work in a different way. However, it has been shown that inflammatory processes, mediated by particulates, could be measured *in vitro* (Alfaro-Moreno et al., 2008; Brandenberger et al., 2010; Hussain et al., 2009; Müller et al., 2010; Waldman et al., 2007). In addition to the lack of mechanistic knowledge, the task to find a representative cell composition, relevant to serve as a partial *in vivo* replacement, is difficult in respect to the complex cell composition of the respiratory system. Furthermore, a potential *in vitro* model system should consider the properties of the nanomaterial that is to be tested, e.g. solubility, protein reactivity or surface structure, in order to produce reliable results.

Assessing the potential of new compounds to cause sensitization is indispensable to assure people's health at home as well as at work. In order to achieve this task, it is necessary to be able to distinguish between contact and respiratory sensitizers, because it is important to know whether one has to prevent contact with possible sensitizers via the dermal or respiratory route. At the moment, a differentiation of both classes is neither possible *in vitro* nor *in vivo*. Regarding the fact that contact sensitizers tend to trigger a T_H1 response, on the contrary respiratory sensitizers mostly induce a T_H2 orchestrated response (Fig. 5) (De Jong et al., 2009; Goutet et al., 2005; Kimber et al., 2011; Plitnick et al., 2003), it is intended to exploit this pattern to design new assays by distinguishing contact from respiratory sensitizers by measuring the differences in the cytokine spectra or the activated genes (Verstraelen et al., 2008, 2009a,b,c). Such methods would contribute a lot to the improvement of safety at work, because companies could better protect their workers and minimize the risk for occupational sensitization.

Summarizing the information given so far, even a complex coculture system is still far away from mimicking the whole spectrum of processes *in vivo*.

The use of cell culture based model systems seems to be a major task for future toxicological studies but also to serve as a tool to design aerosol-based inhalable drugs (Agu and Ugwoke, 2011). Furthermore, such model systems could be exploited for more mechanistical studies leading to a broader knowledge about the involvement of the epithelium during inflammation not only in connection with particles or allergens.

The increased use of cell-based systems will further push the development of more alternative *in vitro* methods for other fields of toxicology, e.g. inhalation toxicology and additionally lead to a broader understanding of core steps in inflammation and sensitization in humans. However, it is important to realize that the *in vitro* approaches presented in this review are still in the early stages. In addition, none of the presented models considers the influence of physical stress, such as stretching, on the epithelium. An interesting approach to include this has been recently published, showing

that physical stress can significantly alter the output of an *in vitro* experiment (Huh et al., 2010). The successful implementation into an integrated test strategy will be dependent on further research in respect to extensive standardization, referring to *in vivo* endpoints, and also collaboration between researchers.

7. Conclusions

Respiratory sensitization, as a consequence of the continuous exposure to PM, eNPs and chemicals at home or in the occupational setting, is increasing the risk for allergic respiratory diseases. Evaluating the potential of new compounds, including chemicals and eNPs present in many consumer and personal care products, is an important task. Due to the large amount of different chemicals and particles that need to be tested, it is necessary to develop fast and reliable models to study the mechanisms of sensitization processes. The *in vitro* model systems to assess the hazard potential of particles should consider the properties of the nanomaterials, such as solubility, protein reactivity, surface structure or possible agglomeration. Considering the large number of potential candidates for testing, applying *in vivo* methods may be not possible because of the huge number of laboratory animals needed. *In vitro* coculture systems have high potential to support the unravelling of some of the involved mechanisms, and could serve as a partial replacement for *in vivo* studies. However, the presented *in vitro* systems need further development and standardization in order to properly forecast complex hazards such as respiratory sensitization.

The field of inhalation toxicology, studying the toxic potential of particles and chemicals on the human body, is just one possible application area, but similar models are under development for other parts of the human body as well. In the future it is also possible, that one of the presented models may contribute to the development of aerosol-based inhalable drugs (Agu and Ugwoke, 2011).

8. Conflict of interest

The authors state no conflict of interest.

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