Assessment of chemical-induced local irritation and inflammation in organotypic lung tissue model - Precision-cut lung slices (PCLS)

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Zusammenfassung

Das Ziel dieser Arbeit war die Analyse immuntoxischer Effekte bei Chemikalien-induzierter respiratorischer Irritation und Sensibilisierung in einem organotypischen Gewebekulturmodell aus Präzisionslungenschnitten (PCLS). Dafür wurden relevante LMW Chemikalien, darunter respiratorische Sensibilisatoren, Kontaktsensibilisatoren und nicht-sensibilisierende Reizstoffe, an PCLS getestet.

EC_{50}-Werte für die Chemikalien-induzierte Toxizität in humanen PCLS wurden mit EC_{50}-Werten aus *in vitro* Daten von THP-1 und NCTC Zelllinien, mit Hilfe derer sich verschiedene Aspekte von Monozyten und Epithelzellen darstellen lassen, korreliert. Weiterhin wurde eine signifikante Korrelation mit LD_{50}-Werten aus *in vivo* Inhalationsstudien an Ratten ermittelt, die für *in vivo* Dosisfindungsstudien relevant ist. Zur Quantifizierung lokaler Entzündungsreaktionen wurde die Freisetzung relevanter Zytokine und Chemokine quantitativ erfasst. TNF-α und IL-1α Konzentrationen waren in humanen PCLS nach der Exposition mit den respiratorischen Sensibilisatoren Trimellithsäureanhydrid und Ammonium Hexachloroplatinat in subtoxischen Konzentrationen signifikant erhöht, während Kontaktsensibilisatoren und nicht-sensibilisierende Reizstoffe die Freisetzung dieser Zytokine nicht im gleichen Ausmaß induzierten. Die vorläufigen Ergebnisse zeigen die Eignung von PCLS als *ex vivo* Modell für die Untersuchung von Immunreaktionen. Dieses Modell kann für die Vorhersage von Chemikalien-induzierter Toxizität verwendet werden, ist allerdings aufgrund der Komplexität der Funktions- und Reaktions-Veränderungen von unter anderem z.B. dendritischen Zellen (DCs) und T-Zellen für ein einfaches Screening der Allergene ungeeignet.

Um PCLS als ein geeignetes alternatives *ex vivo* Modell zur Reduktion der Tieranzahlen von inhalationstoxikologischen Untersuchungen zu bewerten haben drei unabhängige Laboratorien (Fraunhofer ITEM, BASF SE und der RWTH Aachen) parallel das toxische Potenzial von 20 Chemikalien mittels PCLS bestimmt. Über 900 Dosis-Wirkungskurven wurden generiert und analysiert. Die ermittelten $Log_{10}[IC_{50}$ (µM)] Werte ergaben für alle Daten, die mittels WST-1 und BCA-Tests erhoben wurden, die beste Interlabor-Konsistenz. Während sich durch WST-1 und LDH toxische Wirkungen nach der Zugabe der meisten Substanzen darstellen ließen, konnten in mindestens einem der drei beteiligten Laboratorien signifikante Erhöhungen der extrinsischen IL-1α Konzentration mit respiratorischen Sensibilisatoren, nicht mit nicht-sensibilisierenden Reizstoffen beobachtet werden. Die Reproduzierbarkeit unter den teilnehmenden Laboratorien zeigte hinreichend niedrige Variationen bei den Daten der WST-1 und BCA Tests. Die Etablierung der PCLS als akutes Toxizitätsmodell wurde mit einem kleinen Datensatz erfolgreich durchgeführt und mit 20 verschiedenen Chemikalien verifiziert.

Schlagworte: Präzisionslungenschnitte; respiratorische Toxizität; Vorhersagemodell

Abstract

The aim of these studies was to assess the immunotoxicity occurring in chemical-induced irritation and sensitization by using an organotypic tissue culture model precision-cut lung slices (PCLS). For that, LMW human health relevant chemicals, including respiratory sensitizers, contact sensitizers and non-sensitizing irritants were tested in PCLS.

EC_{50} values from chemical-induced toxicity in human PCLS were correlated significantly on the one hand with *in vitro* data published for THP-1 and NCTC cell lines, which have different aspects of monocyte-derived cells and skin-derived epithelial cells, and on the other hand with LD_{50} values from *in vivo* rat inhalation studies, which could be interesting for *in vivo* dose-finding studies. Local respiratory inflammation was quantified by measuring the production of cytokines and chemokines. TNF-α and IL-1α were increased significantly in human PCLS after exposure to the respiratory sensitizers trimellitic anhydride and ammonium hexachloroplatinate at subtoxic concentrations, while contact sensitizers and non-sensitizing irritants failed to induce the release of these cytokines to the same extent. The preliminary results show an *ex vivo* model for studying immunotoxicity responses which might be used for prediction of chemical-induced toxicity, but is far too complex for a simple screening of allergens based on functional and behavior changes of e.g. dendritic cells (DCs) and T-cells.

In order to assess PCLS as a suitable alternative *ex vivo* approach to reduce animal numbers of inhalation toxicology, three independent laboratories (Fraunhofer ITEM, BASF SE and RWTH Aachen) have analyzed parallel toxic potential on PCLS exposed to 20 chemicals. More than 900 dose-response curves have been fitted and analyzed. $Log_{10}[IC_{50}$ (μM)] obtained for all assay endpoints showed best inter-laboratory consistency for the data obtained by WST-1 and BCA assays. While WST-1 and LDH indicated toxic effects for majority of the substances, significant increases in extrinsic IL-1α could be observed in many respiratory sensitizers but not in non-sensitizing irritants at one of the three participating laboratories at least. The reproducibility within the participating laboratories appeared to have acceptably low between-lab variations for WST-1 and BCA assay. The assessment of PCLS as an acute toxicity model was successfully established with a small training data set and verified by 20 different chemicals.

Keywords: precision-cut lung slices; respiratory toxicity; prediction model

Preamble

This doctoral thesis was prepared at the Fraunhofer Institute for Toxicology and Experimental Medicine (ITEM) in the Department of Pre-clinical Pharmacology and In Vitro Toxicology under the supervision of Prof. Dr. Armin Braun and Dr. Katherina Sewald. Human lung tissue for the preparation was obtained in cooperation with Klinikum Region Hannover. The prevalidation of the *ex vivo* model PCLS for the prediction of respiratory toxicology was conducted in three independent laboratories (Fraunhofer ITEM, BASF SE, RWTH Aachen). BfR was partner for scientific advice and efficient conduction of the project. *Ex vivo* experiments were performed to evaluate the potential of PCLS to reflect the immune responses described in occupational asthma.

The present work refers to the following publications:

Lauenstein L, Switalla S, Prenzler F, Seehase S, Pfennig O, Förster C, Fieguth HG, Braun A, Sewald K.
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1 Introduction

This doctoral thesis describes the use of an alternative *ex vivo* model of precision-cut lung slices (PCLS) to implement local respiratory toxicity by assessing a broad variety of toxicological and immunological endpoints. Therefore, animal and human PCLS were exposed to LMW chemicals including well-known industry sensitizers and non-sensitizing irritants. At the beginning of this study chemical-induced irritation and inflammation in the airway were tested by using cytotoxicity testing and the determination of cytokine patterns. After that chemical-induced irritation was prevalidated with a training set of twenty chemicals in three independent laboratories in order to reduce animal testing numbers of inhalation toxicology with regard to dose-finding.

1.1 Immune mechanisms of airway responses to injury

The respiratory tract is daily exposed to 10.000 litres of inhaled air containing a wide variety of exogenous potentially harmful agents [Holt *et al.*, 2008]. The inhaled agents can be either infectious like bacteria, fungi and viruses or non-infections like allergens and chemicals [Hammad *et al.*, 2008]. In order to protect the body from inhaled agents, the respiratory immune system composes a complex network of interacting cells including inflammatory and structural cells in the airway tissue which play an effective host defense system against harmful inhaled irritants.

The respiratory airway epithelium assemble the first line of defense against inhaled agents [Hammad *et al.*, 2008]. Airway epithelial cells can for example express many pattern recognition receptors (PRRs) like Toll-like receptors (TLRs) for a quick detection of pathogens and response to pathogen-associated molecular patterns (PAMPs). These are present in pathogens like gram-negative bacteria as lipopolysaccharides. PRRs are also present on cells like macrophages, dendritic cells (DCs), endothelial cells, mucosal epithelial cells, and lymphocytes. The activation of PRRs leads to NF-κB activation, release of pro-inflammatory cytokines and chemokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1α and IL-8. The released cytokines and chemokines support on the one hand the inflammation in the airways and the contractility of airway smooth muscle cells [Adner *et al.*, 2002; Berry *et al.*, 2007], on the other hand the activation of for example DCs in both conducting airways and

lung parenchyma which coordinates the adaptive immune responses [Veres *et al.*, 2007].

After the activation of DCs, they migrate to the draining lymph nodes and process antigen presentation to $CD4^+$ or $CD8^+$ T cells. These cells belong to the major T cell subsets that play a central role in immune system function. If the antigen presentation is mediated by MHC class II, it will be recognized by naive $CD4^+$ T cells. Naive $CD4^+$ T cells will be primed by the communication and differentiate into effector and memory cells. The phenotypes of effector $CD4^+$ T cells can differ into various subsets like T helper cells (T_H)1, T_H2, T_H17, and regulatory T cells (Treg) depending on the stimulation conditions [Chen *et al.*, 2013] (Figure 1). T helper cells can activate B cells to differentiate into plasma cells and memory B cells which can produce high-affinity antibodies and circulate into tissues or mucosa to reach sites of local infection and render their effector role. The circulating antibodies could provide immediate protection against infections with the same antigen [Ron *et al.*, 1981]. In this case, memory B cells are activated by the recall antigen resulting in a secondary response which provides high level of protection. All the interactions mentioned above are via soluble mediators for the crosstalk between structural cells, innate and adaptive immune cells to provide the local immunoregulation which eventually controlled immunological homeostasis in the airway [Bao *et al.*, 2014].

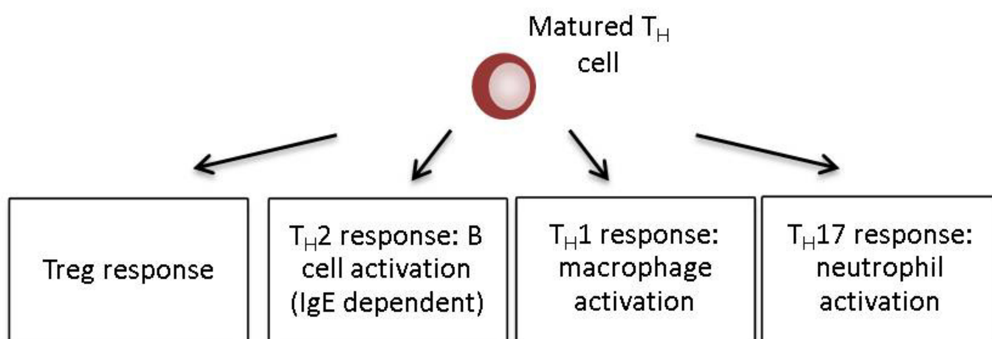

Figure 1: The phenotypes of effector $CD4^+$ T cells after stimulation with different antigens. T_H cells: T helper cells, Treg: regulatory T cells.

1.2 Inhalation of toxicants can induce respiratory toxicity

Inhalation of environmental and industrial chemical substances can cause respiratory toxicity. Damage may occur in the upper and lower airways including the nasal passages, pharynx, trachea, and bronchi [McKay, 2014]. Respiratory toxicity leads to a

variety of acute and chronic pulmonary diseases, including local irritation, pulmonary edema, acute respiratory dysfunction syndrome (ARDS), bronchitis, emphysema, asthma and cancer [McKay, 2014]. Most respiratory irritants are also toxic to the lung parenchyma if inhaled substances reach the deep lung in sufficient amount [Saric *et al.*, 2000]. However, many inhaled substances have also systemic toxic effects after being absorbed and passed through the lungs. Body responses to inhalation of toxicants include not only injury in the respiratory system but also blood and other organs such as spleen which can induce severe organ injury [Hayes *et al.*, 2010]. Acute responses of lung to injury could also induce cell necrosis exposed to e.g. acidic or alkaline agents which can alter cell membrane permeability, and lead to cell death.

A typical disease example of acute lung injury is the acute respiratory distress syndrome (ARDS), which is characterized by noncardiogenic pulmonary edema, severe hypoxemia and decreased lung compliance after exposure to high-dose inhalation of toxicants [McKay, 2014]. A common feature of acute lung injury and ARDS is increased alveolar membrane permeability followed by epithelial and endothelial disturbance and a diffuse inflammation in the pulmonary parenchyma. [Saguil *et al.*, 2012; Wang *et al.*, 2008]. The onset of ARDS occurs within 12-72 hours after initial lung injury caused by exposure to direct or indirect respiratory toxicants which leads to activation and accumulation of neutrophils [Ware *et al.*, 2000]. Consequently, released multiple pro-inflammatory cytokines such as IL-8, IL-1, TNF-α, released reactive oxygen species (ROS), and migration of large numbers of neutrophils across endothelial and type I pneumocyte surfaces lead to pulmonary edema, increased permeability, gaps in the alveolar epithelial cells, and necrosis of type I and II pneumocyte cells [Tsushima *et al.*, 2009]. At the end, formation of fibrosis in pulmonary parenchyma, loss of surfactant, and decrease of pulmonary compliance accelerate severe respiratory failure [Meduri *et al.*, 1998].

Prolonged exposure to respiratory toxicants can cause structural damage in the lungs, resulting in chronic diseases such as pulmonary fibrosis, emphysema, and cancer [Goldkorn *et al.*, 2013; Mulla *et al.*, 2013]. Oxidative stress is the best understood pathophysiological component of airway diseases such as chronic obstructive pulmonary disease (COPD) [Kirkham *et al.*, 2013]. Oxidative stress is derived from increased burden of inhaled oxidants like cigarette smoke, and from the increased amounts of ROS generated by inflammatory, immune and various structural cells of the

airways leading to specific loss of ciliated cells of the airway epithelium and of type I pneumocyte cells, and subsequently disturbance of the tight junction interface causing subepithelial and submucosal damage and further inflammatory response and bronchoconstriction [Tuder *et al.*, 2012].

Certain compounds, such as trimellitic anhydride, glutaraldehyde, and some metals like platinum can act not only as non-specific irritants in high concentrations, but can also induce allergic sensitization like asthma as well [Kimber *et al.*, 2014].

1.3 Pathogenesis of asthma and occupational asthma

1.3.1 Mechanisms of immunologically mediated asthma

Asthma is a heterogeneous chronic inflammatory disease. Common asthma symptoms include recurrent attacks of shortness of breath, wheezing, chest tightness and coughing. It is characterized by reversible airflow obstruction, airway hyperresponsiveness, mucus hyersecretion, and airway remodeling [NHLBI Guideline, 2007]. Airway inflammation is elemental to asthma pathogenesis supported by the presence of inflammatory cells such as eosinophils, allergen-specific T_H2 cells, immunoglobulin (Ig) E, and mast cells [Galli, 1997; Lane *et al.*, 1996; Leckie *et al.*, 2000; NHLBI Guideline, 2007].

Asthma can be classified as atopic and non-atopic asthma. Although non-atopic asthma has a different clinical profile than atopic asthma, however, both diseases are determined by concerned immunopathological characters which are dominated by T_H2 cells and IgE [Kudo *et al.*, 2013]. In non-atopic asthma bronchial hyperreactivity is mostly in response to stimuli such as cold air or exercise, whereas atopic asthma is induced by inhaled allergens such as pollen, house dust mites, or chemicals [Corren, 2013]. In atopic asthma, T cells are mostly differentiated into T_H2 cells with expression of T_H2 type cytokines, such as IL-4, IL-5, and IL-13 which initiate sensitization and the subsequent immune responses to the specific allergen by accumulation and infiltration of eosinophils, mast cells, and recruitment T_H2 cells and further recruitment of mast cells and eosinophils [Finiasz *et al.*, 2011]. IgE production by B cells and recruitment of T cells during the late phase of allergic reactions causes a secondary immune response with high-affinity and antigen-specific antibodies that can act rather quickly if the same type of antigen appears in the future [Eckl-Dorna *et al.*, 2013]. Additionally, chemokines such as eotaxin, IL-8, RANTES, MCP-1 are released to elevate the

migration of further immune cells which keep the allergic reaction and their symptoms ongoing [Wong *et al.*, 2005].

The causes of asthma are not completely understood and are considered as multifactorial in origin [Wills-Karp, 2004]. Genetic and environmental factors are the most important risk factors that influence the development and expression of asthma [Tsicopoulos *et al.*, 2013]. In conclusion, asthma mechanisms are complicated and the features of asthma are an outcome of the complexities of human genome including gene-gene as well as gene-environmental interactions [Lovinsky-Desir *et al.*, 2012].

Occupational asthma (OA) is defined as asthma initiated by an airborne agent encountered in the workplace [Bernstein, 2003; Cromwell *et al.*, 1979; Di Stefano *et al.*, 2004; Tarlo *et al.*, 2003; Wild *et al.*, 2003; Zeiss, 2002]. This includes also development or exacerbations of pre-existing asthma [Holsapple *et al.*, 2006; Mapp *et al.*, 1999]. OA prevalence was reported to range between 9% and 15% among the asthmatic population [Nicholson *et al.*, 2005]. The onset of the disease can be induced by an immunologic response in the lung sensitized to an airborne agent inhaled at workplace, named sensitizer-induced OA [Birdi *et al.*, 2013]. Sensitizer-induced OA which could be induced by industrial high molecular weight (HMW) substances such as proteins or polysaccharides [Wild *et al.*, 2003], low molecular weight (LMW) chemicals, or metals [Bernstein, 2003; Di Stefano *et al.*, 2004]. HMW proteins (more than 10 kDa) are generally derived from microorganisms, plants, or animals. Sensitization caused by HMW is initiated through classical IgE antibody and eosinophil inflammation [Wild *et al.*, 2003]. In contrast to HMW allergens, LMW agents are less than 10 kDa and are mostly electrophilic, which have to form covalent bonds with nucleophilic amino acids such as lysine, cysteine, and histidine in the respiratory mucosa to become immunogenic [Sastre *et al.*, 2003] (Figure 2).

The traditional classification for hypersensitivity reactions was proposed by Gell and Coombs in 1963 and is currently the most commonly known classification system, which is divided into four types [Gell *et al.*, 1963]. HMW substances are considered to act as common airborne allergens through IgE mediated responses, while the mechanisms by which LMW substances induce asthma may be different, for example isocyanates, acid anhydrides may lead to airway sensitization through non-IgE-mediated immunologic mechanisms that are only partially elucidated [Kenyon *et al.*, 2012].

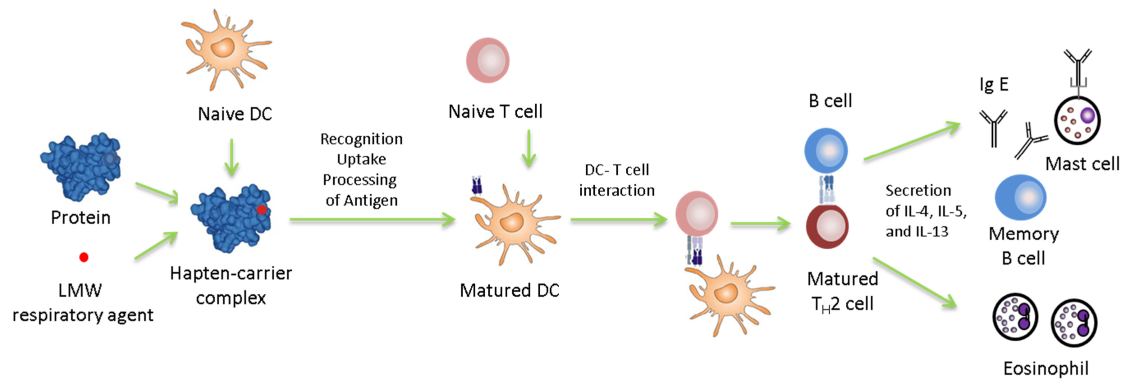

Figure 2: Sensitization caused by a LMW respiratory agent. The green pathway describes the molecular mechanism of sensitization.

1.3.2 Mechanisms of non-immunologically mediated asthma

The pathogenesis of OA can also be non-immunologic which is induced by rapid exposure to high concentrations of irritant at workplace or by chronic exposure to inhaled irritants, both of them named irritant-induced OA [Brooks *et al.*, 2011; Verstraelen *et al.*, 2008]. Irritant-induced asthma is a type of non-immunologic occupational asthma that prolonged bronchial hyperresponsiveness and airflow obstruction by inhaled substances. It can be induced by a single or multiple high- or low-dose exposures to injurious agents [Labrecque, 2012]. Reactive airway dysfunction syndrome (RADS) is the best known and characterized form of irritant-induced asthma caused by a single or multiple high-doses of respiratory injuries such as heated acids, toluene diisocyanates, and chlorine dioxide [Labrecque, 2012]. This type of asthma has the same symptoms as the immunological type whereas no latency period occurs. The onset of RADS symptoms occurs within 24 hours after exposure [Brooks *et al.*, 2011]. It is dominated by an inflammatory reaction with unspecific immune cells eliminating the chemicals. Pathogenesis of RADS involves innate, non-adaptive immune responses and starts with bronchial epithelial injury and release of pro-inflammatory mediators which are also able to result in airway remodelling [Brooks *et al.*, 2011].

1.4 Test methods for the identification of different sensitizers

1.4.1 In vivo test methods for the identification of respiratory and contact sensitizers

Respiratory sensitization is an immunological state of the respiratory tract that results from specific adaptive immune responses to antigenic exposure leading to hypersensitivity after subsequent exposure to the sensitizing substance.

One of the well-established *in vivo* models for respiratory allergy research was the guinea pig model, which has been used for nearly a century [Magnusson *et al.*, 1969]. In this model, the lung of guinea pig is the target organ for the identification of hypersensitivity exposed to respiratory sensitizer. It has been reported that the responses in guinea pigs are in a similar way as observed in human regarding lung injury, antibody responses, and respiratory sensitization [Briatico-Vangosa *et al.*, 1994; Karol, 1987]. However, there are a number of drawbacks in the use of guinea pig model of respiratory sensitization. For example, in guinea pigs the major class of antibody response is IgG, whereas in human IgE is the predominant class [Pauluhn *et al.*, 2005; Ritz *et al.*, 1993; Sarlo *et al.*, 1992]. Furthermore, guinea pig has a prominent lung eosinophilia without antigen sensitization [Rothenberg *et al.*, 1995]. Therefore, these disadvantages prohibited the development of guinea pig model as a prediction model for respiratory sensitizer.

At present, the widely used alternative approach which based on the immunological events and responses that are provoked by contact allergens is the murine local lymph node assay (LLNA). The LLNA is a predictive test that uses *in vivo* cell proliferation in the draining lymph nodes for assessment of the contact sensitization of chemicals [Basketter *et al.*, 2002; Magnusson *et al.*, 1969; OECD Guideline 429, 2010]. The LLNA is the unique model which has been regulatory accepted and validated. The observed degree of lymphocyte proliferation has been shown to correlate well with the sensitization potency of the test material [Kimber *et al.*, 1994]. This assay possessed several advantages over guinea pig sensitization assays, including the generation of quantitative dose-response data for addressing sensitization potency, the reduction and refinement of animal testing, and decreases of experimental time and costs [Anderson *et al.*, 2011]. Originally, the LLNA was designed for predicting skin sensitizers, however, most respiratory sensitizers tested have also been shown to be positive [Boverhof *et al.*, 2008]. It appears that respiratory allergens can induce sensitization when applied

topically to the skin [Hilton *et al.*, 1998]. In the modified LLNA, referred to as respiratory LLNA (which has not been validated yet), chemicals are inhaled, and enhanced proliferation of lymphocytes as well as the cytokine profile in the draining lymph nodes can be used to identify and distinguish strong contact and respiratory sensitizers [Arts *et al.*, 2008; Basketter *et al.*, 1992; de Jong *et al.*, 2009; Kimber *et al.*, 2007; OECD Guideline 429, 2010; Van Och *et al.*, 2002]. However, dermal application is the only route of exposure validated for the LLNA, which excluded allergens that cannot pass through the skin. There are currently no accepted and validated test methods to identify chemicals with a potential to cause respiratory sensitization [Holsapple *et al.*, 2006; Kimber *et al.*, 1996; van Loveren *et al.*, 2008]. The prevalence of both contact and respiratory sensitizations tends to grow proportionally to an expanding variety of chemicals. Therefore, further models are still required which can be used to assess the respiratory sensitizing potential of work-related agents.

1.4.2 In vitro, in chemico, and in silico alternative test methods for the identification of sensitizers

In vivo studies are very complex, cost-intensive and especially associated with pain and suffering of many laboratory animals. A continued challenge has been led to the development of *in vitro* approaches such as cell culture systems according to reduce, refine and replace the use of animals. The use of cultured DCs or DC-like cells is the most common method for the identification of sensitizers and for the further characterization of response through *in vivo* studies to differentiate respiratory from contact sensitizers. Human peripheral blood mononuclear cell-derived dendritic cells (PBMC-DC) have been utilized commonly to monitor the response to various sensitizers through studies on e.g. surface marker expression or gene expression responses, which support the identification of sensitizing potential [Hansen *et al.*, 2005; Hulette *et al.*, 2002; Larsson *et al.*, 2009]. A number of dendritic-like cell lines have also been investigated including e.g. the human myeloid cell lines MUTZ-3 and THP-1 for induction of respiratory sensitization reactions [Megherbi *et al.*, 2009; Mitjans *et al.*, 2008; Nelissen *et al.*, 2009; Python *et al.*, 2009]. In the past years several *in vitro* assays have been already validated for screening toxicity in the context of REACH [Marx *et al.*, 2007; Vitale *et al.*, 2009]. The majority of validated and accepted alternative test methods were published for topical and oral applications. For example, skin irritation

test methods were applied not only *in vivo* methods but also *in vitro* testing (Episkin^{TM}, Epiderm^{TM} and SkinEthic^{TM}) [Alepee *et al.*, 2013; Curren *et al.*, 2006; Hoffmann *et al.*, 2005; Tornier *et al.*, 2010; Yuki *et al.*, 2013]. Since July 2013 Regulation (EC) No 1223/2009 of the European Parliament and of the Council on cosmetic products provides the prohibition of animal testing on cosmetic compounds which should be fully replaced by alternative methods [European Parliament, 2009]. But there is still no validated test method for the respiratory toxicity.

Chemical substances (haptens) can trigger a sensitization reaction through binding to a protein by stable covalent bonds or coordination bonds. The study of chemical-protein reactivity in the sensitization process is an additional non-cell-based *in vitro* approach which is defined as *in chemico* study. A number of studies have used peptide reactivity assays for the identification of sensitizers by determining chemical-protein conjugates or by measuring the depletion of peptides [Gerberick *et al.*, 2004; Gerberick *et al.*, 2007; Kato *et al.*, 2003; Maxwell *et al.*, 2011]. For example trimellitic anhydride and maleic anhydride have chemical characteristics containing functional groups such as anhydrides which are usually used to modify primary amines on proteins and providing reactive groups for subsequent reactions with e.g. proteins in biological environments [Lalko *et al.*, 2012]. In summary, current *in vitro* and *in chemico* approaches for assessing the sensitization potential of substances have advantages in the understanding of chemical-induced and regulated cellular and molecular mechanisms. However, due to the complexity of the immune system, most *in vitro* or *in chemico* assays can only assess some aspects of the multi-step procedures required to achieve sensitization.

Another alternative to animal model names *in silico* approaches, which identify allergens by using structure-activity relationships (SAR) [Seed *et al.*, 2008]. SAR models are primarily based on chemical structure alerts and reactivity of functional groups with proteins by using many software applications like ChemProp and TIMES-SS [Meinert *et al.*, 2010; Patlewicz *et al.*, 2007]. For example, a number of studies have described structural alerts with molecular sub-structures or fragments that contain an electrophilic or pro-electrophilic group within a molecule which can covalently bind to self-proteins for contact sensitization and contact irritation as for example glyoxal [Patlewicz *et al.*, 2007]. Glyoxal is an extremely reactive Schiff base former that has been shown to be able to cross-link protein chains. This chemical is capable of cross-linking proteins due to the two carbonyl groups, which allows mechanistic information to be used for hazard identification [Enoch *et al.*, 2012; Marquie, 2001]. However, *in*

silico models show limitations due to complexity of the available databases and the use of software for unknown allergens [Thomas *et al.*, 2005].

1.5 PCLS reflect the natural immunological responses of the intact organ

Organ slices have been used in biochemistry for studying basic pathways of intermediary metabolism for several decades [Warburg, 1923]. However, the production of identical slices in many of the early studies using manual techniques was difficult, being highly dependent on the experience of the investigator. The development of the Krumdieck tissue slicer, which improved culturing and slicing technologies by the production of consistent dimensions of slices with reduced stress for the tissue for the first time [Brendel *et al.*, 1987; Krumdieck *et al.*, 1980; Smith *et al.*, 1985]. Live circular tissue slices of nearly identical diameter and thickness of thin circular sections can be generated from most tissues firstly by using a coring tool which cut the tissue into cylindrical tissue cores and subsequently by a microtome that cut by a rapidly oscillating disposable blade to the vertical main axis of the tissue core [Krumdieck, 2013]. Krumdieck tissue slicer operates submerged in a cold isotonic medium that carries the cut slices outside the microtome. All the instruments can be sterilized and slices can be obtained at a constant rate [Krumdieck, 2013].

The advantages of this organotypic lung tissue model are diverse. Firstly, thin slices of precise sectioning are able to generate reproducible results [Martin *et al.*, 1996]. Due to improvements of the model by resting slices or better conditions like suitable culture medium along with others, it shows good reproducibility of findings [Switalla *et al.*, 2010b]. Secondly, PCLS provide physiological complexity of high biological relevance [Sewald *et al.*, 2013; Switalla *et al.*, 2010b]. They provide a unique approach to investigate integrated physiology that links the cellular and organ responses. They retain many aspects of the cellular and structural organization of the lung. The immune response of PCLS could be investigated on different levels (e.g. protein production, gene expression, and mediator release) and results could be extrapolated to *in vivo* immune responses [Sewald *et al.*, 2013]. And thirdly, according to 3R concept, there is a public demand to limit the number of animals and reduce distress of laboratory animals. PCLS can produce copious numbers of slices which obtained from one lung lobe of different laboratory animals (e.g. mouse, rats, guinea pigs) and humans. Each experiment can be performed with each own internal control. Moreover, the dynamics

of macroscopic changes of bronchoconstriction associated with the airways could be observed with conventional microscopy [Schleputz *et al.*, 2012; Seehase *et al.*, 2011]. The microscopic changes associated with cellular events could be also observed with confocal or two photon microscopy [Sanderson, 2011].

In the last years, the slice technique was established for a wide range of species such as rat, mouse, guinea pig, non-human primate, and human [Sewald *et al.*, 2013]. Tissue slices could also be produced from a variety of different organs such as liver, kidney, heart, and lung [Bergner *et al.*, 2002; De Kanter *et al.*, 2004; Pushparajah *et al.*, 2007]. Vital lung slices were used for the assessment of responses to immunomodulators like LPS and dexamethasone [Henjakovic *et al.*, 2008; Seehase *et al.*, 2012; Switalla *et al.*, 2010b], determination of genotoxicity using Comet assay [Switalla *et al.*, 2013], studies on xenobiotics-mediated metabolism [De Kanter *et al.*, 2004; Groothuis *et al.*, 2013; Niu *et al.*, 2013; Umachandran *et al.*, 2004], investigations of small airways in the early allergic response, calcium signalling combined with bronchoconstriction, bronchoconstriction in non-human primate [Bergner *et al.*, 2002; Seehase *et al.*, 2011; Wohlsen *et al.*, 2003], and other pharmacological and toxicological studies [Fisher *et al.*, 2013; Monteil *et al.*, 1999; Nassimi *et al.*, 2009; Sturton *et al.*, 2008]. PCLS have the ability to observe changes in cell physiology and subsequently these responses could be manifested themselves at the level of the organ, lung slices have become a standard tool for the investigation of lung disease.

All these above mentioned aspects supported the development of PCLS in this doctoral thesis as an *ex vivo* model which could offer an appropriate way to assess local immunotoxicity. The aim of this doctoral thesis was firstly to analyse chemical-induced irritation and inflammation by assessing a variety of immunotoxic endpoints in human PCLS, and secondly to prevalidate the PCLS model as an organotypic tissue model for *in vitro* testing of starting concentrations for acute inhalation toxicity studies, which may ultimately lead to a refinement and reduction of animal testing.

2 Hypothesis

A variety of *in vitro* and *in vivo* models were investigated for respiratory toxicity studies with different aspects. The most *in vitro* and *in vivo* models did not have the capability to extrapolate to the human situation due to lacking organ complexity and human physiology, respectively. Alternative methods for the assessment of chemicals for respiratory toxicity were only established through dermal route application *in vivo* or cell lines and three-dimensional (3D) models of respiratory tract *in vitro* in the last years, however, there are still no accepted and validated alternative methods for the identification of respiratory toxicity.

Hypothesis of this doctoral thesis was that the identification of respiratory toxicants could be characterized by means of chemical-induced cytotoxicity and specific cytokine pattern in an organotypic lung tissue model PCLS.

For proving this hypothesis, two different studies were investigated:

1. Acute local respiratory irritation and inflammation were assessed in PCLS after exposure to well-known industry sensitizers and non-sensitizing irritants. Aim was to identify chemical-induced toxicity for the risk assessment of occupational asthma.
2. The second study was focused on the prediction of chemical-induced toxicity, which aimed to prevalidate PCLS as a standardized tool of dose-finding at the beginning extrapolated to *in vivo* situation for acute inhalation toxicity studies.

3 Assessment of immunotoxicity induced by chemicals in human precision-cut lung slices (PCLS)

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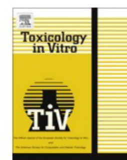

Assessment of immunotoxicity induced by chemicals in human precision-cut lung slices (PCLS)

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A B S T R A C T

Occupational asthma can be induced by a number of chemicals at the workplace. Risk assessment of potential sensitizers is mostly performed in animal experiments. With increasing public demand for alternative methods, human precision-cut lung slices (PCLS) have been developed as an *ex vivo* model.

Human PCLS were exposed to increasing concentrations of 20 industrial chemicals including 4 respiratory allergens, 11 contact allergens, and 5 non-sensitizing irritants. Local respiratory irritation was characterized and expressed as 75% (EC_{25}) and 50% (EC_{50}) cell viability with respect to controls. Dose–response curves of all chemicals except for phenol were generated. Local respiratory inflammation was quantified by measuring the production of cytokines and chemokines. TNF-α and IL-1α were increased significantly in human PCLS after exposure to the respiratory sensitizers trimellitic anhydride (TMA) and ammonium hexachloroplatinate (HClPt) at subtoxic concentrations, while contact sensitizers and non-sensitizing irritants failed to induce the release of these cytokines to the same extent. Interestingly, significant increases in T_H1/T_H2 cytokines could be detected only after exposure to HClPt at a subtoxic concentration.

In conclusion, allergen-induced cytokines were observed but not considered as biomarkers for the differentiation between respiratory and contact sensitizers. Our preliminary results show an *ex vivo* model which might be used for prediction of chemical-induced toxicity, but is due to its complex three-dimensional structure not applicable for a simple screening of functional and behavior changes of certain cell populations such as dendritic cells and T-cells in response to allergens.

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1. Introduction

Asthma is one of the most common chronic inflammatory lung diseases based on a hypersensitivity reaction to environmental stimuli such as proteins or chemical xenobiotics (Martinez, 2007; Di et al., 2011). It is characterized by recurrent attacks of breathlessness and wheezing, reversible airflow obstruction and bronchial spasm, supported by the presence of inflammatory cells such as eosinophils, allergen-specific T_H2 lymphocytes, and activated dendritic cells (DCs) or mast cells (Galli, 1997; Lane and Lee, 1996; Leckie et al., 2000; NHLBI Guideline, 2007). These immune cells produce inflammatory mediators including cytokines and chemokines, thereby perpetuating the inflammatory process. In bronchial asthma, cytokines such as TNF-α, interleukin (IL)-1, IL-4, IL-5, and IL-13 are produced, for example, by DCs supporting the inflammation and the allergen-specific T_H2 response (Dearman et al., 2005). Additionally, chemokines such as eotaxin, IL-8, RANTES, and MCP-1 are released to elevate the migration of further immune cells which keep the allergic reaction and its symptoms going (Wong et al., 2005).

Occupational asthma (OA) is defined as asthma initiated by workplace exposures (Bernstein, 2003; Cromwell et al., 1979; Wild and Lopez, 2003; Tarlo and Liss, 2003; Zeiss, 2002; Di Stefano et al., 2004). This includes development or exacerbations of pre-existing asthma (Mapp et al., 1999; Holsapple et al., 2006). The onset of the disease can be induced by industrial high-molecular-weight (HMW) substances such as proteins or polysaccharides (Wild and Lopez, 2003), low-molecular-weight (LMW) chemicals, or metals (Bernstein, 2003; Di Stefano et al., 2004). LMW chemicals play an

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important role, since a high number of them is known or suspected to cause respiratory allergy in humans (Zhang et al., 2009, 2002). These include platinum compounds (e.g. ammonium hexachloroplatinate) (Ban et al., 2010), reactive dyes, and acid anhydrides (e.g., trimellitic anhydride). LMW chemicals mostly act as haptens due to their small size (e.g. anhydrides). They become allergenic after conjugation with native carrier proteins (e.g. human serum albumin) to form a complete antigen which is able to provoke a specific immune response (Sastre et al., 2003). Additionally, the sensitization process is also associated with irritating capabilities of the chemical. In this case, the disease is referred to as irritant-induced asthma, which on the one hand can be induced by repeated-dose application of low doses of irritants or on the other hand by high doses of irritants where the exposures are in the irritant dose range (Verstraelen et al., 2008; Brooks and Bernstein, 2011).

Due to increasing incidences of OA induced by LMW agents, the imperative emerged to identify LMW chemicals which could trigger a sensitization at workplaces, in particular with regard to risk assessment (Malo and Chan-Yeung, 2009; Orriols et al., 2006). To date, risk assessment of potentially sensitizing chemicals plays an important role in the registration of chemicals to protect human health. At present, the local lymph node assay (LLNA) and the guinea pig maximization tests are used for the detection of sensitizers (OECD Guideline 429, 2010; Basketter et al., 2002; Magnusson and Kligman, 1969). Originally, these assays were designed for predicting skin sensitizers, however, most respiratory sensitizers tested have also been shown to be positive (Boverhof et al., 2008). It appears that respiratory allergens can induce sensitization when applied topically to the skin (Hilton et al., 1998). In the modified LLNA, referred to as respiratory LLNA (which so far has not been validated), chemicals are inhaled, and enhanced proliferation of lymphocytes as well as the cytokine profile in the draining lymph nodes can be used to identify and distinguish strong contact and respiratory sensitizers (Basketter and Scholes, 1992; Arts et al., 2008; Kimber et al., 2007; Van Och et al., 2002; de Jong et al., 2009; OECD Guideline 429, 2010).

However, there are currently no accepted and validated test methods to identify chemicals with a potential to cause respiratory sensitization (van Loveren et al., 2008; Kimber et al., 1996; Holsapple et al., 2006). It is also clear that the use of animal models has limitations, because of the physiological and biochemical dissimilarities (Hartung, 2009). Moreover, in the context of REACH (European Community regulation for the Registration, Evaluation, Authorisation and Restriction of Chemicals) (Foth and Hayes, 2008) and the principle of the 3Rs (Vitale et al., 2009), there is an increasing public and legal demand for alternative methods. Since July 2013 Regulation (EC) No 1223/2009 of the European Parliament and of the Council on cosmetic products provides the prohibition of animal testing on cosmetic compounds which should be fully replaced by alternative methods (European Parliament, 2009). Therefore, further models are required which can be used to assess the sensitizing potential of work-related agents. In particular, new human-relevant *in vitro* models are needed which reproduce the acute cell responses to sensitizers and allow a distinction between respiratory allergens and contact allergens.

Human precision-cut lung slices (PCLS) are an *ex vivo* model consisting of all relevant cell types of the respiratory tract situated in their microanatomical environment (De Kanter et al., 2002; Henjakovic et al., 2008b). PCLS are well-established in pharmacological testing and thus offer the opportunity to gain insight into chemical-induced effects in lung tissue (Henjakovic et al., 2008a,b; Switalla et al., 2010). Furthermore, lung tissue models have also been used for research on xenobiotic metabolism (De Kanter et al., 2004; Umachandran et al., 2004), the early allergic response (Wohlsen et al., 2003), and calcium signaling (Bergner and Sanderson, 2002).

The present study using the *ex vivo* model PCLS was part of the European Union project Sens-it-iv, which involved 28 partners from across Europe. The overall goal of Sens-it-iv was to develop strategies to replace animal testing by alternative methods for risk assessment of potential skin or lung sensitizers for the chemical and cosmetics industries. The goal in our workgroup was to develop and establish an alternative strategy using the *ex vivo* technique PCLS for the analysis of effects after acute exposure to chemicals. Since some chemical-induced irritations may also induce sensitization in skin or lung tissue, both irritation and inflammation after submerged exposure to chemicals were assessed by a variety of immunotoxic endpoints. Therefore, human PCLS were exposed to LMW chemicals, including respiratory sensitizers, contact sensitizers and non-sensitizing irritants. Chemical-induced toxicity, resulting in 50% and 75% of viability (EC_{50} and EC_{25}) compared to vehicle control, was calculated for all chemicals investigated. In order to compare the data of chemical-induced toxicity *ex vivo* to *in vitro* and *in vivo* situations, EC_{25} values of human PCLS were correlated on the one hand with *in vitro* data published for THP-1 and NCTC cell lines, which have different inspects of monocyte-derived cells and skin-derived epithelial cells, and on the other hand with LD_{50} values from *in vivo* rat inhalation studies, which could be interesting for *in vivo* dose-finding studies. Furthermore, the initial response to respiratory and contact allergens was marked firstly by the coordinated release of pro-inflammatory mediators such as TNF-α and IL-1α at subtoxic concentrations. Secondly, local responses of T cells in patient lung tissue were determined by analyzing T_H1/T_H2 cytokine release for the identification of respiratory and contact sensitizers.

The present paper describes the performance of tests for the irritant potency and sensitization potential of a selection of LMW allergens in human PCLS and compares the results with data obtained in different cell models and with data from *in vivo* tests retrieved from online databases.

2. Materials and methods

2.1. Human lung explant culture

Human lung explants were obtained from male and female patients who underwent lung resection for cancer at Oststadt-Krankenhaus, Hanover, Germany. All patients gave written consent. The performance with human lung tissue was approved by the ethics committee of the Hanover Medical School. Only lung tissue containing no tumors as qualified by medical pathologists was used for the experiments. Tissue was processed immediately on the day of resection as described below. The age of patients was 60 ± 10 years, and 80% of them were smokers.

2.2. Media, reagents and chemicals

Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 Ham (DMEM) with L-glutamine, 15 mM HEPES without phenol red, pH 7.2–7.4 was supplied by Sigma Aldrich (Munich, Germany) and supplemented with 7.5% w/v sodium bicarbonate, without fetal calf serum. Medium for cultivation was prepared with penicillin and streptomycin (Sigma Aldrich, Munich, Germany). Low-melting agarose, Earle's Balanced Salt Solution (EBSS), Triton X-100, dimethyl sulfoxide (DMSO), and protease inhibitor cocktail were also purchased from Sigma Aldrich (Munich, Germany). PBS (0.1 M sodium phosphate and 0.15 M NaCl, without Ca^{2+} and Mg^{2+}) was obtained from Lonza (Verviers, Belgium). Enzyme-linked

immunosorbent assay kits (ELISA DuoSets) were supplied from R&D Systems (Wiesbaden-Nordenstadt, Germany). WST-1 assay was purchased from Roche (Mannheim, Germany). BCA Protein Assay Kit was obtained from Pierce (Rockford, IL, USA). MSD® 96-Well MULTI-ARRAY® and MULTI-SPOT® were purchased from Meso Scale Discovery (Gaithersburg, Maryland, USA).

2.3. Preparation of PCLS and tissue cultures

Human lung slices were prepared as described before (Ressmeyer et al., 2006; Wohlsen et al., 2003; Switalla et al., 2010). In brief, human lung lobes were firstly cannulated with a flexible catheter and the selected lung segments were inflated with 1.5% low-melting agarose medium solution. The inflated lungs were immediately solidified on ice. After polymerization the tissue was cut into 1-cm-thick slices. Tissue cores were stamped and cut into approx. 250-μm-thick sections in EBSS using a special microtome (Krumdieck tissue slicer; Alabama Research and Development, Munford, AL, USA). Tissue slices were washed and incubated in DMEM containing 100 U/mL penicillin and 100 μg/mL streptomycin. PCLS were cultured at 37 °C, 5% CO_2, and 90–95% air humidity under cell culture conditions and used for experiments as described below.

The following acceptance criteria were used to control the quality of the tissue: (i) only lung tissue containing no tumors as qualified by medical pathologists was used, (ii) the tissue was sufficiently viable [WST-1 assay: minimum OD of the mean (duplicate-averaged) tissue control (incubated only with medium) or vehicle control had to be 0.6.], (iii) the tissue was sufficiently sensitive to a toxicant (1% Triton X-100), iv) the tissue was sufficiently sensitive to 100 ng/mL LPS which had to result in an increase of pro-inflammatory cytokine IL-1α to minimum of 150% compared to untreated control, (v) and most importantly the tissue was sensitive to a positive treatment control. Therefore, 50 μg/mL dexamethasone were used. The results of an individual donor were only excepted if dexamethasone decreased LPS-induced IL-1α to baseline levels.

2.4. Incubation of PCLS with chemicals

PCLS were incubated with 20 chemicals selected within the Sens-it-iv consortium, including 4 respiratory allergens: trimellitic anhydride (TMA), ammonium hexachloroplatinate (HClPt), maleic anhydride (MA), and glutaraldehyde (GA); 11 contact allergens: 1,2-dibromo-2,4-dicyannobutane (2-Bro), cinnamic aldehyde (CinAld), cinnamic alcohol (CinOH), 2,4-dinitrochlorobenzene (DNCB), eugenol (Eug), *p*-phenylenediamine (PPD), resorcinol (Res), tetramethylthiuramdisulfide (TMTD), glyoxal (Glyo), 2-mercaptobenzothiazole (2-Mer), and 4-nitrobenzyl bromide (4-Nit); and 5 non-sensitizing irritants: phenol (Phe), lactic acid (LA), salicilic acid (SA), sodium lauryl sulfate (SLS), and glycerol (Glyc).

Sections were incubated with the chemicals diluted in DMEM at 37 °C, 5% CO_2 and 90–95% air humidity under standard submerged cell culture conditions. Lung slices without addition of chemicals were incubated as negative control. Each chemical was tested in at least 3 biological donors (biological replicates) in five increasing concentrations. Within one run, every concentration of a chemical was tested at once. The number of slices obtained from one donor was sufficient to test the entire range of concentrations of a chemical, including all technical duplicates and controls. After 24 h total incubation time, cell culture supernatants as well as the PCLS tissue were analyzed. The supernatant was collected for extrinsic cytokine and chemokine determination. To this end, 0.2% protease inhibitor cocktail was added to the supernatant. PCLS were either used for WST-1 assay or for determination of intrinsic cytokine levels and protein content. For protein and intracellular cytokine measurement the tissue was permeabilized with 1% Triton X-100 in PBS plus 0.2% protease inhibitor cocktail at 37 °C for 60 min. Samples were stored at −80 °C and cytokine contents were measured either by MSD or ELISA.

2.5. Preparation of chemicals

HClPt, GA, CinAld, Res, Glyo, Phe, LA, SLS, and Glyc were dissolved in sterile pre-warmed (37 °C) DMEM. TMA, MA, 2-Bro, CinOH, DNCB, Eug, PPD, TMTD, 2-Mer, 4-Nit, and SA were dissolved in DMSO at 100-fold of the desired final concentration. The solvent DMSO was present at a constant volume in the vehicle controls and in all test concentrations. The highest concentration of test chemicals was intended to induce a cytotoxic effect of more than 50% dead cells.

2.6. WST-1 reduction

WST-1 Proliferation Assay Kit provides an easy-to-use tool for studying changes in cell viability. The assay is based on the reduction of tetrazolium salt WST-1 to soluble formazan in the mitochondria of metabolically active cells. The amount of formazan dye formed directly correlates with the number of metabolically active cells in the culture. After incubation of PCLS, the medium was removed and PCLS were incubated for 1 h at 37 °C with 0.125 mL freshly prepared WST-1 solution per slice (diluted 1:10 in culture medium). Absorbance of the formazan solution was determined at 450 nm with a reference wavelength of 630 nm.

2.7. Calcein AM/ethidium homodimer-1 staining

Viability of the tissue slices was directly checked by calcein acetoxymethyl/ethidium homodimer-1 (calcein AM/EthD-1) staining (Invitrogen, Karlsruhe, Germany) using a confocal laser scanning microscope Meta 510 (Zeiss, Jena, Germany). Live cells were distinguished by enzymatic conversion of calcein AM to intensely green fluorescent calcein. EthD-1 binds to DNA and therefore produces intracellular orange/red fluorescence in nuclei of dead cells. Lung slices were incubated with 4 μM calcein AM and 4 μM EthD-1 for 45 min at room temperature. PCLS were washed in DMEM and investigated by confocal laser scanning microscopy (40× water immersion objective, excitation wavelengths 488 nm and 543 nm, emission filters BP 505–550 nm and LP 560 nm, thickness 40 μm). Image stacks of a defined volume were analyzed with Bitplane IMARIS 7.4.0.

2.8. Quantitative image analysis with IMARIS 7.4.0

Three-dimensional fluorescence images of PCLS were quantitatively analyzed with the IMARIS 7.4.0 software. Confocal datasets with a dimension of 900 × 900 × 40 μm were processed via "surface rendering", which allowed semi-automated counting of EthD-1-labeled, red fluorescent nuclei of dead cells. This was achieved by counting spots ⩾5 μm in diameter. In a second step, the total volume of the cytoplasm of viable cells was determined by calculating the full volume of calcein (green) fluorescent structures, expressed as μm^3. Subsequently, the ratio of counted cell nuclei and volumes of cytoplasm of live cells stained with calcein was calculated. Threshold levels for the calculation of "surface objects" and "spots" were set once for each channel and the same thresholds were used for all datasets. The viability of PCLS is expressed as the number of $EthD^+$ red spots/10^5 μm^3 $calcein^+$ green volume.

2.9. ELISA: quantification of IL-1α and TNF-α

IL-1α and TNF-α were measured in tissue supernatants and lysis extracts of PCLS using commercially available enzyme-linked

immunosorbent assay kits (ELISA DuoSets, R&D Systems, Wiesbaden-Nordenstadt, Germany). ELISA was performed according to the manufacturer's specifications. The lower limit of quantification was 31 pg/mL for human TNF-α and IL-1α. Absorbance was determined at 450 nm with a reference wavelength of 570 nm.

2.10. Measurement of cytokines and chemokines by Meso Scale Discovery Assays

Levels of cytokines and chemokines were determined in supernatants and lysis extracts of PCLS exposed to TMA, HClPt, GA, DNCB, CinAld, 2-Bro, CinOH, SLS, Phe, and LA using MSD technology. Here, detection of cytokines is based on the detection of electrochemiluminescence. A 96-well multi-spot plate for the detection of 8 human cytokines (IFN-γ, IL-10, IL-12 p70, IL-13, IL-1β, IL-2, IL-4, and IL-5) and single-spot plates for the detection of RANTES and eotaxin, respectively, were obtained from MSD. The assay was performed according to the manufacturer's instructions. Twenty-five µL of each standard and sample were incubated for 1.5 h with vigorous shaking (1000 rpm) at room temperature. Detection antibody solution was dispensed at 25 µL into each well of the MSD plate. The plate was also incubated for 1.5 h with vigorous shaking (1000 rpm) at room temperature. Finally, the plate was washed three times with PBS + 0.05% Tween-20 and analyzed immediately after addition of Read Buffer. Calculation of cytokines was performed using a 4-fold serial diluted standard. Data analysis was performed using the discovery workbench software.

2.11. Protein determination

Protein concentrations were determined by the BCA method using bovine serum albumin (BSA) as standard. Twenty-five µL of sample or BSA were incubated with 200 µL BCA reagents for 30 min at 37 °C. Absorbance was measured at a wavelength of 570 nm.

2.12. Statistical analysis

Data in the figures are given as means ± SEM. Statistical analysis was performed by non-parametric *t*-tests (Mann–Whitney test) (software: GraphPad Prism 4, version 4.03). Correlation of data generated *in vivo* and *ex vivo* was evaluated using a linear regression analysis model. Differences between treated samples and control were considered statistically significant at a level of $p < 0.05$.

2.13. Curve fitting

EC_{50} (effective concentration at 50% reduction of cell viability) and EC_{25} values (effective concentration at 25% reduction of cell viability) were evaluated for results of WST-1 assay. Data are presented as mean ± SEM (%) compared to tissue or vehicle control converted from measured absorbance. Percentage calculations were normalized and analyzed by "nonlinear regression" using "sigmoidal dose–response" with defined constrains for the determination of EC_{50} values and EC_{25} values (software: GraphPad Prism 4, version 4.03).

3. Results

3.1. Determination of 50% and 75% cell viability in human PCLS

In order to measure chemical-induced cytotoxicity in human PCLS, viability of the tissue was determined by detection of released lactate dehydrogenase (LDH) using the LDH assay, by measurement of metabolic enzyme activity using the WST-1 assay, and by quantitative image analysis of LIVE/DEAD® staining. To this end, tissue sections were incubated with the selected set of chemicals for 24 h. Fig. 1 shows examples of the determination of toxic and subtoxic concentrations obtained in human PCLS after submerged exposure to the respiratory agents HClPt, TMA, GA, and MA using the LDH (Fig. 1A–D) and WST-1 assays (Fig. 1E–H). EC_{50} and EC_{25} values were evaluated primarily by the results of the WST-1 assay, since exposure to HClPt, TMA, GA, and MA failed to increase lactate dehydrogenase release (Fig. 1E–H). In order to ensure that viability was reliably measured, a microscopy-based approach was chosen. Therefore, tissue was stained by LIVE/DEAD® staining and images were obtained by confocal laser scanning microscopy (CLSM). Fig. 1 shows examples of LIVE/DEAD® staining of human PCLS after incubation with HClPt (Fig. 1I), TMA (Fig. 1J), and GA (Fig. 1K), which resulted in 25% reduction of cell viability in comparison to respective controls.

Dose–response curves of respiratory allergens (TMA, HClPt, MA, and GA), contact allergens (2-Bro, CinAld, CinOH, DNCB, Eug, PPD, Res, TMTD, Glyo, 2-Mer, and 4-Nit), and non-sensitizing irritants (LA, SA, SLS, and Glyc) were generated by determination of the number of metabolically active cells in the PCLS using WST-1 assay (Supplement 2). Concentration-dependent toxicity could be shown for all tested chemicals, with EC_{25} values ranging from 0.051 µg/mL to 1895 µg/mL, and EC_{50} values ranging from 0.16 µg/mL to 5625 µg/mL except for Phe (Table 1). Thus, the most toxic substance was Glyo with an EC_{25} value of 0.051 µg/mL and an EC_{50} value of 0.16 µg/mL. The least toxic substance was Res with an EC_{25} value of 1895 µg/mL and an EC_{50} value of 5625 µg/mL. No dose–response curve could be generated for Phe, since no clear metabolic reduction of WST-1 was observed. Subtoxic concentrations were confirmed by using LIVE/DEAD® staining for CLSM. Subsequently, the microscopic images were analyzed by IMARIS and the ratio of nuclei of dead cells in relation to the volume of live cells in human PCLS was quantified with a maximum concentration of 250 µg/mL exposed to Phe (Supplement 1).

3.2. Comparison of chemical-induced cytotoxicity in the PCLS model to other in vitro or in vivo studies

Recently published EC_{25} values ranging from 5 µg/mL for TMTD to 500 µg/mL for TMA were calculated from *in vitro* studies using the THP-1 cell line, which was exposed to HClPt, TMA, CinAld, DNCB, PPD, TMTD, Phe, SA, and SLS (Mitjans et al., 2008). Other recently published EC_{25} values ranging from 2.5 µg/mL for 4-Nit to 1000 µg/mL for Res as well as for Glyc were calculated from the NCTC cell line, which was exposed to HClPt, TMA, CinAld, CinOH, DNCB, Eug, PPD, Res, TMTD, 2-Mer, 4-Nit, Phe, LA, SA, SLS, and Glyc (Mitjans et al., 2008; Corsini et al., 2009). Additionally, *in vivo* deposited doses converted from LD_{50} values, ranging from 0.01 mg for Glyo to 19.10 mg for LA published from rat inhalation toxicity studies (Online databank, 2011), could be used as reference values for the correlation with EC_{50} values of human PCLS (Table 1).

To determine the degree of correlation between the results obtained in human PCLS and the *in vitro* findings, EC_{25} values of human PCLS and corresponding EC_{25} values from data generated with the human cell line THP-1 exposed to HClPt, TMA, CinAld, DNCB, Eug, PPD, TMTD, Phe, SA, and SLS and the human cell line NCTC exposed to HClPt, TMA, CinAld, CinOH, DNCB, Eug, PPD, Res, TMTD, 2-Mer, 4-Nit, Phe, LA, SA, SLS, and Glyc were entered in linear regression analysis models. Individual EC_{25} values of human PCLS correlated significantly with data from the THP-1 and NCTC cell lines (Pearson $r = 0.86$ and 0.78; $p\text{-value} = 0.0033$ and 0.0004, respectively) (Fig. 2A and B).

In the same way as the comparison with the *in vitro* findings, EC_{50} values of human PCLS were also entered in linear regression

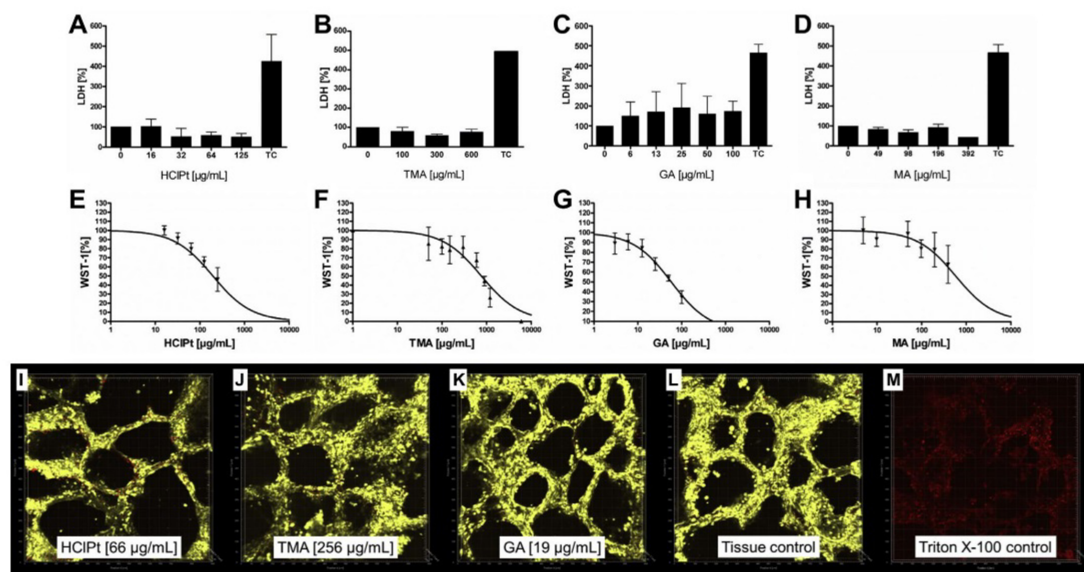

Fig. 1. Toxic and subtoxic concentrations of respiratory agents as shown here for HClPt, TMA, GA, and MA were determined in human PCLS by measurement of (A–D) released LDH [$n = 5_{(HClPt, TMA, GA)}$, $n = 3_{MA}$]; (E–H) reduced mitochondrial activity using WST-1 assay ($n = 16_{HClPt}$, $n = 9_{TMA}$, $n = 7_{GA}$, and $n = 4_{MA}$). LIVE/DEAD® staining of human PCLS for confocal microscopy after exposure to HClPt, TMA, and GA (no picture was taken for MA). Tissue sections were stained with calcein AM/EthD-1 after incubation with (I) 66 µg/mL HClPt, (J) 256 µg/mL TMA, (K) 19 µg/mL GA, (L) without chemicals (tissue control), or (M) after cell lysis (Triton X-100 for positive control). Fluorescent staining in PCLS was analyzed by confocal laser scanning microscopy (20× objective, excitation wavelengths 488 nm and 543 nm, emission filters BP 505–550 nm and LP 560 nm, thickness circa 30 µm, grid spacing = 50 µm).

analysis models with deposited doses converted from LD_{50} values from the online databank including values where the LD_{50} is likely to be greater than the highest tested dose (Online databank, 2011) for HClPt, TMA, MA, GA, Eug, Res, PPD, TMTD, Glyo, 2-Mer, Phe, LA, SA, and Glyc, with Pearson $r = 0.70$ (p-value = 0.0052) (Fig. 2C).

3.3. Respiratory allergens TMA and HClPt significantly increase pro-inflammatory cytokines TNF-α and IL-1α in human PCLS

In the present study, respiratory sensitizers (TMA, HClPt, GA, and MA), contact sensitizers (DNCB, 2-Bro, CinAld, CinOH, 2-Mer, and 4-Nit), and non-sensitizing irritants (Phe, LA, and SLS) were investigated for their ability to induce an acute inflammatory response by cytokine release in human PCLS (Figs. 3 and 4). Two or three subtoxic concentrations (approximating EC_{25} values) were selected for 24-h submerged exposure under standard cell culture conditions. The respiratory sensitizers TMA and HClPt induced a dose-dependent and significant increase in IL-1α by up to 855% and 338%, respectively (Fig. 3A and B), whereas GA and MA failed to induce IL-1α (Fig. 3C and D). The respiratory sensitizers HClPt and GA induced dose-dependent and significant production of TNF-α, increased by up to 440% and 241%, respectively (Fig. 4B and C), whereas TMA and MA failed to induce TNF-α (Fig. 4A and D). The contact sensitizers (DNCB, 2-Bro, CinAld, CinOH, 2-Mer, and 4-Nit) and the non-sensitizing irritants (Phe, LA, and SLS) failed to induce any of the pro-inflammatory cytokines IL-1α and TNF-α (Figs. 3E–L and 4E–L).

3.4. Production of T_H1/T_H2 cytokines induced by respiratory allergens (TMA and HClPt) in human PCLS in comparison to contact allergens (2-Bro and CinAld) and non-sensitizing irritants (Phe and SLS)

In the present study, human PCLS were incubated with either respiratory sensitizers, contact sensitizers, or non-sensitizing irritants to test the effect on T_H1/T_H2 cytokine production in human PCLS as an approach mimicking the *in vivo* situation (Van Och et al., 2002). Total IL-10 production was significantly increased (3.0-fold) after exposure to both respiratory allergens TMA and HClPt (Fig. 5A). Furthermore, significant increases after exposure to HClPt could be detected for IL-2 with up to 1420%, for IL-13 with up to 1320%, for IL-5 with up to 620%, and for eotaxin-2 with up to 203% (Fig. 5C–F). However, the T_H1 cytokine IFN-γ was also significantly induced by HClPt with up to 2810% (Fig. 5B). The contact allergens 2-Bro and CinAld as well as the negative controls Phe and SLS did not induce production of IL-10, IL-2, IL-13, IL-5, eotaxin-2, and IFN-γ (Fig. 5).

3.5. Different levels of cytokine production induced by HClPt in human and mouse PCLS

Both human and mouse PCLS were exposed under submerged conditions for 24 h to HClPt at subtoxic concentrations of 64 µg/mL and 32 µg/mL, respectively. Extracellular and intracellular production of IFN-γ, IL-10, IL-8, TNF-α, RANTES, eotaxin-2, and IL-1α were determined. Cytokine and chemokine levels were represented percentage-wise to compare the expression in human and mouse PCLS. Significant increases could be detected for IFN-γ with up to 4237%, RANTES with up to 530%, and eotaxin-2 with up to 251% in human PCLS after exposure to 64 µg/mL HClPt in comparison with mouse PCLS after exposure to 32 µg/mL HClPt. Total IL-10, IL-8, TNF-α, and IL-1α production was increased by up to 257%, 166%, 149%, and 270%, respectively, in human PCLS exposed to 64 µg/mL HClPt compared to mouse PCLS exposed to 32 µg/mL HClPt (Supplement 3).

4. Discussion

The present study addressed two major issues: Firstly, acute chemical-induced respiratory injury was assessed by determination

Table 1
Human PCLS were exposed to 20 chemicals including 4 respiratory allergens, 11 contact allergens, and 5 non-sensitizing irritants under submerged conditions for 24-h in serum-free DMEM. EC_{25} (effective concentration at 25% reduction of cell viability) and EC_{50} (effective concentration at 50% reduction of cell viability) values of chemicals were calculated on the basis of dose–response curves assessed by WST-1 reduction (Supplement 2). EC_{25} values published for a human promyelocytic cell line (THP-1) and a human keratinocyte cell line (NCTC), and LD_{50} values from published data of inhalation studies with rats exposed to the same chemicals were found (online databanks: ChemIDplus Lite, TOXNET, and NIOSH Pocket Guide to Chemical Hazards). The deposited doses were calculated. abbr: abbreviation; inhal: inhalation.

Chemical abbr.	Chemical name	Category	Human PCLS EC_{25} (μg/mL)	Human PCLS EC_{50} (μg/mL)	THP-1 EC_{25} (μg/mL)	NCTC EC_{25} (μg/mL)	Rat inhal LD_{50} (mg/m³)	Rat inhal deposited dose (mg)
HClPt	Ammonium hexachloroplatinate	Respiratory allergen	66	193	15	15	565/8 h	1.36
GA	Glutaraldehyde	Respiratory allergen	19	58	Unknown	Unknown	480/4 h	1.15
TMA	Trimellitic anhydride	Respiratory allergen	256	761	>500	>500	>2330/4 h	5.60
MA	Maleic anhydride	Respiratory allergen	188	576	Unknown	Unknown	>4350/4 h	10.45
2-Bro	1,2-Dibromo-2,4-dicyanobutane	Contact allergen	8.1	24	Unknown	Unknown	Unknown	Unknown
CinAld	Cinnamaldehyde	Contact allergen	48	147	20	40	Unknown	Unknown
CinOH	Cinnamyl alcohol	Contact allergen	97	286	Unknown	300	Unknown	Unknown
DNCB	2,4-Dinitrochlorobenzene	Contact allergen	1.7	5.2	5	5	Unknown	Unknown
Eug	Eugenol	Contact allergen	55	159	Unknown	225	2580/4 h	6.20
PPD	*p*-Phenylenediamine	Contact allergen	12	36	22	108	920/4 h	2.21
Res	Resorcinol	Contact allergen	1895	5625	Unknown	>1000	>7800/4 h	18.75
TMTD	Tetramethylthiuram disulfide	Contact allergen	6	19	5	32	500/4 h	1.20
Glyo	Glyoxal	Contact allergen	0.051	0.16	Unknown	Unknown	2.440/4 h	0.01
2-Mer	2-Mercaptobenzothiazole	Contact allergen	32	93	Unknown	125	1270/4 h	3.05
4-Nit	4-Nitrobenzyl bromide	Contact allergen	0.6	1.9	Unknown	2.5	Unknown	Unknown
Phe	Phenol	Irritant	>250	>250	70	70	316/4 h	0.76
LA	Lactic acid	Irritant	256	761	Unknown	>500	7940/4 h	19.10
SA	Salicylic acid	Irritant	200	585	250	250	900/1 h	2.16
SLS	Sodium dodecyl sulfate	Irritant	19	55	30	30	Unknown	Unknown
Glyc	Glycerol	Irritant	25	77	Unknown	>1000	570/1h	1.37

of cytotoxic effects of 20 industrial chemicals on human PCLS. Our results clearly showed that toxicity was concentration-dependently induced by nearly all tested chemicals, leading to cell death or tissue injury in lung parenchyma. Secondly, chemical-induced inflammation in human PCLS was assessed by measurement of pro-inflammatory and T_H1/T_H2 cytokines at subtoxic concentrations. Here, it was assumed primarily that these cytokines/chemokines could initially be released, for example, by activated resident macrophages, mucosal dendritic cells and structural cells such as fibroblasts, epithelial cells, and endothelial cells which subsequently could influence T cell activation and differentiation occurring at the first contact with potential sensitizers. Although manifestation of the sensitization process after uptake of allergens in the lungs and migration of antigen-presenting cells to the lymph nodes cannot be mimicked in live lung sections due to missing connections to the blood and lymph system, the onset of the process is orchestrated by cytokines and chemokines which indeed can be induced in PCLS (Switalla et al., 2010).

Inhalation of chemicals can induce respiratory irritation, inflammation, and sensitization (Wanner et al., 2010; Calzetta et al., 2011). In case of a single exposure to high levels of an irritating chemical, an immediate response of the nervous system prevents tissue damage (Mariussen, 2012). Nevertheless, health outcomes after long-term exposure to low levels of a chemical can be even worse (Brimfield, 2012). In the present study, we determined the cytotoxicity of 20 selected case chemicals in human lung tissue after acute exposure (Table 1). Chemical-induced toxicity with a minimum reduction of viability by 25% was observed in human PCLS with all chemicals except phenol. The most toxic chemical was glyoxal, which is known to attack amino groups of proteins, nucleotides and lipids. This leads to inactivation of enzymes, disturbance in the cellular metabolism, impaired proteolysis, and inhibition of cell proliferation and protein synthesis (Shangari and O'Brien, 2004). Other chemicals such as sodium lauryl sulfate, which is used as an anionic detergent, also induced cell death in human lung tissue. It totally disrupts cell membranes and denatures proteins at higher concentrations (Chaturvedi and Kumar, 2011). The least toxic chemical was resorcinol with EC_{25} value of 1895 μg/mL, which is due to its lower reactivity in aqueous solutions (Hahn, 2006). Some chemicals were able to change the pH of the medium. However, they were not neutralized in order to mimic real exposure at workplace. As all case chemicals were incubated with human PCLS under submerged conditions, chemicals with limited water solubility such as trimellitic anhydride were initially dissolved in the solvent DMSO. The solvent was not toxic at the final concentration used, but an influence on cells cannot be excluded.

The multicellular microanatomy of PCLS makes this *ex vivo* model very attractive for a first assessment of acute local respiratory toxicity induced by chemicals. Other published studies investigated cellular responses of single cell lines such as human keratinocyte cells (NCTC 2455) and human promyelocytic cells

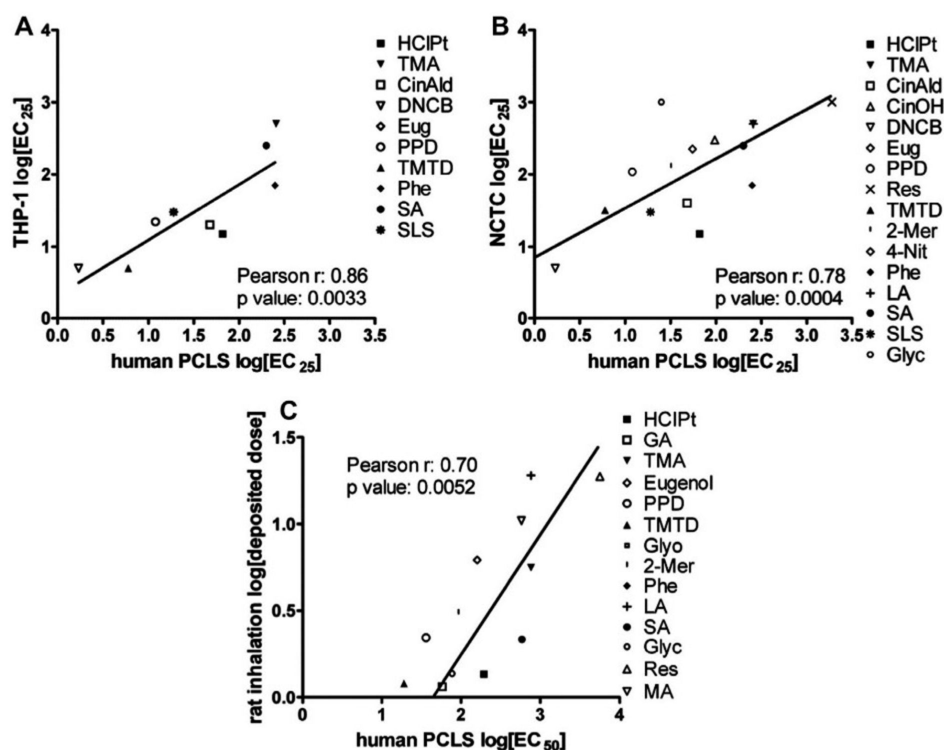

Fig. 2. Correlations were determined between EC_{25} in human PCLS and (A) the human cell line THP-1 for HClPt, TMA, CinAld, DNCB, Eug, PPD, TMTD, Phe, SA, and SLS with Pearson's rank of 0.86, and (B) the human cell line NCTC for HClPt, TMA, CinAld, CinOH, DNCB, Eug, PPD, Res, TMTD, 2-Mer, 4-Nit, Phe, LA, SA, SLS, and Glyc with Pearson's rank of 0.78, using a linear regression analysis model. Data of the human cell lines THP-1 and NCTC were taken from Corsini et al. (2009) and Mitjans et al. (2008), respectively. Correlations were also determined between EC_{50} in human PCLS and (C) *in vivo* data taken from online inhalation studies (ChemIDplus Lite, TOXNET, ECHA, and NIOSH Pocket Guide to Chemical Hazards) with Pearson's rank of 0.70, using a linear regression analysis model.

(THP-1) to some of the chemicals used in the present study (Corsini et al., 2009; Mitjans et al., 2008). Comparison of the *ex vivo* model with *in vitro* models showed that the EC_{25} values in human PCLS correlated better with the EC_{25} values in THP-1 cells than in NCTC cells (Fig. 2), suggesting that cells in human PCLS behave more like monocyte-derived cells regarding their resistance to chemicals than skin-derived epithelial cells. Remarkable is the spreading of the toxicity data, which is less the case for single-cell cultures, where all EC_{25} values are very close together. This wide range distribution can also be observed for *in vivo* toxicity data of several inhalation toxicity studies after exposure to the same chemicals. Here, the most and least toxic chemical were glyoxal and resorcinol, which were also found to be the most and least toxic chemical in human lung sections. The less toxic chemicals after testing in human PCLS were trimellitic anhydride and lactic acid, of which lactic acid was reported to be the least toxic chemical with about 7940 mg/m^3 after 4 h of exposure in rats. Altogether, the toxicity of chemicals in the human *ex vivo* model correlated significantly with LD_{50} values from *in vivo* rat inhalation studies. However, comparison of the toxicological data from animal experiments with *ex vivo* data obtained in human tissue remains critical due to differences in (1) the species used in the models (rodent *versus* human), (2) the type of model, with the *in vivo* models providing organ functionality but no human physiology and the *ex vivo* model providing human physiology but no organ functionality, and (3) the site of action, which can be different from the site of exposure. In particular the site of action *versus* the site of exposure has to be taken into consideration. For some inhalable toxicants, the lungs are the main route of exposure but not the main target and toxicity is observed mainly in other organs such as liver and kidneys. Moreover, for most chemicals toxicity data for humans are only available if published in case reports. Although for some chemicals human toxicity data after acute and chronic exposures of, for example, workers who had contact with glyoxal are available, the actual exposure doses are unknown. Hence, whether the obtained human respiratory toxicity data for the selected chemicals reflects the *in vivo* situation very closely or not remains highly speculative. The utility of human PCLS as a prediction model for cytotoxicity should be part of different *in vitro* or *in vivo* models in order to reflect the various toxicological processes (Sauer et al., 2013).

In the second part of our study, human PCLS were exposed under submerged conditions to chemicals including respiratory sensitizers (TMA, HClPt, GA, and MA), contact sensitizers (DNCB, 2-Bro, CinAld, CinOH, 2-Mer, and 4-Nit), and non-sensitizing irritants (Phe, LA, and SLS) for 24 h. This incubation of human tissue with allergens induced production of a variety of cytokines and chemokines. The early response comprised in particular cytokines such as TNF-α and IL-1α produced by both inflammatory and structural cells. Of course, this induction of cytokines could be due to chemical-induced loss of viability, which can be accompanied by the production of pro-inflammatory cytokines. Within this work, however, special attempts were made to use only subtoxic

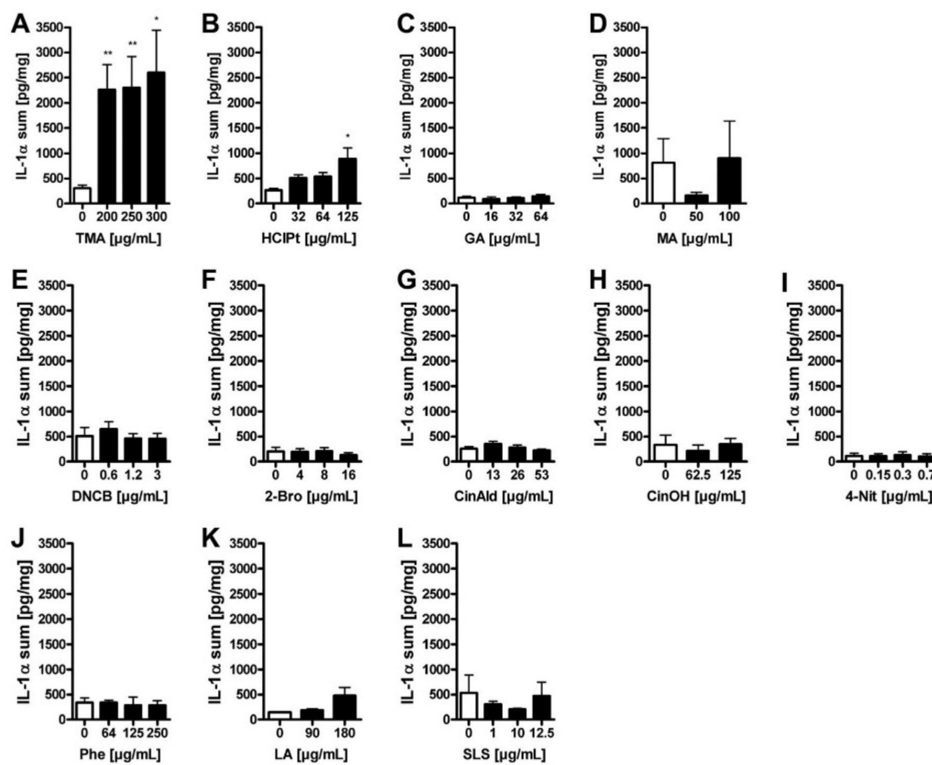

Fig. 3. Total production of IL-1α induced by the respiratory sensitizers (A) TMA, (B) HClPt, (C) GA, and (D) MA, the contact sensitizers (E) DNCB, (F) 2-Bro, (G) CinAld, (H) CinOH, and (I) 4-Nit, and the non-sensitizing irritants (J) Phe, (K) LA and (L) SLS in human PCLS after 24-h submerse exposure to two or three subtoxic concentrations. It shows induction of significant increases by the respiratory sensitizers (A) TMA and (B) HClPt. Cytokine levels in culture supernatants and lysates were determined by ELISA. Data for PCLS are presented as mean ± SEM, $n = 3_{(MA,\ LA,\ 4\text{-}Nit,\ CinOH)}$, $n = 4_{GA}$, $n = 5_{2\text{-}Bro}$, $n = 9_{(TMA,\ SLS,\ Phe,\ DNCB)}$, $n = 14_{HClPt}$, $n = 16_{(CinAld)}$. $^{*}p < 0.05$; $^{**}p < 0.01$ (Mann–Whitney test). Sum: sum of extracellular and intracellular of IL-1α.

concentrations in order to avoid effects mediated by direct toxicity. Interestingly, respiratory sensitizers increased TNF-α or IL-1α, while contact sensitizers and non-sensitizing irritants failed to induce release of these cytokines to the same extent. However, TNF-α and IL-1α cannot be considered as biomarkers for the differentiation between respiratory and contact sensitizers. There are other biomarkers such as IL-8 which were already described in recent publications and were used for identification of allergens (Mitjans et al., 2008). Unfortunately, in human PCLS solvents such as DMSO, which was mostly used to dissolve respiratory sensitizers, also induced high amounts of IL-8. Thus, IL-8 could not be used for the screening of potential chemical allergens in human lung tissue. In this context, we also have to consider that the use of lungs obtained from (i) old donors (60 ± 10 years), and (ii) 80% of whom were smokers are additional factors to affect the tissue reactivity. Efforts have been made to guarantee adequate reactivity of the lung tissue by using e.g. positive controls such as LPS. Here, if the reactivity of the lung tissue was below 150% compared to non-exposed samples the tissue was not accepted for further evaluation. Nevertheless, cells that respond to LPS might be others than cells that respond to allergens. Furthermore, adapted reactions of the immune system against foreign agents show age-related differences in mature and young organisms. Adults and young children are displaying marked differences in the immune system leading to increased susceptibility of the young organism. Using lung tissue of rather elder people might be a further reason for failure.

In conclusion, production of at least one of the pro-inflammatory cytokines TNF-α or IL-1α was elevated significantly after exposure to respiratory sensitizers at subtoxic concentrations in human PCLS. This could influence the local respiratory cytokine milieu, leading for example to acute inflammation, stimulation of phagocytosis in macrophages, maturation of DCs, and migration of cells. As a result, the sensitizers that enter the lungs change not only the functional and phenotypic behavior of immune cells, but may also influence structural cells, leading to diverse effects such as epithelial changes, increased smooth muscle mass, increased number of activated fibroblasts, and vascular changes (Araujo et al., 2008; Holgate, 2008). Both inflammatory and structural cells in the airway tissue play an active role in modulating the immune response and thus in the outcome of the lung immune response by release of multiple cytokines, chemokines, and growth factors (Al-Muhsen et al., 2011).

In case of the onset of an allergic reaction, it has been shown *in vivo* that respiratory sensitizers induce predominantly T_H2-type immune responses with cytokine release of IL-4 and IL-10, whereas contact sensitizers are usually characterized by T_H1-type responses and IFN-γ release (Dearman and Kimber, 1999, 1991; de Jong et al., 2009; Plitnick et al., 2002). In our study, we investigated the release of cytokines/chemokines (IL-10, IL-2, IL-13, IL-5, IFN-γ, and eotaxin-2) in human lung slices after acute exposure to respiratory allergens such as trimellitic anhydride and ammonium hexachloroplatinate in comparison to contact allergens and non-sensitizing

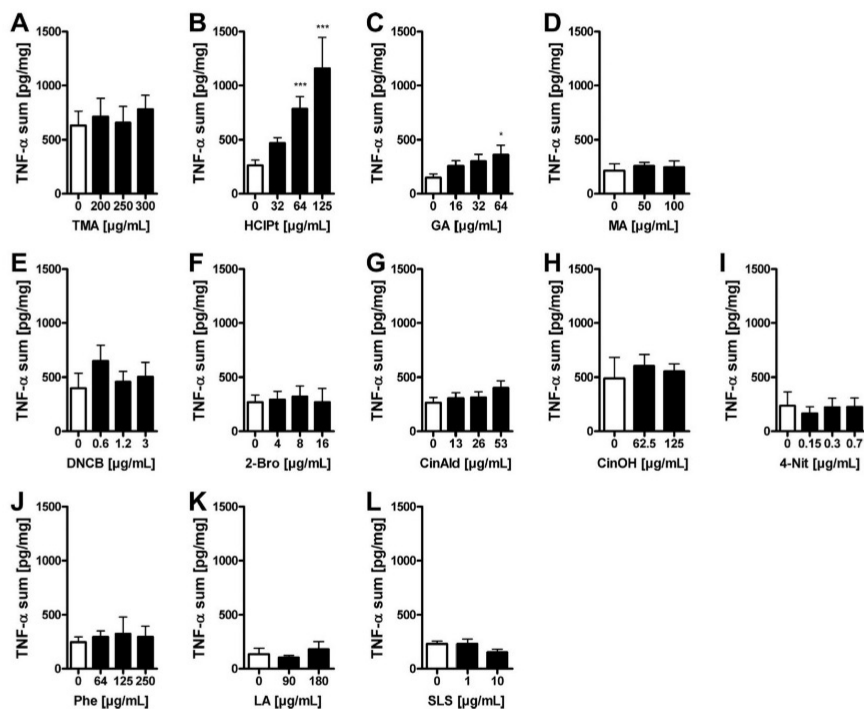

Fig. 4. Total production including extracellular and intracellular TNF-α induced by the respiratory sensitizers (A) TMA, (B) HClPt, (C) GA, and (D) MA, the contact sensitizers (E) DNCB, (F) 2-Bro, (G) CinAld, (H) CinOH, and (I) 4-Nit, the non-sensitizing irritants (J) Phe, (K) LA and (L) SLS in human PCLS after 24-h submerse exposure at two or three subtoxic concentrations. It shows a significant increase induced by the respiratory sensitizers (B) HClPt and (C) GA. Cytokine levels in culture supernatants and lysates were determined by ELISA. Data for PCLS are presented as mean ± SEM, $n = 3_{(MA,\ 4\text{-Nit},\ LA)}$, $n = 4_{(GA,\ CinOH)}$, $n = 6_{2\text{-Bro}}$, $n = 8_{DNCB}$, $n = 9_{(TMA,\ Phe,\ SLS)}$, $n = 14_{HClPt}$, $n = 16_{CinAld}$, $^{*}p < 0.05$; $^{**}p < 0.01$ (Mann–Whitney test). Sum: total production of TNF-α.

irritants due to the possible early immune responses. Our results could not show a T_H2-specific response after exposure to allergens such as trimellitic anhydride. One explanation is that trimellitic anhydride could lose allergenic potential if it is converted to trimellitic acid in aqueous environments (OECD SIDS, 2002). Interestingly, we found that ammonium hexachloroplatinate was able to induce the T_H2-type cytokines IL-5, IL-13, and IL-10 as well as the T_H1-type cytokine IFN-γ. It was suspected that the human lung tissue used had been pre-sensitized by platinum, a well-known allergen. Platinum group elements are emitted from automobile exhaust catalysts in the environment and have caused concern for their environmental and biological accumulation (Ek et al., 2004; Rauch et al., 2005a,b). To prove our suspicion, extracellular and intracellular production of IFN-γ, IL-10, IL-8, TNF-α, RANTES, eotaxin-2, and IL-1α were measured in both mouse and human PCLS (Supplement 3). Again, cytokine release was measured after exposure to subtoxic concentrations to avoid unspecific initial increase and later decrease due to loss of cell viability. No increase in cytokines could be detected in mouse PCLS after 24-h exposure to ammonium hexachloroplatinate. Since the donors of human lung material in the present study might already have been exposed to some of the test substances or components before, as outlined above for platinum compounds, activation of haptenated peptide-specific memory T cells was followed by an increase in T_H1/T_H2 cytokines in lung tissue (Schuppe et al., 1998). Production of pro-inflammatory cytokines could be directly induced by receptor-mediated endocytosis of dendritic antigen-presenting cells (Schuppe et al., 1998). It has also been reported recently that there are numerous resident memory T cells in human lung tissues which could respond to re-call antigens (Purwar et al., 2011). This re-call effect was inducible due to the manifestation of the sensitization process before. Thus, the sensitization risk from automobile catalysts in the environment could be regarded as a potential health concern due to allergenic reactions in susceptible individuals.

In summary, the underlying mechanisms of chemical-induced irritation and inflammation are diverse and complex and only partly understood. According to our current understanding, persistent irritation and inflammation are intertwined local processes having a crucial impact on sensitization. In this context, it becomes obvious that the development of a risk assessment model for chemical-induced sensitization should be based on the monitoring of irritation and inflammation. Therefore, a tissue culture model with physiologically highly relevant cell–cell communication between different cell types for the assessment of irritation and inflammation is needed. For all approaches mentioned above, PCLS offer a novel and unique way for studying the response of human lung tissue at the level of protein production, enzyme activities, and mediator release without animal testing. Furthermore, extrapolation of this information to the human *in vivo* situation is possible. The high degree of complexity provides a physiologically more relevant model and circumvents drawbacks of simple single-cell cultures (Roggen et al., 2006). Within our work, we have shown that different and intertwined mechanisms of immunotoxicity take place: (1) a direct toxic effect and (2) an immune-mediated mechanism. Nevertheless, as with any other assay for chemical-induced

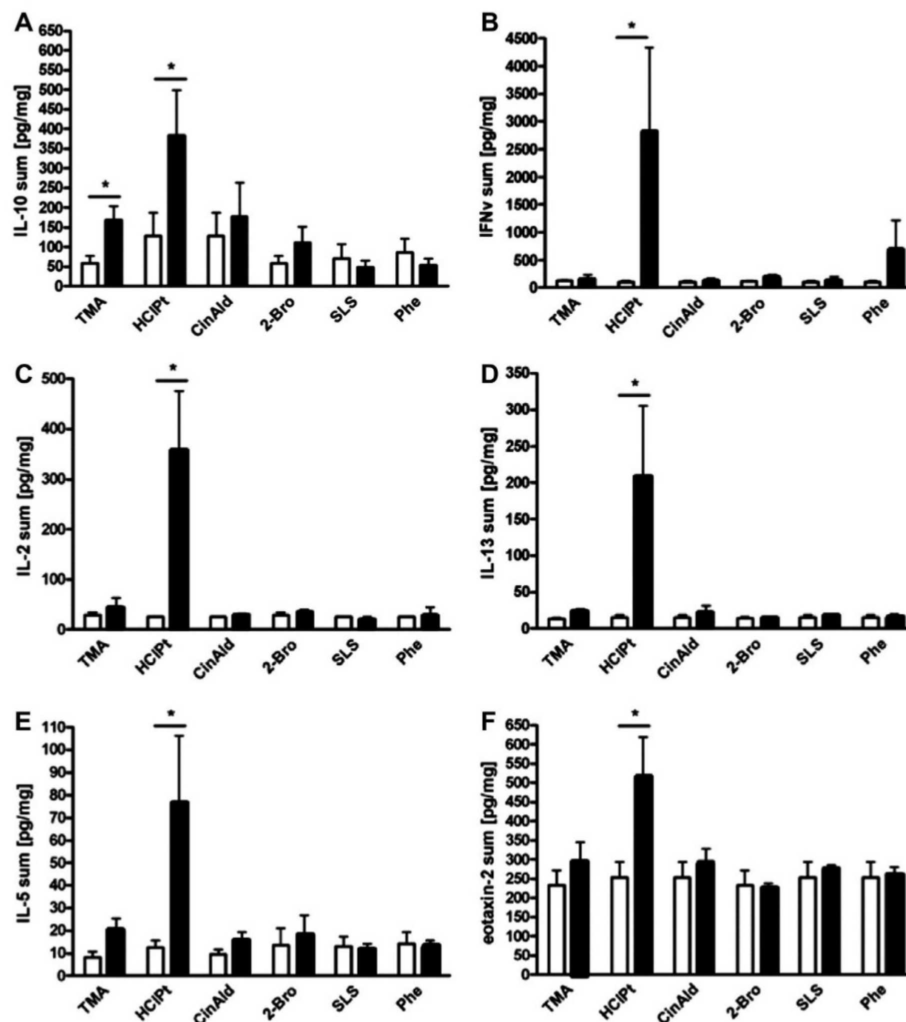

Fig. 5. Total production of (A) IL-10, (B) IFN-γ, (C) IL-2, (D) IL-13, (E) IL-5, and (F) eotaxin-2 induced by the respiratory sensitizers TMA and HClPt, the contact sensitizers CinAld and 2-Bro, and the non-sensitizing irritants SLS and Phe in human PCLS after 24-h submerse exposure at one subtoxic concentration (TMA 200 μg/mL, HClPt 64 μg/mL, CinAld 53 μg/mL, 2-Bro 8 μg/mL, SLS 12.5 μg/mL, and Phe 250 μg/mL) and without chemicals (controls with the same dilutions). Cytokine levels in culture supernatants and lysates were determined by MSD technology. Data for PCLS are presented as mean ± SEM, $n = 3$, $^{*}p < 0.05$; $^{**}p < 0.01$ (Mann–Whitney test). Sum: sum of extracellular and intracellular cytokine amounts.

sensitization, the limitations of the test system have to be considered.

Limitations of the PCLS model are the following: (i) Trafficking of cells from blood into the lungs cannot be assessed. Similarly, migration of cells from the lung into the blood or lymph nodes cannot be evaluated. Both limit the use of *ex vivo* lung tissue for the assessment of adaptive immune responses. (ii) Although PCLS show good reproducibility of findings in one assay, variations within human donors are high. These results were to be expected and reflect the genetic diversity of individuals. (iii) Although the technique is not more cost intensive than other *in vitro* models it is clearly limited by availability of human donors. (iv) There are several publications reporting the metabolic activation of compounds (Lake et al., 2003; Pushparajah et al., 2007). Nevertheless, metabolic activation of substances might also be limited. (v) In the project described here we used a distinctive set of chemicals. The exposure was done submersely. Whether or not it can be used for testing of wide range of chemical classes e.g. nanomaterials, pesticides, metal compounds and other industrial substances remains open. In particular the use for the testing of nanomaterials might be limited. Since 2009 it has been studied whether PCLS can be used for the prediction of chemical-induced toxicity and thereof applied for replacement of dose range finding experiments before *in vivo* acute inhalation toxicity studies are performed (project funded by the German Ministry for Education and Research, project number 0315720 A-C).

In conclusion, allergen-induced cytokines were observed but not considered as biomarkers for the differentiation between respi-

ratory and contact sensitizers. Our preliminary results show an *ex vivo* model which might be used for prediction of chemical-induced toxicity, but is due to its complex three-dimensional structure not applicable for a simple screening of functional and behavior changes of cell populations such as dendritic cells and T-cells although these cells are present in lung section.

5. Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tiv.2013.12.016.

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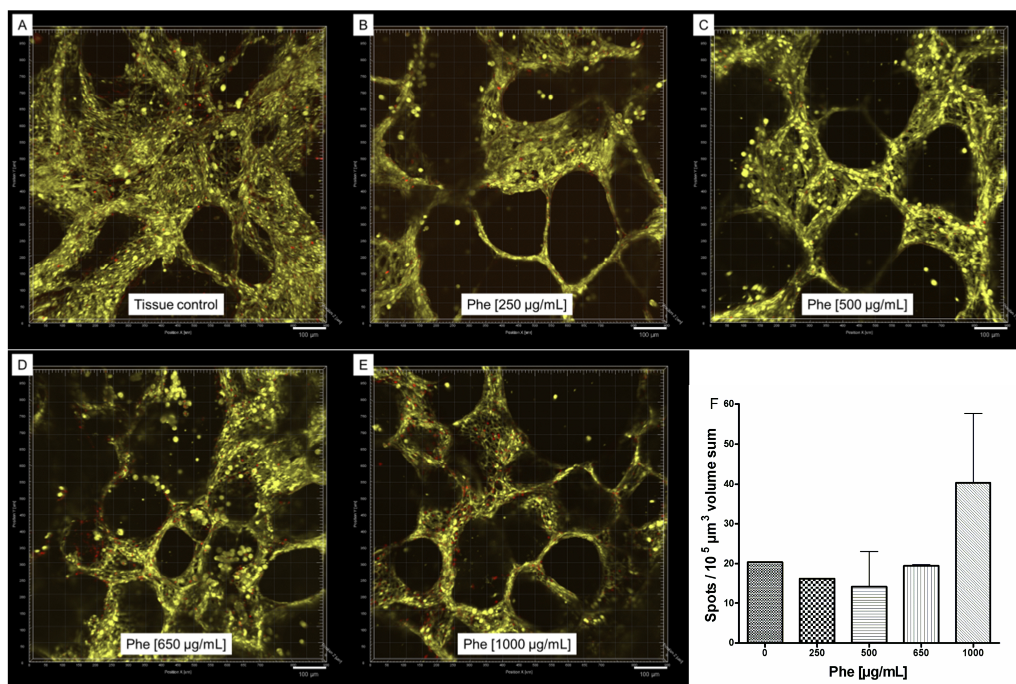
Legends for the Online Supplement

Supplement 1: Image analysis of concentration-dependent cell death induced by Phe in human PCLS. Tissue slices were stained with 4 µM calcein AM and 4 µM EthD-1 after 24-h incubation **A)** without Phe or with **B)** 250 µg/mL Phe, **C)** 500 µg/mL Phe, **D)** 650 µg/mL Phe, and **E)** 1000 µg/mL Phe. Images were examined by confocal laser scanning microscopy (20× objective, excitation wavelengths 488 nm and 543 nm, emission filters BP 505–550 nm and LP 560 nm, thickness circa 30 µm, grid spacing = 50 µm) and **F)** analyzed with IMARIS 7.4.0. Red color shows cell nuclei (Ø 5 µm) of dead cells and yellow color the cytoplasm of viable cells. Concentration-dependent changes in human PCLS viability after cultivation with Phe or following cell lysis with Triton X-100. Results are given as numbers of 5 µm Ø spots (nuclei of dead cells) in 10^5 μm^3 total tissue volume (cytoplasm of living cells).

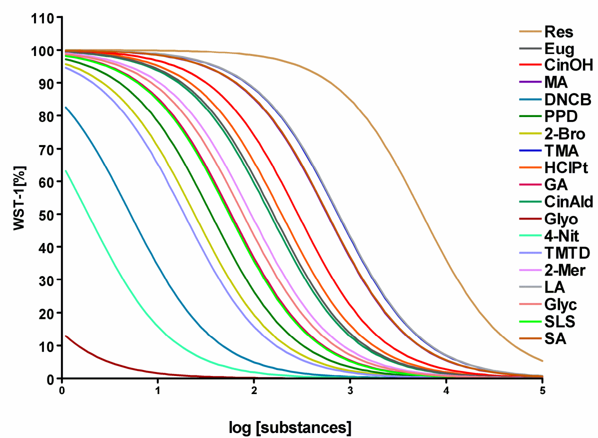
Supplement 2: Dose-response curves of the respiratory allergens TMA, HClPt, MA, and GA, the contact allergens 2-Bro, CinAld, CinOH, DNCB, Eug, PPD, Res, TMTD, Glyo, 2-Mer, and 4-Nit, and the non-sensitizing irritants Phe, LA, SA, SLS, and Glyc were generated for metabolic reduction of WST-1 to soluble formazan measured at 420-480 nm. Data were normalized and the EC_{50} values were determined by nonlinear regression using sigmoidal dose-response.

Supplement 3: Cytokine and chemokine production of IFN-γ, IL-10, TNF-α, RANTES, eotaxin-2, and IL-1α in **A)** human PCLS and **B)** mouse PCLS after 24-h submerse exposure to 64 µg/mL and 32 µg/mL HClPt, respectively. **C)** Cytokine and chemokine levels were represented percentage-wise to compare expression of cytokines and chemokines in different species (human and mouse). Cytokine and chemokine levels in culture supernatants and lysates were determined by MSD technology and ELISA. Data are shown as sum of extracellular and intracellular cytokine and chemokine production. Data for PCLS are presented as mean ± SEM, n=3, $^*p < 0.05$; $^{**}p < 0.01$ (Mann-Whitney test).

Supplement 1



Supplement 2



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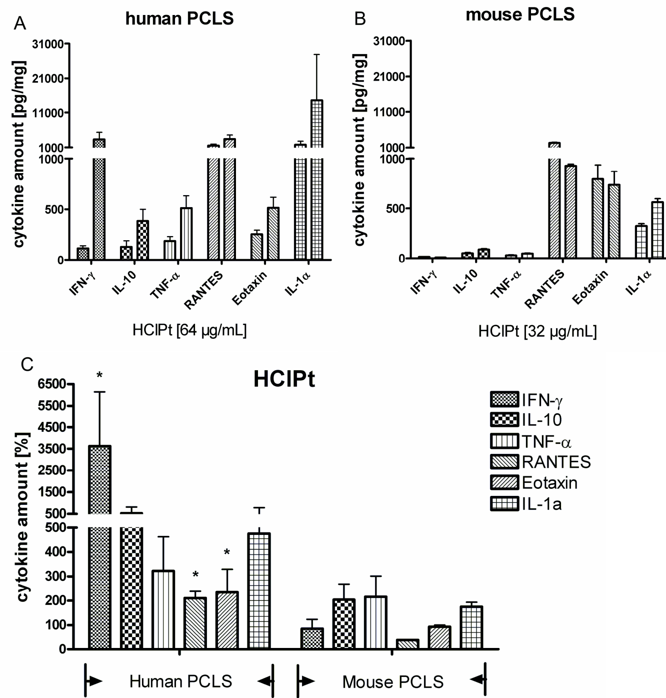

4 Prevalidation of the ex-vivo model PCLS for prediction of respiratory toxicity

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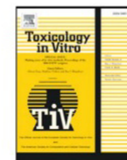

Prevalidation of the ex-vivo model PCLS for prediction of respiratory toxicity

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ABSTRACT

In acute inhalation toxicity studies, animals inhale substances at given concentrations. Without additional information, however, appropriate starting concentrations for in-vivo inhalation studies are difficult to estimate. The goal of this project was the prevalidation of precision-cut lung slices (PCLS) as an ex-vivo alternative to reduce the number of animals used in inhalation toxicity studies. According to internationally agreed principles for Prevalidation Studies, the project was conducted in three independent laboratories. The German BfR provided consultancy in validation principles and independent support with biostatistics.

In all laboratories, rat PCLS were prepared and exposed to 5 concentrations of 20 industrial chemicals under submerged culture conditions for 1 h. After 23 h post-incubation, toxicity was assessed by measurement of released lactate dehydrogenase and mitochondrial activity. In addition, protein content and pro-inflammatory cytokine IL-1α were measured. For all endpoints IC_{50} values were calculated if feasible. For each endpoint test acceptance criteria were established.

This report provides the final results for all 20 chemicals. More than 900 concentration–response curves were analyzed. $Log_{10}[IC_{50}$ (μM)], obtained for all assay endpoints, showed best intra- and inter-laboratory consistency for the data obtained by WST-1 and BCA assays. While WST-1 and LDH indicated toxic effects for the majority of substances, only some of the substances induced an increase in extracellular IL-1α. Two prediction models (two-group classification model, prediction of LC_{50} by IC_{50}) were developed and showed promising results.

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1. Introduction

For many substances, inhalation is the most relevant route of occupational exposure. Regulatory application of alternative methods to animal testing, for both acute and repeated-dose inhalation toxicity studies, however, has lagged behind compared to other routes of administration, due to the complexity of the respiratory system and the diversity of local and systemic responses (Sullivan et al., 2014). It is further complicated by the fact that for some substances, the lungs are the main route of exposure but not the main target. Toxicity is then observed in other organs such as the liver, spleen, and kidney (Hope and Hope, 2012; Kennedy, 2012; Vandebriel and De Jong, 2012). Nevertheless, the respiratory tract is frequently the most sensitive and thus most important target in inhalation studies (Escher et al., 2010).

Alternative test methods accepted by the regulatory authorities have been published for in-vivo studies with topical and oral administration of substances (BeruBe et al., 2009). For acute oral toxicity, for example, several validated in-vivo alternatives are internationally accepted (e.g. acute toxic class method, fixed-dose procedure, up-and-down procedure), optionally in combination with in-vitro testing of starting concentrations (OECD TG423, 2001; OECD TG425, 2002; Guidance Document, 2001; OECD, 2010). Similar approaches – merely based on refined and reduced animal testing – have been developed for acute inhalation toxicity testing (acute toxic class method, TG436) (OECD TG433, 2004; OECD TG436, 2009). There is, however, no validated in-vitro alternative currently available for the respiratory system that is accepted by the regulatory authorities.

Inhalation of substances can induce various local and systemic effects such as respiratory irritation, acute and chronic inflammation,

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and sensitization (Calzetta et al., 2011; Wanner et al., 2010). Possible health outcomes include impaired respiratory function, severe organ injury, hyperplasia, fibrosis, and respiratory allergy (Hayes and Bakand, 2010). In view of this large diversity of effects, a single (non-animal testing) alternative predicting the entire diversity of biological responses of the respiratory tract is very unlikely to be found. On the other hand, many in-vitro models resembling different parts of the respiratory tract have been reported for scientific and industrial purposes (Hansen et al., 2005; Hulette et al., 2002; Lalko et al., 2012; Larsson et al., 2009; Megherbi et al., 2012; Mitjans et al., 2008; Nelissen et al., 2009; Patlewicz et al., 2007; Python et al., 2009). Among them are single cell lines forming monolayers, such as A549, BEAS-2B, and Calu-3, as well as three-dimensional (3D) models of human-derived epithelium, such as EpiAirway™ (MatTek Corporation, Ashland, MA, USA) and MucilAir™ (Epithelix Sarl, Geneva, Switzerland) (Hirakata et al., 2010; Huang et al., 2013; Ren and Daines, 2011; Reus et al., 2013). Moreover, research has remarkably changed the general perception of organotypic tissue models such as precision-cut lung slices (PCLS), parenchymal strips, and isolated vessels and bronchi (Kroigaard et al., 2012; Trifilieff et al., 2009). These models are considered to be of great importance, since the microanatomy of the respiratory tract comprises widely varying cell types that may respond differently to the same substance (Guilliams et al., 2013). Some in-vitro and ex-vivo models have been established for prediction of organ injury, respiratory sensitization and inflammation (Huang et al., 2009; Huang et al., 2011).

The goal of this BMBF-funded project was the scientific prevalidation of rat PCLS (rPCLS) as an alternative test method for in-vivo dose range finding experiments in acute inhalation toxicity studies. The project was conducted in three independent laboratories (Fraunhofer ITEM, BASF SE, and RWTH Aachen) according to internationally agreed principles for Prevalidation Studies (Curren et al., 2006; OECD GD34, 2005) in consecutive phases over three years, aiming at a first assessment whether the rPCLS method is reliable and relevant for the intended purpose. To achieve this, the study phases comprised (i) successful transfer of methods from experienced to naïve laboratories, (ii) refinement of methods, (iii) development of robust test acceptance criteria, (iv) assessment of reproducibility within and between laboratories, and (v) proposal of a preliminary prediction model (PM) to allow prediction of expected in-vivo toxicities from the in-vitro data (Archer et al., 1997; Worth and Balls, 2001). To develop the PM, a training set of twenty chemicals was used representing different chemistry and different modes of toxicological action. The German Federal Institute for Risk Assessment (BfR) provided advice in conformity with agreed validation principles and performed independent biostatistical analyses.

2. Materials and methods

2.1. Participants and organization

The three participating laboratories of the project were the Fraunhofer Institute for Toxicology and Experimental Medicine ITEM (Germany, coordinating laboratory), BASF SE (Germany), and RWTH Aachen (Germany). Coordinator of the project was A. Braun (Fraunhofer ITEM, Germany). The German Federal Institute for Risk Assessment (BfR) provided support with biostatistics (Fig. 1). Team meetings were organized twice per year during the term of the project (2010–2013) in order to share relevant information, develop details of the protocols, discuss results, and decide on the next steps. Practical training meetings for the laboratory staff were performed to ensure correct use of the protocols. Laboratory names were anonymized for this paper.

2.2. Media, reagents and chemicals

Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 Ham (DMEM) with L-glutamine, 15 mM HEPES without phenol red, pH 7.2–7.4, penicillin/streptomycin (P/S), low melting agarose, Earle's Balanced Salt Solution (EBSS), triton X-100 (TX-100), ammonium hexachloroplatinate (AHCP), lipopolysaccharide (LPS, from *Escherichia coli* 0111:B4) and protease inhibitor cocktail were supplied by Sigma Aldrich (Munich, Germany). Phosphate buffered saline (PBS, without Ca^{2+} and Mg^{2+}) was obtained from Lonza (Wuppertal, Germany). Ammonium hexachloroplatinate, aniline, ethanol, formaldehyde, n-hexyl chloroformate, methyl methacrylate, paracetamol, paraquat, sodium dodecylsulfate, trimellitic anhydride, triton-X 100, toluene diisocyanate, zinc oxide were purchased from Sigma Aldrich (Munich, Germany). Isophorone diisocyanate and octanoyl chloride were obtained from ABCR GmbH (Karlsruhe, Germany). Acetic anhydride was supplied by Bernd Kraft GmbH (Duisburg, Germany). Glutaraldehyde and lactose were purchased from Fisher Scientific GmbH (Schwerte, Germany). Acetone and N,N-dimethylformamide were obtained from Honeywell Riedel de Haën (Seelze, Germany). Enzyme-linked immunosorbent assay (ELISA) kits were supplied by R&D Systems (DuoSets, Wiesbaden-Nordenstadt,

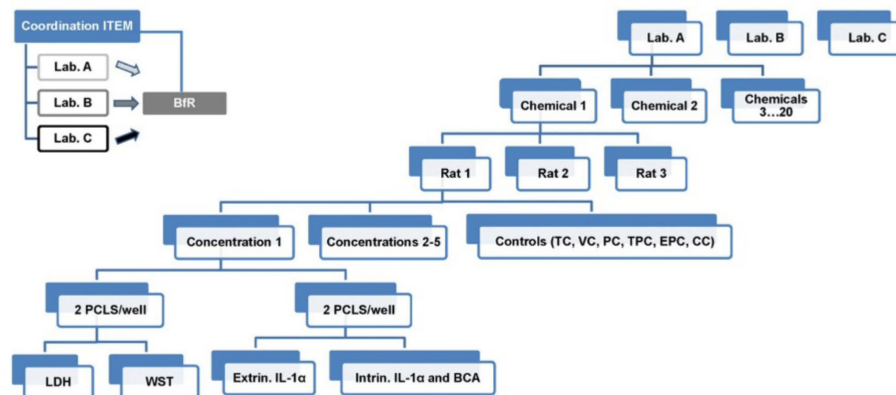

Fig. 1. The figure in the top left corner shows the management of the study. Each laboratory submitted its results to BfR for biostatistics. The study was coordinated by Fraunhofer ITEM. The big figure shows the experimental design of inter-laboratory prevalidation of rat PCLS. Each participating laboratory performed identical experiments. Each substance was tested in three independent biological runs. Twenty substances were tested. Each substance was applied at five concentrations. Experiments included controls for every endpoint method. Each sample was measured in duplicate (technical replicates). Cytotoxicity was measured by LDH and WST-1 assay. Extrinsic and intrinsic IL-1α and protein content were determined by ELISA and BCA assays. TC: tissue control with culture medium only; VC: vehicle control, if necessary; CC: chemical control of the highest tested concentration without PCLS for chemical interference test; PC: positive control for WST-1 assay with AHCP; TPC: technical positive control for LDH assay with TX-100; EPC: positive control for ELISA with LPS; extr.: extrinsic; intr.: intrinsic.

Germany). Cell proliferation reagent WST-1 and LDH cytotoxicity detection kit were purchased from Roche (Mannheim, Germany). BCA protein assay kit was obtained from Pierce (Rockford, IL, USA).

2.3. Laboratory equipment

Commonly used equipment was different in each laboratory. Major equipment was as follows: laboratory A — Krumdieck tissue slicer (Alabama Research & Development, USA), incubator (HeraCell, Thermo Scientific Heraeus, Germany), ELISA Reader (MRX, Dynatech Laboratories, USA); laboratory B — Krumdieck tissue slicer (Alabama Research and Development, USA), incubator (BDD 6220, Thermo Scientific Heraeus, Germany), Multilabel counter (Wallac 1420, Perkin Elmer, USA), ELISA reader (Sunrise, Tecan, Switzerland); laboratory C — Krumdieck tissue slicer (Alabama Research and Development, USA) or microtome (#06.12.07, Wissenschaftliche Werkstatt, RWTH Aachen, Germany), incubator (Innova CO-170, New Brunswick Scientific Co., Inc., USA), GENios ELISA Reader (Tecan, Switzerland).

2.4. Animals

Female rats [Wistar, Crl:WI (Han) nulliparous and non-pregnant] were housed under conventional and certified laboratory conditions in a regular 12-hour dark/light cycle at ambient temperature of 22 ± 2 °C and a relative air humidity of 55 ± 15%. Diet and drinking water were available ad libitum. Animals were acclimated for at least one week and sacrificed at the age of 8–10 weeks.

2.5. Preparation of rat precision-cut lung slices (rPCLS)

Animals were sacrificed by an i.p. overdose (~100 mg/kg body weight) of pentobarbital-Na and the lung was immediately dissected without damaging the lung tissue. The trachea was cannulated and the lung was filled with 10 mL/200 g body weight 37 °C-warm 1.5% low-melting agarose medium solution, either before or after the lung was separated from the animal. After polymerization of agarose to gel, lung lobes were separated, and tissue cores (8 mm in diameter) were prepared and cut into about 200–300 μm thick slices in 4 °C-cold EBSS using a Krumdieck microtome (Alabama Research and Development, Munford, AL, USA). Subsequently, tissue slices were incubated for 2 h in DMEM, supplemented with 100 units/mL penicillin (P) and 100 μg/mL streptomycin (S) in a petri dish under standard cell culture conditions (37 °C, 5% CO_2). During this incubation period the medium was exchanged every 30 min in order to remove cell debris.

2.6. Test substance selection

In this study, twenty chemicals were chosen for determination of their cytotoxic and pro-inflammatory potential using rPCLS: paraquat, isophorone diisocyanate (IPDI), triton X-100 (TX-100), toluene diisocyanate (TDI), AHCP, glutaraldehyde (GA), sodium dodecyl sulfate (SDS), octanoyl chloride (OC), n-hexyl chloroformate (HCF), formaldehyde (FA), trimellitic anhydride (TMA), acetic anhydride (Ac2O), aniline, zinc oxide (ZnO), N,N-dimethylformamide (DMF), methyl methacrylate (MMA), ethanol, acetone, paracetamol, and lactose. Out of these substances, lactose, which is well tolerated by inhalation and shows no toxic effect in-vivo, and paracetamol, which is pharmacologically active in liver, were proposed as negative controls. TX-100 was chosen as positive control substance. All other substances have known modes of toxicological action via inhalation and can chemically be divided into acrylates, aldehydes, amines, anhydrides, chloroformates, chlorides of organic acids, isocyanates, solvents, and detergents. Two metal compounds were also included. All investigated chemicals including CAS numbers, molecular weights, and tested concentrations are listed in Table 1. All compounds were purchased by one laboratory and distributed to the others. Supplement Table 1 presents – as far as known – inhalation median lethal concentration (LC_{50}) and oral median lethal dose (LD_{50}) values and the corresponding GHS classification for all chemicals. No in-vivo experiment had to be performed for the present study.

In some cases only in-vivo studies with more or less than 4 h inhalation were found. To classify these substances into GHS categories LC_{50} values were converted according to following formulas: LC_{50} value B for A hours was converted into LC_{50} estimate value D for C hours using for Gas/vapor: $D = B\sqrt{A} / \sqrt{C}$; and for Dust/mist: $D = BA / C$. In the case of performing GHS classification, enter 4 (hours) for C (GHS Guidance, 2005).

Regarding acute inhalation toxicity and according to the Globally Harmonized System (GHS), 15% of the substances (3/20) fall into category 1 ($LC_{50} \leq 0.05$ mg/L for aerosols, $LC_{50} \leq 0.5$ mg/L for vapors, and $LC_{50} \leq 100$ ppm for gases), 25% (5/20) into category 2 ($0.05 < LC_{50} \leq 0.5$ mg/L for aerosols, $0.5 < LC_{50} \leq 2.0$ mg/L for vapors, and $100 < LC_{50} \leq 500$ ppm for gases), 15% (3/20) into category 3

Table 1
Twenty test substances were selected for prevalidation of rPCLS. Substances were tested in rPCLS in all three participating laboratories. Table shows for each substance: number according to known order in inhalation toxicity, chemical name, abbreviation, chemical class, CAS-number, molecular weight, used vehicle, tested concentrations, and dilution factor. Abbr.: abbreviation; DMSO: dimethyl sulfoxide; EGDE: ethylene glycol dimethylether.

Substances	Abbr.	Class	CAS #	M [g/mol]	Vehicle	Tested concentrations [mM]	Dilution factor	Purity
Acetic anhydride	Ac2O	Anhydrides	108-24-7	102.1	–	2.3, 5.0, 10.8, 23.3, 50.0	2.15	≥99.5%
Acetone	Acetone	Solvents	67-64-1	58.1	–	458, 732, 1172, 1875, 3000	1.6	≥99.9%
Ammonium hexachloroplatinate	AHCP	Metal compounds	16919-58-7	443.9	–	0.05, 0.1, 0.2, 0.5, 1.0	2.15	≥99.9%
Aniline	Aniline	Amines	62-53-3	93.1	–	4.7, 10.1, 21.6, 46.5, 100.0	2.15	≥99.5%
Ethanol	Ethanol	Solvents	64-17-5	46.1	–	534, 854, 1367, 2188, 3500	1.6	≥99.8%
Formaldehyde	FA	Solvents	50-00-0	30.3	–	0.8, 2.0, 5.0, 12.4, 31.0	2.5	37 wt.%
Glutaraldehyde	GA	Aldehydes	111-30-8	100.1	–	0.2, 0.5, 1.1, 2.3, 5.0	2.15	25 wt.%
Isophorone diisocyanate	IPDI	Isocyanates	4098-71-9	222.2	0.5% DMSO	0.1, 0.3, 0.5, 1.2, 2.5	2.15	≥97.5%
Lactose	Lactose	Carbohydrate	63-42-3	342.3	–	4.7, 10.1, 21.6, 46.5, 100.0	2.15	≥99.0%
Methyl methacrylate	MMA	Acrylates	80-62-6	100.1	–	4.7, 10.1, 21.6, 46.5, 100.0	2.15	≥98.5%
n-Hexyl chloroformate	HCF	Chloroformates	6092-54-2	164.6	0.5% DMSO	4.7, 10.1, 21.6, 46.5, 100.0	2.15	≥96.5%
N,N-dimethylformamide	DMF	Solvents	68-12-2	73.1	–	381, 610, 977, 1563, 2500	1.6	≥99.9%
Octanoyl chloride	OC	Chlorides of organic acids	111-64-8	162.7	1% ethanol	4.6, 7.3, 11.7, 18.8, 30.0	1.6	≥98.5%
Paracetamol	Paracetamol	Analgetics	103-90-2	151.2	–	4.2, 9.1, 19.5, 41.9, 90.0	2.15	
Paraquat	Paraquat	Pesticides	1910-42-5	257.2	–	0.4, 1.3, 4.0, 12.7, 40.0	3.16	≥97.5%
Sodium dodecyl sulfate	SDS	Detergents	151-21-3	288.4	–	0.05, 0.1, 0.2, 0.5, 1.0	2.15	≥99.0%
Trimellitic anhydride	TMA	Anhydrides	552-30-7	192.1	0.5% DMSO	3.1, 4.9, 7.9, 12.5, 20.0	1.6	97%
Triton X-100	TX-100	Detergents	9002-93-1	647.0	–	0.02, 0.05, 0.11, 0.23, 0.50	2.15	≥99.0%
Toluene diisocyanate	TDI	Isocyanates	584-84-9 & 26471-62-5	174.2	1% EGDE	4.7, 10.1, 21.6, 46.5, 100.0	2.15	≥94.5%
Zinc oxide	ZnO	Metal compounds	1314-13-2	81.4	0.1% acetic acid	0.1, 0.3, 0.5, 1.2, 2.5	2.15	≥99.0%

($0.5 < LC_{50} \leq 1.0$ mg/L for aerosols, $2.0 < LC_{50} \leq 10.0$ mg/L for vapors, and $500 < LC_{50} \leq 2500$ ppm for gases), 15% (3/20) into category 4 ($1.0 < LC_{50} \leq 5.0$ mg/L for aerosols, $10.0 < LC_{50} \leq 20.0$ mg/L for vapors, and $2500 < LC_{50} \leq 20{,}000$ ppm for gases), and 30% (6/20) into category 5. The criteria for category 5 are intended to enable identification of substances which are of relatively low acute toxicity hazard, but under certain circumstances may present a danger to vulnerable populations. These substances are anticipated to have an oral or dermal LD_{50} in the range of 2000–5000 mg/kg body weight and equivalent doses for inhalation.

2.7. Exposure of rPCLS to substances

Twenty substances were tested in all participating laboratories, each in three independent experiments minimum (biological replicates, $N \geq 3$). Endpoints were evaluation of membrane integrity via LDH assay, mitochondrial activity via WST-1 assay, protein content via BCA assay, and intrinsic and extrinsic IL-1α content via ELISA.

Substances were treated as "unknown" and tested according to the following procedure: (i) checking of solubility and dilution steps, and if necessary (ii) finding of appropriate solvents for heavily soluble or insoluble chemicals without additional effects of the solvent on tissue. Thus, substances were dissolved in DMEM + P/S, if necessary with an appropriate vehicle, in five different concentrations using dilution factors as given in Table 1. Then 24-well cell culture plates were filled with 500 μL of pre-warmed substance solution per required well. Two visually round and intact rPCLS per well were added. Four replicates were used for each substance concentration: two technical replicates for LDH and WST-1 assay and two technical replicates for measurement of protein and IL-1α content. The experimental design is presented in Fig. 1. After 1 h of exposure under standard cell culture conditions, a three-fold washing step was performed to remove the substances from the tissue (except for the technical positive control, see below), followed by a post-incubation period of 23 h with DMEM + P/S. Different exposure time periods have been compared before start of the study, including pulse exposure vs. permanent exposure. Finally, the combination of 1 h pulse exposure plus 23 post-incubation was selected. For this approach we obtained optimal effects on cytotoxicity using WST-1 and LDH assay without e.g. loss of LDH activity due to interferences with substances. Plates were sealed with an adhesive film to account for volatility of substances for the entire incubation time.

In parallel with the chemically treated tissue samples, a tissue control (TC) cultivated only with culture medium, a chemical control (CC) without rPCLS (substance interference test for WST-1 and LDH assays), a positive control (PC) for WST-1 assay with 200 μM or 500 μM AHCP, a technical positive control (TPC) for LDH assay with 0.1% TX-100, a positive control for ELISA (EPC) with 100 ng/mL LPS and, when necessary, a vehicle control (VC) were prepared. VC tissue slices were treated with the vehicle selected for the test item using the same concentration as in the tissue slices exposed to the test item.

2.8. Cytotoxicity assays

Cytotoxicity of substances was determined using the cell proliferation kit WST-1 and LDH cytotoxicity detection kit. For the WST-1 assay the medium was removed from the rPCLS and 250 μL of the WST-1 working reagent per well were added. After 1 h of incubation under standard cell culture conditions, 100 μL of supernatant were transferred to a 96-well plate in duplicates. Absorbance was measured at 450 nm with a reference wavelength of 600–700 nm.

For the LDH assay 50 μL of tissue culture supernatant were transferred to a 96-well plate in duplicates. Fifty μL of LDH reaction mixture were added and plates were incubated for 20 min at room temperature, protected from light. Absorbance was measured at 490 nm with a reference wavelength of 690 nm.

2.9. Protein determination

The BCA assay is based on the colorimetric detection of a bicinchoninic acid/Cu^{+} complex which is influenced by the presence of protein bonds. It is widely used for the determination of protein concentrations.

At the end of culture period, supernatant was removed and the total protein content of the remaining tissue was subsequently measured using the BCA assay. The tissue was therefore lysed by incubation with 500 μL 1% TX-100 in PBS, pH 7.4 plus 0.2% protease inhibitor cocktail at standard cell culture conditions for 1 h. The extracted total protein of the tissue was measured by using the protocol of the Pierce® BCA protein assay kit. Bovine serum albumin (included in the assay kit) was used for the standard curve.

2.10. ELISA: quantification of IL-1α

Culture supernatant was removed at the end of culture period. The remaining tissue was lysed by incubation with 500 μL 1% TX-100 in PBS, pH 7.4 plus 0.2% protease inhibitor cocktail at standard cell culture conditions for 1 h. IL-1α was measured in supernatants and lysis extracts of rPCLS using commercially available ELISA kits according to the manufacturer's protocol. For the eight-point standard curve recombinant rat IL-1α, included in the kit, was used. The lower and upper limits of quantification were at 16 pg/mL and 1000 pg/mL, respectively.

2.11. Definition of acceptance criteria

At the beginning of the project initial protocols based on existing protocols and experience were used. According to Prevalidation principles, during the pilot phase six substances shown in Table 1 (aniline, GA, lactose, MMA, paracetamol, TX-100) were used to refine the rPCLS protocols and to develop robust test acceptance criteria (ACs) for each endpoint based on data from three different laboratories. Both, the final rPCLS endpoint protocols and the biostatistically developed ACs for each endpoint protocol were then included in final standard operating procedures (SOPs) and later applied to the remaining 14 test chemicals. The next section gives a detailed description of the ACs established for each of the individual endpoints in order to assure reliable and valid assay performance in each laboratory. Only if each acceptance criterion for each individual endpoint was met the data were accepted for further evaluation and curve fitting. An experiment had to be repeated, if one of the endpoint-specific controls did not meet the established acceptance criteria.

Depending on the toxicological endpoint measured three to four different ACs per endpoint assay were defined (Table 2). In general, the ACs for all endpoint assays were established to ensure a minimum technical quality, to define endpoint related values for the negative and positive reference samples and – for the BCA and IL-1α assay – to ensure a minimum quality of the standard curves.

In detail, AC1, controlling the magnitude of technical errors, was generally used for all endpoints (except IL-1α) and was set to ≤0.3. For the cytotoxicity assays ACs were defined so as to assure sufficiently high tissue viability of non-substance-exposed reference samples. For the WST-1 assay this was achieved by defining of a minimum optical density of TC and VC equal to or greater than 0.6 (WST-1 AC2). For the LDH assay AC2 assured cellular tissue integrity under untreated conditions, thus release of LDH in TC and VC had to be ≤30% in comparison with the TPC (LDH AC3). Further ACs (WST-1 AC3; LDH AC2) demonstrated responsiveness of the tissue to an active substance. Therefore, AHCP was used in laboratory-specific concentrations for the WST-1 assay. This PC had to reduce viability to 66% minimum in comparison with the TC. To demonstrate a maximum response of rPCLS in the LDH assay, TX-100 was used as TPC (Table 2). Here, TPC had to have a minimum optical density of 1.0. The standard curve of BCA was evaluated by analysis of eight calibration standards using a three-parameter logistic

Table 2
Endpoint specific acceptance criteria. AC: acceptance criteria; OD: optical density; TC: tissue control only with culture medium; VC: vehicle control; PC: positive control for WST-1 assay with AHCP; TPC: technical positive control for LDH assay with TX-100; EPC: positive control for ELISA with LPS; BSA: bovine serum albumin; $S_{y,x}$: residual standard error; CV_{adj}: offset adjusted coefficient of variation.

Assay	Acceptance criteria	Definition
WST-1	AC1	Difference between duplicate OD measurements has to be ≤0.3
	AC2	Minimum OD of the TC and VC has to be ≥0.6
	AC3	PC has to be ≤66% of the TC and VC
LDH	AC1	Difference between duplicate OD measurements has to be ≤0.3
	AC2	Minimum OD of the TPC has to be 1.0
	AC3	TC and VC have to be ≤30% of the TPC
BCA	AC1	Difference between duplicate OD measurements has to be ≤0.3
	AC2	Mean OD of the maximum BSA concentration of standard curve has to be ≥0.8
	AC3	$S_{y,x}$ of the fit of the standard curve has to be ≤0.1
	AC4	Mean OD of the TC and VC has to be ≥0.3
IL-1α	AC1	Restricting CV_{adj} has to be ≤20%
	AC2	Mean OD of the maximum concentration of standard curve has to be ≥1.0
	AC3	$S_{y,x}$ of the fit of the standard curve has to be ≤0.1
	AC4	EPC has to be ≥140% of the TC and VC for intrin. IL-1α

regression algorithm. The upper limit of detection was defined to be higher than 0.8, which provides a standard curve covering a sufficiently high dynamic range (BCA AC2). The quality of standard curve fit was controlled by the residual standard error ($S_{y,x}$), which had to be equal to or lower than 0.1 (BCA AC3). The total protein content of two untreated tissue sections (TC and VC) was defined at a minimum optical density of 0.3 (BCA AC4). Similar quality control checks were used for IL-1α (Table 2). AC4 here was used to assure a sufficiently high anti-inflammatory cytokine response of tissue exposed to LPS. It had to result in 140% or more intrinsic IL-1α for the TC or VC. All ACs are summarized in Table 2.

2.12. Data management

All laboratories used the same EXCEL spreadsheet template, developed by BfR and Fraunhofer ITEM, to capture all test results. The structure and functions of this template were write-protected. To allow *ad hoc* decisions about the test validity at the laboratory level, the results for all acceptance criteria were calculated by the implemented functions. All data entered were submitted to the project partner BfR responsible for further analysis across laboratories. Curve fitting and statistical analyses were performed as described below.

2.13. Curve fitting

For all endpoints a sigmoid concentration–response model was fitted to the data and IC_{50} values were calculated. Curve fitting and analysis were performed automatically using the statistical computing environment R (R Development Core Team, 2010). The functions “loess” for non-parametric fitting, “drm” (Ritz, 2005) for parametric fitting, and “mrdrm” for a combination of the two were employed. For curve fitting a model-robust approach was used (Nottingham and Birch, 2000). The response (y) is predicted by a weighted sum of a parametric fitting ($\hat{y}_p$) and a non-parametric fitting ($\hat{y}_{np}$):

$$\hat{y} = (1-\lambda)\hat{y}_p + \lambda\hat{y}_{np}.$$

The non-parametric fitting applied was a local linear regression procedure. The mixing parameter λ is determined in the generalized cross-validation procedure PRESS (Nottingham and Birch, 2000). For the endpoints WST-1, BCA, and intrinsic IL-1α a parametric model was used, either a three-parametric log-logistic model or the Brain–Cousens model (Ritz, 2005). The Brain–Cousens model is given by the following equation:

$$y(x) = \frac{d-fx}{1+\exp(b(\log(x)-\log(e)))},$$

where x denotes the dose and y(x) the response, the shape of which is determined by the parameters *b*, *d*, *f* and *e*.

Akaike's information criterion (AIC) (Akaike, 1998) was calculated for each fitting of a data set according to both models. This approach adds to the logarithm of the residual sum of squares the number of parameters used, thus penalizing smaller residual sum of squares with the number of parameters. The model yielding the smaller AIC was chosen as parametric part of the approach. For the endpoints LDH and extrinsic IL-1α a four-parametric log-logistic model was chosen (Ritz, 2005). The non-parametric fitting was performed by local linear regression (Nottingham and Birch, 2000).

2.14. Calculation of laboratory-specific and overall mean and median IC_{50} and log IC_{50}

For WST-1, BCA, and IL-1α assay the IC_{50} value refers to the concentration, at which curve fitting as described above predicts half the value of the corresponding non-exposed control. For LDH assay the IC_{50} was defined as concentration, which predicts the average of the corresponding non-exposed control and the mean value of the TPC. To determine this point a bisection procedure was applied using the R function *bisect* from the package pracma, which finds roots of univariate functions in bounded intervals (Borchers, 2015). The search for IC_{50} was performed in an interval from 0 to 5 times the maximum concentration applied. IC_{50} values were first calculated endpoint-specifically for each substance and laboratory. Majority rules were applied in this process for the three independent runs of a single laboratory. Laboratory-independent overall IC_{50} values were calculated as mean or median of the individual laboratory-specific IC_{50} values.

Median log IC_{50} values of each substance for each endpoint and laboratory were used for regression and classification of the substances. The median was employed, because it is a robust estimate of the central tendency, which minimizes potential biases originating from outliers. Additionally, in contrast to the mean the median can be calculated, even if for some curves only minimal (>) IC_{50} values have been estimated. In case only minimal IC_{50} values could be estimated for the majority of replicates, i.e. the only information obtained was that the IC_{50} was greater than a certain value, this value was used for further analysis.

2.15. Analysis of intra- and inter-laboratory variances

The standard deviation (Stdev) and coefficient of variation (Cv = Stdev / mean ∗ 100%) of the logarithmically (decimal) transformed IC_{50} values, denoted as log-transformed (log IC_{50}), were used as measures of dispersion to describe variability of IC_{50}. This provides a first estimation of the variance per endpoint and substance within (intra-laboratory variance) and across (inter-laboratory variance) laboratories.

2.16. Two-group classification model

In the two-group classification model, substances were divided into two classes based on their in-vivo acute inhalation toxicity (toxic and non-toxic). Substances with GHS categories 1, 2, and 3 were assigned to the toxic class and substances with GHS categories 4 and 5 to the non-toxic class. To classify the results of the present study the median of log IC_{50} values from the WST-1 assay was calculated for each laboratory and each substance. The in-vitro class center of the toxic class was then calculated as of the median for all substances that had been assigned to this class in-vivo. The same was done for the non-toxic class. Finally, the mean of these two medians was defined as the

threshold for binary classification. Each substance whose median was below this threshold was classified as "toxic", whereas substances with median values above the threshold were classified as "non-toxic".

2.17. Prediction of the LC_{50} by IC_{50}

The in-vivo lethality values of the substances used in this project were used to develop a prediction model (see also Supplement Table 1). For some chemicals only inhalation LC_{50} values from studies with exposure durations different from 4 h were found. In these cases, the LC_{50} values were converted to 4-h LC_{50} values.

In order to develop a prediction model, which allows for estimation of LC_{50} by IC_{50} a linear regression analysis was performed with values from laboratory C. The log IC_{50} values of WST-1 assay were regarded as predictors and the log-transformed LC_{50} values were regarded as response:

$$\log_{10}(LC_{50}) = \beta_0 + \beta_1 \log_{10}(IC_{50}).$$

2.18. Sensitivity and specificity

Sensitivity and specificity provide measures for the ability of an assay to correctly identify positive and negative results (in the present study to correctly classify substances as toxic or non-toxic). The sensitivity was calculated as the number of correct positives (correctly identified as toxic) / (number of correct positives + number of false negatives (incorrectly identified as non-toxic)). Specificity was calculated as the number of correct negatives (correctly identified as non-toxic) / (number of correct negatives + number of false positives (incorrectly identified as toxic)).

2.19. Statistics

Statistical analysis was performed by using the one-way ANOVA (software: GraphPad Prism 4, version 4.03). The outcomes of the post-hoc treatment to control comparisons with Bonferroni adjustment of p-values to account for multiple testing are reported. Differences between treated samples and controls were considered statistically significant at the level of $p < 0.05$. Comparison of data was analyzed as indicated in the appropriate section using either parametric correlation (Pearson's product moment correlation and two-tailed P value) or non-parametric correlation (Spearman's rank correlation r).

3. Results

3.1. Acceptance criteria

Absorbance values represented the raw data in the different endpoint assays. Consequently, most of AC referred to the optical density of the samples (Table 2). Deviations from any of the acceptance criteria indicated a problem likely related to assay quality or experimental conduct. In the cytotoxicity assays, quality controls indicated when viability of the tissue was insufficiently low. Responsiveness of the tissue to an effective toxic substance was included as positive control. Based on concentration–response curves, minimum and maximum values of absorption were defined and had to be met for subsequent experiments. Further ACs were used to control the quality of standard curves as generated in the BCA and ELISA assays. These ACs (AC2 and AC3) guaranteed sufficient spreading of absorption values, providing a high dynamic range as well as a close fitting between predicted and observed responses of standard curves. An important issue was the definition of a minimum absorption value for non-treated tissue sections (tissue and vehicle controls). Instead of measuring and standardizing the thickness of slices, measurement of total protein content was used to indirectly monitor thickness of lung slices.

To ensure that the requirements (Table 2) were fulfilled, all samples were entered for evaluation, automatically checked and accepted for further analysis only if the defined criteria were met. Supplement Table 2 gives the number of samples entered for evaluation and the proportion [%] of finally accepted cases for the lastly tested substances (DMF, ethanol, acetone, formaldehyde). AC1, which represents the magnitude of technical errors such as pipetting errors for every assay, was met in ≥95% of all experiments. In the WST-1 and LDH assays AC2 and AC3 were also met in most cases (≥83%). For BCA assay the quality requirement for the standard curves (AC2 and AC3) was fulfilled in all experiments in all laboratories. The minimum optical density of untreated samples (AC4) of the BCA assay was also reached in all experiments. Fulfillment of AC2 and AC3 as quality criteria for the ELISA standard curves ranged from 92% to 100%. Experiments that did not meet all endpoint-specific AC were not used for calculation of the IC_{50} and correlation with in-vivo. These experiments were repeated and the data used for subsequent determinations of IC_{50} values. In general, fulfillment of test acceptance criteria improved over the entire project time.

3.2. Substance-induced cytotoxicity in rPCLS: log IC_{50} results for all endpoints

In each participating laboratory rPCLS were prepared and exposed to five concentrations of 20 preselected substances (Table 1). This paper reports the final results for all substances. Concentration–response curve data from the various qualified assays were used for non-linear sigmoidal curve fitting using mathematical models. Logarithmically transformed IC_{50} [μM] values (log IC_{50}) were calculated for each substance, laboratory, and endpoint (Supplement Tables 3 to 6). More than 900 curves have been fitted. Data meeting acceptance automatically were used to determine overall mean and median log IC_{50} values for each endpoint and most of the substances (Table 3).

For 18 of 20 substances defined median log IC_{50} values could be calculated for WST-1 assay for all laboratories (Supplement Table 3, Fig. 2A, Table 3). For MMA and lactose, only minimal (>) IC_{50} values were examined except for laboratory C. Median log IC_{50} values constantly covered a wide range of approximately four orders of magnitude from 1.91 (corresponding IC_{50} value: 81 μM) for TX-100 to 6.17 (corresponding IC_{50} value: 1.45 M) for ethanol. The majority of log IC_{50} values calculated by each laboratory was within the measured concentration range, with few exceptions where IC_{50} values were found to be above the maximum applied concentrations (IPDI, TMA, TDI, paracetamol, see Supplement Table 3). In three cases outliers were detected with a Grubbs test (Grubbs, 1950) (laboratory C: SDS, OC, acetone). For the calculation of the median the outliers were not excluded. For a single laboratory a defined log IC_{50} for lactose could only be obtained by extrapolation.

The second cytotoxicity assay used was the LDH assay. It finally yielded a less complete data set than the WST-1 assay. Concentration–response curves provided defined log IC_{50} values for only 11 out of 20 substances in all laboratories (Supplement Table 4, Fig. 2B, Table 3). Of those 11 defined IC_{50} values three could only be determined in one laboratory and two additional values in only two laboratories. Median log IC_{50} values covered approximately four orders of magnitude from 2.46 for SDS (corresponding IC_{50} value: 288 μM) to 6.52 for acetone (corresponding IC_{50} value: 3.3 M). After lactose and MMA exposure no effect was observed on LDH release. The reason why many substances failed to be fitted must be a matter of some considerable concern and needs explanation. Please refer to the discussion chapter.

Total protein content was quantified using BCA assay. Log IC_{50} values could also be calculated for 20 substances (Supplement Table 5, Fig. 2C, Table 3). Median log IC_{50} values ranged from 2.25 for TX-100 (corresponding IC_{50} value: 178 μM) to 6.59 for acetone (corresponding IC_{50} value: 3.9 M) (Table 3). MMA treatment in most cases had no effect on the total protein content of lung slices. IPDI and lactose treatment provided defined values in one laboratory.

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Table 3
Mean and median of endpoint specific log-transformed IC_{50} [log IC_{50} (μM)] for all tested chemicals ± inter-laboratory standard deviation (Stdev) and coefficient of variation (Cv). Log IC_{50} values are given for A) WST-1 assay, B) LDH assay, C) BCA assay, and D) intrinsic IL-1α (intIL-1α) as measured by ELISA. Chemicals are ranked by increasing median log IC_{50} values. The number of each substance refers to known order in inhalation toxicity (see also Table 1). Abbreviations of chemicals are given according to Table 1. ">" values are described in the Materials and methods section.

	Chemical	Median log IC_{50}	Mean log IC_{50}	Inter-lab. Stdev	Inter-lab. Cv
A	WST-1 assay				
3	TX-100	1.91	1.89	0.05	2
7	SDS	2.42	2.39	0.06	2
5	AHCP	2.61	2.60	0.03	1
14	ZnO	2.86	2.99	0.12	4
6	GA	3.14	3.10	0.17	5
2	IPDI	3.16	3.25	0.21	7
1	Paraquat	3.53	3.34	0.15	4
10	FA	3.67	3.70	0.12	3
11	TMA	4.00	4.16	0.43	11
8	OC	4.18	4.19	0.04	1
12	Ac2O	4.19	4.19	0.04	1
9	HCF	4.32	4.23	0.26	6
13	Aniline	4.77	4.61	0.31	6
4	TDI	4.83	4.84	0.27	6
20	Paracetamol	4.94	4.92	0.20	4
16	MMA	>5.70	>5.70	NA	NA
19	Lactose	>5.70	5.56	0.07	1
18	Acetone	6.11	6.16	0.19	3
15	DMF	6.14	6.11	0.11	2
17	Ethanol	6.17	6.18	0.04	1
B	LDH assay				
7	SDS	2.46	2.46	–	–
5	AHCP	2.94	2.90	0.10	3
14	ZnO	2.99	3.09	0.32	11
2	IPDI	3.64	3.71	–	–
6	GA	3.71	3.71	–	–
1	Paraquat	4.24	4.20	0.45	11
10	FA	4.38	4.31	0.04	1
13	Aniline	4.94	5.01	0.06	1
20	Paracetamol	5.15	5.12	0.03	1
15	DMF	6.29	6.29	0.48	8
18	Acetone	6.52	6.52	0.08	1
C	BCA assay				
3	TX-100	2.25	2.24	0.04	2
7	SDS	2.63	2.63	0.08	2
5	AHCP	3.13	3.17	0.09	3
14	ZnO	3.46	3.42	0.33	9
6	GA	3.68	3.76	0.28	8
11	TMA	4.06	4.06	0.54	13
2	IPDI	4.10	2.93	0.68	16
8	OC	4.24	4.26	0.01	0
12	Ac2O	4.29	4.33	0.05	1
10	FA	4.50	4.58	0.19	4
9	HCF	4.64	4.65	0.03	1
1	Paraquat	4.84	4.80	0.31	6
13	Aniline	4.93	4.85	0.10	2
4	TDI	4.98	4.88	0.49	10
20	Paracetamol	5.28	5.25	0.25	5
19	Lactose	5.70	5.59	0.06	1
16	MMA	>5.70	>5.70	0.00	0
15	DMF	6.37	6.35	0.07	1
17	Ethanol	6.43	6.52	0.01	0
18	Acetone	6.59	6.45	0.44	7
D	intIL-1α ELISA				
3	TX-100	2.21	2.17	0.13	6
7	SDS	2.40	2.21	0.57	24
5	AHCP	2.83	2.83	0.23	8
14	ZnO	3.08	3.02	0.09	3
6	GA	3.23	3.21	0.18	6
2	IPDI	4.10	3.40	0.20	6
10	FA	3.59	3.67	0.20	5
11	TMA	4.03	4.00	0.58	14
9	HCF	4.17	4.10	0.44	11
8	OC	4.23	4.21	0.10	2
12	Ac2O	4.29	4.23	0.12	3
1	Paraquat	4.58	4.72	0.24	5

Table 3 (*continued*)

	Chemical	Median log IC_{50}	Mean log IC_{50}	Inter-lab. Stdev	Inter-lab. Cv
D					
13	Aniline	4.75	4.46	0.64	13
4	TDI	4.8	4.49	0.58	12
20	Paracetamol	4.84	4.88	0.29	5
16	MMA	5.42	5.09	0.49	9
19	Lactose	5.70	5.20	0.29	5
15	DMF	6.13	6.14	0.04	1
17	Ethanol	6.27	6.24	0.06	1
18	Acetone	6.30	6.20	0.57	9

3.3. IL-1α as a biomarker of inflammation

IL-1α as a biomarker of inflammation was assessed using ELISA. Intracellular IL-1α was either unchanged or decreased to levels below tissue control (Table 3D). Only paraquat very significantly increased intracellular IL-1α levels of up to 140% vs. controls (laboratory A). As intracellular IL-1α decreased with increasing cytotoxicity, it was used to determine mean and median log IC_{50} values (Supplement Table 6, Fig. 2D, Table 3D). Median values hardly covered 5 orders of magnitude from 2.21 for TX-100 (corresponding IC_{50} value: 162 μM) to 6.30 for acetone (corresponding IC_{50} value: 2.0 M). Extracellular IL-1α was very low in control tissue. Even if tissue was stimulated with LPS, which is an activator of the innate immune system, most of the induced IL-1α could only be detected intracellularly after 24 h. Nevertheless, the results showed extracellular IL-1α to be increased significantly after exposure to Ac2O, AHCP, aniline, ethanol, FA, GA, paracetamol, paraquat, SDS, TMA, and TX-100 in at least one laboratory. No increase or decrease could be observed after exposure with lactose, MMA, HCF, and TDI. Other chemicals induced a non-significant increase of extracellular IL-1α in at least two laboratories (Fig. 3). The increases were observed in the herein reported three independent experiments.

3.4. Intra- and inter-laboratory variability

Experiments were performed under as near identical conditions as possible. Beside all steps that were performed to minimize variability and are already described in the Materials and methods (e.g. material, equipment, SOPs), the staff was intensively trained — on site if necessary. We performed no staff swapping and blind evaluations but all data were submitted to and evaluated by an independent partner.

Intra-laboratory variability of log IC_{50} was evaluated for each of the biological replicates (N = 3) for each substance and laboratory. Results of the three laboratories were used to determine inter-laboratory variances. Mean values, standard deviation (Stdev), and coefficient of variation (CV) of log IC_{50} were calculated for each substance within each laboratory (Supplement Tables 3 to 6) and across the laboratories (Table 3).

Log IC_{50} showed best inter-laboratory consistency and good agreement for both the WST-1 and BCA assay (Table 3, Supplement Tables 3 and 5). For 52 combinations (out of 60 for three laboratories and 20 substances) log IC_{50} values for WST-1 could be estimated numerically, enabling calculation of intra- and inter-laboratory CV. About two thirds of the inter-laboratory CV values were lower than 5%; the maximum value was 16%. For 7 substances an intra-laboratory CV greater than 10% was determined. All inter-laboratory CV values were lower than 10%. For all experiments conducted with lactose and MMA log IC_{50} values were estimated greater than the maximum applied concentration (except one replication of laboratory A). Similar results have been observed for the BCA assay where the intra- and inter-laboratory CV values were only in few cases between 10% and 20%. Thus, these results were (at least) qualitatively reproducible.

Analysis of inter-laboratory correlation of log IC_{50} values reached satisfactory levels of agreement (Spearman $r > 0.9$, Fig. 4). BCA assay

proved to be as robust as WST-1 assay. Consistency between laboratories was also good ($CV < 20\%$) for LDH assay. Intra- and inter-laboratory variability was high for intracellular IL-1α ELISA with intra- and inter-laboratory CV values reaching up to 50% and 25%, respectively. In general, agreement between the readings of laboratory A versus B was higher (Spearman) for all endpoints than between laboratories A versus C and B versus C.

3.5. Correlation of in-vitro (ex-vivo) IC_{50} with in-vivo inhalation LC_{50} and oral LD_{50} values

The substances used in this project were selected to represent different chemical classes and modes of action for which acute inhalation and oral lethality data are available (i.e. Sigma, RTECS, ChemIDplus Lite, TOXNET, ECHA, NIOSH Pocket Guide to Chemical Hazards, and unpublished BASF studies, Supplement Table 1). For some chemicals we found only inhalation LC_{50} values from studies with exposure durations different from 4 h. In these cases, the LC_{50} values were converted to 4-hour LC_{50} values (see also material and method section). The in-vivo data were then converted to molar concentrations, ranging from 0.01 μM for paraquat to 1.2 mM for acetone. Published oral rat LD_{50} values were also converted to molar-based doses which ranged from 222 μmol/kg for paraquat to 153 mmol/kg for ethanol and (Supplement Table 1). Thus, the most toxic substance via inhalation and oral uptake was paraquat. The least toxic substances were acetone for inhalation and ethanol for oral administration.

The results of the WST-1 assay were used for development two prediction models (prediction of LC_{50} by IC_{50} and two-group classification model). The WST-1 assay was selected because (i) it provides toxicologically relevant mechanistic evidence for cell injury, and ii) delivered a more complete data set as the LDH assay. Secondly we wanted to establish a model which has highest prospects for further validation. All endpoints were quite similar (high correlation) and the combination of two assays provided no additional information.

For correlation analysis, IC_{50} values i.e. the medians of the laboratory means obtained by WST-1 assay in rPCLS and/values of acute inhalation (LC_{50}) and oral lethality (LD_{50}) in rats were used for linear regression. No in-vivo inhalation toxicity data were available for paracetamol (inhalation), lactose (inhalation), and HCF (oral) (Supplement Table 1). Medians [μM] of IC_{50} values of rPCLS correlated highly significantly with rat LC_{50} values [μM] from inhalation studies (Pearson correlation coefficient $r = 0.87$, $p < 0.0001$) and with rat LD_{50} values [μmol/kg] from oral studies (Pearson correlation coefficient $r = 0.75$, $p = 0.0002$) (Fig. 5).

3.6. Two-group classification model

In the two-group classification model, substances were divided into two classes (toxic and non-toxic) based on their in-vivo inhalation toxicity. Substances with GHS categories 1, 2, and 3 were assigned to the toxic class. Substances with GHS categories 4 and 5 were classified as "non-toxic". Subsequently, the results of the WST-1 assay were used to determine thresholds. Each substance whose median was below this threshold was classified as "toxic", whereas substances with median values above the threshold were classified as "non-toxic. The goodness of prediction of the two-group classification model was assessed in terms of specific measures of the Cooper statistics, namely: sensitivity and specificity.

Median log IC_{50} values of each substance were used for classification. Since for lactose and MMA no effect could be determined up to a logarithmic dose of 5.7 on a μM scale, the median of log IC_{50} values of these two substances could not be expressed precisely. Therefore, we assumed for lactose and MMA the highest applied concentration as minimal possible IC_{50} value. To test robustness of the model even lower values were assumed for both substances which finally resulted only in small differences in classification. For the two-group classification model, IC_{50} values were transformed to mg/L units, considering the molecular weights of each substance. Thresholds of log IC_{50} for two-group classification were calculated to be 3.83 for laboratory A, 3.88 for laboratory B, and 3.91 for laboratory C. Classification of the substances using the two-group classification model is presented in Table 4 and Fig. 6. Sixteen out of 20 substances were classified correctly in all laboratories, thereof six substances of the non-toxic class and ten of the toxic class. Four substances, namely three non-toxic and one toxic

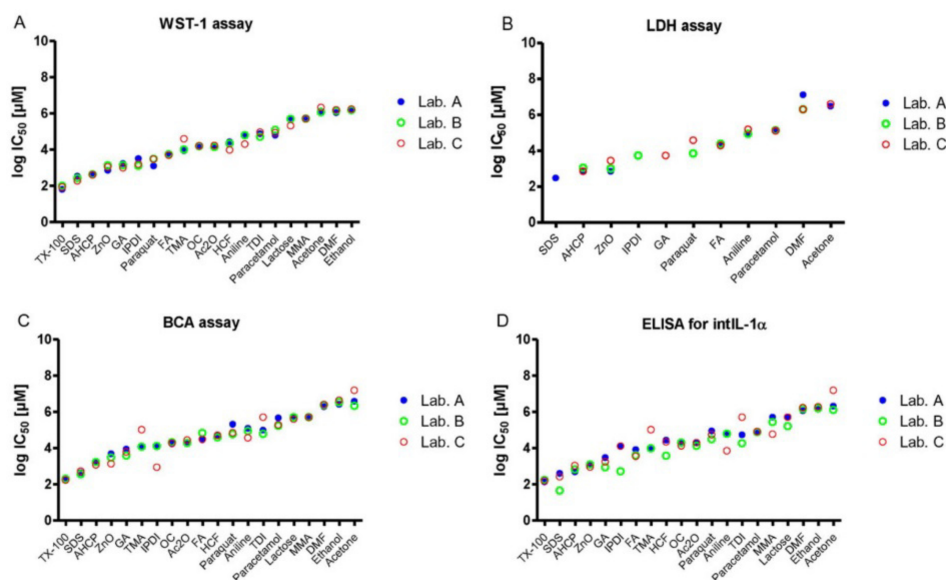

Fig. 2. Mean of log transformed IC_{50} [μM] values (log IC_{50}) for all chemicals ordered by magnitude is shown for (A) WST-1 assay, (B) LDH assay, (C) BCA assay, and (D) intracellular IL-1α (intIL-1α). Lab.: laboratory. For ">" values, minimum values have been entered.

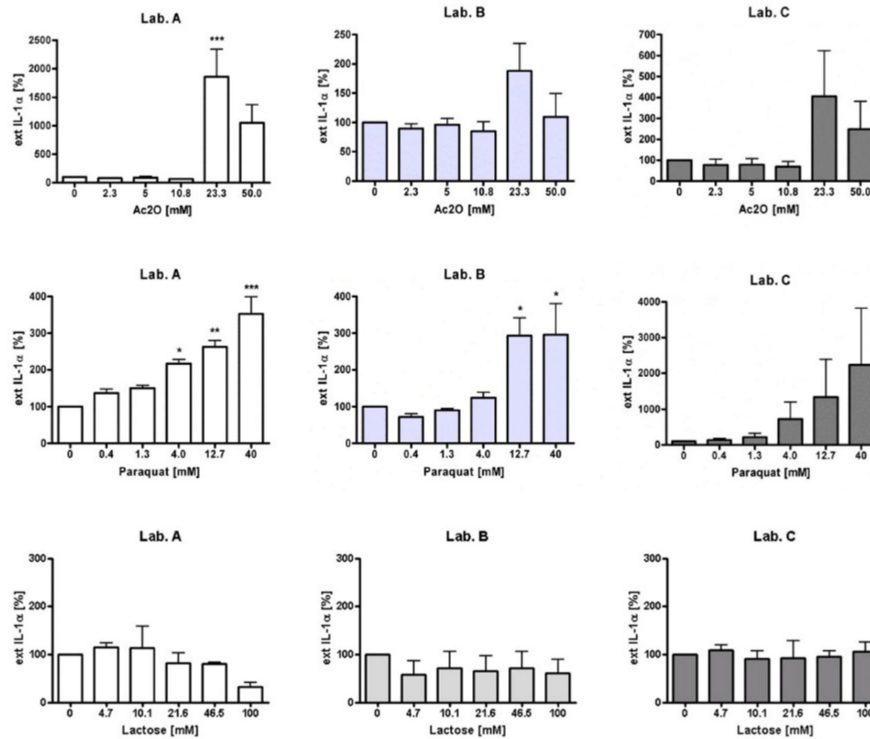

Fig. 3. Extracellular IL-1α (extIL-1α) of rPCLS after 1 h exposure to increasing concentrations of indicated substances. Cytokine levels were determined by ELISA. Data are presented as mean ± S.E.M. n = 3. *p < 0.05, **p < 0.01, and ***p < 0.001. Lab.: laboratory.

in-vivo, were classified falsely in all laboratories. TDI was falsely classified as non-toxic, whereas TMA, AHCP, and ZnO were classified falsely as positive in all laboratories (Table 4, Fig. 6). Thus, only one substance was false negative. Consequently, sensitivity was 91% and specificity was 66% in all participating laboratories. Assuming lower values for lactose and MMA changed the sensitivity of laboratory A to 82% and the specificity of laboratory C to 78%.

All results were obtained in leave-one-out cross-validation which is a model technique for evaluating how the results of a statistical analysis will generalize to an independent data set. This gives the possibility to estimate how accurately a predictive model will perform in practice (Geisser and Seymour, 1993; Kohavi, 1995). Thus, the toxicity of each substance per endpoint and laboratory combination was predicted by a model trained on subsets of data of all other substances for the same endpoint and laboratory combination without the subset data of the same substance.

3.7. Prediction of the LC_{50} by IC_{50}

The two-group classification model used in this study is a simplification of the actual toxicity. When using only toxic versus non-toxic class, the steepness of toxicity is not included, i.e. highly toxic (GHS category 1) and less toxic (GHS category 3) are all combined in the toxic class. Therefore, we have also viewed on how highly toxic substances (e.g. members of GHS category 1 such as TDI, IPDI, and paraquat) came out.

We used linear regression analysis to develop a prediction model which allows predicting in-vivo toxicity based on observed in-vitro cytotoxicity. In order to obtain a prediction model allowing estimation of LC_{50} by $IC_{50,}$ linear regression analysis was performed with values from every laboratory. The log IC_{50} values of the WST-1 assay were regarded here as predictors and the log-transformed LC_{50} values as response. The obtained formula is given in the material and methods section. The coefficients were estimated by the least-square method as $\beta_0 = -3.11$ and $\beta_1 = 0.9252$ for laboratory A, $\beta_0 = -3.12$ and $\beta_1 = 0.9287$ for laboratory B, and $\beta_0 = -2.53$ and $\beta_1 = 0.7993$ for laboratory C. Variances explained (i.e. the square of correlation coefficient) by this model were 63% for laboratory A, 63% for laboratory B, and 0.55% for laboratory C.

4. Discussion

4.1. Application of ACs to the different endpoint assays

At the beginning of this project, a standardized rPCLS protocol was established and variances in assay performance were determined. The basic protocol and technique for preparation of rat lung tissue sections closely followed those published in previous reports (Held et al., 1999; Ressmeyer et al., 2006). Initial sets of experiments using a first selection of six substances were focused on the definition of AC and subsequently applied to the remaining substances. When applying the ACs to each endpoint, WST-1, LDH, and BCA assays showed good validity, as the ACs were met by more than 95%. More difficulties in meeting the ACs were seen with the ELISA. The endpoint was highly sensitive to experimental conditions (e.g. differences in technical equipment, see also Materials and methods) beyond description in SOPs. Standardization was therefore not entirely possible. Experiments that did not meet all AC were not used for calculation of IC_{50} values but, instead, they were repeated and the data used for subsequent determinations of IC_{50} values.

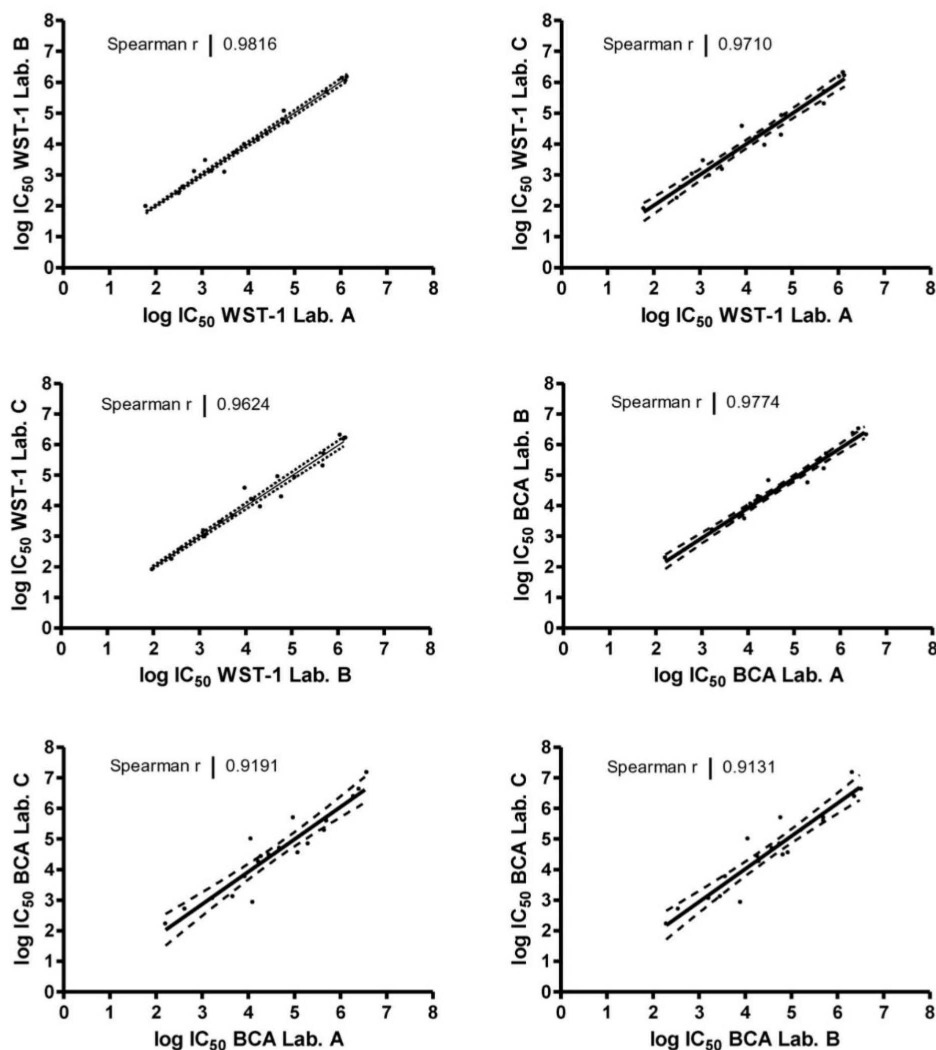

Fig. 4. Inter-laboratory correlation of mean log-transformed IC_{50} values [μM] obtained by WST-1 assay and BCA assay. The data of every laboratory was plotted against the data of every other laboratory. Comparison was performed by non-parametric Spearman correlation. For illustration only result of linear regression is shown as line and 95% confidence intervals are shown as dashed lines. Lab.: laboratory.

4.2. WST-1 assay revealed to be the best endpoint assay

Two endpoint assays were used for cytotoxicity: (i) determination of cell death as measured by leakage of the cytosolic enzyme LDH into culture medium (Legrand et al., 1992), and (ii) loss of metabolic enzyme activity assessed with the water-soluble dye WST-1 (Vistica et al., 1991).

Initially we thought that comparison of several viability endpoints could provide useful complementary data for (mechanistic) cytotoxicity, avoiding misinterpretation of chemicals interfering with the assay system. WST-1 assay provided the most complete and promising data set. LDH assay also proved to be responsive, ranking the chemicals in nearly the same order as the WST-1 assay but high substance concentrations induced wash-out effects. Thus, changing the medium after 1 h of incubation influences the outcome of the LDH assay. The wash-out effects were observed at high concentrations of AcO2, ethanol, HCF, OC, SDS, TDI, TMA, and TX-100. It was the main reason why these substances failed to be fitted. The use of later time points for LDH is often hampered by interferences of substances (e.g. AHCP, TMA) with the LDH activity (Lauenstein et al., 2014). Although the LDH assay was performed exactly the same as the WST-1 assay, it was actually not less predictive but less "practicable". Manual analysis and fitting of the big data set was not feasible. Altogether, the LDH assay was as responsive – also for 1 hour exposure – as the WST-1 assay but its further use was hindered by the limited efficiency during automatic data analysis.

It is worth mentioning here that BCA assay was standardized for determination of total protein content, but also reflected substance-induced cytotoxicity. The BCA assay was less responsive, as shown by the fact that WST-1 assay indicated cytotoxicity for some substances at lower concentrations already.

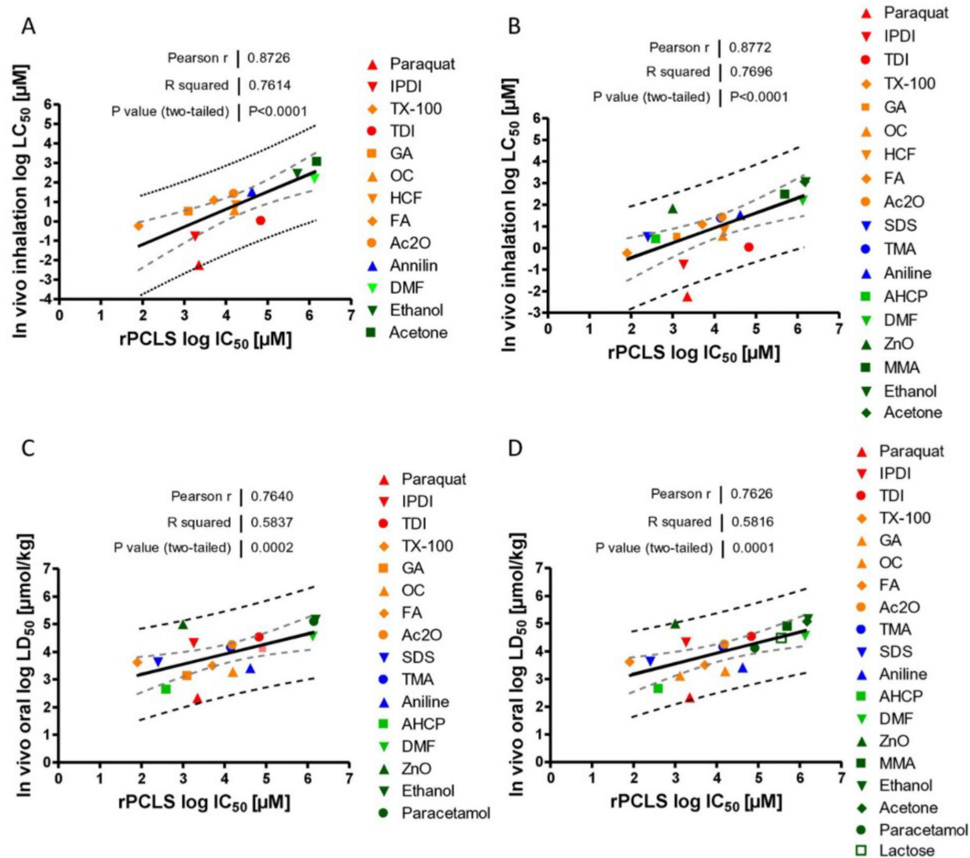

Fig. 5. Correlation of ex-vivo IC_{50} (WST-1 assay) with in-vivo inhalation LC_{50} (A, B) and oral LD_{50} values (C, D). In-vivo data were taken from online databases (Sigma, RTECS, ChemIDplus Lite, TOXNET, ECHA, and NIOSH Pocket Guide to Chemical Hazards) and converted to molar amounts (details are given in Supplement Table 1). IC_{50} values obtained by WST-1 assay in rPCLS and LC_{50}/LD_{50} values of acute inhalation and oral lethality in rats were used for linear regression analysis. A) Correlation shown for all substances except lactose, paracetamol (unknown LC_{50} values), MMA (no defined IC_{50} value), ZnO, TMA, AHCP, SDS (no defined LC_{50} values). B) Correlation shown for all substances except lactose, paracetamol (unknown LC_{50} values). For MMA, ZnO, TMA, AHCP, and SDS minimum values were used. C) Correlation shown for all substances except lactose, MMA (no defined IC_{50} values), and HCF (unknown LD_{50} value). D) Correlation shown for all substances except HCF (unknown LD_{50} value). For MMA and lactose minimum values were used. 95% confidence interval is shown as gray dashed line. 95% prediction interval is shown as black dashed line. Symbol colors refer to GHS classes as follows: 1 = red, 2 = orange, 3 = blue, 4 = light green, 5 = dark green.

Finally, we conclude that the combination of two different enzyme assays (LDH and WST-1) for cytotoxicity (with or without BCA assay) was not more sensitive and reliable than the WST-1 assay alone. The combination of the two assays did not provide additional complementary data. All substances that reduced the metabolic activity of the tissue also induced an increase in LDH activity in supernatant. Using the twenty described substances we found no case where it was contrary, e.g. decrease of tissue activity without detectable LDH leakage (e.g. due to substance interference).

4.3. Can IL-1α be used as biomarker for inflammation?

For inflammation we used only one endpoint. Substance-induced pro-inflammatory responses were assessed by quantification of IL-1α. This is a protein of the interleukin-1 family that has strong pro-inflammatory effects in the body, promoting the development of sepsis and fever (Dinarello, 2009; Frank et al., 2008; Sims and Smith, 2010). In lung tissue, IL-1α is produced mainly by monocytic cells such as activated macrophages, but also by epithelial and endothelial cells (Suwara et al., 2014). IL-1α has been reported to be an indicator of inflammation in lung tissue, e.g. due to exposure to LPS, adjuvants, or chemicals (Cao et al., 2011; Henjakovic et al., 2008; Xing et al., 1994). In our study, IL-1α responsiveness of rPCLS to LPS and substance treatments was quantitatively extremely variable within all laboratories. In rPCLS IL-1α peaks intracellularly at 24 h after permanent exposure to LPS (Henjakovic et al., 2008). Interestingly, some substances such as Ac2O and AHCP, known to induce respiratory inflammation accompanied by cellular infiltration and edema, significantly increased extracellular IL-1α levels in rPCLS (Fig. 3). On the other hand, substances such as lactose and MMA showed no effect on IL-1α levels. Although IL-1α could not be used to derive IC_{50}, these results suggest that IL-1α could be a marker for the prediction of substance induced inflammatory effects. Further efforts are needed to assess the usefulness of this information, and to establish it as valid and reliable endpoint as the inter-laboratory concordance was insufficient.

4.4. Intra- and inter-laboratory variabilities

The results from each laboratory were evaluated concerning intra- and inter-laboratory variability. Variability within and between

Table 4
Classification of substances using the binary prediction model. The number of each substance refers to known order in inhalation toxicity (see also Table 1). Lab.: laboratory; tox: toxic; non: non-toxic.

#	Substance	In vivo	Ex vivo		
			Lab. A	Lab. B	Lab. C
1	Paraquat	Tox	Tox	Tox	Tox
2	IPDI	Tox	Tox	Tox	Tox
3	TX-100	Tox	Tox	Tox	Tox
4	TDI	Tox	Non	Non	Non
5	AHCP	Non	Tox	Tox	Tox
6	GA	Tox	Tox	Tox	Tox
7	SDS	Tox	Tox	Tox	Tox
8	OC	Tox	Tox	Tox	Tox
9	HCF	Tox	Tox	Tox	Tox
10	FA	Tox	Tox	Tox	Tox
11	TMA	Non	Tox	Tox	Tox
12	Ac2O	Tox	Tox	Tox	Tox
13	Aniline	Tox	Tox	Tox	Tox
14	ZnO	Non	Tox	Tox	Tox
15	DMF	Non	Non	Non	Non
16	MMA	Non	Non	Non	Non
17	Ethanol	Non	Non	Non	Non
18	Acetone	Non	Non	Non	Non
19	Lactose	Non	Non	Non	Non
20	Paracetamol	Non	Non	Non	Non

laboratories were evaluated for each constellation. Best consistency, low variability, and strong agreement were found for WST-1 and BCA assays. Both assays were comparably robust. Results for LDH assay were found to be moderately matching, whereas intra- and inter-laboratory variability was high for IL-1α determination by ELISA. In summary, WST-1 assay seems to be best suited for analysis of cytotoxicity in rPCLS. The assay was transferable between the laboratories and provided relevant and reproducible results. The same was true for BCA assay used for determination of total protein content. The transferability of the ELISA based quantification of IL-1α was only limited, and results were less reproducible and very sensitive to laboratory specific conditions. In general, with a sample size of three biological replicates, the results have to be interpreted very carefully and need further validation.

Ranking of substances in the ex-vivo assay correlated well with underlying toxicological mechanisms. TX-100 is one of the most toxic compounds in-vivo and was ranked as most toxic in the ex-vivo model. Among the most toxic substances was also SDS, which is used as detergent, too. Both chemicals are known to disrupt cell membranes. SDS denatures proteins at higher concentrations (Chaturvedi and Kumar, 2011). Other chemicals such as isocyanates are known to attack proteins which lead to disturbance of cellular processes in e.g. metabolism, proteolysis, proliferation and protein synthesis. Substances such as Ac2O change their chemical structure. In aqueous media, Ac2O hydrolyses to acetic acid within few minutes. In our study the effects of Ac2O and acetic acid cannot be distinguished. In-vivo toxic threshold concentrations for Ac2O were found to be lower (about half) than for acetic acid. This is suggesting initial toxic effect by the anhydride before it hydrolyzed to acetic acid (OECD SIDS, 1997). Moreover, some chemicals were able to change the pH of the medium. They were not neutralized before they were applied to the test system in order to mimic real-life exposure. As test substances were incubated with rPCLS under submerged conditions, chemicals with limited water solubility such as TMA were initially dissolved in appropriate solvents. These solvents showed no effects at the final concentrations used.

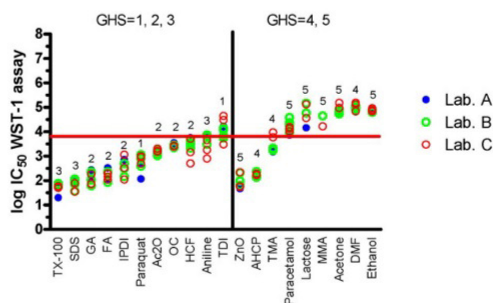

Fig. 6. Classification of substances using the binary prediction model. Means of log IC_{50} as obtained by WST-1 assay were converted to mg/L and ranked according to GHS category. GHS categories 1, 2, and 3 were sorted in toxic class. GHS categories 4 and 5 were sorted as non-toxic class. Red line indicates the ex-vivo threshold value. Small numbers above the symbols refer to the GHS class of each substance. Lab.: laboratory.

4.5. The two prediction models

For comparison with in-vivo data we developed two prediction models. The first prediction model was a two-group classification model where substances were divided into two classes based on their in-vivo acute inhalation toxicity. Substances with GHS categories 1, 2, and 3 were assigned to the toxic class and substances with GHS categories 4 and 5 to the non-toxic class. With this approach, 16 out of 20 test substances were classified correctly in all laboratories (Fig. 6). The reason why TDI and ZnO were falsely classified is most probably the same as that explaining the outliers in the second prediction model proposed in the next section below. A sensitivity of 91% was found for all laboratories, whereas the specificity was calculated at 66% for all laboratories.

The second prediction model was based on linear regression analysis of ex-vivo and in-vivo data. IC_{50} values obtained by WST-1 assay in rPCLS showed significant correlation with LC_{50} of in-vivo inhalation rats. The results can potentially be used to predict starting concentrations of acute inhalation toxicity studies. Significant correlation was also observed with LD_{50} values of oral studies in rats. Nevertheless, some outliers were obvious: (Akaike, 1998) Although paraquat is the substance with the lowest LC_{50} among the selected substances, it is ranked only on seventh position in the ex-vivo model (WST-1 assay) and was about 30 times less toxic than the most toxic substance ex-vivo TX-100. The lung is the primary target organ of paraquat. However, the primary underlying mechanism of paraquat is oxidative damage, which – and this is remarkable – leads to delayed toxic damage of lung tissue via pulmonary fibrosis. In the end, this condition is the usual cause of death after exposure to paraquat and which most commonly occurs 7–14 days after acute exposure (EPA Handbook, 2006). It shows that substances with long term effects such as paraquat can be tested only to a limited extent in acute models. (Archer et al., 1997) ZnO is less toxic in in-vivo studies (GHS category 5). In the rPCLS model, it ranked among the five most toxic substances. The difference is that ZnO was dissolved in acetic acid before application to rPCLS, whereas in-vivo it is inhaled in the undissolved form as dust (Klimisch et al., 1997). Toxicity of dissolved ZnO has been reported to be due to zinc ion concentrations leading to mitochondrial dysfunction, caspase activation, and apoptosis (Kao et al., 2012). Therefore, it can also not be compared to Sauer et al., 2014 where ZnO was applied as nanomaterial for 24 h. (Balls and Fentem, 1999) The diisocyanates IPDI and TDI are highly toxic in-vivo (GHS category 1) and ranked among the five most potent substances used in this study, but showed only minor cytotoxic effects ex-vivo. TDI in particular reacts quickly with water and its half-life time in water is only a few minutes (Collins, 2002). IPDI is more stable and remains in aqueous solutions for some hours (OECD SIDS, 2006). This might be the reason why the ex-vivo IC_{50} value of IPDI is about 30 times lower than that of TDI, whereas in-vivo both compounds differ only by a factor of six. It shows that substances being unstable in solution such as

isocyanates have only limited applicability for testing at submerse exposure conditions.

Both prediction models were based on the use of the median IC_{50} values. In general, models using concentrations different from IC_{50} may be of interest in future. Benchmark Dose (BMD) for example can also rank potencies of substances based on in-vitro dose–response curves even when no IC_{50} can be calculated (Louisse et al., 2010). With this approach doses for low effect levels such as a 5–10% change from baseline can be estimated and subsequently converted into predicted in-vivo doses. Although it was not applied in this study it could be an interesting approach in future.

4.6. How does the results compare to other in-vitro models?

In the following discussion our results are compared with other published in-vitro models. A recently published study (Sauer et al., 2013) investigated responses of the two commercially available 3D models, MucilAir™, and EpiAirway™, and of two monolayer cell lines, human A549 epithelial cells and 3T3 mouse embryonic fibroblasts, to the same chemicals as assessed in the present study. IC_{50} values using the MTT and WST-1 assays have been reported. We used these published IC_{50} values for direct correlation to in-vivo lethality data by linear regression analysis. The IC_{50} values of each model correlated significantly with oral LD_{50} and inhalation LC_{50} values (Supplement Fig. 1). Remarkable is the dynamic range spreading the data, which for the same chemicals was less pronounced for single-cell cultures (4 orders of magnitude for A549and 3T3) than for in-vivo toxicity data (6 orders of magnitude). Among these models, rPCLS was the first model used for prevalidation. In comparison with the other models, rPCLS correlated best with in-vivo data (Pearson $r = 0.87$). But also A549, MucilAir™, and EpiAirway™ showed good correlation with in-vivo (Pearson r 0.85, 0.84 and 0.83, respectively) (Supplement Fig. 1). Only 3T3 cells did not correlate well (Pearson $r = 0.71$). Interestingly, all models showed similar outlier pattern, as discussed already above for the results of the present study.

The question needs to be discussed whether for endpoints such as cytotoxicity single cell lines can be taken. The results of the study show that among the tested cell lines only alveolar epithelial cells such as A549 correlated well with in-vivo whereas the fibroblast cell line 3T3 did not. Lauenstein et al. (2014) showed that cells in human lung tissue behave more like monocyte-derived cells regarding their resistance to chemicals than skin-derived epithelial cells. This supports the theory that different cell populations have different responsiveness to chemicals. In the lung more than 45 different cell types can be found. Epithelial cells, nerve fibers, monocytes/macrophages, dendritic cells are in the first line of defense. All of them may respond differently to the same substance, some cell types even possess receptors that provide a chemical sensation (Guilliams et al., 2013). In the context of likely exposed cells the advantage of an ex-vivo tissue culture model is its multicellular microanatomy which is very attractive for a first assessment of acute local respiratory toxicity.

Moreover, we want to develop a model in future that is not merely based on cell injury but also provide early biomarkers for respiratory inflammation and chronic diseases, such as sensitization, proliferation, and fibrosis. The use of cell cultures is limited as published by Lauenstein et al. (2014). Here it was shown that biomarkers developed in cell culture were not proven to be predictive and reliable in human lung tissue. Switalla et al. (2010) showed that biomarkers in human lung tissue ex-vivo correlated very well with in-vivo response of probands. Nevertheless, in face of the different adverse effects after inhalation of toxicants it seems to be unlikely to develop a single approach – be it cell lines or tissue models – predicting the entire diversity of biological responses of the respiratory tract in acute toxicity studies.

4.7. Translation from in-vitro to in-vivo

The comparison of animal toxicity data with in-vitro/ex-vivo data remains critical due to the following issues: i) Differences can occur, for example, in the site of action, which can be different from the site of exposure. ii) Only a small number of substances was used in our study. Retrospective analysis of more than 300 chemicals tested according to the guidance document for oral toxicity has shown that a larger number of test chemicals provides different outcomes than the initial studies using less substances (Schrage et al., 2011). iii) Biotransformation in rPCLS remains questionable. Although, the lungs have been reported to have considerable capacity for metabolism (De Kanter et al., 2004), the rPCLS model has to be characterized using substances requiring biotransformation. Among the so far twenty tested substances only paracetamol requires biotransformation. Its metabolite is known to cause liver toxicity. Paracetamol showed weak toxicity in rPCLS suggesting somewhat biotransformation in rPCLS. iv) Exposure was done under submersed conditions. Whether or not rPCLS can be used to test, for example, nanomaterials, pesticides, metal compounds, and other industrial substances could not be addressed in this study. In particular, the use of rPCLS for the testing of chemicals quickly reacting with aqueous solutions might be limited (Landsiedel et al., 2010; Monopoli et al., 2011; Sauer et al., 2014). v) The model was set up as an acute exposure model. Long-term effects leading to development of fibrosis cannot be studied (Bonfield et al., 1995; Gwyer Findlay and Hussell, 2012; Yankaskas et al., 2004). Nevertheless, within this pre-validation study the respiratory toxicity data obtained for the selected substances reflected the in-vivo situation very closely. Issues that need to be reflected and one has to be aware of have been discussed above.

4.8. Conclusions and perspective

In conclusion, rPCLS as a test system for cytotoxicity can be part of a test battery of different in-vitro or in-vivo models to reflect the various processes associated with inhalation toxicity (Sauer et al., 2013). Whether or not this alternative can eventually be used to reduce or even replace in-vivo studies can only be answered by analyzing a larger set of new reference chemicals different from the set used in our study. However, according to the internationally recognized principle of a modular approach to validation (Hartung et al., 2004; OECD GD 34, 2005), the current study may be accepted already as a sufficient proof of intra- and interlaboratory reproducibility, and reproducibility over time. Thus, a new, independent data set might not necessarily have to be tested again in three laboratories. The main goal of such a special study would be a robust verification of the prediction models developed here post hoc with the data obtained from twenty reference chemicals in three laboratories. Since results of statistical cross-validation applied here to the training data set of twenty chemicals suggest robustness of the prediction models also when applied to new data, the current study offers a particularly good basis for a special follow-up study focused only on verification of the prediction models as one important part of the validation module "relevance". A pre-submission of this approach to EURL/ECVAM and a check by the independent Boards of EURL/ECVAM (PARERE, ESTAC and ESAC) could aid deciding about the design of follow-up Validation Study (module).

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Transparency document

The Transparency document associated with this article can be found, in the online version.

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Legends for the Online Supplement

Supplement table 1: In-vivo toxicity data of each substance used in the prevalidation of rPCLS. Table shows for each substance: abbreviation according to table 1, in-vivo acute inhalation (inhal.) LC_{50} in µM, corresponding GHS classification, proposed mechanism of acute inhalation toxicity, in-vivo acute oral LD_{50} values in µmoL/kg body weight.

Supplement table 2: Fulfillment of acceptance criteria. The table shows the number of samples entered for evaluation and the finally accepted numbers [%]. AC: acceptance criterion; N: number of analyzed samples. Lab.: laboratory.

Supplement table 3: Intra-laboratory variability is shown for mean of log-transformed IC_{50} values [µM] of metabolic activity as assessed by WST-1 assay in each laboratory (A, B, C) with corresponding intra-laboratory standard deviation, coefficient of variation, and numbers of values included. Substances are ranked according to toxicity. Cv: coefficient of variation; Lab.: laboratory; Stdev: standard deviation; log IC_{50}: log-transformed IC_{50} [µM].

Supplement table 4: Intra-laboratory variability is shown for mean of log-transformed IC_{50} values [µM] of LDH activity as assessed by LDH assay in each laboratory (A, B, C) with corresponding intra-laboratory standard deviation, coefficient of variation, and number of values included. Absolutely no values were obtained for TX-100, TDI, ethanol, Ac2O, lactose, MMA, HCF, OC, and TMA. Substances are ranked according to toxicity. Cv: coefficient of variation; Lab.: laboratory; Stdev: standard deviation; log IC_{50}: log-transformed IC_{50} [µM].

Supplement table 5: Intra-laboratory variability is shown for mean of log-transformed IC_{50} values [µM] for total protein as assessed by BCA assay in each laboratory (A, B, C) with corresponding intra-laboratory standard deviation, coefficient of variation, and numbers of values included. Substances are ranked according to toxicity. Cv: coefficient of variation; Lab.: laboratory; Stdev: standard deviation; log IC_{50}: log-transformed IC_{50} [µM].

Supplement table 6: Intra-laboratory variability is shown for mean of log-transformed IC_{50} values [µM] of intrinsic IL-1α (intIL-1α) as assessed by ELISA in each laboratory (A, B, C) with corresponding intra-laboratory standard deviation, coefficient of

variation, and numbers of values included. Substances are ranked according to toxicity. Cv: coefficient of variation; Lab.: laboratory; Stdev: standard deviation; log IC_{50}: log-transformed IC_{50} [µM].

Supplement figure 1: Correlation of recently published in-vitro studies using 3D models A) PCLS, B) MucilAir™, D) EpiAirway™ and monolayer cell lines, C) human A549 epithelial cells and E) 3T3 mouse embryonic fibroblasts (Sauer et al., 2013). IC50 values were taken from Sauer et al., 2013, converted to molar mass and used for linear regression analysis with in-vivo inhalation LC50 values. In-vivo data were taken from online (Sigma, RTECS, ChemIDplus Lite, TOXNET, ECHA, and NIOSH Pocket Guide to Chemical Hazards) and converted to molar amounts (details are given in Supplement Table 1). For better comparison data sets were reduced to the lowest common number of chemicals. Fifteen identical chemicals provided values in every model. Hence, TX-100, AHCP and ZnO were not included.

Supplement table 1

#	Substance	*In vivo* inhal. LC_{50} [µM]	GHS classification	Biological mechanisms**	*In vivo* oral LD_{50} [µmol/kg]
1	Paraquat	0.01[2]	1	Oxidative stress, inhibition of respiratory chain	222
2	IPDI	0.18[2]	1	Respiratory irritation, respiratory sensitization	21715
3	TX-100	0.62[2]	2	Cell lysis	4328
4	TDI	1.05[1]	1	Respiratory irritation, respiratory sensitization	33295
5	AHCP	>2.55[2]	4	Respiratory irritation, respiratory sensitization	439
6	GA	3.20[2]	2	Respiratory irritation	1339
7	SDS	>3.38[2]	3	Cell lysis	4466
8	OC	3.87[1]	2	Lung edema	1961
9	HCF	7.11[1]	2	Lung edema	Unknown
10	FA	13.05[3]	2	Respiratory irritation	3300
11	TMA	>23.95[2]	4	Respiratory irritation, respiratory sensitization	13977
12	Ac2O	26.00[1]	2	Respiratory irritation, respiratory sensitization	17434
13	Aniline	35.12[1,2]	3	Respiratory irritation, bronchoconstriction	2685
14	ZnO	>70.02[2]	5	Transient pulmonary inflammation	103649
15	DMF	164.00[1]	4	Respiratory irritation	38304
16	MMA	297.70[1]	5	Respiratory irritation	78641
17	Ethanol	1149.67[1]	5	Respiratory irritation	153145
18	Acetone	1217.04[1]	5	Respiratory irritation	122633
19	Lactose	Unknown	5	Unknown	29214
20	Paracetamol	Unknown	5	Liver toxicity	12857

[1] tested as vapor; [2] tested as aerosol; [3] tested as gas; * For Ac2O, TDI, Paraquat and acetone only LC_{50} values from studies with exposure durations different from 4 hours were found. In these cases, the LC_{50} values were converted to 4-hour LC_{50} values as described in the material section.**including cross-references from other toxicity studies

Supplement table 2

Endpoint assay	**Acceptance criteria**	**Lab. A**		**Lab. B**		**Lab. C**	
		N entered	N [%] accepted	N entered	N [%] accepted	N entered	N [%] accepted
WST-1	AC1	216	**100**	216	**100**	216	**100**
	AC2	12	**100**	12	**100**	12	**100**
	AC3	12	**100**	12	**100**	12	**100**
LDH	AC1	216	**100**	216	**100**	216	**100**
	AC2	12	**83**	12	**100**	12	**100**
	AC3	12	**100**	12	**100**	12	**92**
BCA	AC1	48	**100**	32	**100**	56	**98**
	AC2	6	**100**	4	**100**	7	**100**
	AC3	6	**100**	4	**100**	7	**100**
	AC4	12	**100**	12	**100**	12	**100**
ELISA	AC1	96	**95**	32*	**100**	96	**100**
	AC2	12	**100**	4	**100**	12	**100**
	AC3	12	**100**	4	**100**	12	**92**
	AC4	12	**83**	12	**50**	12	**100**

* AC1 for ELISA was not applied for laboratory B (equipped with pipetting robot)

Supplement table 3

			Intra-laboratory variability WST-1 assay				
#	Substance	Lab.	Median log IC_{50}	Mean log IC_{50}	Stdev	Cv [%]	N
3	TX-100	A	**1.91**	**1.79**	0.27	15	3
		B	**1.98**	**1.98**	0.05	3	5
		C	**1.89**	**1.91**	0.04	2	3
7	SDS	A	**2.52**	**2.51**	0.08	3	3
		B	**2.42**	**2.40**	0.19	8	5
		C	**2.42**	**2.25**	0.24	11	2
5	AHCP	A	**2.58**	**2.59**	0.02	1	3
		B	**2.63**	**2.61**	0.12	5	4
		C	**2.61**	**2.60**	0.03	1	3
14	ZnO	A	**2.86**	**2.84**	0.09	3	3
		B	**3.06**	**3.11**	0.26	8	3
		C	**2.85**	**3.03**	0.34	11	3
6	GA	A	**3.14**	**3.21**	0.19	6	3
		B	**3.16**	**3.12**	0.27	9	4
		C	**2.86**	**2.98**	0.23	8	3
2	IPDI	A	**3.50***	**3.49***	NA	NA	3
		B	**3.13**	**3.09**	0.27	9	3
		C	**3.16**	**3.18**	0.51	16	3
1	Paraquat	A	**3.27**	**3.08**	0.38	12	3
		B	**3.54**	**3.47**	0.21	6	4
		C	**3.53**	**3.46**	0.17	5	3
10	FA	A	**3.61**	**3.72**	0.27	7	3
		B	**3.84**	**3.72**	0.24	6	3
		C	**3.67**	**3.67**	0.13	3	3
11	TMA	A	**3.89**	**3.92**	0.06	1	3
		B	**4.00**	**3.99**	0.05	1	3
		C	**4.68***	**4.58***	NA	NA	2
8	OC	A	**4.18**	**4.21**	0.1	2	3
		B	**4.14**	**4.17**	0.06	1	3
		C	**4.21**	**4.19**	0.03**	1**	2
12	Ac2O	A	**4.25**	**4.22**	0.07	2	3
		B	**4.18**	**4.13**	0.09	2	5
		C	**4.19**	**4.21**	0.07	2	3
9	HCF	A	**4.42**	**4.41**	0.12	3	3
		B	**4.32**	**4.32**	0.14	3	5
		C	**3.93**	**3.96**	0.51	13	3
13	Aniline	A	**4.77**	**4.77**	0.01	0.2	3
		B	**4.83**	**4.78**	0.16	3	5
		C	**4.27**	**4.29**	0.38	9	3

4	TDI	A	**4.83**	**4.86**	0.1	2	3
		B	**4.71**	**4.70**	0.19	4	4
		C	**5.23***	**4.95**	0.63	13	3
20	Paracetamol	A	**4.68**	**4.78**	0.18	4	3
		B	**5.07***	**5.07***	0.24	5	5
		C	**4.94**	**4.92**	0.09	2	3
16	MMA	A	**>5.70**	**>5.70**	-	-	3
		B	**>5.70**	**>5.70**	-	-	4
		C	**>5.70**	**>5.70**	-	-	3
19	Lactose	A	**>5.70**	**>5.70**	-	-	3
		B	**>5.70**	**5.68***	-	-	4
		C	**5.58***	**5.30***	0.4	7	2
18	Acetone	A	**6.11**	**6.11**	0.1	2	3
		B	**6.05**	**6.05**	0.1	2	3
		C	**6.41**	**6.31**	0.15	2	2
15	DMF	A	**6.04**	**6.03**	0.09	1	3
		B	**6.14**	**6.13**	0.07	1	3
		C	**6.25**	**6.18**	0.19	3	3
17	Ethanol	A	**6.15**	**6.14**	0.02	0.3	3
		B	**6.17**	**6.17**	0.05	1	3
		C	**6.22**	**6.22**	0.07	1	3

NA, not analysed; * Value(s) found by extrapolation above the maximum applied concentration; ** For one run only a lower bound could be determined. This data point was detected as outlier by the Grubbs test (Grubbs, 1950) and not considered for the calculation of standard deviation and cv.

Supplement table 4

			Intra-lab. variability LDH assay				
#	Substance	Lab.	Median	Mean	Stdev	Cv [%]	N
7	SDS	A	**2.46**	**2.46**	0.02	1	2
		B	**-**	**-**	-	-	-
		C	**-**	**-**	-	-	-
5	AHCP	A	**2.83**	**2.83**	0.15	5	2
		B	**3.03***	**3.03***	0.02	1	4
		C	**2.94**	**2.84**	0.25	9	3
14	ZnO	A	**2.80**	**2.84**	0.09	3	3
		B	**2.99**	**2.99**	0.18	6	3
		C	**3.43***	**3.43***	0.13	4	2
2	IPDI	A	**-**	**-**	-	-	-
		B	**3.64***	**3.71***	0.12	3	3
		C	**-**	**-**	-	-	-
6	GA	A	**-**	**-**	-	-	-
		B	**-**	**-**	-	-	-
		C	**3.71***	**3.71***	0.16	4	2
1	Paraquat	A	**-**	**-**	-	-	-
		B	**3.92**	**3.83**	0.5	13	4
		C	**4.56**	**4.56**	0.04	1	2
10	FA	A	**4.38**	**4.29**	0.18	4	3
		B	**4.39**	**4.36**	0.09	2	3
		C	**4.31**	**4.27**	0.08	2	3
13	Aniline	A	**4.92**	**4.93**	0.04	1	3
		B	**4.94**	**4.92**	0.03	1	5
		C	**5.20***	**5.17***	0.15	3	3
20	Paracetamol	A	**5.15***	**5.15***	0.13	2	3
		B	**5.08***	**5.11***	0.13	3	6
		C	**5.19***	**5.1***	0.19	4	3
15	DMF	A	**>7.10**	**>7.10**	-	-	2
		B	**6.29**	**6.29**	0.03	0.4	2
		C	**6.26**	**6.28**	0.04	1	3
18	Acetone	A	**6.46**	**6.46**	0.08	1	2
		B	**-**	**-**	-	-	-
		C	**6.58***	**6.58***	0.18	3	2

* Value(s) found by extrapolation above the maximum applied concentration

Supplement table 5

			Intra-lab. variability BCA assay				
#	Substance	Lab.	Median log IC_{50}	Mean log IC_{50}	Stdev	Cv [%]	N
3	TX-100	A	**2.20**	**2.21**	0.16	7	3
		B	**2.25**	**2.29**	0.12	5	7
		C	**2.27**	**2.22**	0.09	4	3
7	SDS	A	**2.63**	**2.63**	0.02	1	3
		B	**2.55**	**2.55**	0.01	0.3	2
		C	**2.71**	**2.71**	0.15	5	2
5	AHCP	A	**3.25***	**3.23***	0.17	5	3
		B	**3.11***	**3.21***	0.29	9	3
		C	**3.13***	**3.06***	0.12	4	3
14	ZnO	A	**3.67***	**3.67***	0.29	8	2
		B	**3.46***	**3.46***	0.06	2	2
		C	**3.03**	**3.12**	0.21	7	3
6	GA	A	**4.04***	**3.93***	0.19	5	3
		B	**3.49**	**3.57**	0.36	10	3
		C	**3.68**	**3.77***	0.23	6	3
11	TMA	A	**4.06**	**4.06**	0.01	0.2	3
		B	**4.06**	**4.06**	0.04	1	3
		C	**>5.00**	**>5.00**	-	-	2
2	IPDI	A	**>4.10**	**>4.10**	-	-	2
		B	**>4.10**	**>4.10**	-	-	1
		C	**2.93**	**2.93**	0.12	4	2
8	OC	A	**4.22**	**4.22**	0.01	0.1	3
		B	**4.24**	**4.30**	0.10	2	3
		C	**4.24**	**4.26**	0.08	2	3
12	Ac2O	A	**4.29**	**4.28**	0.02	0.5	3
		B	**4.25**	**4.27**	0.05	1	3
		C	**4.35**	**4.43**	0.15	3	3
10	FA	A	**4.45**	**4.45**	0.03	1	2
		B	**4.80***	**4.82***	0.14	3	3
		C	**4.50***	**4.48***	0.05	1	3
9	HCF	A	**4.64**	**4.68**	0.18	4	3
		B	**4.61**	**4.58**	0.19	4	5
		C	**4.67**	**4.70**	0.43	9	3
1	Paraquat	A	**>5.30**	**>5.30**	-	-	3
		B	**4.71***	**4.75***	0.09	2	3
		C	**4.84***	**4.84***	0.46	10	2
13	Aniline	A	**5.08***	**5.08***	0.04	1	2
		B	**4.93**	**4.93**	0.01	0.2	4
		C	**4.88**	**4.55**	0.70	15	3

4	TDI	A	**4.98**	**4.98**	0.10	2	2
		B	**4.77**	**4.77**	0.31	7	2
		C	**>5.70**	**>5.70**	-	-	2
20	Paracetamol	A	**>5.65**	**>5.65**	-	-	3
		B	**5.17***	**5.21***	0.18	3	5
		C	**5.28***	**5.28***	0.30	6	2
19	Lactose	A	**>5.70**	**>5.70**	-	-	3
		B	**>5.70**	**>5.70**	-	-	6
		C	**5.59***	**5.59***	0.01	0.1	2
16	MMA	A	**>5.70**	**>5.70**	-	-	3
		B	**>5.70**	**>5.70**	-	-	3
		C	**>5.70**	**>5.70**	-	-	3
15	DMF	A	**6.26**	**6.28**	0.04	1	3
		B	**6.37**	**6.37**	0.01	0.1	3
		C	**6.38**	**6.39**	0.02	0.3	3
17	Ethanol	A	**6.42**	**6.40**	0.03	1	3
		B	**6.43**	**6.52**	0.24	4	3
		C	**6.43**	**6.63**	0.38	6	3
18	Acetone	A	**6.59***	**6.57***	0.24	4	3
		B	**6.31**	**6.32**	0.07	1	3
		C	**>7.18**	**>7.18**	-	-	2

* Value(s) found by extrapolation above the maximum applied concentration

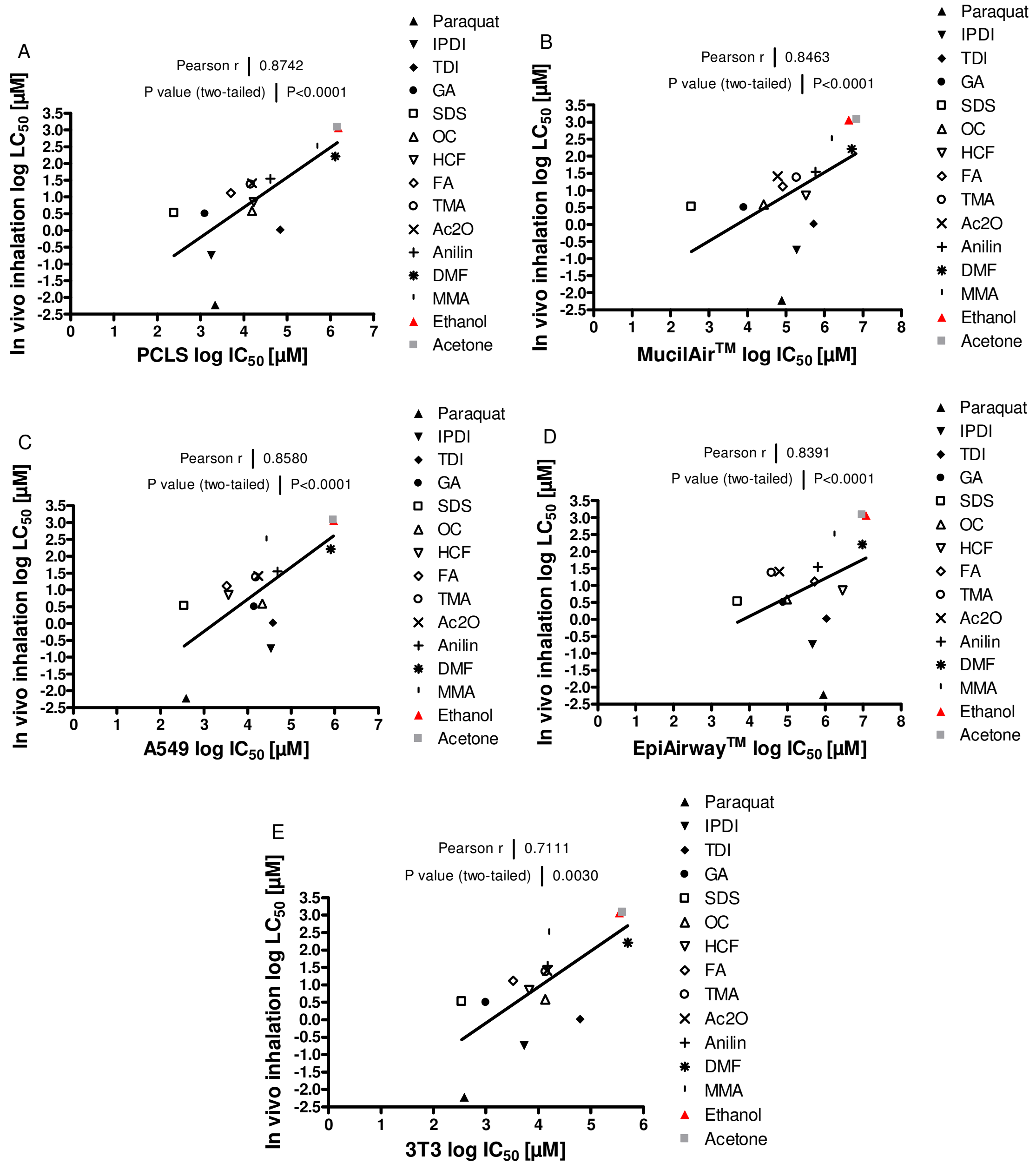
Supplement table 6

			Intra-lab. variability intIL-1α ELISA				
#	**Substance**	**Lab.**	**Median log IC_{50}**	**Mean log IC_{50}**	**Stdev**	**Cv [%]**	**N**
3	TX-100	A	**2.03**	**2.12**	0.22	10	3
		B	**2.29**	**2.22**	0.12	5	3
		C	**2.21**	**2.18**	0.08	3	3
7	SDS	A	**2.62**	**2.59**	0.10	4	3
		B	**1.55**	**1.64**	0.20	12	4
		C	**2.40**	**2.40**	0.03	1	2
5	AHCP	A	**2.65**	**2.67**	0.12	4	3
		B	**2.83**	**2.81**	0.18	6	4
		C	**3.10***	**3.02***	0.16	5	3
14	ZnO	A	**3.18**	**3.04**	0.38	12	3
		B	**3.08**	**3.08**	0.21	7	2
		C	**3.00**	**2.94**	0.34	12	3
6	GA	A	**3.46**	**3.46**	0.11	3	2
		B	**3.10**	**2.93**	0.47	16	3
		C	**3.23**	**3.23**	0.06	2	2
2	IPDI	A	**>4.10**	**>4.10**	-	-	2
		B	**2.76**	**2.70**	0.11	4	3
		C	**>4.10**	**>4.10**	-	-	2
10	FA	A	**3.90**	**3.90**	0.3	8	2
		B	**3.59**	**3.59**	-	-	1
		C	**3.53**	**3.53**	1.78	50	2
11	TMA	A	**4.03**	**4.02**	0.04	1	3
		B	**3.97**	**3.97**	0.05	1	3
		C	**>5.00**	**>5.00**	-	-	2
9	HCF	A	**4.49**	**4.42**	0.14	3	3
		B	**3.62**	**3.56**	0.32	9	3
		C	**4.17**	**4.33**	0.30	7	3
8	OC	A	**4.23**	**4.24**	0.07	2	3
		B	**4.33**	**4.29**	0.38	9	4
		C	**4.13**	**4.11**	0.05	1	3
12	Ac2O	A	**4.29**	**4.28**	0.04	1	3
		B	**4.09**	**4.11**	0.05	1	3
		C	**4.29**	**4.29**	0.04	1	2
1	Paraquat	A	**4.94***	**4.94***	0.19	4	2
		B	**4.58**	**4.48**	0.67	15	3
		C	**4.49**	**4.73***	0.46	10	3
13	Aniline	A	**4.75**	**4.75**	0.12	3	2
		B	**4.79**	**4.78**	0.07	1	3
		C	**3.67**	**3.84**	0.45	12	3

4	TDI	A	**4.80**	**4.72**	0.28	6	3
		B	**4.61**	**4.25**	0.98	23	3
		C	**>5.70**	**>5.70**	-	-	2
20	Paracetamol	A	**4.83**	**4.86**	0.06	1	3
		B	**4.87**	**4.87**	0.00	0	2
		C	**4.84**	**4.90**	0.15	3	3
16	MMA	A	**>5.70**	**>5.70**	-	-	2
		B	**5.42***	**5.42***	0.29	5	2
		C	**4.75**	**4.75**	0.53	11	2
19	Lactose	A	**>5.70**	**>5.70**	-	-	2
		B	**5.20***	**5.20***	0.06	1	2
		C	**>5.70**	**>5.70**	-	-	2
15	DMF	A	**6.07**	**6.05**	0.04	1	3
		B	**6.15**	**6.15**	0.08	1	2
		C	**6.13**	**6.22**	0.17	3	3
17	Ethanol	A	**6.28**	**6.28**	0.03	0.5	2
		B	**6.18**	**6.18**	0.04	1	2
		C	**6.27**	**6.27**	0.06	1	2
18	Acetone	A	**6.30**	**6.30**	0.18	3	2
		B	**6.10**	**6.10**	-	-	1
		C	**>7.18**	**>7.18**	-	-	2

* Value(s) found by extrapolation above the maximum applied concentration

Supplement figure 1



5 General discussion

The data presented in this thesis addressed two different functional issues: at the beginning, the aim was to identify chemical-induced acute irritation and inflammation in the airway by using the organotypic lung model PCLS. Our preliminary results show that chemical-induced irritation could be predicted by the assessment of local respiratory toxicity in PCLS. However, chemical-induced inflammation in the airway could not be implicated by a simple screening of respiratory allergens in PCLS due to missing connections to the blood and lymph system in response to allergens (publication: "Assessment of immunotoxicity induced by chemicals in human precision-cut lung slices (PCLS)"). Subsequently, PCLS was standardized and prevalidated as an alternative *ex vivo* approach to reduce the number of animals used in inhalation toxicity studies in three independent laboratories (publication: "Prevalidation of the ex-vivo model PCLS for the prediction of respiratory toxicology").

5.1 Chemical-induced toxicity in the development of acute lung injury

5.1.1 Inhalation of toxicants leading pulmonary epithelial injury

Pulmonary epithelial injury including pulmonary microvascular endothelial cell injury and barrier dysfunction could lead ongoing effects of acute lung injury that can subsequently induce pulmonary edema and migration of circulating neutrophils into the alveolar spaces [Lucas *et al.*, 2009; Razavi *et al.*, 2004; Wang *et al.*, 2013; Wang *et al.*, 2002]. Inhalation of toxicants has been shown to cause epithelial death in sensitive cells regulated either by necrosis or apoptosis [Vyas *et al.*, 2013]. Apoptosis and necrosis could be recognized based on differences in the morphological, biochemical, and molecular changes of dying cell [Vyas *et al.*, 2013]. Necrosis is mostly directly induced by an overdose of toxic substance and responded to a massive injury [Vyas *et al.*, 2013]. The factors which orchestrate the apoptotic process are diverse including noxious agents, oxidative stress, and other numerous intrinsic or extrinsic factors [Elmore, 2007]. For example, chemicals like glyoxal leads to cell toxicity by the production of ROS, which degrades the mitochondrial membrane leading to cell apoptosis or induces lipid peroxidation and formaldehyde formation [Shangari *et al.*, 2004]. Formaldehyde

interferes with airway integrity and functions and induces airway inflammation [Kastner *et al.*, 2011]. Chemicals like eugenol could generate ROS and phototoxicity in solutions with various pH and trigger apoptosis [Atsumi *et al.*, 2001]. Nevertheless, the precise molecular requirements that activate the apoptotic pathway remain largely unknown. For example, ROS could be produced in the metabolism of industrial chemicals by e.g. cytochrome P450 to free radical products and initiates apoptosis response [Parke *et al.*, 1996].

5.1.2 Assessment of toxic features of acute lung injury in live lung sections

Recently, in regard to respiratory system several 3D models of human-derived epithelium which closely resembles the epithelial tissue of the respiratory tract than single cell line were used as *in vitro* assays for the assessment of toxic features of acute lung injury, such as EpiAirway^{TM} (MatTek Corporation, Ashland, MA, USA) and MucilAir^{TM} (Epithelix Sarl, Geneva, Switzerland). However, the cellular anatomy of the respiratory tract comprises at least 45 extensively varying cell types with different functions, individual cell types may respond quite differently to the same substance. The ongoing development of *in vitro* toxicological test methods led to the advanced investigation of the potential application of new test methods as an alternative for toxicity testing of common workplace harmful substances. Therefore, the organotypic model PCLS was investigated in the present study by measuring different cytotoxic endpoints. In our workgroup, an air-liquid interface culture of murine PCLS was already established as a model system exposed to O_3 and NO_2 as model gas compounds to mimic the acute toxicity in acute lung injury [Switalla *et al.*, 2010a]. In the present study, we tried to understand mechanism of chemical-induced respiratory toxicity to mimic the toxic features of acute lung injury. The endpoints were (a) the released cytosolar enzyme lactate dehydrogenase in the culture medium, (b) the remaining mitochondrial enzyme activities in the lung tissue slices, and (c) imaging analysis by live/dead fluorescence staining.

Firstly, cell death was measured by the release of lactate dehydrogenase in the culture medium, however, accurate detection was not successful for all chemicals since exposure to a lot of chemicals failed to increase lactate dehydrogenase release due to chemical properties. In the modified protocol chemicals was exposed for one hour and changing the culture medium for further 23 hours post-incubation. Some substances led

to membrane damage during the exposure time in such amount, that a large part of the LDH was washed out together with the substance after the one hour exposure. Hence not all LDH could be determined after the post-incubation time.

Acute respiratory toxicity of test chemicals was assessed mainly by the measurement of quantified number of metabolically active cells in human and rat PCLS. Our results clearly showed that chemical-induced respiratory toxicity was concentration-dependently. Rather than single chemical concentration studies, dose-response curves of test chemicals were achieved and EC_{50} values were estimated for all tested chemicals. The most toxic chemical was glyoxal and triton X-100 among different selected test chemicals in human and rat PCLS, respectively. Glyoxal is known to attack amino groups of proteins, nucleotides and lipids. This leads to inactivation of enzymes, disturbance in the cellular metabolism, impaired proteolysis, and inhibition of cell proliferation and protein synthesis [Shangari *et al.*, 2004]. Triton X-100 is an nonionic detergent, which is mostly used for lysing cells to extract protein and other cellular organelles and also used as membrane permeabilizing agents [Koley *et al.*, 2010].

Four chemicals (trimellitic anhydride, ammonium hexachloroplatinate, sodium dodecyl sulfate, and glutaraldehyde) were assessed either in human or in rat PCLS (Table 1). The most and least toxic chemicals were sodium dodecyl sulfate and trimellitic anhydride in both species by using WST-1 cell proliferation assay, respectively. Sodium dodecyl sulfate is mostly used as anionic detergent which can totally disrupt cell membranes and denature proteins at higher concentrations [Chaturvedi *et al.*, 2011]. It was reported trimellitic anhydride exhibits low acute toxicity by the oral, dermal, and inhalation routes [OECD SIDS, 2002]. Respiratory toxicity of these four test chemicals could be translated from rat to human situation in PCLS for human toxicological risk assessments.

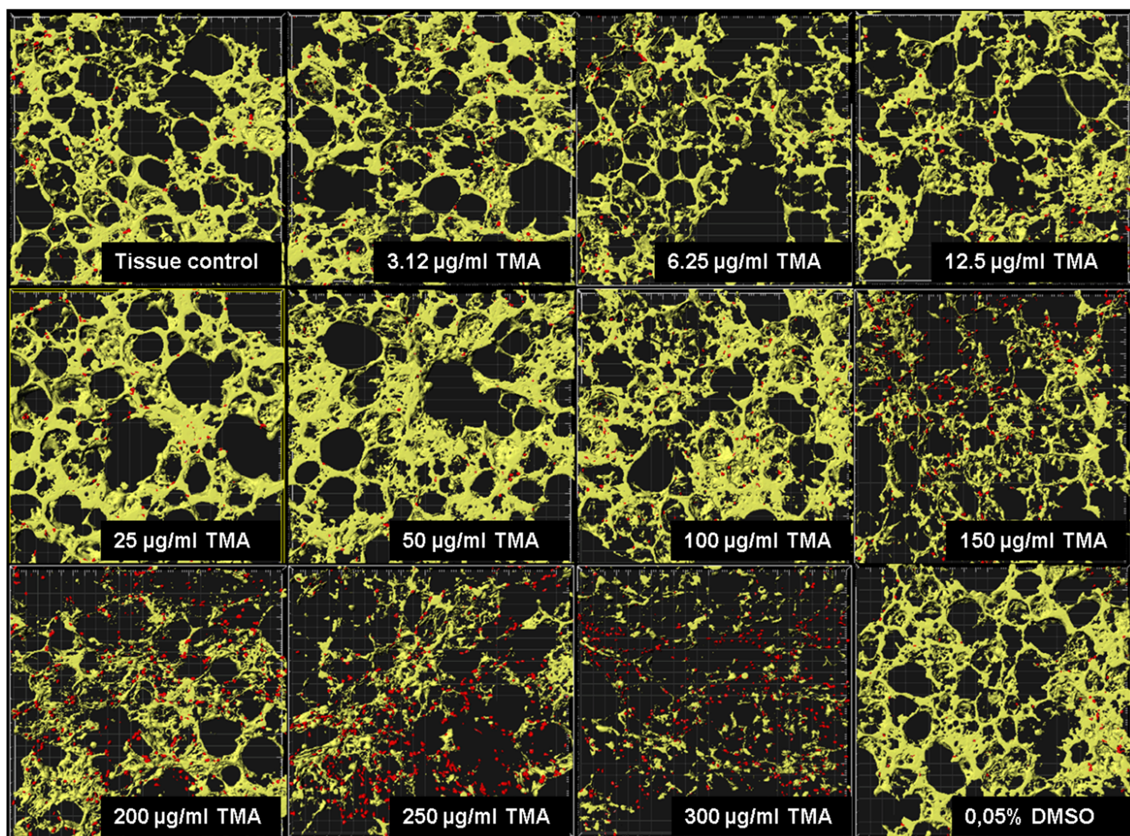
Chemical name	**CAS #**	**M [g/mol]**	**Class**	**Rat PCLS EC_{50} [µg/mL]**	**Human PCLS EC_{50} [µg/mL]**
Trimellitic anhydride	552-30-7	552-30-7	Anhydrides	3119	761
Ammonium hexachloroplatinate	16919-58-7	443.9	Metal compounds	177	193
Glutaraldehyde	111-30-8	100.1	Aldehydes	127	58
Sodium dodecyl sulfate	151-21-3	288.4	Detergents	96	55

Table 1: Four test substances were tested either in rat PCLS or in human PCLS model. Table shows for each substance: chemical name, CAS-number, molecular weight, chemical class, EC_{50} (effective

concentration at 50 % reduction of cell viability) values of chemicals were calculated on the basis of dose-response curves assessed by WST-1 reduction

In order to ensure the reliability of measured cell viability, a microscopy-based approach was chosen. Live cells were visualized by calcein acetoxymethyl (calcein AM), a lipid soluble fluorogenic diester that can passively cross the cell membrane in an electrically neutral form. Calcein AM can be converted by intracellular esterases into negatively charged fluorescent analogue [Neri *et al.*, 2001]. In cells undergoing apoptosis, early chromatin condensation could be visualized as increased signal intensity, since non-specific esterase is also present in nuclei [Böcking, 1974]. Figure 3A showed one example in mouse PCLS exposed to increased concentrations of trimellitic anhydride, a stronger signal intensity could be observed at 25 µg/mL and 50 µg/mL, which could indicate the beginning of apoptosis [Palma *et al.*, 2008]. Subsequently, cell membrane permeability increased, esterase activity decreased and ethidium homodimer (EthD-1) reacted with nucleic acids (red fluorescence), which also reflected the physical alteration in the epithelial [Gatti *et al.*, 1998]. The morphology of tissue slices could be regarded as a sufficient tool for adequate identification of cell death with regard to apoptosis. Dead cells could be quantified in percentage terms by the total volume of viable cells with the help of a software (IMARIS 7.4.0.) (Figure 3B).

A



B

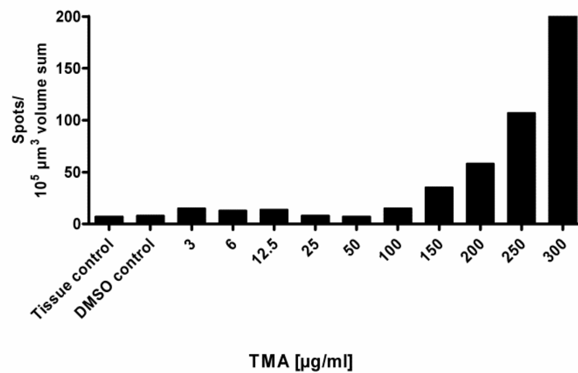


Figure 3: A) Image analysis of mouse PCLS exposed to TMA with at different concentrations (3.12 µg/ml – 300 µg/ml). The first and the last images were tissue control with DMEM and 0.5% Triton X-100 control, respectively. Tissue slices were stained with 4 µM calcein AM and 4 µM EthD-1 after 24 h of submerged cultivation; B) The images were examined by two-colour confocal laser scanning microscopy and analyzed with IMARIS 5.5.3. Red colour shows cell nuclei (Ø 5 µm) of dead cells and green colour the cytoplasm of viable cells (grid spacing = 20 µm). Concentration-dependent changes in mouse PCLS viability after cultivation with TMA. Results are given as numbers of 5 µm Ø spots (nuclei of dead cells) in 10^5 μm^3 total tissue volume (cytoplasm of living cells).

5.2 Chemical-induced toxicity in the development of non-immunologically mediated occupational asthma

5.2.1 Measurement of cytokines for the assessment of chemical-induced inflammation in PCLS model

It is known that airway epithelial cells, alveolar macrophages are capable to secret early chemoattractant and pro-inflammatory cytokines like IL-8, IL-1α and TNF-α in direct response to viral and bacterial infections, noxious gases, and sensitizing chemicals [Parker *et al.*, 2011]. Chemokine IL-8 was described recently as biomarker for identification of allergens in human promyelocytic cell line THP-1 [Mitjans *et al.*, 2008]. Unfortunately, solvent DMSO which was used to dissolve respiratory sensitizer glutaraldehyde, trimellitic anhydride, and maleic anhydride induced high amounts of IL-8 in human PCLS. The solvent was not toxic at the final concentration used, but an influence on cells cannot be excluded. Beside the influence of DMSO on cells, the human tissue reactivity could be another possible factor because 80% of the lungs were

obtained from smokers of 60 ± 10 years old donors. Therefore, IL-8 is not suitable to be used as biomarker for the screening of potential chemical sensitizers in human lung tissue. It has been shown that both early response pro-inflammatory cytokines TNF-α and IL-1 contribute to alveolar epithelial barrier dysfunction in acute lung injury [Frank *et al.*, 2008; Patel *et al.*, 2013; Pugin *et al.*, 1996]. In human PCLS, pro-inflammatory cytokine TNF-α or IL-1α was increased significantly after exposure to respiratory allergens at subtoxic concentrations, while contact sensitizers and non-sensitizing irritants failed to induce release of these cytokines to the same extent. Respiratory allergen maleic anhydride could induce neither TNF-α nor IL-1α production. The basic control used for the measurement of IL-1α was too high due to the reactivity of human lung donor mentioned before. In conclusion, production of pro-inflammatory cytokines TNF-α or IL-1α exposed to respiratory sensitizers could amplify ongoing inflammatory processes via the recruitment and activation of specific subsets of inflammatory cells as well as changes of structural cells in the form of increased smooth muscle mass, subepithelial fibrosis, vascular proliferation in the airway tissue [Laberge *et al.*, 2004; Osorio *et al.*, 2013]. However, pro-inflammatory cytokines TNF-α and IL-1α cannot be considered as biomarkers for the differentiation between respiratory and contact sensitizers. Moreover, we have seen positive results of cytotoxicity-induced increase of extracellular IL-1α in rat PCLS after exposure to acetic anhydride and ammonium hexachloroplatinate, which are known to induce respiratory inflammation. On the contrary, non-sensitizing substances such as lactose and MMA showed absolutely no effect on extracelluar IL-1α. These results encourage the vision that pro-inflammatory cytokine like IL-1α could be a valuable marker for prediction of substance induced inflammatory effects.

It has been reported that respiratory sensitizers favor association with soluble proteins whereas contact allergens preferentially bind to cellular proteins before the allergic response begins [Hopkins *et al.*, 2005]. The selective chemical-protein binding ability may indicate an early determinant of the form that allergic responses to respiratory or contact sensitizers will take place. The selective haptenation of cellular or extracellular protein by chemical allergens is associated with cytokine polarization, which was described *in vivo* that respiratory sensitizers caused statistically significant increases in T_H2-type cytokine release including IL-4, IL-5, and IL-13, whereas contact sensitizers induced predominantly T_H1-type cytokine response and mainly IFN-γ production [de Jong *et al.*, 2009; Dearman *et al.*, 1999; Mori *et al.*, 2012]. In human lung slices, typical

T_H1/T_H2 cytokine profiles could not be detected by measurement of the cytokines/chemokines production of IL-10, IL-2, IL-13, IL-5, IFN-γ, and eotaxin-2 exposed to respiratory allergens trimellitic anhydride and ammonium hexachloroplatinate compared to contact allergens and non-sensitizing irritants. It has been shown *in vitro* that enriched DC from atopic donors showed no significant alteration in either cytokine production or T cell stimulatory capacity after exposure to respiratory and contact sensitizers due to human donor variability [Holden *et al.*, 2008]. Lack of T cell responses in human tissue after transplantation is one possible explanation for sensitization deficiency to both respiratory and contact sensitizers. Additionally, loss of allergenic potential in aqueous environment like trimellitic anhydride should be considered as another cause of defect [OECD SIDS, 2002].

Interestingly, all the cytokines/chemokines including T_H1/T_H2 cytokines IL-5, IL-13, IL-10, IFN-γ, regulatory T cells (Tregs) cytokine IL-2, and eosinophil chemotactic protein (eotaxin-2) were increased significantly after exposure to ammonium hexachloroplatinate at subtoxic concentration. The hypothesis is that human lung tissue from patients had been pre-sensitized by soluble platinum salt belonged to platinum group elements (PGEs), in which the greater proportion of PGE are emitted from automobile vehicle exhaust catalysts (VECs) in the environment. PGE emissions can be deposited on road dusts consequently, it enters into water, soil, and food chain. Several studies have reported that PGE may be transformed into ions solubilized in human body fluids and tissues like hexachloroplatinate and tetrachloroplatinate which belong to the most potent allergens associated with asthma and other serious health problems in human [Konieczny *et al.*, 2013; Ravindra *et al.*, 2004; Wiseman *et al.*, 2009]. In the present study, we compared the sensitization potential of ammonium hexachloroplatinate in both mouse and human PCLS through the measurement of extracellular and intracellular production of IFN-γ, IL-10, IL-8, TNF-α, RANTES, eotaxin-2, and IL-1α. Mouse tissue was used as control without any pre-treatment. Test concentrations of ammonium hexachloroplatinate have been controlled to avoid unspecific cytokine changes regarding reduced cell viability. In mouse PCLS, cytokines remained unchanged after 24-hour exposure to ammonium hexachloroplatinate whereas significant increases of IFN-γ, RANTES, and eotaxin-2 could be detected in human PCLS. Therefore, the donors of human lung material used might have been exposed to some of the platinum group elements, which conjugated with human carrier proteins and provoked a specific immune response through the complete haptenated antigen. The

haptenated antigen could induce firstly pro-inflammatory cytokine production and subsequently circulate dendritic antigen-presenting cells and regulatory T lymphocyte functions leading production of memory T cells in the body [Burastero *et al.*, 2009]. Antigen-specific memory T cells persist at many sites in the body, infection of the lung could result the migration of a greater numbers of recall memory T cells at lung peripheral sites [Woodland *et al.*, 2009]. Moreover, it has been reported that numerous resident memory T cells are present in human lung tissues where they provide a first line of defense against secondary antigens [Purwar *et al.*, 2011]. Once the antigen specific memory T cells activated, increases of T_H1/T_H2 cytokines could be detected in the lung tissue [Ndejembi *et al.*, 2007]. Thus, the sensitization potential of the accumulation of PGEs from automobile catalysts indicates that environmental exposures to metal may pose human health risk and elicit allergenic reactions.

5.2.2 Live lung sections for the risk assessment of non-immunologically mediated occupational asthma

Non-immunologically mediated occupational asthma is dominated by an inflammatory reaction with immune cells and cytokine release aiming to eliminate the irritant chemicals [Tarlo, 2014]. RADS is one typical form of non-immunological asthma which starts with bronchial epithelial injury and release of pro-inflammatory cytokines leading to airway hyperresponsiveness [Brooks *et al.*, 2011]. The mechanism of irritant-induced asthma may be mediated by neural reflexes [Alarie, 1973; Nielsen, 1991]. Substantial properties, the concentration and the duration of exposure enable a substance to bind to and activate irritant receptors of specific ion channels of sensory neurons innervating the airways, for example irritant-sensing ion channel (e.g. TRPA1) [Caceres *et al.*, 2009; Bautista *et al.*, 2006]. The sensory nerves can secrete pro-inflammatory neuropeptides such as Substance P and cause a neurogenic inflammation at the side of contact with the chemical by stimulating different immune cells as well as epithelial cells to release pro-inflammatory cytokines [Meggs, 1994] (Figure 4). If an irritant chemical is corrosive or inhaled at a very high concentration it could also directly damage the respiratory epithelial cells and therefore enhance the potential for developing neurogenic inflammation [Lutz *et al.*, 2004]. In chapter 5.1 acute lung injury induced by chemicals could be assessed in live lung sections. As mentioned above, the pro-inflammatory cytokine TNF-α or IL-1α was increased significantly after exposure

to respiratory chemicals at subtoxic concentrations. In summary, the live lung sections could be used for the first risk assessment of toxic chemicals for non-immunological asthma at the workplace.

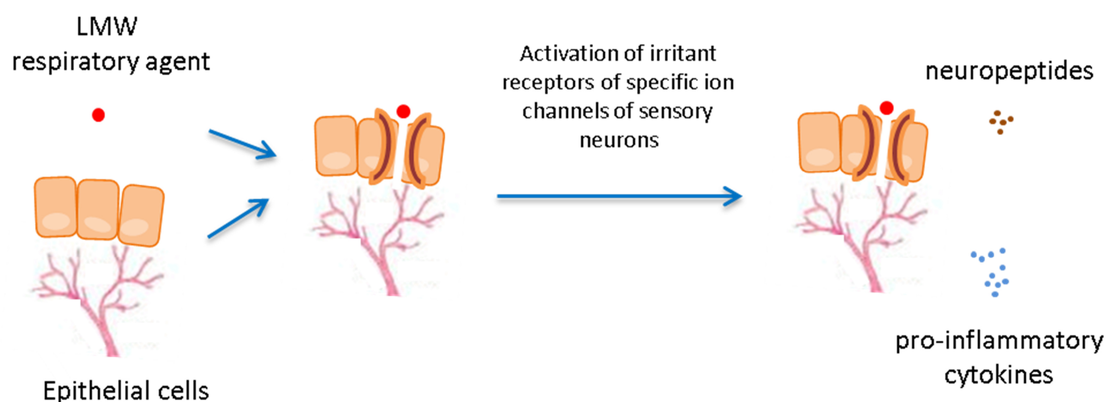


Figure 4: Irritation caused by a LMW agent. The blue pathway shows the molecular mechanism of irritant-induced neurogenic inflammation.

5.3 Predictivity of the ex vivo model PCLS for respiratory toxicology

5.3.1 Reproducibility of PCLS in rat

Generally, alternative toxicity testing methods have to be validated which is crucial for the reliability and reproducibility for the intended purpose before they can receive regulatory approval. We have investigated a standard process using rat PCLS exposed to twenty chemicals with optimized protocols in three independent laboratories. After testing of the first six substances, variability of different toxicological endpoints within and between laboratories was evaluated, in which different acceptance criteria (ACs) were defined for each different assay. Four assays including WST-1 and LDH assays for the cytotoxicity, BCA assay for the measurement of total protein in slices, and IL-1α as biomarker for inflammation have been used as endpoints in rat PCLS. After setting of protocols with defined ACs, standard operation procedures (SOPs) for all endpoints were distributed to each laboratory for further testing of the rest fourteen industrial chemicals.

Intra- and inter-laboratory variability was evaluated for each endpoint. Log10[EC_{50} (μM)] obtained for all assay endpoints showed best intra- and inter-laboratory consistency for the data obtained by WST-1 and BCA assays, in which WST-1 assay

was supported to be the best suitable analysis for cytotoxicity. The reason is that BCA assay was less sensitive to cytotoxicity shown by the fact that for some substances (isophorone diisocyanate, dimethylformamide, ethanol) cytotoxicity was found by WST-1 assay but the maximum concentration was not high enough to induce loss of total protein in the BCA assay. And LDH assay was not suited in the short-term exposure with post-incubation due to washout effect. IL-1α as biomarker for inflammation was less reproducible and sensible due to specific laboratory conditions. In summary, WST-1 assay was the best transferable assay for chemical-induced cytotoxicity and showed the least intra- and inter-laboratory variability that can provide valid and reproducible results. The test protocol used in this study is adequately transferable for practical use.

5.3.2 Correlation of PCLS to published in vitro models

The microanatomy of PCLS for the assessment of acute local respiratory toxicity induced by chemicals was comparable with *in vitro* different cell lines and 3D models. Comparison of the human PCLS with *in vitro* models showed that the EC_{25} values in human PCLS correlated significantly with the EC_{25} values in human promyelocytic cells (THP-1) and in human keratinocyte cells (NCTC 2455), in which higher value of correlation was shown in THP-1 cells than in NCTC cells, which suggested that cells in human PCLS behave more like monocyte-derived cells regarding their resistance to chemicals than skin-derived epithelial cells.

The EC_{50} values in rat PCLS correlated also significantly with two 3D models: MucilAir™ and EpiAirway™, and two other single cell lines: adenocarcinomic alveolar type II basal epithelial cells (A549) and mouse embryonic cells (3T3). Two 3D models correlated better than single cell line models, suggesting that cells in rat PCLS behave more like bronchial epithelial cells isolated from biopsies than primary human tracheal or bronchial epithelial cells.

Remarkably, the spreading of the toxicity data in all tested models has a common feature that EC_{50} values were much closer in single-cell cultures than PCLS model and 3D models. The wide range of distribution for PCLS and 3D models could also be found for *in vivo* toxicity data of inhalation toxicity studies after exposure to the same substances.

5.3.3 Assessment of in vivo relevance of PCLS model

The EC_{50} values of test chemicals in both human and rat PCLS were compared to acute inhalation LC_{50} values for rat taken from published toxicological databases. Regression analysis indicated significant correlations between *ex vivo* and published *in vivo* inhalation toxicity data for selected industrial chemicals ($p < 0.01$). Pearson's correlation coefficient with *in vivo* toxicity data from online inhalation studies was less comparable to human PCLS (Pearson $r = 0.7$) than rat PCLS (Pearson $r = 0.9$). And the toxicity in the rat PCLS model correlated also highly significantly with LD_{50} values from *in vivo* rat oral studies (Pearson $r = 0.8$). The correlations showed less variability in the same species compared to human. In principle, for *in vivo* relevant assessment it was necessary to compare and correlate the data obtained from human studies since the physiological and biochemical dissimilarities of animal models [Hartung, 2009]. For this reason, another study in our group have compared data from a clinical study performed by Schaumann *et al.*, 2008 in cytokine levels after instillation of endotoxin with data from human PCLS exposed to LPS. The calculated coefficient of correlation was 0.9, indicating high resemblance and high relevance to the "human *in vivo*" situation [Schaumann *et al.*, 2008; Switalla *et al.*, 2010b]. Therefore, the predictive capacity for toxicity classification should also be more relevant after exposure to human PCLS with further investigations of large numbers of applied chemical substances.

In summary, the obtained results from human PCLS could be used for studying the response of human lung tissue at the level of protein production, enzyme activities, and mediator release. Moreover, this information could be extrapolated to the human *in vivo* situation. The high degree of complexity provides a physiologically more relevant model for the prediction of chemical-induced toxicity in human situation which provides a big potential for ranking, screening and risk assessment of chemical toxicants [Roggen *et al.*, 2006]. The model could not completely be used for the simple screening of respiratory sensitizers for the occupational asthma since the missing connection with the blood and lymph system. However, the neurogenic inflammation of respiratory epithelial cells could be used as danger signal of potential respiratory toxicants which could lead to asthma syndromes.

6 Outlook

According to the presented data it could be shown that the multicellular microanatomy of PCLS provides a suitable model to assess chemical-induced acute local respiratory toxicity which can be used to reflect different adverse health effects and exposure scenarios. However, *ex vivo* PCLS model is limited to be used for the assessment of adaptive immune responses due to missing connections to the blood and lymph system. Early biomarkers for acute respiratory toxicity like cytokines and chemokines, functional changes of certain cell populations such as epithelial cell still need to be identified. Moreover, the use of submerged PCLS for the testing of nanomaterials and chemicals quickly reacting with aqueous solutions might be improved with regard to free contact between native atmospheres and the biological test system.

7 List of abbreviations

3D	Three-dimensional
3Rs	Refinement, reduction and replacement
ACs	Acceptance criteria
ARDS	Acute respiratory distress syndrome
AM	Calcein acetoxymethyl
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BCA	Bicinchoninic acid
B cell	Bone marrow cell
CD	Cluster of differentiation
COPD	Chronic obstructive pulmonary disease
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC_{50}	Effective concentration, 50%
EthD-1	Ethidium homodimer
GHS	Globally harmonized system
HMW	High molecular weight
IFN-γ	Interferon-gamma
IC_{50}	Inhibitory concentration, 50%
Ig	Immunoglobulin
IL	Interleukin
LC_{50}	Lethal concentration, 50%
LD_{50}	Lethal dose, 50%
LDH	Lactate dehydrogenase
LLNA	Local lymph node assay
LPS	Lipopolysaccharide
LMW	Low molecular weight
MCP-1	Monocyte-chemotactic protein-1
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NK cell	Natural killer cell

NCTC	National collection of type cultures
OA	Occupational asthma
OECD	Organization for Economic Co-operation and Development
PAMP	Pathogen-associated molecular pattern
PRRs	Pattern recognition receptors
PBMC	peripheral blood mononuclear cell
PCLS	Precision-cut lung slices
PGEs	Platinum group elements
RADS	Reactive airway dysfunction syndrome
RANTES	Regulated on activation, normally T cell expressed and secreted
REACH	Registration, evaluation, authorization and restriction of chemicals
ROS	Reactive oxygen species
SAR	Structure activity relationships
SOPs	Standard operation procedures
T cell	Thymus cell
T_H cell	T helper cell
TLR	Toll-like receptor
TG	Test guideline
TMA	Trimellitic anhydride
TNF-α	Tumor necrosis factor alpha
Tregs	Regulatory T cells
TRPA	Transient receptor potential cation channel, subfamily A, member 1
UN	United Nations
VECs	Vehicle exhaust catalysts
WST-1	Water soluble tetrazolium-1

8 Appendix I: List of publications

Switalla S., **Lauenstein L.,** Prenzler F., Knothe S., Förster C., Fieguth H.-G., Pfennig O., Schaumann F., Martin C., Guzman C. A., Ebensen T., Muller M., Hohlfeld J.M., Krug N., Braun A., Sewald K.
"Natural innate cytokine response to immunomodulators and adjuvants in human precision-cut lung slices".
Toxicol Appl Pharmacol 2010 Apr 14, Accepted

Lauenstein L, Switalla S, Prenzler F, Seehase S, Pfennig O, Förster C, Fieguth HG, Braun A, Sewald K.
"Assessment of immunotoxicity induced by chemicals in human precision-cut lung slices (PCLS)".
Toxicol in vitro 2013 Dec 24, Accepted

Wang-Lauenstein L, Hess A, Braun A, Kolle S, Landsiedel R, Liebsch M, Ma-Hock L, Pirow R, Schneider X, Steinfath M, Vogel S, Martin C, Sewald K.
"Prevalidation of the ex-vivo model PCLS for prediction of respiratory toxicology".
Toxicol in vitro 2016 Jan 07, Accepted

9 Appendix II: Curriculum Vitae

Personal details

Name:	Lan Wang-Lauenstein
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Postgraduate studies

03/2008 – 03/2013	Doctoral thesis at the Fraunhofer Institute of Toxicology and Experimental Medicine, Hannover, Germany

Undergraduate studies

10/2003 - 07/2007	Studies in biology, degree: Diplom-Biologin Gottfried Wilhelm Leibniz Universität Hannover, Germany; Studies of biology
09/1998 - 07/2002	Studies in educational biology, degree: Bachelor Tianjin Normal University, China

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09/1995 - 07/1998	Secondary school, Di Sishisan, Tianjin, China
09/1991 - 07/1995	Secondary school, Zhongshan, Tianjin, China
09/1986 - 07/1991	Primary school, Zhongying, Tianjin, China

10 Appendix III: First author´s contribution to the papers of this thesis

1. **Lauenstein L**, Switalla S, Prenzler F, Seehase S, Pfennig O, Förster C, Fieguth HG, Braun A, Sewald K. "Assessment of immunotoxicity induced by chemicals in human precision-cut lung slices (PCLS)". *Toxicol in vitro 2013 Dec 24, Accepted*
   Lan Lauenstein performed the half of the experiments concerning chemicals in PCLS, analyzed all data and wrote the manuscript.

2. **Wang-Lauenstein L**, Hess A, Braun A, Kolle S, Landsiedel R, Liebsch M, Ma-Hock L, Pirow R, Schneider X, Steinfath M, Vogel S, Martin C, Sewald K. "Prevalidation of the ex-vivo model PCLS for prediction of respiratory toxicology". *Toxicol in vitro 2016 Jan 07, Accepted*
   Lan Wang-Lauenstein and Annemarie Hess, performed the experiments concerning chemicals in rat PCLS, analyzed all data and wrote the manuscript.

11 Appendix IV: Acknowledgements

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My special and sincere thanks go to Dr. Katherina Sewald. Her unlimited patience, her constant encouraging, and personal guiding were the foundation of this thesis. The scientific support and the inspirations have been invaluable for the success of this work. I learned indescribable much and I'm very grateful for that.

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