Characterization of local respiratory irritation and inflammation after acute exposure to biological and chemical substances in PCLS

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Zusammenfassung

Die chronisch obstruktive Lungenerkrankung (COPD) und die akute Lungenschädigung/akutes Lungenversagen (ARDS) sind Erkrankungen, die aufgrund ihrer Kortikosteroidresistenz durch hohe Mortalitätsraten gekennzeichnet sind. Für das Verständnis der zugrunde liegenden immunologischen und toxikologischen Mechanismen ist die Entwicklung humanrelevanter präklinischer *in vivo*- und *in vitro*-Modelle von hoher Dringlichkeit. Ziel dieser Arbeit war es, ein Modell zu etablieren, das die Immunantwort in COPD und die vorrangig toxischen Aspekte der akuten Lungenschädigung/ARDS widerspiegeln kann. Dazu wurde die organtypische Gewebekultur Präzisionslungenschnitte (PCLS) mit den Modellsubstanzen Lipopolysaccharid (LPS), Ozon (O_3) und Stickstoffdioxid (NO_2) exponiert.

Die Stimulation humaner PCLS mit dem Toll-like-Rezeptor-4 (TLR4) Agonist LPS aktivierte das angeborene Immunsystem und induzierte eine pro-inflammatorische Immunantwort, wie sie in COPD-Patienten beschrieben wird. Zytokine wie z. B. IL-1β, MCP-1 und GM-CSF zeigten einen signifikanten Anstieg, der durch das synthetische Kortikosteroid Dexamethason inhibiert werden konnte. Dagegen bewirkte eine akute Exposition mit hohen Dosen O_3 und NO_2 vorrangig eine Schädigung des Gewebes, wie sie bei akuter Lungenschädigung/ARDS auftritt. Eine pro-inflammatorische Immunantwort konnte nicht gezeigt werden. Zytokine wie IL-12, Eotaxin, MCP-1 oder MIP-1β blieben nahezu unverändert und ein Anstieg konnte lediglich für IL-1α nach O_3-Exposition nachgewiesen werden.

Damit konnte abschließend gezeigt werden, dass PCLS ein geeignetes Model sind, um die Immunantwort in COPD und die Gewebetoxizität in akuter Lungenschädigung/ARDS als Teilaspekte dieser Erkrankungen darzustellen.

Abstract

Severe lung diseases including chronic obstructive pulmonary disease (COPD) and acute lung injury/acute respiratory distress syndrome (ARDS) result in high mortality rates with resistance to nearly all anti-inflammatory therapies. To understand the underlying immunological and toxicological mechanisms the need emerged to develop adequate preclinical *in vivo* and *in vitro* models that extrapolate the human *in vivo* situation. The aim of these studies was to provide a model reflecting the immune responses in COPD and reflecting the toxicological events occurring in acute lung injury/ARDS. For that, widely used model compounds lipopolysaccharide (LPS), ozone (O_3), and nitrogen dioxide (NO_2) were added to the organotypic tissue culture model precision-cut lung slices (PCLS).

Treatment of human PCLS with the toll-like receptor 4 (TLR4) agonist LPS activated the innate immunity resulting in a pro-inflammatory cytokine profile similar to that in COPD patients. Cytokines such as IL-1β, MCP-1, and GM-CSF were significantly increased and could be inhibited by co-treatment with the synthetic corticosteroid dexamethasone. In contrast, air-liquid interface (ALI) exposure of PCLS to the reactive gas compounds O_3 and NO_2 induced damage of lung parenchyma – described as a main feature in acute lung injury/ARDS. However, the pro-inflammatory immune response was not prominent. Cytokines such as IL-12, eotaxin, MCP-1, and MIP-1β remained unchanged. IL-1α expression was only increased upon O_3 exposure.

Concluding it could be shown that PCLS represent a suitable model to reflect immune responses in COPD, respectively tissue damage in acute lung injury.

Schlagwörter: Präzisionslungenschnitte (PCLS), Chronisch obstruktive Lungenerkrankung (COPD), akute Lungenschädigung/akutes Lungenversagen (ARDS), cytokine, toxicity

Key words: Precision-cut lung slices (PCLS), chronic obstructive lung disease (COPD), acute lung injury/ acute respiratory distress syndrome (ARDS), Zytokine, Toxizität

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Preamble

This cumulative doctoral thesis was prepared at the Fraunhofer Institute for Toxicology and Experimental Medicine (ITEM) in the Department of Immunology, Allergology and Immunotoxicology under the supervision of PD Dr. Armin Braun and Dr. Katherina Sewald. The experiments with precision-cut lung slices (PCLS) were mainly performed in the Department of Immunology, Allergology and Immunotoxicology. Human lung tissue for the preparation was obtained in cooperation with Klinikum Region Hannover. The air-liquid interface (ALI) exposure model was implemented in cooperation with the Department of In Vitro Toxicology of the Fraunhofer ITEM. *Ex vivo* experiments were performed to evaluate the potential of PCLS to reflect the immune response described in COPD and the acute lung damage occurring in acute lung injury/ARDS.

The present work refers to the following 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.
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"Effects of acute *in vitro* exposure of murine precision-cut lung slices to gaseous nitrogen dioxide and ozone in an air-liquid interface (ALI) culture".
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1 Introduction

This doctoral thesis describes the use of PCLS as a model to reflect the immunological and toxicological responses described in the lung diseases COPD and acute lung injury/ARDS, respectively. For that, widely used model compounds LPS, NO_2, and O_3 were used. Special emphasis was placed on the induction of cytotoxic or pro-inflammatory responses, which finally were compared to *in vitro* and *in vivo* data.

1.1 Homeostasis of the lung

1.1.1 Interplay of resident and migrating cell types under homeostasis

The lung plays a pivotal role in gas exchange. It is lined by a monolayer of epithelial cells with a surface of approximately 70 m^2 in adults. Each minute, 6-7.5 L of air are inhaled (Holt *et al.*, 2008). The elaborate branching network of airways with its extensive surface area is directly connected to the external environment. It is thus possible that inhaled molecules may pass the thin air-blood barrier and can rapidly be absorbed into the systemic circulation. This renders the lung also to a portal of entry into the body for a multitude of potentially harmful environmental agents (Patton and Byron, 2007). These inhaled agents comprise chemicals like irritant gases or agents of biological origin. The latter include dangerous infectious particles such as bacteria, viruses, and fungi, and non-infectious particles such as allergens, plant pollen, and animal dander (Hammad and Lambrecht, 2008). Keeping the local respiratory homeostasis is a sophisticated challenge for the organism. It involves maintenance of the integrity of the mucosal epithelial barrier and the immunological balance. In particular, cells of the immune system such as macrophages and dendritic cells (DCs), but also structural cells like epithelial or endothelial cells fulfil these requirements, to protect the lung from noxious agents and preserve the function of the organ.

Epithelial and endothelial cells are resident structural cells that are no typical effector cells of the innate immunity; but they contribute to microbial defense mechanisms by their barrier function. They form a paracellular barrier via tight junctions, thereby preventing agents and pathogens from entering the body or the systemic circulation as

applied to endothelium. Upon inhalation and successful colonization of pathogens which is followed by disturbance of the homeostasis, epithelial type II pneumocytes support inflammatory responses by the secretion of cytotoxic and anti-microbial factors. These include surfactant proteins and lysosomes or reactive oxygen species (ROS) (Kinnula *et al.*, 1992; Suzuki *et al.*, 2008). The important role of the epithelium for the interplay and for modulating the activity of adjacent cell populations can be seen, e.g., in the secretion of the cytokine IFN, which amplifies the host response to infectious particles. Produced growth factors such as granulocyte colony-stimulating factor (G-CSF) and granulocyte/macrophage colony-stimulating factor (GM-CSF) improve local survival of neutrophils and regulate DC and B cell functions (Bleck *et al.*, 2006; Cox *et al.*, 1992; Kato *et al.*, 2006). Other mechanisms are used by resident endothelial cells: They reorganize their cytoskeleton and alter the expression of surface molecules such as E- and P-selectins or integrins such as Leukocyte Function-Associated Antigen-1 (LFA-1) and Very-Late Antigen-4 (VLA-4) to actively regulate the immigration of, e.g., leukocytes and the re-establishment of homeostasis. Common to both epithelial and endothelial cells is the production of cytokines and chemokines in response to external stimuli. Depending on the stimulus, the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1α (IL-1α) as well as chemokines such as MIP-1/2, RANTES, or IL-8 are released (Suzuki *et al.*, 2008). The latter are responsible for the chemoattraction of migrating monocytes and lymphocytes (Bless *et al.*, 2000).

The function of the epithelium and the endothelium to prevent invasion of inhaled agents is supported by resident but also migrating immune cells belonging to the innate immunity and regulating the primary immunological balance. These are leukocytes including macrophages and DCs which link the innate and the adaptive immunity. They possess the capability of regulating immune responses by, e.g., production of pro- and anti-inflammatory cytokines thus balancing lung homeostasis. Both cell types are located in interstitial tissues and alveolar spaces. Macrophages are mainly located on mucosal surfaces whereas DCs are below mucosal surfaces (Lohmann-Matthes *et al.*, 1994; Veres *et al.*, 2009). Here, alveolar macrophages phagocyte microbial components, particles, and cellular debris, and secrete proteases or ROS due to activation (MacNee, 2001; Russel *et al.*, 2002). The activation of macrophages is furthermore supported by the direct interaction with epithelial cells (Frank *et al.*, 2006). Beneath epithelial

surfaces, DCs take up antigens which have succeeded in passing the epithelial barrier and present them by major histocompatibility complex (MHC) class II molecules to naïve T cells in the lymph nodes, thereby activating the adaptive immunity. However immature DCs also contribute to lung homeostasis by identifying pathogenic components. A viral infection e.g. results in the destruction of infected cells by natural killer (NK) cells. The resulting production of type I IFNs leads to activation of surrounding cells such as epithelial cells. In this context, DCs support the defense mechanisms by recognition of viral nucleotides due to TLR3 ligation. The crosstalk between NK cells and maturing DCs results in an activation and cytokine production by both cell types, including NK cell proliferation and further DC maturation (Gerosa *et al.*, 2002). Macrophages and DCs both secrete pro-inflammatory mediators such as TNF-α and IL-1β as well as chemokines such as monocyte chemotactic protein-1 (MCP-1) or MIP-1. These chemoattractants induce the recruitment of cells responsible for the second phase of the immune response such as neutrophils, NK cells, and monocytes, but additionally support activation of epithelial and endothelial cells (Clark *et al.*, 2000). The release of IL-10 mediates an opposing anti-inflammatory effect which regulates the balance of pro- and anti-inflammatory cytokines, thus preventing tissue injury (Ogawa *et al.*, 2008).

An imbalance between pro- and anti-inflammatory mediators can be the result of acute but also chronic exposure to biological or chemical agents including irritating gas compounds or cigarette smoke. This disturbed homeostasis is a feature of lung diseases like chronic obstructive pulmonary disease (COPD) and acute lung injury/acute respiratory distress syndrome (ARDS).

1.2 Disturbance of homeostasis in the respiratory tract leads to the severe lung diseases COPD and acute lung injury/ARDS

1.2.1 Permanent inhalation of the main risk factor cigarette smoke can lead to COPD

Chronic obstructive pulmonary disease (COPD) is a complex inflammatory lung disease that has become a major and increasing global health problem. It is currently ranked as

the sixth leading cause of mortality worldwide (Barnes, 2007; Rabe *et al.*, 2007). According to published data of the World Health Organization WHO, approximately 210 million people suffer from COPD. As a consequence of this an estimated 3 million died of the disease in 2005. In developed countries, COPD is mainly induced by smoking; other risk factors include occupational exposure, air pollution, and especially in developing countries exposure to biomass fuels (Smith, 2000, Barnes *et al.*, 2003).

The disease is characterized by clinical and pathological outcomes such as chronic obstructive bronchiolitis, emphysema, and irreversible airflow limitation. Multiple mediators and inflammatory cells are involved and orchestrate the development of the disease. Regarding the immunological aspects of COPD, they differ between the four stages that were defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD). The early stage I is characterized by the generation of oxidative stress including, e.g., superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) (Barnes, 2004; Rahman *et al.*, 1996). Occurring breakdown of connective tissue mainly affects epithelial and endothelial cells. Degradation products such as hyaluronate and biglycan are generated and activate macrophages as well as DCs through TLR2/4 ligation. Subsequently, inflammatory mediators such as MCP-1, TNF-α, IL-1β, GM-CSF, and IL-8 are released (Barnes *et al.*, 2003; Barnes, 2004; Cosio *et al.*, 2009). The early stage is followed by the stage II, which is characterized by DC-mediated activation of $CD8^+$ T cells and T_H1 cells. DCs release IL-12 which induces STAT-4-dependent production of IFN-γ in $CD4^+$ T cells. The amount of activated T cells correlates with the degree of airflow limitation in COPD. $CD8^+$ cytotoxic T cells are the predominant cells in the chronic stages III and IV. Their presence correlates with the number of apoptotic cells and the degree of emphysema and airflow obstruction (Cosio *et al.*, 2009). In these stages, cells die of necrosis and apoptosis induced by the release of proteolytic enzymes such as perforin or granzymes A and B (Chrysofakis *et al.*, 2004; Lieberman, 2003). The chronic pulmonary inflammation also implies in systemic effects. These include loss of muscle function and muscle mass, especially in the progressive course of the disease. Furthermore, an increased number of neutrophils and activated leukocytes as well as increased cytokine production can be detected in the peripheral blood (Oudijk *et al.*, 2003).

1.2.2 Widely used model compounds such as LPS and cigarette smoke mimic COPD in animal and cell-based models

Substantial knowledge of the clinical and pathological outcomes of the disease and its progression has been obtained from studies with COPD patients (Dekhuijzen *et al.*, 1996; Dentener *et al.*, 2001; Tumkaya *et al.*, 2007). Thereby, the impact of the disturbed balance between oxidants and antioxidants with regard to the development of emphysema has been demonstrated (Dekhuijzen *et al.*, 1996). In particular, the released oxidants O_2^- and H_2O_2 exceeded the amounts of endogenous enzymatic antioxidants such as catalase, superoxide dismutase (SOD), and glutathione peroxidase (Barnes, 2004). Anti-inflammatory corticosteroids, however, were ineffective in redressing homeostasis and in protecting the lung from disruption (Culpitt *et al.*, 1999).

These clinical and pathological outcomes are the basis of most experimental exposure models that are performed in animals such as rats (Lee *et al.*, 2005), rabbits (Terashima *et al.*, 1997), guinea pigs (Wright and Churg, 1990), monkeys (Plopper and Hyde, 2008), and mice (Shapiro *et al.*, 2003). Different components are being used to elicit parts of the disease: e.g., tissue-grading enzymes such as papain or neutrophil elastase (Fox and Fitzgerald, 2009; Stone *et al.*, 1993), coal mine dust (Coggon and Newman, 1998), lipopolysaccharide (LPS) (Brass *et al.*, 2008), and, most importantly, cigarette smoke. Chronic exposure (> 6 months) of mice and guinea pigs to cigarette smoke produces lesions that morphologically resemble human emphysema of the chronic stages III and IV (Shapiro *et al.*, 2003; Wright and Churg, 1990). The same lesions could be generated by instillation of the plant protease papain, human neutrophil elastase, and LPS (Brass *et al.*, 2008; Fox and Fitzgerald, 2009). Systemic outcomes such as an increased number of granulocytes in the blood or stimulated bone marrow that are similar to those in humans could be shown in rabbits in response to cigarette smoke (Terashima *et al.*, 1997). The direct comparison of LPS-induced human COPD models to animal models revealed that in both humans and animals, an inflammatory response is evoked (Hohlfeld *et al.*, 2007; Vernooy *et al.*, 2002). Although cell types or the magnitude of effects differed, pro-inflammatory cytokines such as TNF-α, IL-6, or IL-18, which are present, e.g., in the early stage I, were released in all these models (Hohlfeld *et al.*, 2008; Jagielo *et al.*, 1996; Vernooy *et al.*, 2002).

The background of the cigarette smoke-induced cytokine production, especially the influence of mitogen-activated protein kinase (MAPK) or the transcription factors activating protein-1 (AP-1), extracellular signal-regulated protein kinase ERK1/2, and NF-κB, was additionally elucidated in cell culture models. In particular, THP-1 cells, DCs, human lung macrophages, and bronchial as well as alveolar epithelial cells were investigated (Birrell *et al.*, 2008; Demirjian *et al.*, 2006; Hellermann *et al.*, 2002, Vassallo *et al.*, 2005). In isolated macrophages and parenchymal explants directly received from COPD patients the immune responses regarding expression of IL-1, TNF-α, IL-5, GM-CSF, and IL-10 upon LPS and dexamethasone treatment were analyzed (Culpitt *et al.*, 2003; Hackett *et al.*, 2008). Unresponsiveness to dexamethasone in alveolar macrophages from COPD patients, as it is known in patients, could be confirmed. Furthermore, a reduced ability to respond to stimuli, killing of bacteria, and phagocytosis was found (King, Jr. *et al.*, 1988).

In all above mentioned studies on COPD, it became clear that it is a disease which is based on a chronic pro-inflammatory immune response regulated by the interplay of immune cells. In cell cultures it is nearly impossible to investigate immune reactions that are predicated on cell interactions. However, these interactions and the physiological structure can be displayed in 3D lung tissue culture models which comprise all cell types of the respiratory tract. Such models additionally offer the possibility to study toxic events occurring, e.g., in lung diseases that are mainly characterized by an acute toxicological response with a diffuse uncoordinated inflammatory response in the background. These lung diseases are acute lung injury and ARDS.

1.2.3 High-dose exposure to chemicals and toxic agents leads to acute lung injury/ARDS

Acute lung injury or the severe form ARDS are lung diseases characterized by devastating disorders of the pulmonary immune system. In these diseases, a diffuse damage to pulmonary parenchyma occurs within hours to days. Responsible insults are divided into direct and indirect insults. Direct insults comprise high-dose exposure to chemicals, inhalation of toxic drugs or smoke. Indirect or extra-pulmonary insults

include severe sepsis, acute pancreatitis, and trauma (Tsushima *et al.*, 2009; Wang *et al.*, 2008). In the USA, an estimated 190,000 cases are described each year with an in-hospital mortality of approximately 40% (Rubenfeld *et al.*, 2005). The main causes of the high mortality rate are emerging systemic disorders such as systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS), or multiple organ failure (MOF). A common feature of all forms of acute lung injury and ARDS is increased alveolar membrane permeability owing to endothelial and epithelial disruption and a diffuse inflammation in the pulmonary parenchyma.

In general, the disease is characterized by two major phases: the acute exudative phase and the later fibroproliferative phase. The exudative phase occurs within 12-72 hours and is associated with edema and increased permeability in endothelial cells and type I pneumocytes. Consequently, the structural barrier is lost, resulting e.g. in an electrolyte imbalance. Multiple pro-inflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-α and ROS are released, surfactant activity is impaired by the disruption of type II pneumocytes, and the blood flow is disturbed by thrombus formation leading to hypoxemia. In the fibroproliferative phase, fibroblasts proliferate and differentiate, arteries are destructed, and finally consolidation and fibrosis of the pulmonary parenchyma become apparent (Tsushima *et al.*, 2009).

1.2.4 Model compounds LPS and irritant gases O_3 and NO_2 mimic acute lung injury/ARDS in animal and cell-based models

Human *in vivo* studies performed with LPS as a main inducer of acute lung injury/ARDS are critical, especially with regard to arising systemic disorders (Andreasen *et al.*, 2008). For this reason, the study of endotoxin-induced inflammation and lung injury in humans is limited to few models. Administration of endotoxin into the whole lung was used to study pulmonary function (Michel *et al.*, 1997; Rylander *et al.*, 1989). Intrapulmonary local segmental challenges with LPS made it possible to determine local lung inflammation by quantifying immigrated cells such as neutrophils and pro-inflammatory cytokines such as IL-1β, TNF-α, IL-8, and G-CSF (O´Grady *et al.*, 2001). Similar results were obtained after intravenous administration of low-dose LPS. Low-grade systemic inflammation indicated by increased release of pro-

inflammatory cytokines could be measured (Erikstrup *et al.*, 2006; Starkie *et al.*, 2003), but the application of higher doses of LPS was too critical so that severe sepsis could not be imitated. Besides LPS, further direct insults such as ozone were administered to assess induced lung injury and impaired pulmonary function in humans (Samet *et al.*, 2001).

Given the inadequacy of human models, multiple models of acute lung injury and ARDS as well as endotoxemia models for systemic inflammation were generated in mice, rats, pigs, hamsters, sheeps, and rabbits (Beutler *et al.*, 2008; Doherty *et al.*, 1992; Gonzalez *et al.*, 1996; Heinzel *et al.*, 1994; Howard *et al.*, 1993; Meulenbelt *et al.*, 1994; Peng *et al.*, 2004). In most cases, LPS was used to elicit a similar phenotype of the disease, but additionally further direct insults such as acid, oleic acid, bleomycin, saline lavages, or the irritant gases ozone (O_3) and nitrogen dioxide (NO_2) were applied (Januszkiewicz and Mayorga, 1994; Kenyon *et al.*, 2002; Lehnert *et al.*, 1994; Matute-Bello *et al.*, 2008).

The above mentioned insults have been selected to reflect local and systemic features of acute lung injury/ARDS. While acids disrupt the alveolar barrier accompanied by neutrophil infiltration, bleomycin causes inflammatory injury and fibrosis (Matute-Bello *et al.*, 2008). LPS-induced acute lung injury/ARDS models allow the investigation of pulmonary hypertension, changes in dynamic pulmonary compliance, and pulmonary edema (Gonzalez *et al.*, 1996). Microvascular permeability based on disruption of the epithelial and endothelial barriers and inflammation were studied after 6 and 24 hours, time points that play an important role in the exudative phase of the disease (Peng *et al.*, 2004). Additionally released cytokines such as IL-1β, TNF-α, MCP-1, and IL-8 have high relevance in acute lung injury (Bao *et al.*, 2010; Xu *et al.*, 2010). O_3-/NO_2-based acute lung injury/ARDS models were used to investigate time courses of cells such as macrophages or neutrophils, edema, epithelial cell damage, the role of inducible nitric oxide synthase, and imbalanced glutathione metabolism (Bassett *et al.*, 1988; Hochscheid *et al.*, 2005; Kenyon *et al.*, 2002; McElroy *et al.*, 1997). Inhalation of very high and toxic amounts (up to 250 ppm) of NO_2 gave insight into the dependence and balance between exposure concentrations and exposure time in lung injury and development of edema (Lehnert *et al.*, 1994). Systemic outcomes including multiple organ failure and multiple organ dysfunction have mostly been investigated in animal

models of endotoxemia (Angelova *et al.*, 2004; Beutler *et al.*, 2008). In these studies, the role of cytokines such as IFN-γ, TNF-α, and IL-10, or the chemokines MCP-1 and RANTES in systemic inflammation were investigated during lethal LPS-induced endotoxemia (Angelova *et al.*, 2004; Heinzel *et al.*, 1994; Howard *et al.*, 1993; Wysocka *et al.*, 1995).

The importance of the occurring endothelial and epithelial barrier disruption as well as the production of cytokines and proliferation of fibroblasts in the progression of the disease is incontestable. Therefore, the underlying mechanisms have been investigated in *in vitro* models including alveolar epithelial cells, macrophages, and neutrophils (Geiser, 2003; Geiser *et al.*, 2004; Jacobson, 2009; McVerry and Garcia, 2005; Perkins *et al.*, 2007; Yin *et al.*, 2010). In one of these studies, alveolar epithelial repair mechanisms were investigated in an *in vitro* model of wound repair using A549 cells and lung distal epithelial cells in the presence of oxidative stress (Geiser *et al.*, 2004). Actin cytoskeletal rearrangement and cell signaling were shown to be influenced in endothelial cells (Jacobson, 2009; McVerry and Garcia, 2005) and activated macrophages were implicated to modulate inflammatory immune responses by production of TNF-α or macrophage migration inhibitory factor (MIF4) (Yin *et al.*, 2010).

Understanding the underlying mechanisms to avoid the generation of apoptotic and necrotic cells as well as an inflammatory response can eventually prevent systemic disorders, organ failure, and death (Lucas *et al.*, 2009). These mechanisms have been comprehensively studied in the presented *in vivo* and *in vitro* models to reflect COPD and acute lung injury/ARDS stages. *In vivo* animal models, however, lack human physiology, and human models are ethically unacceptable because of the systemic outcomes. *In vitro* models furthermore lack organ complexity, resulting in the use of organ-based tissue cultures.

1.3 PCLS combine organ complexity and human relevance in an *ex vivo* lung model

The limited value and inadequacy of current *in vivo* and *in vitro* lung models have led to an augmented use of tissue cultures during the last years. Major limitations concerning the suitability of a model already begin with the structure of the lung. Laboratory animals, in particular rodents, display essential differences in structure such as missing respiratory bronchioles or thickness of the epithelium, which is only one fifth that of humans (Plopper and Hyde, 2008). In addition, life expectancies are low (for rodents an average of 2 years), making it difficult to reflect lung diseases that need to develop over many years such as COPD. Furthermore, limitation of cell lines is the reflection of immune responses that are based on one single cell type. While the human bronchial epithelial cell line BEAS-2B release chemokines or growth factors such as MIP-3α or GM-CSF in response to LPS (Sha *et al.*, 2004), DCs produce T cell polarizing cytokines such as IL-12 or IFN-γ (van Riet *et al.*, 2009). Organotypic cultures thus offer the unique possibility to overcome some of these discrepancies and to investigate organ-specific effects in a three-dimensional (3D) model (MacGregor *et al.*, 2001).

Precision-cut lung slices (PCLS) constitute an organotypic tissue culture model that consists of all cell types of the respiratory tract. Basically all PCLS consist of the same cell types. But the exact composition of lung cells in PCLS mainly depends on the species. Human PCLS contain cells of non-respiratory and respiratory bronchioli which start at the fourth generation of the bronchial tree and additionally of the surrounding alveoli. Bronchi can not be prepared because of their dimension. In contrast, rodent lungs can be prepared completely due to their small dimension. Thus, PCLS start at the first generation of the bronchial tree and are composed of lobar and segmental bronchi, non-respiratory bronchioli, and alveoli. The alveolar epithelium is formed by type I and type II pneumocytes. Type I pneumocytes are mainly responsible for gas exchange and type II pneumocytes produce surfactant. The alveoli are separated by thin *septa interalveolari* that contain a compact network of capillaries, fibers of collagen, fibroblasts, leukocytes, mast cells, macrophages, DCs, contractile cells, and nerve fibers. In PCLS all these cells are situated in their physiological arrangement, thereby reflecting the functionality and morphology of the intact organ. Further differences in

the existing cell types but also in the states of activation between, e.g., humans and mice are mainly based on their contact to external antigens. In this context, humans who constantly inhale antigens possess additional immune cells in the respiratory tract, such as memory T and B cells that are lacking in naïve mice.

The method of preparing precise organ slices was initially developed in 1980 by Krumdieck *et al.*, who for the first time used a special microtome (Krumdieck *et al.*, 1980). In the 90s, Martin *et al.* refined the method and succeeded in generating very thin slices with a constant thickness of approximately 250 ± 20 µm without loss of viability, with high precision, and high reproducibility (Martin *et al.*, 1996). The low variability is mainly based on the fact that internal controls can be performed within one animal, which furthermore reduces the number of required animals and subsequently confirms the requirements for an alternative method. In the following years, the technique was established for a wide range of species such as rat, mouse, guinea pig, primate, and human (Bergner and Sanderson, 2002b; Cooper and Panettieri, Jr., 2008; Henjakovic *et al.*, 2008; Ressmeyer *et al.*, 2006; Joad *et al.*, 2009), but also for a variety of different organs such as liver, kidney, heart, and lung (Bergner and Sanderson, 2002a; De Kanter *et al.*, 2004; Pushparajah *et al.*, 2008). Vital lung slices were used for microscopic observations to investigate in particular functional responses of the airways and their contribution to outcomes of sensitization. For this purpose, bronchoconstriction as an early allergic response was determined after treatment of passively sensitized lung slices with different stimuli (Wohlsen *et al.*, 2001; Wohlsen *et al.*, 2003; Cooper *et al.*, 2009). The combination of bronchial contractility and calcium signaling was analyzed in slices with a thickness of 75 µm after addition of adenosine triphosphate (ATP) or acetylcholine (Bergner and Sanderson, 2002a; Bergner and Sanderson, 2002b). Close to the use of PCLS in pharmacological studies (Moreno *et al.*, 2006; Nassimi *et al.* 2009; Sturton *et al.*, 2008), evaluation of the biological response of PCLS to toxic chemicals has frequently been described. Xenobiotics-mediated metabolism and detoxification mechanisms have been evaluated in response to diesel exhaust particles, aroclor 1254, fuel, acrolein, solid lipid nanoparticles, and paraquat (Hays *et al.*, 2003; Lake *et al.*, 2003; Le Prieur *et al.*, 2000; Monteil *et al.*, 1999; Morin *et al.*, 1999; Nassimi *et al.*, 2009; Price *et al.*, 1995). Others have reported the use of lung slices for several infection studies with viruses and bacteria to obtain insights into

inflammatory processes or cytopathic effects (Chakrabarty *et al.*, 2007; Goris *et al.*, 2009).

For all the above mentioned approaches, PCLS display a suitable model to study immunological and toxicological issues in a tissue culture. The preservation of the pulmonary structure in PCLS and the possibility to generate lung slices from nearly all species, including humans, allow an extrapolation to the *in vivo* situation. This furthermore offers the unique possibility to reflect stages of lung diseases in a model relevant for the *in vivo* situation.

2 Hypothesis

The reflection of aspects of COPD and acute lung injury/ARDS demands adequate *in vitro* and *in vivo* models that offer the capability to extrapolate the *in vivo* situation. *In vitro* models, however, lack organ complexity and *in vivo* animal models lack human physiology. A variety of 3D models were developed imitating organ and tissue function, but to date no lung tissue culture models have been described which can reflect the immune response in COPD and the lung destruction in acute lung injury/ARDS.

Hypothesis of this doctoral thesis is that PCLS display a tissue model which reflects immunological and toxicological components of the lung diseases COPD and acute lung injury/ARDS, respectively.

For proving this hypothesis, two different aspects were investigated:

1. The potential of the organotypic lung model PCLS to provide a suitable model for mimicking the immune response in COPD was assessed. For this purpose, lung slices were exposed to the widely used model compound LPS, a component of gram-negative bacteria. Aim was to activate the innate immunity and to induce a prevailing immunological response by TLR4 ligation (Ishii *et al.*, 2008). Its immunological effects are based on the activation of NF-κB. Subsequently, an immediate release of cytokines such as TNF-α and IL-1β (Bailly *et al.*, 1990) occurs which should reflect the release of pro-inflammatory mediators described in COPD.

2. O_3 and NO_2 were applied to primarily induce toxicity of lung parenchyma which was already shown e.g. *in vitro* in epithelial cells (Ayyagari *et al.*, 2004; Bakand *et al.*, 2007) and which is described to be a main acute feature in acute lung injury/ARDS. The immunological response was expected to be weak. Very high concentrations of both gas compounds were used, since lung injury and toxication are associated with high-dose exposure to O_3 (1–5 ppm) and NO_2 (100–500 ppm). A special issue of these exposures was the application of both gaseous compounds in an ALI culture – representing a physiologically relevant exposure with an unobstructed contact of lung tissue and chemical.

3 Natural innate cytokine response to immunomodulators and adjuvants in human precision-cut lung slices

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Natural innate cytokine response to immunomodulators and adjuvants in human precision-cut lung slices

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ABSTRACT

Prediction of lung innate immune responses is critical for developing new drugs. Well-established immune modulators like lipopolysaccharides (LPS) can elicit a wide range of immunological effects. They are involved in acute lung diseases such as infections or chronic airway diseases such as COPD. LPS has a strong adjuvant activity, but its pyrogenicity has precluded therapeutic use. The bacterial lipopeptide MALP-2 and its synthetic derivative BPPcysMPEG are better tolerated. We have compared the effects of LPS and BPPcysMPEG on the innate immune response in human precision-cut lung slices. Cytokine responses were quantified by ELISA, Luminex, and Meso Scale Discovery technology. The initial response to LPS and BPPcysMPEG was marked by coordinated and significant release of the mediators IL-1β, MIP-1β, and IL-10 in viable PCLS. Stimulation of lung tissue with BPPcysMPEG, however, induced a differential response. While LPS upregulated IFN-γ, BPPcysMPEG did not. This traces back to their signaling pathways via TLR4 and TLR2/6. The calculated exposure doses selected for LPS covered ranges occurring in clinical studies with human beings. Correlation of obtained data with data from human BAL fluid after segmental provocation with endotoxin showed highly comparable effects, resulting in a coefficient of correlation >0.9. Furthermore, we were interested in modulating the response to LPS. Using dexamethasone as an immunosuppressive drug for anti-inflammatory therapy, we found a significant reduction of GM-CSF, IL-1β, and IFN-γ. The PCLS-model offers the unique opportunity to test the efficacy and toxicity of biological agents intended for use by inhalation in a complex setting in humans.

Introduction

The respiratory tract has a large surface which is in constant contact with the external environment. From an immunological point of view, inhaled air contains a wide range of antigens. Most of them are harmless, some can be dangerous in certain circumstances, e.g. allergens, and others are harmful, such as infectious bacteria or viruses. Maintenance of homeostasis in the lung thus represents an ambitious challenge for the immune system (Holt et al., 2008). Discrimination between non-pathogenic and dangerous antigens requires the identification of pathogen-specific molecular patterns. This is facilitated through so-called pattern recognition receptors (PRR), which are able to distinguish different classes of pathogenic molecules. PRRs, such as Nod-like receptors, C-type lectin receptors, or Toll-like receptors (TLRs), recognize bacterial components that have entered the lung and evoke an inflammatory response upon binding to their ligands. Such immune responses support the prevention of microbial colonization. PRRs present on immune cells can be effectively exploited to enhance the protective immunity triggered by vaccination through co-administration of antigens with their ligands, which thereby act as adjuvants.

The endotoxin LPS is a potent inducer of inflammation and is ubiquitous in the environment, e.g. in ambient aerosols (Heinrich et al., 2003), but also appears in cigarette smoke (Hasday et al., 1999). Inhalation of high amounts of endotoxins can induce severe airway inflammation and thus contributes to airway diseases such as chronic obstructive pulmonary disease (COPD). Main characteristics of such inflammations are airway remodeling, tissue damage, and often emphysema, leading to destruction of the parenchyma (Hogg and Senior, 2002; Hogg, 2004). These disease features have been described to be mediated by infiltrated neutrophils, activated macrophages, T lymphocytes, endothelial and epithelial cells (Hunninghake and Crystal, 1983). All cell types contribute to the alveolar destruction, e.g., by secreting proteases or reactive oxygen species (ROS) (Barnes

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et al., 2003; MacNee, 2001; Russell et al., 2002). Furthermore, activation of these cells results in an increased release of pro-inflammatory cytokines such as IL-1, TNF-α, IL-8, and MCP-1, eventually leading to recruitment and activation of even more cells (van Eeden and Sin, 2008).

The involvement of cytokines is a characteristic hallmark of acute and chronic inflammation and results from activation of the signal transduction pathway NF-κB. For example, ROS mediate inflammatory responses via direct contact with cells as well as by activation of the signal transduction pathway NF-κB itself (Adcock et al., 1994; Doz et al., 2008). After release of the inhibitory subunit IκB, activated NF-κB binds to DNA and thus switches on a multiplicity of inflammatory genes, resulting in an amplification of the immune response. NF-κB can also be activated by other stimuli such as UV light, cytokines, or additional immune modulators such as MALP-2.

MALP-2 is derived from *Mycoplasma fermentans* and is a potent stimulator of macrophages, dendritic cells, and B-cells, thereby inducing pro-inflammatory immune responses (Morr et al., 2002; Muhlradt and Schade, 1991; Muhlradt and Frisch, 1994; Takeuchi et al., 2000). In contrast to LPS, which acts via TLR4, MALP-2 activates NF-κB through TLR2 and TLR6 (Takeuchi et al., 2000; Takeuchi and Akira, 2001). In general, MALP-2 is used as an adjuvant coadministered with an actual antigen to enhance the humoral or cell-mediated immune response (Rharbaoui et al., 2002, 2004). In this connection, it could be shown that MALP-2 results in enhanced host defense against bacterial infection (Jorgens et al., 2009; Kerber-Momot et al., 2010; Reppe et al., 2009). Its synthetic bisacycloxypropylcysteine derivative BPPcysMPEG is conjugated with polyethylene glycol and shows improved stability and solubility. MALP-2 and BPPcysMPEG have been used for pharmacological interventions in allergic lung diseases. Their administration in these cases resulted in an augmentation and a shift towards a T_H1 immune response (Weigt et al., 2004, 2005).

The importance of regulating innate immune responses in the lung leads to the urgent need for predictive pre-clinical models. For this purpose, the development and improvement of human organotypic cultures or *in vitro* models for the prediction of pharmacological or toxicological outcomes were intensified (MacGregor et al., 2001; Martin et al., 2008; Resnick et al., 1974). We used human precision-cut lung slices (PCLS) to establish a physiologically relevant human acute inflammation model.

The rationale of the present study was to explore how closely the innate cytokine response of a human lung tissue model resembles the *in vivo* situation. Therefore, human PCLS were treated with immunomodulators to demonstrate their capability to respond to well-known adjuvants of the innate immune system. Firstly, we explored whether an LPS-induced inflammatory response in living lung tissue is also characterized by rapid accumulation of pro-inflammatory cytokines, as seen in human *in vivo* studies using segmental LPS challenge of the lung. Secondly, we characterized the innate cytokine response to the TLR ligand BPPcysMPEG. Furthermore, by using the corticosteroid dexamethasone we investigated whether any therapeutic efficacy of immunosuppressive drugs against LPS-induced inflammatory immune responses could be shown in lung slices.

Materials and methods

Media, reagents and chemicals. PBS (0.1 M sodium phosphate and 0.15 M NaCl, without Ca^{2+} and Mg^{2+}) was obtained from Lonza (Verviers, Belgium). 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, but without fetal calf serum. Medium for cultivation was prepared with penicillin and streptomycin (Sigma Aldrich, Munich, Germany). Both low-melting agarose and Earle's Balanced Salt Solution (EBSS) were also purchased from Sigma Aldrich (Munich, Germany). LPS (*E. coli*, serotype 0111:B4) was supplied in lyophilized form by Sigma Aldrich (Munich, Germany) and dissolved in PBS, pH 7.4. WST-1 was purchased from Roche (Mannheim, Germany).

Endobronchial endotoxin challenge in patients with mild asthma. The experimental setup of the human *in vivo* study is briefly summarized according to Schaumann et al., 2008. In the study described there, endobronchial segmental instillation of endotoxin (2 ng/kg) was performed in saline solution in subjects with mild asthma. The study population consisted of 17 nonsmoking subjects (nine women and eight men; mean age 28 ± 5.2 years) who were allergic to house dust mites. None of the patients received therapeutic drugs such as corticosteroids or theophylline. A second bronchoscopy with BAL of the same lung segment (6 × 20 mL saline solution pre-warmed to 37 °C) was performed 24 h later. BAL fluid samples were filtered and centrifuged. Supernatants were stored at −80 °C. Determination of cytokine and chemokine levels was performed using a multiplex assay kit (Lincoplex; Linco Research Inc., St. Charles, MO, USA) according to the manufacturer's specifications.

Human lung explant culture. The experiments performed with human lung tissue were approved by the ethics committee of the Hannover Medical School. Patients gave written informed consent. Human lung lobes were obtained from male and female patients who underwent lung resection for cancer. Only lung tissue containing no tumors 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. The number of patients has been indicated in each figure.

Preparation of PCLS and tissue cultures. Lung slices were prepared essentially as described before (Wohlsen et al., 2003; Ressmeyer et al., 2006). For preparation of human lung slices, lung lobes were initially cannulated with a flexible catheter and selected lung segments were inflated with 1.5% low-melting agarose medium solution. Agarose-inflated lungs were solidified on ice and chipped into 1-cm-thick slices. Eight-mm tissue cores were stamped and sliced into approx. 250 µm thick sections in Earle's balanced salt solution using a special microtome (Krumdieck tissue slicer; Alabama Research and Development, Munford, AL, USA). Tissue slices were washed and cultivated in Dulbecco's modified eagle's medium/nutrient mixture F-12 Ham (DMEM) with L-glutamine and 15 mM HEPES supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin. PCLS were maintained for 1 day at 37 °C, 5% CO_2, and 100% air humidity under normal cell culture conditions.

Incubation of PCLS with BPPcysMPEG or LPS, in combination or not with dexamethasone. PCLS were incubated with up to 5 µg/mL LPS, up to 50 ng/ml BPPcysMPEG, and 50 µg/mL dexamethasone in DMEM under standard submerged cell culture conditions. Tissue slices without addition of substances were incubated as controls. After incubation, culture supernatants were collected for determination of extracellular cytokine levels. PCLS were lysed with 1% Triton X-100 in PBS for measurement of intracellular cytokine levels. Samples were stored at −80 °C after addition of 0.2% protease inhibitor cocktail (Sigma Aldrich, Munich, Germany) and cytokine contents were measured either by Meso Scale Discovery (MSD) and Luminex assays or by ELISA.

Measurement of cytokines and chemokines by Meso Scale Discovery Assays or Luminex technology. Levels of the cytokines IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 (p70), IL-13, TNF-α, IFN-γ, MCP-1, GM-CSF, RANTES, and MIP-1β were determined in supernatants and lysis extracts of PCLS that were exposed to LPS, BPPcysMPEG, and dexamethasone using MSD technology or Luminex technology. For Luminex measurements, a bead-based 9-plex kit (Bio-Rad Laboratories, Munich, Germany) was used to

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measure IL-1α, IL-4, IL-5, IL-10, IL-12 (p40), TNF-α, RANTES, eotaxin, and MIP-1β, and a 4-plex kit was purchased to measure MCP-1, GM-CSF, IL-6, and IL-8. The assays were performed according to the manufacturer's specifications. Cytokines were quantified using an eight-point calibration curve constructed from the provided standard. Data analysis was performed using the Bio-Plex Manager Software version 4.0. Additionally, cytokines were quantified using MSD technology, which is based on the detection of electrochemiluminescence. A 96-well multi-spot plate for the detection of 9 human cytokines (IFN-γ, IL-1β, IL-2, IL-5, IL-10, IL-12 (p70), IL-13, MCP-1, and MIP-1β) was obtained from MSD (Gaithersburg, USA). The assay was performed according to the manufacturer's instructions. The time of incubation for standard and samples was 1.5 h. Calculation of cytokines was performed using a 4-fold serial diluted standard. Data analysis was performed using the discovery workbench software.

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), performed according to the manufacturer's specifications. The lower limit of quantification was 31 pg/mL for human TNF-α and IL-1α.

Protein determination. Protein concentrations were determined by the BCA method using bovine serum albumin (BSA) as standard (BCA Protein Assay Kit, Pierce, Rockford, IL, USA). 25 µL of sample or BSA was incubated with 200 µL BCA reagent for 30 min at 37 °C. Absorbance was measured at a wavelength of 590 nm.

Determination of lactate dehydrogenase (LDH) activity. Release of LDH is widely used to assess changes in cell culture viability. LDH is an intracellular protein that is released into the cell culture supernatant after disintegration of the cell membrane. Its activity is measured by conversion of a tetrazolium salt to formazan, which is formed proportionally to the amount of necrotic cells. Changes in LDH activity were determined in culture supernatant using a commercial enzymatic assay obtained from Roche (Mannheim, Germany). Triton X-100 (1% in PBS)-permeabilized PCLS were investigated as reference (100% dead cells). Results were calculated as percentages of the total LDH content.

WST-1 reduction. The cell proliferation reagent WST-1 is a tetrazolium salt commonly used for spectrophotometric quantification of changes in cellular viability. The assay measures metabolic activity/proliferation of cells and is based on enzymatic cleavage of WST-1 to a water-soluble formazan dye that can be detected in the supernatant of tissue cultures without further solubilization of formazan crystals. After incubation of PCLS, the medium was removed and PCLS were incubated for 1 h at 37 °C with 0.125 mL WST-1 solution per slice (diluted 1:10 in culture medium, prepared freshly). Absorbance of the formazan solution was determined at 420–480 nm with a reference wavelength of 690 nm.

Assay validation: intra– and inter–assay variations in human PCLS. Intra-assay variability was evaluated by determination of at least five replicate samples from one donor under the same exposure conditions (LPS-exposed versus non-exposed). Samples from at least ten independent patients were measured to determine the inter-assay variance. All samples received were analyzed for viability (LDH and WST-1 assay), protein content (BCA assay) as well as for pro-inflammatory cytokines IL-1α and TNF-α. Each validation sample was analyzed in duplicate in every assay. Mean values, standard deviation (SD), and the coefficient of variation (CV = SD/mean × 100%) were calculated for each sample.

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 5.5.3.

Analysis of CD68 immunofluorescent staining. PCLS were fixed in 2% paraformaldehyde and permeabilized in 0.3% Triton X-100. Blocking was performed with donkey serum. Lung slices were incubated at 4 °C overnight with 0.4 µg/mL mouse anti-human CD68 antibodies (clone KP1, Abcam, Cambridge, UK) and subsequently incubated at 4 °C overnight with 7.5 µg/mL Cy5-conjugated $F(ab')_2$-fragments of donkey anti-mouse IgG (Dianova, Hamburg, Germany). The appropriate isotypes were used as controls for staining specificity. The PCLS were mounted with ProLong Gold anti-fade to avoid bleaching of the fluorescent dyes. Staining of the surface marker was investigated by confocal laser microscopy (40× water immersion objective, 633 nm, emission filter LP 680 nm, thickness 20 µm) and quantitatively analyzed with Bitplane IMARIS 5.5.3.

Hematoxylin–eosin (HE) staining. Lung samples were fixed in 10% formalin, embedded in paraffin, and processed using the paraffin slice technique. Briefly, thin sections (3–6 µm thickness) were dyed with hematoxylin–eosin (HE staining) for routine histology. Sections were stained with hematoxylin, washed in water, differentiated in 70% alcohol containing HCl, then again washed in water, and stained with 0.5% eosin. Sections were washed in 1% acetic acid, dehydrated, cleared, and mounted for microscopy. The degree of lung damage was examined by a pathologist using light microscopy.

Quantitative image analysis with IMARIS 5.5.3. Three-dimensional fluorescence images of PCLS were quantitatively analyzed with the IMARIS 5.5.3 software. Confocal datasets with a dimension of 900 × 900 × 40 µm were processed via "surface rendering", which allowed a 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.

Statistical analysis. Data in the figures are given as means ± SEM. Statistical analysis was performed by unpaired *t*-test, one-way ANOVA Dunnett's, and Tukey 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. The number of patients is indicated in the figures. Differences between treated samples and control were considered statistically significant at a level of $p < 0.05$.

Results

Fluorescent staining reveals high viability of human lung tissue and allows the identification of target cells

Lung slices were cultured for 24 h with LPS, dexamethasone, and BPPcysMPEG and were subsequently stained with calcein AM and ethidium homodimer-1. CD68 staining was performed in fixed tissue after culturing. To estimate viability the three-dimensional structure

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of PCLS was analyzed with the IMARIS software. Viability staining revealed that submerse exposure of human lung slices to selected agents resulted in no loss of viability within a time period of 24 h (Fig. 1). Compared to the untreated tissue control quantitative analysis with IMARIS evidenced that the viability of lung slices was not affected after stimulation with LPS, BPPcysMPEG, and in combination with dexamethasone (Fig. 2). The macrophage-specific CD68 staining showed the structure and spatial arrangement of alveolar macrophages within this three-dimensional space (Fig. 1). The obtained data showed that none of the substances had toxic effects on human PCLS.

Inter- and intra-assay variances for human PCLS show variability for inflammation markers from donor to donor

Inter- and intra-assay variances were determined to assess the different baseline levels and reactions after stimulation. LPS exposure was used to assess variations in human tissue. Samples were measured each as an untreated tissue control and after LPS treatment. Each validation sample was analyzed for LDH release, WST-1 metabolic activity, and protein content as well as for pro-inflammatory cytokines IL-1α and TNF-α.

Intra-assay variances for protein content and WST-1 were less than 20%. Inter-assay variabilities ranged between 14 and 33% (Table 1). Results for ELISA showed that intra-assay variances for TNF-α were between 19 and 34%, whereas the inter-variances ranged between 9 and 46%. IL-1α reached 14 to 64% for intra-variance and 48 to 76% for inter-variance from donor to donor.

LPS triggers a dose-dependent increase in pro-inflammatory cytokines in human PCLS

Studies by Schaumann et al. (2008) were able to show that provocation with LPS induced a significant release of pro-inflammatory

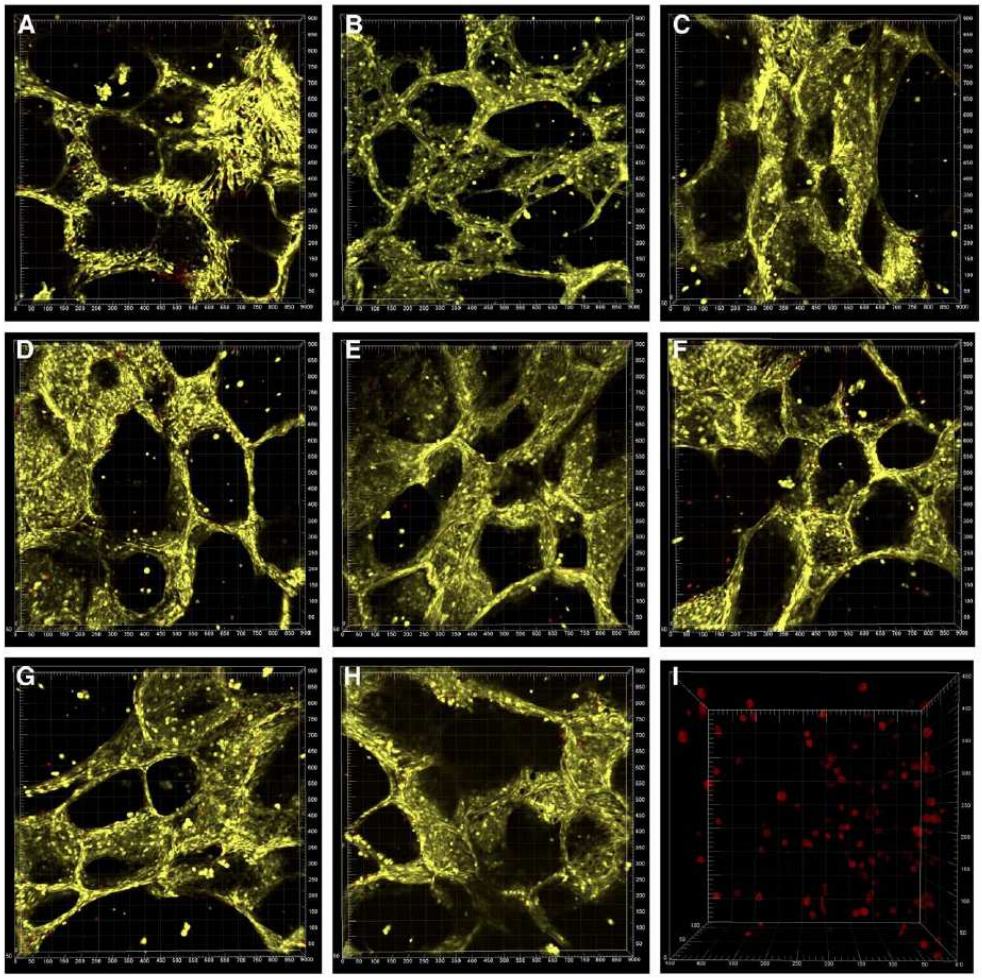

Fig. 1. Viability staining after 24 h of incubation with LPS, dexamethasone and BPPcysMPEG, and CD68 staining in human PCLS. Lung slices were treated without (A) or with 5 ng (B) or 100 ng (C) LPS, 50 μg dexamethasone (D), the combination of 5 ng LPS (E) or 100 ng LPS (F) with 50 μg dexamethasone, and 25 ng (G) or 50 ng (H) BPPcysMPEG and stained with calcein and ethidium homodimer-1. Untreated slices were stained against CD68 (I). Fluorescent staining in PCLS was detected by confocal laser scanning microscopy.

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cytokines IL-1α and TNF-α in patients (Figs. 3B/D). In these studies, the inflammatory responses in the lungs of mild asthmatics resulted in a pattern of inflammatory cells in the alveolar space after LPS challenge similar to that described by O'Grady et al. (2001) in healthy subjects. Based on these data, human (Schaumann et al., 2008) PCLS were implemented to test their ability to respond to the well-known modulator of the innate immune system LPS. To this end, human PCLS were exposed to LPS and the effects on cytokine and chemokine contents were analyzed. The LPS-induced acute inflammatory response in viable lung tissue was characterized by rapid accumulation of pro-inflammatory cytokines such as TNF-α and IL-1α, representing a fast inflammatory response of the lung tissue. LPS induced a dose-dependent and significant increase in extracellular TNF-α (Fig. 3), and this effect already started at LPS amounts of 10 ng/mL. Intracellular IL-1α was significantly increased (Fig. 3), while extracellular amounts remained lower but also exhibited concentration dependency (data not shown). Furthermore, significant increases could be detected for the chemokines MIP-1β, RANTES, and MCP-1 (Figs. 4 and 5). These increases were up to 1100% for MIP-1β, up to 720% for RANTES, and up to 670% for MCP-1. Further cytokines such as pro-inflammatory IL-1β and IFN-γ, growth factor GM-CSF, and anti-inflammatory IL-10 showed significantly increased accumulation after LPS treatment (Figs. 4 and 5). Increased values were detected for IL-1β to 1900%, for IFN-γ to 1700%, for IL-10 to 3600%, and for GM-CSF to more than 6000%.

Therapeutic intervention with dexamethasone prevents LPS-induced cytokine burst

In the present study, the anti-inflammatory steroid dexamethasone was used as an immunosuppressive drug to demonstrate the inhibition of immune responses in the human *ex vivo* model. Human PCLS were exposed to LPS with or without addition of dexamethasone, and the effect on cytokine and chemokine release was determined.

Treatment with dexamethasone significantly reduced LPS-elicited cytokine levels of pro-inflammatory cytokines IL-1β and IFN-γ, growth factor GM-CSF, and chemokine MCP-1 (Figs. 4 and 5). A similar tendency ($p<0.1$), albeit not statistically significant, could also be shown for the cytokines IL-10 (up to 65%) and IL-12 (up to 55%) and additionally for the chemokines MIP-1β (up to 50%) and RANTES (up to 50%).

Table 1
Inter- and intra-assay variances of human PCLS after 24 h of culture without (A) and with (B) LPS. Intra- and inter-assay variances were determined for total protein, enzyme activity, and extracellular TNF-α and intracellular IL-1α by using ELISA. $n=13$.

	Endpoints			
24 h	BCA protein (%)	WST-1 (%)	ELISA: TNF-α (%)	ELISA: IL-1α (%)
A				
Intra CV	3–19	<7	23	19–64
Inter CV	22	24	13–46	76
B				
Intra CV	8–19	6	19–34	14–29
Inter CV	14	33	9	48

BPPcysMPEG induces a marked pro-inflammatory response

The acute inflammatory immune response to the MALP-2 derivative BPPcysMPEG was analyzed after 24 h of treatment using ELISA and MSD technology to measure the amounts of cytokine produced. Indeed, treatment with BPPcysMPEG was associated with significantly elevated levels of IL-1β, IL-10, and MIP-1β and showed a maximum of immune stimulation for both concentrations tested (Fig. 6). In contrast, IL-1α, TNF-α, and IFN-γ were not significantly influenced by the treatment. Apart from the pro-inflammatory response, T-cell cytokines such as IL-2, IL-5, and IL-13 were determined and found to be unchanged after stimulation with BPPcysMPEG (data not shown).

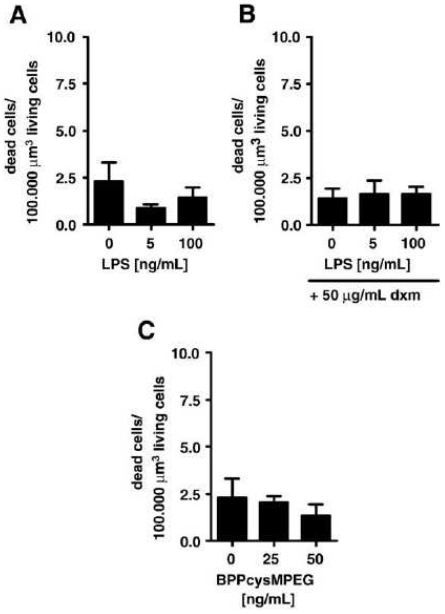

Fig. 2. Quantitative analysis of vitality staining of human PCLS incubated with LPS, dexamethasone and BPPcysMPEG. Three-dimensional images of PCLS, stained with calcein and ethidium homodimer-1 and generated by two-color immunofluorescence confocal microscopy, were analyzed with IMARIS 5.5.3. Viability of PCLS is expressed as quantity of spots (>5 µm diameter) in 10^5 µm³ green tissue volume. $n=3$.

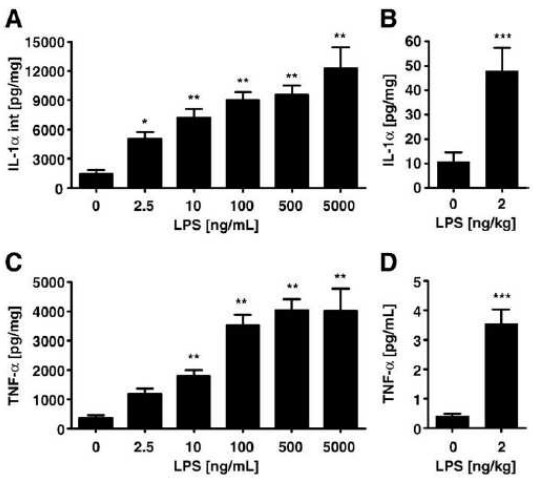

Fig. 3. IL-1α and TNF-α in human PCLS (A, C) and BAL fluid from asthmatic patients after provocation with LPS (B, D). TNF-α levels in culture supernatants and IL-1α levels in lysates were determined by ELISA in human PCLS after 24 h of culture. Cytokine amounts in BAL fluid were determined using Luminex technology (Schaumann et al., 2008). Data are presented for PCLS as mean ± SEM, $n=12$, $^*p<0.05$; $^{**}p<0.01$; $^{***}p<0.005$ (one-way ANOVA Dunnett's test for PCLS, *t*-test for BAL).

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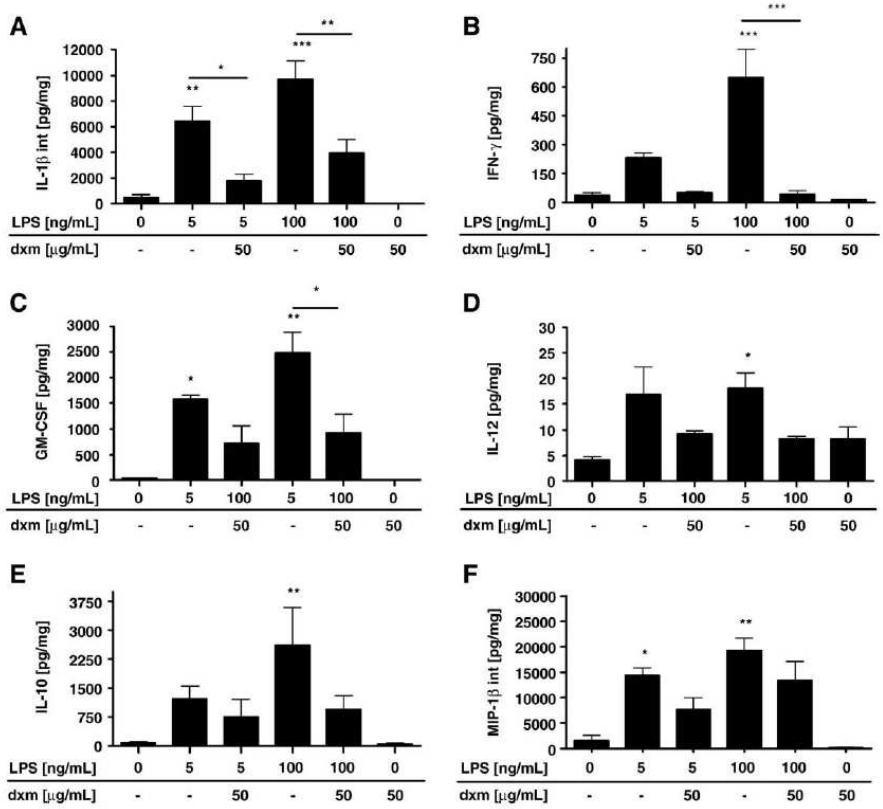

Fig. 4. Total production of GM-CSF, IL-12, IL-1β, IL-10, MIP-1β, and IFN-γ in PCLS after 24 h of treatment with LPS and dexamethasone. Cytokine levels in culture supernatants and after lyses were determined by MSD technology. Data are presented as mean ± SEM, $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.005$ (GM-CSF, IL-12, IFN-γ, and MIP-1β: $n=3$, IL-1β and IL-10: $n=6$). dxm: dexamethasone, int: intracellular.

Comparison of endotoxin-induced cytokine burst in vivo and ex vivo

To ascertain the degree of correlation of the results obtained in PCLS with *in vivo* results, the data from an earlier clinical study from our laboratories (see Schaumann et al., 2008) were utilized in linear regression analysis models. Cytokine levels in BAL fluid from subjects who had undergone segmental instillation of 2 ng endotoxin/kg body weight/10 mL resulted in an averaged concentration of 15 ng/mL used for human patients with an assumed averaged weight of 75 kg. These data were used for comparison with the results of the 100 ng/mL LPS treatment in our study to get an impression of the rate of correlation between both systems. Correlation of results generated in both studies was evaluated using a linear regression analysis model for 12 cytokines (IL-6, IL-1α, MIP-1β, IL-10, GM-CSF, RANTES, IL-5, IL-2, IL-13, TNF-α, IL-8, and MCP-1). There was good correlation of the results for 10 cytokines (IL-6, IL-1α, MIP-1β, IL-10, GM-CSF, RANTES, IL-5, IL-2, IL-13, and TNF-α) with a coefficient of correlation of 0.931 and a slope of $y=0.0009x$ (Fig. 7). MCP-1 and IL-8 reached the upper detection limits, but as there was poor correlation between *in vivo* and *ex vivo* results, they were excluded from the calculation.

Discussion

This study addressed two major aspects: firstly, we compared the effects of bacterial LPS and BPPcysMPEG on the innate immune cytokine response of human lung tissue *ex vivo*. Our experiments clearly demonstrated the potency of LPS and BPPcysMPEG, which are considered to be TLR4 and TLR2/6 agonists with adjuvant activity, respectively, to induce a pronounced production of IL-1α, TNF-α, IL-1β, IL-10, GM-CSF, and MIP-1β in the extracellular compartment of lung cells. Although recruitment of cells such as neutrophils and lymphocytes from blood and lymphoid tissue into the lung parenchyma and vice versa cannot be studied directly, a complex sequence of events is initiated that takes place in early inflammatory processes and are responsible for immune activation. Secondly, this paper describes the feasibility of using organotypic cultures from lungs of human donors to evaluate the desired effect of drugs in the therapy of respiratory diseases. Infectious diseases and COPD in particular are related to internal or environmental exposure to LPS, thus leading to an enhanced inflammatory condition of the lungs (Kitz et al., 2008; Hill et al., 2000). At this point, the anti-inflammatory effect of dexamethasone was shown to suppress *ex vivo* the LPS-induced local pro-inflammatory response, monitored by IL-1α, TNF-α, IL-1β, GM-CSF, and MIP-1β in tissue of human donors.

In human PCLS, the initial response to endotoxin exposure was marked by a coordinated release of cytokines. Rapid release of IL-1β, MIP-1β, and IFN-γ is known to contribute to immunostimulation by activating epithelial or airway smooth muscle cells. These "early-response cytokines" are produced mainly by macrophages, epithelial cells, fibroblasts, and/or endothelial cells (Fiorentino et al., 1991;

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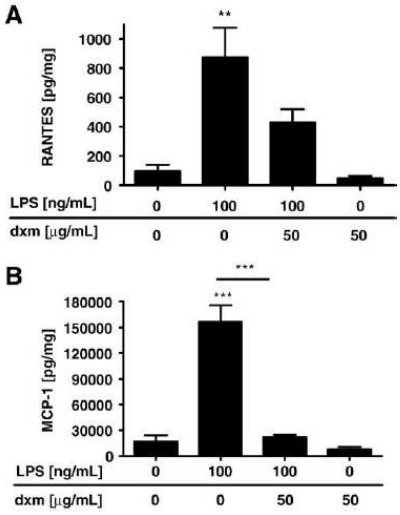

Fig. 5. Extracellular cytokine release of RANTES and MCP-1 in human PCLS after 24 h of treatment with LPS and dexamethasone. Cytokine levels in culture supernatants were determined by Luminex technology. Data are presented as mean ± SEM, $^{*}p<0.05$; $^{**}p<0.01$ (RANTES and MCP-1: $n=3$). dxm: dexamethasone.

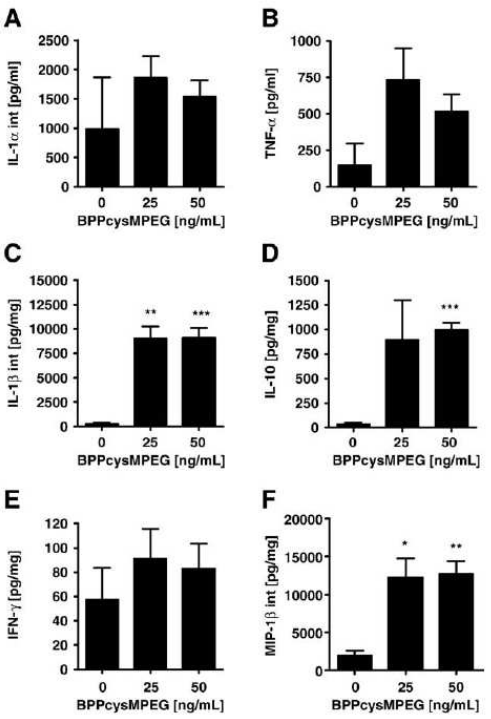

Fig. 6. Extracellular and intracellular production of IL-1α, TNF-α, IL-1β, IL-10, MIP-1β, and IFN-γ in PCLS after 24 h of treatment with BPPcysMPEG. Cytokine levels in culture supernatants and after lyses were determined by ELISA and MSD technology. Data are presented as mean ± SEM, $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.005$ (TNF-α, IL-1α: $n=2$; IFN-γ, IL-1β, IL-10, and MIP-1β: $n=3$). int: intracellular.

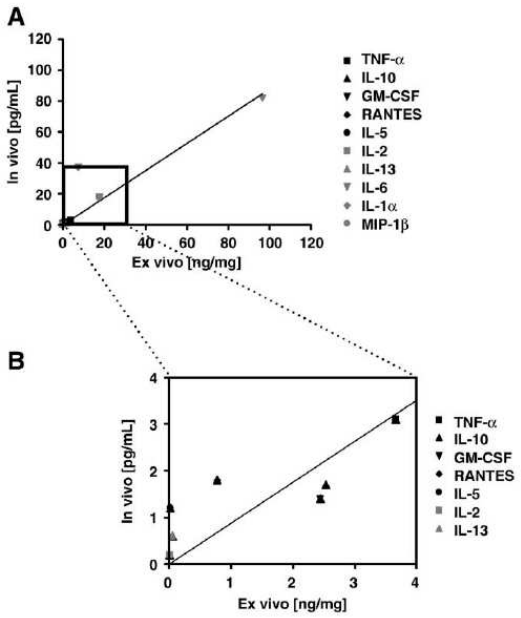

Fig. 7. Correlation of cytokine production in human lung slices and human BAL fluid from *in vivo* studies after treatment with LPS (Schaumann et al., 2008). The cytokines IL-6, IL-1α, MIP-1β, IL-10, GM-CSF, RANTES, IL-5, IL-2, IL-13, and TNF-α were correlated. The enlarged detail (B) shows cytokines of low abundance.

Olszewska-Pazdrak et al., 1998). They influence other immune cells such as macrophages or T cells, thus leading to production of further cytokines such as TNF-α or to recruitment of other cells such as neutrophils, macrophages, dendritic cells, and T cells (Bless et al., 2000; Dinarello et al., 1996; Bracke et al., 2007). Resident $CD68^{+}$ alveolar and tissue macrophages in particular are present in high numbers in lung tissue (Fig. 1). As the balance of pro- and anti-inflammatory cytokines determines the outcome of an immune response, we found not only secretion of pro-inflammatory cytokines but also of anti-inflammatory mediators, as evidenced by the significant increase in IL-10. IL-10 downregulates the expression of pro-inflammatory mediators *in vivo* and subsequently prevents tissue injury by inhibiting p38 MAP kinase activity during AP-1 signaling in antigen-presenting cells, monocytes, or macrophages (Rajasingh et al., 2006; Howard et al., 1993). Nevertheless, the strong immunostimulatory effect of LPS and, therefore, its extreme pyrogenicity has precluded therapeutic use. This has led to the search for alternative biological agents with similar immunostimulatory properties but less pyrogenicity, which would be better tolerated in humans.

Bacterial MALP-2 is a powerful lipopeptide with adjuvant activity (Rharbaoui et al., 2002). It is an activator of innate immunity and a potent inducer of localized inflammatory responses (Kaufmann et al., 1999; Knorr et al., 2008). Fibroblasts have been shown to react to MALP-2 by releasing chemokines (Morr et al., 2002), and this could also be shown *in vivo* (Deiters and Muhlradt, 1999; Rharbaoui et al., 2004). Dendritic cells responded to MALP-2 with an upregulation of genes for several cytokines involved in the induction of inflammation (Deiters et al., 2004; Weigt et al., 2003). The synthetic MALP-2 derivative BPPcysMPEG is a compound that has been demonstrated to exert immunomodulatory activity in animal models (Fuchs et al., 2009; Schulze et al., 2008; Cazorla et al., 2008; Bosch et al., 2009). In these studies, BPPcysMPEG was administered intranasally with an

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antigen-adjuvant mixture. In our study, topic exposure of human lung tissue to BPPcysMPEG also induced a specific immune response. The overall activation here was similar to LPS, but not all cytokines were elevated to the same extent by BPPcysMPEG. We clearly found qualitative and quantitative discrepancies between LPS and BPPcysMPEG. For example, release of IFN-γ was greatly different between LPS and BPPcysMPEG. While LPS enhanced IFN-γ production, activation with BPPcysMPEG did not (Figs. 4 and 6). These opposed effects might be caused by their different use of TLRs and the involved signaling pathways. LPS is known to act via TLR4 (Bihl et al., 2001), whereas BPPcysMPEG acts through TLR2 and 6 (Takeuchi et al., 2000; Takeuchi and Akira, 2001). A prerequisite for such activation is the presence in the lung tissue of TLR4 and TLR2/6 which could readily detect the LPS and MALP derivative, respectively. It has been shown that TLR4, TLR2, and TLR6 are present in lung epithelial cells (Sha et al., 2004). Stimulation of TLRs by pathogen-associated molecular patterns (PAMPs) induces several genes involved in immune responses through a MyD88-dependent signaling pathway. In this case, the signaling cascade ends with the transcription of genes via the transcription factor NF-κB (Kumar et al., 2009). On the other hand, IFN-γ is transcribed via a MyD88-independent way. This pathway is switched on by the co-produced cytokine IL-12. The binding of IL-12 to its receptor (IL-12R) leads to the activation of a signaling cascade via the transcription factor STAT4 and subsequent transcription of IFN-γ (Heinzel et al., 1994). In previous studies, the lipopeptide MALP-2 – the scaffold molecule of BPPcysMPEG – failed to induce IL-12 itself, which is an explanation for the lack of IFN-γ in human lung tissue after exposure to BPPcysMPEG (Weigt et al., 2003). In contrast, LPS triggers the production of IL-12 via TLR4, which has also been verified in living lung tissue. The biological action of MALP-2, however, can be potentiated by additional IFN-γ treatment (Weigt et al., 2004). Furthermore the lymphokine IFN-γ appears 4–5 h after the pro-inflammatory cytokines TNF-α or IL-1α, supporting the hypothesis that previously produced cytokines stimulate the release of IFN-γ in NK cells (Carrega et al., 2008; Doherty et al., 1992). Comparing the maximum cytokine releases induced by the fever inducers IL-1β and TNF-α, we observed that BPPcysMPEG induced levels of IL-1β as high as those observed for LPS. In contrast, we detected only a moderate increase in TNF-α after exposure to BPPcysMPEG — a further explanation for the lacking IFN-γ. Indeed, the parent molecule MALP-2 might be involved in the development of fever in rats (Knorr et al., 2008), but it did not lead to sepsis.

In the present study, the range of exposure concentrations selected for LPS lay between 5 ng/mL and 5 μg/mL and also comprised the concentration of 15 ng/mL which was used in clinical trials (Schaumann et al., 2008). The local pulmonary effects on the cytokine pattern *in vivo* and *ex vivo* were highly comparable (Fig. 7). On the one hand, this provides additional safety information about new leading candidate adjuvants before possible inhalation in humans. But on the other hand, it also provides the chance to assess new drugs for lung diseases in human organotypic tissue in a model of acutely inflamed lung. This point leads to the anti-inflammatory therapy of e.g. infectious diseases, inflammatory airway diseases such as COPD, and allergies. In this context, a variety of immunosuppressive drugs such as corticosteroids are frequently used to block pulmonary stress in patients. Corticosteroids such as dexamethasone in particular are known to reduce cell numbers *in vivo* by suppressing chemotactic and pro-inflammatory mediators (Barnes, 2005). Dexamethasone activates the transcription of anti-inflammatory proteins such as IκBα through the GRE promoter region and inhibits the transcription of pro-inflammatory mediators, thus inducing gene silencing (Barnes, 2005). Indeed, dexamethasone was highly effective *ex vivo* and diminished the local cytokine release of IL-1β and IFN-γ in human PCLS. It also inhibited the enhanced secretion of IL-10, RANTES, MCP-1, and MIP-1β. Therapeutic intervention with dexamethasone thus clearly prevented endotoxin-induced cytokine burst in human lung tissue.

In the present study, human tissue was obtained from patients who suffered from non-small-cell carcinoma (squamous cell lung carcinoma, adenocarcinoma, and large-cell lung carcinoma). Each patient has his or her own characteristics (i.e., sex, age, weight, and height) and medical history. In order to make this highly varying and perishable part of the model more assessable, human PCLS were prepared only from organs obtained from patients who had not received any treatment for cancer. Only macroscopically and microscopically tumor-free tissue was used (Fig. S1). Further acceptance criteria for the use of human tissue were as follows: 1) baseline release of pro-inflammatory mediators was low, 2) each experiment was performed with reference stimulation (e.g. 100 ng/mL LPS) resulting in a significant increase in TNF-α, 3) tissue with diminished total protein content was excluded, and 4) viability was controlled by e.g. WST-1 and LDH viability assays. Furthermore, the inflammatory effect of LPS on tumor tissue was determined in samples from more than ten donors. Depending on the particular parameter considered, inter-individual differences from donor to donor can exceed 20% (Table 1). These results were to be expected and reflect the genetic diversity of human cells. Nevertheless, the induction of local cytokine responses offers an opportunity to test the efficacy and toxicity of biological agents intended for administration by inhalation such as adjuvants, cytokines, monoclonal antibodies, and proteins in the most relevant species: in humans. High-risk agents in particular can be applied in doses that are high enough to achieve sufficient local concentrations and information regarding their pyrogenicity — either as a desired effect or as an adverse side effect. Although such a model may contribute to the evaluation of substances for their biological activity in humans before first-in-man clinical trials, their *in vivo* relevance must be carefully considered. It is important to highlight that this approach exhibits substantial advantages, since it is based on human lung tissue rather than on cell lines (i.e., offers the complexity of a full multicellular system embedded in a human matrix). In fact, although cell lines allow signaling pathways to be studied (Eder et al., 2009), the regulation of cytokines that are dependent on the immune response of different cells, as it was shown for IFN-γ and IL-12, will otherwise remain a mystery.

Conflict of interest statement

None of the authors has any financial interest in relation to the submission.

The Fraunhofer ITEM, however, is a public non-profit contract research institution. There are existing contracts with private and public institutions regarding the use of PCLS for testing purposes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.taap.2010.04.010.

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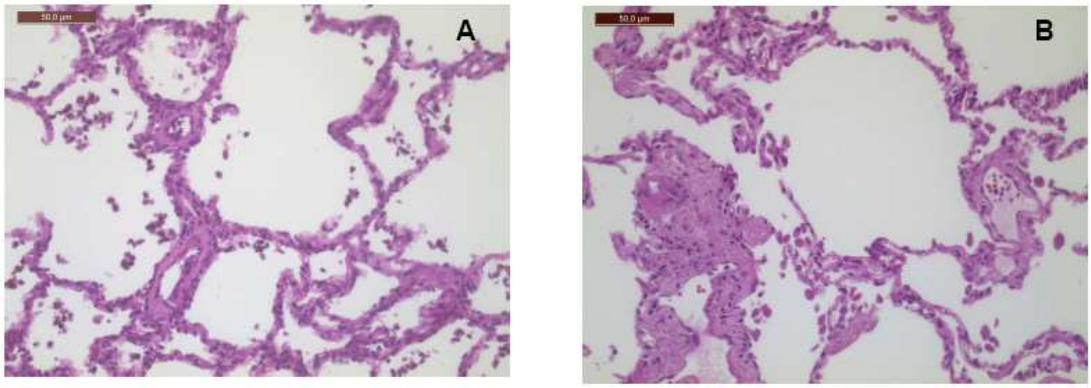
Figure S1. Histopathological sections of lung tissue received from healthy trauma patient (A) and cancer patient (B). Illustrated are lung slices stained with hematoxylin/eosin and detected by light microscopy (magnification 20×).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4 Effects of acute *in vitro* exposure of murine precision-cut lung slices to gaseous nitrogen dioxide and ozone in an air-liquid interface (ALI) culture

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Effects of acute *in vitro* exposure of murine precision-cut lung slices to gaseous nitrogen dioxide and ozone in an air–liquid interface (ALI) culture

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ABSTRACT

The aim of this study was to establish an air–liquid interface (ALI) culture of precision-cut lung slices (PCLS) for direct exposure of lung cells to gaseous contaminants. Nitrogen dioxide (NO_2) and ozone (O_3) were selected as model gas compounds. Acute pro-inflammatory and toxic effects of NO_2 and O_3 on live lung tissue were investigated. Murine PCLS were exposed to different flow rates (3–30 mL/min) of synthetic air, O_3 (3.5–8.5 ppm), or NO_2 (1–80 ppm). Tissue survived *ex vivo* in ALI culture and resisted exposure to NO_2 (1–10 ppm) and O_3 (3.5–8.5 ppm) for 1 h. Longer exposure to NO_2 resulted in a clear loss of viability, whereas exposure to O_3 was less effective. Exposure to NO_2 dose-dependently induced release of the pro-inflammatory IL-1α (40%), whereas RANTES, IL-12, and eotaxin remained unchanged. Early secretion of IL-1α (80%), RANTES (>800%), MIP-1β (44%), and MCP-1 (60%) was already detected after 1 h of exposure to O_3. The obtained data showed that direct exposure to O_3 and NO_2 induced cytotoxicity and pro-inflammatory responses in PCLS with ALI culture. This provides a model that more closely resembles *in vivo* exposure of airborne contaminants, and thus should be appropriate for toxicity testing.

1. Introduction

The lung is a major conduit for inhalable toxic substances. Inhalation toxicology serves the purpose of assessing the health hazard for humans, mostly by animal experiments (as described e.g. in the OECD guideline for testing of chemicals 403/433/436, acute inhalation toxicology, adopted May 12, 1981). The health risks have to be determined by establishment of dose–effect relationships to estimate the toxic potency of compounds. The imperative to develop alternative methods in the field of acute inhalation toxicology in the context of REACH and the 3Rs (Bakand et al., 2007; Russell and Burch, 1959, reprinted 1992) is currently the basis for the employment of live lung tissue (also called precision-cut lung slices, PCLS). With the use of PCLS as an *ex vivo* model of "acute inhalation injury" chemicals can be tested for respiratory toxicity without animal experiments.

Characteristic features of chemically induced injury to the lung include cellular changes and respiratory inflammation. Both, cellular alterations and release of inflammatory cytokines as quantifiable parameters can be reproduced in the PCLS model (Henjakovic et al., 2008; Nassimi et al., 2009). Other features of the human disease, such as changes in the histopathological picture due to chronic inflammation, cannot be studied in organotypic cultures of lung tissue. This includes the trafficking of cells from blood into the lungs during injury that cannot be assessed due to the lack of circulatory systems. Nevertheless, a model based on PCLS offers the possibility to study nearly all naturally occurring cell types of the respiratory tract situated in their physiological environment, without animal experimentation. This fact has attracted the use of the technique over the past years for purposes such as calcium signaling, analysis of processes of detoxification or bronchoconstriction (Bergner and Sanderson, 2002; De Kanter et al., 2004; Martin et al., 1996; Wohlsen et al., 2003). A common obstacle to all these *ex vivo* models is the necessity to apply soluble chemical fractions to the submerged organotypic cultures. Hence, analyzing the characteristics and mechanisms of the toxicity induced by gaseous compounds or other airborne material using an *in vitro* or *ex vivo* technique requires an air–liquid interface (ALI) exposure technique to enable free contact between native atmospheres and the biological test system. A great advantage of PCLS compared to cell lines is the chance to expose alveolar ducts and alveoli containing many cell types required for immune responses, such as macrophages, dendritic cells, endothelial and epithelial cells, directly to the gaseous compound. Contrasting with submerged treatment a wide range of insoluble chemicals can be further analyzed. Moreover, particulate matter (PM), a component of urban air pollution consisting of

Abbreviations: ALI, air–liquid interface; BCA, bicinchoninic acid; COPD, chronic obstructive pulmonary disease; CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; LPS, lipopolysaccharide; NO_2, nitrogen dioxide; O_3, ozone; OD, optical density; PBS, phosphate-buffered saline; PCLS, precision-cut lung slices.

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solid and liquid particles, can be analyzed prospectively by deposition from the aerosol phase. First experiments with diesel PM were performed in PCLS by Morin et al. and Bion et al. in a rolling system supplying slices with culture medium and additionally exposing them to complex gases alternately (Bion et al., 2002; Morin et al., 1999). These complex rolling systems can be substituted by an ALI exposure which offers the opportunity of an improved comparability to *in vivo* exposures.

Ozone (O_3) and nitrogen dioxide (NO_2) were selected as model compounds for direct exposure of lung tissue to gaseous airborne contaminants. Both gases are well known environmental oxidant air pollutants to which humans can be exposed. Exposure to high levels of O_3 (1–5 ppm) and NO_2 (100–500 ppm) is associated with lung injury and toxication. NO_2 is a precursor of photochemical smog and also leads to generation of O_3. It facilitates sensitization at least at high exposure concentrations in animal studies (Moldeus, 1993), and consequences of inhalation of lower doses (<0.5 ppm) are associated with exacerbation of asthma, COPD, or pneumonia in humans (Belanger et al., 2006; Bernstein et al., 2004; Cheng et al., 2007; Lee et al., 2007; Schelegle et al., 2003; Strand et al., 1997). Modulation of airway inflammation in asthma patients is ascribed to elevated neutrophil levels (Scannell et al., 1996), increasing epithelial permeability after exposure to those gaseous compounds, and the release of pro-inflammatory mediators IL-1α, IL-8, and TNF-α *in vitro* (Bayram et al., 2001). Exposure to O_3 leads to deciliation and disruption of epithelial cells with increased transmucosal permeability (Bhalla, 1999). Acute effects of O_3 and NO_2 at relatively high concentrations of 5 ppm are observed within 4 h in studies with mouse and rat lung alveolar type II cells, while an exposure to NO_2 induced morphological changes like shedding of epithelial cells into the airways, proliferation of these cells, or pulmonary edema, as shown *in vivo* (Hajela et al., 1990; Persinger et al., 2001).

The purpose of this study was to reproduce known biological effects of the irritant gases NO_2 and O_3 in PCLS in a gas-phase exposure system using an ALI technique. Special emphasis was placed on establishing the *in vitro* exposure of organotypic lung cultures to gaseous compounds without inducing harm to the tissue by the procedure itself. It could thus be shown that PCLS can be adapted for air–liquid interface culture. Furthermore, our results conclusively confirmed that acute exposure of live lung tissue to single high doses of NO_2 and O_3 was associated with tissue injury and inflammation, offering the chance to use lung slices in a testing approach that more closely reflects natural conditions and responses.

2. Materials and methods

2.1. Animals and husbandry conditions

Female mice (BALB/cAnNCrl, 8–10 weeks) were obtained from Charles River (Sulzfeld, Germany). Animals were kept under conventional housing conditions (22 °C, 55% humidity, and 12-h day/night cycle).

2.2. Preparation of PCLS and tissue culture

Lung slices were prepared as previously described (Held et al., 1999; Henjakovic et al., 2008; Nassimi et al., 2009; Ressmeyer et al., 2006). Briefly, animals were sacrificed with an i.p. overdose of pentobarbital-Na. Extraction of lung tissue was performed directly *post-mortem* to conserve vitality of the tissue. Lungs were filled *in situ* with 1.5% low-melting agarose medium solution. Lungs were cooled *in situ* with ice, lung lobes were separated and cut in EBSS into approximately 250-μm thick slices using a special microtome (Krumdieck tissue slicer; Alabama Research and Development, Munford, AL, USA). Tissue slices were incubated in Dulbecco's modified eagle's medium/nutrient mixture F-12 Ham (DMEM) with L-glutamine and 15 mM HEPES. PCLS were washed with DMEM for 1 h. Medium for incubation contained 100 units/mL penicillin and 100 μg/mL streptomycin. PCLS were cultured for 1 day at 37 °C, 5% CO_2, and 100% air humidity under cell culture conditions.

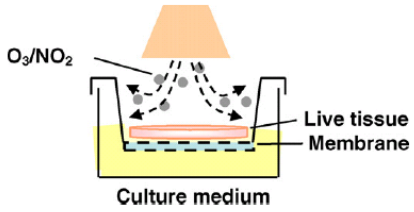

Fig. 1. Experimental set up of exposure conditions using air–liquid interface culture. Vacuum was generated to direct synthetic air, O_3, or NO_2 over the slices. PCLS were supplied with medium from below the membrane.

2.3. Media, reagents and chemicals

Pentobarbital-Na was purchased from Merial (Hallbergmoos, Germany). PBS (0.1 M sodium phosphate and 0.15 M NaCl, without Ca^{2+} and Mg^{2+}) was obtained from Lonza (Verviers, Belgium). Dulbecco's modified eagle's medium/nutrient mixture F-12 HAM (DMEM) with L-glutamine, 15 mM HEPES, and 7.5% (w/v) sodium bicarbonate, pH 7.2–7.4 was supplied by Sigma–Aldrich (Munich, Germany). Medium for cultivation was prepared with penicillin and streptomycin (Sigma–Aldrich, Munich, Germany). Low-melting agarose, Earle's Balanced Salt Solution (EBSS), and Triton X-100 were also purchased from Sigma–Aldrich (Munich, Germany). LPS (*Escherichia coli*, serotype 0111:B4) was supplied lyophilized by Sigma–Aldrich (Munich, Germany) and dissolved in PBS, pH 7.4. WST-1 was purchased from Roche (Mannheim, Germany).

2.4. In vitro exposure of PCLS using air–liquid interface (ALI) conditions

Immediately before exposure, lung tissue slices (250-μm thick) were washed with DMEM and placed onto polyethylene terephthalate (PET) membranes with pore sizes of 3 μm, 1.6×10^2 pores/cm^2, and an area of approximately 1 cm^2 (3181, Becton Dickinson, Germany).

Tissue was exposed to synthetic air (20.5% O_2 in N_2, Messer Griesheim, Germany), O_3, or NO_2 (see below) using gas flow rates of 10 mL/min per exposed PCLS. During exposure the tissue slices were fed from beneath the membrane with pre-warmed (37 °C) DMEM medium supplemented with L-glutamine, HEPES, and penicillin/streptomycin alone (Fig. 1). Changes of pH-value after exposure to gaseous compounds were not observed. The air–liquid interface exposure lasted either for 1 h or 3 h. After exposure membranes were transferred to companion plates. Fresh medium was added on top of the slices and PCLS were post-incubated for 21 h or 23 h.

Following incubation, culture supernatants were collected for determination of extracellular cytokine levels. PCLS were lysed with 1% Triton X-100 in PBS for measurement of intracellular cytokine levels. Samples were stored at −80 °C after addition of 0.2% protease inhibitor cocktail (Sigma–Aldrich, Munich, Germany), and cytokines were measured either by Luminex technology or by ELISA.

2.4.1. Exposure to nitrogen dioxide (NO_2) and ozone (O_3)

NO_2 concentrations were diluted from a stock of 100 ppm NO_2 in synthetic air using mass flow controllers for different flow ranges (Analyt, Germany) in a gas flow system. O3 was generated in situ by photolysis of synthetic air using a PenRay-lamp (Oriel Sarl, Paris) and diluted with synthetic air. Neither carbon dioxide nor any humidification was added to the test atmospheres.

2.5. Incubation of PCLS with LPS

PCLS were incubated with 5 ng/mL LPS in DMEM using standard submerged cell culture conditions. As negative control tissue slices were incubated without addition of LPS. Medium was replaced after 1 h and tissue was further incubated for 23 h in DMEM without LPS. Culture supernatant was collected, PCLS were lysed as described above, and samples were stored at −80 °C for further analysis.

After incubation, culture supernatants were collected for determination of extracellular cytokine levels. PCLS were lysed with 1% Triton X-100 in PBS for measurement of intracellular cytokine levels. Samples were stored at −80 °C after addition of 0.2% proteinase inhibitor cocktail (Sigma–Aldrich, Munich, Germany), and cytokines were measured either by Luminex technology or by ELISA.

2.6. WST-1 reduction

The viability reagent WST-1 is a tetrazolium salt commonly used for spectrophotometric quantification of cellular viability. After incubation of PCLS, the medium was removed and PCLS were incubated for 1 h at 37 °C with 0.125 mL WST-1 solution per slice (diluted 1:10 in culture medium, prepared freshly). Absorbance of the formazan solution was determined at 420–480 nm with a reference wavelength of 690 nm.

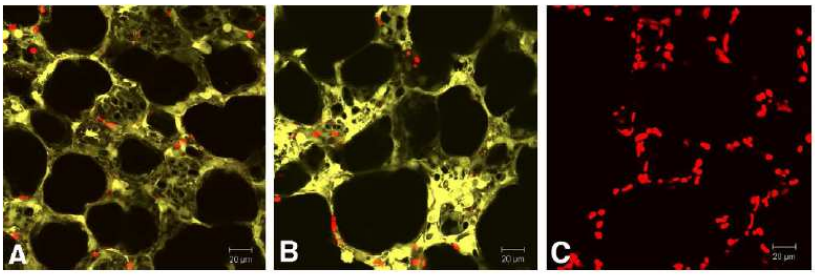

Fig. 2. Image analysis of PCLS after cultivation with different culturing systems. Tissue slices were stained with 4 μM calcein AM and 4 μM EthD-1 after 24 h of submerged cultivation (A), 24 h of air–liquid interface cultivation (B), and after cell lysis with Triton X-100 (C). The images were examined by confocal laser scanning microscopy and analyzed with IMARIS 5.5.3. Red colour shows cell nuclei (⌀ 5 μm) of dead cells and green colour the cytoplasm of viable cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2.7. Calcein AM/ethidium homodimer-1 staining

Vitality of the tissue slices was also 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 20 μm). Image stacks of a defined volume were analyzed with Bitplane IMARIS 5.5.3 (Henjakovic et al., 2008).

2.8. Quantitative image analysis with IMARIS 5.5.3

Image stacks of PCLS were quantitatively analyzed with IMARIS 5.5.3 software. In a defined volume the ratio of numbers of EthD-1-labeled cell nuclei to volume of calcein in cytoplasm of live cells was determined. Cell nuclei of dead cells were counted as spots ≥5 μm diameter, and volumes of cytoplasm of live cells stained with calcein were determined. Thresholds were set once for each channel and used for all datasets. Viability of PCLS is expressed as quantity of spots in 10^5 μm^3 green tissue volume.

2.9. Measurement of cytokines and chemokines by multiplex bead array assay

Levels of the cytokines IL-1α, TNFα, RANTES, eotaxin, MCP-1, MIP-1β, IL-6, IL-10, IL-12p40, KC, and G-CSF in supernatants and lyses extracts of PCLS exposed to O_3 and NO_2 were determined using Luminex technology. The cytokine multiplex bead array assay kit was purchased from BioRad (Bioplex cytokine assay). This kit was used according to the manufacturer's specifications. Lower limits of detection were 6 pg/mL for TNFα, 2 pg/mL for IL-1α, 5 pg/mL for RANTES, 148 pg/mL for eotaxin, 2 pg/mL for MIP-1β, 2 pg/mL for IL-10, 2 pg/mL for IL-12p40, 2 pg/mL for IL-6, 1 pg/mL for G-CSF, 3 pg/mL for KC, and 14 pg/mL for MCP-1. The measured cytokine levels of TNF-α and IL-10 were below detection limits.

2.10. ELISA: quantification of IL-1α and MIP-1β

IL-1α and MIP-1β were measured in tissue culture supernatants and lyses extracts of PCLS using commercially available enzyme-linked immunosorbent assay kits (ELISA DuoSet, R&D Systems, Wiesbaden-Nordenstadt, Germany). ELISA was performed according to the manufacturer's specifications. The lower limit of detection was 31.25 pg/mL for both cytokines.

2.11. Protein determination

Protein concentrations were determined by the BCA method, with bovine serum albumin (BSA) as standard (BCA Protein Assay Kit, Pierce, Rockford, IL, USA). Twenty-five microliters of sample or BSA were incubated with 200 μL BCA reagent for 30 min at 37 °C. Absorbance was measured at a wavelength of 592 nm.

2.12. Statistical analysis

Data in the figures are given as means ± SEM. Statistical analysis was performed by unpaired *t*-test, by One-way ANOVA Dunnett's test or by One-way ANOVA Tukey test (software: GraphPad Prism 4, version 4.03). Differences between treated samples and control were considered statistically significant at the level of $p < 0.05$.

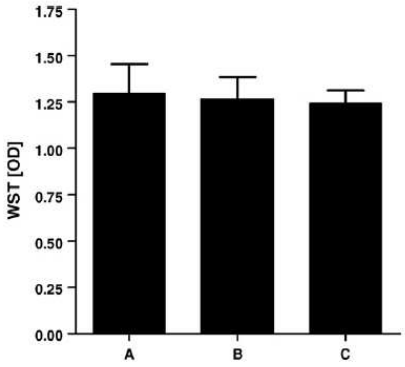

Fig. 3. Metabolic activity of lung tissue after submerged culture, and permanent ALI culture versus lung tissue exposed to synthetic air. Enzymatic activity in PCLS was measured via WST-1 assay after 24 h of submerged cultivation (A), 24 h of permanent ALI cultivation (B), and after 1 h exposure to synthetic air with 23 h submerged post-incubation (C). $n = 9$. OD = optical density at 420–480 nm.

3. Results

3.1. Air–liquid interface culture of PCLS versus submerged culture

In a first set of experiments, conditions for an ALI cultivation of murine lung slices on microporous membranes were defined for further use in exposures to gaseous compounds. Viability of lung tissue should not be affected by the procedure itself. Therefore, a membrane with a defined pore size and density was chosen which allowed a humidified microclimate around the tissue and enabled nutrification from the basal side of the membrane alone (Fig. 1).

According to quantitative image analysis, viability of ALI-cultivated PCLS remained stable at about 100% compared to standard submerged cultivation, indicating no progressive cellular damage during the first 24 h of exposure. Calcein AM/EthD-1 staining demonstrated tissue slices with alveolar walls remaining intact, whereas cytotoxic Triton X-100 clearly increased the number of dead cells (Fig. 2). Similar results were obtained for metabolic activity of lung tissue after submerse and ALI culture (Fig. 3). Here, WST-1 assay showed no differences of mitochondrial enzyme activity between tested culture conditions. Hence, live lung tissue tolerated ALI cultivation using these conditions and was compatible with a culture situation needed for an *in vitro* exposure of lung parenchyma to gaseous compounds.

While viability was unaffected by the mode of cultivation, it needed to be demonstrated that the tissue was still able to release

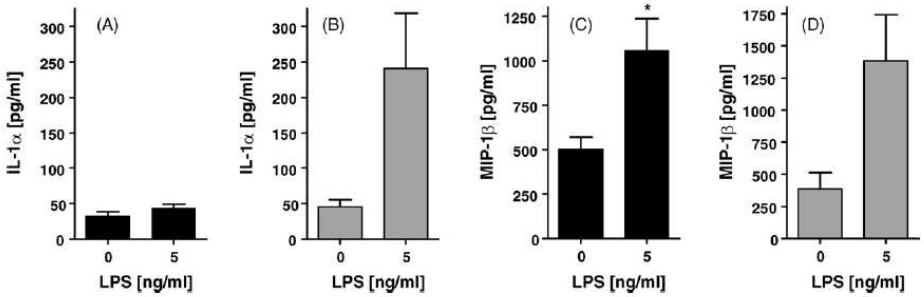

Fig. 4. Extracellular and intracellular accumulation of IL-1α (A and B, respectively) and MIP-1β (C and D, respectively) in PCLS after 1 h air–liquid interface cultivation followed by 23 h post-incubation with 5 ng/mL LPS (one PCLS/insert). Cytokine levels in culture supernatants were determined by ELISA. Data are presented as mean ± S.E.M. IL-1α: 0 ng/mL LPS: $n = 5$, 5 ng/mL LPS: $n = 8$, MIP-1β: $n = 4$, $^*p < 0.05$ (unpaired t-test).

soluble mediators such as pro-inflammatory cytokines. Therefore, PCLS were ALI-cultivated and post-incubated in the presence of LPS. Treatment with 5 ng/mL LPS induced a strong increase in the intracellular pro-inflammatory cytokine IL-1α and the chemokine MIP-1β (Fig. 4). Extracellular MIP-1β was also elevated, whereas the extracellular concentration of IL-1α remained constantly low. After continuous air–liquid cultivation of PCLS in the presence of LPS, cytokine concentrations in the basal medium were below the lower limit of detection.

3.2. *In vitro exposure to synthetic air: influence of the gas flow rate on tissue viability*

PCLS were exposed to synthetic air (20.5% O_2 in N_2) using flow rates between 3 and 30 mL/min to investigate the effects of the gas flow rates on tissue viability. The test atmospheres were not humidified and no carbon dioxide was added. Control tissue was either not exposed ("submerged control") or exposed for the same time under the same conditions but without gas flow ("air–liquid interface").

Viability of the exposed tissue was unaffected at gas flow rates from 3 to 10 mL/min. The number of dead cells (EthD-1$^+$ cells) and the volume of live cells (calcein$^+$ cells) were similar to those observed with no flow, that is 'air–liquid interface' culture (Fig. 5A–C). However, by using a flow rate of 30 mL/min per microporous membrane the ratio between stained cell nuclei of dead cells and stained living cytoplasm doubled after 1 h of exposure from 5.5 to 13.1 compared to a flow rate of 3 mL/min (Fig. 5D). Production of MIP-1β and IL-1α remained absolutely unchanged over four experiments at all flow rates. Furthermore, variability of repeated experiments regarding the production of cytokines was between 14% and 30%. These results show that a constant flow exposure of PCLS with direct contact of tissue and test atmosphere is possible. A gas flow rate of 10 mL/min was used for each of the subsequent experiments.

3.3. *Effect of in vitro exposure to NO_2 and O_3 on PCLS*

Live lung tissue of mice was exposed to synthetic air (exposure control), NO_2, or O_3 (both in synthetic air) using the ALI technique. Quantitative analysis of live/dead stained tissue revealed that 1 h of exposure resulted in no changes of viability. But exposure to 80 ppm NO_2 for 3 h, however, resulted in decreasing amounts of calcein stained parenchyma and more necrotic cell death (as evidenced by bright nuclear fluorescence of orange/red Eth-D1 stain). For O_3 we tested concentrations between 3.3 and 8.5 ppm. An effect on viability of the tissue could be observed after 3 h of exposure to 8.5 ppm O_3 (Fig. 6, Figs. S1 and S2).

Exposure to 10 ppm of NO_2 modulated the inflammatory response of live lung tissue and induced a dose-dependent trend for release of the pro-inflammatory cytokines IL-1α (40% at 10 ppm/1 h), whereas RANTES (Fig. 7), IL-12, eotaxin, and MCP-1 remained more or less unchanged at approximately 25 pg/mg for IL-12, 300 pg/mg for eotaxin, and 25 ng/mg for MCP-1. Changes between the control and the highest concentration lay at a maximum of approximately 30% for all cytokines. Brief exposure to O_3 also induced a significant increase in the pro-inflammatory cytokine IL-1α and a more than 8-fold increased expression for RANTES (Fig. 8). MIP-1β and MCP-1 were elevated dose-dependently (MIP-1β 44% and MCP-1 60%), while IL-12 and eotaxin remained nearly unchanged at approximately 25 pg/mg for IL-12 and 1030 pg/mg for eotaxin.

4. Discussion

The application of an *in vitro* exposure model was developed and evaluated for testing of respiratory inflammatory mediators and toxicity induced by airborne contaminates. Gaseous compounds have been subject to various toxicological studies *in vitro* using different experimental setups. Some setups included submerged cultures with the test gas directed over the adherent cells by shaking them at certain angles so that the cells oscillated between exposure and immersion in a culture medium (Bion et al., 2002; Bombick et al., 1998; Rusznak et al., 1996). Others passed the gas over the cell suspension (Ayyagari et al., 2007). In our studies exposure of PCLS to NO_2 and O_3 was performed on microporous membranes using ALI conditions combined with a continuous flow of the test atmosphere to each individual slice to favor an unobstructed contact of PCLS and the gas phase. In contrast, a submerged exposure situation always leads to intensive mixing of the test gas and the culture medium. Thus, the gaseous compound reacts with components of the culture medium and direct contact with cells is hindered. Therefore, such a testing strategy would document the toxicity of the locally generated reaction products of the test gas and the culture medium, rather than represent the toxic properties of the gas itself (Devalia et al., 1993; Tarkington et al., 1994). Moreover, the use of isolated or cultured cells includes several disadvantages such as changing phenotypes (e.g. activation of dendritic cells) as a result of cultivation (Swanson et al., 2004) and a lack of connective tissue that allows interaction and communication between cells. PCLS possess these capabilities and represent a unique and promising biological test system providing a useful tool to study the complexity of gas-induced lung alterations *in vitro*.

In the present study, particular attention was paid to efficient adjustment of the exposure conditions for complete preservation of the nature of living tissue. The lung tissue stayed viable dur-

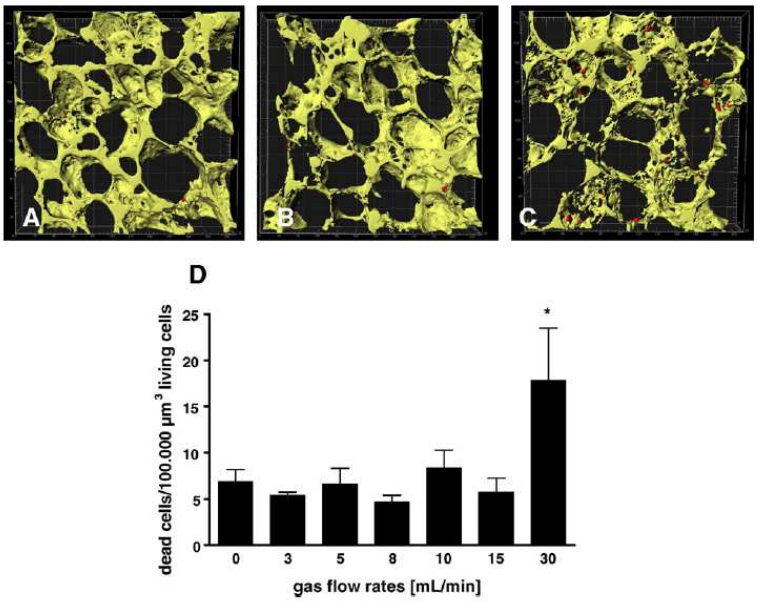

Fig. 5. Image analysis of PCLS after exposure to synthetic air at gas flow rates of 10 mL/min (B) and 30 mL/min (C) for 1 h followed by 23 h post-incubation under submerged conditions. Control tissue (A) was not exposed but air–liquid interface cultivated. Tissue slices were stained with 4 µM calcein AM and 4 µM EthD-1. The images were examined by two-colour confocal laser scanning microscopy and analyzed with IMARIS 5.5.3 (D). Red colour shows cell nuclei (⌀ 5 µm) of dead cells and green colour the cytoplasm of viable cells (grid spacing = 20 µm). $n = 4$, $^{*}p < 0.05$ (One-way ANOVA Dunnett's test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

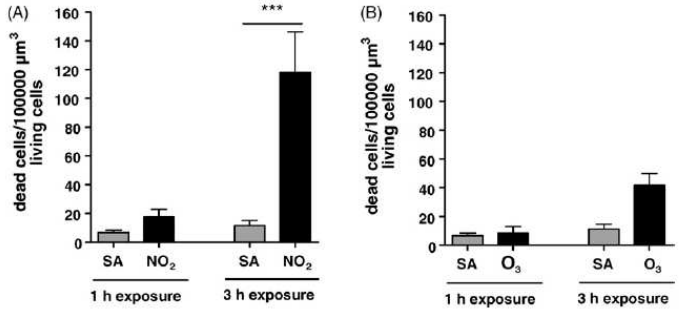

Fig. 6. Quantitative image analysis of murine PCLS exposed to 80 ppm NO_2 and 8.5 ppm O_3. The quantitative analysis with IMARIS was performed after 1 and 3 h of exposure to NO_2 and O_3 followed by 23 h of submerged post-incubation versus exposure to synthetic air (SA). $n = 4$; $^{***}p < 0.005$ (One-way ANOVA Tukey).

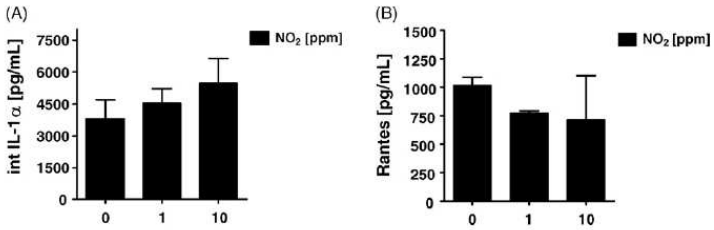

Fig. 7. Intracellular increase of IL-1α (A) and extracellular increase of RANTES (B) in PCLS exposed in air–liquid interface culture to NO_2 for 1 h followed by 23 h submerged post-incubation. Cytokine levels in culture supernatants and tissue lysates were determined by ELISA or Luminex technology. Data are presented as mean ± S.E.M. int IL-1α: $n = 8$, RANTES: $n = 3$ (One-way ANOVA Dunnett's test).

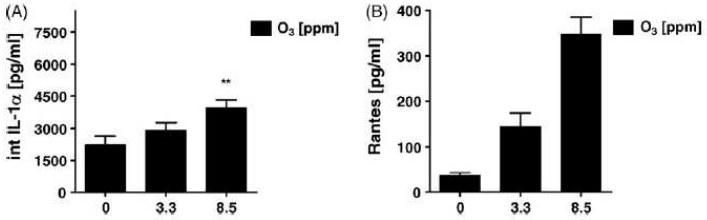

Fig. 8. Intracellular increase of IL-1α (A) and extracellular increase of RANTES (B) in PCLS exposed to O_3 in air–liquid interface culture for 1 h followed by 23 h submerged culture post-incubation. Cytokine levels in culture supernatants and tissue lysates were determined by ELISA or Luminex technology. Data are presented as mean ± S.E.M. int IL-1α: $n = 5$, RANTES: $n = 3$, ** $p < 0.01$ (One-way ANOVA Dunnett's test).

ing ALI exposure with nutrification from the basolateral side of the microporous membrane for at least 24 h, which until now could be shown only for submerged cultures or roller systems (Held et al., 1999; Martin et al., 1996; Richter et al., 2000). Live slices showed high metabolic enzyme activity in the WST-1 assay, and the morphology remained unaltered as indicated by fluorescence staining, as previously shown (Henjakovic et al., 2008). Comparable viability was achieved after exposure of murine PCLS to synthetic air at flow rates below 15 mL/min, which did not seem to induce a drying and therefore dying of the tissue. Higher flow rates, however, induced a decrease in cell viability. These results correlate with data shown in studies using *in vitro* single cell systems (human lung fibroblasts and human bronchial epithelial cells) under ALI conditions (Ritter et al., 2001). Therefore, both cell lines and PCLS resist physical forces that occur during exposure to gaseous compounds. Moreover, since the exposure flow rates tolerated by PCLS (about 10 mL/min/cm^2) are even higher compared to *in vitro* single-cell systems (about 3–8 mL/min/cm^2) they can be characterized as a robust and reliable biological testing material under these conditions (Ritter et al., 2001). After adjustment of the exposure conditions the model was used to expose live lung tissue to gaseous compounds. O_3 and NO_2 were selected as model gases for unobstructed exposure of lung tissue to gaseous compounds. Both gases are well-known ubiquitous indoor and outdoor oxidant pollutants.

The toxicant NO_2 induces respiratory irritation, acute inflammation, pulmonary edema, and pneumonia (Winder, 2004). Its poor water solubility and high reactivity cause cytotoxicity and inflammation in the terminal conducting airways, the alveolar ducts, and the alveoli (Tu et al., 1995). Cell types such as ciliated cells of the bronchial epithelium, types I and II cells of the alveolar epithelium, and alveolar macrophages seem to be very sensitive to NO_2 exposure. NO_2 is hydrolyzed to give nitric acid and reaches concentrations of about 65% in the culture medium (Devalia et al., 1993; Postlethwait and Mustafa, 1989). Brief high-dose exposure to NO_2 induced cell death in murine PCLS. Such a NO_2-mediated cell injury in lung parenchyma may be causally related to the early generation of pro-inflammatory cytokines. Indeed, cytokine expression after exposure to NO_2 was previously reported in human bronchial epithelial cells or alveolar macrophages (Dandrea et al., 1997; Devalia et al., 1993). Exposure to 45 ppb to 45 ppm NO_2 for 30 min to 6 h led to an upregulation of TNF-α, IL-1β, IL-8, and GM-CSF (Ayyagari et al., 2004; Dandrea et al., 1997; Devalia et al., 1993). Pre-existing inflammation in atopic and asthmatic individuals make cells more susceptible to injury upon exposure to NO_2, after which significant upregulation of the chemokine RANTES was observed (Bayram et al., 2001). But contrary to our expectations, although there was a trend, we did not observe a significant initial increase in the pro-inflammatory cytokine IL-1α in live lung tissue, whereas RANTES was not changed at all after short-term exposure to NO_2. Long-term exposure and subsequently continuous damage of lung cells can lead to chronic inflammation which cannot be observed in the experimental set up in PCLS due to limited exposure and culturing time. But both cytokines are known to be increased and therefore also to be involved in chronic inflammatory lung diseases (Greally et al., 1993; Zhu et al., 2001). They belong to the IL-1 gene family, but in contrast to IL-1β IL-1α is an intracellular regulator and mediator of local inflammation (Dinarello, 1996). Even if the release of cytokines is commonly thought to be one of the earliest events, our data suggest that IL-1α and RANTES in non-inflamed lung tissue under normal basal conditions may not be involved to the same extent as with other environmental oxidants such as O_3.

O_3 is a very reactive gas and penetrates deeply into the lungs. It induces acute inflammation by stimulating cytokine secretion by macrophages and epithelial cells (Manzer et al., 2008). Damage of ciliated and alveolar type I cells occurs, and squamous metaplasia as well as rapid influx of neutrophils were observed in humans (Bascom, 1996; Uysal and Schapira, 2003). Single acute exposure to O_3 did not induce immediate toxicity in parenchyma, but survival of tissue *ex vivo* was reduced after prolonged exposure to 8.5 ppm O_3. O_3 is highly reactive with a variety of organic biomolecules such as proteins, unsaturated fatty acids, or nucleic acids. It is known that the time of diffusion of O_3 into the tissue is 3-fold higher than its half-life (Pryor, 1992). Hence, most of the O_3 gas cannot reach deeper areas of the lung tissue and subsequently does not induce a clear cytotoxic effect, as observed for NO_2. Early substantial secretion of the pro-inflammatory cytokine IL-1α and a trend for the chemokine RANTES could already be seen after 1 h of exposure. Similar results after exposure to up to 100 ppb O_3, including upregulation of RANTES and IL-1α, were obtained in bronchial epithelial cell cultures (Bayram et al., 2001) or rat NR8383 alveolar macrophage culture (Manzer et al., 2008). These data are supported by the description of the early phase which is initiated during 2–24 h after acute exposure to O_3. Here, chemotactic factors like RANTES are synthesized and released to direct the migration and activation of neutrophils (Leikauf et al., 1995). Influx of inflammatory cells like neutrophils cannot be quantified in PCLS because of the lack of cell migration from the circulation, but was shown in *in vivo* studies with patients exposed to 0.25 ppm O_3 for 3 h (Alexis et al., 2008).

Toxicity data of several inhalation toxicity studies after acute, subacute, and chronic exposure to NO_2 and O_3 are available from the Registry of Toxic Effects of Chemical Substances (RTECS) (http://www.cdc.gov/niosh/). For example, the LC_{50} for NO_2 in rats was reported to be about 220 mg/m^3 (117 ppm) following 1 h of exposure to NO_2 (NIOSH, 2008). For human beings, first symptoms of toxication have been reported after acute exposure to NO_2 (40–70 min) at doses between 56 mg/m^3 and 169 mg/m^3 (30–90 ppm) (NIOSH, 2008). In comparison, 1-h exposure to 11 ppm NO_2 induced loss of cell viability by up to 50% in both A549 and fibroblasts (Bakand et al., 2006, 2007). In our study, the range of exposure doses was selected from 10 to 80 ppm NO_2 to cover concentrations that can occur in scenarios where human beings are exposed to brief high doses of NO_2. Our results demonstrated significantly more necrotic cells at 80 ppm NO_2, indicating that

acute NO_2 exposure leads to cell damage. This suggests that a high sensitivity of single cell type-based *in vitro* test methods may not entirely reflect the *in vivo* situation after exposure to brief high doses of NO_2. Similarly, the LC_{50} for O_3 in mice has been shown to be about 12.6 ppm (23.6 mg/m^3/3 h) and for inhalation in humans was 50 ppm (98 mg/m^3/30 min). In a single monocyte cell culture 0.5 ppm O_3 (0.98 mg/m^3) was lethal to at least 60% of the cells (Klestadt et al., 2002). This discrepancy between *in vivo* and *in vitro* studies is a subject of debate and controversy in the scientific community (Bakand et al., 2005; Blaauboer, 2002); although according to our understanding most of the mentioned models have advantages in certain applications. Moreover, historical *in vivo* data that are available vary strongly from study to study and can hardly be used as reference for the validation of *in vitro* models.

In summary, our studies demonstrated that PCLS offer a suitable model to study the cytotoxic and pro-inflammatory effects of inhaled irritant compounds such as O_3 and NO_2 under direct ALI conditions. It can be used to study the effects of gaseous compounds on lung cells in organotypic cultures. The advantage of combining of PCLS with ALI conditions is it should decrease the need for animal experiments for risk assessment. It also more closely reflects real life inhalation of airborne contaminants, and may be more suitable for toxicity testing in this context.

Conflict of interest

None declared.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.toxlet.2010.04.004.

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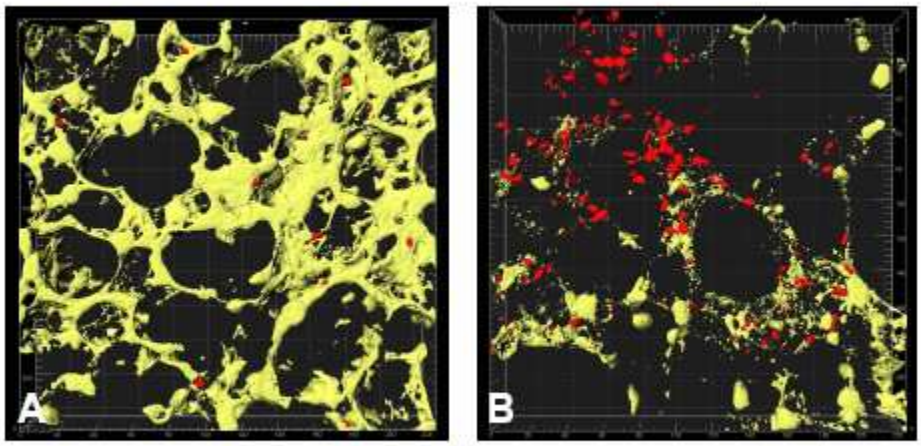
Legends for the Online Supplement

Supplementary figure I. Image analysis of PCLS after 3 h of exposure to NO_2. Lung slices were exposed to synthetic air (A) and 80 ppm NO_2 (B) for 3 h at gas flow rate of 10 mL/min followed by 21 h of submerged post-incubation. Tissue slices were stained with 4 µM calcein AM and 4 µM EthD-1, examined by 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).

Supplementary figure II. Image analysis of PCLS after 3 h of exposure to O_3. Lung slices were exposed to synthetic air (A) and 8.5 ppm O_3 (B) for 3 h at gas flow rate of 10 mL/min followed by 21 h of submerged post-incubation. Tissue slices were stained with 4 µM calcein AM and 4 µM EthD-1, examined by 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).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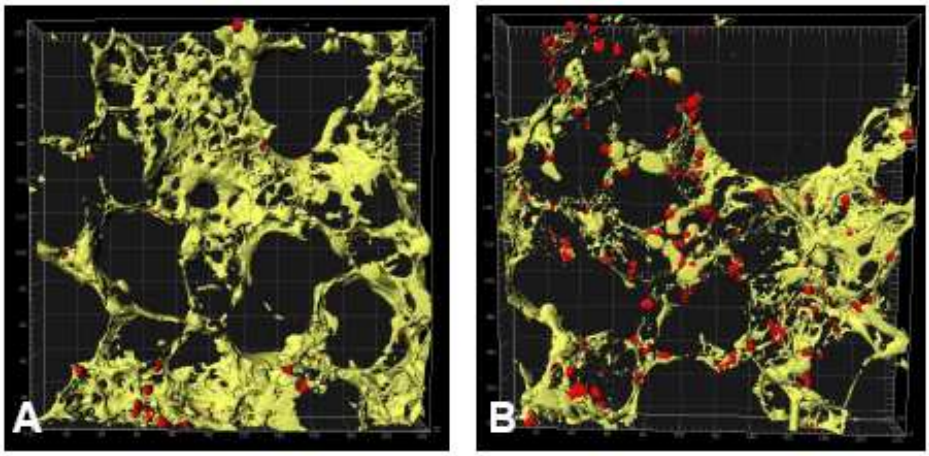
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5 General discussion

The data presented in this thesis addressed different functional issues: most importantly, the aim was to provide an *ex vivo* organotypic lung model which reflects immunological and toxicological aspects of the human lung diseases COPD and acute lung injury/ARDS, respectively. To this end, pro-inflammatory markers of the innate immune system in response to widely used model compound LPS were studied in human PCLS (publication: "Natural innate cytokine response to immunomodulators and adjuvants in human precision-cut lung slices"). Furthermore, O_3 and NO_2 were applied to primarily induce cytotoxicity in an ALI culture to mimic the acute toxicity in acute lung injury/ARDS. In this context, a physiologically relevant model should furthermore be established to enable unobstructed contact of the chemical and the lung tissue (publication: "Effects of acute *in vitro* exposure of murine precision-cut lung slices to gaseous nitrogen dioxide and ozone in an air-liquid interface (ALI) culture").

5.1 Reflection of the pro-inflammatory response in COPD in the organotypic lung model PCLS

COPD as a lung disease with increasing prevalence in especially industrial countries is commonly caused by cigarette smoking with LPS as one important smoke component (Barnes *et al.*, 2003; Hasday *et al.*, 1999). Its function as an inducer of acute inflammation and neutrophil influx was supported by its capability to mimic the development of emphysema-like lesions and the cytokine profile of COPD – reasons why LPS is being widely used as a model compound (Brass *et al.*, 2008; Hackett *et al.*, 2008, Savov *et al.*, 2002). LPS itself derives from the cell wall of gram-negative bacteria and ubiquitous in the environment, e.g. in ambient aerosols (Heinrich *et al.*, 2003). Its highly conserved structure is a so-called pathogen-associated molecular pattern (PAMP) and provides the basis for its recognition by one of 13 already known toll-like receptors: TLR4 (Ishii *et al.*, 2008; Kumar *et al.*, 2009). In this process, a complex is built consisting of LPS itself, LPS-binding protein, and the cell surface glycoprotein CD14 (Chow *et al.*, 1999). This complex subsequently binds to the extracellular leucin-rich repeated domain of TLR4 on various cell types such as DCs,

mast cells, eosinophils, monocytes, macrophages, B-cells, or epithelial cells, thereby initiating the intracellular NF-κB signaling (Iwasaki and Medzhitov, 2004; Takeuchi *et al.*, 2001; Zhang *et al.*, 2010). This signaling pathway and the resulting cytokine immune responses induced by LPS are highly organized, even when looking at lung diseases like COPD. This involves the release of multiple mediators including TNF-α, IL-1β, MCP-1, GM-CSF, and IL-8 (Barnes, 2004).

A variety of cell cultures are used to study the release of cytokines as a response of the innate immunity to stimulation of different TLR like TLR3 and TLR4. In this context, bronchial epithelial cells such as BEAS-2B showed up-regulation of MIP-3α and GM-CSF in response to LPS (Sha *et al.*, 2004). They furthermore were used as an *in vitro* COPD model to investigate the role of LPS-induced expression of connective tissue growth factor (CTGF) (Nishioka *et al.*, 2010). Human monocytes, human alveolar and bronchial epithelial cells lines, but also lung tissue explants released TNF-α, IL-1α, IL-1β, IL-8, and IL-6 in response to LPS or cigarette smoke (Bailly *et al.*, 1990; Hackett *et al.*, 2008; Hellermann *et al.*, 2002). Alveolar macrophages isolated from COPD patients are suitable to investigate anti-inflammatory effects of dexamethasone (Culpitt *et al.*, 1999). Single-cell cultures, however, are artificial systems that do not represent the complexity of the lung, in contrast to 3D *in vitro* models such as organotypic lung slices (Martin *et al.*, 1996; Nassimi *et al.*, 2009). They combine all immune and structural cells and allow the complex response to LPS to be studied.

We established this human-based tissue model based on the production of cytokines in response to LPS to mimic the immunological response in COPD. Observations in COPD patients showed that increased amounts of cytokines such as TNF-α or IL-8 are produced compared to healthy subjects or healthy smokers (Aaron *et al.*, 2001; Keatings *et al.*, 1996). Animal models are often used to investigate features of COPD (Howard *et al.*, 1993; Kaneko *et al.*, 2007; Nadadur *et al.*, 2005; Poynter *et al.*, 2006). In these and similar studies, local inflammatory processes in the lung but also systemic outcomes were analyzed (Brass *et al.*, 2008; Terashima *et al.*, 1997). In general, however, COPD is a human-based disease that cannot fully be depicted in animals. For this reason, the focus of these models lies on the reflection of features of the disease such as early or chronic phases and their association with, e.g., the development of emphysema or the

production of pro-inflammatory mediators. Studies using mouse models observed elevation of the pro-inflammatory cytokines TNF-α, IL-1α, IL-1β, GM-CSF, RANTES, or MCP-1 using LPS and cigarette smoke as model compounds (Bracke *et al.*, 2007; Van der Deen *et al.*, 2007; Ogino *et al.*, 2009; Vernooy *et al.*, 2002). We could show that stimulation of human PCLS with LPS resulted in a significant increase in the same cytokines that were reported in the outcomes of COPD such as TNF-α, IL-1β, GM-CSF, RANTES, and MCP-1. The induced cytokine secretion observed in patients and after treatment with LPS are both based on activation of the signaling pathway NF-κB in a MyD88-dependent manner (Kumar *et al.*, 2009; Hellerman *et al.*, 2002). This NF-κB induction was shown to be dependent on TLR4 in both cases (Bihl *et al.*, 2001; Doz *et al.*, 2008). These data support the hypothesis that PCLS are suitable to display signaling pathways and the release of pro-inflammatory mediators which play an important role in the immunological response of COPD.

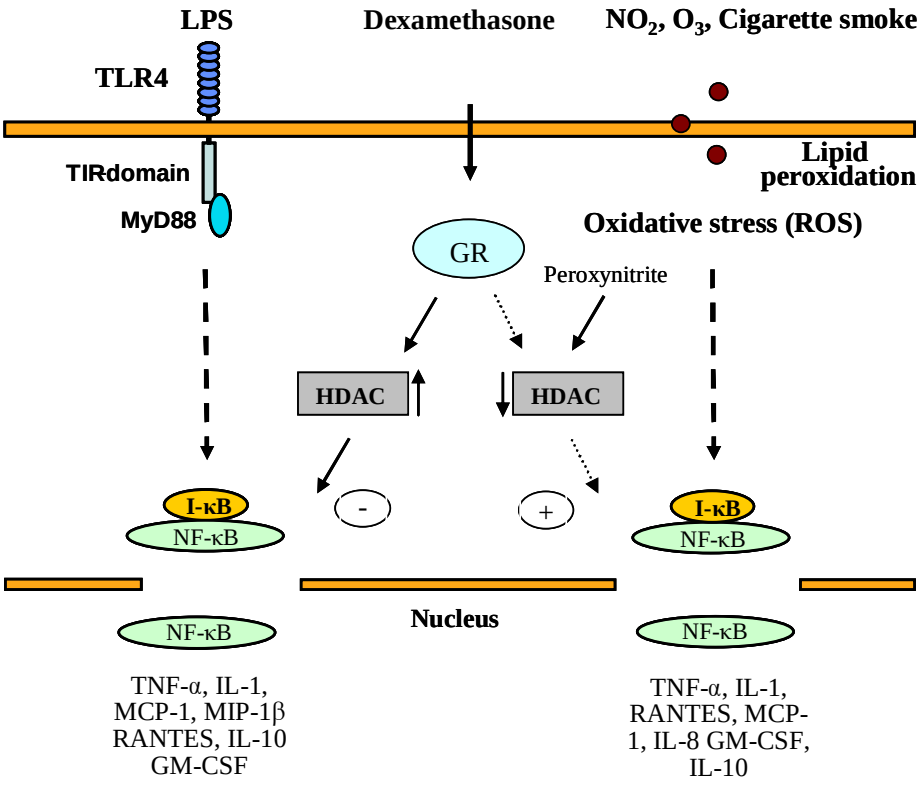

Figure 1: Signaling cascades induced in PCLS after treatment with model compounds LPS, NO_2, O_3, dexamethasone, and cigarette smoke.

Abbreviations: LPS: Lipopolysaccharide, TLR: Toll-like receptor, TIR-domain: Toll/Interleukin-1 receptor domain, MyD88: Myeloid differentiation primary response gene 88, NF-κB: Nuclear factor of kappa light polypeptide gene enhancer in B-cells, I-κB: NF-κB inhibitor beta, GR: Glucocorticoid receptor, HDAC: Histone deacetylase, NO_2: Nitrogen dioxide, O_3: Ozone, ROS: Reactive oxygen species, TNF-α: Tumor necrosis factor alpha, IL-1: Interleukin 1, MCP-1: Monocyte-chemotactic protein-1, MIP-1β: Macrophage-inflammatory protein-1 beta, RANTES: Regulated on activation, normally T cell expressed and secreted, GM-CSF: Granulocyte/macrophage colony-stimulating factor

In PCLS, treatment with dexamethasone successfully prevented LPS-induced production of pro-inflammatory cytokines and chemokines such as IL-1β, IFN-γ, GM-CSF, and MCP-1 (figure 1). Tendencies for an inhibition could also be seen for RANTES or IL-10. Studies in macrophages, peripheral blood mononuclear cells (PBMCs), or whole blood showed similar results (Bhavsar *et al.*, 2010; Giamarellos-Bourboulis *et al.*, 2010). Cytokines such as TNF-α, IL-1β, IL-10, and MIP-1β were clearly reduced by dexamethasone. This is based on the fact that corticosteroids inhibit the production of pro-inflammatory cytokines by recruiting histone deacetylase-2 (HDAC), which furthermore suppresses the activation of cytokines (figure 1) by gene

silencing through deacetylation of DNA-bound histones. However, corticosteroids alone are nearly ineffective when used as a therapeutic drug in COPD (Culpitt *et al.*, 1999; Culpitt *et al.*, 2003; Keatings *et al.*, 1997). There was no benefit in lung function, in cell counts, or in reduction of cytokines such as IL-8 and TNF-α in human studies. In COPD, permanent exposure to cigarette smoke induces strong oxidative stress which impairs the activity of histone deacetylases (figure 1), so that pro-inflammation and a destruction of lung alveoli are perpetuated (Barnes, 2004; Ito *et al.*, 2001). Lung tissue additionally can be destroyed directly by free radicals released in response to cigarette smoke (Sun *et al.*, 1995). The breakdown of connective lung tissue and the resulting degradation products such as hyaluronan from epithelial cells can furthermore act as ligands for TLR4 or TLR2 and enhance the inflammatory process (Jiang *et al.*, 2006). It was already shown that one of the main differences between lung injury induced by LPS and cigarette smoke is the fact that LPS does not induce oxidative stress in a direct way (Valenca *et al.*, 2008). While cigarette smoke which contains free radicals itself additionally induces superoxide dismutase, catalase, or neutrophil elastase (Shapiro *et al.*, 2003; Valenca *et al.*, 2008), LPS does not. These findings could be an explanation for the inhibitory function of dexamethasone in PCLS, which contradicts the *in vivo* situation.

Contrary to the pro-inflammatory response of COPD that was successfully reflected in the established PCLS model, toxic feature that predominantly characterize acute lung injury/ARDS were subsequently analysed and discussed in comparison to models of lung injury and endotoxemia.

5.2 PCLS mimic early toxic features of acute lung injury/ARDS

Acute lung injury/ARDS is mainly characterized by a diffuse damage of lung parenchyma induced, e.g., by inhalation of toxic agents or smoke. Edema and increased permeability in endothelial cells and type I pneumocytes occur within hours. For depicting cellular damage in acute lung injury/ARDS the irritant gas compounds O_3 and NO_2 have been widely used as model compounds. Both agents are common air pollutants which have been described to be associated with exacerbations of lung diseases such as bronchial asthma (Bayram *et al.*, 2001; Hiltermann *et al.*, 1995; Jenkins

et al., 1999). But most importantly, both gas compounds cause acute toxic effects in the airways as have been described for acute lung injury/ARDS, in particular when administered at high doses.

In vivo studies in rhesus monkeys showed increased necrotic airway epithelial cell death after exposure to 0.8 ppm O_3, with injured epithelial cells being removed by immigrated neutrophils (Hyde *et al.*, 1999). Additionally, a close association between the O_3 dose, the degree of epithelial injury, and glutathione depletion could be shown (Plopper *et al.*, 1998). High toxic doses ranging from 2 ppm to 5 ppm induced immediate toxicity in rats, which was furthermore supported by gene array analysis in rats and alveolar type II cells derived from rats (Nadadur *et al.*, 2005; Wang *et al.*, 2006). Besides apoptotic proteins like growth arrest and DNA-damage inducible 45 alpha, stress proteins such as heat shock proteins but also antioxidants like glutathione S transferase and superoxide dismutase were additionally up-regulated.

The association of toxicity and epithelial lung disruption has been widely studied in *in vitro* models of inhalation injury. To this end, O_3- and NO_2-induced cytotoxicity was investigated, e.g., in A549, NHBE cells, HeLa cells, THP-1 cells, and HUVE cells (Ahmad *et al.*, 2005; Ayyagari *et al.*, 2007; Bakand *et al.*, 2006; Brink *et al.*, 2008; Fakhrzadeh *et al.*, 2004; Tu *et al.*, 1995). The background of the toxic effects that are induced by gas compounds are occurring necrosis and apoptosis. Kinetics revealed caspase-3-independent apoptotic cell death during the early period after exposure to NO_2. However, necrotic cell death occurred prevalently at later time points (Ayyagari *et al.*, 2007). In this context, generated nitric oxides (NO) were shown to be involved. Besides extracellular damage like that of epithelial cells, intracellular damage of DNA was found (Brink *et al.*, 2008). Different mechanisms which are responsible for toxicity exist, such as induction of oxidative stress through production of ROS such as peroxynitrite, nitric oxides, or superoxides (Fakhrzadeh *et al.*, 2004; Klestadt *et al.*, 2002; Laskin *et al.*, 2001). In this context, lipid peroxidation occurs mainly based on ROS which directly attack either cellular membrane lipids or circulating lipoprotein molecules, leading to cytotoxicity. This could be shown in human studies investigating the association of short- and long-term exposure with induced oxidative stress and lipid peroxidation (Chen *et al.*, 2007).

In PCLS, treatment for 3 hours with NO_2 and O_3 resulted in toxic effects. In particular, the highest concentrations used, reaching 80 ppm for NO_2 and 8.5 ppm for O_3, induced cell death. Vitality staining revealed an elevated number of stained dead nuclei and decreased esterase activity indicated by lacking calcein staining. Apart from lipid peroxidation through which membrane lipids are directly disrupted, free radicals are also able to activate pro-inflammatory mediators by degrading the IκBα subunit, resulting in constitutive expression of NF-κB (Bar-Shai and Reznick, 2006; Bar-Shai *et al.*, 2008). As described, toxic events can also be triggered by pro-inflammatory mediators (Shimabukuro *et al.*, 2003). In this case, cytokines such as TNF-α, IFN-γ, and IL-6 exacerbate O_3- and NO_2-induced cytotoxicity (Ayyagari *et al.*, 2004; Cho *et al.*, 2001; Fakhrzadeh *et al.*, 2004; Johnston *et al.*, 2005). The pro-inflammatory cytokine IL-1 plays an important role in the early phase after exposure to O_3, which is initiated after 2 to 24 hours (Leikauf *et al.*, 1995), but it was also described to be involved in toxic events leading to lung leakage. Similar results could be shown for cytokines such as MCP-1 and MIP-1β (Leikauf *et al.*, 1995; Zisman *et al.*, 1997). One underlying mechanism is the extrinsic pathway which is based on the binding of, e.g., the pro-inflammatory cytokine TNF-α to its death receptor TNF-R1 possessing a death domain. Subsequently, apoptosis is induced by this complex and procaspase 8 (Galani *et al.*, 2010).

Exposure to both gas compounds induced cytotoxicity in PCLS, but only exposure to O_3 induced an early release of IL-1α and a tendency for RANTES. Cytokines such as eotaxin, IL-8, or granulocyte colony-stimulating factor (G-CSF) were not induced. This was also shown for concentrations of up to 100 ppb O_3 in bronchial epithelial cell cultures (Bayram *et al.*, 2001) or rat NR8383 alveolar macrophage cultures (Manzer *et al.*, 2008). Exposure to NO_2 resulted in a dose-dependent production of pro-inflammatory IL-1α (40%), whereas RANTES, IL-12, and eotaxin remained unchanged. In acute lung injury/ARDS toxicity of epithelial and endothelial cells is a dominating process, although production of cytokines could be demonstrated (Galani *et al.*, 2010). In this context, release of, e.g., the pro-inflammatory cytokine IL-1, which was also detected in PCLS after exposure to O_3, increases chemotaxis of neutrophils, diminishes apoptosis of activated neutrophils, and subsequently supports the ongoing destruction of structural cells.

PCLS turned out to be a suitable organotypic model to reflect lung diseases. Its relevance for the *in vivo* situation is a critical point and has to be assessed and discussed in the context of further complex established models.

5.3 Status of the development of organ complexity of organotypic cell- and tissue-based 3D-models

The evaluation of cellular complexity and structural integrity has been widely used over at least two decades. At this, physiological models were developed to mimic organ and tissue function in a 3D arrangement as it was shown for capillaries or mammary glands (Enami and Nandi, 1977; Ingber and Folkman, 1989). The most simplistic model was the application of single or immortalized cells with tissue-specific differentiated functions. Cells were embedded on membranes to study, e.g., adhesion of fibroblasts, type II pneumocyte function and morphology, or induction of apoptosis in breast cell lines (Cukierman *et al.*, 2001; Kirshner *et al.*, 2003; Rannels *et al.*, 1987). These models approximated tissue-specific function, but did not comprise different cell types as they are present in an organ. The reflection of, e.g., apoptosis induced by adhesion molecules can be studied in single cells, but the impact of apoptosis-regulating cell types including NK cells and $CD8^+$ T cells on affected cells cannot be analyzed (Kirshner *et al.*, 2003; Smyth *et al.*, 2005). For this reason, 3D-organotypic co-cultures constituted the next step in the development. In epidermal biology, 3D models have been successfully developed by culturing epidermal cell suspensions on dermis or on fibroblasts (Prunieras *et al.*, 1983; Bell *et al.*, 1981). In inhalation toxicology, 3D co-culture models were used to simulate the respiratory tract. Macrophages, epithelial cells, and DCs were analysed in a triple model to study the outcomes of exposure to polystyrene particles (Rothen-Rutishauser *et al.*, 2005; Brandenberger *et al.*, 2010). Furthermore, dissociated lung cells were cultured on Matrigels to study rearrangement and polarization of lung cells (Schuger *et al.*, 1990; Schuger et al., 1996). But none of these models reflects the individuality and function of a complete organ which is based on the surrounding environment like the extracellular matrix and neighbouring cell interactions. Consequently, tracheal ring preparations, isolated perfused lungs, or lung parenchymal strips were developed for the analysis of, e.g., smooth muscle function (de Jongste *et*

al., 1985; Jongejan *et al.*, 1990; Finney *et al.*, 1985; Wang *et al.*, 1992). However, the responses of parenchymal strip preparations were not consistent between different species and not even within one animal (Finney *et al.*, 1985; Ghelani *et al.*, 1980). In contrast, the lung tissue model PCLS possesses a uniform thickness of 250 µm due to precise slicing by the Krumdieck Tissue Slicer, which can be provided for almost all species. The presence of the relevant structure and architectural organization is supported by the manifold cells, their cell-cell and cell-matrix interactions as shown by smooth muscle and airway contractions, or by staining of antigen-presenting cells (Bergner and Sanderson, 2002a; Cooper *et al.*, 2009; Henjakovic *et al.*, 2008). But neither PCLS nor one of the previously mentioned models can overcome the discrepancy of the missing blood or lymphatic circulation. Despite the lack of immigrating cells, they possess a complex and organ-relevant structure. But relevant and complex organotypic models, however, should not only imply the possibility to study complex immune responses induced in different cells simultaneously; relevant organotypic models are required to cover another main aspect: measured responses have to be physiologically relevant and have to reflect the *in vivo* situation.

5.4 Assessment of *in vivo* relevance of PCLS

Finally, the conditions for complexity of the organ are confirmed in PCLS; but to assess the *in vivo* relevance of the lung slices it was necessary to compare and correlate the data obtained by using PCLS with already known data from human studies. To this end, data from a clinical study performed by Schaumann *et al.*, 2008 were utilized in a linear regression analysis model. Twelve cytokines including TNF-α, IL-1α, MIP-1β, IL-10, GM-CSF, and RANTES from bronchoalveolar lavage (BAL) fluid from subjects who had undergone segmental instillation of 2 ng endotoxin were correlated with data from human PCLS. The calculated coefficient of correlation was 0.931, indicating high resemblance and high relevance to the *in vivo* situation. Nevertheless, careful consideration is necessary here, given that the lung tissue for the generation of PCLS was received from cancer patients.

For the relevance of a model it is not only important to have a system that imitates the *in vivo* situation of the immune response very closely, but the uptake of a chemical or a

drug also has to resemble the *in vivo* exposure. In general, airway epithelial cells are the first line of defense in the lung. Their barrier function is supported by the secretion of mucins (Girod *et al.*, 1992), surfactant (Hohlfeld *et al.*, 1997), and anti-microbial peptides such as defensins or lysozymes (Schnapp and Harris, 1998). Furthermore, macrophages and DCs take up antigens for presentation. Subsequently, these are the first cells and molecules that get in contact with inhaled antigens. The unobstructed exposure of insoluble compounds such as gaseous compounds or particles has been the subject of various toxicological *in vitro* studies using different experimental setups. Some of these included submerged cultures with the test gas being directed over the adherent cells by shaking them at certain angles, so that the cells alternated between exposure and immersion in the culture medium (Bion *et al.*, 2002; Bombick *et al.*, 1998; Rusznak *et al.*, 1996). Others passed the gas over the cell suspension (Ayyagari *et al.*, 2007). Submerged exposure conditions, however, always lead to intensive mixing of the test gas and the culture medium. In this case, the gaseous compound such as O_3 reacts with components of the culture medium and is very effectively hindered from reaching the cells. The ALI culture exposure model we used, performed with O_3 and NO_2 in PCLS, in the future might offer new insights into effects induced by airborne or inhaled chemicals which up to date have only been studied under conditions that do not resemble the *in vivo* uptake of antigens.

In conclusion, the obtained results indicated that organotypic lung model PCLS approaches the *in vivo* situation very closely and most importantly it displays a suitable model to reflect immunological responses of COPD and toxic aspects of acute lung injury/ ARDS.

6 Outlook

By means of the presented data it could be shown that PCLS provide a suitable model to reflect the immunological response of COPD. One main characteristic of COPD, however, is its unresponsiveness to corticosteroids which generally inhibit a pro-inflammatory immune response. LPS-induced pro-inflammatory mediators could be inhibited in PCLS by the synthetic corticosteroid dexamethasone, thus contradicting the *in vivo* situation. One of the main limitations of the LPS-induced COPD model is the lacking oxidative stress that was described after contact to cigarette smoke. Consequently, PCLS should be exposed to cigarette smoke to deliver a more relevant COPD-model. For direct quantification of oxidative stress, the amounts of glutathione should be determined in PCLS. In a cigarette smoke-induced COPD model, co-treatment with dexamethasone should fail in reducing the cigarette smoke-induced pro-inflammatory response of PCLS. Roflumilast, a PDE-4 inhibitor which is actually successfully used as a therapeutic drug in COPD, could additionally be used for co-exposure with cigarette smoke, expected to prevent the pro-inflammatory response. But cigarette smoke also contributes to DNA fragmentation as one risk factor for cancer. Such a fragmentation could be quantified by using a comet assay.

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8 List of abbreviations

ALI	Air-liquid interface
AP-1	Activator protein-1
ARDS	Acute respiratory distress syndrome
ATP	Adenosine triphosphate
BAES-2B	Human bronchial epithelial cell line
BAL	Bronchoalveolar lavage
B cell	Bone marrow cell
CD	Cluster of differentiation
COPD	Chronic obstructive pulmonary disease
CTGF	Connective tissue growth factor
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ERK	Extracellular signal-regulated kinase
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte/macrophage colony-stimulating factor
GOLD	Global initiative for chronic obstructive lung disease
GR	Glucocorticoid receptor
H_2O_2	hydrogen peroxide
HBEC	Human bronchial epithelial cell line
HDAC	Histone deacetylase
HeLa cell	Human epithelial cervical cancer cell
HUVE cell	Human umbilical vein endothelial cell
IFN-γ	Interferon-gamma
IκBα	Inhibitor of NF-κB
IL	Interleukin
LFA-1	Leukocyte function associated antigen-1
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte-chemotactic protein-1
MHC	Major histocompatibility complex

MIF4	Macrophage migration inhibitory factor 4
MIP-1β	Macrophage-inflammatory protein-1 beta
MODS	Multiple organ dysfunction syndrome
MOF	Multiple organ failure
MyD88	Myeloid differentiation primary response gene (88)
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NHBE cell	Normal human bronchial epithelial cell
NK cell	Natural killer cell
NO	nitric oxides
NO_2	Nitrogen dioxide
NOS	Nitrogen species
O_2^-	superoxide anion
O_3	Ozone
PAMP	Pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PCLS	Precision-cut lung slices
Ppm	parts per million
RANTES	Regulated on activation, normally T cell expressed and secreted
ROS	Reactive oxygen species
SIRS	Systemic inflammatory response syndrome
SOD	Superoxide dismutase
STAT	Signal transducer and activator of transcription
T cell	Thymus cell
T_H cell	T helper cell
THP-1 cell	Human monocyte leukemia cell line
TIR	Toll/IL-1R
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TNF-R	Tumor necrosis factor receptor
VLA	Very-late-antigen-4
WHO	World Health Organization

9 Appendix I: List of publications

Switalla S., Knebel J., Ritter D., Krug N., Braun A., Sewald K.
"Effects of acute *in vitro* exposure of murine precision-cut lung slices to gaseous nitrogen dioxide and ozone in an air-liquid interface (ALI) culture".
Toxicol Letters. 2010. 196: 117-124

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. in press

Nassimi M., Schleh C., Lauenstein H.D., Hussein R., Lubbers K., Pohlmann G., **Switalla S.**, Sewald K., Muller M., Krug N., Muller-Goymann C.C., Braun A.
“Low cytotoxicity of solid lipid nanoparticles in in vitro and ex vivo lung models”.
Inhal. Toxicol. 2009. 21:104-109.

Henjakovic M., Sewald K., **Switalla S.**, Kaiser D., Muller M., Veres T. Z., Martin C., Uhlig S., Krug N., Braun A.
“Ex vivo testing of immune responses in precision-cut lung slices”.
Toxicol Appl Pharmacol. 2008. 231:68-76.

10 Appendix II: Curriculum Vitae

Personal details

Name:	Simone Switalla
Date of birth:	13th November, 1980
Place of birth:	Neustadt am Rübenberge, Germany

Postgraduate studies

2006-2010	Doctoral thesis at the Fraunhofer ITEM, Hannover, Germany

Undergraduate studies

04/2006	**University degree: diploma**
4/2005 - 2/2006	Diploma thesis at the Fraunhofer ITEM, Hannover, Germany
11/2000 - 4/2006	Gottfried Wilhelm Leibniz Universität Hannover, Germany; Studies of biology

School education

1993-2000	Secondary school, Gymnasium Gaußstraße, Neustadt am Rübenberge, Germany
1991-1993	Secondary school Orientierungsstufe Süd, Neustadt am Rübenberge, Germany
1987-1991	Primary school Stockhausenstraße, Neustadt am Rübenberge, Germany

11 Appendix IV: First author´s contribution to the papers of this thesis

1. **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 Pharm. 2010.*

   Simone Switalla established the method of human PCLS and performed the experiments concerning LPS and dexamethasone in PCLS, analyzed all data and wrote the manuscript.

2. **Switalla S.**, Knebel J., Ritter D., Krug N., Braun A., Sewald K. "Effects of acute *in vitro* exposure of murine precision-cut lung slices to gaseous nitrogen dioxide and ozone in an air-liquid interface (ALI) culture". *Toxicol Letters. 2010.*

   Simone Switalla established the ALI culture model, performed the PCLS culture, analyzed all data and wrote the manuscript.

12 Appendix V: Acknowledgements

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My thanks go to Klinikum Region Hannover, especially to Prof. Fiehguth, Dr. Christine Förster, and Dr. Olaf Pfennig for providing us with human material. Their constant dedication made this cooperation possible. In this context, I have to thank Prof. Hohlfeld who made this contact and helped us with all ethical questions.

I really appreciated the cooperation with the department of *In Vitro* Toxicology. I especially acknowledge Dr. Jan Knebel and Dr. Detlef Ritter for providing the technical installations for the Air-liquid interface exposures, for all the efficient scientific discussions, and for this funny ESTIV congress in Sweden. I enjoyed it.

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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Last but not least, I would like to thank my family and friends for their unlimited support. Completion of this thesis would not have been possible without their help!

Erklärung zur Dissertation

gemäß §6(1) der Promotionsordnung der Naturwissenschaftlichen Fakultät der Gottfried Wilhelm Leibniz Universität Hannover

für die Promotion zum Dr. rer. nat.

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel

„Characterization of local respiratory irritation and inflammation after acute exposure to biological and chemical substances in PCLS"

selbständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Hannover, 22.02.2011

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Simone Switalla