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Marmoset monkeys as a preclinical model in respiratory research

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Table of contents

Preamble

This cumulative thesis was prepared at the Department of Airway Immunology of the Fraunhofer Institute for Toxicology and Experimental Medicine under supervision of Prof. Dr. Armin Braun and Dr. Katherina Sewald and at the Pathology Unit of the German Primate Center under supervision of Prof. Dr. Franz-Josef Kaup. The measurements of bronchoconstriction in precision-cut lung slices (PCLS) of different non-human primate species were performed in cooperation with PD Dr. Christian Martin and Dr. Marco Schlepütz from the Department of Pharmacology and Toxicology of the University Hospital Aachen, RWTH Aachen.

The present work refers to the following publications:

1. Seehase S., Schlepütz M., Switalla S., Mätz-Rensing K., Kaup F.-J., Zöller M., Schlumbohm C., Fuchs E., Lauenstein H.D., Winkler C., Kuehl A.R., Uhlig S., Braun A., Sewald K., Martin C. **Bronchoconstriction in non-human primates: a species comparison**, *J Appl Physiol. 2011 Sep; 111(3):791-8. Epub 2011 Jun 23.*
2. Seehase S., Lauenstein H.D., Schlumbohm C., Switalla S., Neuhaus V., Förster C., Fieguth H.G., Pfennig O., Fuchs E., Kaup F.-J., Bleyer M., Hohlfeld J.M., Braun A., Knauf S., Sewald K. **LPS-induced lung inflammation in marmoset monkeys – an acute model for anti-inflammatory drug testing**, *PLoS ONE, 2012: 7(8): e43709. Epub 2012 Aug 28.*

1. General introduction

This doctoral thesis focuses on the evaluation of a preclinical non-human primate (NHP) model for human respiratory diseases in the marmoset monkey. Special emphasis was placed on the investigation of the mechanisms of airway smooth muscle (ASM) constriction and lipopolysaccharide (LPS)-induced airway inflammation by using the *ex vivo* model of precision cut lung slices (PCLS). Since not all features of inflammation can be mirrored in PCLS, an *in vivo* model of unilateral LPS provocation was developed and evaluated by using the recently approved anti-inflammatory drug roflumilast, a phosphodisterase-4 (PDE4) inhibitor. The data of this newly developed NHP model were compared to the human situation.

1.1. Respiratory diseases

Inflammatory lung diseases such as acute lung injury (ALI), chronic obstructive pulmonary disease (COPD), and asthma bronchiale, cause significant morbidity and mortality worldwide and display a major public health impact (BRAMAN, 2006; MANNINO and BUIST, 2007; RUBENFELD et al., 2005). On a cellular level, these respiratory diseases are based on an inflammation which can be either acute, such as in the case of ALI, or chronic, such as in the case of asthma bronchiale and COPD. Altogether, the two major features of these respiratory diseases are inflammation accompanied by bronchoconstriction.

Acute direct exposure of the lung to noxious fumes or other toxicants, viral or bacterial respiratory infections, and gastric aspiration can lead to ALI, as well as its more severe form – the acute respiratory distress syndrome (ARDS) (BRUN-BUISSON et al., 2004; RUBENFELD et al., 2005; WARE and MATTHAY, 2000). A non-pulmonary indirect lung injury due to setting of systemic processes including sepsis, major trauma, and drug overdose can also cause ALI/ARDS. In general, ALI/ARDS represent acute inflammatory conditions of the respiratory tract, which are

characterized by hypoxemia and non-cardiogenic pulmonary edema. This severe pathology is commonly accompanied by a multi-organ involvement and severe extra-pulmonary inflammation (RAGHAVENDRAN et al., 2008).
Within 72 hours after an acute noxious stimulus, an initial acute exudative phase occurs, followed by a fibro-proliferative phase which lasts for more than 7 days (PIERRAKOS et al., 2012). However, these two stages can overlap. The initial acute phase is associated with diffuse alveolar damage and microvascular injury with subsequent pulmonary edema. Moreover, an influx of inflammatory cells including neutrophil granulocytes, and a profound release of mediators such as pro-inflammatory cytokines, reactive oxygen species (ROS), and proteases occurs (LIN et al., 2010; GROMMES and SOEHNLEIN, 2011; CROSS and MATTHAY, 2011). In response to the damaged epithelial cells, rapid fibroblast and myofibroblast proliferation takes place within the followed fibro-proliferative phase (CROSS and MATTHAY, 2011). Thus, new collagen-rich extracellular matrix is deposited in the pulmonary interstitium and fibrosis can become apparent (WARE and MATTHAY, 2000; RAGHAVENDRAN et al., 2008). In addition to this, also type II pneumocytes proliferate, which can further represent a chronic source of pro-inflammatory mediators (RAGHAVENDRAN et al., 2008; SHARMA et al., 2007).

Chronic exposure of the lungs to noxious airborne particles such as cigarette smoke, exposure to occupational dusts and chemicals, as well as exposure to polluted air as a result from burning biomass fuels contribute to the development of COPD (RABE et al., 2007). Of all these harmful substances, especially cigarette smoking exhibits the main risk factor. Interestingly, only 25 % of smokers develop COPD, indicating an additional involvement of genetic, epigenetic, and environmental risk factors (LAMPRECHT et al., 2011; LUNDBACK et al., 2003). So far, most of these factors are largely unknown. However, deficiency in alpha1-antitrypsin, an inhibitor of neutrophil elastase and other serine proteases, has shown to be associated with COPD (JANCIAUSKIENE et al., 2011; SITKAUSKIENE et al., 2008).
Clinically, COPD is characterized by chronic airflow limitation and pathological alterations in the peripheral airways and lung parenchyma such as chronic

inflammation, tissue damage, and emphysema resulting in airway fibrosis and alveolar destruction. According to the Global Initiative for Chronic Obstructive Lung Disease (GOLD), the severity of COPD is spirometrically classified in four stages (I-IV) accompanied by an inflammatory immune response (BRUSSELLE et al., 2011; COSIO et al., 2009).

In acute as well as chronic lung inflammations noxious agents lead to the activation of pattern recognition receptors (PRR), either directly or due to injury of epithelial cells. Consequently, damage associated molecular patterns (DAMPs) such as hyaluronic acid or biglycan are released (JIANG et al., 2005; SCHAEFER et al., 2005). These DAMPs as well as airborne particles such as bacteria-derived lipopolysaccharide (LPS), act as ligands for transmembrane Toll-like receptors (TLR)2 and TLR4, which belong to the family of PRRs (KAWAI and AKIRA, 2010; RYLANDER, 2002), and subsequently induce an activation of macrophages and dendritic cells (DCs). As a result, inflammatory mediators such as tumor-necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), granulocyte macrophage colony-stimulating factor (GM-CSF), and IL-8 are released, which orchestrate the inflammation due to an induction of neutrophil, monocyte, and T cell infiltration into the airways (BARNES, 2004c; COSIO et al., 2009; MILLS et al., 1999). Additionally, the lipid mediator leukotriene (LT) B_4 is produced by macrophages, monocytes, and neutrophil granulocytes, and in turn attracts further inflammatory cells including neutrophil granulocytes and CD8+ T cells (BIERNACKI et al., 2003; DRAKATOS et al., 2009; KOSTIKAS et al., 2005). Other lipid mediators derived from arachidonic acid are the prostanoids prostaglandin (PG) E_2 and $PGF_{2\alpha}$ (MONTUSCHI et al., 2003). Both prostanoids sensitize and activate airway sensory nerves to enhance coughing. PGE_2 acts as a bronchodilator and stimulator of mucus secretion, whilst $PGF_{2\alpha}$ acts as a bronchoconstrictor (BORCHERS et al., 1999; STONE et al., 1992). Beside this, activated inflammatory and structural cells produce ROS such as superoxide anions ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2). This endogenous ROS production together with exogenous oxidants derived from cigarette smoke contributes to further damage of the lung tissue (BARNES, 2004c). Moreover, in

patients with emphysema, the macrophage-expressed matrix metalloproteinases (MMP)-1 and MMP-9 are increased (BARNES, 2004a; FINLAY et al., 1997). Regarding the chronic lung disease COPD, COSIO and colleagues (2009) have attributed this chain of events to the early GOLD stage I, which is characterized by mild airflow limitation (forced expiratory volume in one second (FEV_1) ≥ 80% predicted) accompanied by chronic cough and sputum production (BRUSSELLE et al., 2006).

The previously activated DCs promote the differentiation of CD4+ T cells due to activation of the signal transducer and activator of transcription 4 (STAT4) via release of IL-12, a T cell stimulating factor (AGNELLO et al., 2003). Thus, IL-12 polarizes CD4+ T cells towards a T helper type 1 (T_h1) phenotype, which is characterized by increased release of interferon-gamma (IFN-γ) (DI STEFANO et al., 2004). Additionally, material of injured, necrotic, and apoptotic cells is engulfed by DCs and presented via major histocompatibility complex (MHC) class I to CD8+ T cells, which induce cytotoxicity (FREEMAN et al., 2009). This T cell activation and proliferation have been attributed to the moderate GOLD stage II (COSIO et al., 2009), which is marked by worsening airflow limitation with shortness of breath developing on extension (50% ≤ FEV_1 80% predicted) accompanied by cough, sputum production, and dyspnea (RABE et al., 2007).

Recruitment and activation of inflammatory cells including macrophages, neutrophil granulocytes, eosinophil granulocytes, CD4+ and CD8+ T cells, and B cells in the airways generally worsens with disease progression (HOGG et al., 2004; MOLFINO and JEFFERY, 2007). In particular, the presence of these CD8+ (cytotoxic) T cells in COPD patients is more pronounced compared to CD4+ T cells and correlates also with the degree of airflow limitation and emphysema (O'SHAUGHNESSY et al., 1997; HODGE et al., 2007). Activated CD8+ T cells release proteolytic enzymes such as perforins and granzymes causing apoptosis or necrosis of structural airway cells (URBANOWICZ et al., 2010). The late GOLD stages III and IV are, therefore, characterized by fatal airflow limitation (30% < FEV_1 predicted) with life-threatening exacerbations (RABE et al., 2007; HODGE et al., 2007; KEATINGS et al., 1996; MOLFINO and JEFFERY, 2007). The pronounced inflammation in COPD patients

additionally goes along with systemic manifestations concerning the skeletal muscle and the pulmonary vasculature. Thus, COPD-associated co-morbidities include cardiovascular diseases including ischaemic heart disease and heart failure, as well as musculoskeletal impairment (BARNES and CELLI, 2009).

As in COPD, the feature of airway obstruction accompanied by an inflammation displays also a prominent symptom in asthma bronchiale. The molecular pattern of underlying inflammation in asthma and COPD remain, however, highly diverse. Moreover, contrary to COPD, which appears mainly in individuals over the age of 40 years, asthma often develops in early childhood and is characterized by a reversible airflow obstruction.

Asthma is most frequently induced by an imbalanced immune response to normally harmless environmental antigens including pollen derived proteins, dust mite extract, or animal dander (ARSHAD et al., 2001). The inhalation of these allergenic compounds in sensitized individuals immediately triggers off an early phase allergic reaction (EAR) accompanied by a late phase allergic reaction (LAR) (ARVIDSSON et al., 2007; HATZIVLASSIOU et al., 2010).

In the EAR, antigen-specific IgE antibodies cross-link on the high affinity Fc receptor (FcεR) I, which is present on the surface of mast cells and basophils (KINET, 1999; STONE et al., 2010). Subsequently, mast cells and basophils degranulate and release preformed mediators such as histamine or tryptase, and rapidly produce lipid metabolites as well as pro-inflammatory cytokines. Several of these mediators lead either directly or indirectly via the release of acetylcholine from parasympathic nerve endings to the contraction of ASM. Altogether, bronchoconstriction - the main clinical manifestation of the EAR - occurs from a combination of ASM contraction, mucus secretion, and vasodilatation (BOUSQUET et al., 2000). All these pathologies can be induced by arachidonic acid metabolites such as the cysteinyl LTs (cysLT) LTC_4, LTD_4, and LTE_4 (BUSSE, 1998). Moreover, the PGs PGD_2 and $PGF_{2\alpha}$ contribute to bronchoconstriction in asthmatics (MATHE et al., 1973; SAMPSON et al., 1997), whereas PGE_2 acts as a bronchodilator like in COPD (GAUVREAU et al., 1999). Thromboxane (Tx) A_2 displays another potent bronchoconstrictor and also induces

ASM hyperplasia *in vitro* (NOVERAL and GRUNSTEIN, 1992; ALLEN et al., 2006). Beside this, histamine acting via the histamine 1 (H_1) receptor as well as the peptide-mediator endothelin (ET)-1 acting via ET-A and ET-B receptors lead to a contraction of the ASM (GOLDIE, 1998; TOGIAS, 2003). In general, the EAR occurs very fast and usually peaks within 2 hours after allergen contact accompanied by immediate clinical symptoms such as coughing, bronchospasm, and dyspnea.
Subsequently, LAR occurs 4 to 6 hours after allergen provocation, and involves the recruitment and activation of inflammatory cells (SMITH and JOHNSON, 2005). Particularly eosinophils, as well as CD4+ T helper cells of type 2 (T_h2), macrophages, and basophils are plentiful in biopsies of asthmatics (BARNES et al., 1998; STONE et al., 2010). These inflammatory cells release a multitude of inflammatory mediators including the T_h2 cytokines IL-4 and IL-13 that act directly or indirectly on the airways resulting in AHR. Repeated allergen exposure over years causes a chronic inflammation of the respiratory tract accompanied by airway remodeling. Due to the persistent inflammatory activation airway tissue is consistently being injured and healed, resulting in structural changes of the lung such as fibroblast accumulation, mucus gland enlargement, or thickening of the subepithelial basement membrane combined with a decline in airway function (BATEMAN et al., 2008).

Currently, rapid acting inhalable bronchodilators such as β_2-agonists (e.g. salmeterol) as well as antagonists of the muscarinergic acetylcholine receptor subtype 3 (M_3) (e.g. tiotropium) represent the therapeutics of choice for relieving acute bronchoconstriction in asthma, COPD, and ALI/ARDS (BUSSAMRA et al., 2009; MYSORE and RUFFIN, 2011). In combination with inhaled corticosteroids, these bronchodilators display the most effective medication for chronic asthma (GINA REPORT, 2011). Corticosteroids are, however, ineffective at reducing the inflammation in COPD patients (BARNES, 2011). Moreover, in ALI/ARDS they have been shown to be only effective when administered within 14 days after the onset of disease. A later routine administration seemed to be harmful (LAMONTAGNE et al., 2010). So far, no effective pharmacotherapy is available for reducing ALI's/ARDS's complex lung injury pathology (RAGHAVENDRAN et al., 2008). The management of

ALI/ARDS, therefore, primarily emphasizes the non-pharmacologic approach of protective ventilation strategy as well as conservative fluid management (AMATO et al., 1998; FRANK et al., 2002; THE ACUTE RESPIRATORY DISTRESS SYNDROME NETWORK., 2000). Moreover, no therapy has currently the potential to halt the progressive pulmonary inflammation and destruction of lung parenchyma associated with COPD (YAO et al., 2008). Consequently, there is urgent need for broad-spectrum anti-inflammatory treatment of these respiratory diseases. Antagonists against inflammatory mediators display a promising therapeutic approach in the treatment of ALI/ARDS as well as COPD. So far, the identification of potential targets as well as their testing in preclinical models, however, remains difficult due to the lack of predictive animal models exhibiting huge homologies to the respiratory anatomy and physiology of humans (STEVENSON and BELVISI, 2008).

1.2. Preclinical models in respiratory research

Preclinical models facilitate the understanding of disease mechanisms as well as the identification of critical pathways. Thus, new treatments that target the underlying inflammatory mechanisms can be developed. Several *in vitro*, *ex vivo,* and *in vivo* approaches of human or non-human experimental set-ups have been elaborated in this context.

In vitro models include primary cell lines as well as permanent cell cultures such as immortalized cell lines, which enable a high throughput screening, e.g. the discovery of pathways or the prediction of potential toxicological risks (GAITONDE and BALU-IYER, 2011; HATZELMANN and SCHUDT, 2001; TAN et al., 2012). The permanent human bronchial epithelial cell lines NHBE and BEAS-2B have frequently been used to study pulmonary inflammation, cellular function, and differentiation (ERLEMANN et al., 2007; PASCAL and TESSIER, 2004; PICHAVANT et al., 2005; VAN WETERING S. et al., 2007; WONG et al., 2005). The alveolar epithelial cell line A549 has extensively been used to study alveolar reactivity to different stimuli (NAPOLITANO et al., 2012; WISNEWSKI et al., 2000) as well as to assess environmental pollutants (PERSOZ et al., 2012). Additionally, human monocyte/ macrophage cell lines like

Mono-Mac-6 or THP-1 have been used as short term models to evaluate respiratory sensitization (HOFER et al., 2004; VERSTRAELEN et al., 2008; VERSTRAELEN et al., 2009; TIETZE and BLOMEKE, 2008). Altogether, *in vitro* models are useful for the evaluation of cytotoxicity to drugs, solvents, or formulations as well as for the distinct analysis of mechanistic purposes in the different airway zones of the respiratory tree (FERNANDES and VANBEVER, 2009; HATZELMANN and SCHUDT, 2001; ANDERSSON et al., 2010).

Cell cultures are usually grown under monolayer conditions, which display a simplified and artificial model compared to the *in vivo* situation (VAN VLIET E., 2011). To overcome this issue, co-cultivation of different cell types or designing of three-dimensional (3D) tissue models were conducted to obtain more complex information about signal transduction, gene expression, and cell-cell interactions with regard to drug absorption or clearance mechanisms of the lung (BERUBE, 2011; FERNANDES and VANBEVER, 2009; VAN VLIET E., 2011). For example, human monocytes and THP-1 macrophages were co-cultured with monolayers of respiratory epithelial cells (A549) (STRIZ et al., 2011), or human airway epithelial cells (BEAS-2B) were cultured with lung fibroblasts (HFL-1) (LANG et al., 1998). By contrast, *ex vivo* models such as the isolated perfused lung (IPL), tracheal strips, and PCLS represent more complex models compared to *in vitro* cell culture. In particular, the structural integrity and interactions between cells are maintained and this enables the analysis of inflammatory responses underlying respiratory diseases in more detail (MEIRING et al., 2005; NEMMAR et al., 2005; SWITALLA et al., 2010). Moreover, *ex vivo* models allow the investigation of drug absorption without being influenced by other organs (MAERTENS et al., 2010). This advantage, however, displays also a limitation of this model when compared to *in vivo* models. Due to their limited life span, *ex vivo* models can only be used for investigating acute issues of diseases including pro-inflammatory features such as cytokine up-regulation or the mechanism of ASM contraction during EAR in asthma (LANCAS et al., 2006; SWITALLA et al., 2010). Altogether, compared to *in vitro* models the clinical relevance of *ex vivo* models is increased and they can provide more complementary data to studies carried out *in vivo* (SWITALLA et al., 2010). The *ex vivo* models described, however,

still need an involvement of animals. A new “organ-on-a-chip” microdevise, therefore, offers another alternative to animal and clinical studies for drug screening and toxicological applications (HUH et al., 2010). By using only human cells, this biomimetic microsystem has been shown to reproduce a complex, integrated response on organ-level physiology and pathology.
However, despite much progress in the development of alternatives to animal use, no *in vitro* or *ex vivo* model can replace the complexity afforded by *in vivo* studies carried out in various animal species (STEVENSON and BELVISI, 2008).

1.2.1. PCLS for modeling airway diseases *ex vivo*

In line with Russell’s and Burch’s 3 Rs rule “replacement, reduction, refinement” several alternative methods reducing pain, distress, and damage of laboratory animals were established for the investigation of basic mechanisms of respiratory diseases. Various *ex vivo* techniques have been developed in this context (EVANS and ADLER, 1981; SAKAGAMI, 2006; UHLIG, 1998). Due to the fact that PCLS closely resemble the morphology and functionality of the intact respiratory tract, they bridge the gap between cell culture and animal studies (HOLMES et al., 2011). Furthermore, they allow the investigation of pharmacological effects under identical experimental conditions in different species such as rodents (HELD et al., 1999; HENJAKOVIC et al., 2008a), guinea pigs (RESSMEYER et al., 2009), humans (RESSMEYER et al., 2009; WOHLSEN et al., 2003), and rhesus macaques (KOTT et al., 2002).
Various studies explored the ASM response in PCLS to different stimuli such as allergens and drugs or chemicals (HENJAKOVIC et al., 2008a; MARTIN et al., 2000a; MARTIN et al., 1996; MARTIN et al., 2000b; MARTIN et al., 2000c; KOTT et al., 2002). In addition to this, PCLS have been used to investigate the immune response to immunomodulators and adjuvants as well as during adenovirus infection in the context of respiratory diseases such as pneumonia, COPD, and asthma (BOOTH et al., 2004; HENJAKOVIC et al., 2008b; SWITALLA et al., 2010). The mechanisms of EAR as one symptom of the acute asthma attack were studied in

human PCLS (WOHLSEN et al., 2003) and enabled the testing of promising therapeutic candidates targeting the modulation of AHR (BANERJEE et al., 2012; STURTON et al., 2008). Additionally, the pro-inflammatory response of asthma and early COPD have been investigated in this model and facilitated the estimation of a PDE4 inhibitor's anti-inflammatory and airway relaxing properties (MARTIN et al., 2002).

The reductions of animal numbers in pharmaceutical research as well as an economic use of valuable compounds resemble the outstanding advantages of the PCLS method (RESSMEYER et al., 2006). However, like other *in vitro*/*ex vivo* methods, PCLS cannot completely replace *in vivo* models by reasons of limited culturing time and the lack of a circulatory and metabolizing system (HENJAKOVIC et al., 2008a). Thus, for studying inflammatory cell recruitment such as neutrophil influx, the key marker of COPD, *in vivo* models are necessary.

1.2.2. LPS-stimulation for mimicking inflammatory lung diseases

The endotoxin lipopolysaccharide (LPS) is part of the outer membrane of gram-negative bacteria and is also present as a contaminant in cigarette smoke, air pollution, and organic dusts (HASDAY et al., 1999; RYLANDER, 2002). LPS displays the prototype of a microbial trigger that stimulates innate immunity via CD14 and TLR4 resulting in a profound inflammatory response due to activation of nuclear factor kappa B (NF-κB) (FEARON and LOCKSLEY, 1996). This transcription factor NF-κB plays an important role in the regulation of cell activity resulting in an enhanced pro-inflammatory cytokine production (BARNES, 2006). Epidemiologic studies in humans chronically exposed to dusts containing LPS revealed an increase in the prevalence of cough and phlegm, and a decrease in lung function when compared to non-affected specimens (KHARITONOV and SJOBRING, 2007; RYLANDER, 2002; SMIT et al., 2006). On a cellular level, acute respiratory LPS exposure in humans induced an increase in inflammatory mediators such as IL-1β, TNF-α, IL-6, and granulocyte-colony-stimulating factor (G-CSF) as well as a pronounced influx of neutrophil granulocytes in the lung (MICHEL et al., 1997;

O'GRADY et al., 2001). Altogether, these local inflammatory features are also present in human respiratory diseases including early COPD and ALI/ARDS. LPS-induced inflammation models are, therefore, widely-used short-term models for the development and testing of new anti-inflammatory drugs – although they do not reflect all the features of human (especially chronic) respiratory diseases (GERAETS et al., 2010; HOHLFELD et al., 2008; NIALS et al., 2011; TRALAU-STEWART et al., 2011; VACCA et al., 2011).

The induction of a pro-inflammatory response can be performed in *in vitro*, *ex vivo*, and *in vivo* approaches of various species. For instance, LPS-based *in vitro* models using THP-1 cells and human primary macrophages were used to investigate the role of MMP in inflammatory airway diseases (BIRRELL et al., 2006). Moreover, *ex vivo* studies using human and murine PCLS revealed a dose-dependent increase in pro-inflammatory mediators such as IL-1 and TNF-α in response to LPS (HENJAKOVIC et al., 2008b; SWITALLA et al., 2010). Acute *in vivo* models in mice based on LPS-induced lung inflammation resulted in an inflammatory reaction with increases in neutrophil cell counts as wells as TNF-α and IL-1β levels in BAL fluid (ANAS et al., 2010; BIRRELL et al., 2006). The severe endothelial and epithelial injury of human ALI/ARDS cannot, however, be mimicked by a single inhalative LPS stimulation (WIENER-KRONISH et al., 1991). The administration of LPS over several weeks in rodents and guinea pigs resulted in remodeled airways with thickened walls, and an increase of goblet cells resembling human emphysema accompanied by altered lung function (SAVOV et al., 2003; BRASS et al., 2008; TOWARD and BROADLEY, 2001; VERNOOY et al., 2002; WRIGHT et al., 2008). Such a repeated LPS exposure, however, has been shown to attenuate the release of pro-inflammatory cytokines and to increase the response of anti-inflammatory cytokines including IL-10 (DRAISMA et al., 2009; ZEISBERGER and ROTH, 1998). Moreover, it does not reflect the progressive and steroid-insensitive inflammation present in COPD (STEVENSON and BELVISI, 2008).

Despite these limitations, acute and chronic respiratory LPS-induced inflammation models can contribute towards understanding the pro-inflammatory features of

inflammatory lung diseases and are useful when identifying targets for developing new anti-inflammatory therapeutics in COPD or ALI management.

1.3. Commonly used animal species in respiratory research

Animal models act as a bridge between *in vitro* studies and human clinical trials (ZOSKY and SLY, 2007). As such, they represent a useful tool for understanding disease pathology as well as for the development and testing of potential therapeutics (DAWKINS and STOCKLEY, 2001; ZOSKY and SLY, 2007). The interpretation and extrapolation of the results from such models to the human situation is, however, highly dependent on the outcome of interest and the species chosen (ZOSKY and SLY, 2007).

Mice represent the most popular species for investigating the molecular and cellular mechanisms underlying the pathogenesis of ALI/ARDS, COPD, and asthma (BATES et al., 2009; BRUSSELLE et al., 2006; MATUTE-BELLO et al., 2008). Many different genetically modified mouse strains are available as well as a broad spectrum of analysis tools, which offer unprecedented capabilities to explore biological systems under pathological and physiological conditions (BRUSSELLE et al., 2006; PAIGEN, 1995). Therefore, by switching-off, up-regulating, or suppressing single mechanistic pathways a number of potential therapeutic targets have been developed in mice. Unfortunately, some therapeutic approaches have provided only little or no benefit for the treatment of human respiratory diseases so far (TAUBE et al., 2004). For example, an IL-12 antibody revealed no significant effects on LAR or AHR in asthmatics despite initial promise in certain mouse models (BRYAN et al., 2000).

Rats also display common animal models for human respiratory diseases (MARTIN and TAMAOKA, 2006). As in mice, several rat models of antigen sensitization and challenge have been developed in order to induce an allergic response in the airways (ZOSKY and SLY, 2007; HYLKEMA et al., 2002; SINGH et al., 2003). Moreover, certain specific asthma models in rats provide an opportunity to mimic the human EAR and LAR as well as an AHR (TULIC et al., 2002).

Rodent models are, however, often limited in reflecting the human pathology due to anatomical, physiological, and immunological differences (PLOPPER and HYDE, 2008). Differences in the architecture and cellular composition of the tracheobronchial airways such as monopodial branching pattern in mice and rat, and the dichotomous branching pattern in NHP and humans might have an impact on the outcome of pharmaceutical studies (PLOPPER and HYDE, 2008). Furthermore, they may have a significant impact on aerosol deposition, which influences the delivery of an allergen to the airway as well as the distribution of a bronchoconstrictive agent during lung function measurements (ZOSKY and SLY, 2007). Unlike the several generations of respiratory bronchioles in NHP and humans, mice have one or no respiratory bronchiole (PLOPPER and HYDE, 2008). Moreover, cartilage in mice is only present in the trachea lobar bronchi, in contrast to NHP and humans where it is present in the trachea up to the distal bronchiole. Apart from differences in the airway anatomy, there are also differences in cellular composition of the airways such as the thickness of the epithelium. In rhesus macaques, the epithelium in the proximal intrapulmonary airways is half as thick as in humans, and two to three times thicker as in mice. Differences are also apparent with regard to the release of mediators upon an inflammatory stimulus. Mast cells in humans, for example, release histamine which is not the case in mice and rats, where they release serotonin instead (BARNES et al., 1998). Therefore, the efficacy of bronchodilatatory compounds targeting the H_1 receptor is difficult to evaluate in rodents. Further animal models using other species for preclinical testing have been established in guinea pigs (RESSMEYER et al., 2006), dogs (OUT et al., 2002), sheep (ABRAHAM, 2008; SHICHIJO et al., 2009), and NHP (BLACK et al., 2001; GUNDEL et al., 1992; JOAD et al., 2006; JOAD et al., 2008; JOAD et al., 2009; MITCHELL et al., 2010; SCHELEGLE et al., 2001; VAN SCOTT et al., 2004; YOUNG et al., 1999). Due to their close phylogenetic relation, NHPs exhibit various anatomical similarities to humans and possess a high homology to different human target structures including cytokine receptors such as the IL-4 receptor (TOMKINSON et al., 2009). Thus, NHPs emerge as one of the most suitable animal models to reflect human airway pathology. As such, they exhibit the potential to verify findings from preclinical studies

in rodents, before a compound enters clinical trials in humans (COFFMAN and HESSEL, 2005). However, their use displays certain limitations due to ethical issues, high costs of animal use, and the risk of zoonoses (COFFMAN and HESSEL, 2005; QUIGLEY, 2007).

1.3.1. The marmoset monkey as a model for human airway diseases

One promising NHP species to study features of respiratory diseases is the common marmoset (*Callithrix jacchus*), which has already widely been used for biomedical research in neuroscience, reproductive biology, infectious diseases, toxicology or behavioral research (MANSFIELD, 2003). Common marmosets are small, anthropoid, non-endangered New World monkeys residing in the Brazilian rain forest and belonging to the family of Callitrichidae (BRANDON-JONES and GROVES, 2002). Like other NHP, they exhibit an anthropoid gas exchange apparatus and, therefore, have already been used as animal models for inhalational studies (BARBIER and BACHOFEN, 2000; SURRIBAS and VON LAWZEWITSCH, 1987; BERGERS et al., 2004; KURATA et al., 1997). Along with these lung anatomical similarities, marmoset monkeys share huge homologies with humans regarding the genes involved in the immune response, including IL-6 and IL-2 (KOHU et al., 2008). For this reason they have already been used for the preclinical testing of the IL-1β antagonist canakinumab (Ilaris®) (EUROPEAN MEDICINES AGENCY, 2009). Moreover, marmosets exhibit comparable metabolizing enzymes such as the cytochrome P450 (CYP) 3A4, which represents the basis for many clinically relevant “medicine-medicine” interactions (ORSI et al., 2011). Thus, they display a key model for studying metabolic interactions of pharmaceuticals (KOEHLER et al., 2006; ORSI et al., 2011; ZUHLKE and WEINBAUER, 2003).

In general, marmoset monkeys breed well in captivity and require modest husbandry conditions (ABBOTT et al., 2003). Due to their small body size they are easier to handle compared to the larger NHP. In addition, they do not harbor human relevant pathogens such as Herpes B virus, which is often found in laboratory populations of macaques (ELMORE and EBERLE, 2008). Because of their various anatomical and

physiological similarities to humans, as well as their modest requirements in animal husbandry, the marmoset monkeys might represent suitable preclinical animal models for human respiratory diseases.

2. Hypothesis

For the testing of new, highly-specific compounds targeting features of human respiratory diseases such as ALI/ARDS, COPD, and asthma, adequate preclinical animal models are necessary.

The present thesis hypothesizes that PCLS of marmoset monkeys display a suitable model for reflecting human mechanisms of bronchoconstriction. Moreover, it is hypothesized that the pro-inflammatory features of human inflammatory lung diseases including ALI/ARDS and COPD can be mirrored in a tired approach of this small anthropoid NHP. The marmoset monkey, therefore, might represent an appropriate animal model for the testing of drugs which target inflammatory and bronchoconstrictive features of human respiratory diseases.

For providing this hypothesis two different projects were examined:

(1) Bronchoconstriction represents an important feature of inflammatory lung diseases. Thus, pharmacological mechanisms of mediator-induced bronchoconstriction shall be investigated in different NHP species *ex vivo* using the technique of PCLS. An interspecies comparison shall be carried out in order to evaluate the relevance of the New World monkey common marmoset, representing a preclinical model for human mechanisms of bronchoconstriction. In addition, bronchoconstrictive mechanisms of three Old World monkey species including cynomolgus macaque, rhesus macaque, and olive baboon shall be examined and compared with data of humans, rodents, and guinea pigs.

(2) Inflammation displays another prominent feature of ALI/ARDS and COPD. Therefore, inflammatory mechanisms constitute an important target for the development of new therapeutics. Due to the fact that cell recruitment cannot be mirrored *ex vivo*, a tired translational approach for preclinical testing of anti-inflammatory drugs shall be conducted. In a first step, the pro-inflammatory response of the potent immune activator LPS shall be analyzed on marmoset PCLS and compared with equally treated human PCLS. In addition, the

therapeutic efficacy of LPS-induced inflammation shall be assessed using the PDE4 inhibitor roflumilast and the corticosteroid dexamethasone. In the following step, the *ex vivo* approach shall be transferred onto the *in vivo* situation. Thus, an acute unilateral LPS challenge shall be performed to evaluate the therapeutic efficacy of these anti-inflammatory drugs in marmoset monkeys by analyzing bronchoalveolar influx of neutrophil granulocytes and TNF-α release.

3. Bronchoconstriction in non-human primates: a species comparison

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Abstract

Bronchoconstriction is a characteristic symptom of various chronic obstructive respiratory diseases such as chronic obstructive pulmonary disease and asthma. Precision-cut lung slices (PCLS) are a suitable *ex vivo* model to study physiological mechanisms of bronchoconstriction in different species. In the present study, we established an *ex vivo* model of bronchoconstriction in nonhuman primates (NHPs). PCLS prepared from common marmosets, cynomolgus macaques, rhesus macaques, and olive baboons were stimulated with increasing concentrations of representative bronchoconstrictors: methacholine, histamine, serotonin, leukotriene (LT)D_4, U46619, and endothelin-1. Alterations in the airway caliber were measured and compared with previously published data from rodents, guinea pigs, and humans. Methacholine induced maximal airway constriction, varying between 74 and 88 % in all NHP species, whereas serotonin was ineffective. Histamine induced maximal bronchoconstriction of 77 to 90 % in rhesus macaques, cynomolgus macaques, and baboons and a lesser constriction of 53 % in marmosets. LTD_4 was ineffective in marmosets and rhesus macaques but induced a maximum constriction of 44 to 49 % in cynomolgus macaques and baboons. U46619 and endothelin-1 caused airway constriction in all NHP species, with maximum constrictions of 65 to 91 % and 70 to 81 %, respectively. In conclusion, PCLS from NHPs represent a valuable ex vivo model for studying bronchoconstriction. All NHPs respond to mediators relevant to human airway disorders such as methacholine, histamine, U46619, and endothelin-1 and are insensitive to the rodent mast cell product serotonin. Only PCLS from cynomolgus macaques and baboons, however, responded also to leukotrienes, suggesting that among all compared species, these two NHPs resemble the human airway mechanisms best.

4. LPS-induced lung inflammation in marmoset monkeys – an acute model for anti-inflammatory drug testing

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Abstract

Increasing incidence and substantial morbidity and mortality of respiratory diseases requires the development of new human-specific anti-inflammatory and disease-modifying therapeutics. Therefore, new predictive animal models that closely reflect human lung pathology are needed. In the current study, a tiered acute lipopolysaccharide (LPS)-induced inflammation model was established in marmoset monkeys (*Callithrix jacchus)* to reflect crucial features of inflammatory lung diseases.
Firstly, in an *ex vivo* approach marmoset and for the purposes of comparison, human precision-cut lung slices (PCLS) were stimulated with LPS in presence or absence of the phosphodiesterase-4 (PDE4) inhibitor roflumilast. Pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF-α) and macrophage inflammatory protein-1 beta (MIP-1β) were measured. The corticosteroid dexamethasone was used as treatment control. Secondly, in an *in vivo* approach marmosets were pre-treated with roflumilast or dexamethasone and unilaterally challenged with LPS. Ipsilateral bronchoalveolar lavage (BAL) was conducted 18 hours after LPS challenge. BAL fluid was processed and analyzed for neutrophils, TNF-α, and MIP-1β.
TNF-α release in marmoset PCLS correlated significantly with human PCLS. Roflumilast treatment significantly reduced TNF-α secretion *ex vivo* in both species, with comparable half maximal inhibitory concentration (IC_{50}). LPS instillation into marmoset lungs caused a profound inflammation as shown by neutrophilic influx and increased TNF-α and MIP-1β levels in BAL fluid. This inflammatory response was significantly suppressed by roflumilast and dexamethasone.
The close similarity of marmoset and human lungs regarding LPS-induced inflammation and the significant anti-inflammatory effect of approved pharmaceuticals assess the suitability of marmoset monkeys to serve as a promising model for studying anti-inflammatory drugs.

5. Discussion

The data presented in this thesis comprise different experimental approaches to evaluate the marmoset monkey as an animal model to study respiratory diseases. The main findings are summarized as follows: (1) PCLS of marmoset monkeys demonstrate huge similarities compared to human mechanisms of bronchoconstriction as well as the immune response to LPS and the feasibility of anti-inflammatory intervention; (2) LPS-induced lung inflammation in marmoset monkeys can be modulated by the recently approved COPD drug roflumilast in a human-relevant dose, revealing significant reduction of neutrophil numbers and TNF-α levels in BAL fluid. Thus, marmoset monkeys might represent a promising predictive animal model of human lung diseases for testing of new pharmaceuticals.

For the development of new and innovative pharmaceuticals targeting mechanisms of bronchoconstriction, suitable animal models that are closely related to human airway anatomy and physiology are necessary. NHP display such a promising animal model due to their high phylogenetic proximity. Previously, various *in vivo* models of NHP including rhesus macaques, cynomolgus macaques, and baboons have been used to investigate the efficacy of the bronchoconstrictors histamine, methacholine, or LT (MADWED and JACKSON, 1997; PATTERSON et al., 1983; ROEHRS et al., 1981; SCHELEGLE et al., 2001; TOMKINSON et al., 2009; VAN SCOTT et al., 2004). Such *in vivo* experiments especially in NHP are, however, often viewed critically due to ethical concerns and are mostly very cost-intensive (COFFMAN and HESSEL, 2005). Moreover, the utilization of various methods hindered a comparison of the results. To overcome these issues, we used the *ex vivo* technique of PCLS for evaluating mechanisms of bronchoconstriction.

Methacholine and histamine are routinely utilized bronchoconstrictors for diagnosing AHR and for evaluating the efficacy of therapeutic treatments in human clinical trials (O'BYRNE et al., 2009). In most species including guinea pigs, rodents, and humans, methacholine caused constriction of the ASM via binding on the M_3 receptor (FISHER et al., 2004; HELD et al., 1999; O'BYRNE et al., 2009). During this study we

were able to show that methacholine was a very effective bronchoconstrictor in PCLS of olive baboon, cynomolgus macaque, rhesus macaque, and common marmoset too. Histamine also displayed a strong bronchoconstrictive efficacy in the analyzed NHP species as well as in guinea pigs and humans but not in rodents (CHURCH, 1975; HELD et al., 1999; RESSMEYER et al., 2006). Serotonin, however, is a potent bronchoconstrictor in rodents and guinea pigs, but revealed no or only weak bronchoconstrictive efficacy in the studied NHP. This finding is in line with human clinical trials, where serotonin induces no significant changes in FEV_1 after challenge with a maximum of 20 mg/mL serotonin (BROCKLEHURST, 1958; RESSMEYER et al., 2006; TONNESEN, 1985). Additionally, studies using human derived PCLS revealed no changes in airway caliber after treatment with increasing concentrations of serotonin (RESSMEYER et al., 2006).

Other bronchoconstrictors with clinical relevance are cysLTs and Tx as well as ET-1. All of them are released from immune cells or injured epithelial cells during the inflammatory response in respiratory diseases such as asthma and COPD (BARNES et al., 2003; BARNES et al., 1998). CysLTs including LTC_4, LTD_4, and LTE_4, are increased in the BAL fluid of asthmatics and have been correlated with the pathophysiology in asthma (KIM et al., 2006; PETERS-GOLDEN, 2008; RESSMEYER et al., 2006). Because cysLTs mediate ASM contraction via the $cysLT_1$ receptor, several $cysLT_1$ receptor antagonists such as montelukast or zafirlukast have been developed for inhibiting bronchoconstriction in asthma. These antagonists are, however, only successful in half of asthmatics due to variants in genes of the leukotriene pathway such as a polymorphism in *ALOX5* encoding for the arachidonate 5-lipoxygenase (HAWKINS and PETERS, 2008; KLOTSMAN et al., 2007; LIMA et al., 2006; PORTELLI and SAYERS, 2012; TELLERIA et al., 2008). Patients with repeated polymorphism in the ALOX5 promoter display a limited ability to initiate the cascade of LT synthesis. Thus, LTs are not responsible for airway obstruction in these patients which also implicates the limited efficacy of $cysLT_1$ receptor antagonists (TELLERIA et al., 2008). Such genetic variability seems to be present in NHP species, as well. Previous studies showed that the LTD_4-receptor antagonists SK&F 104353 and ICI 198615 significantly reduced antigen-induced as

well as LTD_4-induced bronchoconstriction in cynomolgus macaques and humans (CHRISTIE et al., 1991; OSBORN et al., 1992; ROVATI et al., 1992; TURNER et al., 1996). On the contrary, in rhesus macaques the *Ascaris suum* antigen-induced airway response could not be inhibited by the LTD_4 receptor antagonist ICI 198615 (PATTERSON et al., 1988). However, LTD_4-challenge in anesthetized healthy rhesus macaques by using an unphysiologically high concentration of 1 mM LTD_4 induced changes in pulmonary function (PATTERSON et al., 1983). Our data revealed no response to LTD_4 in PCLS of rhesus macaques as well as common marmosets. This might be due to the 1000-fold lower concentration in comparison to the dosage used by PATTERSON (1983). In PCLS of baboons and cynomolgus macaques, LTD_4 was a more potent bronchoconstrictor than histamine as described before in human subjects (BARNES et al., 1984).

Another metabolite of the arachidonic acid pathway contributing to bronchoconstriction is TxA_2 (JONES et al., 1992a). Elevated levels of TxA_2 have been reported in various respiratory diseases including asthma and COPD (DAVI et al., 1997; DOGNE et al., 2002a). TxA_2 is produced by platelets and a number of cell types including epithelia cells, ASM cells, and resident macrophages (NUSING et al., 1990). Since the action of TxA_2 is mediated through the Tx prostanoid receptor, several antagonists have been developed such as seratrodast and ramatroban for preventing and reducing bronchoconstriction in human asthma (DOGNE et al., 2002b). In line with previous findings in humans, the stable TxA_2 mimetic U46619 caused bronchoconstriction in all studied NHP and revealed a more potent broncho-constriction capacity compared to methacholine (JONES et al., 1992b).

Furthermore, ET-1 levels are also increased in sputum of asthma and COPD patients (CHALMERS et al., 1997; ROLAND et al., 2001). Previously, it was demonstrated that ET-1 was a 100-fold more potent bronchoconstrictor in asthmatics compared to methacholine (CHALMERS et al., 1997). In PCLS of humans, guinea pigs, and rodents ET-1 revealed a 25- to 100-fold increase in bronchoconstrictive efficacy compared to methacholine (HELD et al., 1999; RESSMEYER et al., 2006). We were able to show that this also holds true for PCLS of NHPs, except baboons.

Altogether, mechanisms of bronchoconstriction in NHPs shared great analogies with human lungs due to the fact that all human relevant bronchoconstrictors, except LTD_4, induced ASM constriction in all the NHPs studied. Compared to rodents, the PCLS of NHPs generally responded more sensitively to the bronchoconstrictors tested. Among these NHPs, the marmoset monkey and the baboon were the most susceptible species concerning methacholine-induced and histamine-induced bronchoconstriction. For ET-1-induced bronchoconstriction, in particular marmoset monkeys as well as cynomolgus macaques responded most sensitively. Moreover, all the NHP species tested represent useful models for investigating pharmaceuticals targeting TxA_2 in the context of preventing bronchoconstriction in asthmatics. Pharmaceuticals targeting cysLT receptors, however, have to be investigated in cynomolgus macaques or in baboons due to their high ASM sensitivity to LTD_4 which seems to be absent in marmoset monkeys and rhesus macaques. Regarding the high homologies as well as moderate housing costs, easy handling, and breeding conditions, the marmoset monkey represents a more interesting species among all the NHPs for testing bioactive pharmaceuticals targeting ASM response than rodents or guinea pigs.

Besides the mechanisms of bronchoconstriction of NHP`s, we evaluated a tiered LPS-induced acute lung-inflammation model in marmoset monkeys for the testing of anti-inflammatory pharmaceuticals. For predictivity and biological relevance, we performed a comparison to human lung tissue regarding the LPS-induced inflammation. Furthermore, we compared the anti-inflammatory potential of the PDE4 inhibitor roflumilast on LPS-stimulated PCLS of marmoset monkeys and humans.

Ex vivo acute LPS exposure in human PCLS for reflecting features of respiratory diseases such as ALI/ARDS and COPD has previously revealed a dose-dependent increased of pro-inflammatory cytokines (SWITALLA et al., 2010). This effect was also observed in marmoset PCLS as indicated by increased TNF-α and MIP-1β levels. Moreover, the LPS-induced TNF-α release in marmoset and human PCLS correlated significantly, suggesting a similar LPS-induced pro-inflammatory response

in the lung tissue of both species. Compared to human PCLS, marmoset PCLS showed a five times stronger increase of TNF-α secretion after LPS stimulation. This might be due to the fact that human lung tissue was received from patients undergoing resection for cancer, whereas marmoset lung tissue was obtained from naïve animals getting euthanized for other purposes. Similarly to human PCLS (SWITALLA et al., 2010), the commonly used corticosteroid dexamethasone significantly reduced LPS-induced TNF-α and MIP-1β secretion in PCLS of marmoset monkeys. Thus, these findings additionally approve that the discussed steroids resistance in New World monkey species, such as the squirrel monkey (*Samiri sciureus*), is not appearing in marmoset monkeys (CHROUSOS et al., 1986).

Despite high doses of corticosteroids having been widely used in the management of COPD, they failed to reduce disease progression or mortality (BARNES, 2004b). Corticosteroids switch-off NF-κB regulated genes encoding for pro-inflammatory cytokines due to deacetylating the hyperacetylated histones through the recruitment of the nuclear enzyme histone deacetylase-2 (BARNES et al., 2004). This process becomes impaired as a result of oxidative stress in patients with COPD, severe asthma, as well as in asthmatics who smoke (ITO et al., 2004; THOMSON et al., 2004; BARNES, 2010). Increasing corticosteroid dosage cannot overcome this steroid resistance, hence, alternative anti-inflammatory therapeutics have to be developed. A first success was achieved with the approval of the PDE4-inhibitor roflumilast as a treatment for COPD by the US Food and Drug Administration (FDA) in 2011. PDE4 is the major metabolizing enzyme of the second messenger cyclic adenosine monophosphat (cAMP) and further attenuates cell activation in inflammatory cells including neutrophil granulocytes (SCHUDT et al., 1999), macrophages (GANTNER et al., 1997), eosinophil granulocytes (HATZELMANN et al., 1995), T-lymphocytes (ESSAYAN et al., 1997), mast cells, and epithelial cells (TORPHY, 1998). Inhibition of PDE4 activity results in an increase of intracellular cAMP and is accompanied by down-regulation of inflammatory cell activity (BUNDSCHUH et al., 2001).

We have used this compound to investigate the modulation capacity in a tiered acute LPS-induced lung-inflammation approach of marmoset monkeys. The corticosteroid

dexamethasone served as a therapeutic control as it represents a group of potent anti-inflammatory and immunosuppressant drugs being very effective. In marmoset and human PCLS roflumilast revealed similar anti-inflammatory potency as shown by almost analogue pIC_{50} of 8.88 and 8.97, respectively. These pIC_{50} values calculated are in line with a previous study performed by TRALAU-STEWART and colleagues (2011) revealing pIC_{50} of 8.30 and 7.71 for human peripheral blood mononuclear cells (PBMC) and whole blood (WB) assays (TRALAU-STEWART et al., 2011). However, TRALAU-STEWART et al. (2011) used 1 ng/mL LPS of *Escherichia coli* serotype O127:B8 and an incubation period of 20 hours whereas we used 100 ng/mL LPS of *E. coli* serotype O111:B4 for 24 hours. Thus, a more detailed comparison of the results from these studies remains difficult due to the fact that various *E. coli* lipopolysaccharides have been suggested to have different levels of efficiency for releasing pro-inflammatory cytokines including TNF-α (AKARSU and MAMUK, 2007). The organotypic lung model PCLS has been shown to closely reflect the human *in vivo* situation. We also uncovered a high correlation between LPS-induced TNF-α release of marmoset and human PCLS. Based on this, a deposit dose of 500 ng LPS was applied into the lung of marmoset monkeys derived from the EC_{50} of the *ex vivo* set up. Furthermore, due to analogue pIC_{50} in human and marmoset PCLS, the human therapeutically effective dosage of 500 µg roflumilast was orally administered in marmoset monkeys once daily for up to 5 days.

Since LPS is a contaminant found in cigarette smoke, the LPS concentration applied is comparable to the smoking of 3-4 cigarettes, each containing 120 ± 64 ng bioactive LPS. By smoking one pack of cigarettes per day smokers easily inhale an amount of 2.4 µg LPS (HASDAY et al., 1999). This LPS dose is similar to the level of LPS exposure of cotton textile workers suffering from adverse health effects (KENNEDY et al., 1987). Additionally, potentially pathogenic bacteria colonizing the airways of COPD patients also produce a small amount (36 mEU/mL) of endotoxins, which contributes to the progression of the disease (SETHI and MURPHY, 2008). An estimation of the deposited LPS dose is difficult due to the fact that a part of the inhaled LPS gets lost due to exhalation.

In vivo models of acute respiratory LPS challenge in animals as well as in humans are widely used for the testing of anti-inflammatory drugs (BUNDSCHUH et al., 2001; HOHLFELD et al., 2008; MITCHELL et al., 2010; TRALAU-STEWART et al., 2011). Although these short time models do not reflect all features of human disease, they are useful in evaluating the anti-inflammatory potential of a drug. In this context, several human clinical trials conducting a segmental LPS challenge have been used. By doing so, a precise LPS-administration of a specific lung region was of great advantage due to the possibility of using an unchallenged lung lobe or segment in the same individual as control (ATOCHINA-VASSERMAN et al., 2011; HOHLFELD et al., 2008). The unilateral LPS-application further allows the sampling from more peripheral lung regions and was reported to show relevant therapeutic effects with newly developed therapeutics (HOHLFELD et al., 2008). Related to these studies, we used a unilateral LPS-application with subsequent BAL 18 hours later in the marmoset monkeys. In addition to the advantage of a specific LPS administration, we did not euthanize the NHP for sample taking due to ethical reasons. This feature is in strong contrast to studies commonly conducted in small laboratory animals, which are normally final trials (NADER and BARAKA, 2012; TOWARD and BROADLEY, 2001).

O'GRADY and colleagues (2001) first showed local endotoxin-induced lung inflammation in healthy human subjects resulting in a strong influx of neutrophil granulocytes and an increase in inflammatory mediators including TNF-α, IL-1β, and MIP-1β 6 hours after challenge (O'GRADY et al., 2001). After 24 hours, however, most mediators returned to baseline levels, while neutrophil granulocytes, macrophages, monocytes, and lymphocytes were still increased (O'GRADY et al., 2001). HOHLFELD and colleagues (2008) performed a segmental bronchial endotoxin challenge with following BAL after 24 hours to investigate anti-inflammatory properties of the PDE-4 inhibitor roflumilast on inflammatory cell level. They found significant increases in TNF-α, IL-6, IL-8, MMP-9, and MCP-1 in BAL fluid compared to baseline samples. However, no significant differences between roflumilast and placebo group were noticed (HOHLFELD et al., 2008). For the purposes of our study, a time point of 18 hours post LPS-challenge was defined, in

order to cover changes on cellular level as well as on cytokine level. In line with human data, roflumilast-pretreatment prevented an influx of total cells in LPS-challenged marmoset monkeys. As observed in the human clinical trial, roflumilast-pretreatment significantly reduced the LPS-induced neutrophilic influx and TNF-α increase in comparison to the sham-treated positive group. This was also detectable in the treatment control group. Oral dexamethasone treatment significantly reduced the relative numbers of neutrophil granulocytes, while absolute neutrophil numbers were only reduced by trend. Inhaled corticosteroids have also been shown to diminish LPS-induced neutrophilia and TNF-α expression in a cynomolgus macaque model of lung inflammation (MITCHELL et al., 2010). The use of corticosteroids for the management of patients with COPD has, however, been a controversial matter: While some human clinical trials revealed no protective outcome of oral corticosteroids on LPS-induced inflammation in sputum of healthy subjects (CULPITT et al., 1999; MICHEL et al., 2007), others reported a positive effect on exacerbations as well as on markers of lung inflammation including a reduced percentage of neutrophil granulocytes after treatment with inhaled corticosteroids alone (DAVIES et al., 1999; OZOL et al., 2005). Because of this heterogeneity and the strong systemic side effects, oral corticosteroids in particular, are no longer recommended for the treatment of COPD over a longer period of time. Inhaled corticosteroids, however, are still recommended for patients with frequent exacerbations especially in combination with bronchodilators for improving lung function (ABDOOL-GAFFAR et al., 2011). Altogether, these facts emphasize once again the urgent need of potent innovative drugs for the treatment of respiratory diseases such as COPD.

We used an acute LPS challenge to model pro-inflammatory cytokine release and neutrophilia of complex respiratory diseases such as ALI/ARDS and COPD. However, COPD represents a slowly progressive chronic respiratory disease comprising airflow limitation and a variety of pathological changes in the peripheral airways and lung parenchyma (BARNES, 2004b). Some features including structural changes such as emphysema and small airway remodeling can, therefore, only be displayed in chronic studies (WRIGHT et al., 2008). In rodents and guinea pigs,

chronic LPS administrations over several weeks lead to COPD-related pathological changes such as enlarged air spaces, remodelled airways with thickened walls, and increased goblet cells (TOWARD and BROADLEY, 2001; VERNOOY et al., 2002; WRIGHT et al., 2008). A chronic LPS challenge over an extended period would also be conceivable in marmoset monkeys with the benefit of a repeated unilateral stimulation in a small anthropoid animal.

A notable limitation of the LPS-induced inflammation model in marmoset monkeys is the use of a relatively poorly-characterized NHP species. This becomes particularly obvious in the restricted scope of marmoset-specific analytics. Despite cell differentiation with common cytology methods is sufficient to evaluate a neutrophilic inflammation in marmoset monkeys *in vivo*, further endpoints such as MMPs or additional cytokines including IL-10 would be desirable (HOHLFELD et al., 2008; MICHEL et al., 1997; O'GRADY et al., 2001; WRIGHT et al., 2008). Reliable analyses of alterations on the cytokine level only comprise TNF-α and MIP-1β, contrary to the variety on murine and human commercially available tools. The marmoset genome is, however, available as a draft assembly suggesting the identification, development, and marketing of further analytics in the near future. As shown in the first project, we were able to uncover vast analogies in marmoset monkeys to human mechanisms of bronchoconstriction. In addition to the vast similarities in lung anatomy (SURRIBAS and VON LAWZEWITSCH, 1987), important analogies in immunity related genes including cytokines like IL-6, IL-8, and IL-1β, as well as cell surface molecules such as CD40 and CD86, have been reported for marmoset monkeys and humans (KOHU et al., 2008). Further, SASAKI et al. (2009) published the establishment of a transgenic marmoset monkey. The use of NHP species for biomedical research is, however, often viewed critically due to ethical concerns (COFFMAN and HESSEL, 2005). But the evidence of the scientific value of marmoset monkeys as a predictor for efficacy and adverse effects in humans due to the closer similarities to human metabolism and enzyme structures compared to rodents, dogs, and pigs is increasing (IGARASHI et al., 1997; SAKUMA et al., 1997). Moreover, the marmoset monkey exhibits similar pharmacodynamic effects as humans (SMITH et al., 2001).

Our acute LPS-induced lung inflammation model in marmoset monkeys might be useful for the study of new pharmaceuticals targeting neutrophilic inflammations in the lung. As shown for roflumilast, especially the testing of inhibitors targeting cell signaling pathways of inflammatory mediators as well as therapeutics directed against chemokines, cytokines, and adhesion molecules will be promising targets. The close immunological and metabolical similarities of marmoset monkeys to humans might, therefore, be in great demand for the preclinical testing of such highly specific biological therapeutics. This advantage has already been taken into account for pharmacokinetic and toxicity testing of the IL-1β antagonist canakinumab (Ilaris®), which was neither cross-reactive with IL-1β from rodents nor cynomolgus macaques. The marmoset monkey represented the only non-human species that was specifically recognized by canakinumab due to a high analogue in the IL-1β amino acid sequence (EUROPEAN MEDICINES AGENCY, 2009). Moreover, the testing of a new human IgG1-κ antibody targeting human IL-12p40, a shared subunit of IL-12 and IL-23, was executed in a marmoset monkey autoimmune encephalomyelitis (EAE) model of multiple sclerosis ('T HART et al., 2005; 'T HART and MASSACESI, 2009; BROK et al., 2002).

In conclusion, the obtained results indicate that PCLS of marmoset monkeys closely reflect human mechanisms of bronchoconstriction. Furthermore, the tiered LPS-induced inflammation model in this small anthropoid NHP, mirrored pro-inflammatory features of human inflammatory lung diseases such as ALI/ARDS and COPD. The marmoset monkey, therefore, represents a suitable model for the preclinical testing of potential therapeutics targeting inflammatory and bronchoconstrictive features of human respiratory diseases.

6. Outlook

The marmoset monkey represents an animal model which is suitable for the study of human respiratory diseases such as ALI/ARDS, COPD, and asthma. We were the first to show data, comprising mechanisms of bronchoconstriction of various NHP species to commonly used bronchoconstrictors including histamine, methacholine, ET-1, serotonin, LTD_4, and U46619. However, we did not perform pharmacological intervention studies for determining mediators involved in allergen-induced bronchoconstriction. Therefore, future experiments should address mechanisms of allergen-induced bronchoconstriction using receptor antagonists or inhibitors, such as montelukast targeting the $cysLT_1$ receptor, tripolidin targeting the H_1 receptor, salbutamol targeting the β_2-receptor, or tiotropium targeting the M_3 receptor.

Secondly, we designed a tiered model for LPS-induced lung inflammation in marmoset monkeys. Primarily this model was developed for testing of novel, highly specific, and biologically-active drugs. Despite intense efforts, however, it was not possible to use such pharmaceuticals due to demanding access. Moreover, the limited amount of read-out parameters makes the reliable detection of cytokines others than TNF-α and MIP-1β difficult. Therefore, alternative approaches for monitoring the LPS-induced inflammation will have to be considered such as analysis of cytokines on mRNA level or *in vivo* imaging. Due to the fact that the inflammatory respiratory disease COPD is also an obstructive disease it would be useful to establish lung function measurements for investigating LPS-induced acute exacerbations in marmoset monkeys.

7. Summary

Marmoset monkeys as a preclinical model for respiratory research

Sophie Seehase

Increasing incidence and substantial morbidity and mortality of respiratory diseases including ALI/ARDS, COPD, and asthma bronchiale requires the development of new effective therapeutics. In this context, highly human-specific and biologically-active substances targeting cell signaling pathways or directed against cytokines and adhesion molecules display the most promising treatment strategies. For the preclinical testing of these biologically active substances, however, suitable predictive human relevant animal models are needed.

The aim of the present work was to evaluate the suitability of the small anthropoid New World monkey common marmoset to serve as a preclinical animal model for respiratory diseases. Airway obstruction and inflammation represent important features of these diseases and have been analyzed in *ex vivo* and *in vivo* approaches. Using PCLS, the mechanisms of bronchoconstriction were investigated in marmoset monkeys, as well as in three Old World monkey species including, cynomolgus macaques, rhesus macaques, and olive baboons. The results were then compared to published data of humans and commonly used small laboratory animals. Furthermore, in accordance with previously published inflammation models in humans, a tiered approach of acute LPS-induced lung inflammation was designed in marmoset monkeys *ex vivo* and *in vivo*.

The treatment of marmoset PCLS with various bronchoconstrictors including methacholine, histamine, serotonin, ET-1 and the Tx-prostanoid agonist U46619 displayed huge similarities compared to human mechanisms of bronchoconstriction. Additionally, exposure of marmoset PCLS to LPS, a strong activator of the innate immune system, revealed huge analogies to equally treated human PCLS regarding TNF-α release. This LPS-induced TNF-α release was significantly reduced by the PDE4 inhibitor roflumilast with the same potency as in human PCLS resulting in closely related pIC_{50}. The pre-treatment of marmoset monkeys with the human

dosage of roflumilast for 5 consecutive days revealed a significant lower influx of neutrophil granulocytes, as well as TNF-α in BAL fluid 18 hours after LPS challenge. Furthermore, as in human PCLS the corticosteroid dexamethasone also revealed strong immunosuppressive effects in LPS-induced lung inflammation in marmoset monkeys.

Along with these findings, we were able to show that PCLS of marmoset monkeys are a suitable model for investigating mechanisms of bronchoconstriction in the context of obstructive lung diseases. Furthermore, the tiered approach of LPS-induced inflammation in marmoset monkeys represents a new preclinical model for evaluating therapeutic efficacy of new-class pharmaceuticals targeting inflammatory features of respiratory diseases.

8. Zusammenfassung

Weißbüschelaffen als präklinisches Modell für respiratorische Erkrankungen
Sophie Seehase

Atemwegserkrankungen wie ALI/ARDS, chronisch obstruktive Lungenerkrankung (COPD) und Asthma bronchiale sind weltweit verbreitet und stellen ein großes ökonomisches Problem für das öffentliche Gesundheitssystem dar. Auf Grund der bisher nur unzureichenden Therapiemöglichkeiten besteht ein großer Bedarf zur Entwicklung neuer Therapeutika. Eine wichtige Rolle spielen hierbei biologisch aktive Substanzen, die hochspezifisch bestimmte Zielstrukturen, z.B. Rezeptoren angreifen und so die Signalkaskade inhibieren. Zur Testung dieser Substanzen sind Tiermodelle mit hoher Prädiktivität notwendig, die sowohl in Anatomie als auch Physiologie große Homologien zum Menschen aufweisen.
Das Ziel dieser Arbeit war es abzuschätzen, ob die Neuweltaffenspezies Weißbüschelaffe ein geeignetes Tiermodell für Lungenerkrankungen des Menschen repräsentieren kann. Hierzu wurden die Aspekte Entzündung und Atemwegskonstriktion in *ex vivo* und *in vivo* Ansätzen untersucht. Unter Verwendung von Präzisionslungenschnitten (PCLS) wurden die Mechanismen der Bronchokonstriktion im Weißbüschelaffen, sowie in den drei Altweltaffenspezies Javaneraffe, Rhesusaffen und Anubispavian erforscht und mit bereits publizierten Daten vom Menschen und kleinen Labortieren verglichen. In Anlehnung an ein bereits publiziertes Atemwegsentzündungsmodell im Menschen, wurde ein abgestufter Ansatz der akuten LPS-induzierten Lungenentzündung im Weißbüschelaffen konzipiert.
Die Behandlung von Weißbüschelaffen PCLS mit verschiedenen Bronchokonstriktoren zeigte große Übereinstimmungen zu humanen Bronchokonstriktionsmechanismen. Die Stimulation von Weißbüschelaffen PCLS mit Lipopolysaccharid (LPS) wies bezüglich der induzierten TNF-α Sekretion große Übereinstimmung mit gleichbehandelten humanen PCLS auf. Diese LPS-induzierte TNF-α Sekretion konnte mit dem Phosphodiesterase-4 Inhibitor Roflumilast sowohl in

humanen als auch Weißbüschelaffen PCLS bei annähernd gleichen pIC_{50} Werten reduziert werden. Ebenso wie in humanen PCLS, wirkte zudem das Glukocorticoid Dexamethason im Weißbüschelaffen immunsupprimierend. Die orale Vorbehandlung von Weißbüschelaffen mit der humanen Dosis an Roflumilast über 5 Tage reduzierte im Vergleich zur Scheinbehandlung den LPS-induzierten Influx an neutrophilen Granulozyten sowie einen Anstieg der TNF-α Konzentration in der bronchoalveolären Lavageflüssigkeit 18 Stunden nach unilateraler bronchialer LPS Provokation.

Damit konnte gezeigt werden, dass Weißbüschelaffen ein vielversprechendes Tiermodell zur Abschätzung der therapeutischen Wirksamkeit neuartiger Pharmazeutika zur Behandlung von entzündlichen und obstruktiven Lungenerkrankungen darstellen.

9. List of Abbreviations

A549	Adenocarcinomic human alveolar basal epithelial cell line
AHR	Airway hyperresponsiveness
ALOX5	Arachidonate 5 lipoxygenase
ASM	Airway smooth muscle
BAL	Bronchoalveolar lavage
BEAS-2B	Human bronchial epithelial cell line
cAMP	Cyclic adenosine monophosphat
CD	Cluster of differentiation
COPD	Chronic obstructive pulmonary disease
CYP	Cytochrome P450
CysLT	Cysteinyl leukotriene
DAMP	Damage associated molecular patterns
DC	Dendritic cell
EAE	Autoimmune encephalomyelitis
EAR	Early allergic reaction
EC_{50}	Half maximal effective concentration
E. coli	*Escherichia coli*
ET	Endothelin
FcεR	High affinity Fc receptor
FDA	US Food and Drug Administration
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
GM-CSF	Granulocyte macrophage colony-stimulating factor
GOLD	Global initiative for chronic obstructive lung disease
H_1	Histamine 1
IFN-γ	Interferon-gamma
IC_{50}	Half maximal inhibitory concentration
IL	Interleukin
IPL	Isolated perfused lung

LAR	Late allergic reaction
LPS	Lipopolysaccharide
LT	Leukotriene
MCP-1	Monocyte chemotactic protein-1
MHC	Major histocompatibility complex
MIP-1β	Macrophage inflammatory protein-1 beta
MMP	Matrix metalloproteinase
mRNA	Ribonucleic acid
NFκB	Nuclear factor “kappa-light-chain-enhancer” of activated B cells
NHBE	Normal human bronchial epithelial cell line
NHP	Non-human primates
PBMC	Peripheral blood mononuclear cell
PCLS	Precision cut lung slices
PDE4	Phosphodisterase-4
pIC_{50}	Negative logarithm of the half maximal inhibitory concentration
PRR	Pattern recognition receptors
STAT4	Signal transducer and activator of transcription 4
T cell	Thymus cell
T_H cell	T helper cell
THP-1	Human monocyte leukemia cell line
TLR	Toll like receptor
TNF-α	Tumor necrosis factor alpha
TxA_2	Thromboxane A2
WB	Whole-blood

10. List of References

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