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HABILITATIONSSCHRIFT

Molecular mechanisms of the interaction of *L. pneumophila* with the pulmonary epithelium

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von

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1. Gutachter:

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For my parents:
Dje Claude N'Guessan and N'Taho Pauline Affa

For my son:
Lukas Cyrill N'Guessan

For my 11 brothers and sisters

For my grandfather:
N'Guessan N'Golè Philippe

Abstract (engl.)

Legionella pneumophila is a major cause of sporadic and epidemic community- and hospital - acquired pneumonia (CAP and HAP) that leads to frequent deaths worldwide. To cause these diseases, *L. pneumophila* has to interact with the pulmonary epithelial surface of the lung, which is known not only to be a mechanical barrier against invading pathogens, but also to play an important role in innate immunity. Thus, it is essential to understand the molecular mechanisms underlying this interaction. The present work will give an overview on the pathogenesis of *L. pneumophila*-induced pulmonary infections. Focus will be put on mechanisms of target cell activation via putative host cell receptors. Furthermore activated signal transduction pathways in *L. pneumophila*-infected pulmonary epithelial cells will be presented in detail.

In the studies addressed here, we demonstrate that *L. pneumophila* infects pulmonary epithelial cells. Identification of virulence factors of *L. pneumophila* by pattern recognition receptors such as Toll-Like Receptor (TLR)2, TLR5 and TLR9 strongly induce the expression of diverse cytokines, antimicrobial peptides, and prostaglandin E2 (PGE_2). The expression of the above mentioned mediators depends on the activation of Mitogen-Activated Protein Kinases (MAPKs), Protein Kinase C (PKC), NF-κB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells), and the Activator Protein 1 (AP-1). Furthermore, upon recognition of *L. pneumophila* by intracellular receptors such as Neuronal Apoptosis Inhibitory Protein (NAIP) and ICE protease-activating factor (Ipaf), the intracellular bacterial replication in pulmonary epithelial cells is strongly reduced. Taken together, we investigated relevant issues of innate immunity activation in *L. pneumophila*-infected pulmonary epithelial cells. These findings will contribute to the understanding of the pathogenesis of Legionnaires' disease.

Abstract (dtsch.)

Legionella pneumophila ist ein wichtiger Erreger der sporadischen und endemischen ambulant erworbenen („community acquired pneumonia“, CAP) und nosokomialen Pneumonien („hospital acquired pneumonia“, HAP). Diese Erkrankungen führen weltweit zu einer erhöhten Sterblichkeit. Um eine Pneumonie auszulösen, muss *L. pneumophila* mit den Lungenepithelzellen interagieren. Diese stellen nicht nur eine physikalische Barriere für Atemwegserreger dar, sondern spielen auch eine wichtige Rolle in der angeborenen Immunität. Es ist daher von großer Bedeutung die molekularen Mechanismen dieser Interaktion zu verstehen. Die vorliegende Arbeit gibt eine Übersicht über die Pathogenese der durch *L. pneumophila* hervorgerufenen pulmonalen Infektionen und fokussiert hierbei insbesondere auf die Mechanismen der Zielzellinfektion über potentielle Wirtszellrezeptoren, sowie die nachfolgend aktivierten Signalübertragungswege in *L. pneumophila*-infizierten Lungenepithelzellen.

Es konnte gezeigt werden, dass *L. pneumophila* Lungenepithelzellen infiziert. Die Erkennung der Virulenzfaktoren dieses Erregers durch Mustererkennungsrezeptoren wie Toll-Like Rezeptor (TLR)2, TLR5 und TLR9 führte zu einer starken Induktion unterschiedlicher Zytokine, sowie antimikrobieller Peptide und Prostaglandin E2 (PGE_2). Der Produktion obengenannter Mediatoren ging eine Aktivierung der Mitogen-Aktivierten Protein Kinase (MAPK), Protein Kinase C (PKC), NF-κB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells), und dem Aktivator Protein 1 (AP-1) voraus.

Darüber hinaus reduzierte die intrazelluläre Erkennung von *L. pneumophila* durch das Neuronal Apoptosis Inhibitory Protein (NAIP) und den ICE protease-activating factor (Ipaf) das Wachstum dieser Erreger in Lungenepithelzellen stark.

Zusammenfassend, wurden wichtige und relevante Mechanismen der Aktivierung der angeborenen Immunität in *L. pneumophila* infizierten Lungenepithelzellen adressiert. Diese neuen Erkenntnisse werden zum Teil dazu beitragen, die komplexe Pathogenese der Legionärskrankheit zu verstehen.

Index of contents with the compilation of selected publications

1 Introduction

1.1 Pneumonia

Pneumonia is an inflammatory disease of the lung. This disease is a common cause of morbidity and mortality among infectious diseases worldwide (1-4). It consumes a great deal of health, social and economic resources (2-4). Pneumonia is still the third leading cause of death in Germany (4). Furthermore, children under five years, elderly, and patients with co-morbidities (COPD, asthma, diabetes, HIV etc…) and impaired immune system are disproportionately vulnerable for this infectious disease (4). A major concern in the management of pneumonia are the emerging antibiotic-resistant bacterial strains (2;4). The current clinical classification includes community-acquired pneumonia (CAP), hospital-acquired pneumonia (HAP), ventilator-associated pneumonia (VAP), and nursing home-acquired pneumonia (NHAP) (2). The most commonly identified bacterial agents causing pneumonia are *Streptococcus pneumoniae* (also called pneumococcus; 35 - 50%), *Haemophilus influenzae* (20%), *Mycoplasma pneumoniae* (12-13%) and *Legionella pneumophila* (5%) (2;4). The studies presented here, focus on the mechanisms of interaction of *Legionella pneumophila* with pulmonary epithelial cells. Therefore, this pathogen and its related disease will be presented in detail.

1.2 *L. pneumophila* induces pneumonia

L. pneumophila is the causative infectious agent of the so called Legionnaires' disease. It is also one of the most common causative agents of severe CAP and HAP with high mortality (5). In Germany, *L. pneumophila* accounts for 4% of all CAP. However, both geographic and seasonal variations in incidence are characteristics of Legionnaires' disease (6). The Germany wide network for CAP demonstrated that the proportion of *L. pneumophila* induced pneumonia was similar for CAP and HAP (1). Nevertheless the mortality rate was considerably higher in patients with HAP (1). *L. pneumophila* is transmitted through inhalation of aerosols of contaminated water (5;7). Air conditioners and cooling systems are known to be the major source of the mentioned community-acquired outbreaks. Furthermore, smoking, age, co-morbidity (COPD, asthma, diabetes, HIV etc…), and organ transplantations are associated with a higher risk of infection (8). However, severe Legionnaires' disease has also been reported in previously healthy people (9). *L. pneumophila* has been recognized as a significant cause of hospital-acquired pneumonia in both healthy and immunosuppressed hosts (8).

Legionnaires' disease and Pontiac fever [an acute self-limiting non-fatal illness caused by *Legionella* ssp. without pneumonia but with a short incubation period and a short duration (5;7)], both known as legionellosis, have been reported in children and adults with similar clinical symptoms. Legionnaires' disease begins generally after an incubation period of 2–10 days or longer (10). Early nonspecific symptoms are a moderate cold, mild fever, malaise, anorexia, and muscle aches (5;10). Approximately half of the patients produce puss-forming sputum and about one third develops blood-streaked sputum or cough up blood. Later symptoms are strong fever, bronchiolitis, alveolitis, and lung damage with infiltrated lung areas. Since most of the symptoms are similar, it is difficult to clinically distinguish patients with Legionnaires' disease from patients with other types of pneumonia caused by bacteria such as *S. pneumoniae*, *Mycoplasma pneumoniae*, and *Chlamydophila pneumoniae* (11).

Since Legionnaires' disease is characterized by a high mortality rate and often requires hospitalization, early diagnosis that enables appropriate antimicrobial treatment is life-saving. Diagnosis of Legionnaires' disease in patients is mostly based on culture, serology or antigen detection in urine. In addition, the tendency to identify *L. pneumophila* using molecular techniques such as the detection of nucleic acid is increasing. A sensitivity of 25% to 75% concerning cultures of *Legionella* from respiratory secretions has been reported (6;11). If combined with the urinary antigen tests, sensitivity might reach 99% or higher, while specificity would remain around 50% to 90%, depending on the severity of disease. For the treatment of Legionnaires' disease Macrolides (Clarithromycin, Roxithromycin and Azithromycin) and Fluorchinolone (Moxifloxacin) remain the gold standards (11).

1.3 Microbiology of *L. pneumophila*

L. pneumophila is a Gram-negative, intracellular coccobacillus, which is a member of the gamma-subgroup of proteobacteria (5). The family *Legionellaceae* includes only the single genus *Legionella*, but contains 56 species/subspecies with over 70 serogroups (5). *Legionella* species are ubiquitous in aquatic environments as well as in moist soil and mud.

L. pneumophila usually parasitizes its natural host *Acanthamoeba castellanii*, which lives in the same environment(5). Man-made modifications of the environment render the contact of this pathogen with a new host (human macrophages) possible (5). This occurs by inhalation of parasitized amoebae that enable *L. pneumophila* to infect the human host causing thereby legionellosis (12). Apart of the amoebae, *L. pneumophila* can directly infect human pulmonary cells (12-14). After internalization into a host cell, *L. pneumophila* persists inside the phagosome and prevents its fusion with the lysosomes (12;15). In addition, it recruits

endoplasmic reticulum-derived vesicles and mitochondria to the phagosome, forming a replication vacuole, in which the bacteria multiply (12;15). In case of nutrients deficiency, *L. pneumophila* enters the transmissive phase and expresses virulence proteins (12;15). This results in the lysis of host cells, the escape of *L. pneumophila* and the initiation of a new infection cycle (12;15). A broad number of virulence factors of *L. pneumophila* have been identified so far. Among them the type IV bacterial protein secretion system (T4SS) encoded by the dot/icm genes. After host cell infection, these genes allow the bacterium to hinder the fusion of the phagolysosome and to replicate in an endoplasmic–reticulum-derived vacuole (12;15).

It has also been suggested that the T4SS and the type II bacterial secretion system (T2SS) enable the bacteria to export proteins into the cytoplasm of host phagocytic cells, activating thereby diverse cell signalling pathways (12;15). Apart from the virulence factors of *L. pneumophila* mentioned above, flagellin, peptidoglycans and bacterial nucleic acids have been shown to activate host cells (12-15). Further virulence factors of *L. pneumophila* have been described, but here we will only focus on the factors cited above, since they are relevant to the studies presented.

1.4 Pulmonary immunity

The alveolar surface is the largest of the body in contact with the outside environment. Like the skin and the gastrointestinal mucosa, the lung is permanently in contact with a broad array of microorganisms as well as organic and inorganic materials. Therefore, the organism developed strategies to recognize and to distinguish friends or foes from the outside environment. Higher vertebrates possess two interactive protective systems: the innate and the adaptive immune systems. The innate immune system is older and is composed of soluble proteins (e.g. cytokines, antimicrobial peptides and surfactant protein), and phagocytic leukocytes (16). While the soluble proteins ligate and neutralize microbial products, phagocytic cells float through the bloodstream and migrate into tissues at sites of inflammation, or reside in tissue waiting for foreign material. The innate immune system is permanently active and is immediately responsive. It is all the time ready to recognize and inactivate microbial products entering the lung. The innate immune system is activated through the recognition of common microbial motifs, pathogen-associated molecular patterns (PAMPs). Higher vertebrates have developed an adaptive immune system made of specific cells such as lymphocytes that respond specifically to signals from the innate immune system. This leads to the production of high-affinity antibodies. These antibodies bind microbes and

viruses and enable their elimination by leukocytes which are localized in tissue and lymph nodes. Cytokines and growth factors produced by macrophages, dendritic cells, and epithelial cells of the innate immune system direct the specialized antibody responses of the adaptive immune system. In contrast to the innate immune system, the adaptive immune system has a memory component. Both systems together, enable the host to respond with adequate accuracy to a broad array of microorganisms and other particles inhaled in everyday life (16).

1.5 The pulmonary innate immunity

Innate immunity is an important system that plays a major role in lung antimicrobial defences (16;17). Latest research focussing on mechanisms of recognition of microbial products by cells of the innate immune system has revolutionized the understanding of host defences in the lung as well as in other organs. The innate immune system comprises epithelial cells lining the alveolar surface, lung leukocytes, and the conducting airways. The innate immune system activates adaptive immunity in the lung. Furthermore, it interacts with other systems, such as apoptotic pathways and diverse cellular signalling pathways (18). Human diversity in innate immune responses is a major factor of some variability seen in the responses of patients to pathogens and particles in the lung (19). Therefore, new strategies to modulate innate immune responses could be of great importance in limiting the adverse consequences of some inflammatory reactions in the lung.

1.6 The lung epithelium

Apart of being a physical barrier, the epithelial cells on mucosal surfaces of the lung are important components of innate and adaptive immunity (16;20). They initiate, regulate, and resolve the innate and adaptive immune responses at pulmonary mucosa (16;20). Epithelial cells express a broad array of molecules, which are essential for immune response. These are for example chemokines, cytokines, antimicrobial peptides, surfactants, prostaglandins, major histocompatibility complexes (MHC) of class I and II, and various co-stimulatory molecules (16;20). A requirement for the initiation of a host response is the recognition of pathogens by means of pattern recognition receptors.

1.6.1 Pattern recognition receptors (PRRs)

The innate immune system recognizes invading pathogens by using pattern recognition receptors (PRRs), which detect conserved signature molecules, such as bacterial cell wall components or microbial nucleic acids (21;22). PRRs relevant for the detection of *L. pneumophila* comprise the Toll-Like Receptors (TLRs), NOD-like receptors (NLRs), and the RIG-I-like receptors (RLRs) (15;22). These PRRs are expressed in different host cells of the lung, such as bronchial and alveolar epithelial cells, as well as alveolar macrophages (22;23).

1.6.2 Toll-Like Receptors (TLRs)

In mammals, 12 members of the TLR family have been identified so far (21;22). TLRs recognize various pathogen-associated molecular patterns (PAMPs) derived from bacteria, viruses, fungi and parasitic-protozoa (21;22). TLRs are expressed in distinct cellular compartments (21;22)While TLR-1, -2, -4, -5, -6, -10, and -11 are located in the extracellular membrane, TLR-3, -7, -8, and -9 are found in the membranes of endocytic vacuoles (21;22). The recognition of pathogens by the TLRs (apart of TLR3) initiates a MyD88-dependent complex signalling pathway leading to activation of NF-κB-dependent pro-inflammatory gene expression. In addition, TLR3 and TLR4 (via the TIR-domain-containing adapter-inducing interferon-β (TRIF)) as well as TLR7-9 (by MyD88) are able to activate the type I interferon (IFN) pathway through the interferon regulatory factors (IRFs) (21;22). While the exact ligand and function of TLR10 and TLR12 are unknown, TLR11 specifically mounts an immune response to *Toxoplasma gondii* and uropathogenic *Escherichia coli* by activating MyD88 (21;22).

TLR and (co-receptors)	Cellular localization	TLR ligands
TLR1/2	Cell surface	Triacyl lipopeptides
TLR2 (Dectin-1, C-type lectin)	Cell surface	Peptidoglycan, lipoarabinomannan, hemagglutinin, phospholipomannan, glycosylphosphophatidyl inositol mucin, zymosan
TLR3	Endosome	ssRNA virus, dsRNA virus, respiratory syncytial virus, murine cytomegalovirus
TLR4 (MD2, CD14, LBP)	Cell surface	Lipopolysaccride, mannan, glycoinositolphospholipids, envelope and fusion proteins from mammary tumor virus and respiratory syncytial virus, respectively, endogenous oxidized phospholipids produced after H5N1 avian influenza virus infection, pneumolysin from *streptococcus pneumonia*, paclitaxel.
TLR5	Cell surface	Flagellin from flagellated bacteria
TLR6/2 (CD36)	Cell surface	Diacyl lipopeptides from mycoplasma), lipoteichoic acid
TLR7	Endolysosome	ssRNA viruses, purine analog compounds (imidazoquinolines). RNA from bacteria from group B streptococcus
TLR8 (only in human)	Endolysosome	ssRNA from RNA virus, purine analog compounds (imidazoquinolines).
TLR9	Endolysosome	dsDNA viruses herpes simplex virus and murine cytomegalovirus, CpG motifs from bacteria and viruses, hemozoin malaria parasite
TLR11 (only in mouse)	Cell surface	Uropathogenic bacteria, profillin-like molecule from *Toxoplasma gondii*

Table 1: TLR ligands and cellular location(21)

1.6.3 NOD-like receptors (NLRs)

The NLR family, which detects invasive pathogens and initiates an appropriate immune response, comprises more than 20 members that are barely investigated. Two of the best characterized members of the NLRs, NOD (Nucleotide-binding Oligomerization Domain)1 and NOD2 are known to detect bacterial peptidoglycan fragments within the cytosol, which leads to transcriptional regulation of NF-κB-dependent genes (22). Furthermore, recent studies suggest that NOD1 and NOD2 are capable of recognizing internalized *L. pneumophila* and thereby control the innate immune response in a mouse pneumonia model (24). Moreover, the NLR family member hNAIP (human Neuronal Apoptosis Inhibitor Protein) and Ipaf (ICE-protease activating factor) recognize and control *L. pneumophila* replication in human cells (22).

1.6.4 RIG-I-like receptors (RLRs)

The retinoic acid-inducible gene I (RIG-I), a part of the RNA helicase domain and the melanoma differentiation-associated gene 5 (MDA5) are a group of cytoplasmic receptors which recognize viral nucleic acids. Upon recognition of pathogen factors by RIG-I and MDA5, IRF3 is activated via MAVS (Mitochondrial Antiviral Signaling). This pathway leads to increased production of type I interferons (IFN). Recent studies have demonstrated that these receptors can induce the production of type I IFN via ligation with intracellular bacterial nucleic acids (22;25). Furthermore, it has been demonstrated that the recognition of DNA by RIG-I/MDA5 is dependent on cytosolic RNA polymerase III (26).

1.7 Signalling pathways activated by *L. pneumophila*

Recognition of this airborne pathogen by immune receptor initiated complex signalling pathways leads to the activation of Mitogen-Activated Protein Kinase (MAPK) pathways, Phosphoinositide 3 Kinase (PI3K), Protein Kinase C (PKC) isoforms, NF-κB and the Activator Protein-1 (AP-1) pathways (14;27;28).

Activation of these pathways may result either in the liberation or the suppression of cytokines, chemokines, and antimicrobial substances (14;27;28). Furthermore, activation of IRF3 by *L. pneumophila* induced the expression of type I IFN (29). *L. pneumophila* induced the activation of IL-1β and and IL-18 via caspase-1 (30). In recent unpublished work, we demonstrated a similar mechanism leading to the reduction of the expression of the antimicrobial peptide cathelicidin in *L. pneumophila*-infected alveolar epithelial cells. We showed that DNA as well as RNA from *L. pneumophila* reduced the expression of cathelicidin in human lung epithelium. The RNA-TLR3 and DNA-TLR9 signalling pathways merged to the activation of PI3K, which mediates interferon regulatory factor (IRF)3 or IRF7 activation. Subsequently, reduced retinoid X receptor alpha (RXRα) expression impeded the RNA polymerase II-recruitment to the cathelicidin promoter thereby shutting down the expression of this peptide.

2 Objectives

Infectious diseases of the lung are a major cause of death. Although *L. pneumophila* is a major cause of pneumonia, the interaction between *L. pneumophila* and pulmonary cells is sparely understood. The presented studies, characterize the molecular mechanisms of the interaction of *L. pneumophila* with pulmonary epithelial cells.

3. Results (Summary of publications 1-9)

3.1 Mechanisms of *L. pneumophila*-dependent induction of antimicrobial peptides in infected pulmonary cells

Publication 1

Scharf S, Hippenstiel S, Flieger A, Suttorp N, and **N'Guessan PD**. Induction of human {beta}-Defensin-2 in pulmonary epithelial cells by *Legionella pneumophila*: The Involvement of TLR2 and TLR5, p38 MAPK, JNK, NF-{kappa}B and AP-1. ***Am J Physiol Lung Cell Mol Physiol***. 2010; 298(5):L687-95.

Legionella pneumophila is an important causative agent of severe pneumonia in humans. Human alveolar epithelium is an effective barrier for inhaled microorganisms and actively participates in the initiation of innate host defense. Induction of antimicrobial peptide human beta-Defensin-2 (hBD-2) by various stimuli in epithelial cells has been reported. However, the mechanisms by which bacterial infections enhance hBD-2 expression remains poorly understood. In this study, we investigated the effect of the pulmonary pathogen *L. pneumophila* on induction of hBD-2 in human pulmonary epithelial cells. Infection with *L. pneumophila* markedly increased hBD-2 production and the response was attenuated in TLR2- and TLR5-transient knock-down cells. Furthermore, pre-treatment with SB202190 (an inhibitor of p38 mitogen-activated protein kinase, p38 MAPK) and JNK II (an inhibitor of c-Jun N-terminal kinase, JNK), but not U0126 (an inhibitor of extracellular signal-regulated kinase, ERK) reduced *L. pneumophila*-induced hBD-2 release in A549 cells. *L. pneumophila*-induced hBD-2 liberation was mediated via recruitment of NF-kappaB and AP-1 to the hBD-2 gene promoter. Additionally, we showed that exo- and endogenous hBD-2 elicited a strong antimicrobial effect towards *L. pneumophila*. Together, these results suggest that *L. pneumophila* induces hBD-2 release in A549 cells, and the induction seems to be mediated through TLR2 and TLR5 as well as activation of p38 MAPK, JNK, NF-kappaB, and AP-1.

Publication 2

Scharf S, Vardarova K, Lang F, Schmeck B, Opitz B, Flieger A, Heuner K, Hippenstiel S, Suttorp N, and **N'Guessan PD**. *Legionella pneumophila* induces human beta defensin-3 in pulmonary cells. ***Respir Res.*** 2010; 11:93.

Legionella pneumophila is an important causative agent of severe pneumonia in humans. Human alveolar epithelium and macrophages are effective barriers for inhaled microorganisms and actively participate in the initiation of innate host defense. The beta defensin-3 (hBD-3), an antimicrobial peptide is an important component of the innate immune response of the human lung. Therefore, we hypothesized that hBD-3 might be important for immune defense towards *L. pneumophila*.

We investigated the effects of *L. pneumophila* and different TLR agonists on pulmonary cells in regard to hBD-3 expression by ELISA. Furthermore, siRNA-mediated inhibition of TLRs as well as chemical inhibition of potential downstream signaling molecules was used for functional analysis.

L. pneumophila induced release of hBD-3 in pulmonary epithelium and alveolar macrophages. A similar response was observed, when epithelial cells were treated with different TLR agonists. Inhibition of TLR2, TLR5, and TLR9 expression led to a decreased hBD-3 expression. Furthermore, expression of hBD-3 was mediated through a JNK-dependent activation of AP-1 (c-Jun) but appeared to be independent of NF-κB. Additionally, we demonstrated that hBD-3 elicited a strong antimicrobial effect on *L. pneumophila* replication.

Taken together, human pulmonary cells produce hBD-3 upon *L. pneumophila* infection via a TLR-JNK-AP-1-dependent pathway, which may contribute to an efficient innate immune defense.

3.2 Mechanisms of *L. pneumophila*-dependent induction of cytokines and chemokines in infected pulmonary epithelial cells

Publication 3

Vardarova K, Scharf S, Lang F, Schmeck B, Opitz B, Eitel J, Hocke AC, Slevogt H, Flieger A, Hippenstiel S, Suttorp N, and **N'Guessan PD**. PKC(alpha) and PKC(epsilon) differentially regulate *Legionella pneumophila*-induced GM-CSF. ***Eur Respir J.*** 2009; 34(5):1171-9.

Legionella pneumophila is an important causative agent of severe pneumonia in humans. The human alveolar epithelium is an effective barrier for inhaled microorganisms and actively participates in the initiation of innate host defense. Although secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) is essential for the elimination of invading Legionella spp., mechanisms of *Legionella pneumophila*-induced release of this cytokine are widely unknown. In this study, we have demonstrated a Toll-Like Receptor (TLR)2- and TLR5-dependent release of GM-CSF in *L. pneumophila*-infected human alveolar epithelial cells. GM-CSF secretion was not dependent on the bacterial type II or type IV secretion system. Furthermore, an increase in protein kinase C (PKC) activity, particularly PKC(alpha) and PKC(epsilon), was noted. Blocking of PKC(alpha) and PKC(epsilon) activity or expression, but not of PKC(beta), PKC(delta), PKC(eta), PKC(theta), and PKC(zeta), significantly reduced the synthesis of GM-CSF in infected cells. While PKC(alpha) was critical for the initiation of a nuclear factor-kappaB-mediated GM-CSF expression, PKC(epsilon) regulated GM-CSF production via activator protein 1. Thus, differential regulation of GM-CSF by PKC isoforms contributes to the host response in Legionnaires' disease.

Publication 4

Lorenz J, Zahlten J, Pollok I, Lippmann J, Scharf S, **N'Guessan PD**, Opitz B, Flieger A, Suttorp N, Hippenstiel S, Schmeck B. *Legionella pneumophila*-induced IκBζ-dependent expression of interleukin-6 in lung epithelium. ***Eur Respir J.*** 2011; 37(3):648-57.

Legionella pneumophila causes severe community- and hospital-acquired pneumonia. Lung airway and alveolar epithelial cells comprise an important sentinel system in airborne infections. Although interleukin-6 (IL-6) is known as a central regulator of the immune response in pneumonia, its regulation in the lung is widely unknown. Herein, we demonstrated that different *L. pneumophila* strains induced delayed expression of IL-6 in comparison to IL-8 by human lung epithelial cells. IL-6 expression depended at early time points on flagellin recognition by TLR5, the activity of both MEK1 and p38 MAP kinase, and at later time points on the type IV secretion system. In the same manner, but more rapidly, the recently described transcription factor IkappaBzeta was induced by *Legionella* infection and – binding to the NF-kappaB subunit p50 – recruited to the il6 promoter together with C/EBPbeta and phosphorylated AP-1 subunit cJun. Similarly, histone modifications and NF-kappaB subunit p65/RelA appeared at the ikappabzeta and subsequently at the il6 gene promoter, thereby initiating gene expression. Gene silencing of IkappaBzeta reduced *Legionella*-related IL-6 expression by 41%. Overall, these data indicate a sequence of flagellin/TLR5- and type IV-dependent IkappaBzeta expression, recruitment of IkappaBzeta/p50 to the il6 promoter, chromatin remodeling and subsequent IL-6 transcription in *L. pneumophila*-infected lung epithelial cells.

Publication 5

Schmeck B, Lorenz J, **N'Guessan PD**, Opitz B, van Laak V, Zahlten J, Slevogt H, Witzenrath M, Flieger A, Suttorp N, Hippenstiel S. Histone acetylation and flagellin are essential for *Legionella pneumophila*-induced cytokine expression. ***J Immunol.*** 2008; 181(2):940-7.

Legionella pneumophila causes severe pneumonia. Acetylation of histones is thought to be an important regulator of gene transcription, but its impact on *L. pneumophila*-induced expression of pro-inflammatory cytokines is unknown. *L. pneumophila* strain 130b induced the expression of the important chemoattractant IL-8 and genome-wide histone modifications in human lung epithelial A549 cells. We analyzed the IL-8-promoter and found that histone H4 was acetylated and H3 was phosphorylated at Ser(10) and acetylated at Lys(14), followed by activation of the transcription factor NF-kappaB. Recruitment of RNA polymerase II to the IL-8 promoter corresponded to the increase in gene transcription. Histone modification and IL-8 release were dependent on p38 kinase and NF-kappaB pathways. Legionella-induced IL-8 expression was decreased by histone acetylase (HAT) inhibitor anacardic acid and enhanced by histone deacetylase (HDAC) inhibitor trichostatin A. After Legionella infection, HATs p300 and CREB-binding protein were time-dependently recruited to the IL-8 promoter, whereas HDAC1 and HDAC5 first decreased and later reappeared at the promoter. Legionella specifically induced expression of HDAC5, but not of other HDACs in lung epithelial cells. The knockdown of HDAC1 or 5 did not alter IL-8 release. Furthermore, Legionella-induced cytokine release, promoter-specific histone modifications, and RNA polymerase II recruitment were reduced in infection with flagellin-deficient mutants. Legionella-induced histone modification as well as HAT-/HDAC-dependent IL-8 release could also be shown in primary lung epithelial cells. In summary, histone acetylation seems to be important for the regulation of pro-inflammatory gene expression in *L. pneumophila*-infected lung epithelial cells. These pathways may contribute to the host response in Legionnaires' disease.

Publication 6

N'Guessan PD, Etouem MO, Schmeck B, Hocke AC, Scharf S, Vardarova K, Opitz B, Flieger A, Suttorp N, Hippenstiel S. *Legionella pneumophil*a-induced PKCalpha-, MAPK-, and NF-kappaB-dependent COX-2 expression in human lung epithelium. ***Am J Physiol Lung Cell Mol Physiol.*** 2007; 292(1):L267-77.

Legionella pneumophila causes community- and hospital-acquired pneumonia. Lung airway and alveolar epithelial cells comprise an important barrier against airborne pathogens. Cyclooxygenase (COX) and microsomal PGE(2) synthase-1 (mPGES-1)-derived prostaglandins like prostaglandin E(2) (PGE(2)) are considered as important regulators of lung function. Herein, we tested the hypothesis that *L. pneumophila* induced COX-2 and mPGES-1-dependent PGE(2) production in pulmonary epithelial cells. *Legionella* induced the release of PGE(2) in primary human small airway epithelial cells and A549 cells. This was accompanied by an increased expression of COX-2 and mPGES-1 as well as an increased PLA(2) activity in infected cells. Deletion of the type IV secretion system Dot/Icm did not impair *Legionella*-related COX-2 expression or PGE(2) release in A549 cells. *L. pneumophila* induced the degradation of IkappaBalpha and activated NF-kappaB. Inhibition of IKK blocked *L. pneumophila*-induced PGE(2) release and COX-2 expression. We noted activation of p38 and p42/44 MAP kinase in *Legionella*-infected A549 cells. Moreover, membrane translocation and activation of PKCalpha was observed in infected cells. PKCalpha, p38 and p42/44 MAP kinase inhibitors reduced PGE(2) release and COX-2 expression. In summary, PKCalpha, p38 and p42/44 MAP kinases controlled COX-2 expression and subsequent PGE(2) release in *Legionella*-infected lung epithelial cells. These pathways may significantly contribute to the host response in Legionnaires' disease.

Publication 7

Lippmann J, Rothenburg S, Deigendesch N, Eitel J, Meixenberger K, van Laak V, Slevogt H, **N'guessan PD**, Hippenstiel S, Chakraborty T, Flieger A, Suttorp N, Opitz B. IFNbeta responses induced by intracellular bacteria or cytosolic DNA in different human cells do not require ZBP1 (DLM-1/DAI). ***Cell Microbiol.*** 2008; 10(12):2579-88.

Intracellular bacteria and cytosolic stimulation with DNA activate type I IFN responses independently of Toll-Like Receptors, most Nod-Like Receptors and RIG-Like Receptors. A recent study suggests that ZBP1 (DLM-1/DAI) represents the long anticipated pattern recognition receptor, which mediates IFNalpha/beta responses to cytosolic DNA in mice. Here we showed that *Legionella pneumophila* infection, and intracellular challenge with poly(dA-dT), but not with poly(dG-dC), induced the expression of IFNbeta, full-length hZBP1 and a prominent splice variant lacking the first Zalpha domain (hZBP1DeltaZalpha) in human cells. Overexpression of hZBP1, but not hZBP1DeltaZalpha, slightly amplified poly(dA-dT)-stimulated IFNbeta reporter activation in HEK293 cells, but had no effect on IFNbeta and IL-8 production induced by bacteria or poly(dA-dT) in A549 cells. We found that mZBP1 siRNA impaired poly(dA-dT)-induced IFNbeta responses in mouse L929 fibroblasts at a later time point, while multiple hZBP1 siRNAs did not suppress IFNbeta or IL-8 expression induced by poly(dA-dT) or bacterial infection in human cells. In contrast, IRF3 siRNA strongly impaired the IFNbeta responses to poly(dA-dT) or bacterial infection. In conclusion, intracellular bacteria and cytosolic poly(dA-dT) activate IFNbeta responses in different human cells without requiring human ZBP1.

Publication 8

Opitz B, Vinzing M, van Laak V, Schmeck B, Heine G, Günther S, Preissner R, Slevogt H, **N'Guessan PD**, Eitel J, Goldmann T, Flieger A, Suttorp N, Hippenstiel S. *Legionella pneumophila* induces IFNbeta in lung epithelial cells via IPS-1 and IRF3, which also control bacterial replication. ***J Biol Chem.*** 2006;281(47):36173-9.

Legionella pneumophila, a Gram-negative facultative intracellular bacterium, causes severe pneumonia (Legionnaires' disease). Type I interferons (IFNs) have been so far associated with antiviral immunity, but recent studies also indicate a role of these cytokines in immune responses against (intracellular) bacteria. Here we showed that wild-type *L. pneumophila* and flagellin-deficient *Legionella*, but not *L. pneumophila* lacking a functional type IV secretion system Dot/Icm, or heat-inactivated *Legionella* induced IFNbeta expression in human lung epithelial cells. We found that the transcription factors (IRF)-3 and NF-kappaB-p65 translocated into the nucleus and bound to the IFNbeta gene enhancer after *L. pneumophila* infection of lung epithelial cells. RNA interference demonstrated that in addition to IRF3, the caspase recruitment domain (CARD)-containing adapter molecule IPS-1 (interferon-beta promoter stimulator 1) is crucial for *L. pneumophila*-induced IFNbeta expression, whereas other CARD-possessing molecules, such as RIG-I (retinoic acid-inducible protein I), MDA5 (melanoma differentiation-associated gene 5), Nod27 (nucleotide-binding oligomerization domain protein 27), and ASC (apoptosis-associated speck-like protein containing a CARD) seemed not to be involved. Finally, bacterial multiplication assays in small interfering RNA-treated cells indicated that IPS-1, IRF3, and IFNbeta were essential for the control of intracellular replication of *L. pneumophila* in lung epithelial cells. In conclusion, we demonstrated a critical role of IPS-1, IRF3, and IFNbeta in *Legionella* infection of lung epithelium.

Publication 9

Vinzing M, Eitel J, Lippmann J, Hocke AC, Zahlten J, Slevogt H, **N'Guessan PD**, Günther S, Schmeck B, Hippenstiel S, Flieger A, Suttorp N, Opitz B. NAIP and Ipaf control *Legionella pneumophila* replication in human cells. ***J Immunol.*** 2008; 180(10):6808-15.

In mice, different alleles of the mNAIP5 (murine neuronal apoptosis inhibitory protein-5)/mBirc1e gene determine whether macrophages restrict or support intracellular replication of *Legionella pneumophila*, and whether a mouse is resistant or (moderately) susceptible to *Legionella* infection. In the resistant mice strains, the nucleotide-binding oligomerization domain (Nod)-like receptor (NLR) family member mNAIP5/mBirc1e, as well as the NLR protein mIpaf (murine ICE protease-activating factor), are involved in recognition of *Legionella* flagellin and in restriction of bacterial replication. Human macrophages and lung epithelial cells support *L. pneumophila* growth, and humans can develop severe pneumonia (Legionnaires' disease) after *Legionella* infection. The role of human orthologs to mNAIP5/mBirc1e and mIpaf in this bacterial infection has not been elucidated. Herein, we demonstrated that flagellin-deficient *L. pneumophila* replicated more efficiently in human THP-1 macrophages, primary monocyte-derived macrophages, and alveolar macrophages, and in A549 lung epithelial cells compared with wild-type bacteria. Additionally, we noted expression of the mNAIP5 ortholog hNAIP in all cell types examined, and expression of hIpaf in human macrophages. Gene silencing of hNAIP or hIpaf in macrophages or of hNAIP in lung epithelial cells lead to an enhanced bacterial growth. On the other hand, the overexpression of both molecules strongly reduced *Legionella* replication. In contrast to experiments with wild-type *L. pneumophila*, hNAIP or hIpaf knock-down affected the (enhanced) replication of flagellin-deficient *Legionella* only marginally. In conclusion, hNAIP and hIpaf mediate innate intracellular defense against flagellated *Legionella* in human cells.

4 Discussions

4.1 Recognition of *L. pneumophila* by extra- and intracellular PRRs in pulmonary epithelial cells

L. pneumophila is an environmental, opportunistic, and accidental pathogen of humans. As a result, no selective pressure on the evolution of *L. pneumophila* from the mammalian immune system had occurred. In general, a strong early inflammatory response is believed to control bacterial replication while cell-mediated immunity contributes to the resolution of the infection and bacterial clearance. The pulmonary epithelium as an important part of the innate immune system that is equipped with germ-line-encoded receptors recognizing microbe specific molecular structures (also called pathogen-associated molecular patterns or PAMPs). *L. pneumophila* can be recognized by extracellular as well as intracellular PAMPs. Accordingly, we demonstrated that *L. pneumophila* is recognized by TLR2, TLR5 and TLR9 in human alveolar epithelial cells (13;14;28). While the atypical LPS of *L. pneumophila* is recognized by TLR2, flagellin and the nucleic acids of the pathogen are recognized by TLR5 and TLR9 respectively (13;14;28). This strongly activates innate immune responses through the release of cytokines (IL-6, IL-8, GM-CSF), PGE_2 and antimicrobial peptides, such as hBD-2 and hBD-3 (13;14;27;28).

Recognition of *L. pneumophila* by TLRs leads to the activation of myeloid differentiation primary response gene 88 (MyD88), an important adaptor molecule for most TLR signalling (22;31). MyD88-deficient mice infected with *L. pneumophila* have an increased bacterial burden in the lung and decreased survival rates, develop more severe lung pathology, and suffer from disseminated bacterial infections in the spleen compared to wild-type mice (31). However, inbred strains of mice are largely resistant to *L. pneumophila* infection, with the notable exception of the A/J mouse strain, which, when inoculated intratracheally with *Legionella* bacteria, develops acute lung inflammation (32).

In humans, TLR polymorphisms have been associated with increased susceptibility to Legionnaires' disease independently of other risk factors (33). In that regard, a common TLR5 polymorphism, which is characterized by a premature stop codon (TLR5392STOP), present in around 10% of the population, has been associated with a significantly increased risk of Legionnaires' disease (33). Individuals with the TLR5392STOP polymorphism have an impaired production of pro-inflammatory cytokines. As the recognition of bacterial flagellin by TLR5 on alveolar epithelial cells is a major driver of an IL-8 and IL-6 response, deficiencies in TLR5 or its dysfunction likely increase the individuals' risk of Legionnaires'

disease by weakening the cytokine response (33). Thus, TLRs might be essential to trigger an appropriate immune response in Legionnaires' disease.

Apart from the TLR system, we and others have demonstrated that the nucleotide-binding oligomerization domain (Nod)-like receptor (NLR) family member hNAIP5, as well as the NLR protein Ipaf (ICE protease-activating factor), are involved in recognition of *Legionella* flagellin(34). This procedure does strongly restrict intracellular replication of *Legionella.* Furthermore, a study done by Frutuoso and colleagues, has demonstrated that other NLR such as NOD1 and 2 are important for the innate immune response of *L. pneumophila* (24). Thus, NLR are also essential for an adequate innate immune response in Legionnaires' disease.

4.2 Pulmonary epithelial cells response upon infection with *L. pneumophila*: signal transduction pathways

After recognition of *L. pneumophila* through the above mentioned pathogen-associated molecular patterns, a complex network of signalling pathways can be activated. Indeed, we have demonstrated that *L. pneumophila* causes a marked increase in the level of phosphorylated ERK, p38 MAPK, and JNK in pulmonary epithelial cells. The implication of such activation on different components of innate immunity is diverse. While the activation of p38 MAPK and JNK pathways strongly induce the expression of hBD-2, only JNK have been found to be essential for the expression of hBD-3. In the case of PGE_2 release and COX-2 expression, the p38 MAPK, p42/44 MAPK, and PKCα pathways were important.

The IL-6 expression depends on activation of p42/44 MAPK, p38 MAPK transcription factor, IκB zeta NF-κB, and AP-1. Moreover, the expression of GM-CSF was differentially controlled by the activation of PKCα and PKCε.

Upon infection of pulmonary epithelial cells by *L. pneumophila*, we have also observed a strong activation of the transcription factors NF-κB and AP-1. While both factors are important for the induction of GM-CSF and hBD-2, expression of hBD-3 is solely controlled by AP-1. Furthermore, the expression of PGE_2 and COX-2 is controlled by NF-κB. We have also found an interconnection between the above mentioned pathways. Increasing evidence indicates that histone modifications, such as acetylation are important for the transcriptional activation state of genes in many cellular processes. Indeed, we have demonstrated that in *L. pneumophila*-infected pulmonary epithelial cells, induction of IL-8 has been dependent on p38 MAPK and the NF-κB activated histone modifications. The observed histone acetylation depends on the virulence factor flagellin of *L. pneumophila*. Concerning the induction of IL-6, we also emphasis the impact of histone modifications in that process. Thus, complex specific

activation of signal transduction pathways in regard to different major components of the innate immune system has to be considered in the course of infection of pulmonary epithelial cells by *L. pneumophila*. Therefore, selective targeting or enhancing of these specific pathways may help to develop new therapeutic strategies in *Legionella* diseases.

4.3 Mechanisms of *L. pneumophila* replication blockade in infected pulmonary epithelial cells

L. pneumophila has been shown to replicate in human cells, including alveolar macrophages and epithelial cells(13;28). *Legionella* are enclosed in a vacuole during their intracellular replication. They possess the type IVB secretion system Dot/Icm that enables them to inject proteins and nucleic acids into the host cell cytoplasm (12). We have demonstrated that wild-type *L. pneumophila*, but not *Legionella* deficient of the Dot/Icm system, induce IFNβ (Type I interferons (IFNs)) expression through IPS-1 (interferon-β promoter stimulator 1) and IRF3 (transcription factors of the IFN regulatory factor (IRF)). In addition, we have observed a negative regulatory effect of IPS-1, IRF3 and IFNβ on the intracellular replication of *L. pneumophila* in lung epithelial cells. As already mentioned in 4.1, detection of *L. pneumophila* by hNAIP5 and Ipaf, results in the restriction of intracellular replication of *Legionella* in pulmonary epithelial cells. We have also demonstrated that hBD-2 as well as hBD-3 strongly reduces intracellular growth of *L. pneumophila* in pulmonary epithelial cells. The mechanisms by which defensins kill or inactivate bacteria is not precisely understood, but is generally thought to be related to a perforation of the peripheral microbial membrane (35). A recently published study has shown a co-localisation of endogenous hBD-2 with the bacterial cell wall of extra- and intracellular replicating *Mycobacterium tuberculosis* in A549 cells (36). For hBD-3 a similar antimicrobial mechanism can be assumed, since a keratinocyte cell line engineered to overexpress hBD-3 has demonstrated significant antimicrobial activity against *Staphylococcus aureus* (37). On the other hand, hBD-3 can activate the NF-κB pathway via TLR-triggered mechanisms (38;39). This may induce secondary effector molecules, which may reduce intracellular replication of *Legionella*. Since we have observed an antimicrobial effect within four hours, we presume that hBD-3 kills *L. pneumophila* via direct perforation of the bacterial membrane.

4.4 Relevance of cytokines and PGE_2 for *L. pneumophila*-induced pneumonia

In published studies we have demonstrated that *L. pneumophila* induces a broad array of cytokines as well as PGE_2 in pulmonary epithelial cells. We have focussed on the molecular mechanisms leading to the expression of Interleukin (IL)-6, -8 and GM-CSF. Concerning IL-6 and IL-8, concentrations in blood and bronchoalveolar lavage fluids of patients suffering from pneumonia are strongly associated with disease severity (40). Furthermore, gene polymorphisms associated with cytokines such as interleukin-6 is also associated with extra-pulmonary dissemination of pneumococcal pneumonia (41). Moreover, a prominent role of IL-6 as well as IL-8 in bacterial pneumonia has been elucidated with mice deficient of each gene (42). IL-6 and IL-8 have an influence on important innate immune mechanisms, for example, reducing neutrophil apoptosis, increasing their cytotoxic capabilities, as well as enhancing their recruitment and activation at the site of inflammation in lung diseases (23). Since we have noticed a strong induction of IL-6 as well as IL-8 in *L. pneumophila*-infected cells, we assume that both cytokines might be of great importance in the course of Legionnaires' disease. Our data show that *L. pneumophila* infection of pulmonary epithelium led to a strong GM-CSF secretion. To clear *L. pneumophila* from the lung, a functionally intact innate immune system, particularly macrophages and polymorphonuclear leukocytes (PMNs), must be present. Epithelial cells have been shown to liberate mediators such as GM-CSF, IL-6, IL-8, IFN-β, and PGE_2 upon infection with *L. pneumophila*. GM-CSF is a 23-kDa haematopoietic growth factor that is able to stimulate *in vitro* survival, proliferation, differentiation and function of myeloid cells and their precursors, particularly PMNs, eosinophils, granulocytes, and monocytes/macrophages (43). Furthermore, GM-CSF plays a critical role in surfactant homeostasis (44) and for stimulating the terminal differentiation of alveolar macrophages (AMs) (45). The important function of GM-CSF for the pulmonary immune responses has been confirmed in vivo by the use of gene knock-out mice, demonstrating a pivotal role of this cytokine for host defense function. GM-CSF-/- mice are more susceptible to several pulmonary bacterial infections, such as *Pseudomonas aeruginosa* (46), group B *Streptococcus* spp. (47), and *Pneumocystis jiroveci* (48). Furthermore, AMs from GM-CSF-/- mice are defective for *Escherichia coli* phagocytosis (49), as well as in the production of tumor necrosis factor (TNF)-α (49) and IFN-γ (49). Taken together, we assume that GM-CSF might be important for the elimination of invading *L. pneumophila* and thus be important for immune response in Legionnaires' disease.

We have also observed a strong induction of PGE_2 in *L. pneumophila*-infected cells. Increased PGE_2 in the lung has been shown to stimulate the secretion of surfactant by alveolar type II

cells and enhance wound closure in airway epithelium (50). It has also been reported that PGE_2 down-regulates the production of important inflammatory cytokines, such as IL-8, IL-12, monocyte chemoattractant protein-1, and GM-CSF, which are essential for leukocyte migration (50). In addition, Aronoff and colleagues have demonstrated that PGE_2 also inhibits alveolar macrophage phagocytosis through the increase of intracellular cAMP (51). Apart from lung epithelium, a recent report by Neild and colleagues demonstrated an increased COX-2 expression and PGE_2 production in *Legionella*-infected macrophages (52). They also demonstrate that macrophage-derived PGE_2 inhibits T cell activation. Since an effective immune response that results in the clearance and growth control of intracellular pathogens often requires the activation of T cells (53), the *Legionella*-induced COX-2 expression and PGE_2 release from alveolar epithelium may interfere with the host response in Legionnaires' disease. The observation that *Chlamydia pneumonia* (54), pneumococci (55), respiratory syncytial virus (56), as well as influenza virus (57), also induce COX-2 expression in pulmonary epithelium underlines the potential role of COX-2 in lung infection.

Since control of the immune response is crucial to assure bacterial clearance and prevent excessive tissue damage in pneumonia, the mechanisms described above could be important in Legionnaires' disease. Thus, our studies provide new mechanisms to elucidate how infected lung epithelial cells might control bacterial replication during the course of Legionnaires' disease.

4.5 Relevance of *L. pneumophila* virulence factors for activation of innate immunity in infected pulmonary epithelial cells

Detection of *Legionella* flagellin, DNA, as well as heat inactivated *L. pneumophila* by host cell pattern recognition receptors (PRRs) seems to be a critical step in legionellosis. This leads to a strong expression of GM-CSF, hBD-2, hBD-3 and PGE_2. Consequently, pointing out an important role of these virulence factors in the activation of an innate immune response. While the Dot/Icm system was important for the *L. pneumophila*-induced IL-6, this virulence factor was not important for the expression of GM-CSF, IFNβ, hBD-2, hBD-3 and PGE_2 in human pulmonary epithelial cells. Thus, a differential role of the Dot/Icm system in regard to the activation of the innate immune system has to be considered. Further studies are needed to investigate the role of other major virulence factors in the activation of innate immunity.

5 Outlooks

In our *in vitro* studies we have addressed the molecular interaction between *L. pneumophila* and the pulmonary epithelial cells in detail. *L. pneumophila* can be detected through various pathogen-associated molecular patterns, which lead to selective activation of signalling pathways and activation of major components of innate immunity. The mechanisms described in our different studies are probably relevant in the course of the Legionnaires' disease, but the data have to be verified in *in vivo* studies. Therefore, viable animal models of Legionnaires' disease have to be established. This is a great challenge, since common mouse pneumonia models can hardly be established due to the fact that these vertebrates are resistant to pulmonary infections with *L. pneumophila*. It has been published that A/J mice are moderately susceptible to *Legionella* infection, when this pathogen is flagellin deficient.

This is a huge restriction in research concerning Legionnaires' disease. Nevertheless, mice strains like A/J mice, which are moderately susceptible to *Legionella* infection offer some limited opportunities. Gene knock-out systems and other pathways addressed in our *in vitro* studies, need to be further investigated *in vivo*. Furthermore, diverse functional Single Nucleotide Polymorphisms (SNPs) in the genes encoding some of the target genes addressed in our studies have been described. These SNPs might be relevant to susceptibility and diseases progression in pneumonia. In representative patients' collectives for *Legionella* pneumonia, the relevance of these SNPs should be addressed. The *in vivo* as well as *ex vivo* approaches are being addressed in new studies, which are currently conducted in our research group.

To summarize, our studies will provide important information on the pathogenesis of *Legionella* pneumonia and may help to develop novel therapeutic strategies for the treatment of this important infectious lung disease.

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8 Statutory declarations (Erklärung)

§ 4 Abs. 3 (k) der HabOMed der Charité

Hiermit erkläre ich, daß
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