

The induction and function of the transcription factor c-myc in macrophages during pneumococcal pneumonia and Legionellosis

vorgelegt von
Diplom-Ingenieurin der Biotechnologie
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von der Fakultät III – Prozesswissenschaften
der Technischen Universität Berlin
zur Erlangung des akademischen Grades

Doktorin der Ingenieurwissenschaften
– Dr.-Ing. –

genehmigte Dissertation

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Tag der wissenschaftlichen Aussprache: 30. Juni 2017

Berlin 2017

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1 SUMMARY

For infectious diseases, effective defense mechanisms which constitute an efficient innate host response are the keys to eliminate invading pathogens. According to different studies, the pathogenicity of bacteria is due to different virulence factors that contribute to overcome those mechanisms. Against that, the immune system evolved a large repertoire to fight the pathogen. Nevertheless, an overly strong host response bears the risk of cell and tissue damage. Especially in infections of the lung, the delicate balance between the robust inflammatory response and the control of it to prevent loss of organ function determines the outcome of the disease. In the therapy of infectious diseases of the lung, antibiotics are used, whereby this treatment is facing increasing resistances. To find an alternative treatment, still remaining gaps in knowledge about the activation of the target cells and thus the signaling pathways of the immune response need to be filled.

Alveolar macrophages are the target cells of *Legionella pneumophila* (*L. pneumophila*), the causing agent of the legionnaire's disease, and one of the first targets of *Streptococcus pneumoniae* (*S. pneumoniae*), introducing severe pneumonia among others. Lower respiratory tract infections are the third most common cause of death worldwide and the second largest infectious source of death in children worldwide. At this *S. pneumoniae* is the most common agent of community acquired pneumonia (CAP), whereas the incidence of community acquired Legionnaire's disease caused by *Legionella* is at 4-5 % in Germany. Nevertheless, it is the subspecies *L. pneumophila* which leads to the infection in 70-90 % (depending on the region) of the cases.

Macrophages are important immune cells for the first defense against invading pathogens. With the recognition of pathogens by different receptors of the innate immune system, macrophages mainly contribute to the defense against pathogens due to cytokine and chemokine secretion and recruitment of adaptive immune cells. Furthermore, they belong to the phagocytes and directly contribute to the clearance of pathogens.

The transcription factor c-myc is known for its role in embryogenesis and regulation of the cell cycle as well as its effect on proliferation and differentiation of cells. Taking into account that c-myc regulates approximately 15 % of the genome including promoter regions of important molecules of the innate immune system; it seems to have a crucial impact on the immune response.

The results presented in this work could show that c-myc is induced by *L. pneumophila* and *S. pneumoniae* in human lung (*ex vivo*) and particularly in human alveolar macrophages (*ex vivo*) and in a macrophage-like cell line (*in vitro*). This induction of c-myc is dependent on

the TLR adaptor molecule MyD88 and the Toll-like receptor (TLR) 2 seems to be involved.

Furthermore, the *Legionella* and *Pneumococci* induced cytokine release is decreased by inhibition or knockdown of c-myc, respectively.

The phagocytosis of *Legionella* by macrophages is compromised by inhibition or knockdown of c-myc. Thereby, it has an effect on the internalization. However, it does not alter the growth of *Legionella*. Interestingly, the impact of c-myc on the phagocytic ability of macrophages regarding *Pneumococci*, including internalization and killing, was only visible by knockdown of c-myc, not by inhibition. This might be due to different modes of action or due to unspecific effect of the inhibitor, respectively.

Taken together, the results of this study indicate a regulatory role of c-myc in the immune response to infections of the lung with *L. pneumophila* and *S. pneumoniae*, upon which the protein is induced over a TLR2 and MyD88 pathway. This leads to increased pro-inflammatory cytokine and chemokine release as well as increased internalization of bacteria.

2 ZUSAMMENFASSUNG

Bei Infektionskrankheiten ist eine effiziente angeborene Immunantwort und damit effektive Verteidigungsmechanismen der Schlüssel zu Eliminierung von eindringenden Pathogenen. Studien zufolge sind für die Pathogenität von Bakterien verschiedene Virulenzfaktoren verantwortlich, welche dazu dienen, die Mechanismen des Wirtsorganismus zu umgehen. Im Gegensatz dazu hat das Immunsystem ein großes Repertoire an Abwehrmechanismen entwickelt, um den Erreger dennoch zu bekämpfen. Allerdings birgt eine zu starke Immunantwort die Gefahr von Zell- oder Gewebeschäden. Besonders bei Infektionen der Lunge bestimmt dieses empfindliche Gleichgewicht zwischen einer robusten Immunantwort und deren Eindämmung zum Schutz vor Gewebeschäden den Ausgang einer Krankheit.

In der Therapie von Bakterien-induzierten Krankheiten werden häufig Antibiotika eingesetzt, wobei sich diese Behandlung mit immer weiter steigender Resistenzen konfrontiert sieht. Um einen alternativen Behandlungsweg zu finden, gilt es die Wissenslücken über die Aktivierung der Zielzellen und damit die Signalwege der Immunantwort zu schließen.

Alveolarmakrophagen sind die Zielzellen von *Legionella pneumophila* (*L. pneumophila*), dem Erreger der Legionellose, und eine der Zielzellen von *Streptococcus pneumoniae* (*S. pneumoniae*), welches unter anderem schwere Pneumonien verursacht. Infektionen des unteren Respirationstraktes sind die dritthäufigsten Infektionen weltweit, wobei diese unter Kindern bis 5 Jahre sogar die zweithäufigste Infektionsform darstellen. Dabei ist die Infektion mit *S. pneumoniae* die häufigste Ursache der ambulant erworbenen Pneumonie (CAP). Die Inzidenz der ambulant erworbenen Legionellose liegt in Deutschland zwar nur bei 4-5 %, dennoch ist die Subspezies *L. pneumophila* mit 70-90 % (je nach Region) der häufigste Erreger.

Makrophagen sind wichtige Immunzellen des angeborenen Immunsystems für die erste Abwehr gegen eindringende Pathogene. Durch die Erkennung dieser anhand von verschiedenen Rezeptoren, der darauffolgenden Sekretion von Chemokinen und Zytokinen und der Rekrutierung von weiteren Immunzellen, tragen Makrophagen wesentlich zur Eliminierung des Erregers bei. Außerdem gehören Makrophagen zu der Gruppe der Phagozyten, womit sie direkt für die Eliminierung sorgen.

Der Transkriptionsfaktor c-myc ist bekannt für seine Rolle in der Embryogenese und der Regulation von Zellzyklusprozessen wie Proliferation und Differenzierung. Beachtet man, dass c-myc als Transkriptionsfaktor etwa 15 % des Genoms reguliert, darunter auch wichtige Promotorregionen von Molekülen des angeborenen Immunsystems, ist es wahrscheinlich, dass c-myc einen Einfluss auf die Immunantwort hat.

Die Ergebnisse dieser Arbeit zeigen, dass c-myc sowohl durch *L. pneumophila* als auch durch *S. pneumoniae* in humaner Lunge (*ex vivo*) und spezieller in humanen Alveolarmakrophagen

(*ex vivo*) und in einer Makrophagen-ähnlichen Zelllinie (*in vitro*) induziert wird. Diese Induktion ist abhängig von dem Toll-like Rezeptor- (TLR-) Adaptormolekül MyD88 und vermutlich ebenfalls von TLR2.

Des Weiteren konnte ein Einfluss von c-myc auf die Zytokin- und Chemokinsekretion festgestellt werden. Diese ist bei Inhibierung oder durch Knockdown von c-myc reduziert.

Die Phagozytose von Legionellen durch Makrophagen ist ebenfalls reduziert, sobald c-myc inhibiert wird oder ein Knockdown vorliegt. Dieser Effekt beschränkt sich auf die Internalisierung der Bakterien, nicht aber auf die Abtötung der Legionellen bzw. die Replikationsfähigkeit. Bei der Phagozytose von Pneumokokken ist ein Einfluss von c-myc anhand der Knockdownversuche zu erkennen, allerdings nicht durch Inhibierung von c-myc. Dies könnte ein Hinweis auf unterschiedliche Wirkungsweisen geben bzw. durch unspezifische Effekte des Inhibitors hervorgerufen sein.

Zusammenfassend weisen die Ergebnisse dieser Arbeit auf eine regulatorische Rolle von c-myc bei der Immunantwort auf Infektionen der Lunge mit *L. pneumophila* bzw. *S. pneumoniae*, wobei auf beide Erreger eine Induktion des Proteins über MyD88 und TLR2 erfolgt, welche einen pro-inflammatorischen Einfluss auf die Zytokin- und Chemokinproduktion, sowie eine erhöhte Internalisierung der Bakterien zur Folge hat.

3 INTRODUCTION

3.1 The human respiratory system

The human respiratory system includes all areas which are necessary for respiration. The upper tract thereby is mainly responsible for movement of the air to the lower tract, specifically the alveoli for gas exchange, but it also holds several mechanisms of protection. In the following, the structure and function of the human respiratory system will be presented briefly.

3.1.1 Structure and function

The human respiratory system is divided in the upper and lower respiratory tract. The upper tract refers to the nostrils, nasal cavity, the pharynx and the larynx. It is, as mentioned, mainly responsible for conduction of the air to the lower tract, thereby filtering, moistening and warming the air (Figure 3.1)^{1,2}.

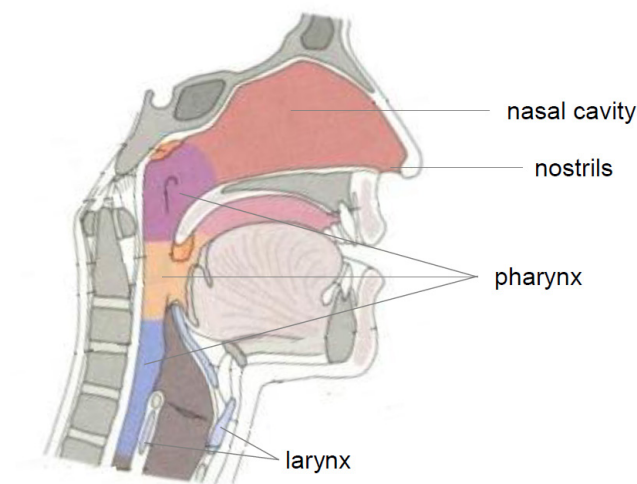


Figure 3.1: Overview of the physiology of the upper respiratory tract.

The upper respiratory tract refers to the nostrils, nasal cavity, pharynx and larynx. Figure was adapted and modified from ³.

The lower respiratory tract guides the air further towards and from the gas exchange surface. The term refers to the trachea, and within the lungs, the bronchi, bronchioles and alveoli. The lungs are sectioned in the pulmonary lobes, which are again subdivided in the pulmonary sections. The trachea divides around the fourth or fifth thoracic vertebra into the primary bronchi (Figure 3.2A), which branch over 23 generations into the second and third bronchi, further into bronchioles which in turn end in air sacs called alveoli (Figure 3.2B and C). Until reaching the bronchioles, the air gets further cleaned by ciliated cells and mucus-producing goblet cells, which mainly compose the respiratory epithelium, layering this part of the respiratory tree. The mucus functions as a special protection by preventing direct contact between particles and the

epithelial cells. Furthermore, it promotes together with the ciliated cells to mucociliary clearance, whereby trapped particles get orally transported and will be swallowed or expelled due to coughing. The alveoli – 170 per cubic millimeter lung parenchyma – consist of two different cells types: type-I-pneumocytes, covering 95 % of the alveoli. They are responsible for the gas exchange. Secondly, there are the type-II-pneumocytes, producing the surfactant reducing the surface tension. This surfactant serves also for clearance of deposited particles, which get transported into the bronchial system by the surfactant pump for mucociliary clearance. The subepithelial layer, the interstitium, contains of a proteoglycan matrix in which elastic fibres, collagen bundles and a tight capillary network are embedded. The interstitium gets very thin at the side of gas exchange, where type-I-pneumocytes are in direct contact with the endothelium of pulmonary vessels. Inside the alveoli and at the alveolar walls reside the alveolar macrophages. They play a key role in the defense mechanisms of the lung by phagocytosis of particles and pathogens and during antigen recognition^{2,4-6}.

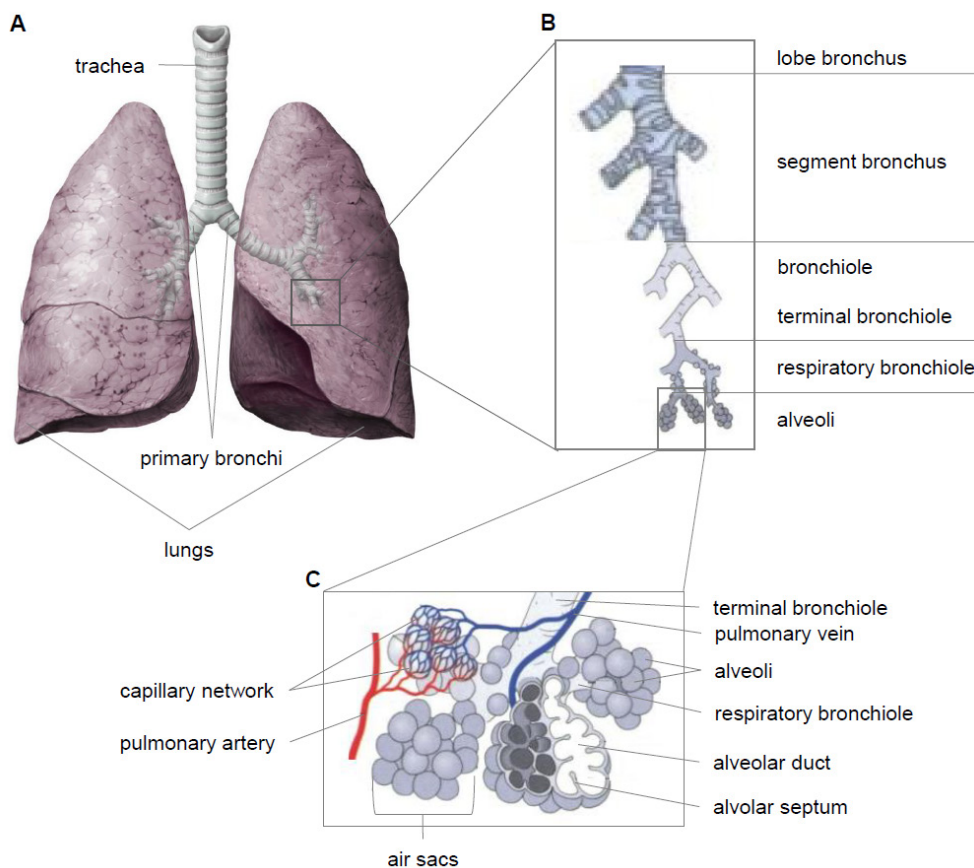


Figure 3.2: Overview of the anatomy of the lower respiratory tract.

A: The trachea divides into the primary bronchi, which divide into the second (lobe) bronchi. **B:** The lobe bronchi further divide into the third (segment) bronchi. They branch further into the bronchioles, terminal bronchioles, respiratory bronchioles and finally into the alveoli. **C:** The terminal bronchioles branch into the respiratory bronchioles, leading to the air sacs, consisting of numerous alveoli connected by alveolar ducts. Alveoli are surrounded by a capillary network coming from the pulmonary artery and vein, respectively. The figure was adapted and modified from ³ and ⁷.

3.2 Infections of the respiratory system

Infections of the respiratory system include all infections located in the respiratory tracts mainly caused by bacteria and viruses, but also by fungi. They are categorized according to their anatomic position in upper and lower respiratory tract infections. Common cold, sinusitis, pharyngitis, tonsillitis, epiglottitis and laryngitis as well as influenza belong to the group of upper respiratory infections. Those infections alone are self-limited and benign. Otitis, especially otitis media is a common complication of respiratory tract infections and therefore often mentioned with upper respiratory tract infections. Infections of the lower respiratory tract include bronchitis, bronchiolitis and pneumonia (Figure 3.3). Bacteria are the main causative agent of lower respiratory tract infections^{8–11}.

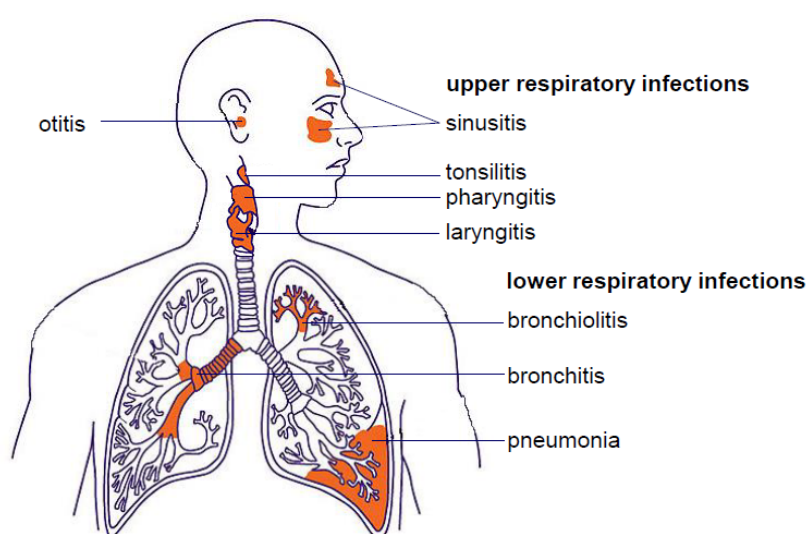


Figure 3.3: Categorization of infections of the respiratory system in upper and lower respiratory tract infections.

Upper respiratory tract infections refer to sinusitis, tonsillitis, pharyngitis, laryngitis and otitis as a common complication during respiratory tract infections. Lower respiratory tract infections are bronchitis, bronchiolitis and pneumonia. Figure adapted from ¹¹.

According to the World Health Organization (WHO), lower respiratory tract infections have been again the deadliest communicable disease in 2015, causing 3.2 million deaths worldwide. They rank the third leading cause of death worldwide, among children under 5 years old, even the second. At that, pneumonia is the single largest infectious cause of death of children younger than 5 years accounting for 16 % of all death^{12–14}.

3.2.1 Pneumonia

Diseases associated with the inflammation of the lung parenchyma and with that consolidation of cells and fluid inside the alveoli are defined as pneumonia. Infections, acute and chronically, are primarily the reason for pneumonia, but also uncommon causes such as environmental contaminants and autoimmune diseases can lead to this severe and potentially lethal illness^{11,15,16}.

Bacterial pneumonia occurs due to the replication of a pathogen and the subsequent immune response of the host. The pathogens reach the lower respiratory tract in different ways. Most common is the aspiration from the pharynx or by droplet infection. Usually, greater particles get restrained by nostril hair and the nasal concha. If smaller particles reach the lower respiratory tract, mucociliary clearance together with cough reflex serve as an effective first defense mechanism. If pathogens can overcome those defense lines, they normally become eliminated by alveolar macrophages and other phagocytes, which get supported by the host-defense proteins of the pulmonary surfactant. Manifestation of pneumonia occurs, if the number of pathogens is higher than the capacity of alveolar macrophages to phagocyte those. Subsequently, an inflammatory reaction will be initiated, where secreted cytokines and chemokines will lead to infiltration of further leukocytes. This inflammatory cascade is important for an efficient defense, but an excessive response can lead to leukocytosis, which is one of the diagnostic indicators of pneumonia, causing compromised gas exchange and consequently to cough and chest pain^{9,17–19}.

Depending on the acquisition of the disease, pneumonia is classified in community-acquired pneumonia (CAP) and hospital-acquired pneumonia (HAP). In connection with ventilation of a patient, HAP is specified as ventilator-associated pneumonia (VAP)^{9,10,17,20}. In the past years, not only patients suffering from HAP, but also patients hospitalized because of CAP, were infected with multi-resistant strains. This led to the specification of CAP introducing the category healthcare-associated pneumonia (HCAP), which gives important criteria for the assessment and treatment of the patient. However, this new category is still discussed⁹.

CAP is the most diagnosed infectious disease worldwide. The incidence in Germany is estimated at 300 000 cases yearly, from which 180 000 patients are hospitalized. Among these, children under 5 years old and elderly are most affected. The mortality rate differs between <1 % for patients treated at home to ≤20 % for hospitalized patients^{9,16,17}. The broad range of infecting agents include bacteria, fungi, respiratory viruses and protozoa, whereby, bacteria are the main cause of CAP-cases with 60-80 %. The most common bacterium is of the species *Pneumococcus*, in particular *Streptococcus pneumoniae*. Therefore, pneumococcal pneumonia and the pathogen will be described in detail in the following sections. Other bacteria caus-

ing CAP are *Haemophilus influenza*, *Staphylococcus aureus*, *Legionella* species, gram-negative bacteria such as *Klebsiella pneumoniae* or *Pseudomonas aeruginosa* and less common *Mycoplasma pneumoniae* and *Chlamydia* species^{9,10,17,21}.

Since the incidence of the Legionellosis – a severe pneumonia – is increasing yearly and its causing pathogen *Legionella* is with up to 13 % of all cases the fourth most infecting agent, the also called Legionnaires' disease and the bacterium will be further explained in the following sections^{9,22}.

3.3 *Streptococcus pneumoniae*

3.3.1 Microbiology

S. pneumoniae, is a 1 µm facultative anaerobic gram-positive bacterium, which forms lancet-shaped diplococci or short chains (Figure 3.4). It belongs to the family of *Streptococcaceae* and the genus *Streptococcus*. It was first isolated in 1881 and known as pneumococcus since 1886 due to its role as the main pneumonia infectious agent. Because of its morphology, it was renamed *Diplococcus pneumoniae* in 1920. Its final name was given in 1974, when many similarities with streptococci were discovered^{23–25}. Historically, it is one of the most studied bacteria which set important cornerstones for molecular biology. Possible transformation of the genetic information about the capsule was observed on *S. pneumoniae*, even though, it was discovered much later that the carrier is DNA. Further discoveries resulting from studies with *S. pneumoniae* were for example the therapeutic effect of penicillin and the resisting function of the capsule in respect to phagocytosis²⁶.



Figure 3.4: Morphology of *Streptococcus pneumoniae*.

Morphology of the unencapsulated derivate *Streptococcus pneumoniae* R6x grown in THY medium at 37°C and 5% CO₂.

S. pneumoniae is a member of the lactic acid bacteria, fermenting glucose to lactic acid²⁷. A capsular polysaccharide forms the outer layer of the bacterium with a thickness of 200-400 nm.

Currently, 94 different capsular antigens have been isolated on the basis of which the serotypes are defined. In all but serotype 3, the capsule is linked covalently to the cell wall peptidoglycan. Because the serotypes can be distinguished by their serological response, it plays an important role in pathogen determination as well as vaccine development^{28–31}. Another important property is the hemolytic behavior. *S. pneumoniae* carries out α -hemolysis, which leads to green surrounded colonies, when grown on blood agar. The color change of the blood agar is caused by oxidation of the hemoglobin of the erythrocytes present in the medium due to secreted H_2O_2 ^{23,32}.

The genome of *S. pneumoniae* (2 - 2.1 million base pairs), arranged in a circular DNA structure, contains of a core set of 1553 genes which are necessary for viability. Another 153 genes are responsible for pneumococcal virulence. In accordance with the role of the capsule as the most important virulence factor, a lot of genetic space encodes for capsular synthesis. Some strains contain transposons, mediating for antibiotic resistance. A high genetic variance up to 10 % within the genome exists between the different strains. This high variability could be explained by the genetic competence of *S. pneumoniae*, which is provided by recombination hotspots due to many copies of direct-repeat DNA elements. The insertion sequences make up to 5 % of the genome. Studies have demonstrated that the genome variation is important for the interaction with the host^{24,33,34}.

3.3.2 Pathogenicity

The successful invasion of bacteria into a host niche is dependent on the activity of several adaptor molecules referred to as virulence factors. Those factors are involved in the disease process. For *S. pneumoniae*, as an opportunistic pathogen of animals and humans and leading cause of morbidity and mortality worldwide, the understanding of the mode of action of virulence factors is very important in regard of intervention points in treatment or vaccine development^{24,34,35}.

The most important virulence factor of *S. pneumoniae in vivo* is probably the capsule. This important role in virulence is on the one hand due to its anti-phagocytotic activity. By masking the antigens on the cell wall surface, the capsule inhibits antigen recognition, acting as an inert shield, because it prevents the interaction between the phagocyte's receptors and the Fc-region of IgG or the complement component iC3b, respectively, because those antigens are anchored deeper into the structure of the cell wall. This inhibition subsequently leads to impaired bacterial opsonization and thus to impaired classical and alternative complement pathway activation^{36–38}. On the other hand, the capsule has an inhibitory effect on mucus binding, which allows the bacterium to transit to the epithelial surface, where it is able to colonize. This

effect is not only explained by the inhibition of opsonophagocytosis but also by not being agglutinated within the luminal mucus. This decrease might be mediated by electrostatic repulsion by the negatively charged capsule, as described by Nelson et al³⁹. Furthermore, the capsule decreases susceptibility to several antibiotics by restriction of autolysis. Commonly used antibiotics such as penicillin or vancomycin do not lead to direct killing of *S. pneumoniae* but induce a death response, including activation of autolysis. Due to expression of the capsule, the process of autolysis is restricted and with that antibiotic-induced lysis. This ability differs between the serotypes due to their different capsular polysaccharides^{24,34,40}.

S. pneumoniae produces the exotoxin Pneumolysin (ply), which is a multifunctional virulence factor, because it interrupts mechanical host defense and limits complement activation and immune response. Ply oligomerizes in the membranes of the host cells which leads to pore formation. It belongs to the thiol-activated group of cytolytic agents, but is primarily found intracellularly in contrast to other members of this group. Upon release, which is coupled with the activity of pneumococcal autolysin, it interacts with the cholesterol in the membrane of the host cell and inserts into the lipid bilayer. Subsequently, lateral diffusion occurs and 20-80 toxin molecules oligomerize, which leads to formation of a ring structure, visible by electron microscopy. The pores lead to lytic activity of the target cells, which allows *S. pneumoniae* to penetrate through the epithelium and continue through alveolar capillary barriers, because of its toxic effect on endothelial cells. This is one explanation for the pulmonary bleedings during pneumococcal pneumonia. Furthermore, ply can disturb host's inflammatory response directly by inhibition of phagocytosis, caused by the cytotoxic effect on these cells, and by subsequent blocking the respiratory burst of phagocytes and chemotaxis^{28,34,41-45}.

Next to the capsule and Pneumolysin, three groups of surface molecules also act as virulence factors: choline-binding enzymes, lipoproteins and proteins bound covalently by a LPXTG-motive. In total, there are 10-15 choline-binding proteins (CBPs), depending on the strain of pneumococcus. These are anchored in the cell wall by the phosphocholine protein (ChoP), which is a composition of cell wall associated acids and lipoteichoic acid (LTA). ChoP is also present in other microorganisms of the upper respiratory tract. It triggers bacterial adherence and activates the innate immune response due to activated signaling pathways downstream of respective receptors²⁸. The choline-binding enzymes include the pneumococcal surface proteins (Psp) A and C. PspA interferes with the C3 component of the complement system and thus inhibits the opsonization of *Pneumococci*. It protects *S. pneumoniae* from the bactericidal effect of apolactoferrin by binding lactoferrin^{24,28}. PspC also contributes to the resistance against the complement by binding the complement regulatory protein H. Furthermore, it is important for adherence of *Pneumococci* to the epithelial surface by binding to the polymeric immunoglobulin receptor⁴⁶. Other important CBPs are the hydrolytic enzymes, the autolysins (Lyt) A, B, C and CbpE. Different studies indicate a pathogenic function for the autolysins. The

36kDa LytA resides inactively in the cytoplasm. During starvation, due to nutrient depletion or because of antibiotic treatment with e.g. penicillin, cell wall association of LytA increases. This active form cleaves the lactyl-amide bond of pneumococcal peptidoglycan, which hydrolyses the cell wall⁴⁷. LytB is an important factor for cell separation. Deletion of this protein leads to deficiency in cell separation and the formation of long chains⁴⁸. For pneumococcal colonization, LytB as well as LytC are required. LytC itself acts as an lysozyme in the upper respiratory tract as it shows its maximal activity at 30°C⁴⁹. Different studies indicate their role in pneumococcal pathogenicity. Not only inflammatory cell wall degradation products are released by the autolysins, but also bacterial DNA and RNA. Furthermore, the lysis leads to the release of harmful bacterial substances as Pneumolysin, H₂O₂ and neuraminidases^{49–51}.

S. pneumoniae produces three surface-associated exoglycosidases: a β -galactosidase BgaA, a β -N-acetylglucosamidase StrH and a neuraminidase NanA. These enzymes remove the N-terminal sugar residues of human glycoconjugates, which serve as a nutrient source. Because of this cleavage, adherence receptors are exposed and the glycosylated defense molecules of the host are impaired. The enzyme NanA, which cleaves the sialic acid residues of glycolipids, glycoproteins and oligosaccharides on the cell surface of the host cell, causing strong cell damage, has a particular role in virulence²⁸. Furthermore, those three proteins are reported to reduce the deposition of complement component C3 which leads to a resistance against opsonization and subsequent killing by macrophages and neutrophils⁵². Those proteins belong to the LPXTG-anchored proteins, which refers to their common LPXTG-motive by which they are recognized and anchored covalently to the peptidoglycan from sortases⁵³.

The expression of up to four zinc metalloproteases has another limiting influence on the host response during pneumococcal infection. They are also anchored to the cell wall by the LPXTG-motif. One of these zinc-metalloproteases is the IgA1 protease, which cleaves immunoglobulin (Ig) A1 on the mucosal membrane, which is the main immunoglobulin isotype in this surrounding. This cleavage leads to compromised phagocytosis via complement and enhanced adherence to the host cell^{54–56}.

Specific ATP-binding cassette transporter systems are important for the nutrient acquisition, e.g. carbon or amino acid substrates, as well as for the export of outer surface adhesions, degradation enzymes, or capsular synthetic components. Furthermore, these transporters contribute to genetic competence (ability to take up homologous strands of naked DNA) and as efflux pumps to resist antibiotics⁵⁷. One component of those transporter systems are metal-ion-binding lipoproteins: pneumococcal surface protein (Psa) A, pneumococcal iron acquisition (Pia) A and pneumococcal iron uptake (Piu) A, which are described as essential for the bacterial virulence. Psa A, for example, mediates the manganese uptake, which results in resistance to oxidative stress due to production of hydrogen peroxide²⁸.

An overview of the important virulence factors of *S. pneumoniae* is visualized in Figure 3.5.

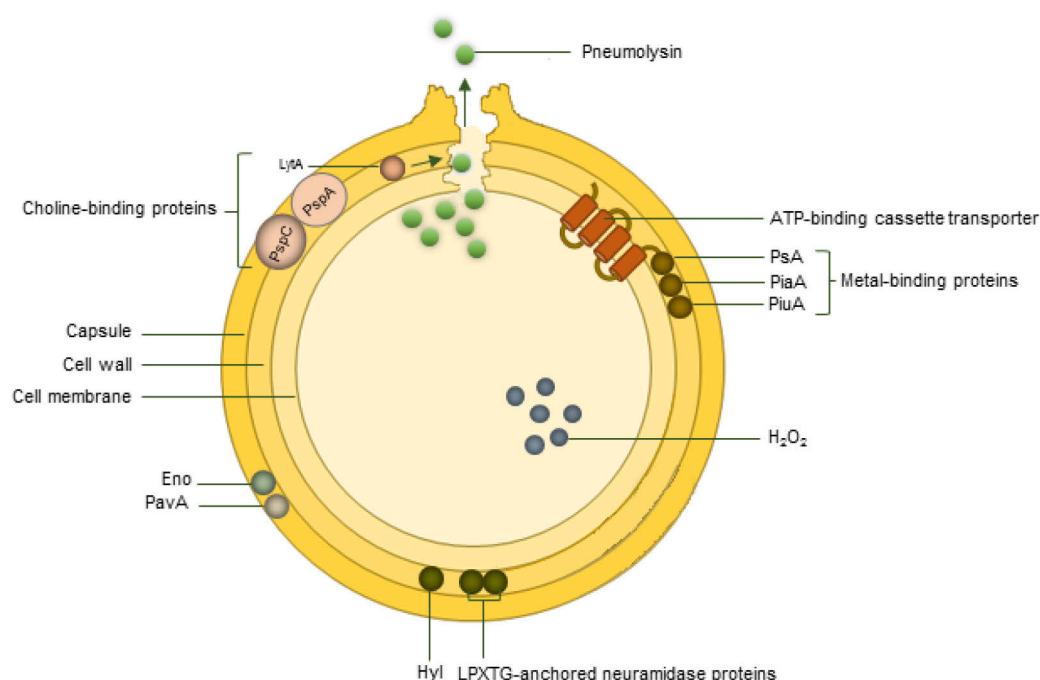


Figure 3.5: Overview of virulence factors of *S. pneumoniae*.

Important virulence factors are the capsule, the cell wall, Pneumolysin, hydrogen peroxide, synthesized by pyruvate oxidase, metal- and choline-binding proteins like PspA, PspC and autolysin (LytA), LPXTG-anchored neuraminidase proteins, enolase (Eno), hyaluronate lyase (Hyl) and pneumococcal adhesion and virulence A (PavA). Figure was adapted and modified from ²⁸.

3.3.3 Pneumonia caused by *Streptococcus pneumoniae*

S. pneumoniae frequently colonizes the upper respiratory tract and is transmitted by direct contact between household members, in day-care centers, jails and other places where crowding is happening. 10 % of adults, especially elderly and 20-40 % of children with a peak at the age of three years are airway carriers of *S. pneumoniae*. This carriage is asymptomatic in most cases and can persist for weeks in adults and even for month in children. But depending on the immune status (immunocompromised or with underlying disease) and on the serotype (invasive or non-invasive), colonization can lead to severe disease^{34,58,59}.

S. pneumoniae is the causative agent of different diseases, namely meningitis, otitis media, sinusitis, pneumonia and can lead to sepsis. Regarding pneumonia, it is one of the leading causes of CAP. People with different predispositions show a higher susceptibility for pneumonia-associated diseases, including pneumonia. For instance, higher incidence for pneumonia, meningitis and bacteremia can be found in children under the age of five years and elderly people older than 65 years. Furthermore, smoking, alcoholism, asthma and immunosuppression are associated with a more severe form of the disease⁵⁹⁻⁶¹.

The development of pneumonia occurs when *Pneumococci* from the nasopharynx reach into adjoining areas or get aspirated into bronchiole or alveoli and if the required elimination of bacteria is not appropriate. To invade those areas and to resist the defense mechanisms of the host's immune response, *S. pneumoniae* developed a high amount on virulence factors, as described before. Again, worth mentioning are the capsule and the Pneumolysin, which contribute especially to their pathogenesis by preventing phagocytosis and breaking through tissue barriers. In most cases of disease development, symptoms after the latency phase are fever, cough with a puerperal discharge and a severe impairment of the overall wellbeing. On the basis of the clinical picture, blood- and sputum cultures and chest radiography occurs the diagnosis of pneumococcal pneumonia. Subsequent treatment is mainly done by application of antibiotics. This treatment faces the problem of emergence of resistant strains, especially for the recommended and mostly used classes penicillin, vancomycin and macrolides. This is reported to be not only caused by clonal dynamics, but it also correlates with antibiotic use including national variations^{62,63}.

Not all of over 90 serotypes are causing disease. Vaccines for children are available against 7 to 13 of the most prevalent serotypes, which work safely and efficaciously for prevention. The WHO recommends the inclusion of the vaccines in immunization programs, especially in countries with high infant mortality. Additionally, it is recommended that elderly >60 years old as well as teenagers and adults with predispositions get vaccinated with the polysaccharide vaccine targeting 23 serotypes^{64–66}.

3.4 *Legionella pneumophila*

3.4.1 Microbiology

The genus *Legionella* and the family *Legionellaceae* were defined with the first isolation in Pennsylvania. *L. pneumophila* is one species out of 50, from which 20 species are reported to be human pathogens. Different immunological behavior is distinguished by the introduction of serogroups. *L. pneumophila* is classified into 14 serotypes. The bacterial cells are thin, measure 2 to 20 µm and appear pleomorphic. Even filamentous forms can appear, especially when grown under laboratory conditions on agar plates, which typically have charcoal as a source of carbohydrates and are supplemented with L-cysteine and ferric iron. As a representative of the gram-negative bacteria, it has the typical structure of an inner and outer membrane and it additionally forms pili. Due to a single polar flagellum, *Legionella* are able to move⁶⁷.

L. pneumophila is an opportunistic intracellular bacterium, causing a severe form of pneumonia, which is also known as Legionnaires' disease. This name refers to the victims of an outbreak of pneumonia in 1976. Attendees at the 58th American Legion Convention in a hotel in Philadelphia, Pennsylvania, suffered from sudden symptoms similar to the flu or other forms

of pneumonia, including fever with chills, cough accompanied with chest pain, headache, and muscle pain. From 182 recorded cases, 147 were hospitalized and 29 died⁶⁸. The causing pathogen, previously unrecognized as a human pathogen, was described only three years later, when the term *Legionella pneumophila* was established reflecting the caused disease, even though it was already isolated in the 50th as an infectious agent of amoebae⁶⁹.

L. pneumophila exists as a parasite of amoebae and ciliated protozoa ubiquitously in natural fresh water environments, but also in man-made water systems. In those, biofilms are required for *L. pneumophila* to survive and spread, where free-living protozoa also benefit the growth⁷⁰. Intracellular replication of *L. pneumophila* is possible in 14 amoebae species, i.a. *Hartmannella* and *Acanthamoeba* as the most prominent ones, and 2 species of ciliated protozoa. These natural hosts not only deliver nutrients and give the possibility of colonizing a habitat, but also protect the bacterium from unfavorable conditions, especially in the cyst form of amoebae. Infection of the amoeba occurs during its trophozoite form, where it proliferates within the *Legionella* containing vacuole (LCV). The function of the LCV will be further explained in the section 3.4.2. Release of *Legionella* from free-living amoebae can occur nonlytic similar to exocytosis of food vacuoles via excretion of vesicles. The other form of release which is more similar to human host cells is the lytic outburst. After rapid replication inside the LCV, *L. pneumophila* exits it and replicates 1-2 more cycles inside the cytosol. Subsequently, phenotypic modulations take place to escape from the used host and invade a new one. This natural lifecycle within amoebae is very similar to the lifecycle of *L. pneumophila* in the human alveolar macrophages, which is thought to be a diversion of its origin. That is why and because person-to-person-transmission has never been reported, which excludes the possibility for a new host for *L. pneumophila*, the infection is also regarded as an accidental infection^{71,72}.

The genome of *L. pneumophila*, which was completely sequenced of three different isolates responsible for different outbreaks, consists of 3.4-3.5 mega base pairs with in average 38 % G and C nucleotides. Species specificity or at least genus specificity is estimated to be at 40 %. Around 2.4 % is phage-derived or an insertion sequence also from eukaryotic hosts. These transfers reflect the evolutionary history of *L. pneumophila*. Furthermore, it implies the ability of this pathogen to adapt to the host. One noteworthy example is the *rafF* gene, which has its origin in a eukaryotic host and plays an important role in establishing the LCV^{73,74}. Different studies report additionally that *L. pneumophila* expresses eukaryotic-like proteins to possibly mimic host cell proteins and by that influence the intracellular life cycle or the hosts signal transduction mechanisms^{75,76}.

3.4.2 Pathogenicity

L. pneumophila can be transmitted by aerosol droplets from contaminated water sources. After those droplets are inhaled by a human host, the alveolar macrophages are the major target as they are used for bacterial replication. The amoebic cells serve as a perfect target, because of their similarities to *L. pneumophila*'s natural host, the amoebae. It has been assumed, that the interaction of *L. pneumophila* with amoebae is responsible for the development of different virulence factors during evolution, which enables *L. pneumophila* to also infect human cells⁷⁷. After inhalation into the lung and engulfment by macrophages, *L. pneumophila* manipulates the endosomal-lysosomal degradation pathway to ensure its survival. In contrast, most phagocytosed microorganisms are trapped inside the phagosome, which fuses with a series of compartments of the endocytic network to the phagolysosome through which an acidification takes place leading to the digestion of the pathogen⁷⁸. *L. pneumophila* utilizes a type IV secretion system (T4SS) to evade phagosome-lysosome fusion and to establish the replication vacuole. This translocation system is encoded by 27 *dot/icm* genes that are expressed constitutively to deliver around 300 molecules altering the host cell processes. Right after internalization, Dot/Icm substrates like VipA, VipD and VipF target the endocytic pathway and interfere with lysosomal protein trafficking. The vacuolar-ATPase (v-ATPase) machinery, which mainly regulates acidification, gets arrested by binding of the Dot/Icm transporter substrate SidK to the catalytic subunit of v-ATPase. In addition, Dot/Icm independent effectors contribute to the inhibition of lysosomal fusion as the multifunctional chaperone HtpB or Lipopolysaccharides (LPS)-rich vesicles^{79,80}. Next to the interference with the endocytic pathway, *L. pneumophila* hijacks the secretory pathway for the recruitment of vesicles originating from the endoplasmic reticulum (ER) to convert the membrane of the LCV into a membrane with ER-characteristics. Early recruitment of the host factor Sec22b – a SNARE protein on ER-derived vesicles regulating the trafficking to the Golgi-apparatus – and Rab1 – a small GTPase necessary for the fusion of ER-derived vesicles to the target membrane – facilitates the formation of the LCV and supports replication. Within the first six hours, more and more ribosomes and ER proteins like calnexin surround the LCV so that the vacuole appears to be comprised by rough ER^{81–83}. Additionally, mitochondria are recruited to the LCV, in which the chaperone HtpB is involved, and replication of the bacterium takes place^{84,85}.

During the first 24 h to 48 h, *L. pneumophila* increases in number within the LCV before it undergoes phenotypic modulations, including flagellation, and exits the LCV. Eventually, lysis of the host cell is initiated, releasing a new generation of bacteria to start a new round of infection. The exit of the LCV and the host cell is mediated through pore formation leading to necrosis and interference with the cell death pathways. Those pathways are induced Dot/Icm-dependently and can be apoptotic as well as non-apoptotic^{79,86}.

The infection cycle of *L. pneumophila* is visualized in Figure 3.6.

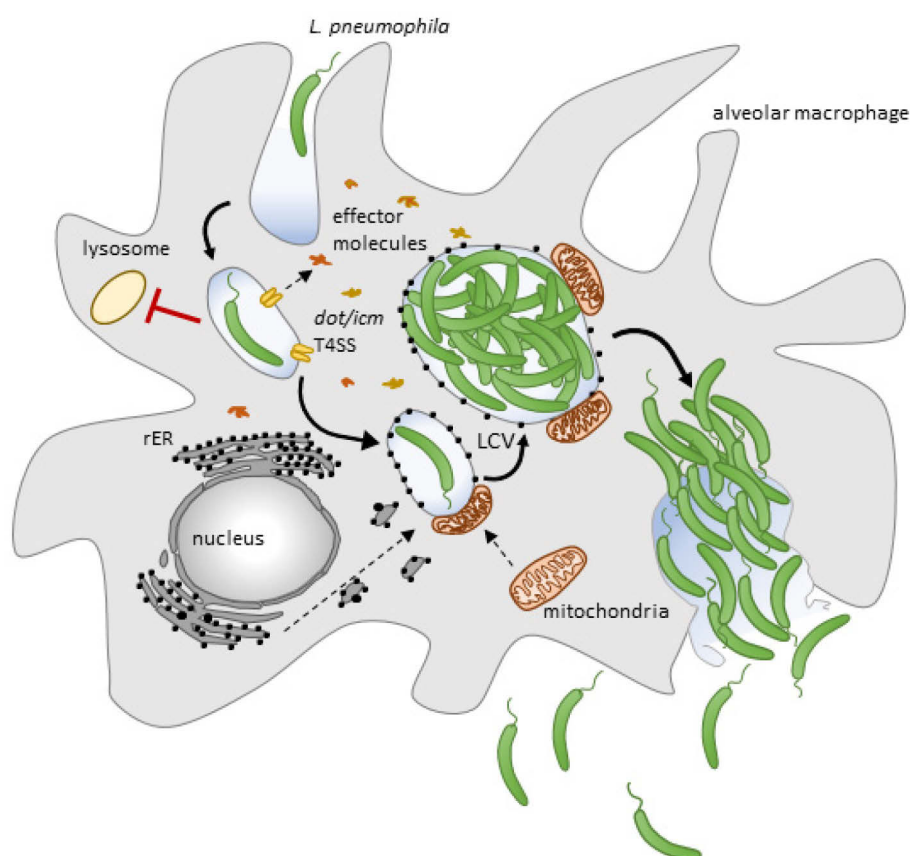


Figure 3.6: Life cycle of *Legionella pneumophila*.

After internalization of *L. pneumophila*, it bypasses endocytosis by blocking lysosomal fusion and recruits vesicles of the rough endoplasmatic reticulum (rER) and macrophages for establishment of the replicative vacuole, the *Legionella*-containing vacuole (LCV). For that it utilizes the Type-IV secretion system (T4SS) which secretes up to 300 effector molecules, interfering with the host's pathways. After termination of replication, *L. pneumophila* is leaving the vacuole, gets phenotypical modified and released from the host cell through lysis. Figure adapted from ^{79,80,87}.

3.4.3 Legionellosis

Legionellosis defines two different clinical syndromes: the Pontiac fever and a severe form of pneumonia which are caused by the pathogen *L. pneumophila*. The onset of the bacterial infection depends on the contaminated water reservoir, which represents the natural environment of *Legionella* and the source of infection. When the bacterium gets into man-made water reservoirs it can persist for years, grow and proliferate. Drinking water pipelines, in particular, represent the source of community-acquired pneumonia in hotels, office or industrial buildings. Additionally, the formation of aerosols containing *Legionella* due to water filled devices like whirlpools, humidifiers and nebulizers have also been associated with infection. The incidence of community-acquired Legionnaires' disease differs, depending on the level of correct diagnosis and reporting, since many countries lack appropriate methods. In general, the incidence depends on extent of contamination of the water reservoir, the immune status of the person in

contact with the water and the extent of exposure. Through different prospective studies, Legionnaires' disease is ranked with 2-13 % of all cases the fourth most cause of CAP. However, the centers of disease control and prevention (CDC) estimated that only 3 % of ambulant-acquired pneumonia are diagnosed correctly. A much higher number of cases was revealed by testing for *Legionella* as the suspected diagnosis. According to the competence center "CAPNETZ", 6 % of all treated pneumonia are caused by *Legionella* which yields 15 000 cases per year. Nevertheless, just around 1 000 cases were reported to the RKI. Hospital acquired pneumonia is caused to 10-50 % by *Legionella* due to contaminated water pipe lines in the hospital. Testing the water quality regularly increases the detection of hospital-acquired Legionellosis and allows faster diagnosis and therapy⁸⁸. Of the reported cases, 75-80 % of the infected persons are over 50 years old and 2/3 are male. Risk factors include smoking, alcoholism, immuno-suppression and chronic respiratory or pulmonary-related disease history. For hospital-acquired pneumonia recent surgery, intubation, mechanical ventilation or aspiration and immuno-suppression for e.g. organ recipients or cancer patients are further risks⁸⁹.

After aspiration or inhalation of aerosols an incubation time of 2 to 10 days is followed by the first symptoms such as fever with chills, headache, loss of appetite and lethargy. This is accompanied by a mild cough, which is unproductive, but patients often have chest pain. Untreated infections worsen during the first week and can lead to respiratory failure and eventually to multi-organ failure. The death rate of Legionellosis is in average 5-10 %. It depends on the hosts status, appropriate treatment and severity of infection. Immuno-compromised patients, that do not get treatment show a death rate between 40-80 %. Appropriate treatment decreases that rate to 5-30 %⁸⁸⁻⁹⁰.

Because there is no vaccine available, prevention can only be done by controlling the water reservoirs, including regular maintenance, cleaning and disinfection of the devices⁹⁰.

3.5 The immune response

There are a lot of cases, in which the contact of microorganisms to multicellular organisms is of benefit for both sides. For instance, a well-established gut microbiota is being recognized as contributing to the overall health to the human host ⁹¹. But in other cases, the microorganisms exploit the host for its purposes and thereby damage the host cells. To defend against these impairing effects and fight the bacteria defined as pathogens, the host develops a defense system. In vertebrates, two different types of systems are distinguished: the innate and the adaptive immune system⁹². The innate immune system is essential for the control of common bacterial infections. If such an infection is not cleared after four to seven days, the adaptive immune system gets activated by different cells of the innate immune system. Those cells are also involved in the elimination of pathogens during the adaptive immune response⁹³.

3.5.1 Recognition of invading bacteria

The innate immune system is the host's first line of defense against invading pathogens. First of all, there are anatomic and physiological barriers including the skin, mucociliary clearance systems, a low stomach pH and anti-bacterial lysozymes in the saliva and other secretions. If pathogens overcome these barriers, the innate immune system takes on defending the host, which is performed by cells of hematopoietic origin, e.g. macrophages and dendritic cells and of nonhematopoietic origin, e.g. epithelial cells⁹⁴. To eliminate invading pathogens, the immune system does not act unspecific, but recognizes them and distinguishes between those and itself. It recognizes pathogens by germ-line encoded pattern recognition receptors (PRRs). Each receptor is detecting highly conserved microbial motives, so-called pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular pattern (MAMPs), if the microbe is not pathogenic⁹⁵. PAMPs are defined by three main criteria: (i) they do not differ between one class of microorganism, which ensures a broad range of microbes to be recognized, (ii) they are a product of signaling pathways, which are unique to microorganisms and thereby allowing to distinguish between self and non-self, (iii) they are essential for microorganisms in regard of physiology and survival, thus limiting the possibility to modify their characteristics to prevent recognition⁹⁶. Different PRRs react on different PAMPs, show specific expression patterns and activate specific signaling pathways which lead to a selected immune response. The underlying machinery is highly conserved from plants to the fruit fly to vertebrates⁹⁵.

3.5.1.1 Pattern recognition receptors (PRRs)

The pattern recognition receptors are next to the complement system involved in the early recognition of pathogens and the subsequent induction of the immune response⁹⁶. Cells of the innate immune system of the hematopoietic origin are macrophages, dendritic cells, mast cells, neutrophilic and eosinophilic granulocytes and natural killer cells. The receptors are expressed both on the cell surface of these cells and in the intracellular space as well as secreted into tissue fluids or into the blood system. After stimulation of the receptors, the aforementioned cell types rapidly mature into short-living effector cells, which are important for the clearance of the infection⁹². In addition, it is also known that epithelial cells play a central role in the innate and adaptive immune response and, after activation by PRRs, secrete pro-inflammatory cytokines and chemokines, as well as recruit immune cells⁹⁷.

PRRs are divided into different classes: Toll-like receptors (TLRs) are stimulated by different components of pathogens and activate the translocation of NF- κ B in the cell nucleus and thus the secretion of cytokines, e.g. interleukin (IL-) 1 β and IL-6, as well as type I interferon (IFN) production⁹². A detailed description of the TLR can be found in chapter 4.3.2.1.

The NOD-like receptor (NLR) family includes 22 intracellular proteins in humans. The structural characteristics like the C-terminal LRR-domain involved in the recognition of the ligands, the central nucleotide-binding oligomerization domain (NOD) and a N-terminal effector domain affiliate them into this class. The effector domain classifies the NLRs into three subfamilies. The NOD-family has a caspase-recruiting domain (CARD), the NALP-family has a pyrin domain (PYD) and the NAIP-family is characterized by a Baculovirus inhibitor of apoptosis protein repeat-containing (BIR) domain^{98–100}.

Intracellular PRRs represent the ones mainly responsible for viral recognition. Viral infections are recognized among others by retinoic acid inducible gene 1- (RIG) like receptors (RLRs) and MDA5. These intracellular receptors recognize viral RNA and induce the production of IFN α and - β for the antiviral immune response⁹². Furthermore, RIG-I and MDA5 are reported to participate in the IFN response to *L. pneumophila*¹⁰¹. Also involved in the recognition of viral infection by viral DNA is the DAI-receptor, but the immune response is still unknown⁹². The cytosol is additionally checked by nucleic acid sensors such as the Interferon-inducing protein AIM2, which does recognize double stranded DNA not only from viruses, but also from bacteria and the host. AIM2 activation was reported to be essential for inflammasome activation and production of IFN γ ^{102,103}.

Different surface receptors on macrophages serve as PRRs and introduce the phagocytosis of microorganisms. There are three noteworthy receptors: first, the macrophage mannose receptor (MMR) of the family of the C-type lectins. It interacts with both gram-positive and gram-negative bacteria as well as fungal pathogens and regulates the transport of the microorganism

into the lysosomal compartment¹⁰⁴. Additionally, the macrophages scavenger receptor (MSR) requires mention. It is considered as a phagocytotic PRR and belongs to the family of type A scavenger receptors. It possesses a particularly high amount of polyanionic ligands as dsRNA, LPS and LTA¹⁰⁵. The third group are the macrophage receptors with a collagen structure (MARCO), which also belong to the type A scavenger receptor family. It binds to the bacterial cell wall and LPS itself and thus induces phagocytosis of bacterial pathogens. MARCO has already been proven to play a role in the immune response to pneumococcal infection. This role was described in more detail in a study of Dorrington et al. in which MARCO is required for the TLR2- and NOD2-regulated immune response^{92,106,107}.

3.5.1.2 Toll-like receptors (TLRs)

Toll-like receptors are considered to be the best characterized pattern recognition receptors. They are evolutionary conserved and were discovered as Toll receptors in the fruit fly *Drosophila* in the first place⁹⁵. Ten years later, the first vertebrate homologue was discovered and subsequently a protein family similar in structure to the one of Toll receptors. Thus, the family was named Toll-like receptors. So far, 13 members of the TLR family have been identified in vertebrates. These are membrane-integrated glycoproteins (type 1) with a cytoplasmic signal domain (TIR), which is homologous to the IL -1 receptor, and an extracellular domain with a different number of leucine-rich repeat (LRR) sequences. LRRs are β -strands and α -helices, linked by loops, creating a horseshoe-shaped domain. The ligand binds to the covalent surface of this structure, whereas an altered motif with a convex surface binding site was discovered in human TLR3⁹⁵. The comparison of the amino acid sequences of the human TLRs show structural similarities, but the TLRs can be subdivided into five subclasses owing to the differences. The common structure reflects the common function in the recognition of pathogenic structures¹⁰⁸. The classes are visualized in Figure 3.7.

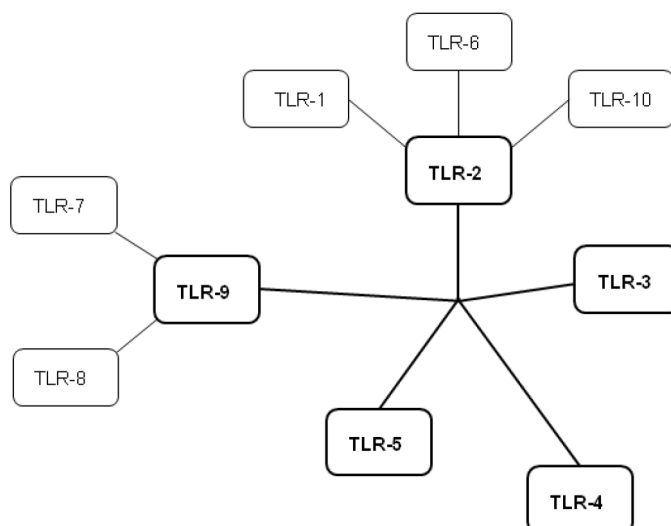


Figure 3.7: Classification of the Toll-like receptors. Figure adapted from ⁹⁵

Toll-like receptors are expressed by different immune cells such as macrophages, dendritic cells, B-cells and specific T-cells, but also from non-immune cells like fibroblasts, endothelial and epithelial cells. The expression is regulated by many of different factors as the microbial invasion, microbial components and cytokines. The expression can be found either intracellular or extracellular: TLR 1, 2, 4, 5 and 6 are localized on the outer membrane of the cell, TLR 3, 7, 8 and 9 inside the endosomal membrane^{108–110}.

Bacterial recognition occurs, among others, by patterns in the bacterial cell wall through which bacteria can be distinguished into two classes: gram-positive and gram-negative bacteria. The components of the cell wall serve as PAMPs.

LPS, which are characteristic for gram-negative bacteria, are directed to the extracellular portion of the TLR4 via the LPS-binding protein (LBP), resulting in oligomerization of the receptor. It should be noted that different bacteria produce different LPS, which differ in phosphate motifs, acyl chain number and composition of the fatty acids. These alterations are the reason for the differently-described biological activities of the lipid A, which is the part of the LPS responsible for the pathogenicity of gram-negative bacteria¹¹¹.

In addition to the detection of LPS, the TLR4 has been described as a recognition receptor for gram-positive *Pneumococci*, since it is intended to detect the pneumococcal exotoxin Pneumolysin¹¹².

TLR2 acts as a major recognition receptor for gram-positive bacteria. It is stimulated by LTA, an important cell wall component. TLR2 is likewise described for the detection of the LTA of the pneumococcal cell wall¹¹³.

Bacterial DNA is distinguished by the endosomal TLR9. The detection is based on the CpG-islands, which are unmethylated compared to the human system. TLR9 plays an important role in the detection of *Pneumococci* via the released microbial DNA. It has been reported that signaling pathways important for the phagocytic activity of macrophages and thus for an early clearing of pneumococcal-infected infections are TLR9-dependent, especially in the respiratory tract¹¹⁴. Furthermore, TLR9 has as well been reported to be involved in the immune response to *L. pneumophila* by stimulating the production of immune-transitioning cytokines like IL-12¹¹⁵.

3.5.2 Signaling pathway upon TLR-stimulation

Recognition of environmental stimuli leads to activation of receptors like TLRs. They in turn trigger signaling cascades, which induce the gene expression of different genes for defense of the host.

In detail, ligand binding by the receptors leads to recruitment of adaptors interacting with the Toll-Interleukin-1 receptor (TIR) domain, which is present at the C-terminus of all TLRs as well as of the adaptors. The known adaptors include the myeloid differentiation factor 88 (MyD88), the TIR domain-containing adaptor inducing IFN- β (TRIF), the TRIF-related adaptor molecule (TRAM), the TIR domain-containing adaptor protein (TIRAP), the sterile alpha and HEAT/Armadillo motif (SARM) and the B-cell adaptor for phosphatidylinositol-4,5-bisphosphate 3-kinase (BCAP). MyD88, TRIF, TRAM as well as BCAP are reported to have activating functions, by which BCAP has a functional N-terminal TIR homology domain, which links TLR-signaling to activation of Phosphoinositide 3-kinase (PI3K). SARM, in contrast, acts as a negative regulator of TRIF-mediated signaling^{116–119}.

All TLRs but TLR3 interact with MyD88. In particular, in addition to the MyD88-dependent signaling pathway, MyD88-independent signaling pathways, especially for TLR2 and TLR4, were also described. TLR3 utilizes TRIF for triggering the signal cascade. However, MyD88 acts as a negative inhibitor downstream of TRIF^{92,117}.

Upon interaction with the adaptors, they recruit and form a complex with IRAK1, IRAK6 and TRAF6, which leads to the activation of I κ B kinase (IKK) via further cascade steps. In consequence, phosphorylated I κ B is degraded and releases NF- κ B which subsequently translocates into the nucleus. This results in the production of pro-inflammatory cytokines, as well as the expression of type 1 IFN⁹⁵.

In addition to the activation of NF- κ B through the MyD88- and TRIF-dependent pathways, TLR-signaling also leads to activation of the AP-1 family by mitogen-activated protein kinases (MAPKs), production of type I IFN, which occurs MyD88-dependent and -independent, and PI3K activation. The latter is also dependent on MyD88 and TIRAP, where BCAP is a possible interaction partner or downstream regulator¹¹⁹.

The stimulation of TLRs leads to the maturation of dendritic cells and to the induction of co-stimulating molecules, chemo- and cytokines, antimicrobial peptides (such as β -defensin 2 by lung epithelial cells¹⁰⁸) and MHC-molecules as well as the decrease of antigen presenting capacities. In addition, the gene expression of the co-stimulatory molecules CD80 and CD86, which activate the adaptive immune system, can be induced by NF- κ B activation⁹². The specific outcome of the receptor stimulation is dependent on the stimulus, the activated TLR itself and the TLR-expressing cell type involved.

In the case of monocyte-derived macrophages and neutrophils, enhanced phagocytosis and increased oxidative burst resulting in a rapid killing of the pathogen account for the immune response. Additionally, resident macrophages respond by increased chemokine secretion to recruit further immune cells to the site of infection. In general, macrophages present in the infiltrated tissue are activated and stimulated to produce pro-inflammatory cytokines such as tumor necrosis factor (TNF), IL-1 β and IL-6⁹⁶.

TNF and IL-1 β in turn, activate the local endothelium to induce vasodilation and to increase permeability of the blood vessels. In addition, the increased fraction of tissue factor (coagulation factor III) on the endothelium leads to local coagulation, which prevents the distribution of the pathogens in the blood system⁹⁶.

IL-1 β and IL-6 also activate hepatocytes for the production of acute phase proteins such as pentraxin and collectin. These activate the complement system and the opsonization of pathogens for phagocytosis by macrophages and neutrophilic granulocytes⁹⁶.

Furthermore, the induction of cytokines activates naïve T-cells and leads to their differentiation¹¹⁹.

3.5.3 Chemokines and Cytokines

A variety of mediators is secreted by the cells of the human host during pathogen defense. These mediators act on the invaders, but also on the host's tissue. This can lead to tissue damage, especially in interactions with products of the pathogens such as Pneumolysin, which can impair the functions of the tissue.

The activation of pattern recognition receptors initiates the release of chemo- and cytokines. Depending on the produced cytokines, the immune response is either cytotoxic, humoral, cell-mediated or allergic¹²⁰. A cascade of responses is activated by cytokines and often several cytokines are needed to orchestrate optimal function.

Cytokines are proteins that affect the maturation and recruitment of leukocytes, resulting in the massive infiltration of polymorphonuclear leukocytes (PMNs) into the lung¹²¹. Furthermore, they act on bone marrow cells and specific immune response cells. They therefore have an influence on the innate and adaptive immune system. Cytokines are divided into four groups³. Among the interleukins are 18 different proteins, which are released from different cells. They act mainly on lymphocytes and activate their maturation and proliferation. Interferons are mainly released from virus-infected cells and have among others virus- and growth-inhibiting functions. They also have an antitumor effect. The tumor necrosis factor acts on different cells. They act deadening on tumor cells or stimulate phagocytosis, which in turn stimulates the immune reaction.

IL-1 β belongs together with IL-1 α and the Interleukin-1 receptor antagonist (IL-1Ra) to the

Interleukin-1-family, a prototypical multifunctional cytokine. IL-1 has an effect on almost all kinds of cell types and often acts together with other cytokines and mediator molecules. IL-1 α and IL-1 β are inflammatory cytokines and are considered beneficial but on the same time toxic. IL-1 β release occurs following the stimulation of pattern recognition receptors and the resulting activation of the inflammasome. This in turn activates Caspase-1, a Cysteine protease, which cleaves the pro-form into the active pro-inflammatory cytokine. The secretion of IL-1 β occurs in almost every organ. This cytokine acts as a protection against infections by the activation of various immune responses such as the rapid recruitment of neutrophil granulocytes to the site of inflammation, the activation of endothelial adhesion molecules, the induction of further cytokines and chemokines, as well as the induction of fever and the stimulation of the specific adaptive immune response. IL-1 β is considered one of the strongest pro-inflammatory cytokines^{122–124}.

Two homologous proteins represent the human TNF. TNF α is primarily produced by mononuclear phagocytes, but also by neutrophils, natural killer cells, endothelial cells and mast cells, whereas mainly lymphocytes produce TNF- β . The induction of TNF is most potentially caused by LPS through the TLR2 and TLR4. Upon stimulation, the active, soluble form of TNF α is released upon cleavage from the membrane-embedded protein by the TNF α converting enzyme. Upon release, TNF binds to its receptors I and II, which in turn induces, on one hand, cytotoxic effects on cancerous cells and antitumoral immune responses. On the other hand, it increases vascular permeability and activates the vascular endothelium, leading to increased entry of complement factors, immune cells and IgG antibodies. This is especially important for the containment of the infection if clearance of the pathogen was not successful^{120,125,126}.

Whereas IL-1 and TNF induce synthesis of each other and IL-6, IL-6 itself terminates this inflammatory cascade and, even more, inhibits the synthesis. It has several more anti-inflammatory effects, but it also acts pro-inflammatory. It is considered to be the most important cytokine for induction of hepatocyte synthesis of acute-phase protein. Because of IL-6 release, B-lymphocytes differentiate into mature plasma cells and T-cells are activated and stimulated for growth and differentiation. IL-6 is then produced by T- and B-lymphocytes, but also by endothelial cells, fibroblasts and mainly by mononuclear phagocytes.

Chemokines are a group of 8-12 kDa molecules with a homology of 20-50 %. Nowadays, there are 47 chemokines known, which regulate activity by interacting with members of the 7 transmembrane G-protein-coupled receptor superfamily, from which 18 chemokine receptors are described. Many of the receptors can bind to more than one ligand. Chemokines were originally described as inflammatory, because they are produced at the site of infection in response or upon a pro-inflammatory stimulus. They have a significant role in chemotaxis in a variety of cells including monocytes, neutrophils, lymphocytes and fibroblasts. Their secretion leads to recruitment of leukocytes. It has furthermore been shown that chemokines have a homeostatic

or housekeeping function, and are involved in the adaptive immune response. For example, chemokines have direct effects on T-cell differentiation and indirect effects on antigen presenting cell (APC)-trafficking. Inflammatory chemokines are secreted by numerous cells in different locations after activation, whereas homeostatic chemokines tend to be expressed by specific tissues or organs^{120,127}.

IL-8, which is systemically named CXCL8, is considered the most important chemoattractant for PMNs, which is primarily secreted by mononuclear phagocytes, endothelial and epithelial cells, but also by T-cells, neutrophils and fibroblasts. In comparison to other chemoattractants, IL-8 appears late. It is also one of the most potent chemoattractant for neutrophils, stimulating neutrophil degranulation, the respiratory burst and adherence to endothelial cells¹²⁰.

3.5.4 Phagocytosis

Cells have established different strategies to internalize particles and solutes. Pinocytosis refers to the uptake of fluid or soluble substances. Endocytosis describes the process, in which macromolecules, small particles and viruses are internalized. Phagocytosis defines the uptake and degradation of particles longer than 5 μm . Phagocytic cells have the capacity to recognize, phagocytose and inactivate invading microorganisms. Therefore, they have a key role in the host defense. Alveolar macrophages represent the resident phagocyte in the lung and play a major role in clearing invading microbes in the lower respiratory tract. Macrophages are, next to neutrophils, considered the professional phagocytic cells, because they are most efficient in internalization, which is among others caused by the presence of phagocytic receptors that increase the range and rate of phagocytosis. Phagocytosis is a very complex process, but can be described in three main steps. Initially, the pathogen is bound either by the cell-surface receptors of the phagocyte, such as the MMR recognizing PAMPs, or by complement receptors after opsonization. Opsonization occurs specifically by IgG antibodies which leads to phagocytosis by Fc receptors or nonspecifically which leads to complement receptor binding. In addition, other PRRs lead to increased phagocytosis, not upon binding their ligand, but by subsequently initiating phagocytosis. Phagocytic receptors are capable of cross-talk and synergy. This collaboration secondly induces rearrangement of the actin cytoskeleton, which in turn leads to the internalization of the pathogen^{128,129}. In detail, ligand binding leads to clustering of the receptors which activates signaling molecules like the tyrosine kinase Syk and Src family members. The collaboration of receptors and the subsequent signals lead to alteration of the Actin cytoskeleton and initiation of membrane motility. This increased motility enables the phagocytic cell to completely engulf or take up the pathogen into the cytosol¹³⁰. The vacuole, now containing the pathogen, is called phagosome. The Actin gets depolymerized again soon after internalization and endosomes can access the phagosome. The phagosome fuses

with a series of compartments of the endocytic network to the phagolysosome through which an acidification takes place leading to the digestion of the pathogen. This formation occurs within 30 minutes^{78,128}. Phagocytosis of pathogens by macrophages initiates the innate immune response, which in turn orchestrates the adaptive response.

3.6 Transcription factors

Signal transduction cascades serve among others to forward signals from the cell membrane into the nucleus. This classical signal transduction starts with the recognition of a signal by specific receptors, followed by the intracellular transmission, which takes place via signal transducer molecules, mainly kinase cascades and ends e.g. in the activation of transcription factors that bind to the DNA after translocating into the nucleus and initiate the expression of various proteins such as adhesion molecules, receptors, enzymes or cytokines. The initiating signal can be amplified or weakened at every step, as there is crosstalk among the various kinases, allowing for a fine regulation of cellular responses that are dependent on the stimulus, the environment and the state of the cell. Those signal transducer kinases include mitogen activated protein kinases (MAPKs), which are subdivided into extracellular-signal regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 MAPK as well as PI3K^{131,132}.

Transcription factors, which are activated upon different stimuli, regulate cellular responses essential for the adaptation of the organism to environmental changes at the genetic level, thereby enhancing or suppressing transcription of their target genes. Transcription factors consist of one or more DNA-binding domains (DBDs), a trans-activating domain (TAD) and an optional signal sensing domain (SSD)¹³³.

One of these factors, worth mentioning, is NF- κ B, which plays a key role in the response to environmental changes and in many biological processes like the inflammatory reaction. NF- κ B has been shown to be expressed in various cells and is present in unstimulated cells bound as homo- or heterodimer to the I κ B protein in the cytosol¹³⁴. Three main routes lead to the activation of NF- κ B: a canonical, a noncanonical and a DNA-induced signal pathway. All signaling pathways share the generation of the DNA-binding dimerization. The second level of NF- κ B regulation is further downstream and includes phosphorylation, acetylation, ubiquitination or prolyl isomerization. There, the NF- κ B-dependent transactivation and the duration and amplitude of the NF- κ B signaling are controlled¹³⁵.

3.6.1 C-Myc

The proto oncogene *c-myc* was discovered 40 years ago as the cellular v-myc avian myelocytomatosis viral oncogene homolog. The myc-family includes B-*myc*, c-*myc*, L-*myc*, N-*myc* and s-*myc*, whereby all but B- and s-*myc* have neoplastic potential. C-*myc* is one of the most-frequently activated oncogenes, being involved in 20 % of all human cancers^{136,137}.

Despite its described role as an oncogene, it belongs to the so-called Yamanaka factors. Shin'ya Yamanaka and John B. Gurdon earned the Nobel Prize in 2012 for breakthrough findings for the re-programming of adult differentiated cells into inducible pluripotent stem cells (iPS). For the generation of the iPS, four transcription factors are needed: c-myc, octamer binding transcription factor 4 (Oct-4), sex determining region Y (SRY) -box 2 (Sox2) and Krüppel-like factor 4 (KLF4)¹³⁸. These factors in common are their strong influence in embryonic development. For instance, it was shown for c-myc that its deletion in murine embryos results in lethality before organ development occurs¹³⁹.

After the embryonic development is finished, a lot of the stem cell factors are nearly no longer detectable anymore, especially in healthy cells. C-myc however remains as a very important transcription factor, regulating up to 15 % of all genes of the human genome. The regulation occurs through different mechanisms including chromatin modulating proteins, recruitment of histone acetylases and DNA methyltransferases. Hence, the targets can be divided in subgroups according to the regulating mechanism¹³⁶. Mainly, c-myc can activate and repress expression of its target genes by binding directly or indirectly as a consequence of expression of the altered c-myc target genes or a c-myc-dependent phenotype.

The *c-myc* gene is comprised of 3 exons, which are translated to (i) a 64 kDa polypeptide or (ii) a 67 kDa polypeptide starting 15 codons further upstream or (iii) a 45 kDa polypeptide. The protein consists of a transactivation domain within the N-terminal side containing the conserved Myc boxes I to IV and a nuclear targeting sequence. A dimerization interface consisting of a helix-loop-helix leucine zipper (HLH/LZ) domain is located at its C-terminal end. The expression of c-myc is induced by mitogens and suppressed by growth-inhibitory signals. Additionally, the activity and function of c-myc is regulated by posttranslational modifications such as phosphorylation, ubiquitylation together with acetylation and interaction with other proteins¹⁴⁰. However, during oncogenic *c-myc* activation, gene expression is directly altered through translocation or amplification or by mutations of upstream signaling pathways. The activation of *c-myc* gene expression is central to signal transduction through the adenomatous polyposis coli (APC) tumor suppressor protein which negatively regulates β -catenin. The inactivation of APC results in activation of β -catenin, a coactivator for the transcription factor Tcf, which is able to directly activate c-myc expression^{136,137,141}.

To facilitate transcription of its target genes, c-myc dimerizes with its binding partner, the myc-

associated protein X (MAX). The human MAX protein also has a HLH/LZ domain, but not a transactivation domain. The c-myc/MAX-heterodimer binds DNA subsequently by recognizing the E-box motif (5'-CACGTG-3'), which results in complex formation of the c-myc N-terminal domain with other transcription factors as TRRAP, GCN5 and TBP. It also leads to recruitment of chromatin-modifying complexes like histone acetylases to open the chromatin for transcription of the respective target gene^{136,142}. In addition to its ability to activate transcription, c-myc is also able to repress transcription. One mechanism is through the initiator or Inr element, which is a consensus transcriptional initiation motif found in certain gene promoters^{143–145}.

MAX homodimers on the contrary are thought to bind competitively to the c-myc/MAX heterodimers to DNA target sites, thus, countering the activation. In addition to this model, the influence of the Mad family proteins has been discovered. These proteins contain a Sin3-interacting domain motif, which recruits Sin3, the transcriptional corepressor N-CoR, and histone deacetylase activity proteins. Histone deacetylation is currently thought to be the major mode of transcriptional silencing by the Mad protein, causing the locking of nucleosomal DNA and, consequently, inhibition of transcription. The Sin3-interacting domain motif, when tethered to an HLH/LZ transcriptional factor, TFEB, that binds myc DNA sites, is able to inhibit c-myc-mediated cellular transformation. Increased expression of Mad proteins is associated with cellular differentiation and growth arrest, suggesting that certain Mad family members behave as tumor suppressors^{146–149}.

Even though 15 % of all genes of the human genome are regulated by c-myc, only a fraction of genes appears to be universally regulated by c-myc independent of cell type or species. There are intriguing differences between the repertoires of c-myc responsive genes identified from different studies. C-myc affects specific classes of genes involving metabolism, protein biosynthesis, cell cycle regulation, including proliferation and differentiation and the cytoskeleton. Additionally, c-myc alterations can lead to apoptosis^{136,150}.

3.6.1.1 C-Myc in the immune response

First studies suggest that c-myc is not only involved in embryogenesis, regulation of the cell cycle and apoptosis and with that tumor development, but that it also has an influence on the immune response, antimicrobial activity or natural resistance of different cells to pathogens. While the stem cell factors are hardly detectable in healthy differentiated cells, studies indicate an increased expression of c-myc after exposure to cytokines or after infections with various viruses, bacteria or parasites^{151–157}.

Viruses require an increased glutamine metabolism for replication, as do cancer cells for growth. During replication of adenoviruses, an enhanced host cell catabolic glucose metabolism through c-myc activation was reported. The adenovirus E4ORF1 was shown to bind to

nuclear c-myc and to induce the activation of specific glycolytic target genes¹⁵⁸. Following their first study, they could furthermore show that the glutamine metabolism is enhanced by c-myc through the increased expression of glutaminase (GLS) as it is to some extent the equivalent to cancer cells. This increased expression promotes an optimal replication of the adenovirus 5 in infected lung epithelial cells. In addition, GLS inhibition leads as well to impaired replication of herpes-simplex virus (HSV) 1 and Influenza A virus¹⁵⁹. Whether HSV-1, Influenza A or other viruses also rely on c-myc for enhancing glutamine metabolism during infection was not shown. However, Higgs et al. published findings, that during chronic infection by Hepatitis-C virus (HCV) the c-myc expression is enhanced. HCV proteins led to Akt activation, which stabilizes β -catenin, of which *c-myc* is a direct target. The enhanced c-myc expression led to increased production of reactive oxygen species (ROS), mitochondrial perturbation, enhanced DNA damage and aberrant cell-cycle arrest. All in all, these results might provide one mechanism, why HCV infection represents a major risk factor of hepatocellular carcinoma (HCC)¹⁵⁴.

In contrast to c-myc being involved in the enhanced replication of viruses or development of tumors, it has also been reported to play a role in host cell death upon infection.

Salmonella typhimurium infection leads to enteritis and typhoid fever, inducing a lymphocytic response which can lead to death. During the infection, macrophages play a key role in fighting and clearing the pathogen. Among other processes, the expression of ornithine decarboxylase (ODC) is activated, which is reported to lead to cell death. Seong et al. related the ODC-induced cell death of macrophages to expression of c-myc. In addition to increased ODC expression upon *Salmonella typhimurium* infection and LPS, by which the bacterium is recognized through TLR4, it was shown that c-myc protein is induced upon infection of bacteria, but not by LPS alone. This induction was dependent on bacterial endocytosis upon invasion, but not on proliferation of the intracellular pathogen and resulted in cell death. The study suggests that in response to the *Salmonella typhimurium* infection, the regulation of c-myc is at a post-translational level and the resulting cell death is not regulated by c-myc activation of ODC but another c-myc dependent pathway¹⁵⁵.

Another infection with a gram-negative bacterium was linked to c-myc. *Helicobacter pylori* infection causes chronic gastric mucosal inflammation, caused by an ineffective immune response. One underlying mechanism describes the induction of macrophage apoptosis. The study by Cheng et al. indicates that c-myc is an important mediator of macrophage activation as well as apoptosis by regulating the ODC activity. Upon infection of macrophages with *Helicobacter pylori*, c-myc expression and nuclear translocation occurred. Without expression and subsequent binding of c-myc to the ODC promoter, mRNA expression and protein activity leading to apoptosis was inhibited¹⁵³.

C-myc is not only described to regulate apoptosis during infection, but also to modulate the

immune response. A study by Yim et al. presents new results showing that c-myc is not only induced upon infection with different *Mycobacterium* species via ERK1/2 and JNK activation, but also that c-myc induction leads to a pro-inflammatory immune response and inhibition of intracellular mycobacterial growth. Upon infection c-myc was induced in a time- and dose-dependent manner on mRNA and protein level. This upregulation seemed to be signaled through ERK1/2 and JNK, but not p38 kinases or NF- κ B. The resulting higher amount of c-myc protein but also the constitutive protein was found to be located in the cytoplasm not in the nucleus. Furthermore, it was shown that c-myc itself enhanced pro-inflammatory cytokine production, in particular TNF α and IL-6, but not the anti-inflammatory cytokine IL-10 upon infection. Moreover, c-myc mediated the suppression of the intracellular mycobacterial growth, which does neither result from cell-death production by c-myc nor the by repression of NRAMP1 transcription, which is reported to be responsible for that inhibition. The suppressing effect seemed to be important in a later stage of infection. However, an involvement of c-myc on cell proliferation and cell cycle regulation, respectively, could not be observed during mycobacterial infection¹⁵⁶.

Na et al. were investigating macrophages and the regulation of their inflammatory role in more detail. They showed that GM-CSF is responsible for upregulation of glycolytic capacity upon LPS-stimulation. The increased glycolytic capacity is required for an inflammatory phenotype of the macrophage, consisting of increased TNF α , IL-1 β , IL-6 and IL-12p70 synthesis. The GM-CSF-mediated upregulation of glycolytic requires de novo c-myc synthesis, resulting in synthesis of glucose transporter 1, -3 and -4. C-myc synthesis thereby involves the mTOR/Akt/ERK signaling pathway¹⁶⁰.

4 AIM OF THIS WORK

The aim of this work was to examine whether and how the protein c-myc is induced by *Streptococcus pneumoniae* and *Legionella pneumophila*, respectively. Furthermore, it should be determined which functions c-myc has during infections caused by the aforementioned pathogens in human macrophages.

4.1 Research questions

1. Do *Streptococcus pneumoniae* and *Legionella pneumophila* induce the expression of the transcription factor c-myc?
2. Due to which signaling pathway is the transcription factor c-myc induced?
3. Does the c-myc expression have any influence on the cytokine secretion?
4. Does this induced c-myc expression have any influence on the phagocytosis ability of macrophages?

5 MATERIALS AND METHODS

5.1 Reagents and instruments

The instruments used in laboratories, such as clean benches, incubators, centrifuges, cooling devices, ice machines, etc. are not listed in this work. They meet the common standards and are regularly maintained.

5.1.1 Instruments

Table 5-1: Instruments.

Instruments	Company	Method
ABI 7300 Real-Time PCR System	Applied Biosystems	RT-qPCR
Bio Photometer	Eppendorf	OD value - bacterial growth
FilterMax F5	Molecular Devices	ELISA (determination OD values)
Multi-Mode Microplate Reader		
Mini Trans-Blot® Cell	Bio-Rad	Western blot
Mini-PROTEAN® Tetra handcast system	Bio-Rad	protein electrophoresis
Mini-PROTEAN® Tetra Vertical Electrophoresis Cell	Bio-Rad	protein electrophoresis
Mr. Frosty Freezing Container	Thermo Scientific	freezing of cells
Nanodrop 2000	Thermo Scientific	RNA/DNA amount and purity
Odyssey Scanner	LI-COR	documentation (Western blot)
Photometer BioMate	Thermo Scientific	determination of protein amount after Bradford
Polymax 1040	Heidolph Instruments	shaking membrane (Western blot)
PowerPac™ Basic power supply	Bio-Rad	protein electrophoresis / blot
RCT basic	Ika-Labortechnik	magnetic stirrer (Western blot)
Thermomixer Comfort	Eppendorf	heating protein samples to 95°C (protein electrophoresis)

5.1.2 Consumable supplies

Table 5-2: Consumable supplies.

Supplies	Company
10cm cell culture dish	BD Biosciences
clear 96-well plate	Nunc™
BD Microlance 3 cannula (Ø = 0.9 mm)	BD Biosciences
BD Plastipak syringe	BD Biosciences
seal plate	Excel Scientific Inc.
cell culture flasks (diff. sizes)	Falcon®
cell culture microplates (diff. formats)	Falcon®
cell culture tube	Falcon®

Supplies	Company
cell scraper	Sarstedt
Columbia agar plate + 5 % sheep blood	BD Mikrobiologie
cotton stick	Applimed SA
cryo tubes	Sigma-Aldrich
cuvettes	Sarstedt
Eppendorf tubes (diff. sizes)	Sarstedt
inoculation loop	Carl Roth
MicroAmp™ optical 96-well reaction plate	Applied Biosystems
MicroAmp™ optical adhesive Film	Applied Biosystems
nitrocellulose hybond membran	Santa Cruz Biotechnologie
petri dish	Falcon
pipette tips (diff. sizes)	Biozym / Sarstedt
serological pipettes (diff. sizes)	Falcon®
Whatman gel blotting paper	Whatmann
µ-Slides 8 Well	Ibidi

5.1.3 Chemicals and reagents

Table 5-3: Chemicals and reagents.

Reagent	Company
3,3',5,5'-tetramethylbenzidine	BD Bioscience
Acrylamid / Bis-Acrylamid, 40 %, Ratio 19:1	Serva
Amersham ECL Plex™ fluorescent Rainbow Marker, full range	Amersham life science
ammonium persulfate	Serva
Ampuwa (RNase-free H ₂ O)	Fresenius Kabi
BioRad Protein Assay	BioRad
bromphenol blue	Biotech Pharmacia
Complete™ Protease Inhibitor Cocktail Tablet	Santa Cruz Biotechnologie
Dulbecco's PBS	PAA
ethanol	Merck Millipore
fetal calf serum	Gibco
glycine	Merck Millipore
H ₂ SO ₄ ; 95-97 %	Merck Millipore
hydrogen peroxide	BD Bioscience
Iso-RNA Lysis Reagent	5 PRIME
Lipofectamine® 2000 Transfection Reagent	Life Technologies
methanol	Merck Millipore
c-myc-inhibitor 10058-F4	Sigma-Aldrich
Nonidet® P-40 99 %	Fluka
Ody Blocking Buffer	LI-COR
PBS	AppliChem

Reagent	Company
PMA	Sigma-Aldrich
polyethylenglycol (PEG)-8000	Sigma-Aldrich
potassium hydroxide	Merck Millipore
saponin	Fluka
SDS	Serva
sodium carbonate	Merck Millipore
sodium fluoride 99 %	Sigma-Aldrich
sodium hydrogen carbonate	Merck Millipore
sodium orthovanadat 98 %	Sigma-Aldrich
sodium pyrophosphate	Sigma-Aldrich
SYTOX™ Red Dead Cell Stain	Invitrogen
TEMED	R&D Systems
Trizma® base	Sigma-Aldrich
TRIS-HCl	Sigma-Aldrich
Triton X-100	Sigma-Aldrich
trypsin/EDTA	Gibco
Tween-20	Sigma-Aldrich
β-mercaptoethanol	Sigma-Aldrich

5.1.4 Stimulants

Table 5-4: Stimulants.

Stimulant	Origin	Usage	Company
CpG (ODN M362)	recombinant	TLR9-ligand	InvivoGen
Flagellin	<i>Salmonella typhimurium</i>	TLR4-ligand	InvivoGen
LPS	<i>Salmonella Minnesota</i> R595	TLR4-ligand	Enzo Liefsciences
MALP-2	recombinant	TLR2-ligand	R&D Systems

5.1.5 Media

Table 5-5: Media and solutions for cell culture

Reagent	Company
DMEM (with high glucose, L-glutamine, phenol red)	Gibco
DMSO	Sigma-Aldrich
fetal calve serum	GE Healthcare
Hank's Balanced Salt Solution (HBSS)	Gibco
L-glutamine GlutaMax™-I	Gibco
Opti-MEM	Gibco
penicillin / streptomycin 100x	PAA
RPMI 1640	Gibco

Table 5-6: Media components for bacterial culture

Reagent	Company
ACES buffer	Sigma-Aldrich
activated charcoal	Carl Roth
agar	Beckton Dickinson
cysteine	Sigma-Aldrich
ferric nitrate	Sigma-Aldrich
glycerol	Sigma-Aldrich
sodium chloride	Merck Millipore
sodium pyruvate	Merck Millipore
Todd-Hewitt bBroth	BD Mikrobiologie
tryptone enzymatic digest from casein	Fluka
yeast extract	Sigma-Aldrich

*After preparation of the medium it was autoclaved for 10 minutes at 115°C and directly poured into petri dishes (agar) or stored at room temperature (broth). Addition of possible selection antibiotic ensued after the agar containing medium was cooled before pouring the plates or right before usage of the broth, respectively.

5.1.6 Antibiotics

Table 5-7: Antibiotics for selection.

Antibiotic agent	Company
ampicillin	Sigma Aldrich
kanamycin	Sigma Aldrich
puromycin	Carl Roth

5.1.7 Antibodies

Table 5-8: Primary antibodies

Antibody	Host	Company
anti- β -Actin	goat	Santa Cruz Biotechnologie
anti-c-myc	rabbit	Santa Cruz Biotechnologie

Table 5-9: Secondary antibodies

Antibody	Host	Company
anti-goat -IgG	mouse, conjugated IRDye800	Rockland
anti-rabbit-IgG	goat, conjugated IRDye800	Rockland

5.1.8 Kits and assays

Table 5-10: Commercially available kits and assays.

Kit	Company	Method
Direct-zol™ RNA MiniPrep	Zymo Research	isolation of total RNA
High Capacity Reverse Transcriptase	Applied Biosystems	reverse transcription from RNA to cDNA
HIV-1 p24 ELISA Kit, 96-Well	BioCat	titer of lentiviral particles
human c-myc TaqMan® Gene Expression Assay (ID: Hs00153408_m1)	Life Technologies	RT-qPCR
human GAPDH TaqMan® Gene Expression Assay (ID: Hs02758991_g1)	Life Technologies	RT-qPCR
human MyD88 TaqMan® Gene Expression Assay (ID: Hs01573837_g1)	Life Technologies	RT-qPCR
LDH Assay	Pierce	determination of toxicity
multiparameter apoptosis assay	Cayman	apoptosis assay
QIAGEN Plasmid <i>Plus</i> Midi Kit	Qiagen	isolation of plasmids for lentiviral production
Recombinant Human IL-1 β /IL-1F2	BD OptEIA	IL-1 β ELISA
Recombinant Human IL-6	BD OptEIA	IL-6 ELISA
Recombinant Human IL-8	BD OptEIA	IL-8 ELISA
Recombinant Human TNF	BD OptEIA	TNF α ELISA
viability assay	Invitrogen	viability assay

5.2 Bacteriology

5.2.1 Bacteria

5.2.1.1 *Streptococcus pneumoniae* strains D39 and R6x

Ex vivo human lung tissue, primary human cells as well as cell lines were infected with *S. pneumoniae* strains of the serotype 2. The wild type strain D39 is a clinical isolate from the year 1916 and is today one of the foremost used model organisms for the investigation of pneumococcal pathogenesis.

For *in vitro* experiments with primary human cells and cell lines the unencapsulated derivate R6x were used for modeling infections next to the wild type strain.

The *S. pneumoniae* strains were kindly provided by Prof. Sven Hammerschmidt, University of Greifswald.

5.2.1.2 *Legionella pneumophila* strain corby

For infections of *ex vivo* human lung tissue, primary human cells and cell lines with the *L. pneumophila* wild type strain corby was used. A subculture was kindly provided by Prof. Bastian Opitz, Department for Infectious Diseases and Pulmonary Medicine at the Charité.

5.2.1.3 *Escherichia coli*

The production and isolation of the used plasmids for the production of lentiviral particles was done with the chemically competent *E. coli* strain “*E. coli*® 5-*alpha*” (Lucigen), containing an *endA1* and a *recA1* mutation. To select positive clones and maintain the plasmids, ampicillin (100 µg/ml) was added to the Luria Bertani (LB) agar or broth, respectively.

5.2.2 Cultivation of bacteria

The work with bacteria was done under sterile conditions using a clean bench. The workplaces are declared as security level S2, where all reagents and instruments are autoclaved, heat sterilized or sterile-filtered.

All materials that came in contact with bacteria were cleaned with Descosept (Dr. Schumacher GmbH), consumables were autoclaved before disposal.

Liquid media and solutions were pre-warmed to 37 °C in the incubator.

5.2.2.1 Storage and cultivation of *S. pneumoniae*

For long-time storage, the strains of *S. pneumoniae* were kept at -80 °C in THY broth (30 g/l Todd-Hewitt broth, 0.5 % yeast extract, pH=7.8 ± 0.2) containing 20 % glycerol. To obtain the glycerol stocks, *Pneumococci* were plated on Columbia agar plates containing 5 % sheep blood, supplemented with selection antibiotics where necessary (2.5 mg kanamycin for D39Δcps). After 12 h incubation at 37 °C with 5 % CO₂, single colonies were transferred on new Columbia agar plates and again incubated until colonies were formed. Those colonies were resuspended in 1 ml of the cryoconservation medium using a sterile cotton tip.

For infection of tissue *ex vivo* or cells *in vitro*, bacteria were freshly plated on Columbia agar plates containing 5 % sheep blood, supplemented with selection antibiotics when necessary (2.5 mg kanamycin for D39Δcps) for 12 h at 37 °C and 5 % CO₂. After the incubation time, single colonies were transferred using a sterile cotton tip into pre-warmed THY broth reaching an OD₆₀₀ = 0.04-0.06. Bacteria were grown in an open system at 37 °C and 5 % CO₂ without shaking until mid-log phase (OD₆₀₀ = 0.2-0.4). Subsequently, bacteria were centrifuged at 1800 g for 10 min at room temperature. The supernatant was discarded and the bacterial pellet resuspended in RPMI 1640 (+2 or 10 % FCS, +1 % glutamine) to a concentration of 1×10⁹ CFU/ml, assuming that an OD₆₀₀ = 1 represents a bacterial concentration of 1×10⁹ CFU/ml. The respective concentration of bacteria for the infection of tissue and cells was achieved by further tenfold dilution.

5.2.2.2 Storage and cultivation of *L. pneumophila*

Stocks of *L. pneumophila* corby were kept at -80 °C in a N-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered yeast extract (AYE) broth (10 g/l ACES buffer, 10 g/l yeast extract, 0.4 g/l cysteine, 0.135 g/l ferric nitrate, pH=6.9), containing 20 % glycerol. Stocks were prepared by incubating *L. pneumophila* corby on buffered charcoal yeast extract (CYE) agar plates (10 g/l ACES buffer, 2 g/l activated charcoal, 10 g/l yeast extract, 10 g/l agar, 0.4 g/l cysteine, 0.135 g/l ferric nitrate, pH=6.9), for 2 to 3 days at 37 °C and 5 % CO₂. With a sterile cotton tip, the culture was transferred in 1 ml cryoconservation medium.

For *in vitro* infections, the bacteria were incubated freshly from the stock for 2 days on CYE agar plates at 37 °C and 5 % CO₂. On the day of infections, bacteria were resuspended in cell culture medium and the bacterial number was calculated on the assumption that an OD₆₀₀ = 1 represents a bacterial concentration of 1×10⁹ CFU/ml. The respective concentration of bacteria for the infection of tissue and cells was achieved by further tenfold dilution.

5.2.2.3 Storage and cultivation of *Escherichia coli*

The cultivation of the commercially acquired competent *E. coli* strain “*E. cloni*® 5-*alpha*” was done in LB broth (10 g/l Tryptone enzymatic digest from casein, 5 g/l yeast extract, 5 g/l sodium chloride) or on LB agar plates (LB broth, 7.5 g/l agar), respectively. The cultivation in LB broth was done in an Unitwist incubator at 37 °C and 5% CO₂ under shaking conditions, whereas the cultivation on agar plates was done in an incubator at 37 °C and 5 % CO₂.

The cultivation of the plasmid clone was done by preparing 5 ml of a pre-culture and a subsequent overnight culture in LB broth. For the overnight culture, 100 µl of the pre-culture were transferred in 50ml LB broth, supplemented with 100 µg/ml Ampicillin for maintenance of the plasmid.

For long-term storage, a stock culture was prepared using 400 µl of the pre-culture and 500 µl glycerol. The clones were kept at -80 °C.

5.2.3 CFU assay

To measure the phagocytotic ability of *S. pneumoniae* or the replication ability of *L. pneumophila* in *in vitro* infected cells, CFU assays were performed.

Therefore 1×10⁵ differentiated THP-1 cells (see section 5.3.1.3) per 96- well were infected with *S. pneumoniae* at an MOI of 100 (1×10⁸ CFU/ml) or *L. pneumophila* at an MOI of 1 (1×10⁶ CFU/ml), respectively (see also section 5.3.2). To ensure simultaneous infection, the plates were centrifuged at 200 g for 5 min and afterwards incubated at 37 °C and 5 % CO₂. After one hour, cells were carefully washed three times with PBS to remove unbound bacteria and the first lysis to determine the intra- and extracellular number of bacteria was done using 0.1 % saponin and frequent pipetting. Subsequently, cells were incubated with RPMI 1640 (+10 % FCS, +1 % glutamine) containing 50 µg/ml Gentamicin for 1 h at 37 °C and 5 % CO₂ to kill extracellular bacteria. Cells were again washed with PBS and incubated again for different time intervals. After those time points, cells were as before lysed with 0.1 % saponin and frequent pipetting. 10 µl of serial dilutions from 1:200 to 1:20 000 of the lysates were plated on Columbia or CYE agar plates, respectively, and incubated for 3 days at 37 °C and 5 % CO₂. Bacterial colonies were counted afterwards and the number of intracellular bacteria was calculated as CFU/ml.

5.3 Cell biology

5.3.1 Cultivation of tissue and cells

The cultivation of human lung tissue and cells was done under sterile conditions using a clean bench. Tissue and cells were cultured in a CO₂ gassed incubator with 5 % CO₂ at 37 °C and a relative humidity of 95 %. The workplaces are declared as security level S2 where all reagents and instruments are autoclaved, heat sterilized or sterile-filtered.

All materials which came in contact with human material or transfected material were cleaned with Descosept (Dr. Schumacher GmbH).

Special security arrangements for the work with lentiviral particles were taken which are described in detail in section 5.4.5.1.

For infection of cells, cells were seeded in a cell culture microplate. The cell concentrations were accorded to the used format of the well plate as shown in Table 5-11.

Table 5-11: Cell concentration/well in different cell culture plates

well plate format	cells/well
6-well plate	1×10^6
24-well plate	2.5×10^5
96-well plate	1×10^5

5.3.1.1 Human lung tissue

Fresh human lung explants were obtained from patients undergoing lung resection at local thoracic surgery clinics. Written informed consent was obtained from all patients and the study was approved by the ethics committee at the Charité – Universitätsmedizin Berlin clinic (protocol number EA2/050/08 and EA2/023/07).

The tumor-free lung explants were directly transported from surgery in sterile RPMI 1640 on ice into the laboratories and processed. Thereby the lung explants were cut into slices and stamped into small cylinders (~8×8 mm) about 100 mg. The lung pieces were incubated in RPMI 1640 (+10 % FCS) medium for 24 h before infection to remove residual amounts of antibiotics and for tissue soothing.

The preparation was done by Dr. Diana Fatykhova and Dr. Katja Zscheppang.

5.3.1.2 Primary human alveolar macrophages

Primary human alveolar macrophages were isolated from human lung tissue, which was obtained from patients undergoing lung resection as described above. Instead of cutting the lung tissue in cylinders, it was directly incubated in RPMI 1640 (+10 % FCS) medium for 24 h. Subsequently, it was filled with Hank's Balanced Salt Solution (HBSS) using a syringe (10ml) with a cannula ($\varnothing=0.9$ mm). Deflating the tissue by massaging with pressure opened the alveoli and released the cells. The tissue was filled with HBSS and deflated repeatedly but at different a position to make sure that no air was injected. Repetitions continued until the rinsing solution stayed clear. The cell solution was then centrifuged at 300 g and 12 °C for 12 min and the cell pellet was resuspended in 1 ml of RPMI 1640 for cell counting (see section 5.3.2.2). After seeding the cells in 24- or 6-well plates, respectively, they were incubated overnight. Afterwards, several washing steps with HBSS were performed to remove erythrocytes. The cells were incubated for at least another three days with RPMI 1640 supplemented with 2 % FCS and 1 % glutamine, at that getting fresh medium every 24 h.

The isolation was also done by Dr. Katja Zscheppang.

5.3.1.3 Monocytic cell line THP-1

The human monocytic cell line THP-1 (DSMZ-number: ACC 16) was cultured as monocytes (suspension cells) in RPMI 1640, supplemented with 10 % FCS, 1 % glutamine and 100 µg/ml Penicillin / Streptomycin in a T₁₇₅ cell culture flask. Splitting was done every two to three days by exchanging 2/3 of the cell suspension with fresh pre-warmed medium.

Once thawed, cells were kept in culture for no longer than three months.

For examination of the role of c-myc, THP-1 monocytes were differentiated to macrophage-similar cells using PMA. Therefore, cells were centrifuged at 300 g for 7 min, washed once with PBS and the pellet was resuspended in 1 ml RPMI 1640 blank medium for counting. Cells were seeded into well plates in RPMI 1640 with PMA (100ng/ml) and incubated for 16-20 h. Afterwards a medium change was done and cells received fresh, pre-warmed RPMI 1640 supplemented with 10 % FCS and 1 % glutamine. Cells were incubated for one more day before infection.

5.3.1.4 Human embryonic kidney cell line 293T (HEK293T)

The cell line HEK293T is a derivate of the human embryonic kidney cell line HEK293 (DSMZ-number: ACC 305; designation 293) with a better transfection efficiency [87].

The cell line was obtained from the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures.

The cultivation of HEK293T cells was done in DMEM, supplemented with 10 % FCS, 1 % glutamine and 100 µg/ml Penicillin / Streptomycin in T₇₅ cell culture flasks until a confluent monolayer was grown out. Subsequently, the cells were split 1:5 or 1:10. Therefore, cells were washed with PBS and afterwards incubated with trypsin for 5 min. By adding 10 ml fresh, pre-warmed medium the trypsinization was stopped. After centrifugation at 300 g for 5 min, cells were resuspended in culture medium and transferred into a new cell culture flask.

For production of viral particles, cells were trypsinized as described and resuspended in a concentration of 5×10^5 cells/ml and seeded in 10 ml-cell culture plates with a concentration of 1×10^7 cells/plate. On the following day, the confluence should be ~80 % for transfection.

For experimental work, cells were used up to passage 20.

5.3.1.5 Cryoconservation

After removal of suspension of THP-1 monocytes or trypsinization of HEK293T-cells, cells were centrifuged at 300 g for 5 to 7 min, resuspended in FCS, supplemented with 10 % DMSO at a concentration of 1×10^7 cells/ml and aliquoted in 1 ml cryo tubes. To ensure a gentle freezing, the cryotubes were put into an isopropanol filled freezing container with a cooling rate of 1 °C per hour. For short term storage, cells were kept at -80 °C and transferred after 1 day from there into a liquid nitrogen tank for long term storage.

5.3.1.6 Thawing of cells

The cryotubes were removed from the liquid nitrogen and placed in a foam holder. 10 ml of pre-warmed medium were ready in a 15 ml-tube. For a gentle thawing of cells to prevent cell stress, 0.5 to 1 ml of the pre-warmed medium were added to the cryogenic tube and withdrawn, the suspension being transferred into the 15 ml-tube. This was repeated until the cells were completely removed from the cryotube. The cells were then incubated overnight in a T₂₅ cell culture flask. On the following day, the optionally trypsinized cell suspension was transferred into a 15 ml tube and centrifuged for 5 min at room temperature at 300 g. The cell pellet was resuspended in fresh cell culture medium and transferred again in a T₂₅ cell culture flask, prepared with culture medium. From there the cells were transferred in bigger flasks when an adequate confluence was reached.

5.3.1.7 Cell count

To determine the cell number a Neubauer chamber was used. Therefore, 10 µl of the cell suspension were given between the cover glass and the chamber. The counting of the cells was done under the microscope, where the cells in all 4 big squares were counted. To obtain the cell number per ml suspension the following formula was used:

$$\text{cells/ml} = \frac{\text{cell count of the 4 corner great-squares}}{4} \times \text{dilution factor} \times 10^4$$

5.3.2 Infection of tissue and cells

5.3.2.1 Infection of human lung tissue

Before infection of the human lung tissue, the lung cylinders were carefully transferred in a new 6-well plate with fresh, pre-warmed RPMI 1640, supplemented with 10 % FCS. To infect the tissue, the bacterial solution (1×10^6 CFU/ml) was slowly injected into the lung tissue, assuring a thorough stimulation. For control, tissue was injected with medium. After 24 h the lung samples were processed for protein extraction.

Actual bacterial load was determined by plating 50 µl bacterial solution from serial dilutions of the applied infection dose on respective agar plates and incubated for 24 h or 72 h, respectively. Afterwards, CFU were counted and the infection dose was calculated in CFU/ml.

The infection of the human lung tissue with *S. pneumoniae* D39 or *L. pneumophila* corby, respectively, as well as the protein extraction (see also section 5.4.1.1) was done by Dr. Diana Fatykhova.

5.3.2.2 Infection of cells

Primary human alveolar macrophages and THP1 cells were infected in RPMI 1640, supplemented with 2 % or 10 % FCS and 1 % glutamine. According to the experiment, time points were ranging from 1 to 48 hours.

If a c-myc-inhibitor (10058-F4, Sigma-Aldrich) was used, it was added to the cells 2 h prior infection at concentrations of 25-100 µM.

To infect the cells, the medium was exchanged with the bacterial solution (1×10^6 CFU/ml or 1×10^8 CFU/ml). For control, medium was added again to the cells. After certain time points, cells were lysed for CFU count, RNA isolation or protein isolation. For determination of cytokine and chemokine release, the supernatant was collected after 16 h and frozen at -20 °C.

Actual bacterial load was determined by plating 50 µl bacterial solution from serial dilutions of the applied infection dose on respective agar plates and incubation for 24 h or 72 h, respectively. Afterwards, CFU were counted and the real infection dose was calculated in CFU/ml.

5.3.2.3 Stimulation with TLR-ligands

To test, whether the induction of c-myc is a response to TLR-activation, TLRs were directly addressed by stimulation THP-1 cells with TLR-Ligands. Therefore MALP-2 (recombinant) was used to address TLR2, LPS from *Salmonella Minnesota* R595 was used as a ligand for TLR4 and Flagellin from *Salmonella typhimurium* to address TLR5. The concentrations used for each ligand are shown in Table 5-12.

Table 5-12: Used concentrations for TLR-ligands.

TLR-ligand	Concentrations
MALP-2	100 ng/ml
LPS	5 µg/ml
Flagellin	10 ng/ml

When stimulating the cells with TLR-ligands, ligands were prepared in respective concentrations in RPMI 1640 (+10 % FCS, +1 % glutamine) and added to the cells by exchanging the medium. A control by just adding medium was carried along.

After 6 h of incubation the cells were processed for protein isolation.

5.3.3 Cytotoxicity assays

5.3.3.1 Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) is a cytosolic enzyme that is released into the supernatant upon cell death and thus can be used as an indicator for cellular toxicity¹⁶¹. The LDH assay (Pierce), was used to examine a toxic effect of the used inhibitor. As a positive control, 1 % Triton X-100 was added to uninfected cells and incubated for 5 min, to obtain the maximum LDH release. The supernatant of uninfected cells was used to determine the spontaneous LDH release which functioned as the negative control. The assay was performed after manufacturer's instructions. The cell death was calculated after the following formula:

$$\text{cytotoxicity [\%]} = \frac{(\text{OD}_{\text{treated}} - \text{OD}_{\text{control}})}{(\text{OD}_{\text{maximum}} - \text{OD}_{\text{control}})} \times 100$$

5.3.3.2 SYTOX™ staining

SYTOX™ Red Dead Cell Stain was used as another method to quantify cell death. The nucleic acid dye with high affinity easily enters the cell if the plasma membrane is affected. The nucleic acids of dead cells will be stained red after a short incubation time¹⁶². The detection of red stained cells was done with the FilterMax F5 Multi-Mode Microplate Reader at 37 °C. Therefore, the treatment of cells was done in clear flat-bottom 96-well plates, where just in every second well cells were seeded. After several time points, ranging from 0 h to 48 h, SYTOX™ Red Dead Cell Stain was added with a dilution factor of 10⁷ from the stock concentration of 5 µM. The detection of red stained cells was done 10 min after addition of the stain over a time of 5 min to generate a kinetic curve, exciting at 640 nm excitation and monitoring emission at 658 nm. As described above, 1 % Triton X-100 was used as a positive control for maximum cell death and medium treated cells as a negative control.

The cell death was calculated in per cent using the mean value:

$$\text{cell death [\%]} = \frac{\text{mean (Emission}_{\text{treated}}) - \text{mean (Emission}_{\text{control}})}{\text{mean (Emission}_{\text{maximum}}) - \text{mean (Emission}_{\text{control}})} \times 100$$

5.3.3.3 LIVE/DEAD® Viability/Cytotoxicity Kit

To visualize the potential cytotoxic effect of the used c-myc inhibitor the LIVE/DEAD® Viability/Cytotoxicity Kit was used for fluorescent microscopy. The kit contains two dyes to determine cell viability depending on the integrity of the plasma membrane and the activity of the enzyme esterase. The green fluorescent Calcein-AM shows intracellular activity of the esterase, which is typical for living cells. The red fluorescent Ethidium-homodimer-1 enters cells with an affected plasma membrane, suggesting cell death¹⁶³. To test the effect of the c-myc-inhibitor, cells were seeded in 8-well µ-Slides and treated with different concentrations of the inhibitor for 24 h and 48 h. Afterwards, the staining was performed according to the manufacturer's protocol and microscopic images were generated.

5.4 Molecular biological methods

5.4.1 Immunoblotting

5.4.1.1 Extraction and determination of total protein

For Western blot purposes, human lung tissue was lysed after infection with *S. pneumoniae* D39, *L. pneumophila* corby by adding 500 µl phosphoprotein lysis buffer to each lung tissue sample after the medium was discarded. Subsequently, the tissue was homogenized using the FastPrep-24 homogenizer on dry ice. After centrifugation at 13 000 g for 15 min at 4 °C, the supernatant was transferred into a new Eppendorf tube and stored at -20 °C. The extraction of protein from human lung tissue was done by Dr. Diana Fatykhova.

Cells were stimulated in 6-well plates with respective strains of *S. pneumoniae*, *L. pneumophila* or TLR ligands for up to 6 h. To stop the infection, the cell culture plates were put on ice. The cells were washed twice with 1 ml phosphoprotein washing buffer and lysed with 50 µl of phosphoprotein lysis buffer using a cell scraper. The lysates were transferred in a 1.5 ml Eppendorf tube and frozen at -20 °C for enhanced lysing efficiency.

The used buffers are listed in Table 5-13.

Table 5-13: Buffers for protein extraction

Buffer	Composition
phosphoprotein washing buffer	5 ml sodiumorthovanadat 98 % (200 mM) 50 ml sodium pyrophosphate (150 mM) 50 ml sodium fluoride 99 % (1 M) 395 ml A. bidest. ad
phosphoprotein lysis buffer	100 µL Tris-HCl, pH 7.4 (500 mM) 50 µl (v/v) Nonidet® P-40 (20 %) 40 µl Complete™ Protease Inhibitor Cocktail (25x) 810 µl phosphoprotein washing buffer

The total protein present in the cell lysate was quantified by a BioRad protein assay after Bradford. Therefore, the lysates were centrifuged at 13 000 g for 10 min at 4 °C to remove cell debris and the supernatant was transferred into a new 1.5 ml Eppendorf tube. Protein concentration was determined with the protein assay according to the manufacturer's protocol. 5 µl of protein lysate were added to 1 ml of 20 % assay reagent in a. bidest., mixed and OD₅₉₅ measured after 10 min of incubation at room temperature by a spectrophotometer. 1 ml of 20 % assay reagent was used for generating the baseline. Protein concentrations were calculated assuming that an OD₅₉₅ of 1 corresponds to 6.59 µg protein per µl, which was obtained by generating a BSA standard curve ranging from 0.2 to 1.5 mg/ml.

5.4.1.2 SDS-PAGE

The extracted proteins were separated by a discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently detected by Western blot. Using SDS-PAGE, proteins are separated by their size and not charge. For that, proteins were boiled for 5 min at 95 °C prior electrophoresis in a sample buffer (ratio 1:1) containing β -mercaptoethanol and SDS, to denature the proteins and apply a negative charge.

After a cooling phase on ice, 40 μ g of each protein sample was loaded into the sample wells of freshly casted 10 % SDS-gels, composed of stacking and separating gel. Electrophoresis was done at 100-120 V for 1.5-2 h. A molecular weight size marker (Amersham ECL Plex™ fluorescent Rainbow Marker, full range) was loaded in a separate lane to determine the approximate molecular mass.

Table 5-14: SDS-PAGE buffer and -solutions

Buffer / solution	Composition
sample buffer (Laemmli)	1.0 ml Tris-HCl, pH 6.8 (500 mM) 0.8 ml glycerol 1.6 ml SDS (10 % w/v) 0.4 ml bromphenolblue (1 % w/v) 0.4 ml β -Mercaptoethanol (1 % v/v) 5.0 ml A. bidest. ad.
SDS-PAGE running buffer	3g Tris-Base 14.4 g glycine 1 g SDS (10% w/v) 1 000 ml A. bidest. ad.
stacking gel (4x)	6 ml Tris-HCl, pH 6.8 500 mM) 100 μ l SDS (10 % w/v) 1.33 ml Acrylamid / Bis-Acrylamid (40 %; Ratio 19:1) 10 μ l TEMED 50 μ l ammoniumpersulfate (10 % w/v) 100 ml A. bidest. ad
separating gel, 10 % (2x)	2.50 ml Tris-HCl, pH8.8 (1.5 M) 100 μ l SDS (10 % w/v) 2.5 ml Acrylamid / Bis-Acrylamid (40 %; Ratio 19:1) 10 μ l TEMED 50 μ l ammoniumpersulfate (10 % w/v) 4.85 ml A. bidest. ad

5.4.1.3 Western blot

To visualize proteins, Western blot was performed. The specific binding of antibodies with conjugated fluorescent dyes was used for the detection.

The proteins were transferred from the gel onto a Nitrocellulose Hybond membrane in a tank blot system at 100 V for 1 h under cooling conditions.

To minimize unspecific binding of the antibodies, the membrane was blocked for 2 h at room temperature with Ody blocking buffer. Afterwards the primary anti-c-myc antibody (rabbit) was applied over night at 4 °C. The antibody was diluted 1:500 in dilution buffer (50 % Ody blocking buffer; 50 % 1x PSB +0.1 % Tween20).

After three washing steps with 1x PBS +0.1 % Tween20 the fluorophore-linked secondary anti-rabbit antibody (goat), was applied for 1h at room temperature.

Excessive present antibody was removed by washing twice with 1x PBS +0.1 % Tween20 and another wash with 1x PBS. The last washing step was carried out without a Tween20 addition, since this could cause a deflection of the signals during the subsequent scanning of the fluorescent dyes.

Blots were analyzed using an Odyssey infrared imaging system (Li-Cor). All buffers used for Western blot analyses are listed in Table 5-15.

Table 5-15: Western blot-buffer and –solutions

Western blot-buffer and –solutions	Composition
transfer buffer	3 g Trizma® base 14.4 g glycine 20 % methanol 1 000 ml A. bidest. ad
dilution buffer	50 % Ody Blocking Buffer 50 % Dulbeccos PBS 1x +0.1 % Tween20

5.4.2 Enzyme-linked Immunosorbent Assay (ELISA)

The release of Chemokines and Cytokines, namely IL-1 β , IL-6, IL-8 and TNF α , upon infection with *S. pneumoniae* D39, *L. pneumophila* corby was examined by sandwich ELISA using R&D System Kits according to manufacturer's instructions.

Infections for ELISA experiments were done in 24-well plates with 5×10^5 cells/well for 16 h. To determine the basic release, the supernatant of uninfected cells was used.

For performance of the ELISA, the samples were diluted as shown in Table 5-16:

Table 5-16: Sample dilutions for ELISA

Chemokine / Cytokine	Dilution of supernatant
IL-1 β	Uninfected control: not diluted Infected with <i>S.p.</i> D39: 1:200 Infected with <i>L.p. corby</i> : 1:100
IL-6	Uninfected control: not diluted Infected with <i>S.p.</i> D39: 1:50 Infected with <i>L.p. corby</i> : 1:50
IL-8	Uninfected control: 1:2 000 Infected with <i>S.p.</i> D39: 1:5 000 Infected with <i>L.p. corby</i> : 1:4 000
TNF α	Uninfected control: not diluted Infected with <i>S.p.</i> D39: 1:100 Infected with <i>L.p. corby</i> : 1:50

The analysis was done with the FilterMax F5 Multi-Mode Microplate Reader, measuring the optical density (OD) at 450 nm. The optical densities measured in the samples were quantified using the respective standard curve carried along and expressed as pg/ml.

The composition of used buffers and solutions is shown in Table 5-17.

Table 5-17: ELISA buffer

ELISA- buffer	Composition
Carbonat buffer, pH 9.5 (1 M)	4.2 g sodium hydrogen carbonat 1.78 g sodium carbonat 500 ml A. bidest. ad
blocking buffer	90 ml Dulbeccos PBS 1x 10 % \triangleq 10ml FCS
wash buffer	1 000 ml Dulbeccos PBS 1x 0.05 % Tween20
TMB Substrate Reagent Set	50 % hydrogen peroxide 50 % 3,3',5,5'-tetramethylbenzidine
stop solution 2N H ₂ SO ₄	27.47 ml H ₂ SO ₄ ; 95-97 % 500 ml A. bidest. ad

5.4.3 Isolation of total RNA and cDNA synthesis

After 6 h infection time in 6-well plates, the cells were lysed by adding 500 μ l Iso-RNA lysis reagent for 5 min and frozen at -20 °C. The isolation of total RNA was done using the Kit "Direct-zol™ RNA MiniPrep" (Zymo Research) after manufacturer's instructions. The RNA concentration and purity was measured photometrical at 260 nm with the NanoDrop2000.

Subsequently, to make the RNA available for real-time qPCR, it was reverse transcribed into cDNA using the high capacity reverse transcriptase (HCRT) kit (Applied Biosystems) according to manufacturer's instructions. All working steps were done on ice.

Briefly, 10 μ l of the isolated RNA were mixed with 10 μ l master mix, composed of 4.2 μ l DNase-/RNase-free water; 2 μ l reverse transcription buffer [10x], 0.8 μ l dNTP mix [100 mM]; 2 μ l RT-random primer [10x]; 1 μ l multiscribe reverse transcriptase [50 U/ μ l]. The reaction was performed in a thermocycler with the following program:

- 10 minutes at 25 °C
- 2 hours at 37 °C
- 5 seconds at 85 °C

The cDNA was diluted 1:5 with 80 μ l DNase-/RNase-free water. Not immediately used cDNA was stored at -20 °C.

5.4.4 Real-time quantitative PCR

Detection of a possible change in the expression pattern was done by real-time quantitative PCR (RT-qPCR) using human TaqMan® Gene Expression Assays (Life Technologies, Table 5-10, section 5.1.8).

For each sample, a 20 μ l RT-qPCR reaction mix was transferred into a 96-well reaction plate. This mix was composed of 10 μ l TaqMan® Gene Expression Master Mix (5x), 4 μ l ddH₂O, 1 μ l respective TaqMan® Gene Expression Assay (20x) – prepared as a RT-qPCR master mix – and 5 μ l cDNA. RT-qPCR was performed on a light cycler (ABI 7300 real-time PCR System) under the following conditions: 2 min at 50 °C, 10 min at 95 °C followed by 40 amplification cycles with 15 sec at 95 °C and 1 min at 60 °C. Automatic baseline and automatic Ct were applied and raw data extracted.

Relative quantification of the respective target genes was done with GAPDH as the endogenous control for normalization of the Ct values and the relative expression of the untreated control was set as 1. Accordingly, the change in the expression pattern was calculated as fold change of control ($\Delta\Delta$ Ct).

5.4.5 Generation of stable knockdown THP-1 cell lines

5.4.5.1 Production of lentiviral vectors

The generation of stable knockdown THP-1 cells was done by transduction of lentiviral particles. Those particles were produced as third generation particles from lentiviral vectors (LV). Lentiviral vectors were developed to achieve a stable gene transduction in separating and resting, non-dividing cells, respectively. The used vectors were derived from modified human immunodeficiency virus 1 (HIV-1) for safe use in laboratories.

Production of lentiviral vectors required the use of four plasmids:

- 2 packaging vectors – pRSV-Rev, pMDLg/pRRE
- 1 envelope vector – pMD2.G: VSV G envelope protein
- 1 transfer vector – pLKO.1

(Maps of all vectors used can be found in the Appendix)

The transfer vector pLKO.1 (Thermo Scientific) was used without any insert as a negative control and with respective shRNA inserts to achieve a knockdown in the target cell line. Furthermore, a puromycin resistance was applied to the cells by using the vector which was used for selecting positively transduced cells. The used shRNA inserts are listed in Table 5-18.

Table 5-18: Sequences of shRNA inserts

Target mRNA	Sequence	<i>E. coli</i> strain	Company
c-myc	ATTGTAGTGCTTTCTGGCTGG	DH5α	Thermo Scientific
MyD88	ACGTTCAAGAACAGAGACAGG	DH5α	Dharmacon

To purify the plasmid DNA, a commercially acquired competent *E. coli* “*E. cloni*® 5-*alpha*” liquid culture as described in section 5.2.2.3 was used. The bacterial culture was centrifuged after overnight incubation in LB medium at 6 000 g for 30 min at 4 °C. The isolation of the plasmids from the bacterial pellets was done using the QIAGEN Plasmid *Plus* Midi Kit (Qiagen) according to manufacturer's instructions. The kit is based on columns with a DNA-binding silica matrix.

The concentration and purity of the isolated plasmids were determined by photometrical measurement at the NanoDrop 2000.

5.4.5.1 Production of infectious lentiviral particles (ILP)

The production of infectious particles was done in the cell line HEK293T. By transfecting the single components (packaging, envelope and transfer vector) into the HEK293T cells, the viral particles are produced by the cells. Therefore, the cells were prepared in 10 cm cell culture plates at a concentration of 1×10^5 cells/ml as described in section 5.3.1.4. Directly before the transfection, the medium was removed and 5ml pre-warmed culture medium was added.

The transfection was done through lipofection with the transfection reagent Lipofectamine® 2000. Therefore, 45 µl Lipofectamine® 2000 were given into 1.5 ml Opti-MEM® and incubated for 5 min at room temperature. In the meantime, a master mix was prepared for the production of the viral particles, composed of the envelope and packaging plasmids in 1.5 ml Opti-MEM® as described in Table 5-19. Thereby, the concentrations are valid for transfection of HEK293T cells in one 10cm cell culture plates.

Table 5-19: Concentrations for transfection of lentiviral vectors

Plasmid	Concentration
pMD2.G	2.7 µg
pMDLg/pRRE	4.5 µg
RSV-Rev	1.8 µg

To this master mix 9 µg of the transfer vector was added with or without a shRNA insert. The master mix was given into the Opti-MEM® / Lipofectamine® 2000 mixture and incubated for another 20 min at room temperature.

Subsequently, the transfection mixture was given dropwise to the cells and incubated at 37 °C and 5 % CO₂ for 16-20 h. On the following day, a medium change was performed with 8 ml DMEM-medium, supplemented with sodium pyrophosphate. Cells were further incubated for another 24 h at 37 °C and 5 % CO₂ followed by harvesting of virus particles on three consecutive days.

For harvesting the viral particles, the medium containing the lentiviral particles, was carefully removed and transferred into 50 ml tubes. Fresh, pre-warmed medium was added carefully to the cells for further incubation. The supernatants were stored at 4 °C for up to 3 days and subsequently centrifuged at 1 500 g and 4 °C for 10 min to remove any cell debris. The viral particles were precipitated overnight at 4 °C with PEG-8000 (10 % w/v) by adding 5 % of the total volume to the supernatants, followed by centrifugation at 1 700g for 15 min. The supernatant was decanted, the pellet resuspended in the remaining liquid and transferred into 1.5ml tubes. After another centrifugation step at 20 000 g the pellet was resuspended in 1/100 PBS. If the transduction of the target cells did not follow right afterwards, the viral particles were

frozen in 100 µl aliquots at -80 °C.

The described work was done under the following special security arrangements:

- wearing a back-closing virus impermeable lab coat, protection goggles and a surgical mask
- wearing two pairs of gloves, the upper one stays under the clean bench
- all work was done under the clean bench
- the work table was covered with a liquid absorbent tissue to collect leaking liquids
- containers were removed from the clean bench only after disinfection with Descosept
- liquid waste was collected under the clean bench in a bottle container with 10% incidin
- waste like plastic ware was collected under the clean bench
- all waste as well as the protection tissue and lab coat were autoclaved right after the work was finished

5.4.5.2 Determination of the viral titer

The determination of the viral titer was done using the HIV-1 p24 ELISA Kit (BioCat) according to manufacturer's protocol. The determination was done after precipitation of the viral particles by diluting the samples 1:10 000.

The optical density (OD) was measured with the FilterMax F5 Multi-Mode Microplate Reader at 450 nm. The viral titer was calculated by using the respective standard curve carried along and expressed as IFU/ml (infectious units per ml).

5.4.5.3 Transduction of THP-1 cells with lentiviral particles

The THP-1 cells were transduced with the lentiviral particles as monocytes in 6-well plates (1×10^6 cells/well). Thereby, the infectious particles were given to the cells at a MOI of 10 (1×10^7 IFU/ml), calculated on the basis of the determined titers. To increase the transduction efficiency, a spin infection was performed at 800 g and 32 °C for 45 min.

After 24 h, the viral particles have been integrated and the medium was exchanged with fresh, pre-warmed culture medium. The cells were incubated for further 6 days, and the medium was changed every two days. On day 6 after transduction, the positively transduced cells were selected by their puromycin resistance with 5 µg/ml puromycin (Carl Roth) for three days and subsequently centrifuged at 300 g for 7 min. The resuspended cells were transferred in cell culture flasks and could be cultured for up to three months as suspension cells.

The knockdown cell line for MyD88 was kindly provided by Dr. Mara Wittig.

In the following, the cells transduced with the transfer vector without insert will be named "THP-

1_pLKO.1" and the cells transduced with the transfer vector with shRNA insert will be named "THP-1_c-myc " or "THP1_MyD88", respectively.

For the functional analysis of the role of c-myc or MyD88 in the investigated infection models, the transduced cells were stimulated with in this study presented strains of *S. pneumoniae* and *L. pneumophila*, as described in Section 5.3.2.

5.5 Statistics

The presented graphs in this work of Western blot quantification, mRNA expression, ELISA-results, phagocytosis and cytotoxicity assays were created using the software PRISM 6.0 (Graph Pad Prism®). All results shown are expressed as a representative graph or as mean \pm SD of at least three independent results.

The statistical analysis was performed with "One way ANOVA" together with the Newmann Keuls posttest. Changes were considered significantly different with a p-value $p < 0.05$, $p < 0.01$, $p < 0.001$ or $p < 0.0001$ and marked with one to four asterisks, respectively.

6 RESULTS

6.1 Induction of c-myc

6.1.1 Induction of c-myc upon *S. pneumoniae* infection

S. pneumoniae is the main cause of community acquired pneumoniae and causes severe lung infection, especially in children and elderly, often with a deadly end. The aim of this study was to examine, if the transcription factor c-myc plays a regulatory role during the immune response. To determine whether *S. pneumoniae* infection leads to any induction of c-myc protein, human lung tissue (HuLu) and macrophages infected with *S. pneumoniae* were analyzed by Western blot.

Therefore, HuLu was infected with the wildtype strain D39 of *S. pneumoniae* (1×10^6 CFU/ml) for 24 h as described in section 5.3.2.1. The obtained data show a 6-fold increase of c-myc upon infection compared to the uninfected control (Figure 6.1A) indicating an involvement of c-myc in *S. pneumoniae* caused lung infection.

Since human lung tissue consists of various cell types, it had to be examined which cell types are specifically leading to a higher c-myc content. Because macrophages are important cells of the innate immune system and hence have great impact on pneumonia outcome, expression of c-myc in human macrophages was further investigated.

Human alveolar macrophages, isolated from human lung tissue samples, were infected with *S. pneumoniae* D39 (1×10^6 CFU/ml) for 6h (Figure 6.1B). The results indicate that alveolar macrophages contribute to the higher c-myc content in human lung tissue. To examine if the capsule has an influence on the c-myc induction, the unencapsulated derivate R6x was also used for infection under the same conditions (Figure 6.1C), which led to an approximately 4-fold higher induction of c-myc. To obtain the highest induction and with that c-myc function, the unencapsulated strain was used additionally for further experiment.

Because primary macrophages from human lungs are not available to an extent that is needed for detailed analysis, the human monocytic cell line THP-1, which can be differentiated in macrophage-like cells by PMA was used. First, the THP-1 cell line was examined for similar c-myc induction upon infection with *S. pneumoniae* R6x. After 6h infection of differentiated cells with 1×10^6 CFU/ml *S. pneumoniae* R6x a comparable (approx. 6-fold) increase of c-myc compared to the uninfected control could be observed (Figure 6.1D). For all further experiments, the immortalized cell line THP-1 was used as a model cell line for human macrophages.

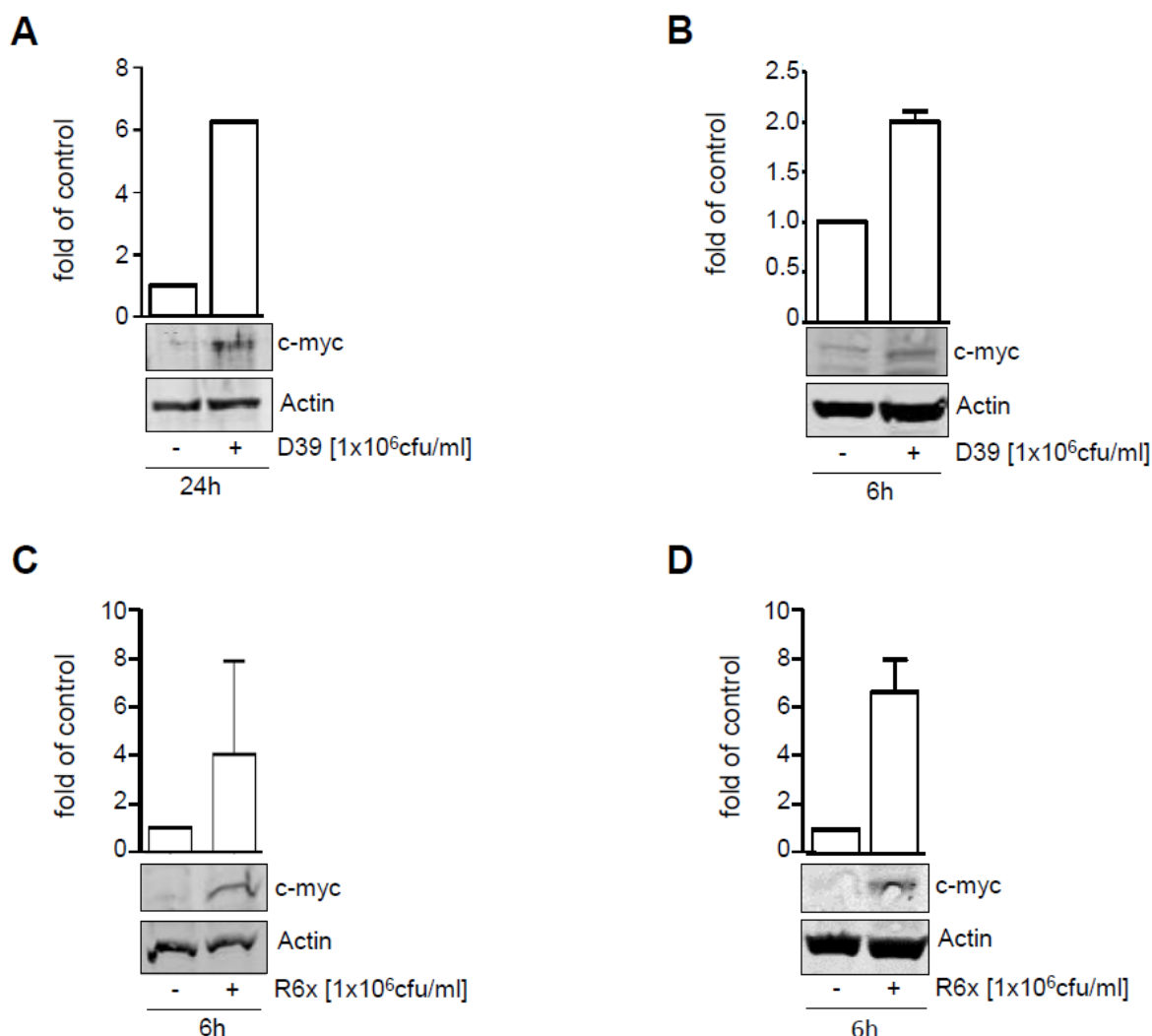


Figure 6.1: Protein biosynthesis of c-myc upon *ex vivo* infection of human lung tissue and *in vitro* infection of macrophages with *S. pneumoniae*

The c-myc expression was determined by Western blot. The constitutive expressed β -actin (Actin) served as a control for equal protein loading. Quantification was done by densitometric analysis using the Licor Odyssey software, comparing infected samples to uninfected control and normalized to β -actin.

A: c-myc protein is induced in HuLu upon infection with *S. pneumoniae* D39 after 24 h. Infection of human lung tissue was done *ex vivo* with 1×10^6 CFU/ml D39. The shown result (Western blot and corresponding quantification) represents the only one experiment of this kind which could be conducted. **B and C:** Human alveolar macrophages were infected *in vitro* in RPMI 1640 +2 % FCS for 6 h with 1×10^6 CFU/ml *S. pneumoniae* wild type strain D39 (B) or *S. pneumoniae* unencapsulated derivate R6x (C). **D:** THP-1 cells were differentiated with PMA and infected with 1×10^6 CFU/ml *S. pneumoniae* R6x. **B-D:** Representative Western blots of at least three independent experiments are shown. Densitometric analysis of Western blots summarize at least three independent experiments. Data are shown as mean value \pm SD.

6.1.2 Induction of c-myc upon *L. pneumophila* infection

The Legionnaire's disease counts between 15 000 to 30 000 cases yearly in Germany. The significantly more often isolated pathogens belong to the serogroup I of *Legionella pneumophila*, which is in total the cause of 90 % of the cases¹⁶⁴.

Part of the aim of this study – examining the regulatory role of the transcription factor c-myc during the immune response – was to compare two different pathogens. In contrast to *S. pneumoniae*, which is an extracellular acting pathogen, *L. pneumophila* acts intracellularly, living and replicating inside the macrophages. To determine whether *L. pneumophila* infection leads to an induction of c-myc protein, as shown for *S. pneumoniae*, human lung tissue (HuLu) and macrophages were infected with *L. pneumophila* corby, which belongs to the serogroup I, and the protein synthesis was analyzed by Western blot.

As it was described in section 5.3.2.1, HuLu was infected with the wildtype strain corby of *L. pneumophila* with an infectious dose of 1×10^7 CFU/ml for 24 h, determining the protein biosynthesis. The obtained data show a 1.5-fold increase of c-myc upon infection compared to the uninfected control (Figure 6.2A).

Because macrophages are the target cells of *L. pneumophila*, expression of c-myc in human macrophages was further investigated. Therefore, human alveolar macrophages, isolated from human lung tissue samples, were infected with *L. pneumophila* corby (1×10^6 CFU/ml) for 6 h. Similar to *S. pneumoniae* infection, alveolar macrophages seem to contribute to a higher c-myc content in human lung tissue caused by *L. pneumophila* (Figure 6.2B).

Also for *L. pneumophila* infection, the human monocytic cell line THP-1 was examined for similar c-myc induction upon infection with *L. pneumophila* corby. After 6 h infection of differentiated cells with 1×10^6 CFU/ml *L. pneumophila* corby, an induction of c-myc twice as high than in alveolar macrophages could be observed (Figure 6.2C).

To obtain the highest induction, the immortalized cell line THP-1 was used for further experiments as a model cell line for human macrophages.

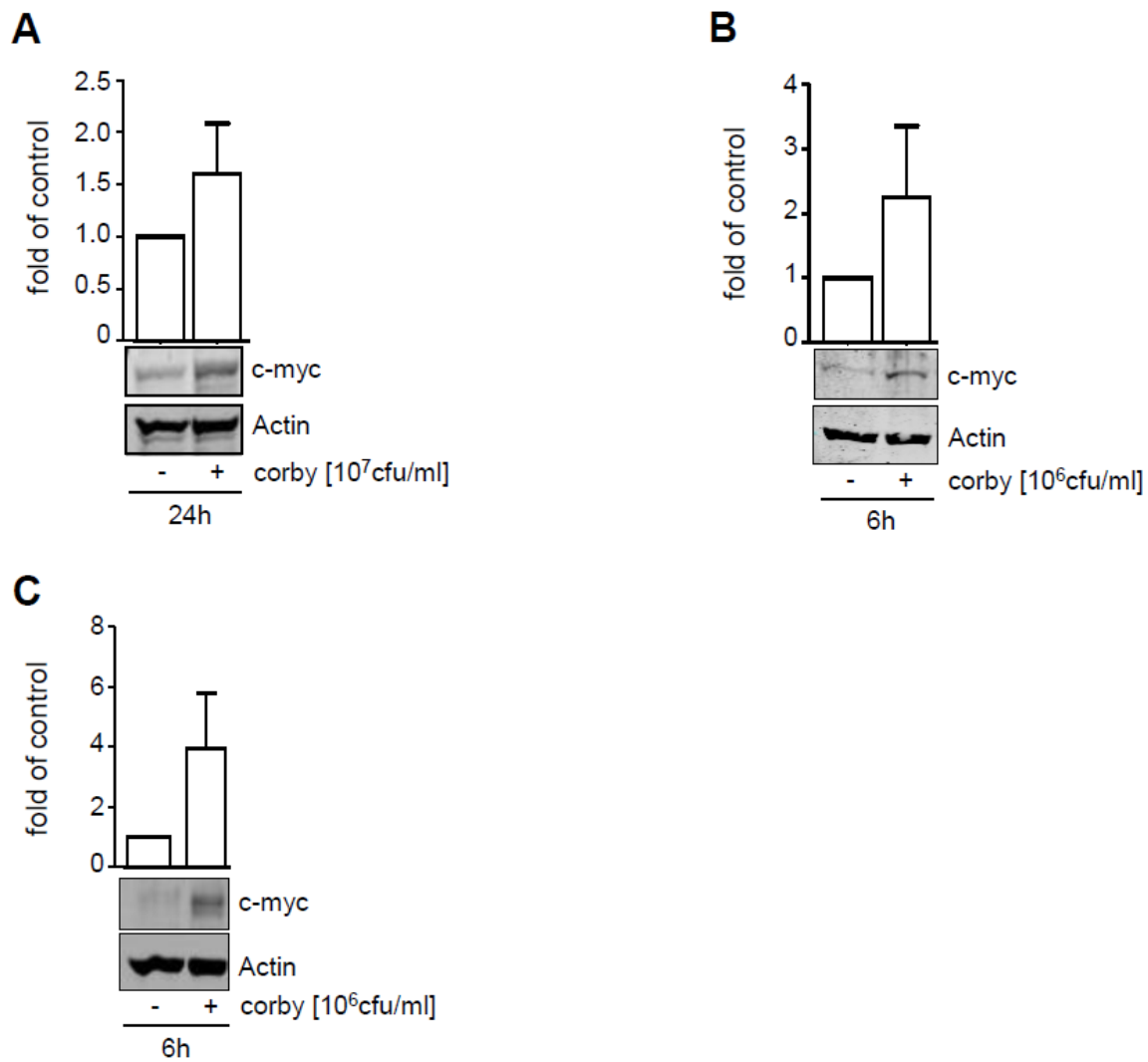


Figure 6.2: Protein biosynthesis of c-myc upon ex vivo infection of human lung tissue and in vitro infection of macrophages with *L. pneumophila*

The c-myc expression was determined by Western blot. As a control for equal protein loading served the constitutive expressed β -actin (Actin). Quantification was done by densitometric analysis using the Licor Odyssey software, comparing infected samples to uninfected control and normalized to β -actin. Representative Western blots of at least three independent experiments are shown. Densitometric analysis of Western blots summarize at least three independent experiments. Data are shown as mean value \pm SD.

A: c-myc protein is induced in HuLu upon infection with 1×10^7 CFU/ml *L. pneumophila* corby after 24 h. **B:** Human alveolar macrophages were infected in vitro in RPMI 1640 +2 % FCS for 6 h with 1×10^6 CFU/ml *L. pneumophila* wild type strain corby. **C:** THP-1 cells were differentiated with PMA and infected for 6 h with 1×10^6 CFU/ml *L. pneumophila* corby.

6.1.3 MyD88 is required for c-myc induction in THP-1 cells

Yim et al. suggested a Toll-like receptor (TLR)-mediated induction of c-myc in *Mycobacteria* infection¹⁵⁶. Hence, the underlying signaling pathway of the c-myc induction in macrophages and its possible dependency of TLR-activation was examined. Instead of testing different TLRs separately, the common adaptor molecule MyD88 was addressed to identify any TLR-dependency.

6.1.3.1 Verification of knockdown of MyD88 in THP-1 cells

Lentiviral shRNA-mediated knockdown of MyD88 was regularly verified by RT-qPCR in *S. pneumoniae* D39 and R6x (Figure 6.3A) or *L. pneumophila* corby (Figure 6.3B) infected cells. Therefore, the control (THP-1_pLKO.1) and knockdown (THP-1_MyD88) cells were infected with the respective bacterium at indicated concentrations for 6 h. Isolated RNA was reverse transcribed into cDNA and analyzed by RT-qPCR. Figure 6.3 verifies the reduction of MyD88 mRNA in the knockdown cells upon infection.

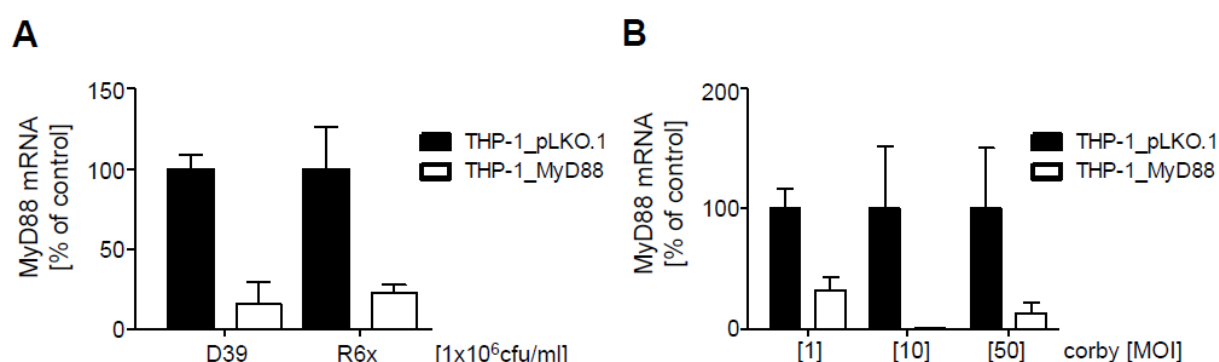


Figure 6.3: Verification of MyD88 knockdown upon infection with *S. pneumoniae* or *L. pneumophila*.

A and B: THP-1 cells were transduced using lentiviral particles with shRNA inserts specific for MyD88 (THP-1_MyD88) or with the backbone vector pLKO.1 (THP-1_pLKO.1) as control. Subsequent puromycin selection, cells were infected after PMA-differentiation for 6 h with 1×10^6 CFU/ml *S. pneumoniae* D39 or R6x (A) or increasing concentrations, ranging from 1×10^6 to 5×10^7 CFU/ml of *L. pneumophila* corby (B). Expression levels of MyD88 were determined by RT-qPCR as technical duplicates. All data sets were normalized to GAPDH and calculated as fold of control relative to the control cells THP-1_pLKO.1. Data are shown as mean value \pm SD and are representative for at least three independent experiments.

6.1.3.2 Knockdown of MyD88 leads to reduced c-myc induction

To examine the dependency of MyD88 for the c-myc induction, pLKO.1 control (THP-1_pLKO.1) and MyD88 knockdown (THP-1_MyD88) THP-1 cells were infected for 6 h with indicated infection doses of *S. pneumoniae* or *L. pneumophila*, respectively. The c-myc protein amount was determined afterwards by Western blot. The obtained results indicate that the *S. pneumoniae*- and *L. pneumophila*-dependent c-myc induction observed in THP-1 cells is dependent on MyD88 during *S. pneumoniae* (Figure 6.4A) and *L. pneumophila* (Figure 6.4B) infection.

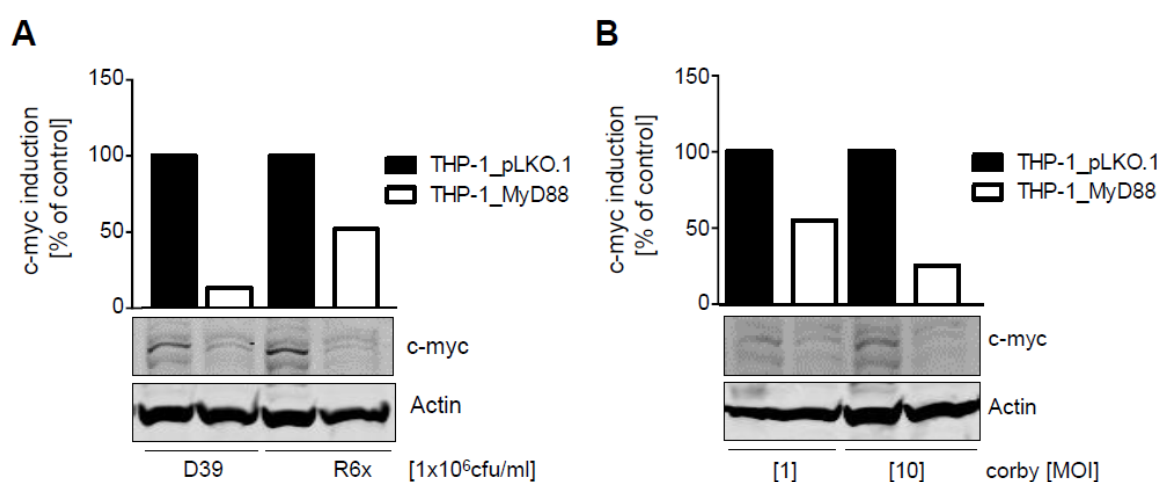


Figure 6.4: C-myc induction upon infection is dependent on the TLR-adaptor molecule MyD88.

A and B: c-myc protein induction is decreased upon bacterial infection after knockdown of MyD88. Infection of THP-1_pLKO.1 and THP-1_MyD88 cells was done *in vitro* after PMA-differentiation with 1×10^6 CFU/ml D39 and R6x for 6 h (A) or with increasing concentrations of *L. pneumophila* corby (B). The c-myc expression was determined by Western blot. The constitutive expressed β -actin (Actin) served as a control for equal protein loading. Quantification was done by densitometric analysis using the Licor Odyssey software, normalizing data to β -actin. The c-myc biosynthesis of MyD88 knockdown THP-1 cells were calculated as % of control relative to the THP-1_pLKO.1 control cells. Blots are representative for at least three independent experiments.

6.1.4 Induction of c-myc seems to be dependent on TLR2

After the dependency of MyD88 for c-myc induction was observed, it was next addressed, which TLR is involved in c-myc induction. Therefore, differentiated THP-1 cells were stimulated with the different TLR-ligands MALP-2 (TLR2), LPS (TLR4) and Flagellin (TLR5) at indicated concentrations for 6 h. The results suggest a role of TLR2, but neither TLR4 (Figure 6.5A) nor TLR5 (Figure 6.5B) seem to be involved.

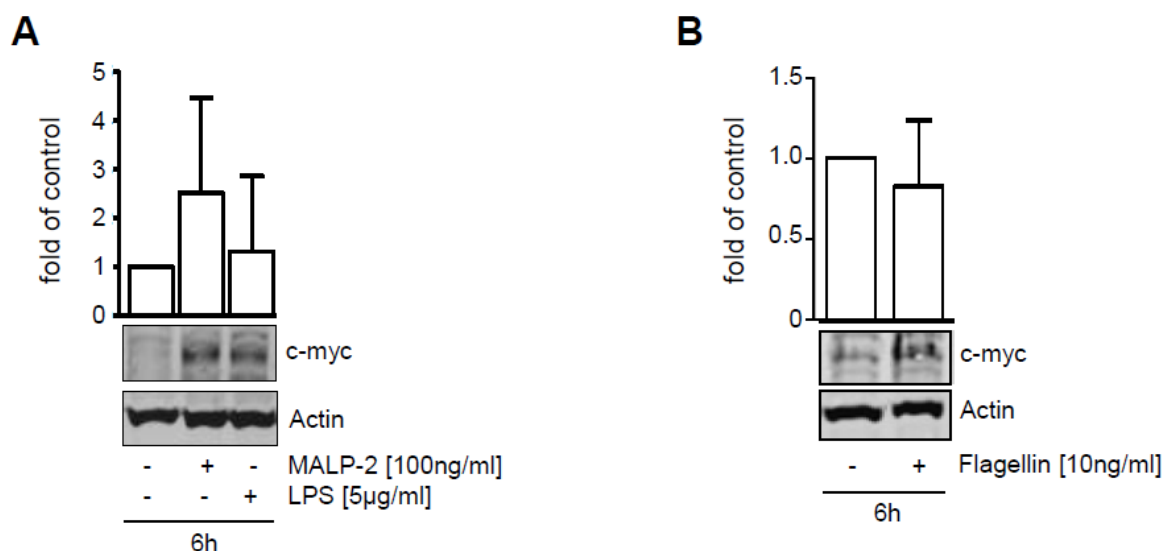


Figure 6.5: C-myc induction in THP-1 cells seems to be dependent on the TLR2.

The c-myc expression was determined by Western blot. The constitutive expressed β -actin (Actin) served as a control for equal protein loading. Representative Western blots of at least three independent experiments are shown. Quantification was done by densitometric analysis using the Licor Odyssey software, comparing infected samples to uninfected control and normalized to β -actin. Densitometric analysis of Western blots summarize at least three independent experiments. Data are shown as mean value \pm SD.

A: THP-1 cells were differentiated with PMA and stimulated for 6 h with 100 ng/ml MALP-2 and 5 μ g/ml LPS, respectively. **B:** Differentiated THP-1 cells were stimulated for 6 h with 10 ng/ml Flagellin.

6.2 Inhibition of c-myc effects the immune response of macrophages

After a c-myc induction was observed upon infection with *S. pneumoniae* as well as with *L. pneumophila*, it was necessary to examine the function of c-myc during such infections. Therefore, c-myc was inhibited and differences to an accompanied control were analyzed.

Inhibition was done using the chemical inhibitor 10058-F4 (Sigma-Aldrich), which blocks the binding of c-myc to its target genes by inhibiting the c-myc/MAX interaction¹⁶⁵. All following results indicate the consequence if c-myc cannot act as a transcription factor.

At first, a possible toxic effect of the inhibitor was examined by three different assays – LDH assay, SYTOX™ staining and LIVE/DEAD® viability/cytotoxicity assay. The LDH release was measured after 16h incubation of differentiated THP-1 cells with the inhibitor at increasing concentrations, ranging from 25 μ M to 100 μ M. A positive and negative control was carried along, to which inhibitor treated cells were compared (Figure 6.6A). The SYTOX™ staining was done after different time points up to 48h and by generating a kinetic curve to ensure best development of the stain. Afterwards, mean values of all time points were generated (Figure 6.6B). The LIVE/DEAD® viability/cytotoxicity assay was used to visualize the possible toxic

effects during 24h and 48h (Figure 6.6C). Overall, cytotoxicity was dose- and time dependent but not significantly high with the used concentrations of the inhibitor. According to these results, all doses were used for all further experiments. Nevertheless, all results gained with the highest applied concentration of 100 μ M must be considered with caution, because the LDH-Assay as well as the microscopic pictures show a higher number of dead cells (Figure 6.6A and C).

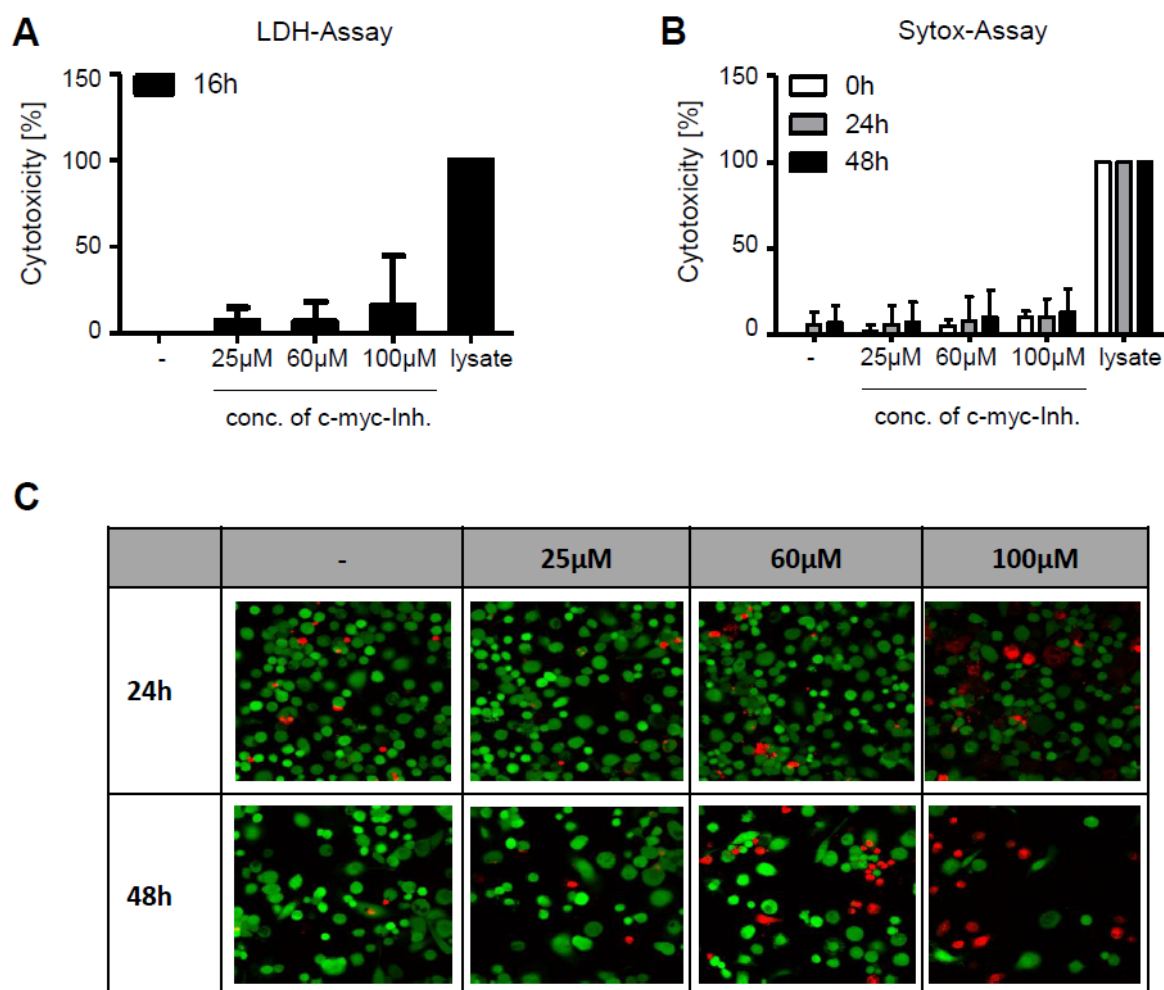


Figure 6.6: Cytotoxic effect of the c-myc inhibitor 10058-F4 on differentiated THP-1 cells.

The cytotoxic effect of the c-myc inhibitor 10058-F4 was determined using three different assays. Therefore, increasing concentrations (conc.) of the inhibitor, ranging from 25 μ M to 100 μ M were applied to differentiated THP-1 for 16h, 24h and 48h, respectively. **A and B:** Data are shown as mean \pm SD of three independent experiments. Cytotoxicity of all applied concentrations is not significant. Data analysis was done with "One way ANOVA" together with the Newmann Keuls posttest. **A:** LDH release of THP-1 cells was measured after 16h of incubation with increasing concentrations of the inhibitor and compared to an untreated control and the positive control, gained by lysing cells with 1% Triton X-100. **B:** SYTOXTM red staining was done immediately after adding the inhibitor (0h) and again after 24h and 48h. The detection followed 10min after addition of the stain over a time of 5min to generate a kinetic curve, exciting at 640nm excitation and monitoring emission at 658nm. Again, 1% Triton X-100 was used as a positive control for maximum cell death and medium treated cells as a negative control. **C:** Cells were seeded in 8-well μ Slides and treated with different concentrations of the inhibitor for 24h and 48h. Afterwards, the staining was performed according to the manufacturer's protocol and microscopic images were generated, whereas green stained cells suggest viable cells and red stained cells suggest dead cells.

6.2.1 Inhibition of c-myc leads to reduced cytokine and chemokine secretion

A variety of mediators are released by human cells during the pathogen defense of the host. At that, macrophages are a major source of a variety of cytokines involved in immune response, for example for the recruitment of other immune cells¹⁶⁶. It was to investigate whether c-myc has an impact on the cytokine release by macrophages. For analysis of the cytokines IL-1 β , IL-6, TNF α and the chemokine IL-8, THP-1 cells were infected with 1×10^6 CFU/ml *S. pneumoniae* D39 or *L. pneumophila* corby for 16 h after pretreatment with different concentrations of the c-myc inhibitor and cell free supernatants were collected and analyzed by ELISA. The IL-1 β production significantly decreased through addition of the inhibitor in *S. pneumoniae* D39 infections (Figure 6.7A), but not in *L. pneumophila* corby (Figure 6.7B) infected THP-1 cells. A significant decrease in IL-6 release during c-myc inhibition in THP-1 cells could be observed upon infection with *S. pneumoniae* D39 (Figure 6.7C). Although there is no significant reduction upon infection with *L. pneumophila* corby, the measured concentrations were considerably lower (Figure 6.7D). A dose-dependent decrease of IL-8 secretion was obtained upon infection of *S. pneumoniae* D39 (Figure 6.7E) and a significant decrease was shown upon infection with *L. pneumophila* corby (Figure 6.7F). For TNF α -production, the same dose dependent decrease as for IL-8 could be observed, which is significant in both infection cases (Figure 6.7G and H).

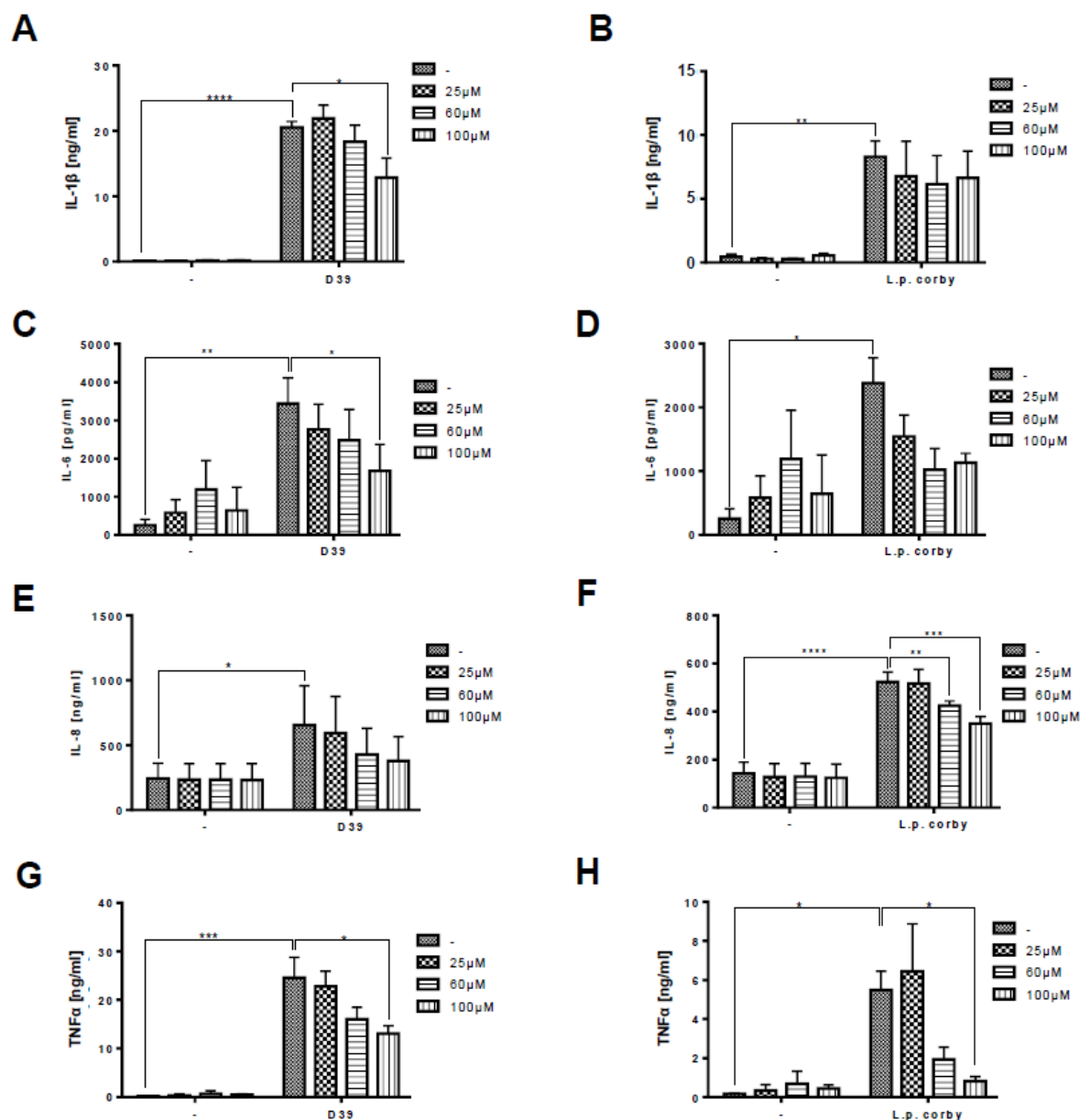


Figure 6.7: Inhibition of c-myc leads to decreased cytokine and chemokine release by THP-1 cells.

ELISA of cell free supernatant after stimulation with *S. pneumoniae* D39 and *L. pneumophila* corby [1×10^6 CFU/ml]. THP-1 cells were pretreated with the inhibitor (25 μ M; 60 μ M; 100 μ M) for 2 h and infected subsequently for 16 h. **A and B:** IL-1 β production upon infection with D39 (A) and corby (B) by pretreated THP-1 cells. **C and D:** IL-6 release after c-myc inhibition in THP-1 cells upon infection with D39 (C) and corby (D). **E and F:** IL-8 secretion upon infection with D39 (E) and corby (F) of c-myc inhibited THP-1 cells. **G and H:** TNF α -production of THP-1 cells after application of c-myc inhibitor and subsequent infection with D39 (G) and corby (H).

Data are shown as mean \pm SD of at least three independent experiments. The statistical analysis was performed with "One way ANOVA" together with the Newmann Keuls posttest. Changes were considered significant for differences with a p-value $p < 0.05$, $p < 0.01$, $p < 0.001$ or $p < 0.0001$ and marked with one to four asterisks, respectively.

6.2.2 Inhibition of c-myc leads to reduced internalization of pathogens

Macrophages serve, next to neutrophils, as major phagocytic cells in the human organism and thus also in the lower respiratory tract. Phagocytosis by macrophages is critical for the uptake and degradation of infectious agents and senescent cells. Hence, it participates in tissue remodeling, the immune response and inflammation¹²⁸. Phagocytosis includes the recognition of pathogens, subsequent internalization and the killing inside the phagolysosome^{167,168}. But depending on the pathogen, the outcome can differ. Whereas bacteria such as *Pneumococci* will be phagocytosed and killed by macrophages, infectious agents like *L. pneumophila* manipulate the vacuolar maturation to replicate inside and thus ensure their survival^{128,169}. Given that c-myc is reported to have a supporting role in phagocytosis of *Mycobacteria* and thereby suppressing mycobacterial growth^{156,167}, the impact on phagocytosis of *S. pneumoniae* and *L. pneumophila* was to investigate.

Phagocytosis ability of THP-1 cells during inhibition of c-myc was tested by a colony forming unit (CFU) assay. Therefore, THP-1 cells were preincubated as described before with the c-myc inhibitor 10058-F4 and incubated with 1×10^8 CFU/ml *S. pneumoniae* R6x (MOI 100) or 1×10^6 CFU/ml *L. pneumophila* corby (MOI 1), respectively. The unencapsulated derivate of the wild type strain of *S. pneumoniae* was chosen, because the capsule might inhibit the binding by the THP-1 cells. CFU assay was performed as described in section 5.2.3, which is schematically shown in Figure 6.8.

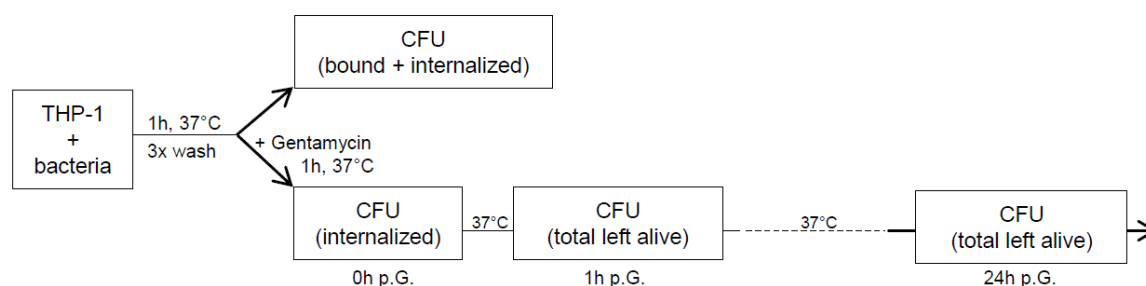


Figure 6.8: Protocol used to analyze phagocytosis ability of THP-1 cells.

THP-1 were preincubated with the c-myc inhibitor and afterwards spin infected with *S. pneumoniae* (MOI=100) or *L. pneumophila* (MOI=1), respectively. After one hour of incubation, cells were first lysed to determine the intra- and extracellular number of bacteria (bound+internalized). Subsequently, the extracellular bacteria were killed by gentamycin treatment and internalized CFU were determined. Cells were incubated again for different time intervals (p.G.), depending on the infecting pathogen (t=1 h; 6 h; 24 h). At each stage, serial dilutions were plated on Columbia or CYE agar plates, respectively, bacterial colonies were counted after adequate incubation and the number of intracellular bacteria total left alive was calculated as CFU/ml.

As shown in Figure 6.9, the phagocytosis of *S. pneumoniae* seems not to be affected by c-myc inhibition, neither the binding, initialization nor the killing (Figure 6.9A). In contrast, *L. pneumophila* is significantly less internalized dose dependent to the applied inhibitor. Although, the binding and the growth of *L. pneumophila* is not affected (Figure 6.9B).

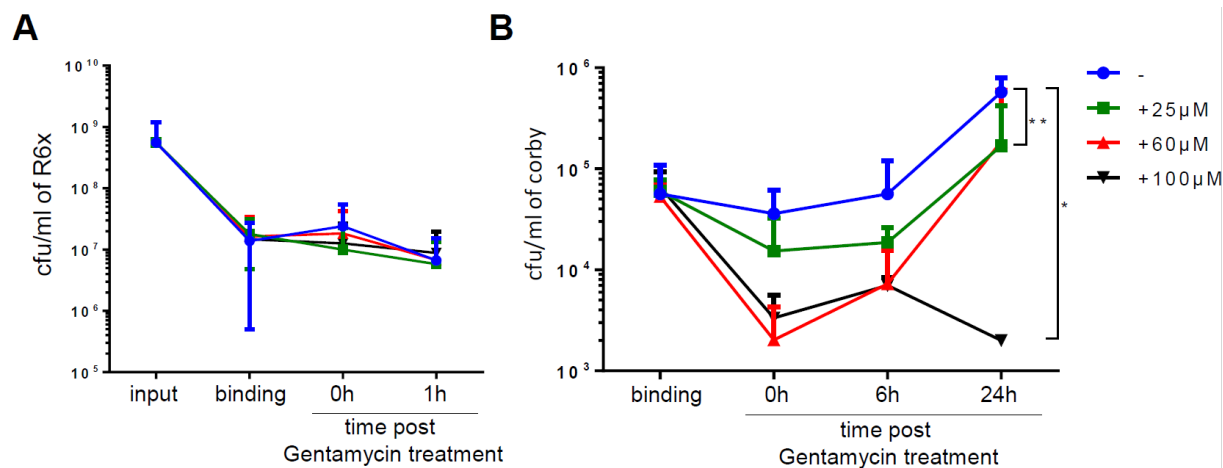


Figure 6.9: Phagocytosis ability of c-myc inhibited THP-1 cells during *S. pneumoniae* or *L. pneumophila* infections.

A and B: 1×10^5 differentiated THP-1 cells were infected with *S. pneumoniae* R6x at an MOI of 100 (A) or *L. pneumophila* corby at an MOI of 1 (B). After spin infection and incubation for 1 h followed Gentamicin (50 μ g/ml) treatment and further incubation for 1 h (A) or 6 h and 24 h (B), bacterial colonies were counted following saponin lysis at indicated time points and the number of intracellular bacteria was calculated as CFU/ml. The number of bound bacteria was calculated by subtracting intracellular bacteria after gentamycin treatment from intra- and extracellular bound bacterial number. Data are shown as mean \pm SD of five independent experiments. Statistical analysis was done with "One way ANOVA" together with the Newmann Keuls posttest. Differences were considered as significant changes with a p-value $p < 0.05$ or $p < 0.01$ and marked with one or two asterisks, respectively.

6.3 Generation of stable knockdown of c-myc in THP-1 cell lines

A chemical inhibitor bears the risk of toxic or unspecific effects. To verify the obtained data presented before, a stable knockdown THP-1 cell line was generated.

The transfer vector pLKO.1 was used without any insert as a negative control and with specific shRNA inserts against c-myc to achieve a knockdown in the target cell line.

THP-1 cells were transduced with the lentiviral particles (1×10^7 IFU/ml) as monocytes in 6-well plates (1×10^6 cells/well). Positive transduced cells were selected by puromycin.

To verify the knockdown of c-myc, the transduced cells were stimulated with *S. pneumoniae* D39 and R6x (1×10^6 CFU/ml) or *L. pneumophila* corby (1×10^6 CFU/ml and 1×10^7 CFU/ml) for 6 h and the knockdown rate of c-myc protein was analyzed by Western blot. Due to the lentiviral transduction, c-myc induction upon infection was reduced by 35-65 % (Figure 6.10). Such verifications were carried along periodically during all further experiments, examining the function of c-myc by comparing the generated knockdown cells to the control cells.

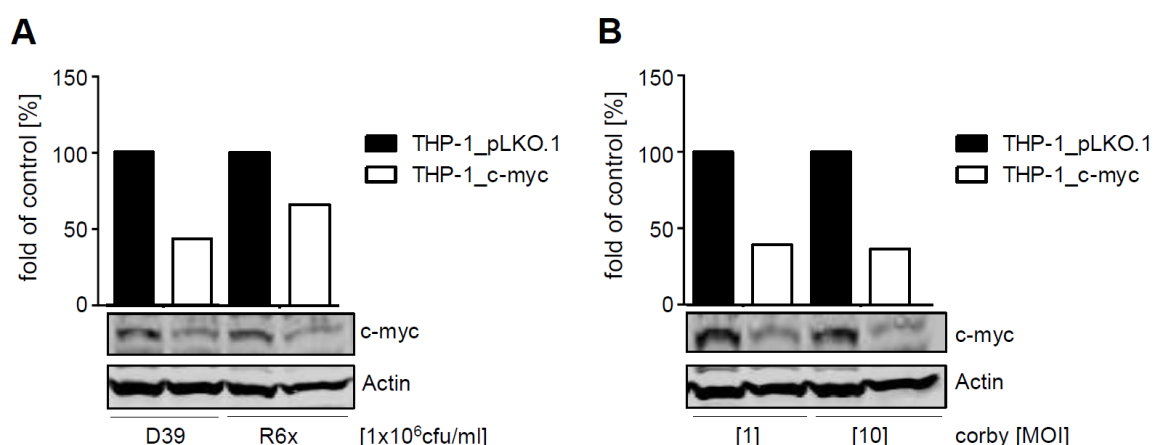


Figure 6.10: Verification of c-myc knockdown upon infection with *S. pneumoniae* or *L. pneumophila*.

A and B: c-myc protein induction is reduced due to lentiviral transduction of specific c-myc shRNA. Infection of THP-1_pLKO.1 and THP-1_c-myc cells were done *in vitro* after PMA-differentiation with 1×10^6 CFU/ml D39 and R6x for 6 h (A) or with increasing concentrations of *L. pneumophila* corby (B). The c-myc expression was determined by Western blot. As a control for equal protein loading served the constitutive expressed β -actin (Actin). Quantification was done by densitometric analysis using the Licor Odyssey software, normalizing data to β -actin. The c-myc biosynthesis of c-myc knockdown THP-1 cells were calculated as % of control relative to the THP-1_pLKO.1 control cells. Blots are representative for at least three independent experiments.

6.3.1 Knockdown of c-myc leads to reduced cytokine and chemokine secretion

The inhibition of c-myc by a chemical inhibitor led to decreased cytokine secretion of differentiated THP-1 cells upon infection with *S. pneumoniae* or *L. pneumophila*, respectively. Because low toxic effects of the inhibitor were detected at a concentration of 100 μ M and the risk of unspecific effects, the obtained results had to be confirmed with a different method. Therefore, THP-1 cells were transduced with lentiviral particles to generate stable knockdown cells, which were infected with 1×10^6 CFU/ml *S. pneumoniae* or increasing concentrations (1×10^6 CFU/ml and 1×10^7 CFU/ml) of *L. pneumophila* (MOI=1 and MOI=10) for 16 h. The cytokine secretion of THP-1_pLKO.1 and THP-1_c-myc cells was determined by ELISA. Again, the four cytokines IL-1 β , IL-6, TNF α and the chemokine IL-8 were measured. Differences in the IL-1 β secretion upon *S. pneumoniae* D39 infections could not be obtained by comparing knockdown to control THP-1 cells (Figure 6.11A). To the contrary, significant differences were determined upon infection with *L. pneumophila* corby (Figure 6.11B). A significant decrease in IL-6 release during c-myc knockdown of THP-1 cells could be observed upon infection with *S. pneumoniae* D39 (Figure 6.11C) and *L. pneumophila* corby (Figure 6.11D). Interestingly, there is no difference in the IL-8 release (Figure 6.11E and F). For TNF α -production, the same decrease as by usage of the inhibitor could be observed, which is significant in both infection cases (Figure 6.11G and H).

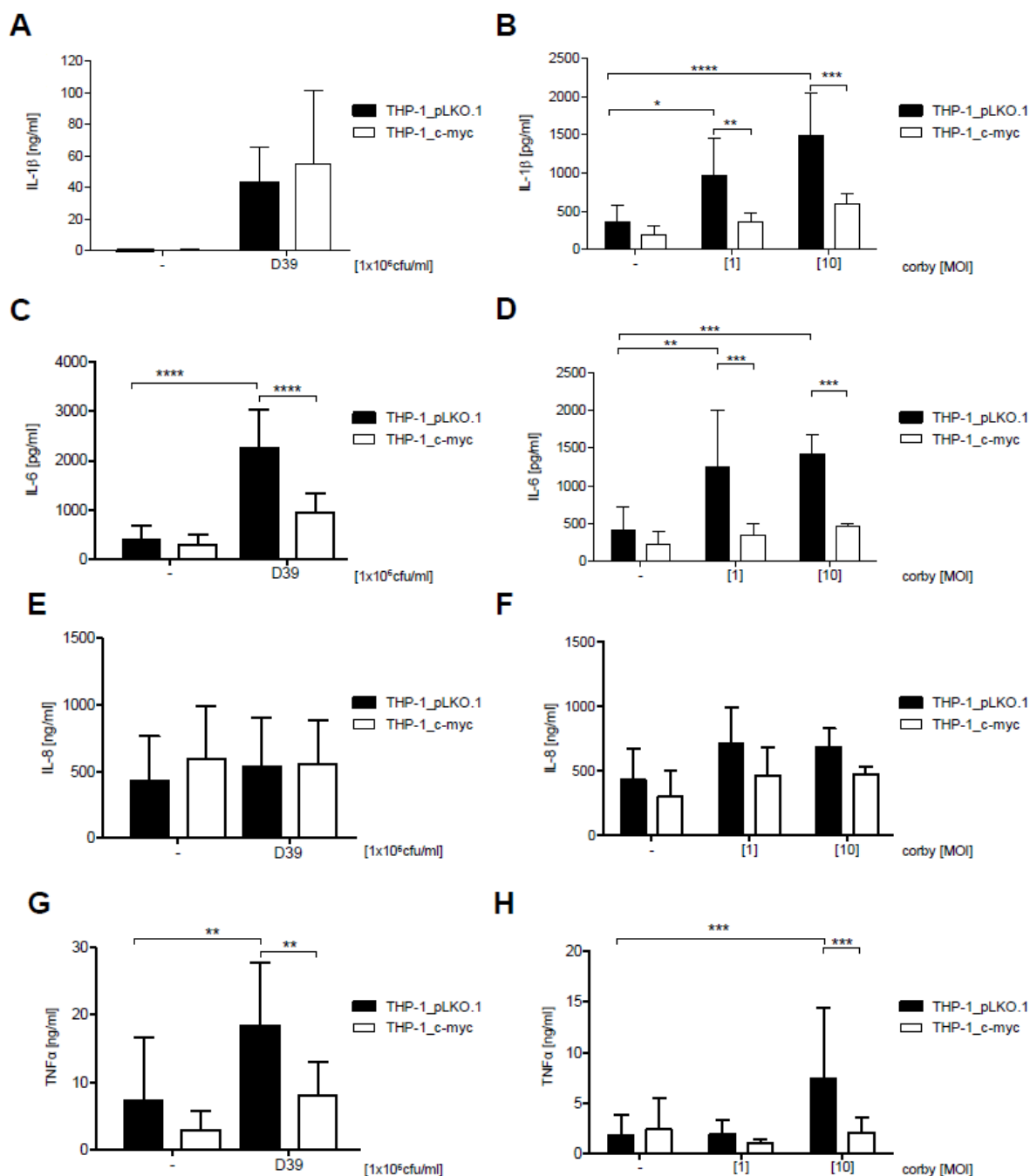


Figure 6.11: Cytokine secretion is decreased in THP-1_c-myc cells compared to THP-1_pLKO.1 control cells.

ELISA of cell free supernatant of THP-1_pLKO.1 and THP-1_c-myc cells upon infection with *S. pneumoniae* D39 and *L. pneumophila* corby with indicated concentrations. Infection time was 16 h. **A and B:** IL-1 β production upon infection with D39 (A) and corby (B) of stable transduced THP-1 cells. **C and D:** IL-6 release of lentiviral manipulated THP-1 cells upon infection with D39 (C) and corby (D). **E and F:** IL-8 secretion upon infection with D39 (E) and corby (F) of lentiviral transduced THP-1 cells. **G and H:** TNF α -production of control and knockdown THP-1 cells after infection with D39 (G) and corby (H).

Data are shown as mean \pm SD of at least three independent experiments. The statistical analysis was performed with "One way ANOVA" together with the Newmann Keuls posttest. Changes were considered as significant differences with a p-value $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$ and marked with one to four asterisks, respectively.

6.3.2 Knockdown of c-myc leads to reduced internalization of pathogens

The phagocytosis ability of macrophages is one of the first defense mechanisms of the innate immune system and leads to an increase in the immune response by follow up activation^{167, 128}. Inhibition of THP-1 cells in c-myc/MAX interaction by a chemical inhibitor (10058-F4, Sigma-Aldrich) and thus its inhibited function as a transcription factor resulted in reduced internalization when infected with *Legionella*. However, it did not affect the internalization of *Pneumococci*. Because low toxic effects of the inhibitor were detected at a concentration of 100 μ M and the risk of unspecific effects, the obtained results had to be confirmed, as it was done before with the results for cytokine secretion with a different method. Hence, the verification was done as well by usage of the c-myc knockdown cells.

Phagocytosis ability of THP-1 control and knockdown cells was tested by a colony forming unit (CFU) assay. Therefore, THP-1_pLKO.1 and THP-1_c-myc cells were incubated with *S. pneumoniae* (1×10^8 CFU/ml) or *L. pneumophila* (1×10^6 CFU/ml), respectively, for one hour to allow macrophages to bind bacteria. Afterwards, CFU assay was performed as described in section 5.2.3, which is schematically shown in Figure 6.12.

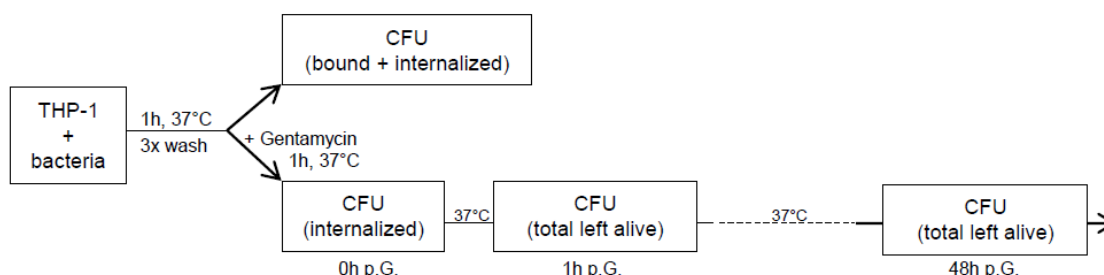


Figure 6.12: Protocol used to analyze phagocytosis ability of THP-1 cells.

THP-1_pLKO.1 and THP-1_c-myc cells were spin infected with 1×10^8 CFU/ml *S. pneumoniae* (MOI=100) or 1×10^6 CFU/ml *L. pneumophila* (MOI=1), respectively. After one hour of incubation, cells were first lysed to determine the intra- and extracellular number of bacteria (bound + internalized). Subsequently, the extracellular bacteria were killed by gentamycin treatment and internalized CFU were determined (0 h p.G.). Cells were incubated again for different time intervals, depending on the infecting pathogen (t=1 h; 24 h; 48 h p.G.). At each stage, serial dilutions were plated on Columbia or CYE agar plates, respectively, bacterial colonies were counted after adequate incubation and the number of intracellular bacteria left alive was calculated as CFU/ml.

In contrast to the results obtained using the c-myc inhibitor, the knockdown of c-myc leads also in *S. pneumoniae* infections to a considerable, though not statistically significant decrease of internalized bacteria and furthermore to a lower killing ability (Figure 6.13A) of the macrophages compared to the control cells, whereas the binding of *S. pneumoniae* to the cells was as obtained with the inhibitor not affected.

In the case of infections with *L. pneumophila*, the internalization of bacteria by c-myc knock-down THP-1 cells compared to the control cells is equally significantly reduced as by untreated cells to inhibitor treated cells at a concentration of 25 μ M. The intracellular growth of bacteria is over a time period of 48 h not affected, either (Figure 6.13B).

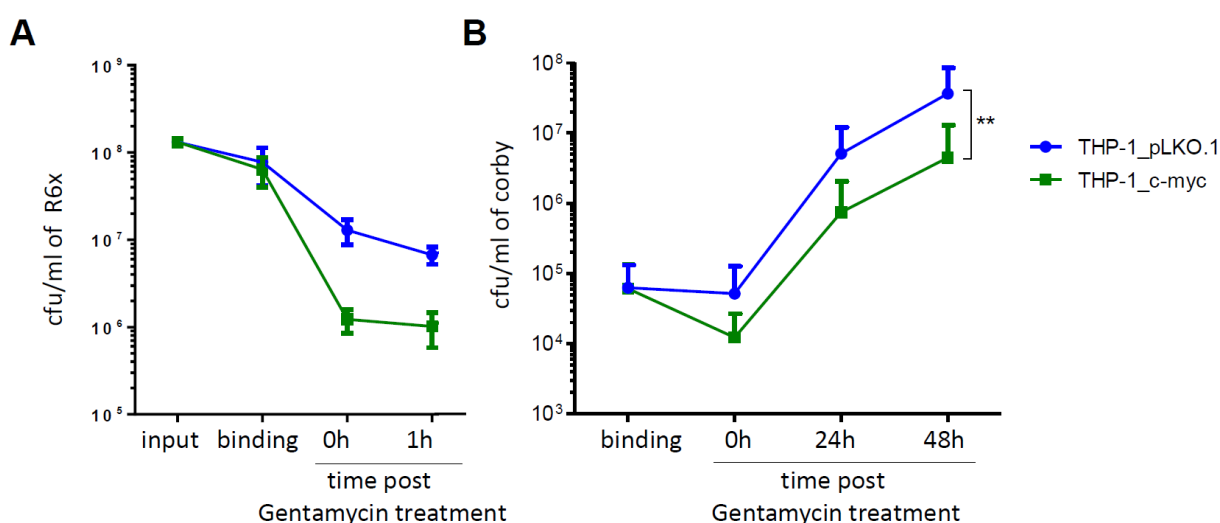


Figure 6.13: Phagocytosis ability decreased in THP-1_c-myc cells compared to THP-1_pLKO.1 control cells during *S. pneumoniae* or *L. pneumophila* infections.

A and B: 1×10^5 differentiated THP-1_pLKO.1 and THP-1_c-myc cells were infected with *S. pneumoniae* R6x at an MOI of 100 (A) or *L. pneumophila* corby at an MOI of 1 (B). After spin infections and incubation for 1 h followed Gentamicin (50 μ g/ml) treatment and further incubation for up to 48 h. Bacterial colonies were counted following saponin lysis at indicated time points and the number of intracellular bacteria was calculated after incubation as CFU/ml. The number of bound bacteria was calculated by subtracting intracellular bacteria after gentamycin treatment from intra- and extracellular bound bacterial number. Data are shown as mean \pm SD of five independent experiments. Statistical analysis was done with "One way ANOVA" together with the Newmann Keuls posttest. Changes were considered as significant differences with a p-value $p < 0.01$ and marked with two asterisks.

7 DISCUSSION

For the past 10 years, c-myc is not only seen as an oncogene after being involved in embryogenesis, but additionally as a regulator of the immune response. Its influence includes the antimicrobial activity or natural resistance of different cells to pathogens. While the stem cell factors are hardly detectable in healthy differentiated cells, studies indicate an induction of c-myc after exposure to cytokines or after infections with various viruses, bacteria or parasites^{151–157}.

This study examined the role of c-myc during the immune response, especially in human macrophages, upon infection with *S. pneumoniae* and *L. pneumophila*, respectively. Hence, the induction of c-myc was examined in *ex vivo* infected human lung tissue, primary human alveolar macrophages and subsequently in THP-1 cells. Results obtained by this study show that upon challenging human lung tissue or macrophages (primary and cell line) with both causative agents of a severe form of pneumonia, c-myc is induced. Furthermore, it could be shown that this induction is regulated via the TLR2/MyD88-dependent pathway. Moreover, the subsequent function of c-myc was observed. It could be shown via a chemical c-myc inhibitor and lentiviral-mediated knockdown experiments, that c-myc modulates the immune response of infected macrophages. It has an influence on the internalization of both pathogens, *S. pneumoniae* as well as *L. pneumophila*, but does not with respect to *L. pneumophila* affect the intracellular growth. In addition to the influence on phagocytosis, c-myc also enhances the secretion of the pro-inflammatory cytokines (Figure 7.1).

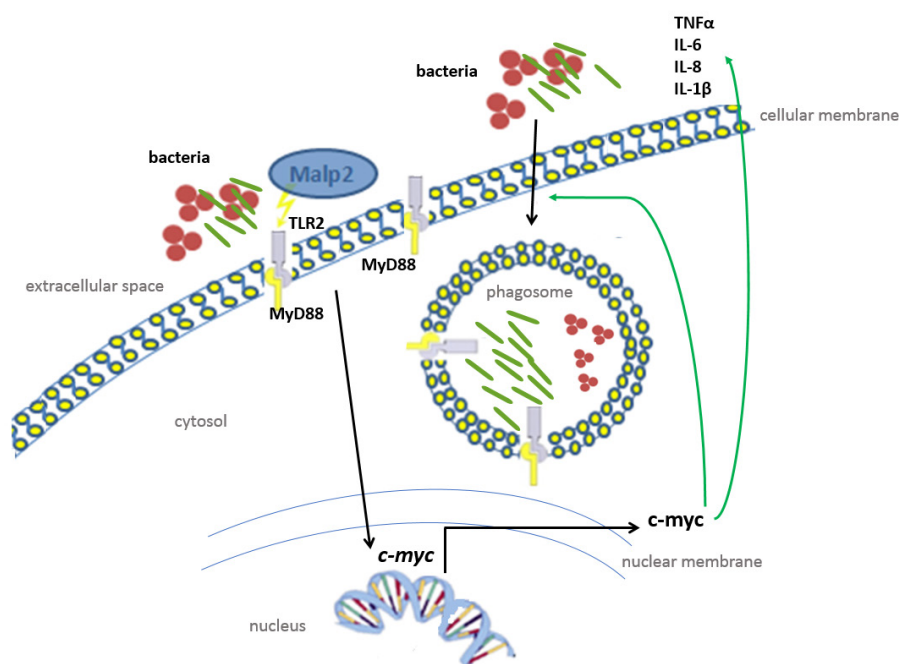


Figure 7.1: Overview of the proposed pathway leading to the induction of c-myc and the resulting increase in cytokine secretion and internalization of pathogens. For detailed description see text.

7.1 Tissue and cells as an infection model

Human pneumonia is modeled with experimental infection of mammals, most frequently mice. But mice and humans differ in many important issues relevant to lung infection, e.g. differences in structural anatomy and cellular composition of the murine respiratory tract^{170,171}. Whereas mouse lungs exhibit only 13-17 generations of respiratory bronchioles, the human respiratory tree branches over 23 generations. Additionally, mouse lungs have a relatively large airway lumen compared to humans¹⁷². Moreover, in regard to immunity, poor translation of murine model studies into the human setting have been reported, e.g. whereas neutrophils are a rich source of leukocyte-derived defensins in humans, no defensins are expressed by neutrophils in mice^{173,174}. Furthermore, mice have notable hematological differences compared to humans, such as lower peripheral blood neutrophil and higher peripheral blood and bone marrow lymphocyte percentages, as well as variable leukocyte morphologies¹⁷⁵. Another important aspect is the use of inbred mouse strains in research. In the case of studying *Legionella* infections, mice models pose the problem, that the bacterium cannot proliferate in peritoneal macrophages from many strains, such as C57BL/6, C3H/HeN, AKR, and BALB/c, but it can proliferate in peritoneal macrophages from A/J mice¹⁷⁶. Additionally, human material reflects the genetic diversity by the donor patients, whereas inbred strains do not as much¹⁷⁷. Another benefit of the human lung tissue model is the preservation of the complex, three-dimensional structure reflecting the different cell types of the human respiratory tract. In explant culture, cells are surrounded by their extracellular matrix and their natural arrangement within the tissue. Additionally, different cell types stay in contact with each other and can interact, whereas cell lines are composed of phenotypically identical cells¹⁷⁸. However, the human lung infection model also has limitations: The recruitment of immune cells from the bloodstream cannot be simulated, which narrows the research on the immune response to the initial and innate response¹⁷⁹. Furthermore, the nutrient content is restricted to the used medium. The alveoli are neither ventilated nor are capillaries perfused, which can lead to processes such as apoptosis or necrosis that may influence the inflammatory response. Other limiting factors are the availability and quality of the tissue sample. The human lung explants in this study were received from patients undergoing lung resection. Although the lung tissue itself was tumor-free, it originated most frequent from cancer patients that were by tendency elderly, prevalently exposed to cigarette smoke and / or suffering from chronic obstructive pulmonary disease (COPD). Thus, it cannot be ruled out that the presence of a tumor in the lung or an existing disease has altered tissue responses¹⁷⁸. Investigation of c-myc induction upon *S. pneumoniae* D39 infection (Figure 6.1A) could only be conducted once, because the available tissue samples of further tryouts either showed impaired response to any stimulation or were not

appropriate due to too many injuries. The obtained result can only be regarded as preliminary data, which should be repeated in future experiments.

Human primary alveolar macrophages represent, as well as human lung tissue, the genetic diversity by the donor patients and there is no need of translation from another system like the murine system. Additionally, observing functions of macrophages in the lung in particular, usage of primary alveolar macrophages has the advantage of originating from the tissue of interest, without having a mixed cell culture, interfering with them or being differentiated *in vitro*. However, modelling an infection with a mono culture always limits the effects resulting from crosstalk with other cells, recruitment of further immune cells by e.g. cyto- and chemokines as well as the activation of the adaptive immune response. Furthermore, the manipulation of primary cells is difficult, because they are more sensible to transfection reagents, and their life span in laboratory cell culture systems is often shorter than needed for such experiments.

The human cell line THP-1 used is a human monocytic cell line derived from an acute monocytic leukemia of a one-year-old boy. THP-1 cells can be differentiated into macrophage-like cells by treatment with phorbol esters (PMA). They then act in several respects as native macrophages developed from monocytes, which are more easily to manipulate in behalf of functional studies. Compared to other human myeloid cell lines such as HL-60 or U937 cells, differentiated THP-1 cells behave much more similar to monocyte-derived macrophages¹⁸⁰. Precisely because of this behavior, this cell line is particularly suitable for studying the physiological functions and mechanisms of macrophages. Furthermore, studies from Cirillo et al. and Takemura et al. evaluated THP-1 cells as a model for studying *L. pneumophila*-related questions. At this, the cell line was reported to behave similarly to human monocytes and macrophages in being invaded by *L. pneumophila* and its intracellular growth^{181,182}. Nevertheless, it should be kept in mind that the cells used are cell lines derived from acute monocytic leukemia¹⁸³. Because proto-oncogenes like c-myc have an important impact on signal transduction, they can have wide ranging effects in basic cellular functions. Nevertheless, c-myc is reported not to change upon differentiation of monocytic THP-1 cells into macrophage-like cells by PMA¹⁸⁴.

7.2 Expression pattern of c-myc upon infection

In this study, it was demonstrated, that both encapsulated and unencapsulated *S. pneumoniae* strains as well as *L. pneumophila* corby are able to induce the c-myc protein in human lung tissue and in particular in alveolar macrophages. It has been shown before, that bacterial or viral infections can lead to c-myc induction^{153,155,156,158}, but only the report by Yim et al. addressed bacterial lung infection¹⁵⁶. The induction of c-myc and its subsequent role in the immune response during pneumococcal pneumonia or Legionellosis, respectively, is reported for the first time in this study. Thereby it was observed for *S. pneumoniae* infection that the induction was significantly weaker in the wt *Pneumococci* than in the case of the unencapsulated mutant. The virulence of the *Pneumococci in vivo* is related to the capsular thickness, which can range from 200 to 400 nm. The capsule forms a protective label against the detection by Fc-receptors and the complement system and thus prevents the phagocytosis of *S. pneumoniae* by phagocytes²⁸. In addition, the capsule covers, among other structures, surface molecules that represent ligands for TLR2 and NOD2. TLR2 is considered as a participating receptor for the detection of gram-positive bacteria to which *S. pneumoniae* belongs. The underlying cell wall component lipoteichoic acid and soluble peptidoglycan serve as ligands¹⁸⁵. But also, the released Pneumolysin can lead to TLR-activation via TLR4¹¹². All in all, the TLR-activation leads to an inflammatory response, which is mediated by MyD88. There are even reports, suggesting a TLR2-independent, but MyD88-dependent mechanism¹⁸⁶. A TLR-initiated immune response is not only reported in the case of *S. pneumoniae* infection, but also for *L. pneumophila* invasion. Recognition of components of *L. pneumophila*, (including LPS, flagellum, and peptidoglycan) by the TLRs, which orchestrates the innate immune responses to *Legionella*, plays an important role in activation of monocytes and alveolar macrophages leading to an inhibition of intracellular proliferation of bacteria. In more detail, it was reported, that the *L. pneumophila* LPS is not effectively recognized by TLR4, but rather TLR2, which is interacting with the lipid A structure¹⁸⁷. Additionally, lipopeptides and lipoproteins of *L. pneumophila* are activated TLR2 as PAMPs. Next to TLR2, TLR5 is activating by the flagellum of the bacterium and TLR9 by its DNA, both important for an effective host response. However, redundancy in the signaling pathways downstream of the mentioned receptors prevents higher susceptibility to *L. pneumophila* in the case of deficiency of one TLR¹⁸⁸. MyD88 as the common TLR-adaptor molecule is suggested as a required participant in the clearance and resistance against *Legionella*. It was for example shown, that MyD88-dependent innate immune responses induced by *L. pneumophila* involve both TLR-dependent responses and IL-18R-dependent production of IFN- γ by natural killer cells. These described MyD88-dependent pathways can function independently, increasing host protection against

an intracellular pathogen^{189–191}.

Consistent with these publications, it was observed in this work, that MyD88 is required for the induction of c-myc upon infection with both bacterial pathogens. So far, the initiating PRR and the mechanisms leading to induction of c-myc have not been described in detail. However, TLR2 seemed to be involved, as it could be observed, that its ligand MALP-2 led to an induction of c-myc, whereas LPS and flagellin were not able to induce c-myc. The involvement of TLR2 needs to be confirmed in further experiments, for example by knockdown of TLR2. Furthermore, the downstream pathway of MyD88-recruitment, leading to the c-myc induction, would be intriguing to investigate.

7.3 Enhanced internalization capacity due to c-myc induction

Phagocytosis is the first step among multiple strategies possessed by macrophages to clear a bacterial infection. Phagocytosis is defined by the recognition, internalization and finally digestion of pathogens at phagolysosome. It was shown in this work that c-myc plays a positive role in phagocytosis and therefore may contribute to the host defense to *S. pneumoniae* as well as *L. pneumophila*. Using the colony forming unit assay, it was observed that pretreatment with the c-myc inhibitor, 10058-F4, could significantly reduce the amount of *L. pneumophila* internalized by macrophages, but neither its intracellular growth nor did it have any influence on *S. pneumoniae* internalization. Verification of the obtained data by lentiviral knockdown of c-myc lead to confirmation of the results regarding *L. pneumophila*. In contrast to the results gained by pretreatment of THP-1 cells with the inhibitor, usage of c-myc knockdown THP-1 cells resulted in an exceptional decrease in internalization of *S. pneumoniae*. Those differences could be, on one hand, explained by the different mode of action. The chemical inhibitor prevents transactivation of c-myc target genes by blocking dimerization of c-myc and MAX¹⁶⁵, whereas the shRNA, induced by lentiviral transduction, leads to degradation of the c-myc mRNA and therefore to the decreased content of c-myc protein. On the other hand, those differences might be explained due to the specificity of the used reagents. Chemical inhibitors have a much higher probability for binding to off-target sequences than shRNAs.

S. pneumoniae possesses several attributes which prevent recognition and subsequent phagocytosis by the host. As mentioned before, the capsule is the most important virulence factor. It masks the antigens on the cell wall surface, and thereby inhibits antigen recognition, acting as an inert shield, because it prevents the interaction between the phagocyte's receptors and the Fc-region of IgG or the complement component iC3b, respectively^{37,38}. Furthermore, three surface-associated exoglycosidases are reported to reduce the deposition of complement

component C3 which leads to a resistance against opsonization and subsequent killing by macrophages and neutrophils⁵². To the contrary, the Autolysin (Lyt) A contributes to an inflammatory host response. This important factor of *Pneumococci* leads to the release of not only inflammatory cell wall degradation products, but also bacterial DNA and RNA⁵¹. Nucleic acids are recognized by TLR8 and TLR9 and it has already been shown that the ability to take up and eliminate *Pneumococci* in macrophages derived from bone marrow and alveolar macrophages is dependent on TLR9 and MyD88^{114,192}.

The results of this work indicate, that a MyD88-mediated induction of c-myc leads to enhanced internalization of *S. pneumoniae*. This conforms to the results Yim et al. obtained. They observed an induction upon *Mycobacterium* infection in macrophages which leads to repression of intracellular microbial growth. This induction is suggested to be TLR-mediated. The demonstrated effect of c-myc was not a result of the oncogene-induced protection from cell death. A previous publication showed that c-myc represses the transcription of *NRAMP1* gene which encodes for a protein responsible for the inhibition of the intracellular mycobacterial growth, but the expression level of *NRAMP1* mRNA in this study was not affected by neither *Mycobacteria* nor c-myc¹⁵⁷.

The results of Yim et al. might be even better comparable with the findings of this study – the positive influence of c-myc on internalization of *L. pneumophila*, because *L. pneumophila* is as well an obligate intracellular living pathogen. It is known of the internalization of *Legionella* that, next to the conventional phagocytosis, engulfment mainly occurs by coiling phagocytosis within pseudopod coil^{193,194}. For phagocytosis, TLR9 seems to be required with respect to macrophages. Additionally, the classical activation via TLR9 triggers expression of nitric oxide and type 1 cytokines¹⁹⁵. Another study observed that non-opsonic-mediated events occur for the internalization of *Legionella*, because of the low serum amounts within the lung and the lack for respective receptor-expression in macrophages. It reported activation of PI3K followed by Akt activation upon virulent *Legionella* infection which leads to enhanced phagocytosis¹⁹⁶. This pathway is typically downstream of TLR activation and MyD88 dependent¹¹⁹.

Anyhow, there are no data available on the influence of c-myc during internalization of any pathogen. Further research should be done to acquire more insight and maybe connect the MyD88-dependent c-myc induction to the enhanced phagocytosis.

7.4 C-myc as a regulatory factor of the immune response

The obtained data in this work suggest pro-inflammatory influence of induced c-myc in regard of cytokine secretion. The comparison of the results gained, on one hand, with the chemical inhibitor of c-myc and on the other hand using lentiviral transduced knockdown cells, generally lead to the same statement, but vary in details. The differences, further described and discussed in the following, can again be explained on the one hand by the different mode of action (c-myc inhibitor versus lentiviral-mediated c-myc knockdown), and on the other hand due to the specificity of the used reagents. The chemical inhibitor prevents transactivation of c-myc target genes by blocking dimerization of c-myc and MAX¹⁶⁵, whereas the shRNA, induced by lentiviral transduction, leads to degradation of the c-myc mRNA and therefore to a decreased content of c-myc protein. Additionally, chemical inhibitors have a much higher probability for binding to off-target sequences than shRNAs.

S. pneumoniae led to the induction of c-myc, which in turn led to an enhanced release of IL-6 and TNF α . It is more difficult to make a declaration towards the release of IL-1 β and IL-8. The inhibition of c-myc, using 100 μ M of the inhibitor, led to a significant reduction of IL-1 β in the supernatant, whereas the knockdown of c-myc did not lead to any changes. It is important to mention that the results gained with highest concentration of the inhibitor should be interpreted with caution, as this concentration showed not significantly but slightly higher toxic effects. Additionally, it is reported that concentrations of the c-myc inhibitor starting from 60 μ M induce cell cycle arrest and apoptosis^{197,198}. The chemokine IL-8 seems to be reduced dose-dependently (but not significantly) during inhibition of c-myc. However, those results could not be obtained by the knockdown of c-myc. A detailed view reveals that the IL-8 secretion in uninfected transduced cells is as high as it is in infected cells during the inhibitor experiments. This suggests that the transduction of lentiviral particles alters the basal IL-8 production of THP-1 cells. To verify the examined influence of c-myc on IL-8 secretion, a different approach should be used. Pulmonary inflammation is an important part of the host defense against *Pneumococci* lung infections^{199,200}. TNF α , together with IL-1 β , belong to the early response cytokines^{201,202}. The TNF-release is i.a. induced by the C-reactive protein (CRP), an acute phase protein, upon its binding to phosphocholine residues on *S. pneumoniae* cell walls and its subsequent interaction with phagocytic cells through Fc γ RI and Fc γ RII which activates the classical complement pathway²⁰³. TNF α is associated with apoptosis and necroptosis of cells²⁰⁴, both mechanisms are important for bacterial clearance without excessive tissue damage²⁰⁵. Furthermore, experiments using cytokine- or cytokine receptor-deficient mice have implicated TNF α and IL-6 as important mediators of this protective inflammatory response^{206,207}. TNF α -inhibition thereby corresponded to decreased neutrophil numbers in the blood and enhanced

bacteremia in mice²⁰⁸. IL-6 does not only contribute to host defense upon its secretion, but is also a counter-regulator of inflammatory responses²⁰⁹. However, as the secretion of both cytokines was downregulated by c-myc discrimination, there seems to be a pro-inflammatory influence of c-myc, which should be further analyzed regarding the mode of action.

The cytokine secretion upon *L. pneumophila* infection is likewise positively regulated by c-myc. Although, the decrease of cytokine release differs in the power between inhibitor-experiments and usage of knockdown cells, the effect observed in this work is generally the same. *L. pneumophila* is known to lead to the induction of TNF α and IFN- γ which in turn enhances bacterial killing of the bacteria²¹⁰. A recent study reported that the type IV secretion system (T4SS) leads to increased pro-inflammatory cytokine production, including IL-1 β , IL-6 and TNF α . This enhanced production involves activation of NF- κ B upon TLR signaling as well as the detection via NOD receptors. Furthermore, a p38 and SAPK/JNK MAPK activated cytokine response independent from TLR signaling but dependent on the T4SS, at which MAPK activation is coordinated with the TLR signaling, was reported²¹¹.

An influence of c-myc on cytokine secretion was until now only reported by Yim et al. during mycobacterial infection. They showed a c-myc dependent upregulation of TNF α and IL-6, which was suggested to contribute to the growth inhibition of *Mycobacteria* during a later phase of infection. Furthermore, they could demonstrate a part of the underlying mechanism: c-myc enhanced IRAK1 degradation, leading to specific activations of ERK1/2 and p38 MAPK but not Akt, and reduced I κ B α protein recovery upon degradation¹⁵⁶. An underlying mechanism on the influence of c-myc on cytokine production could not be examined during this work, but it should be considered in the future as it opens further insight into the mechanisms of host defense, which always gives a possibility for alternative treatment methods.

The review by Liu et al. provides an overview of c-myc interaction with various mediators of inflammation during chronic liver diseases. They provide a regulatory network in which they visualize that c-myc expression is increased by NF- κ B directly, but also by NF- κ B-dependent cytokines as IL-1 β (unknown mechanism), TNF α and IL-4 among others in a possible para- or autocrine loop. IL-6 in contrast negatively regulates c-myc expression according to this network. C-myc in turn positively regulates IL-8 and TNF α , but also IL-10²¹². This network could provide for detailed hypothesis how those interactions might work in bacterial infections and that feedback regulations might be an important part. For example, the shown c-myc-dependent induction of IL-6 during bacterial infection could lead in turn to down-regulation of the c-myc expression. Thus, inflammation might be controlled and further pro-inflammatory cytokine production be reduced if the bacterial clearance went far enough by that point.

The IL-1 mediated induction of c-myc expression is further supported by the study of Kessler et al. They observed an activation of c-myc gene transcription in fibroblasts upon IL-1 treatment via binding of proteins of the NF- κ B-dimers (p50/p65) to these c-myc elements, suggesting a positive c-myc regulation via NF- κ B²¹³. It is as well known, that NF- κ B regulates cytokine and chemokine production during the immune response^{134,135}. Together with the suggested NF- κ B-mediated increase of the c-myc expression, it can be hypothesized that the increased cytokine secretion by c-myc acts as an enhancer of the positive regulated cytokine expression by NF- κ B.

7.5 Concluding remarks

This research project demonstrated induction of c-myc upon infection of human lung tissue (*ex vivo*) and particularly in macrophages (*in vitro*) with *L. pneumophila* and *S. pneumoniae*. This induction of c-myc is dependent on the TLR adaptor molecule MyD88. The exact component serving as PAMP and with that which TLR is responsible for that induction could not be observed in this study. However, it was observed that the TLR2 ligand MALP-2 led to an induction of c-myc which suggests an involvement of that receptor. It should be examined in further studies, if TLR2 is the only initiating PRR or if it leads to c-myc induction by interacting with another receptor. The c-myc induction plays a positive role on the release of pro-inflammatory cytokines and chemokines induced by *Legionella* and *Pneumococci*. Additionally, c-myc leads to enhanced internalization of both bacteria by the phagocytes. Interestingly, there were no differences between the gram-positive *S. pneumoniae* and the gram-negative intracellular living *L. pneumophila*. It is conceivable that the recognition of both bacteria via PRRs (maybe TLR2), which interacts with MyD88, leads to the c-myc induction, which in turn activates a pro-inflammatory phenotype of macrophages. The cytokines released upon this activation, could enhance the internalization of invading bacteria. All in all, this suggests an inflammatory role of c-myc to support the host defense against invading bacteria, which might not be specific for only one family of bacteria, but more pathogens.

In further studies, it should be investigated, by which signaling pathway c-myc is induced downstream of MyD88, how this induction is regulated and how exactly it leads to this pro-inflammatory phenotype – by direct target gene activation or by activating other signaling pathways. This better understanding might help develop novel strategies to treat not only pneumonia but also other infectious diseases. Thereby it should always be kept in mind that c-myc is an oncogene and its unregulated expression could lead to tumor development. Anyhow, knowing

the exact signaling pathways could give the opportunity to not enhance or weaken c-myc expression itself, but simulating its natural induction to support the defensive host response. At the same time, this knowledge could be used to repress a threatening, overwhelming inflammatory response, to prevent tissue damage and therefore sepsis and septic shock.

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 216. Addgene: pMD2.G. Available at <http://www.addgene.org/12259/>.
 217. Addgene: pLKO.1 puro. Available at <http://www.addgene.org/8453/>.

9 APPENDIX

9.1 Vector Maps

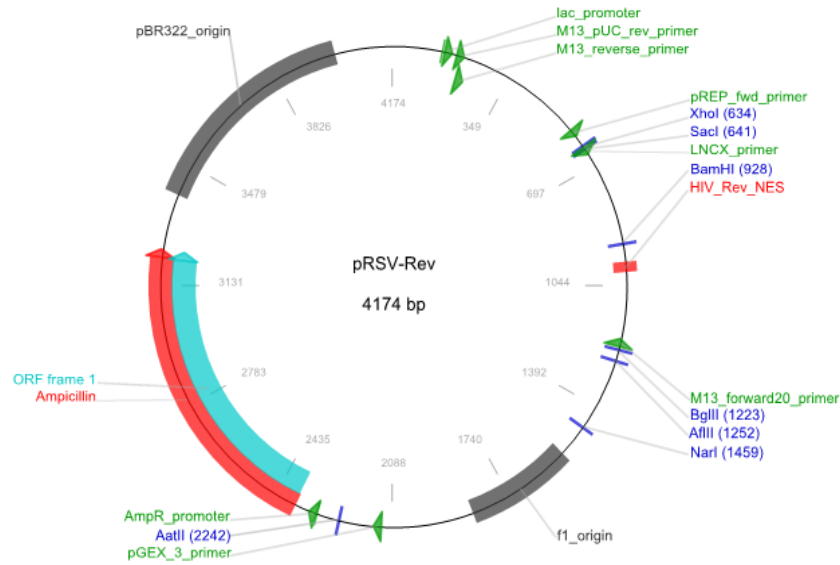


Figure 9.1: plasmid card (schematically) of pRSV-Rev (Addgene plasmid 12253).

Source: <http://www.addgene.org/12253/>; Principal Investigator: Didier Trono; ²¹⁴

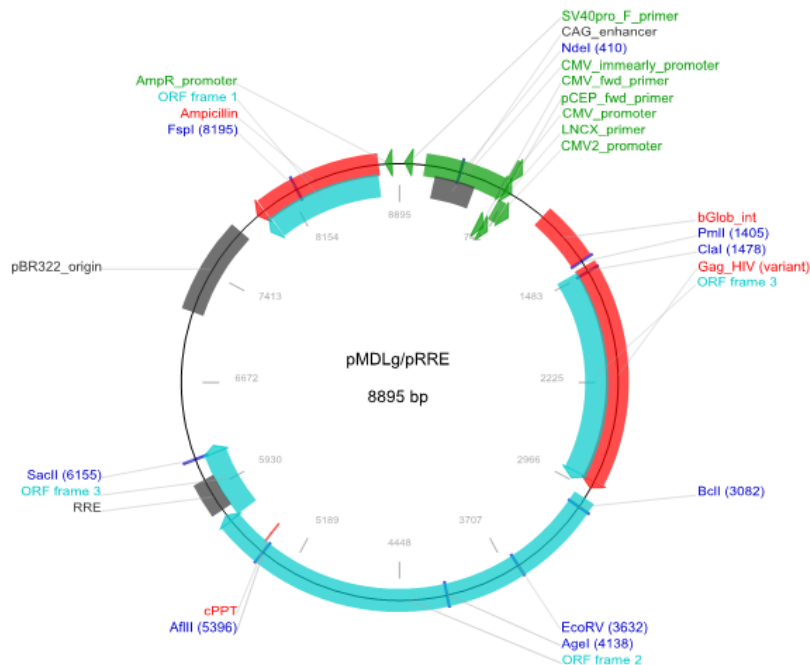


Figure 9.2: plasmid card (schematically) of pMDLg/pRRE (Addgene plasmid 12251).

Source: <http://www.addgene.org/12251/>; Principal Investigator: Didier Trono; ²¹⁵

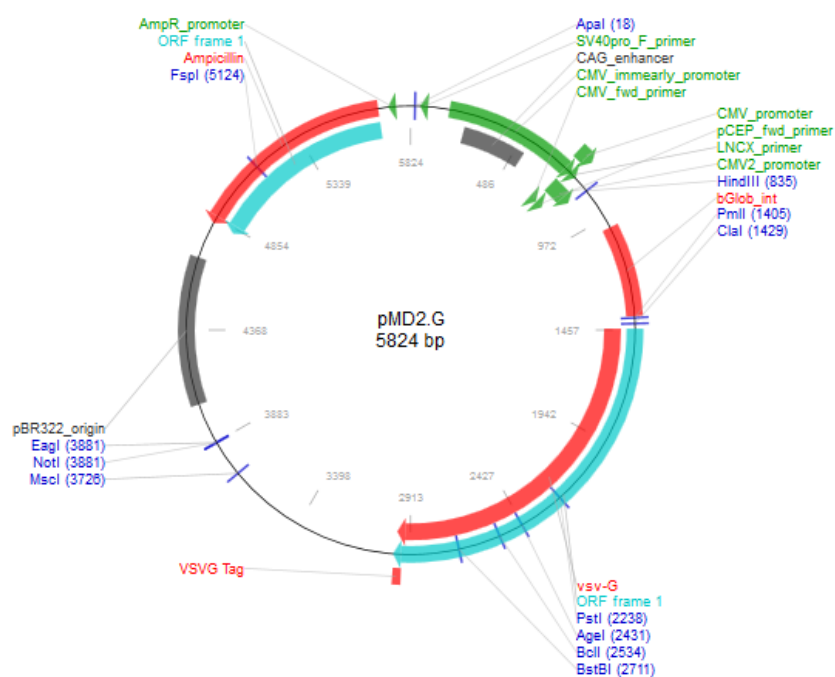


Figure 9.3: plasmid card (schematically) of pMD2.G (Addgene plasmid 12259).

Source: <http://www.addgene.org/12259/>; Principal Investigator: Didier Trono; ²¹⁶

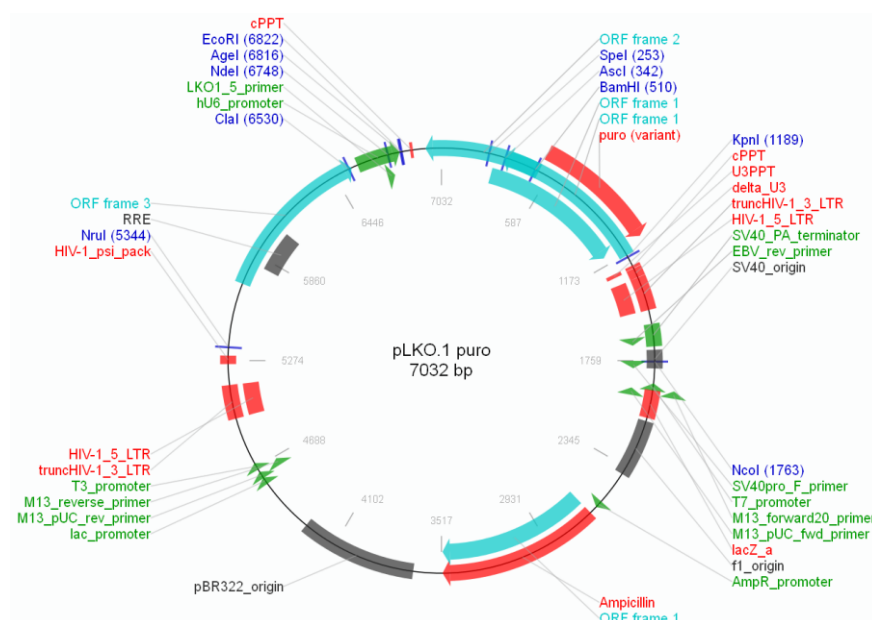


Figure 9.4: plasmid card (schematically) of pLKO.1 (Addgene plasmid 8453).

Source: <http://www.addgene.org/8453/>; Principal Investigator: Bob Weinberg; ²¹⁷

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9.4 List of Abbreviations

This list of abbreviations does not contain common abbreviations and SI-(Système International d'unités) units or their prefixes.

$\Delta\Delta\text{Ct}$	comparative Ct method
A. bidest = ddH ₂ O	twice distilled water
ACES	N-(2-Acetamido)-2-aminoethanesulfonic acid
Akt	protein Kinase B
AP 1	activator protein 1
APC	adenomatous polyposis coli tumor suppressor protein
APS	ammonium persulfate
BCAP	B-cell adapter for phosphatidyl-inositol-4,5-bisphosphate 3-kinase
BIR	Baculovirus inhibitor of apoptosis protein repeat
BSA	bovine serum albumin
CAP	community-acquired pneumonia
CARD	caspase-recruiting domain
CBPs	choline-binding proteins
CDC	centers of disease control and prevention
cDNA	cyclic DNA / cyclic deoxyribonucleic acid
CFU	colony forming units
ChoP	phosphocholine protein
c-myc	v-myc myelocytomatosis viral oncogene homologous
c-myc-Inh.	c-myc inhibitor (100-58-F4)
CYE	charcoal yeast extract
D39	wild type serotype 2 strain of <i>S. pneumoniae</i>
D39 Δ cps	capsule-deficient D39
DBD	DNA-binding domain
DMEM	Dulbeccos modified eagle medium
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
<i>dot/icm</i>	defect of organelle trafficking / intracellular multiplication
DSMZ	German Collection of Microorganisms and Cell Cultures

<i>E. coli</i>	<i>Escherichia coli</i>
ELISA	enzyme-linked immuno sorbent assay
<i>endA1</i>	gene encoding for endonuclease 1
(r)ER	(rough) endoplasmic reticulum
ERK	extracellular-signal regulated kinase
FCS	fetal calve serum
GAPDH	glyceraldehyd-3-Phosphat-Dehydrogenase
GLS	glutaminase
GM-CSF	granulocyte macrophage colony-stimulating factor
H ₂ SO ₄	sulfuric acid
HAP	hospital-acquired pneumonia
HBSS	Hank's buffered Salt Solution
HCAP	healthcare-associated pneumonia
HCC	hepatocellular carcinoma
HCRT	high capacity reverse transcriptase
HCV	Hepatitis C virus
HEK	human embryonal kidney cell
HI-Virus	human immunodeficiency virus
HLH/LZ	helix-loop-helix leucine zipper
<i>H. pilori</i>	<i>Helicobacter pilori</i>
HSV	Herpes simplex virus
HuLu	human lung
Ig	Immunoglobulin
IKK	IκB kinase
IL	Interleukin
iPS	inducible pluripotent stem cells
IRAK	IL-1 receptor-associated kinase
JNK	c-Jun N-terminal kinase
KLF-4	Krüppel-like factor 4
LB	Luria-Bertani
LBP	lipopolysaccharide-binding protein
LCV	<i>Legionella</i> -containing vacuole
LDH	lactate dehydrogenase
LPS	lipopolysaccharide

LRR	leucine-rich repeat
LTA	lipoteichoic acid
LV	lentivirus
Lyt A / B / C	autolysins A / B / C
MALP-2	macrophage-activating lipopeptide
MAMP	microbe-associated molecular pattern
MAP	mitogen-activated protein
MAPK	mitogen activated protein kinase
MARCO	macrophage receptors with a collagen structure
MAX	myc-associated protein X
MHC	major histocompatibility complex
MMR	macrophage-mannose receptor
MOI	multiplicity of infection
mRNA	messenger RNA
MSR	macrophages scavenger receptor
mTor	mechanistic target of rapamycin
MyD88	myeloid differentiation factor 88
NaCl	sodiumchloride
N-Cor	nuclear receptor co-repressor 1
NF- κ B	nuclear transcription factor- κ B
NLR	NOD-like receptor
NOD	nucleotide-binding oligomerization domain
NRAMP	natural resistance-associated macrophage protein
Oct-4	octamer binding transcription factor 4
OD	optical density
ODC	ornithine carboxylase
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethyleneglycol
PI3K	phosphatidylinositol kinase
pLKO.1	mock-vector of the lentiviral transduction
ply	Pneumolysin
PMA	phorbol-12-myristat-13-acetat

PRR	pattern recognition receptor
PiaA	pneumococcal iron acquisition A
PiuA	pneumococcal iron uptake A
PsaA	pneumococcal surface antigen A
Psp	pneumococcal surface proteins
PYD	pyrin domain
R6x	unencapsulated derivate of <i>S. pneumoniae</i> D39
<i>recA1</i>	gene encoding for recombinase 1
RIG-I	retinoic acid inducible gene 1
RLR	RIG-I-like receptor
RNA	ribonucleic acid
RPMI	cell culture medium of the Roswell Park Memorial Institute
RT	room temperature / Reverse Transcriptase/ Real Time
RT-qPCR	real time-quantitative PCR
SARM	sterile alpha and HEAT/Armadillo motif
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
Sin3	homolog of the yeast transcriptional repressor
Sox-2	sex determining region Y -box 2
Src	proto-oncogene tyrosine-protein kinase
SRY	sex determining region Y
SSD	signal sensing domain
<i>S. pneumoniae</i>	<i>Streptococcus pneumoniae</i>
Syk	spleen tyrosine kinase
T4SS	type IV secretion system
TAD	trans-activating domain
Tcf	T-cell specific transcription factor
TEMED	3,3',5,5'-Tetramethylbenzidine
TFEB	transcription factor EB
THP-1	human monocytic cell line, derived from an acute monocytic leukemia patient
THY	Todd Hewitt Broth

TIR	Toll/IL1R homology domain
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TMB	tetramethylbenzidine
TNF α	tumor necrosis factor alpha
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor inducing IFN- β
Tris	tris-(hydroxymethyl)-aminomethane
Tris-HCl	tris-hydrochloride
U	units
v/v	volume per volume
VAP	ventilator-associated pneumonia
w/v	weight per volume
WHO	world health organization
WT	wildtype

9.5 Publications

„Pneumococci LytA-dependent KLF4 expression terminates IL-8 secretion in lung epithelium“

Zahlten J, Herta T, **Kabus C**, Steinfeldt M, Kershaw O, García P, Hocke AC, Gruber AD, Hübner RH, Steinicke R, Doehn JM, Suttorp N, Hippenstiel S.

Am J Respir Cell Mol Biol. 2015;53(4):544-54

Impact Factor: 4.082

Under Review:

“Human beta Defensin-3 alters pneumococci-related innate immune response in macrophages”

Mara M.E. Wittig, **Christin Kabus**, Philippe Dje N'Guessan, Norbert Suttorp and Janine Zahlten

In preparation:

„c-myc is induced in human macrophages during pneumococcal pneumonia which leads to pro-inflammatory immune response.“

Christin Kabus, Mara Wittig, Stefan Hippenstiel and Janine Zahlten

“c-myc regulates the immune response of human macrophages upon *Legionella pneumophila* infection”

Christin Kabus, Mara Wittig, Stefan Hippenstiel and Janine Zahlten

“DNA-release by *Streptococcus pneumoniae* autolysin LytA induced Krueppel-like factor 4 expression controlling pneumococci-related innate immune response in macrophages.”

Toni Herta, Christian Bollensdorf, Aritra Bhattacharyya, **Christin Kabus**, Pedro García, Norbert Suttorp, Stefan Hippenstiel* and Janine Zahlten* (*contributed equally)

“Pneumococci-related hBD2 expression depends on TLR2 and alters innate immune response in macrophages”

Wittig M, N'Guessan DP, **Kabus C**, Suttorp N and Zahlten J.

Oral presentation:

„Induction and Function of the stem cell factor c-myc in Pneumonia caused by *Streptococcus pneumoniae* and *Legionella pneumophila*“

Christin Kabus, Stefan Hippenstiel, Janine Zahlten

20. Symposium „Infektiologie und Immunologie“, Rothenfels 2016

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Christin Kabus, Stefan Hippenstiel, Janine Zahlten

Herbsttagung der DFG-Sektion Zellbiologie, Hannover 2016

Poster presentations:

„Induction and Function of the stem cell factor c-myc in Pneumonia caused by *Streptococcus pneumoniae* and *Legionella pneumophila*“

Christin Kabus, Stefan Hippenstiel, Janine Zahlten

„Tag der Biotechnologie“, TU Berlin 2015 und 2016

„Induction and Function of the stem cell factor c-myc in Pneumonia caused by *Streptococcus pneumoniae* and *Legionella pneumophila*“

Christin Kabus, Stefan Hippenstiel, Janine Zahlten

2nd International Conference „Innate immunity of the lung – Improving pneumonia outcome“, Berlin 2017

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ACKNOWLEDGEMENTS

My PhD project is finally finished, but finishing it required and involved the support and work of many other people, which herewith I would like to thank. I would like to express my gratitude to Prof. Dr. Suttorp for giving me the opportunity to perform my PhD work in the Department for Infectious Diseases and Pulmonary Medicine at the Charité. Moreover, I would like to thank Prof. Dr. Hippenstiel and Dr. Zahlten for developing, together with me, the research questions and thereby giving me the opportunity to work on this fascinating project. Many special and heartfelt thanks go in particular to Dr. Janine Zahlten who helped me finish this thesis. Thank you so much for your input, helpful advice, great ideas and reading of manuscripts. Thank you also for being understanding in work- or not work-related issues and always staying optimistic and hopeful. Thank you very very much!

I thank Prof. Dr. Meyer and Prof. Dr. Lauster for agreeing to be my PhD reviewers and Prof. Dr. Meyer in particular for providing input on my project as well as support and motivation. Further, I would like to thank Prof. Pflugmacher Lima for agreeing to be the chair of my PhD committee.

I would also like to thank the “Manchot Stiftung” for financial support for 3 years - without it, this work would not have been possible.

Furthermore, I would like to thank all former and current PhD students and colleagues of the department for their help, motivating support and stimulating discussions, especially I want to thank Doris and Claudia as well as the members of AG Zahlten! Many thanks go additionally to Mara, Anne R., Kathrin G., Andrea, Jan, Elisa, Elena, Philipp G., Laura, Ling, Moritz, Daniel, Sarah, Iris, Alex and Aritra for creating a great atmosphere at work and afterwards.

I also want to thank my friends outside from the lab. Thank you for your support and encouragement, for listening, sometimes for hours and for putting off my thoughts of the problems bothering me. Thank you for moments full of laughter and fun together next to all the work stress! Laura, Annika and Alena – thank you for reading 120 pages!

Very special thanks go to my beloved family – in particular my parents, but also my brother and my grandparents – who always believed in me and that I can go through with it and overcome any problems! Thank you for your advice and heartwarming words when needed!

Finally, I want to express my deepest gratitude to my husband for holding my back, supporting all the sometimes crazy ideas and plans and being there all the time!

EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit „The induction and function of the transcription factor c-myc in macrophages during pneumococcal pneumonia and Legionellosis“ selbstständig und eigenhändig sowie ausschließlich unter Verwendung der aufgeführten Quellen und Hilfsmittel angefertigt habe.

Ich versichere, dass diese Arbeit in dieser oder anderer Form noch keiner anderen Prüfungsbehörde vorgelegt wurde. Der Inhalt der Promotionsordnung der Fakultät III – Prozesswissenschaften der Technischen Universität Berlin vom 05. Februar 2014 ist mir bekannt.

Berlin, den 14. Juli 2017

Christin Sophia Kabus