hBD-2 AND hBD-3 ARE INDUCED IN *S. PNEUMONIAE* INFECTED HUMAN MACROPHAGES VIA DISTINCT SIGNALING PATHWAYS BUT EXHIBIT SIMILAR IMMUNE MODULATORY FUNCTIONS

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Meiner Familie.

“Difficult roads often lead to beautiful destinations.”

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ABSTRACT

Streptococcus pneumoniae is the most common cause of community acquired pneumonia. This infectious disease is characterized by acute or chronic inflammation of lung tissue which is due to invasion of the pathogen into the lower airway and activation of the host's immune system. Invading pathogens will be combated by macrophages, an important cell type of innate immunity. These cells not only eliminate pathogens but also contribute to tissue repair in the aftermath of infection. The inflammatory response has to be carefully regulated since an overwhelming reaction would lead to excessive tissue damage which would negatively influence the course of the disease. Endogenously produced antimicrobial peptides, such as the human β-defensins, modulate the host's immune response by enhancing or weakening the inflammatory reaction depending on the cellular context in which they are produced. Further, they are directly antimicrobial against various pathogens. Thus, if the mechanisms of induction as well as their effects on *S. pneumoniae* infected cells would be fully understood, exogenous regulation of the β-defensins in pneumococcal pneumonia could improve the outcome of the disease. Results of this study demonstrate that hBD-2 and hBD-3 are induced in *S. pneumoniae* infected human macrophages and that this induction is dependent on the bacterial exotoxin pneumolysin (PLY). Macrophages that are infected with the PLY expressing wild type strain produce hBD-3 whereas hBD-2 was only induced when stimulation was done with a PLY-deficient strain. The induction of hBD-3 involves TLR4, MyD88 and PI3K whereas hBD-2 is induced via TLR2, MyD88 and p38 MAPK / ERK signaling. Furthermore, it became apparent that KLF4, an important transcription factor seems to negatively regulate hBD-3 expression. Interestingly, hBD-3 was bactericidal against unencapsulated *S. pneumoniae* but hBD-2 had no antimicrobial effect at all. But both peptides were able to dose-dependently modulate the production of TNFα in infected macrophages. Lower doses of β-defensins slightly enhanced expression of the cytokine whereas higher doses caused a down-regulation. In conclusion, this study demonstrates that hBD-2 and hBD-3 exhibit similar immune modulating functions in human macrophages infected with *S. pneumoniae* but are regulated differentially via two distinct signaling pathways. It could be shown that this

differential regulation depends on bacterial PLY and that both peptides possess pro- as well as anti-inflammatory properties depending on the context of infection.

ZUSAMMENFASSUNG

Streptococcus pneumoniae ist der häufigste Erreger der ambulant erworbenen Pneumonie. Diese Infektionskrankheit ist charakterisiert durch eine akute oder chronische Entzündung des Lungengewebes, hervorgerufen durch eine Aktivierung des Immunsystems durch eindringende Bakterien. Eindringende Bakterien werden zunächst von Makrophagen bekämpft, einem wichtigen Zelltyp der angeborenen Immunität. Diese Zellen eliminieren nicht nur Pathogene sondern reparieren auch zerstörtes Gewebe nach einer überstandenen Infektion. Die Entzündungsreaktion als solche muss besonders gut reguliert werden, da eine überschießende Immunantwort zu vermehrter Gewebsschädigung führt und damit den Krankheitsverlauf negativ beeinflussen kann. Endogen produzierte, antimikrobielle Peptide, wie die humanen β-Defensine, können die Immunantwort modulieren, indem sie die Entzündungsreaktion weiter forcieren oder abschwächen, je nach dem in welchem zellulären Kontext sie produziert werden. Zusätzlich wirken sie direkt antimikrobiell auf verschiedene Pathogene, daher wäre ein besseres Verständnis der Induktion sowie der verschiedenen Effekte, welche β-Defensine auf *S. pneumoniae* infizierte Zellen ausüben, von Vorteil, um eventuell den Pneumonie-Verlauf durch exogene Regulation dieser Peptide günstig zu beeinflussen. Die Ergebnisse dieser Studie zeigen, dass hBD-2 und -3 in *S. pneumoniae* infizierten humanen Makrophagen induziert werden und das diese Induktion abhängig ist vom bakteriellen Exotoxin Pneumolysin (PLY). Makrophagen welche mit dem PLY-exprimierenden Wild Typ Stamm infiziert wurden, produzierten hBD-3, während hBD-2 nur induziert wurde, wenn eine Stimulierung mit einem PLY-defizienten Stamm erfolgte. Die Induktion von hBD-3 erfolgte über TLR4, MyD88 und PI3K, wohingegen hBD-2 über TLR2/6, MyD88 und p38 / ERK induziert wurde. Weiterhin stellte sich heraus dass KLF4, ein wichtiger Transkriptionsfaktor, die hBD-3 Expression negativ reguliert. Interessanterweise zeigte sich hBD-3 bakterizid gegen unbekapselte *S. pneumoniae* während hBD-2 nicht antimikrobiell wirkte. Beide Peptide waren aber in der Lage, konzentrationsabhängig die TNFα Produktion der infizierten Makrophagen zu modulieren. So stieg die Produktion dieses Zytokins bei geringer β-Defensin Konzentration während sie bei hoher Konzentration

abnahm. Zusammenfassend wird in dieser Arbeit gezeigt, dass hBD-2 und hBD-3 in humanen Makrophagen, welche mit *S. pneumoniae* infiziert sind, ähnliche immun-modulatorische Funktionen erfüllen, dass aber die Regulation dieser beiden Peptide über zwei gänzlich unterschiedliche Signalwege erfolgt. Es konnte gezeigt werden, dass diese differenzielle Regulation abhängig ist vom bakteriellen PLY und dass beide Peptide sowohl pro- als auch anti-inflammatorische Eigenschaften besitzen, je nach dem in welchem infektiologischen Kontext sie produziert werden.

1. INTRODUCTION

1.1 *STREPTOCOCCUS PNEUMONIAE*

1.1.1 BIOLOGY

Streptococcus (S.) pneumoniae is a gram-positive bacterium belonging to the family of *Streptococcaceae* and the genus *Streptococcus*. Since division occurs at a single axis, *S. pneumoniae* usually forms diplococci [1]. The bacteria are aerotolerant, catalase negative anaerobes that ferment glucose to lactic acid. [2]. When grown on blood agar plates, *S. pneumoniae* is inducing hemolysis, the break-down of red blood cells. *S. pneumoniae* is utilizing α-hemolysis which is also called green hemolysis because the blood agar under the bacterial colonies changes its color from red to greenish due to the production of hydrogen peroxide by the bacterium, which oxidizes hemoglobin to methemoglobin. The ability of certain bacteria to induce hemolysis is used to classify different microorganisms and plays a role in diagnostics. For a more detailed classification of bacteria the serotype of the strain needs to be determined. The serotype is especially interesting when it comes to epidemiology because different serotypes differ in their virulence, prevalence and drug resistance [3,4]. In *S. pneumoniae* there are 90 different serotypes known so far which are determined by the pneumococcal capsule (CPS) [5]. The capsule of *S. pneumoniae* consists of polysaccharides and protects the bacteria from the host's immune system [6]. Encapsulated bacteria tend to be more virulent than unencapsulated bacteria [7] that are more frequently found as commensals in the upper respiratoy tract. When compared to gram-negative bacteria, gram-positives are more resistant to environmental influences because they are surrounded by a cell wall, which is built from peptidoglycan with an outer layer of teichoic acid. Although surrounded by cell wall and capsule, *S. pneumoniae* is sensitive against cold, acidic and alkaline pH, and dehydration.

The genome of pneumococci is a closed, circular DNA structure. It consists of around 2 million base pairs [8] and contains a set of 1553 core genes essential for bacterial viability. In addition to the core genes there are 154 genes that contribute to bacterial virulence. There is up to 10% variance between genomes

of different *S. pneumoniae* strains, making the pneumococcal genome a highly variable one. The genes for virulence and drug resistance were acquired throughout evolution by horizontal gene transfer [9].

1.1.2 PATHOGENICITY AND DISEASE

S. pneumoniae is one of the leading cause of morbidity and mortality worldwide [10]. It is the causative agent for diseases such as pneumonia, meningitis, sepsis, otitis media and sinusitis [11–13] but is also found asymptomatically colonizing the nasopharynx of approximately 60% of children attending daycare [14] and 10% of adults [15]. This nasopharyngeal colonization is a prerequisite for spreading and invasion of the pathogen [16,17]. Transmission of the bacteria is achieved by respiratory droplets from person to person in direct contact [14,18]. Usually the bacteria are cleared from the nasopharynx after several weeks to months but if not can invade the lower respiratory tract or migrate into otherwise sterile compartments such as the brain, middle ear and blood where they cause severe diseases [18,19]. The molecular factors that are promoting the transit from the upper to the lower respiratory tract still remain elusive although low-invasive serotypes seem to have a longer duration of colonization than high-invasive serotypes [18]. Community-acquired pneumonia (CAP), one of the invasive diseases caused by *S. pneumoniae*, still has a mortality rate of 10-25% in hospitalized patients [20]. Infants and elderly as well as immune compromised patients are at a higher risk of fatality [21]. Furthermore influenza virus infection predisposes to secondary pneumonia infection accounting for increased cases of pneumonia during and shortly after influenza outbreaks [22,23]. Pneumonia is characterized by excessive inflammation of lung tissue due to invasion of the pathogen into the lower airway of the host. Typical symptoms are a cough, chest pain, fever, and difficulty breathing [24]. Diagnosis is usually done by physiological symptoms and a chest x-ray [25] and for treatment antibiotics are most commonly applied. The use of antibiotics already poses a problem, for drug resistant strains of *S. pneumoniae* are on the rise not only in developing countries but also in Europe [26,27]. This increasing drug resistance is calling for the need to develop new vaccines or antimicrobials for treatment of CAP [28]. Although there are vaccines available, they only target several pathogenic serotypes, preventing the disease from these serotypes

[29–32] but leading to serotype replacement, meaning that non-vaccine serotypes start to predominate in patients [33]. This strongly evokes the need to explore other means of prevention and treatment of pneumococcal pneumonia.

As already mentioned above, different strains of *S. pneumoniae* differ in their ability to cause disease. That is due to various virulence factors and drug resistance genes, which are not shared by all strains. The capsule is one of the most important virulence factors of *S. pneumoniae*. It consists of polysaccharides covalently connected to the peptidoglycan of the bacterial cell wall [34]. Synthesis of the capsule of all serotypes is achieved by the same metabolic pathway [35] and the genes encoding for the capsular components, namely *cpsA*, *cpsB*, *cpsC* and *cpsD*, are largely conserved between strains [36]. Reports state that these genes are also important for the regulation of capsule production [37] although the exact mechanism for regulation of capsule expression is still unknown. Commensal bacteria that colonize the upper respiratory tract are usually unencapsulated or express only a thin capsule to promote colonization by aiding adherence to the respiratory epithelium [38,39]. In general, the amount of capsule surrounding the bacteria differs in each anatomical niche [39,40]. Van der Windt *et al.* [41] report that 15% of strains are unencapsulated and that these strains are mostly found in the nasopharynx. When it comes to invasive disease a thicker capsule is advantageous to evade complement mediated opsonophagocytosis [7,42] and other immunological defense mechanisms [9]. In addition, bacterial antigens are masked by capsular polysaccharides and are thus not “visible” to the host’s immune system. Furthermore, *S. pneumoniae* strains exhibit phase variation [43], a mechanism for dealing with a rapidly changing environment, as occurs when a colonizing strain becomes invasive. Phase variation is the random switching of phenotypes and contributes to the pathogens virulence by generating heterogeneity. A thicker capsule promotes the virulence of the bacteria especially in the blood whereas a thinner capsule aids the binding to epithelial cells by exposing more surface proteins and thereby enhances the colonization of the host [40,42].

Another important virulence factor of *S. pneumoniae* is the exotoxin pneumolysin (PLY). This 53kDa protein is closely associated with development of invasive disease and inflammation since PLY-deficient strains show reduced

virulence, less inflammation and delayed recruitment of neutrophils in animal models [44,45]. PLY is released upon autolysis and is a soluble monomer that binds to cholesterol in the host's cell membrane. Pore-formation occurs by polymerization of up to 50 monomers [46] and induces lysis of the host cells [47–49]. Apart from cell lysis PLY has a wide range of effects at sub-lytic concentrations such as induction of apoptosis [50] and activation of the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome [51] as well as the classical complement pathway [52]. In addition to PLY and the capsule there are several other virulence factors expressed by *S. pneumoniae,* e.g. autolysin (LytA), IgA protease, hyaluronidase, pneumococcal surface antigen A and B (PsaA, PsaB) and pneumococcal surface protein A and C (PspA, PspC) [5,38]. All these virulence factors contribute to either invasiveness or immune evasion of the pathogen.

1.2 THE IMMUNE SYSTEM

On an everyday basis the human body is exposed to a multitude of microorganisms, some of which cause severe diseases. Recognition and elimination of these pathogens is achieved by the immune system. In every multi-cellular organism there is an immune system, albeit there are, of course, differences in complexity. Even in the slime mold *Dictyostelium (D.) discoideum* sentinel cells are present that protect the temporary multi-cellular organism [53]. In addition plants [54], *Drosophila (D.) melanogaster* [55] and mussels [56] have the ability to identify and antagonize pathogens such as viruses, bacteria and fungi. The immune system is challenged with the task of recognizing and eliminating antigens foreign to the body while at the same time tolerance to all structures inherent to the host is of utmost importance. Furthermore, the immune system monitors cellular integrity and eliminates cells that are altered, injured or killed. Throughout evolution the immune system underwent a lot of developmental changes causing vertebrates to have an innate and an adaptive immune system.

1.2.1 THE INNATE IMMUNE SYSTEM

Innate immunity is more ancient than adaptive immunity and is the host's first line of defense against invading pathogens. It includes chemical, physical and cellular barriers. Physical barriers are the skin and mucous membranes; a chemical barrier is e.g. the acidity of the stomach contents - and the cellular barrier consists of an array of cells with receptors able to detect microbial products and to instigate a counterattack [57]. In contrast to the adaptive immune system, the innate immune system reacts to all kinds of pathogens without previous priming of immune cells [58]. An innate immune response towards pathogens is usually accompanied by inflammation of the surrounding tissue. The inflammatory response therefore involves immune cells, blood vessels, and molecular mediators, e.g. eicosanoids. Its purpose is the elimination of the initial cause of cell injury as well as the clearance of necrotic cells and damaged tissue and initiation of tissue repair. Inflammation is a process that requires careful balancing since too much inflammation will cause severe tissue damage whereas weak inflammatory processes could lead to progressive tissue destruction by the pathogen.

The innate immune response in general is usually fast but rather unspecific. Germ-line encoded receptors detect microbes or damaged tissue and cells by highly conserved molecular patterns [59]. For detection of these so called microbe- or pathogen-associated molecular patterns (MAMPs; PAMPs) and damage-associated molecular patterns (DAMPs) specialized receptors are necessary. These pattern-recognition receptors (PRRs) [60] are found for example on macrophages, neutrophils and dendritic cells (DCs), the main effector cells of innate immunity (for further information on PRRs please refer to section 1.2.3). Upon recognition of pathogen the cells mount an innate immune response, resulting in production of cytokines and chemokines such as IL6, IL8, IL12, IL1β and TNFα [61] to recruit further leukocytes such as phagocytic and antigen-presenting cells (APCs) to the site of infection. Pathogens are then eliminated by phagocytosis, secretion of antimicrobial peptides [62], production of reactive oxygen species (ROS), and formation of neutrophil extracellular traps (NETs) [63]. Different classes of pathogens involve recognition via distinct receptors and result in some variation of induced cytokines but in general, cytokines execute important local and systemic effects that contribute to innate as well as adaptive immunity [64]. Moreover, the coordinated functions of the produced cytokines and chemokines not only determine the type of response but are also necessary to mount, shape, maintain and terminate the defense mechanisms of the host. Further, APCs connect innate to adaptive immunity by migration from the site of infection to the draining lymph nodes and subsequent stimulation of residing B- and T-cells. A closely orchestration of the immune response is necessary since all the defense mechanisms applied by the host are highly effective in eliminating pathogens but damage and even kill host cells when the reaction is overwhelming [65–68].

1.2.1.1 MACROPHAGES

All blood cells derive from hematopoietic stem cells in the bone marrow. These cells divide and give rise to two different types of stem cells, a lymphoid progenitor cell and a myeloid progenitor cell. Monocytes derive from the myeloid lineage and are stored as immature cells in the spleen. Monocytes are a subset of circulating white blood cells, able to differentiate into tissue

macrophages [69,70] and showing a remarkable multipotency in different inflammatory environments. Macrophages were first described by E. Metchnikoff in 1893 [71]. They mediate host antimicrobial defense [72] and are implicated in many inflammatory diseases. As one of the most important cell types of innate immunity they are major contributors to the homeostasis of the host by responding to invading pathogens to enhance the immune response and by contributing to tissue repair in the aftermath of infection. Furthermore, tissue resident macrophages are not terminally differentiated but can proliferate to restore cells lost to inflammation [73,74]. This renewal of macrophages is independent of the bone marrow [75] and recent studies suggest a prenatal origin of these special cells [76,77].

Macrophages clearly outnumber other immune cells [78,79] and are professional phagocytes and APCs [80]. For recognition and phagocytosis of pathogens they express a broad variety of PRRs, such as scavenger receptors and toll-like receptors (TLRs). Macrophages are a multitool of the innate immune system as they are able to inhibit proliferation, i.e. kill pathogens and promote proliferation when repairing wounds or damaged tissue. These two distinct functions stem from two possible macrophage phenotypes, called M1 and M2. The M1 macrophage is the classically activated, inflammatory macrophage [81] whereas the M2 macrophage is alternatively activated and has regulatory [82] or anti-inflammatory functions [83,84]. Another important feature of macrophages is the metabolizing of arginine to nitric oxide (NO) (M1 phenotype) or ornithine (M2 phenotype) [85–87]. This balance of NO or ornithine production is regulated by TGFβ, a cytokine found at high levels in the blood serum [88–90]. Interestingly the macrophage phenotype is interchangeable and M1 macrophages are able to transition into M2 macrophages and vice versa. In addition Stables *et al.* found hybrid phenotypes in macrophages from the peritoneum [91]. Although recent studies shed some light on the different macrophage subtypes, the transcription factor profiles of all these subsets are still largely unknown [92].

All macrophages have in common that they do not need activation by other immune cells, rendering innate immunity independent of adaptive immunity [93,94]. Albeit this independence, there is crosstalk between different immune

cells of innate and adaptive origin. For example macrophages are needed to direct T-cells to either become T helper (Th) 1 or Th2 cells by secreting interferon gamma (INFγ) and IL4 respectively [95–97]. Macrophages are not only the first line of defense against invading pathogens but also the driving force of tissue and damage repair as well as the connection to the adaptive immune response, which makes them one of the most important cell types of the immune system in general.

1.2.3 PATTERN RECOGNITION RECEPTORS

PRRs are expressed by various cell types of the immune system, mostly by APCs such as macrophages and DCs. They are germ-line encoded and were first discovered in plants. Their function is the detection of MAMPs, PAMPs and DAMPs, conserved molecular patterns that are essential for microbial survival or molecules that are released from damaged cells and tissues [98]. Thus PRRs detect a wide variety of proteins, saccharides, lipids and nucleic acids [99]. The recognition of pathogen is usually followed by uptake and surface presentation of foreign antigens via major histocompatibility complex (MHC) class I or II [100]. Usually PRRs detect a given pathogen simultaneously or sequentially, thereby activating shared and/or distinct signaling pathways. In general, activation of PRR signaling cascades leads to nuclear translocation of a set of transcription factors such as nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB), activator protein 1 (AP-1) and CCAAT-enhancer-binding protein beta (C/EBPβ) [101]. These regulate the transcription of their target genes that are mostly pro-inflammatory. Hence, the sensing of MAMPs, PAMPs or DAMPs up-regulates the transcription of inflammatory genes that encode e.g. for cytokines, chemokines, type I interferons and antimicrobial peptides. The inflammatory response is necessary for clearance of the pathogen but when overactivated becomes detrimental to the host. A tight regulation of this response is achieved by associated negative feedback loops as well as anti-inflammatory factors such as TGFβ, IL10 and steroid hormones [102,103]. There are four classes of PRRs: TLRs, c-type lectin receptors (CLRs), RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) [104]. Since TLRs are implemented in pneumococci induced immune responses in this study, they are described in more detail in the following section.

1.2.3.1 TOLL-LIKE RECEPTORS

TLRs are a family of transmembrane receptors [100]. They are evolutionary conserved from the worm *Caenorhabditis (C.) elegans*. The toll protein was initially identified as essential for the dorsoventral polarity in *D. melanogaster* [105] where it also plays a critical role in the anti-fungal response [106]. Ten different TLRs have been identified in humans so far. All TLRs are type I integral membrane glycoproteins with an extracellular and a cytoplasmatic functional domain. The extracellular domain contains a varying number of leucine-rich repeat (LRR) motifs whereas the cytoplasmatic domain shows homology to that of the IL1 receptor [98]. Therefore is was named Toll/IL1R homology (TIR) domain [107]. TLRs can be found on various immune cells but also on non-immune cells such as fibroblasts and epithelial cells. The expression of the receptors is not static but can be modulated rapidly in response to cytokines, pathogens or environmental factors [98]. TLRs are expressed extra- or intracellularly with TLR1, TLR2, TLR4, TLR5 and TLR6 on the cell surface and TLR3, TLR7, TLR8 and TLR9 in intracellular compartments [108]. There are several subfamilies of TLRs depending on the receptor ligands. For example TLR1, TLR2 and TLR6 are necessary for detection of lipids and TLR7, TLR8 and TLR9 detect nucleic acids. Some TLRs are even able to recognize several structurally unrelated molecules [109]. Intracellular TLRs are mainly stored in the membrane of the endoplasmic reticulum (ER) to prevent detection of self-nucleotides. In case of phagocytosis of pathogens the receptors are recruited to the endolysosome where they have to undergo processing by proteases before functioning [110]. How this recruitment is done, however, still needs further investigation.

TLR2 senses components from bacteria, mycoplasma, fungi and viruses, including lipoproteins. Recognition of ligands requires heterodimerization with TLR1 or TLR6. The TLR2/1 and TLR2/6 receptors recognize triacyl and diacyl lipoproteins respectively. The ligand-receptor interaction is mediated by ligand binding in a pocket formed by the M-shaped heterodimer [111]. Interestingly the cellular response to TLR2 ligands differs depending on the involved cell type [112]. Due to heterodimerization TLR2 recognizes the broadest range of MAMPs and PAMPs [113].

TLR4 binds lymphocyte antigen 96 (MD2) on the cell surface to recognize lipopolysaccharide (LPS) from gram-negative bacteria [114]. For signaling, two complexes of TLR4-MD2-LPS have to form a functional homodimer [115]. In addition to LPS, TLR4 can bind to viral envelop proteins, as well as to oxidized phospholipids, a category of DAMPs associated with avian influenza virus [116]. Furthermore TLR4 was shown to recognize *S. pneumoniae's* PLY [117].

TLR5 recognizes flagellated bacteria by binding flagellin. Therefore it is highly expressed in DCs from the small intestine. When detecting flagellated bacteria these DCs induce B-cells to differentiate into IgA producing plasma cells [104].

TLR3, TLR7, TLR8 and TLR9 are activated by nucleic acids. TLR3 detects viral dsRNA, TLR7/8 viral ssRNA, TLR7 bacterial RNA and TLR9 unmethylated CpG motifs, a main feature of bacterial DNA. This activation leads to the induction of type I interferons and pro-inflammatory cytokines [118].

TLR10 is structurally related to TLR1. In humans it is functional but although several ligands have been proposed, up until now none of them was confirmed [119,120].

For a full list of TLR ligands please see Kawai *et al.* [121], Schnare *et al.* [122] or Kumar *et al.* [123].

1.2.4 SIGNAL TRANSDUCERS

Sensing of environmental stimuli, including MAMPs, PAMPs or DAMPs, leads to receptor activation and subsequent triggering of signaling cascades that transmit to the nucleus of the cell and enable an adjustment of cellular responses. The transduction of the extracellular stimulus to the nucleus relies on signal transducer molecules which are predominantly kinases. Proteinkinases are special enzymes that are able to phosphorylate and thereby activate other proteins including other kinases. Through this subsequent activation of different proteinkinases a signal can be transmitted from the cell surface to the nucleus. Furthermore, the 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.

1.2.4.1 MITOGEN ACTIVATED PROTEIN KINASES

Mitogen activated protein kinases (MAPKs) are a family of highly conserved proteins found in all eukaryotes. They link receptor activation to induction of gene expression [124] thereby controlling various fundamental cellular processes such as proliferation [125,126], differentiation, cytoskeletal reorganization [127,128], survival, apoptosis [129,130] and inflammation [131–133]. MAPK are serine/threonine-specific kinases that are themselves activated by phosphorylation of threonine or tyrosine (Thr-Xxx-Tyr phosphorylation motif) [134]. The MAPKs are divided into three subgroups, extracellular-signal regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 MAPK.

1.2.4.2 PHOSPHATIDYLINOSITOL KINASE

Phosphatidylinositol kinase (PI3K) is a lipid kinase that catalyzes the transfer of a γ-phosphate residue of ATP to the D3 position of phosphoinositides thereby producing three lipid products.

Phosphatidylinositol (PI) → PI3-phosphate (PIP) → PI3,4-bisphosphate (PIP2) → PI3,4,5-trisphosphate (PIP3) [135]

The resulting product PIP3 is the active form and is anchored to the plasma membrane. It targets AKT, Bruton's tyrosine kinase (BTK), phosphoinositide-dependent kinase (PDK) and others which then accumulate in close proximity to the membrane [136] and target several cytosolic signaling molecules and transcription factors. There are three major classes of PI3Ks, class I, II and III. The subtype IA consists of a heterodimer made up from a regulatory subunit (p85) and a catalytic subunit (p110) and is thought to be the major subtype responsible for convergence of PIP2 to PIP3 *in vivo* [137]. Negative regulation of this pathway is achieved by phosphatase and tensin homolog (PTEN) and SH2-containing inositol phosphatase (SHIP). Both molecules catalyze dephosphorylation of PIP3 to yield PIP2.

1.2.5 TRANSCRIPTION FACTORS

Transcription factors are found in all living organisms. They are proteins that enhance or suppress transcription of their target genes. It was estimated that about 10% of genes in the human genome code for transcription factors [138].

They function as regulatory elements by binding DNA and thereby up- or down-regulating their specific 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). With the DBDs the protein attaches to specific sequences of the DNA that are adjacent to the target gene of the respective transcription factor. There are various families of DBDs including but not limited to helix-turn-helix [139], zinc fingers [140], basic leucine zipper [141], basic helix-loop-helix [142] and homeodomain proteins [143]. The TAD consists of binding sites for other transcriptional regulators and the SSD senses external signals and transmits these to the transcription complex. Transcription factors regulate gene expression by many different mechanisms [144] such as stabilization or blockage of the binding of RNA polymerase to DNA or the recruitment of co-activator or co-repressor proteins to the transcription factor-DNA-complex [145]. Furthermore, they catalyze histone acetylation or deacetylation either directly or by recruiting proteins with catalytic activity to the DNA [146]. Transcription factors and their regulatory functions on DNA level play a very important role in many cellular processes. They are involved in basal transcription regulation [147,148] and in differential enhancement of transcription where they are crucial for development [149], response to extra- and intracellular signals [150–152], cell cycle control [153] and pathogenesis [154]. Transcription factors can bind different related sequences thus a tight regulation of binding and action of transcription factors is needed. This regulation is achieved by various mechanisms such as transcription factor localization. Most transcription factors are “stored” in the cytoplasm and can only translocate to the nucleus when activated [155]. Further, DNA is not always accessible to transcription factors since it is organized in nucleosomes with the help of DNA histones. As most transcription factors need co-factors to successfully recruit the preinitiation complex and RNA polymerase to the DNA, the availability of these co-factors is important in regulation of transcription factor activity. Additional sequence recognition specificity can be found in transcription factors with more than one DBD or through dimerization of two transcription factors that bind to two DNA sequences in close proximity to each other.

1.2.5.1 NF-κB

NF-κB is a specific transcription factor that is found in almost all cells and tissues. It was discovered in 1986 by Sen *et al.* [156]. It controls not only transcription of DNA, cytokine production and cell survival but its activation is also critical for mounting an inflammatory reaction. Structurally there are two classes of NF-κB proteins, class I and class II. The C-terminus of class I proteins has a transrepression activity [157,158] while the C-terminus of class II proteins in contrast has transactivational functions. Proteins belonging to class I are NF-κB1 and NF-κB2, Whereas class II consist of RelA (p65), RelB and c-Rel [159]. These five proteins share a Rel homology domain in their N-terminus and a DBD in their C-terminus [160]. The DBD also functions a dimerization interface as any two of these proteins can interact and form homo- or heterodimers that are able to bind DNA and alter gene transcription. Although many different dimers are possible, usually dimerization of a class I and a class II protein can be observed, with the most classical combination being NF-κB1 and RelA [160].

1.2.5.2 KRÜPPEL-LIKE FACTOR 4

Krüppel-like factor 4 (KLF4) is one of a family of 21 zinc-finger containing transcription factors identified in mammals so far [161]. The zinc-finger proteins are highly conserved among all KLF proteins and they are interspersed with an additional 7aa sequence in between each zinc-finger domain [162]. Three zinc-finger domains are usually found at the C-terminus, they are binding the DNA while the N-terminus is involved in transcriptional regulation and protein/protein interactions. The DBD of the KLF transcription factors is a homologue to the Krüppel protein of *D. melanogaster*, which is needed for correct segmentation of the fly [163]. KLF4 was first identified in 1996 [164] as important factor for the establishment of the skin barrier [165]. Further research revealed that it is involved in regulation of many cellular processes including growth, differentiation, proliferation and apoptosis [166]. In addition, it plays an important role in modulating the inflammatory response in different cell types [163,167,168].

1.2.6 TLR-RELATED SIGNALING PATHWAYS

TLRs play a critical role in the early innate immune response to invading pathogens by sensing MAMPs and PAMPs. Upon ligand binding TLRs initiate a signaling cascade that ultimately triggers an inflammatory response and activation of the cell. The specific outcome of TLR activation depends on the TLR as well as the cell type involved. Macrophages and neutrophils for example enhance phagocytosis and increase the oxidative burst which leads to a rapid uptake or killing of pathogens. Resident macrophages, in contrast, primarily respond with the secretion of chemokines to recruit further immune cells to the site of infection [169]. DCs and macrophages activated by TLR signaling induce pro-inflammatory cytokines that are, for example, important for the regulation of the host's acute phase response [57]. The regulation of the TLR signaling pathways is especially important since an overstimulation could lead to an exaggerated immune response with very high levels of pro-inflammatory cytokines causing septic shock and eventually death of the host. Furthermore, chronically activated TLRs are associated with the development of autoimmune disease in genetically predisposed individuals [170]. Thus regulation of TLR signaling pathways is crucial for a balanced immune response and containment of inflammation as well as infection.

1.2.6.1 INITIATION OF TLR SIGNALING

On a molecular level signaling via TLRs occurs when the LRR domain of the receptor forms a complex with its ligand. The binding of the respective ligand initiates recruitment of adapter molecules through interaction of the C-terminal TIR-domains of the TLR and the adapter molecule. There are six known adapter molecules: myeloid differentiation primary response 88 (MyD88), TIR domain-containing adapter inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM), TIR domain-containing adapter protein (TIRAP), sterile alpha and HEAT/Armadillo motif (SARM) and B-cell adapter for phosphatidylinositol-4,5-bisphosphate 3-kinase (BCAP) [171–173]. All TLRs utilize MyD88 with the exception of TLR3 that uses TRIF for signaling. In contrast to all other TLRs, TLR4 is the only receptor that can either signal through MyD88 or through TRIF. The adaptor proteins TRAM and TIRAP function as shuttling molecules, TIRAP mediating recruitment of MyD88 and TRAM mediating recruitment of

TRIF. TRIF-mediated signaling via TLR3 and TLR4 can be suppressed in human cells by the SARM protein [173,174]. The adapter molecule BCAP has a functional N-terminal TIR homology domain and links TLR signaling to activation of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) [175]. PI3K and its substrate protein kinase B (AKT) are implicated by many studies to be involved in regulation of TLR signaling [176–181]. In addition to PI3K, mitogen-activated protein kinases (MAPKs) are activated through TLR-mediated signaling pathways [169]. Ultimately TLR activation leads to activation of transcription factors. All TLRs activate AP-1 and NF-κB, the latter via activation of the IKK complex and subsequent degradation of the NF-κB inhibitor IκB. In contrast, activation of interferon regulatory factor (IRF) 3 and IRF7 is regulated solely by differential use of the different signaling adapters and the cellular compartment where the signaling originates [169]. Negative regulators of TLR signaling include IL1 receptor-associated kinase (IRAK) 3, single immunoglobulin IL1R-related molecule (SIGIRR) and tumor necrosis factor alpha-induced protein 3 (A20) [182–185]. For a graphic representation of MyD88-dependent TLR signaling please see Fig.1.

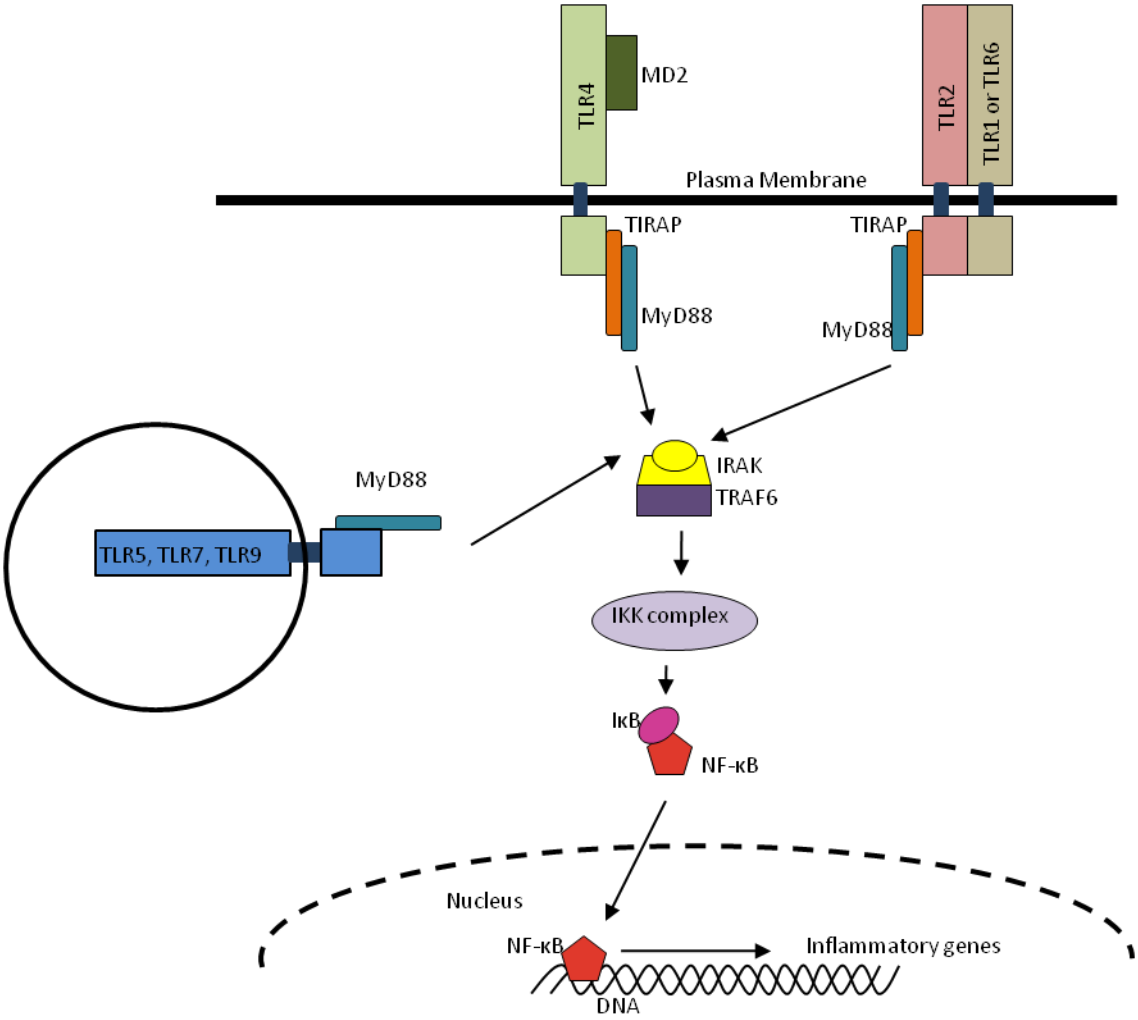

FIGURE 1: MyD88 dependent TLR signaling pathways. MyD88 associates with the cytosolic TIR domain of TLRs via its own C-terminal TIR domain and recruits IRAK to the receptor upon ligand binding. IRAK recruitment is followed by activation of TNF receptor-associated factor 6 (TRAF6) leading to the activation of the IKK complex. Upon activation of the IκB kinase (IKK) complex, IκB is phosphorylated and NF-κB is free to translocate into the nucleus, inducing expression of inflammatory genes. For further details on the IKK complex please refer to section 1.2.6.3.1 (Drawing is modified from Takeda and Akira, 2003 [186])

1.2.6.1 PI3K SIGNALING

Activation of PI3K is, among other stimuli, achieved by different TLR ligands [187] and its activity can be inhibited by several chemical inhibitors, the most prominent being Ly294002 and Wortmannin. There are reports showing that PI3K is activated by TLR2, TLR3, TLR4, TLR5 and TLR9 and that the p85 subunit binds directly to the receptors [188,189]. Furthermore, Rhee *et al.* could show that MyD88 associates with p85 in response to LPS and flagellin [190]. In

addition, MyD88 deficient macrophages have been shown to be defective for AKT phosphorylation [175,191,192], the substrate of PI3K. Although MyD88 has been shown to associate with p85 directly there is another adapter molecule involved in PI3K activation, called BCAP. BCAP binds MyD88 and TIRAP [175] and is critical in mediating PI3K/AKT activation in TLR stimulated cells [169,193,194]. BCAP acts most probably downstream of MyD88 and TIRAP and does not interact with the TLR itself. In macrophages phosphorylation of BCAP is a prerequisite for PI3K recruitment [195–197] (Fig.2).

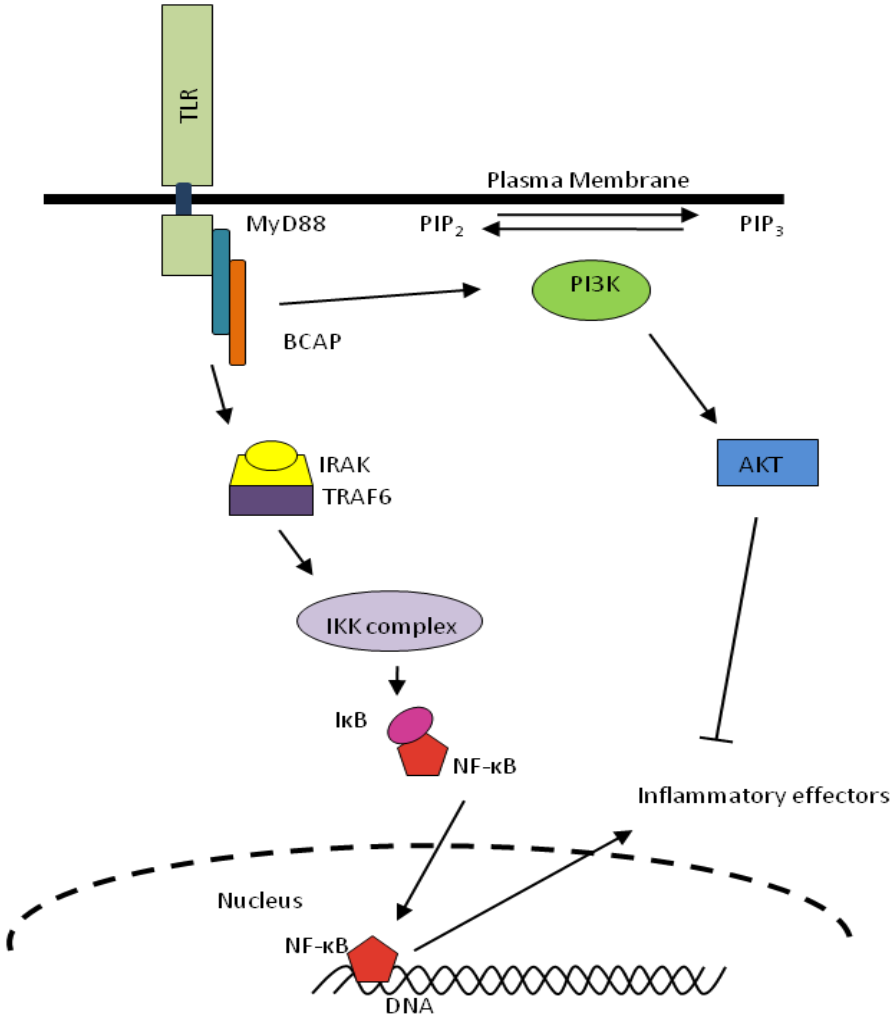

FIGURE 2: TLR-dependent PI3K signaling. Upon stimulation of TLRs MyD88 is recruited to the receptor and mediates the assembly of a complex of IRAK proteins. This complex engages TRAF6 which ultimately activates NF-κB. In addition to NF-κB activation, TLR signaling also leads to activation of PI3K. BCAP binds MyD88 and recruits the PI3K complex which then phosphorylates its substrate PIP2 yielding PIP3. Increased PIP3 activates AKT which is then able to act on its targets. (Drawing is modified from Troutman and Bazan, 2012 [169]).

Apart from TLR signaling it has been shown that PI3K is necessary for phagocytosis of pathogens [198–200] and apoptotic cells [201] as well as engulfment of IgG coated particles [200]. However, the complete underlying signaling pathway is not yet fully understood. Furthermore, there are conflicting results on PI3K signaling being pro- or anti-inflammatory. While Yum *et al.* showed a decreased production of IL1β and TNFα in lung neutrophils when PI3K function was inhibited [202], the same inhibition in DCs and macrophages caused an increased TLR-dependent activation of NF-κB [203,204]. In contrast to this, NF-κB down-regulation by blockage of PI3K has been reported in HeLa, U937, Jurkat and H4 glioma cells [205], as well as in B- [206,207] and T-cells [208,209]. In opposition to these results, it was reported that inhibition of PI3K in monocytes and PBMCs suppresses IL10 but enhances IL12 production when cells were stimulated with ligands for TLR2, TLR4, TLR5 or TLR9 [210,211]. In addition, it was shown in a mouse model for pneumococcal pneumonia that active PI3K and therefore a high amount of PIP3 lead to increased levels of IL10 whereas TNFα and IL6 expression was decreased [212]. In conclusion, there are reports stating that PI3K contributes to NF-κB activation and reports indicating that PI3K inhibits the inflammatory response [178,180,187]. But most probably the mechanism involved is at least cell type specific [213]. Nevertheless, activation of the PI3K signaling cascade seems to represents a major branch of TLR-mediated signaling and thereby contributes to a variety of cellular outcomes, including regulation of the inflammatory response, cell proliferation and cell survival.

1.2.6.2 MAPK SIGNALING

In addition to PI3K, mitogen-activated protein kinases (MAPKs) are activated through TLR-mediated signaling pathways. Signaling and activation through MAPK is organized in a generalized fashion. Upon receptor-dependent and receptor-independent stimulation a MAP-kinase kinase kinase (MAPKKK) phosphorylates a downstream MAP-kinase kinase (MAPKK). When activated the MAPKK in turn phosphorylates a downstream MAPK which then activates other molecules or transcription factors. An overvierw of the MAPK cascades is given in Fig.3.

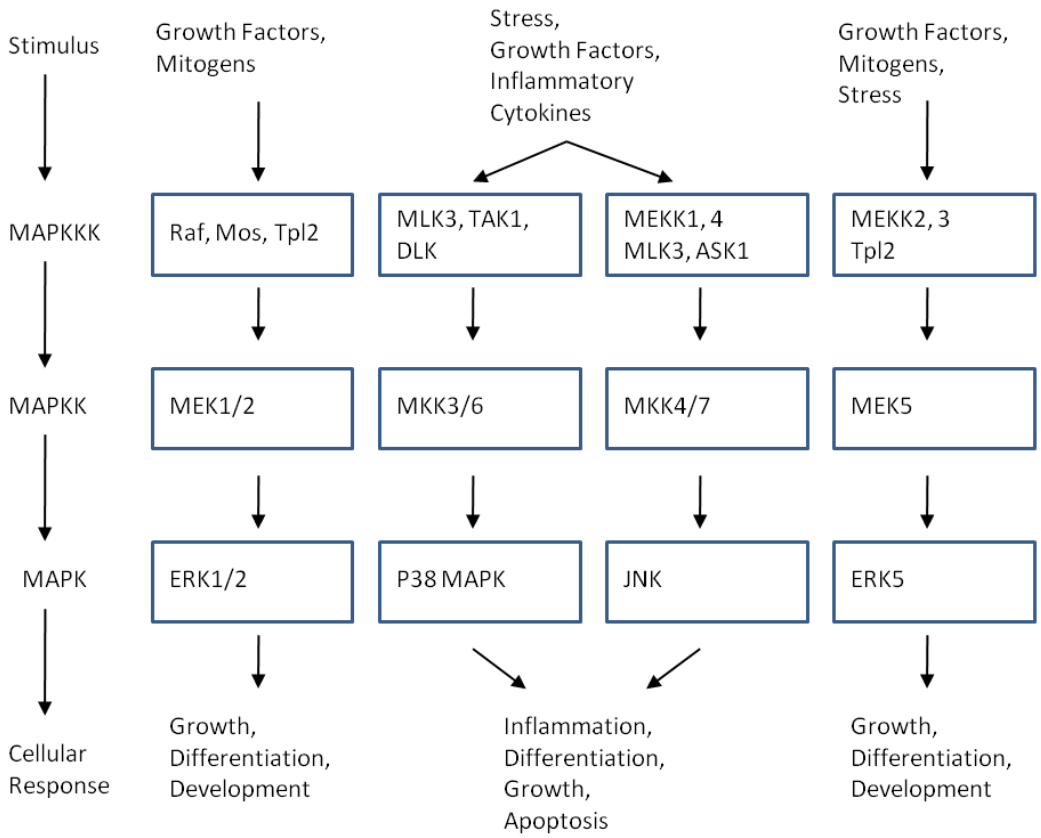

FIGURE 3: MAPK signaling cascades. Receptor dependent and –independent stimulation of MAPKKK leads to phosphorylation of MAPKK which then in turn activates a MAPK by phosphorylation. MAPK activate other signaling molecules or transcription factors and lead to varying cellular responses. (Flow chart modified from cellsignal.com)

Activation of ERK is achieved when proto-oncogene serine/threonine-protein kinase (Raf) phoshorylates MEK1/2 [214] which then transfers a phosphate residue to ERKs tyrosine and threonine residues [215]. In this cascade MEK1/2 is highly selective and will only activate ERK1/2 [216]. Substrates of ERK are found in the cytoplasm as well as in the nucleus, it regulates proliferation, differentiation and survival but also plays a role in inflammation [217]. JNK and p38 MAPK are activated by pro-inflammatory cytokines IL1 and TNFα or by stress related factors including heat shock, endotoxins and ultraviolet (UV) light [218,219]. Their signaling is involved in inflammatory and apoptotic processes as well as in regulation of nitric oxide synthase (NOS) 2 and cyclooxygenase-2 (COX2) [220,221].

Little is known about the inactivation of MAPKs, but in general, inactivation occurs by dephosphorylation of the proteins. A process catalyzed by phosphatases. Activation of MAPKs by *S. pneumoniae* has already been shown in epithelial cells of mouse and human origin [222,223] and in mouse lungs [224]. Apart from their pro-inflammatory activation it has been shown that MAPKs play a role in TLR-dependent anti-inflammation as well [225–227].

1.2.6.3 ROLE AND ACTIVATION OF TRANSCRIPTION FACTORS

1.2.6.3.1 NF-κB

NF-κB is activated by TLR-mediated signaling in many immune cells. It contributes to the pro-inflammatory response by inducing different inflammatory related genes. The activation of NF-κB is a multistep process. Upon stimulation of TLRs, MyD88 or TRIF are recruited to the receptors leading to NF-κB activation [104]. The MyD88-dependent pathway is associated with early activation of NF-κB while the TRIF-dependent pathway is important for the late phase activation of the transcription factor [228]. After recruitment of the proximal adapters, MyD88 engages a complex of IRAK1, IRAK2 and IRAK4 through interaction with their death domains. The IRAK containing complex recruits TRAF6 and TRAF6 mediates the recruitment of TAK binding protein (TAB) 2 and TAB3 which regulate the TGFβ activated kinase 1 (TAK1) [229]. Upon activation TAK1 phosphorylates IκB kinase (IKK) beta which is part of the IκB kinase complex together with IKKα and NF-κB essential modulator (NEMO). The IKK complex then phosphorylates IκBα which is now targeted for proteasome degradation and thereby liberating NF-κB. NF-κB translocates to the nucleus and acts upon its target genes [230] (Please also see Figure 1). Furthermore, NF-κB itself induces IκBα thereby turning off its own activation through a negative feedback loop [231].

1.2.6.3.2 KRÜPPEL-LIKE FACTOR 4

KLF4 is induced in macrophages upon stimulation with LPS, INFγ and TNFα [168] whereas in human endothelial cells only INFγ and TNFα increased KLF4 expression [232]. Induction is dependent on TLR2 in macrophages [233] and on TLR4 in endothelial cells [232]. Interestingly there are controversial results regarding KLF4 being pro- or anti-inflammatory. Although induction of this

transcription factor is connected to pro-inflammatory stimuli, its effects seem to be bidirectional. While there are reports clearly showing anti-inflammatory effects of KLF4 [232,234] there are also researchers who found pro-inflammatory functions of said transcription factor [168,235]. KLF4 was shown to inhibit NF-κB [163] and to induce anti-inflammatory factors such as nitric oxidase (eNOS) [232]. Furthermore, KLF4 induces IL10 in lung epithelial cells stimulated with bacterial DNA [236]. Tetreault *et al.* on the contrary found that overexpression of KLF4 in mouse esophageal cells lead to an induction of pro-inflammatory cytokines [237] and Feinberg *et al.* could show that KLF4 mediates the pro-inflammatory effects of INFγ and TNFα in macrophages through interaction with p65, a member of the NF-κB transcription factor family [168]. These differing reports on KLF4 function testify to a complex role of this transcription factor in inflammation and disease.

1.2.7 CYTOKINES AND CHEMOKINES

Cytokines and chemokines are secreted proteins that regulate and determine the nature of immune responses. Depending on the produced cytokines an immune response develops to be cytotoxic, humoral, cell-mediated or allergic [238]. A cascade of responses is activated by cytokines and often several cytokines are needed to orchestrate optimal function. Here the focus lies on pro-inflammatory cytokines and chemokines as they are produced in response to *S. pneumoniae*.

IL8 is a chemokine that recruits neutrophils, basophils and T-cells to the site of infection. IL8 can be secreted by any cells with TLRs that are involved in the innate immune response. Usually, it is the macrophages that see an antigen first and are the first cells to release IL8.

IL1β is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. The local effects of this cytokine include activation of lymphocytes and the vascular endothelium thereby inducing local tissue damage to increase access of effector cells to the infected tissue.

IL6 promotes lymphocyte activation and antibody production whereas IL12 activates natural killer (NK) cells and induces the differentiation of cluster of differentiation 4 positive ($CD4^+$) cells into Th1 cells [57].

TNFα activates the vascular endothelium and increases vascular permeability [59] leading to increased entry of immunoglobulin (Ig) G antibodies, complement factors and cells to tissues and increases the drainage of lymph into lymph nodes. TNFα is especially important for the containment of infection when clearance of the pathogen is not possible. While the local effects of TNFα are clearly beneficial to the host by successfully limiting the infection, its effects become catastrophic if pathogens overcome the initial defense mechanisms and enter the blood stream. Produced by organ specific macrophages, systemic TNFα causes vasodilation resulting in a loss of blood pressure. In addition, there is induction of increased vascular permeability followed by loss of blood plasma volume. Furthermore, it triggers intravascular coagulation causing many small blood vessels to clot [239,240]. Clotting is accompanied by massive consumption of clotting proteins and therefore the patient's ability to clot blood appropriately is lost. Eventually all these effects cause septic shock which leads to organ failure and thus has a very high mortality rate.

Apart from local effects many cytokines also have long range functions that contribute to host defense. The elevation of body temperature, mainly caused by TNFα, IL1β and IL6 is one example. Fever is generally beneficial to the host since most pathogens grow better at lower temperatures while the adaptive immune response is more intense when body temperatures are elevated. In addition, these three cytokines act on hepatocytes and induce the acute-phase response [241]. In the acute-phase response hepatocytes shift protein synthesis and secretion of different plasma proteins resulting in some plasma protein levels to go up whereas others go down [242]. The induced proteins are called acute-phase proteins and several mimic antibody function but with a broad specificity for PAMPs and only dependent on cytokines for production [243]. Cytokines also induce leukocytosis, an increase in circulating neutrophils. These circulating neutrophils are then recruited to the site of infection by different chemokines. The first chemokine to be characterized was IL8. All chemokines are related in their amino acid sequence and their receptors are all integral

membrane proteins with seven membrane spanning helices. Signaling of these receptors is done via G-coupled proteins. There are mainly two groups of chemokines: CC chemokines with two adjacent cysteins near the amino terminus and CXC chemokines where the two cysteins are separated by a single amino acid. The corresponding receptors are named CCR1-9 and CXCR1-6 [244]. Chemokines function as chemoattractants for leukocytes such as monocytes, neutrophils and other effector cells from the blood with neutrophils usually being the first to arrive in large numbers at the site of infection, whereas monocytes and DCs are recruited in later stages of disease [245]. For recruitment of leukocytes chemokines trigger a conformational change in leukocyte integrins causing the cell to stably bind to the endothelium [246,247]. To exit the blood vessel wall, the leukocytes squeeze between the endothelial cells and are directed to the site of infection by a chemokine gradient bound to extracellular matrix [248,249]. The innate immune response is important in defense against pathogens because it can either clear or contain the infection until an adaptive immune response develops. In contrast to innate immunity, adaptive immunity targets pathogens with greater precision and specificity but needs at least days to develop.

1.3 DEFENSINS

Defensins are antimicrobial peptides that are part of the innate host response in almost all multicellular organisms, including plants, insects, amphibians, birds and mammals [250,251]. They exhibit a broad spectrum antimicrobial activity against gram-positive and gram-negative bacteria, enveloped viruses [252–257] and fungi including *C. albicans* [258]. Defensins consist of 30-40 amino acids and have a molecular weight of 3-6kDa [259,260]. Their three dimensional structure displays three antiparallel β-sheets [261,262] that are stabilized by three intramolecular disulfide bonds [251,252]. They are amphiphilic, meaning that they possess cationic and hydrophobic surfaces [258,263] which are a prerequisite for the disruption of biological membranes [260]. They are cationic with a net charge of +2 or higher [264]. The positive charge makes them highly selective for microbial membranes since bacterial and mammalian membranes differ extensively in their composition. Human membranes are rich in cholesterol while bacterial membranes do not display any sterol at all [265]. Furthermore, human membranes consist of zwitterionic lipids such as phosphatidylcholine and sphingomyelin which render the membrane essentially uncharged [266]. Bacterial membranes on the other hand are rich in anionic lipids such as LPS, peptidoglycan and lipoteichoic acid [251,264,267]. The defensins competitively displace divalent cations (Ca^{2+} and Mg^{2+}) in the membrane, thereby disrupting the barrier properties and facilitating the aggregation of antimicrobial peptides to multimeric pores [252,268,269]. The so formed channels increase membrane permeability resulting in an efflux of potassium that leads to membrane depolarization and loss of membrane integrity eventually [270–273]. This mechanism of membrane permeabilization is relatively unspecific, making the incident of bacterial resistance rare.

Storage of defensins is done as proproteins, meaning that one proteolytic step is necessary to generate the mature peptide [269] but the mechanisms of defensin storage, processing and release are not yet understood. Apart from their antimicrobial function it has been shown that they modulate cytokine responses [274] and are able to bind to chemokine receptors CCR6 [275,276] and CCR2 [277] thereby initiating chemotactic activity in neutrophils, DCs, monocytes and T-cells [275,278,279].

All defensin genes are tightly clustered to a 1Mb region on chromosome 8p22-8p23 and have two or more exons [259]. Their close proximity on DNA level is an indicator that all genes may have developed from one common ancestral gene [280–282]. Defensins are a multigene family and their main gene cluster has been shown to be copy number variable [283] with copy numbers ranging from 2-12. The copy numbers are distributed normally among the population with the most abundant copy number all over the world being 4 [284]. Apparently there is evolutionary pressure on defensin copy number since an increased number of gene copies is associated with psoriasis whereas a decreased copy number facilitates Crohn's disease [285,286]. To add a further layer of complexity there is sequence variation in between the gene copies of the defensin cluster [287,288].

Defensins are classified into three subfamilies based on the arrangement of the intramolecular disulfide bonds, α-defensins, β-defensins and θ-defensins [289]. α- and β-defensins can be found in humans whereas θ-defensins are only present in old-world primates with the exception of human, gorilla, bonobo, and chimpanzee [290,291]. All of the defensins display a similar three dimensional structure with β-sheets and three intramolecular disulfide bonds [262,268,292,293]. Six α-defensins have been characterized so far, they are predominantly expressed in neutrophils and Paneth cells [294,295]. All α-defensins are arginine-rich peptides with a length of 29-35 amino acids [296]. They are synthesized as 93-100 amino acid prepropeptides. They consist of a 19 amino acid signal peptide and a 41-51 amino acid long pro-segment [259]. α-defensins make up 5-7% of total protein in human neutrophils with especially high concentrations in azurophilic granules and phagocytic vacuoles [268]. Since they are mainly found in neutrophils the α-defensins are also named human neutrophil peptides (HNPs). In humans there are 6 HNPs [297,298] and while HNP-1, HNP-3 and HNP-4 are found in neutrophils, HNP-5 and HNP-6 are expressed by Paneth cells [299]. HNP-2 is a truncated defensin that associates to form amphiphilic homodimers [299,300]. HNPs function by directly killing microorganisms intra- or extracellularly [254,301]. HNP-1 and HNP-2 are the most potent of the α-defensins, they are capable of eliminating gram-positive and gram-negative bacteria as well as enveloped viruses at a concentration of 100µg/ml [250,302,303].

In humans six different β-defensins (hBD) have been identified so far although genome based studies suggest at least 28 different hBDs. They are found in epithelial tissue, neutrophils, blood plasma and urine [259,304–307]. β-defensins consist of about 35 amino acids and are synthesized as prepropeptides with 64-68 amino acids [259]. Human β-defensin 1 (hBD-1) was discovered in 1995 and is encoded by the *DEFB1* gene [308]. In contrast to all other hBDs, hBD-1 is expressed constitutively by keratinocytes and other epithelial cells of the respiratory, intestinal and urinary tract [309–312]. It has microbicidal activity against gram-negative bacteria at concentrations of 60-500µg/ml [250] thereby contributing to controlling the microbial flora on epithelial surfaces. Since hBD-1 is expressed constitutively, its predominant role is the defense against pathogens in the absence of inflammation [313]. In contrast, hBD-2, hBD-3 and hBD-4 are inducible in epithelial cells and some leukocytes by different bacterial factors and pro-inflammatory mediators [305,314–316]. hBD-5 and hBD-6 have been identified in 2002, they are localized in the epididymis [307]. Expression of hBDs is highly specific; induction of the peptides depends on a multitude of factors, including the bacterial species, the stimulation conditions and the cell type as well as the state of the stimulated cell [317,318].

1.3.1 HUMAN BETA DEFENSIN 2

Human β-defensin 2 (hBD-2) was discovered in 1997 in lesional skin. It is a 41 residue peptide with homology to hBD-1 [319]. At concentrations of ≤2.4 mM it is monomeric [254] and in humans it is encoded by the *DEFB4* gene on chromosome 8 [320]. Its structure matches that of all defensins, described in more detail above (Section 1.3) and in Fig.4.

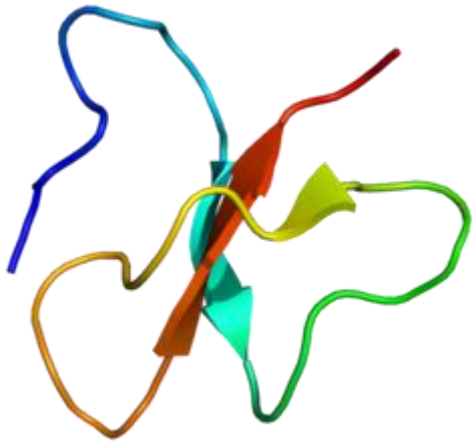

FIGURE 4: Structure of hBD-2. hBD-2 is an amphiphilic peptide with a nonuniform surface distribution of positive charges. It consits of three antiparallel β-sheets and is stabilized by three intramolecular disulfide bonds. (Source: https://en.wikipedia.org/wiki/Beta-defensin_2)

hBD-2 is primarily expressed in keratinocytes, gingival mucosa and epithelium of the skin, the respiratory and the intestinal tract [308,316,321,322]. hBD-2 is expressed at variable levels in healthy cells and is readily induced by IL1β, TNFα and LPS as well as by contact of cells with gram-negative and gram-positive bacteria or *C. albicans* [310,323–325]. Induction is dependent on TLR2, MAPK, NF-κB and AP-1 in different epithelial and keratinocyte cell lines [326–330]. hBD-2 has the ability to block interaction of cells with LPS thereby inhibiting LPS-induced TNFα production that is responsible for an inflammatory response [331]. Apart from that, hBD-2 is highly effective in eliminating gram-negative bacteria and *C. albicans*, with a LD_{90} at 10µg/ml and 25µg/ml respectively [316]. Reports regarding its effect against gram-positive bacteria vary, it was reported to have bacteriostatic [332] or bactericidal activity [333–335] or no effect at all [336]. However, increased expression of hBD-2 is associated with inflammatory diseases, thus it is highly expressed in lung epithelial cells of patients suffering from respiratory infections [313,322,337].

1.3.2 HUMAN BETA DEFENSIN 3

Human β-defensin 3 (hBD-3) was identified in 2001 independently by Harder *et al.* [332], Garcia *et al.* [338] and Jia *et al.* [339]. It is located on chromosome 8, around 15kb distant from hBD-2. Its structure consists of an N-terminal α-helix

followed by three antiparalell β-sheets that are stabilized by three disulfide bonds formed between six canonical cystein residues [340] (Fig.5).

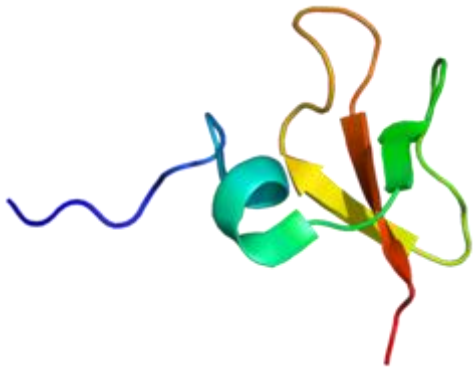

FIGURE 5: Structure of hBD-3. hBD-3 is an amphiphilic peptide with a nonuniform surface distribution of positive charges. It consits of three antiparallel β-sheets and is stabilized by three intramolecular disulfide bonds. (Source: https://en.wikipedia.org/wiki/DEFB103A)

It is synthesized as a 67 amino acid precursor preprotein and processed by proteolytic activation to a mature 45 amino acid peptide [341]. With its net charge of +11 in monomeric form it is highly cationic.

hBD-3 is bactericidal against gram-positive and gram-negative bacteria as well as *C. albicans* [332]. The minimal inhibitory concentration (MIC) ranges from 4-8µg/ml but the antimicrobial activity of hBD-3 is lowered significantly in medium containing 20% heat-inactivated serum [342]. It was shown that hBD-3 killed *S. pneumoniae* at a concentration of 6.25µg/ml [343] but it is not clear if this finding holds true for all *S. pneumoniae* serotypes since only one strain was tested in the aforementioned study. In general, the bactericidal effect of hBD-3 is due to the disruption of cell wall integrity [344]. A permeabilization of *Escherichia* (*E.*) *coli* cell membrane was observed within 20-30min after application of hBD-3. For gram-positive bacteria membrane disruption takes significantly longer [345]. On host cells the cytotoxic effect of hBD-3 largely depends on the peptide concentration. At 10µg/ml hBD-3 has no effect on the viability of THP-1 cells but at 50µg/ml cell viability is decreased significantly [346]. In addition, effects of hBD-3 are not limited to elimination of microorganisms; similar to hBD-2, it is strongly cationic and thus can bind to

the anionic charges of LPS, thereby neutralizing its endotoxic activity [347]. Furthermore, it directly binds to chemokine receptors CCR6 and CCR2, inducing chemotaxis in neutrophils, monocytes and human embryonic kidney (HEK) 293 cells at concentrations of 10-100ng/ml [348].

Expression of hBD-3 is inducible by IFNγ, gram-positive and gram-negative bacteria as well as LPS [349]. There are reports showing induction of hBD-3 via TLR2 [350–352] while others found TLR4 [353,354] to be involved. Induction was observed in keratinocytes, tonsil tissue and epithelia of the respiratory, gastrointestinal and genitourinary tracts [338,355,356]. Garcia *et al.* [338] even described expression of hBD-3 in non-epithelial tissue such as skeletal muscle cells and leukocytes. The induction of hBD-3 is dependent on PI3K [357] and independent of NF-κB [349,358] but reports regarding the peptide's role in inflammation are controversial. Although it was considered pro-inflammatory in some studies [359,360], Semple *et al.* could show that hBD-3 inhibits IL6 and TNFα in human macrophages by blocking liberation of NF-κB [361]. Therefore, hBD-3 modulates and may even attenuate an ongoing immune response. In conclusion, hBD-3 seems to be a multifunctional molecule with a prominent role in inflammation regulation. These multifunctional roles of hBD-3 most certainly need to be further investigated.

1.4 INNATE IMMUNE INTERACTION WITH *S. PNEUMONIAE*

1.4.1 RECOGNITION OF *S. PNEUMONIAE*

When *S. pneumoniae* is not effectively cleared from the nasopharynx it can progress into the lower airway and cause invasive disease. When it reaches the alveoli of the host it is first recognized by alveolar macrophages and DCs. The recognition of *S. pneumoniae* by the innate immune system is largely dependent on PRRs. It has been shown that TLR2, TLR4 and TLR9 play a significant role in detection of the bacteria [117,362,363]. Bacterial cell wall components such as lipoteichoic acid and lipopeptides are sensed by TLR2 [364–366], leading to the induction of pro-inflammatory cytokines such as TNFα and IL6 in addition to the chemokine IL8 via the activation of NF-κB [362,367]. Furthermore, TLR2 involvement in recognition of pneumococci has been analyzed *in vivo* in different mouse models. Mice deficient for TLR2 ($TLR2^{-/-}$) were found to lack the ability to efficiently clear colonizing streptococci from the nasopharynx whereas wild type (wt) mice showed no such impairment [368]. In a model of *S. pneumoniae* induced meningitis $TLR2^{-/-}$ mice were more prone to invasive disease than the wt control animals, had an increased bacterial burden and a higher mortality rate [369,370] while susceptibility to pneumococcal pneumonia was only moderately increased in $TLR2^{-/-}$ mice [362]. Interestingly, infection with PLY deficient strains of *S. pneumoniae* led to elevated mortality rates in $TLR2^{-/-}$ mice, suggesting that in the presence of PLY, detection of pneumococci by TLR2 and TLR4 are somewhat redundant whereas TLR4 signaling becomes especially important in TLR2 deficiency where it compensates for the missing receptor [371].

The role of TLR4 signaling in pneumococcal pneumonia has been studied extensively *in vitro* and *in vivo* but still remains controversial. It has been shown that PLY is detected by TLR4 leading to the production of cytokines [117,372] and that $TLR4^{-/-}$ mice have a higher risk of invasive disease and elevated bacterial numbers in the nasopharynx [117]. Furthermore, bacterial replication in these mice was enhanced during early infection time points when compared to control animals and $TLR4^{-/-}$ mice showed a reduced rate of survival in a model of pneumococcal pneumonia [373]. In contrast to these

findings, van Rossum *et al.* found TLR4 not necessary for the effective clearance of bacterial colonization in mice [368] and in addition, it only plays a limited role in sepsis and meningitis [374,375]. Moreover, it was found that *S. pneumoniae* is able to induce cytokine production independent of TLR4 [51]. Finally, the mechanism of TLR4 activation may include recognition of DAMPs released during lung injury as could be facilitated by PLY, rendering TLR4 signaling only indirectly dependent on the bacterial exotoxin [116]. All these data suggest a role for TLR4 in pneumococcal pneumonia although the molecules and pathways involved still require further investigation.

Another receptor important for recognition of *S. pneumoniae* is the intracellular TLR9. As already mentioned above, this receptor's ligand is CpG-rich, unmethylated bacterial DNA. TLR9 deficient mice had reduced survival rates and impaired bacterial clearance when infected with *S. pneumoniae*. However, the cytokine levels were the same as in the wt control animals [363]. All TLRs mentioned in this section are able to utilize MyD88 for signaling. Thus, mice lacking this very important adapter molecule (MyD88$^{-/-}$) are highly susceptible to infections with *S. pneumoniae*. It was found that MyD88$^{-/-}$ mice had decreased cytokine production while at the same time mortality rates and bacterial loads were significantly increased in models of pneumonia, sepsis and meningitis [376–378]. Compared to the severe phenotype of MyD88$^{-/-}$ mice, the single TLR knockout mice displayed only a mild phenotype which might be due to redundancies in TLR signaling cascades that are needed to ensure a robust innate immune response even in the absence of single receptors or individual receptor ligands.

1.4.2 RESPONSE TO *S. PNEUMONIAE*

Detection of *S. pneumoniae* by PRRs leads to the production of pro-inflammatory cytokines and chemokines including TNFα, IL1β, IL6, and IL8. The chemokines directly stimulate recruitment of neutrophils and monocytes/macrophages to the site of infection [9,379]. This recruitment of innate immune cells is further enhanced by a subsequent Th17 response developed by CD4^{+} and γδ T-cells [380,381].

Neutrophils have been shown to be important for clearance of pneumococci from the airway [382]. They exhibit various killing mechanisms including the release of granules laden with antimicrobial peptides (HNPs) and proteases as well as formation of NETs. Apart from neutrophils, clearance of bacteria largely depends on recruitment of monocytes into the airway lumen. This recruitment is dependent on a Th17 response and activation of CCR2 [383,384]. Furthermore, presentation of antigens by DCs and macrophages is crucial for activation of NK cells, Th1 cells, cytotoxic T-cells and B-cells. Although an antibody response is needed for long time immunization, the development of anti-capsular antibodies does not clear the commensal bacteria from the nasopharynx [385]. However, opsonization of pathogens with antibodies enhances phagocytosis by neutrophils and macrophages through Fc-receptor binding and activation. Phagocytosis is the dominant mechanism for macrophages to eliminate bacteria, either by fusion of the phagosome with the phagolysome which contains lysozyme, proteases and antimicrobial peptides or, when their capacity for bacterial uptake is exhausted, by initiation of apoptosis. Since apoptosis involves production of ROS and NOS it aids killing of ingested pathogens [386,387]. The complement system is another important part of the innate immune system. When activated it facilitates neutrophil recruitment, opsonization and subsequent phagocytosis of bacteria and can directly kill pathogens via the membrane attack complex (MAC) [388]. During infection with *S. pneumoniae* this requires activation of the classical complement pathway [389]. Furthermore even epithelial and endothelial cells of the upper and lower respiratory tract respond to challenging pathogens. After detection via PRRs, they respond by secretion of the antimicrobial peptides hBD-2 and hBD-3 [337,352,356,390,391]. These peptides either directly kill the bacteria or induce chemotaxis in macrophages via their chemokine receptors CCR2 or CCR6 [348]. However, in high risk patients or by evasion of the immune defense by *S. pneumoniae* this response may not be enough to clear the infection [392] leading to enhanced inflammation of the lung and thereby facilitating bacterial dissemination and more severe disease [393,394]. In order to avoid excessive lung injury, anti-inflammatory factors and pathways need to be activated to limit tissue damage inflicted by the inflammatory response. Hence, the host's immune response needs a fine balancing of inflammatory and anti-

inflammatory factors and pathways since inflammation aids the host in clearing the infection while anti-inflammation is crucial for tissue preservation.

1.4.3 INNATE IMMUNE EVASION BY *S. PNEUMONIAE*

S. pneumoniae has evolved various mechanisms to escape the different defense responses applied by the host's innate immune system. By the release of PLY necrosis and apoptosis are induced in neutrophils [395] and macrophages [396,397] leading to the death of these cells whereas the release of extracellular DNAse helps the bacteria to escape from neutrophil NETs [398]. Phagocytosis of the pathogen is severely impaired by a thick capsule surrounding the bacteria which can be generated and altered quite quickly by phase variation [39,399]. In addition, the capsule reduces opsonization of the bacteria with complement factor C3b and by endopeptidase O (PepO) *S. pneumoniae* binds host plasminogen allowing activation and subsequent plasmin-mediated degradation of the remaining C3b on the bacterial surface [400]. Moreover, active plasmin generates fibrin which further prevents C3b deposition [401] and aids bacteria in adhesion to damaged endothelial surfaces [402]. Furthermore, *S. pneumoniae* modifies its cell wall peptidoglycan by O-acetylation, redering it resistant to lysozyme [403]. Evasion of lysozyme degradation by the bacterial cell wall components in the phagolysosome leads to evasion of PRR recognition and limited production of inflammatory cytokines [404,405]. To escape killing by defensins, *S. pneumoniae* decorates its cell surface with highly charged choline thereby increasing membrane charge and decreasing attraction of antimicrobial peptides [406]. Apart from modifying their capsule, *S. pneumoniae* sheds anionic capsular polysaccharides which have been shown to neutralize antimicrobial peptides before they are able to kill the bacteria via pore formation [407]. In addition to these mechanisms, *S. pneumoniae* encodes an efflux pump for macrolides that further confers protection from defensins [408,409]. All in all there is an ongoing co-evolution between *S. pneumoniae* and the host's immune system. While the immune system constantly tries to rid the body from pathogens, the pathogens develop mechanisms to remain and replicate in their ecological niche. Understanding of immune defense and corresponding immune evasion mechanisms are of utmost importance to improve the outcome of pneumococcal induced diseases.

1.5 AIM OF THIS STUDY

The innate immune system is the first line of defense against invading pathogens. Macrophages, the most important effector cells of innate immunity, sense pathogens via PRRs and start producing pro-inflammatory cytokines. At the same time they constrain the infection by phagocytosis and subsequent killing of invading bacteria. When the infection is cleared they take care of the inflammatory response and produce anti-inflammatory mediators to limit tissue damage and facilitate wound healing. In pneumococcal pneumonia a delicate balance of the immune response is especially important since tissue damage caused by inflammation without successful clearance of bacteria leads to more severe disease progression. Antimicrobial peptides have been shown to directly kill bacteria and to aid an immune response by inducing chemotaxis in macrophages. It has been shown that these peptides are produced by endothelial and epithelial cells as well as neutrophils. This study aims to broaden the understanding of hBD-2 and hBD-3 induction in human macrophages during *S. pneumoniae* infection. Therefore, not only the expression patterns of both peptides in infected human lung tissue and human macrophages but also the signaling pathways leading to induction of either defensin were analyzed. Furthermore, the bacterial factors involved in hBD-2 and hBD-3 induction were determined and the functions of both defensins in *S. pneumoniae* infected cells were investigated. This study closes a gap by exploiting if and when hBD-2 and hBD-3 are expressed in human macrophages challenged with *S. pneumoniae* and thereby improving the understanding of the host's immune response in pneumococcal pneumonia. In times of rising numbers of drug resistant *S. pneumoniae* strains this will hopefully contribute to the development of new therapeutic strategies to improve the outcome of *S. pneumoniae* infections.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 REAGENTS

1,4-Dithiothreitol	Merck, Darmstadt
β-Mercaptoethanol	Serva, Heidelberg
Acrylamide/Bisacrylamide 40%	Serva, Heidelberg
Ampuwa (RNase-free H_2O)	Fresenius Kabi, Bad Homburg
Antipain	Sigma Aldrich, Taufkirchen
APS	Serva, Heidelberg
Bacto Todd Hewitt Broth	BD Biosciences, New Jersey
Bacto yeast extract	BD Biosciences, New Jersey
Bradford-Reagent	Bio-Rad, München
Bromphenol blue	Pharmacia Biotech AB, Uppsala
BSA	Sigma-Aldrich, Taufkirchen
Cytochalasin D	Sigma-Aldrich, Taufkirchen
DMEM high glucose	Gibco, Darmstadt
DMSO	Sigma-Aldrich, Taufkirchen
EDTA	AppliChem, Heidelberg
Ethanol	Merck Millipore, Darmstadt
Erythromycin	Sigma Aldrich, Taufkirchen
Ficoll-Paque PLUS	GE Healthcare, München
FCS	GE Healthcare, München
G418 Sulfate/Geneticin	Fischer Scientific, Reinach
Glycerol	Carl Roth, Karlsruhe
Glycoblue	Ambion, Darmstadt
GM-CSF	Sigma-Aldrich, Taufkirchen
Isopropanol	Sigma-Aldrich, Taufkirchen
Kanamycin	Sigma-Aldrich, Taufkirchen

Leupeptin	Sigma Aldrich, Taufkirchen
Lipofectamine 2000	Roche, Mannheim
MALP-2	R&D Systems, Minneapolis
Methanol	Merck Millipore, Darmstadt
Nonidet P40	BioChemika, Duisburg
Odyssey Blocking Buffer	LI-COR Biotechnology, Bad Homburg
Opti-MEM	Gibco, Darmstadt
PBS	Gibco, Darmstadt
PEG-8000	Sigma-Aldrich, Taufkirchen
Penicillin G	Sigma-Aldrich, Taufkirchen
Penicillin/Streptomycin	GE Healthcare, München
Pepstatin	Sigma Aldrich, Taufkirchen
peqGOLD TriFast	PeqLab, Erlangen
Pierce ECL Western Blotting Substrate	Life Technologies, Darmstadt
PMA	Sigma Aldrich, Taufkirchen
PMSF	Sigma Aldrich, Taufkirchen
Ponceau-S	Sigma Aldrich, Taufkirchen
Puromycin	Sigma Aldrich, Taufkirchen
rhBD-2	Biomol, Hamburg
rhBD-3	Biomol, Hamburg
RNase-Free DNase	Promega, Madison
Roti-Aqua-P/C/I	Roth, Karlsruhe
RPMI 1640	Gibco, Darmstadt
SDS	Serva, Heidelberg
Sodiumchloride	Sigma Aldrich, Taufkirchen
Sodiumfluoride	Sigma Aldrich, Taufkirchen
Sodiumorthovanadat	Sigma Aldrich, Taufkirchen
Sodiumpyrophosphate	Sigma Aldrich, Taufkirchen
TaqMan Gene Expression Master Mix	Applied Biosystems, Darmstadt

TEMED	R&D Systems, Wiesbaden
Triton X-100	Boehringer, Ingelheim
Trizma Base	Sigma Aldrich, Taufkirchen
TRIzol	Invitrogen, Darmstadt
Trypsin	Gibco, Darmstadt
Tween-20	Sigma-Aldrich, Taufkirchen

2.1.1.1 MARKER

Precision Plus Protein Kaleidoscope	Bio-Rad, München

2.1.1.2 INHIBITORS

IKK-NBD	Enzo Life Sciences, Lörrach
Ly294002	InvivoGen, Toulouse
U0126	InvivoGen, Toulouse
SB202190	InvivoGen, Toulouse
SP600125	InvivoGen, Toulouse

2.1.2 INSTRUMENTS

7300 Real-Time PCR System	Applied Biosystems, Darmstadt
Autoclave	Systec GmbH, Wettenberg
Tank Blotting System	Bio-Rad, München
FastPrep-24	MP Biomedicals, Santa Ana
FilterMax F5 Multimode Microplate Reader	Molecular Devices, Biberach
Fridges and Freezer	Liebherr, Berlin Heraeus, Hanau
Heracell 240i CO2 Incubator	Thermo Scientific, Braunschweig
Ice machine	Ziegra, Isernhagen
Incubator	Heraeus, Hanau
Laminar Flow	Heraeus, Hanau
Microcentrifuge 5417R	Eppendorf, Hamburg

Mr. Frosty Freezing Container	Thermo Scientific, Braunschweig
NanoDrop 2000	Thermo Scientific, Braunschweig
pH-Meter	Knick GmbH & Co. KG, Berlin
Pipettes	Eppendorf, Hamburg
Photometer	Eppendorf, Hamburg
Power supply	Bio-Rad, München
PTC-200 Peltier Thermal Cycler	MJ Research, St. Bruno
Rotanta 460 R	Hettich, Tuttlingen
Scale	Kern & Sohn GmbH, Balingen
Thermo Mixer	Eppendorf, Hamburg
Vortex Mixer, vv3	VWR, Darmstadt
Water bath	Köttermann, Hänigsen

2.1.3 CONSUMABLES

Amersham Hybond 0.2 PVDF membrane	Amersham, Braunschweig
Cell culture flasks	BD Biosciences, New Jersey
Cell culture tubes	BD Biosciences, New Jersey
Cell scraper	Costar, Cambridge
Columbia agar plates w/ 5% sheep blood	BD Biosciences, New Jersey
Cryo tubes	Nunc, Wiesbaden
ELISA plates	Thermo Scientific, Braunschweig
MicroAmp optical adhesive films	Applied Biosystems, Darmstadt
MicroAmp optical 96-well reaction plate	Applied Biosystems, Darmstadt
Multi-well plates	Falcon, Heidelberg
Nitrile gloves	Ansell, Brüssel
Petri dish	Falcon, Heidelberg
Pipette tips	Sarstedt, Hannover
Serological pipettes	Falcon, Heidelberg
Sterile filters	Merck Millipore, Darmstadt
Urine cup	Sarstedt, Hannover

2.1.4 KITS

Amicon Ultra 0.5 and 2mL Centrifugal Filters	Merck Millipore, Darmstadt
Cytotoxicity Detection Kit (LDH)	Roche, Mannheim
Direct-zol RNA MiniPrep	Zymo Research, Irvine
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems, Darmstadt
HIV-1 p24 ELISA Kit, 96-Well	XpressBio, Frederick
BD OtpEIA Human TNFα ELISA Set	BD Biosciences, New Jersey
BD OtpEIA Human IL1β ELISA Set	BD Biosciences, New Jersey
Human BD-2 ELISA Development Kit	PeproTech, Rocky Hill
Qiagen Plasmid Maxi and Midi Prep	Qiagen, Hilden

2.1.5 CELL LINES

THP-1	ATCC, Wesel
U937	ATCC, Wesel
HEK293T	ATCC, Wesel

2.1.6 q-RT-PCR PRIMERS

TABLE 1: q-RT-PCR Primers

Target Gene	Assay ID	Company
Human β-Actin	Hs00969077_m1	Applied Biosystems
Human IL8	Hs00174103_m1	
Human GAPDH	Hs02758991_g1	
Human S18	Hs01026310_m1	
Human Beta Defensin 2	Hs00823638_m1	
Human Beta Defensin 3	Hs00218678_m1	
Human TLR2	Hs01014511_m1	
Human TLR4	Hs00152939_m1	
Human MyD88	Hs01573837_g1	

2.1.7 LENTIVIRUS

2.1.7.1 VECTORS

pLKO.1	Addgene, Cambridge
pGFP-iLenti	Applied Biological Materials, Richmond
pMD.G	Addgene, Cambridge
pMDLg/pRRE	Addgene, Cambridge
pRSV-Rev	Addgene, Cambridge

2.1.7.2 SEQUENCES

TABLE 2: Sequences of lentiviral shRNA

Target Gene	Sequence	Company
TLR4	TAAGAAAGCTAGACTACTTGG	Dharmacon
MyD88	ACGTTCAAGAACAGAGACAGG	
TLR2	ACTGTCTTTGTGCTTTCTGAAAACTTTGT	ABM
KLF4	ATTGTAGTGCTTTCTGGCTGG	Thermo Scientific
	ATGCCTCTTCATGTGTAAGGC	
	ATTGGAGAGAATAAAGTCCAG	
	ATGAGCTCTTGGTAATGGAGC	
	TACACCGGGTCCAATTCTGGC	

Sequences for KLF4 were pooled.

2.1.8 ANTIBODIES

PRIMARY ANTIBODY	ISOTYPE	COMPANY
anti-β-Actin	Goat IgG	Santa Cruz, California
anti-hBD-3	Rabbit IgG	Santa Cruz, California

SECONDARY ANTIBODY		
anti-goat	Donkey IgG-HRP	Santa Cruz, California
anti-rabbit	Goat IgG-HRP	Santa Cruz, California

2.1.9 BUFFERS, MEDIA AND PLATES

2.1.9.1 SDS-PAGE

LYSIS BUFFER FOR PROTEINEXTRACTION

Tris-HCL, pH 7,4	50mM
Nonidet P-40	1% (v/v)
PMSF	1mM
Antipain	10µg/ml
Leupeptin	10µg/ml
Pepstatin	10µg/ml
EDTA	0.25mM

PHOSPHOPROTEIN WASH BUFFER

Sodiumorthovanadat 98%	200mM
Sodiumpyrophosphate	150mM
Sodiumfluoride 99%	1M
PBS	1x

STACKING GEL BUFFER

SDS	0.4%
Tris-HCL	0.5M
pH	6.8

SEPARATION GEL BUFFER

SDS	0.8%
Tris-HCL	1.5M
pH	8.8

SAMPLE BUFFER	
SDS	0.8%
Glycine	10%
Tris-HCL	0.06M (pH 6.8)
Bromphenol blue	1mg
β-mercaptoethanol	2%

RUNNING BUFFER	
Glycine	0.192M
SDS	0.1%
Tris-HCL	0.025M
pH	8.3

BLOT BUFFER (10X)	
Glycine	1.9M
Tris-HCL	248mM

BLOT BUFFER (1X)	
Blot Buffer (10x)	10%
Methanol	10%

BLOCK BUFFER	
Odyssey Blocking Buffer	50%
PBS	50%

WASH BUFFER	
PBS	
Tween	0.05%

PONCEAU S

Ponceau S	0.1%
Acetic acid	5%

2.1.9.2 MEDIA AND PLATES

THY MEDIUM

bidest. H_2O	65% (v/v)
Todd-Hewitt Broth	30% (w/v)
Yeast extract	5% (w/v)

LB-MEDIUM

Tryptone	1% (w/v)
Sodium chloride	171mM
Yeast extract	0.5% (w/v)

LB AGAR PLATES

Tryptone	1% (w/v)
Sodium chloride	171mM
Yeast extract	0.5% (w/v)
Agar	1.5% (w/v)

CELL FREEZING MEDIUM

FCS	90% (v/v)
DMSO	10% (v/v)

BACTERIA FREEZING MEDIUM

THY	80% (v/v)
Glycerol	20% (v/v)

VIRUS PRODUCTION MEDIUM

DMEM	86% (v/v)
FCS	10% (v/v)
Glutamine	1% (v/v)
Sodium pyruvate (100mM)	1% (v/v)
BSA	1% (v/v)

2.2 METHODS

2.2.1 CYTOLOGICAL METHODS

2.2.1.1 CELL CULTURE

THP-1 and U937 cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 1% Glutamine and Pen/Strep (100µg/ml) at 37°C and 5% CO_2. Splitting was done every two days by replacing 2/3 of the medium with fresh, pre-heated medium. For infection, cells were differentiated into macrophages using PMA. Therefore cells were centrifuged at 300g for 7min, the pellet was resuspended in RPMI 1640 blank medium. Cells were counted and seeded into 12-well plates at a concentration of $5x10^5$ cells/well. Following addition of PMA (100ng/ml) cells were kept at 37°C and 5% CO_2 for 16-20h. Afterwards a complete medium change was done and cells received fresh, pre-heated RPMI 1640 supplemented with 10% FCS, 1% Glutamine and Pen/Strep (100µg/ml). Cells were left at 37°C and 5% CO_2 for 2 more days before infection.

HEK293T cells were cultured in DMEM medium supplemented with 10% FCS, 1% Glutamine and Pen/Strep (100µg/ml) at 37°C and 5% CO_2. Splitting was done every two days. After removal of the old medium cells were washed with PBS. PBS was removed and trypsin was added for 5min. Cells were split 1:5 and received fresh, pre-heated and supplemented medium. In this study cells up to passage 20 were used.

2.2.1.2 ISOLATION OF PBMCs FROM BUFFY COATS

The buffy coats for PBMC isolation were obtained from *DRK-Blutspendedienst*. After arrival the blood was mixed 1:3 with PBS and approx. 25ml of the blood/PBS suspension was layered over 15ml Ficoll Paque. Isolation of human PBMCs was done by density gradient centrifugation (25min, 800g, no brake, RT). Erythrocytes and most polymorphnuclear leukocytes will pass the ficoll-phase because of their higher density whereas PBMCs are found in the interphase. The interphase was carefully transferred to a new 50ml tube and 40ml of cold PBS was added. Cells were centrifuged for 7min at 300g. The supernatant was discarded, cells were resuspended in 10ml of RPMI 1640 blank, counted and seeded at the desired concentrations ($1x10^6$ cells/well in 6-well plates, $5x10^5$ cells/well in 12-well plates). After 120min at 37°C and 5% CO_2,

medium and non-adherent cells were removed. The cell layer was washed three times with PBS by gently swirling the plate. After washing pre-heated RPMI 1640 medium supplemented with 10% FCS, 1% Glutamine, Pen/Strep (100µg/ml) and GM-CSF (10µg/ml) was added to the cells. GM-CSF is needed for differentiation of monocytes into macrophages. Cells were kept in culture at 37°C and 5% CO_2 for 7-10 days. Medium was changed every two days. Fresh medium was always pre-heated.

2.2.1.3 DETERMINATION OF CELL COUNT

Counting of cells was done with a Neubauer chamber. For determination of cell number 10µl of cell suspension were pipetted between the cover glass and the Neubauer chamber. All cells inside of the 4 corner great-squares were counted under the microscope. The mean of the cell number was multiplied by 10^4 and equals the number of cells per ml suspension.

2.2.1.4 PREPARATION AND CULTURE OF HUMAN LUNG TISSUE

Fresh human lung explants were received from patients undergoing lung resection at thoracic surgery centers in Berlin. Written informed consent was obtained from all patients and the study was approved by the ethic committee at the *Charité – Universitätsmedizin Berlin* (project EA2/079/13). The lung pieces originate from the periphery of the resected pulmonary lobes of tumor-free normal lung tissue. Patients with pulmonary or systemic inflammation, tuberculosis, HIV or other chronic infections were excluded from this study. The fresh lung explants were transported in sterile RPMI 1640 blank medium on ice directly from the surgery to the laboratory and prepared immediately. Initially, the lung tissue was cut with a scalpel into little slices (thickness ~3mm) and stamped into small cylinders (diameter 8mm). Each piece weighted about 100mg. To remove residual amounts of antibiotics in the tissue and for tissue soothing, lung pieces were incubated in 24-well plates with RPMI 1640 medium (with 10% FCS) at 37°C and 5% CO_2 overnight. Culture of human lung tissue was done by Dipl. Ing. Andrea Peter.

2.2.1.5 CRYOCONSERVATION

FREEZING OF CELLS

For cryoconservation cells were centrifuged at 300g for 7min. The supernatant was discarded and cells were resuspended in freezing medium at a concentration of $1x10^7$ cells/ml. To gently freeze the cells, cryo tubes were put into a Mr. Frosty freezing container. This container cools down 1°C per h. For short term storage cells were kept at -80°C and for long term storage they were put into liquid nitrogen.

THAWING OF CELLS

For thawing 9ml of pre-heated medium were pipetted into a 15ml tube. 1ml of medium was then put onto the frozen cells, medium and cells were transferred into the tube. Cells were centrifuged at 300g for 7 min. The supernatant was discarded and cells were resuspended in different amounts of medium depending on the following experiments.

2.2.1.6 LACTATE DEHYDROGENASE ASSAY

Quantification of cell death was done with the Cytotoxicity Detection Kit (Roche). This kit measures the amount of lactate dehydrogenase (LDH) in the supernatant. LDH is a stable cytosolic enzyme which is released upon lysis of cells. For determination of cell death, THP-1 were infected with *S. pneumoniae* D39, D39ΔCPS, D39ΔPLY and R6x (MOI 1-100) for 4-16h. As positive control 1% Triton X-100 was added to uninfected cells for 5min to give the maximum LDH release. Negative control was supernatant of uninfected cells. The LDH assay way done according to the manufacturer's instructions. The percentage of specific LDH release was calculated by using the following formula: specific LDH release

$$[\%] = ((OD_{Target} - OD_{Control}) / (OD_{Maximum} - OD_{Control})) \times 100.$$

2.2.2 MICROBIOLOGICAL METHODS

2.2.2.1 BACTERIA

STREPTOCOCCUS PNEUMONIAE

In this study cells were infected with different *S. pneumoniae* serotype 2 strains. Wild type (wt) D39 as well as the isogenic mutants deficient for pneumolysin (D39ΔPLY) and the capsule (D39ΔCPS) were used. Furthermore cells were stimulated with the unencapsulated derivate of D39, R6x.

For long time storage bacteria were frozen at -80°C in THY medium containing 20% glycerol. When used, bacteria were first cultured on Columbia blood agar plates containing 5% sheep blood for 12h at 37°C and 5% CO_2. For cultivation of D39ΔCPS the plates were pre-coated with 2mg/ml kanamycin and for cultivation of D39ΔPLY 2mg/ml erythromycin were applied to the plates. After 12h on the plate, single colonies were transferred into pre-heated THY medium to yield an OD_{600}=0.03-0.06. Liquid cultures were incubated at 37°C and 5% CO2 until they reached OD_{600}=0.2-0.3 which equals a phase of mid logarithmic growth and 10^8 CFU/ml. Bacteria were then centrifuged at 1800g for 10min. After centrifugation the pellet was resuspended in RPMI 1640 blank medium at a concentration of 10^9 CFU/ml. For infection of cells further dilutions of the bacteria were prepared. *S. pneumoniae* strains were kindly provided by Prof. Sven Hammerschmidt, University of Greifswald.

ESCHERICHIA COLI

For transformation with lentiviral vectors *E. coli* XL-10 ultracompetent cells were purchased from Agilent Technologies, Waldbronn.

2.2.2.2 HEAT-INACTIVATION

Bacteria grown as described above were heat-inactivated by incubation at 56°C for 1h. Afterwards cells were aliquoted at a concentration of 10^9 CFU/ml and frozen at -20°C. One sample was streaked out onto Columbia agar plates and incubated at 37°C and 5% CO_2 for 24h. No colonies grew on the plate, confirming that the heat-inactivation worked.

2.2.2.3 STIMULATION OF HUMAN LUNG TISSUE WITH *S. PNEUMONIAE*

Before infection, lung pieces were carefully transferred in 6-well plates with fresh RPMI 1640 medium supplemented with 10% FCS. Human lung tissue was infected with *S. pneumoniae* strain D39 (10^6 CFU/ml) for 24h. Therefore, 0,5ml control or infection medium was slowly injected into lung tissue, thereby assuring thorough stimulation of the tissue. All control tissue samples were injected with RPMI 1640 medium. After 24h lung samples were frozen in TRIzol and stored at -80°C for further processing. Actual bacterial load was determined from serial dilutions of applied infection dose on Columbia agar plates and incubation at 37°C and 5% CO_2 for 24h before colonies were counted and CFUs were calculated. Stimulation of human lung tissue was done by Dipl. Ing. Andrea Peter.

2.2.2.4 STIMULATION OF MACROPHAGES (PBMCs, THP-1, U937) WITH *S. PNEUMONIAE*

Cells were infected in RPMI 1640 blank medium. The medium change was done 90min prior to infection. If inhibitors were used, these were added to the cells 3h before infection at a concentration of 1-10µM. In experiments analyzing phagocytosis, cells were pre-treated with 2µM Cytochalasin D (CytD) 30min before infection. CytD was left on the cells for the whole infection period. Cells were infected with different MOIs of D39, D39ΔPLY, D39ΔCPS and R6x. Heat killed D39 were applied at an MOI of 200. Time points used in this study are ranging from 4-24h. MALP-2 was used in a concentration of 50ng/ml. The actual bacterial load was determined by serial dilutions of the applied infection dose on Columbia agar plates and incubation at 37°C and 5% CO_2 for 24h before colonies were counted and CFUs were calculated.

2.2.2.6 CFU ASSAY OF *S. PNEUMONIAE*

The antimicrobial function of the β-defensins was determined by a growth curve. Bacteria were grown as described above. When they reached an OD_{600} of 0.2-0.3 bacteria were centrifuged at 1800g for 10min. Bacteria were then resuspended in THY medium at a concentration of $1x10^6$ CFU/ml. 900µl of bacterial suspension was transferred into a round-bottom tube. As negative control 100µl PBS were added to one tube. Penicillin G was used in a

concentration of 1µg/ml as positive control. The β-defensins were applied at a concentration of 7µg/ml. Bacteria were left at 37°C and 5% CO_2. After 2, 4 and 6h serial dilutions were plated on Columbia agar plates and after incubation at 37°C and 5% CO_2 for 24h CFU/ml were determined.

2.2.3 MOLECULAR BIOLOGICAL METHODS

2.2.3.1 RNA ISOLATION FROM HUMAN LUNG TISSUE

For mRNA analysis, 1ml TRIzol was added to each lung tissue sample and homogenized using the FastPrep-24 homogenizer on dry ice. The samples then were frozen at - 80°C for at least 24h. The homogenized samples were thawed and kept at room temperature for 5min to permit complete dissociation of the nucleoprotein complex. Subsequently, 0,2ml of chloroform was added and the tubes were shaken vigorously by hand for 15 seconds. After another short incubation at room temperature, the samples were centrifuged at 17900g for 15min at 4°C. Due to centrifugation, the mixture separates into a lower phenol-chloroform phase and an upper aqueous phase, which contains the RNA. The aqueous phase was transferred into a new tube and following, 0,5ml of isopropanol and 1µl of Glycoblue were added to each sample. For precipitation, the samples were stored at - 20°C for at least 30min. After centrifugation at 17900g for 15min at 4°C, the supernatant was discarded and the pellet was washed with 1ml of 75% cold ethanol. For that, the samples were vortexed and centrifuged at 17900g for 10min at 4°C. The supernatant was carefully removed and the pellet was air-dried until all the remaining ethanol was gone. RNA was dissolved in 50µl dd water and RNA concentration was measured with the Nanodrop. To remove potential DNA from RNA samples, RNase-Free DNase was used according to the manufacter's instruction. Following, RNA was precipitated with Roti-Aqua-P/C/I and isolated as described before. 1µg RNA was used for transcription to cDNA. The rest of the RNA was stored at - 80°C. RNA samples from infected human lung tissue were kindly provided by Dipl. Ing. Andrea Peter.

2.2.3.2 RNA ISOLATION FROM CELLS

For mRNA analysis, medium was removed from stimulated cells and 300µl (12-well) or 500µl (6-well) of peqGOLD TriFast was added to the cells. Cells were incubated at RT for 5min before the plates were frozen at -20°C for at least one day. mRNA extraction was either done with the Direct-zol RNA MiniPrep Kit following manufacturer's instruction or according to the following protocol:

Cells were thawed and left at RT for 5min. TriFast reagent was transferred into sterile 1,5ml tubes and $^1/_5$ volume of chloroform was added. Tubes were shaken vigorously for 15sec and left at RT for another 5min. Afterwards samples were centrifuged at 17900g for 15min at 4°C. Due to centrifugation, the mixture separates into a lower phenol-chloroform phase and an upper aqueous phase, which contains the RNA. The aqueous phase was transferred carefully into a new 1,5ml tube. $^1/_2$ volume of isopropanol and 1µl of Glycoblue were added. Samples were briefly vortexed and incubated at -20°C for at least 30min followed by centrifugation at 17900g and 4°C for 15min. The supernatant was discarded and the pellet washed by addition of ice-cold 75% ethanol and centrifugation at 17900g and 4°C for 10min. Washing was repeated thrice. Afterwards the ethanol was removed and the pellet air dried. When fully dried, the pellet was resuspended in 10µl of RNAse-free H_2O and the RNA concentration of the sample was determined by Nanodrop. Samples were stored at -20°C for short term and -80°C for long term storage.

2.2.3.3 cDNA SYNTHESIS

RNA was transcribed using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems. All working steps were done on ice. The master mix consisted of 2µl buffer, 2µl random primers, 0.8µl dNTPs, 1µl reverse transcriptase and 9.2µl ddH_2O, the final volume being 20µl including 5µl of RNA for each sample. The reaction mixture was incubated in a thermo cycler, 10min at 25°C, 2h at 37°C and 5sec at 85°C. Afterwards 80µl ddH_2O was added to the samples that were then stored at -20°C.

2.2.3.4 q-RT-PCR

Relative mRNA expression was measured by q-RT-PCR. cDNA samples were thawed on ice. A master mix was prepared consisting of 10µl Gene Expression

Master Mix, 1µl TaqMan Assay and 4µl ddH_2O for each sample. 15µl of the master mix and 5µl of each cDNA were pipetted per well (96-well plate). Expression of GAPDH, β-Actin or S18 was used as endogenous control for data normalization. The oligonucleotide concentrations in the TaqMan Assays were 18 nmol/ml for the forward and reverse primer and 5 nmol/ml for the probe. The annealing temperature for all assays was at 60°C. The q-RT-PCR was running with the following settings: 50°C for 2min, 95°C for 10min followed by 40x 95°C for 15sec and 60°C for 1min. For analysis of the raw data the 7300 System Sequence Detection Software was used. RQ-values were later on calculated using the ΔΔct-Method.

2.2.4 BIOCHEMICAL METHODS

2.2.4.1 EXTRACTION AND DETERMINATION OF PROTEIN

For protein analysis supernatant and cell lysate were used. Supernatant was collected from the cells (THP-1) after stimulation with *S. pneumoniae* D39 for 16h. Since the defensin proteins are very small (4-5kDa) and most probably very low concentrated, the supernatant was concentrated 25x using Amicon Ultra 0.5 and 2mL Centrifugal Filters. Afterwards 30µl of each sample was loaded onto the SDS-PAGE gel. Cell lysate was harvested by washing the cells (THP-1) after stimulation with *S. pneumoniae* D39 three times with ice-cold phosphoprotein wash buffer. Afterwards 60µl of ice-cold lysis buffer was pipetted onto the cells (12-well format). Cells were scraped on ice and cell lysate was transferred into a fresh 1,5ml tube. Samples were centrifuged at 20800g and 4°C for 10min. The supernatant was transferred into a new tube and the pellet was discarded. Protein concentration in each sample was determined by Bradford protein assay. The Bradford reagent will turn from brown to blue and absorption can be measured in a photometer at OD_{595}. Depending on the absorption protein concentrations can be calculated. For SDS-PAGE 60µg of protein were loaded onto the gel. Before running the gel, loading buffer was added and samples were boiled at 95°C for 5min.

2.2.4.2 SDS-PAGE

SDS-PAGE is used for separation of complex protein suspensions. With this method proteins are separated by their weight and not their charge. By adding SDS and β-mercaptoethanol proteins are unfolded and separated into their subunits. SDS is attaching to the aminoacid chain, bestowing it with a solely negative charge. Before separation of the proteins within the separation gel, the protein suspension is concentrated by a stacking gel with 4% acrylamide.

TABLE 3: Recipe for stacking and separation gel used in SDS-PAGE

	4% Stacking Gel	**17% Separation Gel**
ddH2O	6ml	1.65ml
Stacking Gel/Separation Gel Buffer	2.5ml	2.5ml
SDS 10%	100µl	100µl
Acrylamide	1.33ml	5.7ml
APS 10%	50µl	50µl
TEMED	10µ	5µl

Separation of proteins was done with 70V until samples reached the stacking gel/separation gel border. Afterwards 100V were applied until the dye front reached the bottom of the gel. Due to the loading with SDS all proteins acquire a negative charge and will thus move along the applied electric field.

2.2.4.2.1 WESTERN BLOT

The transfer of proteins that have been separated in SDS-PAGE onto a membrane is a method called Western Blot. The principle is electrophoretic transfer of negatively charged proteins. The proteins are moving along the electrical field and are blotted onto a PVDF-membrane. Therefore the membrane and gel are placed between electrodes and since in this study a tank blotting system was used, the membrane and gel sandwich is completely submerged under blotting buffer within a buffer tank. As there is a lot of heat generated by the electrical field, the buffer needs to be cooled by icepacks within the tank. Blotting of proteins was done for 1h at 100V. After blotting of proteins the membrane was dyed with Ponceau S to prove that the transfer of the

proteins was successful. Ponceau S was then washed off with PBS. To minimize unspecific binding of the antibodies, the membrane was blocked for at least 1h at RT with blocking buffer. Afterwards the primary antibody was diluted in blocking buffer (anti-hBD-3 1:1000; anti-β-actin 1:2000) and left on the membrane for 1h at RT or over night at 4°C. After washing the membrane 3 times with PBS/Tween for 5min the secondary antibody was added (1:2000 in blocking buffer) and left on the membrane for 1h. The secondary antibodies used in this study are coupled with horseradish peroxidase (HRP). After washing the membrane 3x10min with PBS the membrane was developed in a dark room with the Pierce ECL Western Blotting Substrate according to manufacturer's instructions. Luminescence was analyzed on x-ray film. Development time was 5-10min.

2.2.5 IMMUNOLOGICAL METHODS

2.2.5.1 ELISA

Concentrations of IL1β and TNFα were quantified in cell free supernatants by commercially available sandwich ELISA Kits. For analysis of TNFα in supernatants, samples were diluted 1:10 (uninfected control was applied undiluted). For IL1β ELISA a 1:50 dilution was done (uninfected controls were not diluted). For detection of hBD-2 supernatants were concentrated 25x using Amicon Ultra Centrifugal Filters. ELISAs were done according to manufacturer's instructions.

2.2.6 VIROLOGICAL METHODS

2.2.6.1 PRODUCTION OF LENTIVIRAL VECTORS

Vectors containing lentiviral shRNA as well as corresponding control vectors have to be multiplied in bacteria before use. Therefore a 50µl aliquot of *E. coli* XL-10 ultracompetent cells was thawed on ice and 5µl of plasmid were added to the cells. The mixture was incubated on ice for 30min and afterwards put at 42°C for 1min. After addition of 500µl of LB medium to the cells, the liquid culture was incubated at 37°C and 5% CO_2 for 1h. 50-100µl of bacterial

suspension was streaked out onto LB agar plates and incubated over-night at 37°C and 5% CO_2. The next day one colony was transferred into 5ml LB medium supplemented with antibiotics. Depending on the plasmids resistance either kanamycin (50µg/ml) or ampicillin (100µg/ml) was used. Bacteria were incubated for 6-8h at 37°C in a shaker to allow for enough oxygen to reach the proliferating cells. 400µl of this liquid culture were added to 500µl glycerol and frozen at -80°C. For plasmid preparation 100µl bacterial suspension were pipetted into 50ml LB medium containing antibiotics and left shaking in the incubator at 37°C over-night. Plasmids were then isolated from the bacteria using the Qiagen Plasmid Midi- or Maxi-Kit according to manufacturer's instructions. DNA concentration was measured by Nanodrop.

2.2.6.2 PRODUCTION OF LENTIVIRUS

Lentivirus production was done in HEK293T cells. One day before transfection HEK293T cells were seeded into a petri dish (10cm) at a concentration of $1x10^7$ cells. 20ml of DMEM supplemented with 10%FCS. 1% glutamine and Pen/Strep was added to the cells. For transfection a confluence of 60-80% was desirable and only cells below passage 20 were used. Directly before transfection the medium was removed from the cells and 5ml of DMEM were added. For successful production of lentivirus particles, cells needed to be transfected not only with the shRNA or control plasmids but also with packaging plasmids (Maps of all vectors used can be found in the Appendix). A mastermix was prepared containing 2.7µg of pMD.G, 4.5µg of Gag/Pol pMDLg/pRRE and 1.8µg of pRSV-Rev. 9µg of lentivector containing shRNA or scrambled shRNA were added to the mix. Transfection of cells was done with Lipofectamine 2000. Therefore 45µl of Lipofectamine 2000 were pipetted into 1.5ml of Opti-MEM and incubated at RT for 5min. Afterwards the mastermix was added to the Lipofectamine/Opti-MEM mixture and again an incubation of 20min at RT followed. The plasmid/Lipofectamine suspension was then pipetted carefully onto the cells and cells were incubated at 37°C and 5% CO_2 for 16-20h. On the next day a medium change was done, the transfection medium was removed and 8ml of fresh, pre-heated virusproduction medium was given to the cells. Cells were incubated for another 36h at 37°C and 5% CO_2 followed by harvesting of virus particles on three subsequent days. For harvesting the medium was

carefully removed from the cells and they received 8ml of fresh, pre-heated virusproduction medium. The virus containing supernatant was centrifuged at 4°C and 1500g for 10min to remove any cell debris. Afterwards 10% of PEG-8000 solution was added to the supernatant followed by incubation for at least 12h at 4°C. Centrifugation of virus particles was then done at 4°C and 3000g for 30min. Supernatant was discarded and the pellet was resuspended in $^{1}/_{100}$ PBS of the original volume. Virus titer was determined by HIV-1 p24 ELISA Kit according to manufacturer's instructions. Virussuspension was stored at -80°C.

2.2.6.3 TRANSDUCTION OF THP-1 CELLS

For transduction with lentivirus THP-1 cells were seeded into 6-well plates at a concentration of $1x10^6$ cells/well in RPMI 1640 (+FCS, +Glu, +Pen/Strep). The virus was added to the cells at an MOI of 10. Plates were then centrifuged at 32°C and 800g for 45min. Following that cells were incubated at 37°C and 5% CO_2 over night. Medium was changed the next day and after that every two days. Cells were selected for successful transduction on day 6 after transduction with Geneticin (G418-800µg/ml) or Puromycin (5µg/ml) depending on the resistance conferred by the viral plasmid. After one week of selection cells were centrifuged at 300g for 7min and viable cells were transferred into cell culture flasks, concentration of Geneticin was reduced to 400µg/ml for preservation of cells. Successful transduced cells were frozen in liquid nitrogen. The knock-down cell line for KLF4 was kindly provided by Dipl. Ing. Christin Kabus.

2.2.7 STATISTICAL ANALYSIS

Data was analyzed using the GraphPad Prism software, Version 6.00. For all statistical analyses, p values<0.05 were considered significant. Data are expressed as mean ± SEM or as representative graphs of at least three independent experiments. For comparison of two groups the non-parametric, two-tailed Mann-Whitney U Test was used. For comparison of more than two groups Kruskal-Wallis Test followed by a Dunn's post-hoc test was used.

3. RESULTS

3.1 HUMAN BETA DEFENSIN 3

3.1.1 hBD-3 EXPRESSION PATTERNS

S. pneumoniae frequently causes severe lung infection, especially in children and the elderly. One aim of this study was to determine whether induction of hBD-3 in pneumonia patients might improve disease outcome. Therefore the expression patterns of hBD-3 in human lung tissue (HuLu) and macrophages infected with *S. pneumoniae* were analyzed. First of all, the induction of this protein in HuLu upon infection with *S. pneumoniae* was measured. The obtained data show that the expression of hBD-3 is increased 40-fold in infected lungs compared to uninfected samples 24h after stimulation with D39 (Fig.6A) indicating an important role for hBD-3 in *S. pneumoniae* caused lung infection. In a previous study it was described that hBD-3 is induced in human lung epithelial cells [352] and since human lung tissue consists of various cell types expression of hBD-3 in human macrophages was further investigated. Macrophages are important cells of the innate immune system and therefore have huge impact on pneumonia outcome. First of all, there is an induction of hBD-3 in human PBMCs. Furthermore this induction is dose-dependent in PBMCs stimulated with *S. pneumoniae* D39 for 8h and 16h (Fig.6B and Fig.6C). In a next step, the expression pattern of hBD-3 in two different monocytic cell lines, U937 and THP-1, was determined. In both cell lines hBD-3 is induced dose-dependently upon infection with *S. pneumoniae* D39 (Fig.6D and Fig.6E). Additionally, expression of hBD-3 is time dependent in THP-1 cells (Fig.6F). For all further experiments the immortalized cell line THP-1 was used as a model cell line for human macrophages as the expression patterns of hBD-3 are identical in THP-1 and in PBMCs.

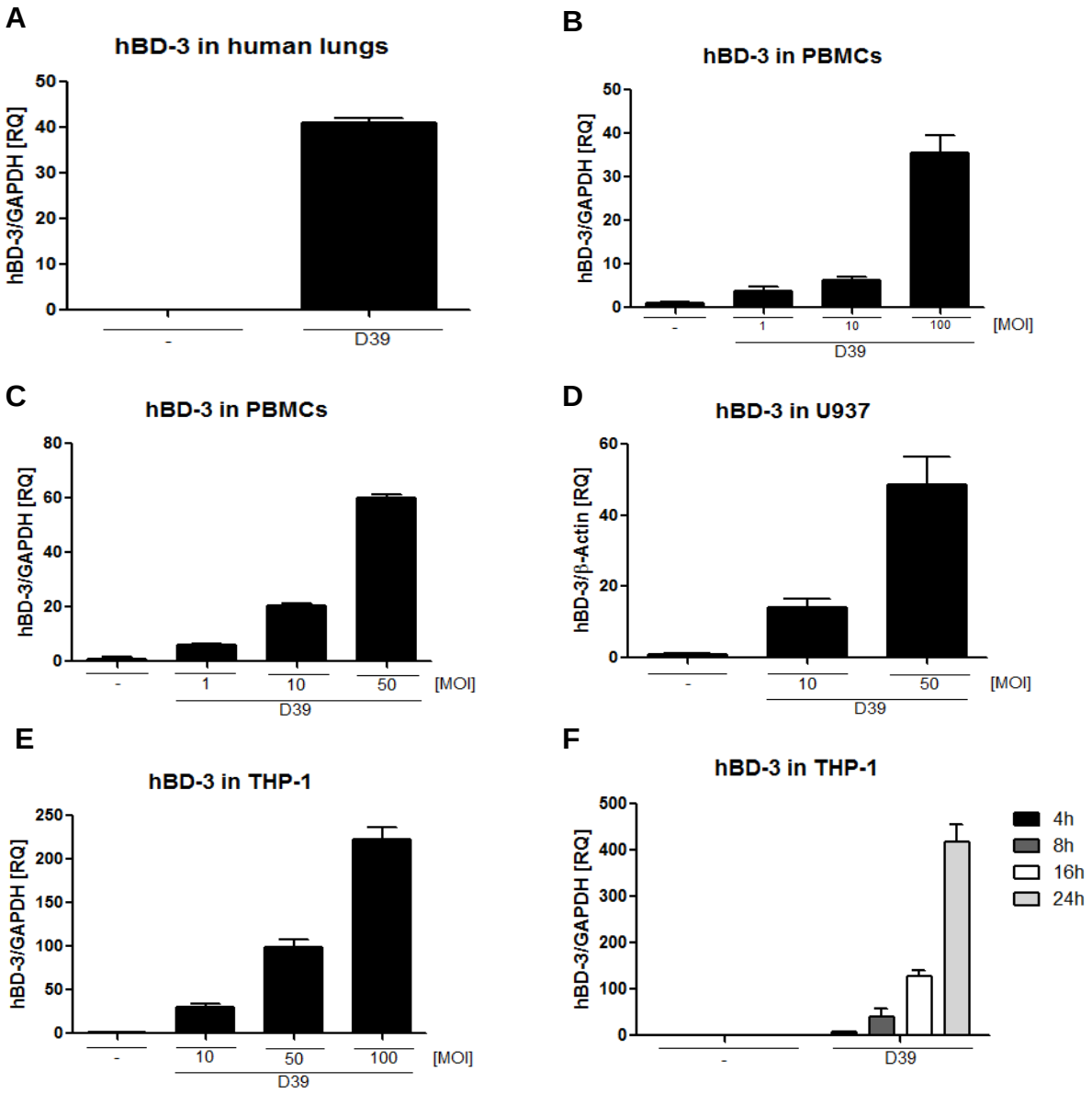

FIGURE 6: Expression of hBD-3 in human lung tissue and macrophages. **A:** Expression of hBD-3 is induced by *S. pneumoniae* D39 in HuLus after 24h of infection. Lungs were infected with 1x10^6 CFU/ml D39 for 24h. **B and C:** Expression of hBD-3 was induced in human PBMCs after 8h (**B**) and 16h (**C**) of infection with D39 in a dose-dependent manner. MOIs used range from 1-100. Cells were infected in RPMI 1640 w/o FCS. **D:** Expression of hBD-3 was induced by D39 in U937 cells after 16h of infection. MOIs 10 and 50 were used. **E:** Expression of hBD-3 induced by D39 in THP-1 cells after 8h of infection. Infection was done in RPMI 1640 w/o FCS. **F:** Expression of hBD-3 in THP-1 cells is time-dependent. D39 MOI 50 was used. Infection time ranges from 4-24h. Expression levels were obtained by q-RT-PCR. All data sets were normalized to GAPDH and β-Actin respectively. Graphs show the mRNA expression of hBD-3 relative to the uninfected control. Data are representative of at least three independent experiments.

The viability of infected THP-1 cells was determined by specific LDH release upon infection. There was no significant LDH release in all infected cells after 4-24h (Fig.7A-D). As expected, the bacterial strains with functioning PLY are more cytotoxic than the PLY-deficient mutant. Furthermore, cytotoxicity was time-dependent but did not show a clear dose-dependency as there were only little differences in cytotoxic effects in MOIs 10-100. According to this data MOI 50 was chosen as the default infection dose since there was the best induction of hBD-3 in all cell types. Infection time points used in this study are 8-16h according to the LDH data where 24h caused the highest cell death in cells stimulated with D39 and D39ΔCPS.

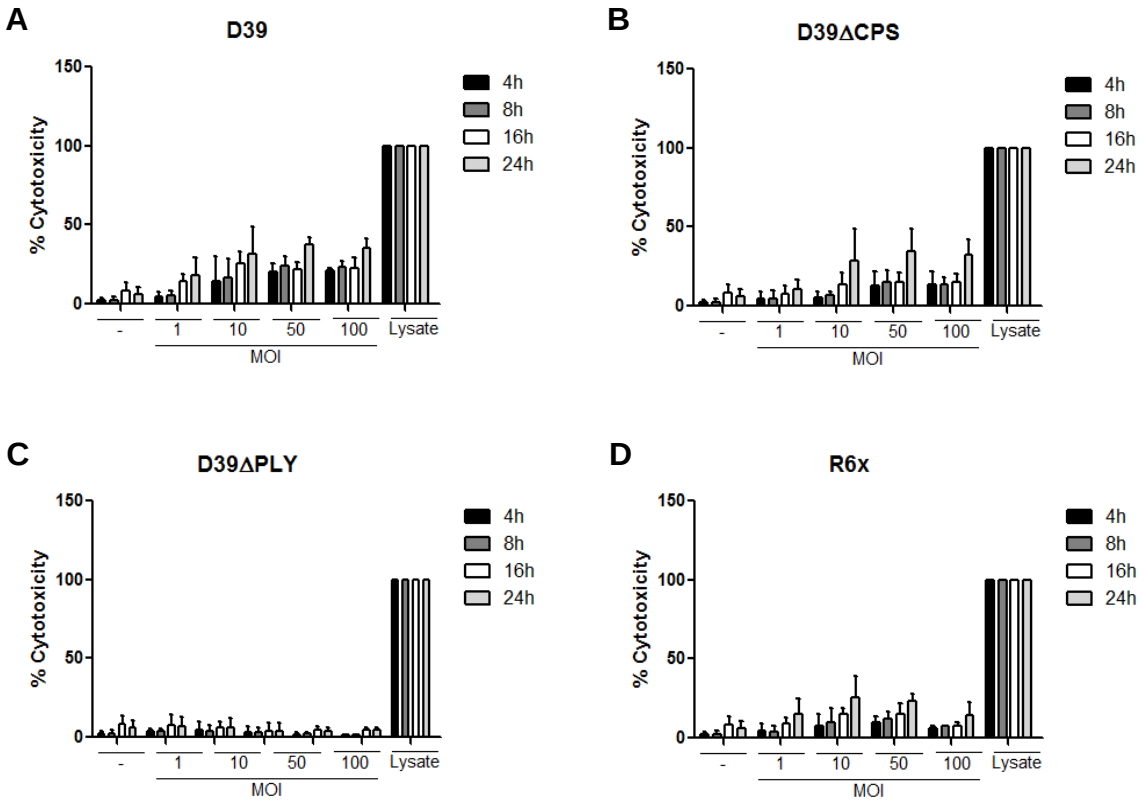

FIGURE 7: Specific LDH release of THP-1 cells infected with different strains of *S. pneumoniae*. A: LDH release of THP-1 infected with different MOIs of D39 for 4-24h. D39 induced cell death of up to 40% (24h) in THP-1. This induction is time and dose dependent. **B:** LDH release of THP-1 infected with D39ΔCPS. Cytotoxicity of D39ΔCPS is similar to D39. **C:** Almost no LDH was released from THP-1 infected with D39ΔPLY, there is no clear time and dose dependency. **D:** R6x induced cell death in THP-1. Cytotoxicity is similar to D39 and D39ΔCPS although there is no clear time and dose dependency. The positive controls in all experiments are cells lysed by 1% triton x-100. Data are shown as mean +/- SEM of three independent experiments. Cytotoxicity of all strains is not significant. Data analysis was done with Kruskal-Wallis Test followed by a Dunn's post-hoc test.

The expression of hBD-3 on protein level was determined by SDS-PAGE. To check if hBD-3 is secreted by the cells, SDS-PAGE was done with supernatants and cell lysates. Since hBD-3 is relatively small and expected concentrations of the protein in the supernatant are rather low, THP-1 cells were infected with D39 (MOI 100) for 16h and the supernatant was concentrated 25x before SDS-PAGE. For detection of hBD-3 in cell lysates MOI 50 was used for 16h to allow for accumulation of the protein. As control for correct handling, blots were stained for β-actin which in case of cell lysate is also the loading control. As expected hBD-3 protein was detected in supernatants of infected cells (Fig.8A) whereas in cell lysates there was no hBD-3 (Fig.8B). This is probably due to the antibody not detecting the preproprotein of hBD-3. There is a slight β-actin signal in supernatant of cells infected with D39. The reason for that β-actin signal is the cytotoxicity of D39 MOI 100 and the 25x concentration of the sample. Consequently there is no β-actin signal in the uninfected cells.

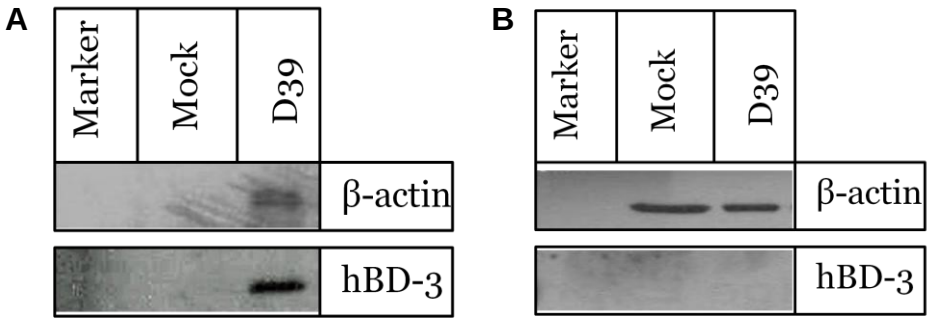

FIGURE 8: hBD-3 protein is detectable in supernatants of THP-1 cells infected with *S. pneumoniae*. A: SDS-PAGE of supernatant of THP-1 cells infected with D39 (MOI 100) for 16h. To increase the amount of hBD-3 the supernatant was concentrated 25x with Amicon Ultra 2ml Centrifugal Filters. For SDS-PAGE 30µl of concentrated supernatant were pipetted onto a 17% gel. **B:** Lysate of THP-1 cells infected with D39 (MOI 50) for 16h. For SDS-PAGE 60µg protein were loaded onto a 17% gel. Blots are representative of three independent experiments.

3.1.2 INDUCTION OF hBD-3 REQUIRES PNEUMOLYSIN AND VIABLE BACTERIA

To identify the underlying signaling pathway of hBD-3 induction in macrophages, different bacterial virulence factors were analyzed. The most important virulence factor of *S. pneumoniae* is its capsule as it protects the bacteria from various immune defense mechanisms and determines the serotype of strain. Hence, PBMCs were infected with encapsulated D39 and the unencapsulated mutants D39ΔCPS and R6x. Interestingly there was no difference in induction of hBD-3 for each strain (Fig.9A). The capsule that saves the bacteria from various immune mediators does not play a role when it comes to the induction of hBD-3.

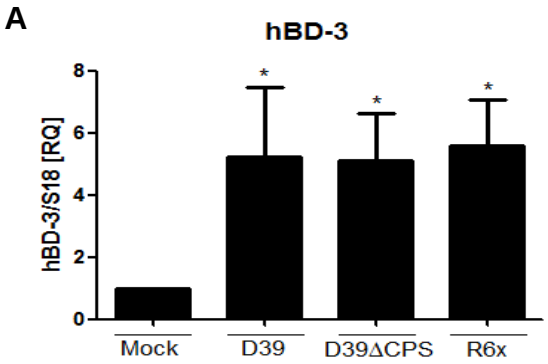

FIGURE 9: Expression of hBD-3 in PBMCs is not dependent on the bacterial capsule. A: PBMCs were infected for 16h with D39, D39ΔCPS and R6x (MOI 10). There is no difference in hBD-3 expression. Expression levels were obtained by q-RT-PCR. All data were normalized to S18 and graphs show the mRNA expression of hBD-3 relative to the uninfected control. Data are shown as mean +/- SEM of four independent experiments. Significance is indicated by asterisks,*=p<0.05. Statistical test used: two-tailed Mann-Whitney-Test.

Another very important virulence factor of *S. pneumoniae* is the exotoxin pneumolysin (PLY). To examine if PLY plays a role in hBD-3 induction, cells were infected with D39 and D39ΔPLY (a pneumolysin deficient mutant of D39). I could show that the induction of hBD-3 was severely decreased when cells were infected with D39ΔPLY compared to wt D39 (Fig.10A). This observation was not due to a failure of infection in general as confirmed by IL8 expression which is similarly increased in D39 as well as in D39ΔPLY infected cells (Fig.10B). Interestingly, recombinant PLY (rPLY) alone was not sufficient to induce hBD-3 (Fig.10A). When rPLY was added to D39ΔPLY the wt induction pattern of hBD-3 was reconstituted (Fig.10A). To further determine which co-

factor could be involved in hBD-3 induction cells were infected with heat-killed D39 (hK D39). As expected hK D39 were not able to induce hBD-3 since PLY is only released during bacterial autolysis [410]. Interestingly, hBD-3 was also not induced by hK D39 in combination with rPLY (Fig.10C). To confirm that infection in general had worked, IL8 was measured in all infected and non-infected cells (Fig.10D). IL8 could be detected in all infected macrophages suggesting that although the cells react to heat-killed bacteria and rPLY, expression of hBD-3 is not up-regulated. Thus, I conclude that not only PLY but also viable bacteria are needed to induce hBD-3 expression in human macrophages.

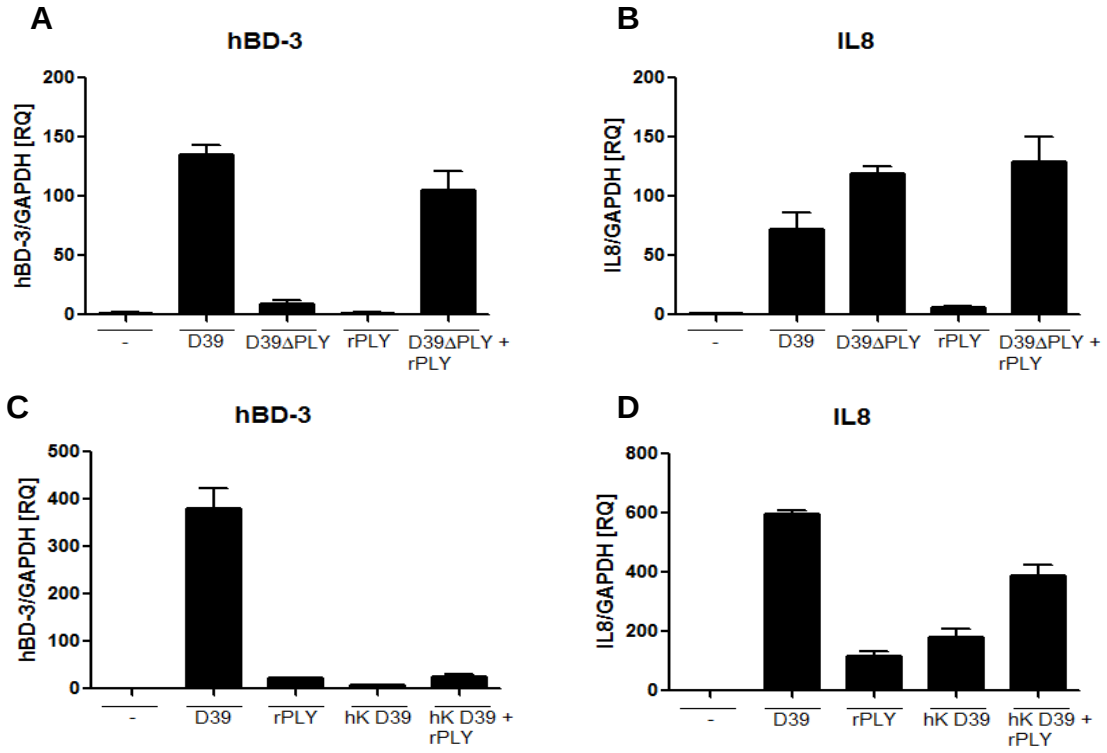

FIGURE 10: PLY-dependent expression of hBD-3 in THP-1 cells. THP-1 cells were left uninfected or infected with D39, D39ΔPLY, rPLY, D39ΔPLY + rPLY, heat-killed (hK) D39 and hK D39 + rPLY for 8h. **A:** Expression of hBD-3 in THP-1 cells infected with D39 or D39ΔPLY (MOI 50). Expression levels of D39 could be reconstituted by adding 0.5µg/ml rPLY to D39ΔPLY. **B:** Corresponding IL8 expression levels in cells infected with D39, D39ΔPLY, rPLY and D39ΔPLY + rPLY. IL8 expression is increased in all infected cells proving that cells are stimulated by the bacteria. **C:** hK D39 (MOI 200) do not induce hBD-3, not even when rPLY is added. **D:** IL8 response of THP-1 cells to D39 and hK D39. IL8 was induced by rPLY, viable as well as hK bacteria. Expression levels were obtained by q-RT-PCR. All data sets were normalized to GAPDH and graphs show the mRNA expression of hBD-3 relative to the uninfected control. Data are representative of at least three independent experiments.

3.1.3 INDUCTION OF hBD-3 IS NOT DEPENDENT ON TLR2

The signaling pathways that lead to hBD-3 induction are still not fully understood. Especially since up to today nobody analyzed the expression of this protein in human macrophages infected with *S. pneumoniae*. To further determine important factors of these pathways toll-like receptor 2 (TLR2) was addressed. This receptor is known to detect gram-positive bacteria via their cell wall components such as lipoteichoic acids [364,411]. When infecting the host, some pneumococci are undergoing a process called autolysis. During that process the bacterial cell wall is degraded into smaller fragments by bacterial enzymes. This degradation may cause an enhanced recognition by TLR2 [412]. To test for TLR2 involvement, THP-1 cells were infected with *S. pneumoniae* D39 and MALP-2, either alone or in combination with rPLY. MALP-2 is a specific agonist of the TLR2/6 heterodimer [413]. The signaling cascade via TLR2/6 involves MyD88 and NF-κB and will lead to the induction of various cytokines such as IL8, IL6 and TNFα [59]. The results obtained in this study suggest that TLR2 does not play a role in hBD-3 induction since neither cells stimulated with MALP-2 alone nor in combination with rPLY showed an increase of hBD-3 mRNA (Fig.11A). That stimulation with MALP-2 does in general have an effect on macrophages is shown in Fig.11B (The graph is identical to Fig.25B; only D39 is shown instead of D39ΔPLY). IL8, a pro-inflammatory cytokine induced via the TLR2- MyD88- NF-κB signaling pathway, is clearly induced in MALP-2, D39 and rPLY treated macrophages. Taken together these data lead to the conclusion that the co-stimulatory molecule needed for hBD-3 induction in human macrophages in addition to PLY is none of the molecules recognized by TLR2. And TLR2, an important receptor when it comes to recognition of bacteria by the immune system, is apparently not involved in the induction of hBD-3 at all.

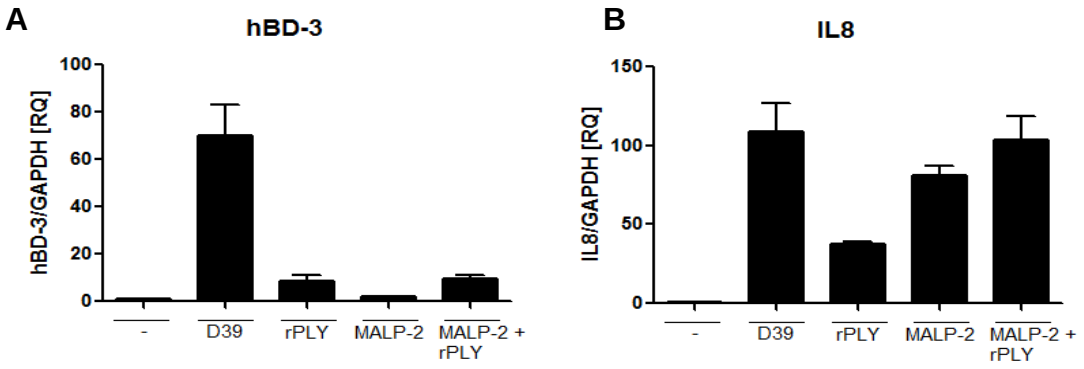

FIGURE 11: TLR2 is not implemented in pneumococci-induced hBD-3 expression in macrophages. Expression of hBD-3 in THP-1 cells uninfected or infected with D39 (MOI 50), rPLY (0.5µg/ml), MALP-2 (50ng/ml) and MALP-2 + rPLY for 8h. **A:** Expression of hBD-3 was induced by stimulation of cells with D39 whereas stimulation with the TLR2/6 agonist MALP-2 alone or in combination with the bacterial PLY did not elicit a strong hBD-3 response in THP-1 cells. **B:** IL8 control for stimulation of the THP-1 cells. D39 as well as MALP-2 and rPLY alone and in combination led to an induction of IL8 mRNA in THP-1 cells. Expression levels were obtained by q-RT-PCR. All data sets were normalized to GAPDH and graphs show the mRNA expression of hBD-3 relative to the uninfected control. Data are representative of at least three independent experiments.

3.1.4 INDUCTION OF hBD-3 DEPENDS ON PHAGOCYTOSIS OF THE BACTERIA BUT IS NOT DEPENDENT ON TLR9

Macrophages are important cells of the innate immune system. They are specialized phagocytes that are able to present antigens to cells of the adaptive immune system. Thereby linking innate to adaptive immunity. Since phagocytosis is one of the main features of macrophages, hBD-3 expression was analyzed in cells where phagocytosis was inhibited by Cytochalasin D (CytD) compared to cells treated with PBS. The induction of hBD-3 as well as the induction of IL8 by D39 and D39ΔCPS was measured after 8h of infection in CytD treated and untreated cells. D39ΔCPS was used in addition to D39 since there are reports stating that encapsulated bacteria are not phagocytosed [414] which would make them an excellent control strain in this experimental set up. Data obtained in this study show that phagocytosis of the bacteria is important for hBD-3 induction in macrophages. When phagocytosis was inhibited, hBD-3 expression levels were around 40-fold lower compared to expression levels in cells with active phagocytosis (Fig.12A). Interestingly, there was almost no

difference between D39 and D39ΔCPS infected cells, indicating that the capsule does not have a significant effect on phagocytosis of the bacteria by THP-1 cells. The expression levels of IL8 were similarly high in D39 and D39ΔCPS infected cells and there was only a slight difference between CytD treated and untreated cells, with treated cells showing a little higher expression levels of IL8 than untreated cells (Fig.12B).

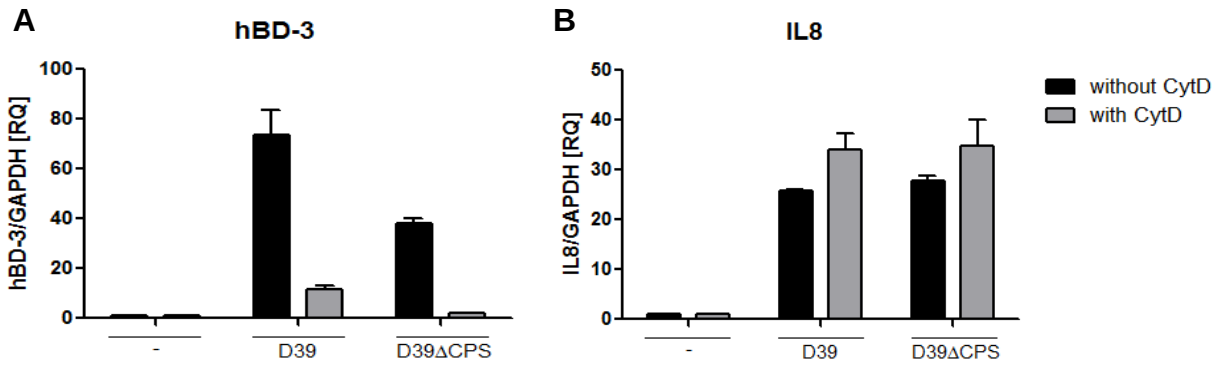

FIGURE 12: Phagocytosis-related expression of hBD-3. Expression of hBD-3 and IL8 in THP-1 cells with or without prior treatment with CytD. Cells were infected with D39 and D39ΔCPS (MOI 50) for 8h. One set of cells was pretreated with CytD at a concentration of 1mM 30min before infection to inhibit phagocytosis. **A:** Expression levels of hBD-3 are clearly decreased in treated cells compared to untreated cells. **B:** IL8 expression levels slightly increased when phagocytosis was blocked. Expression levels were obtained by q-RT-PCR. All data sets are normalized to GAPDH and graphs show the mRNA expression of hBD-3 relative to the uninfected control. Data are representative of at least three independent experiments.

Since bacterial DNA is another important virulence factor released during autolysis of bacteria and detected by internal TLR9, the capacity of the CpG molecule to induce hBD-3 was determined. CpG is an agonist for TLR9 [415] and it was used alone and in combination with rPLY on THP-1 cells. As a negative control the GpC oligonucleotide was used for stimulation. There was no increased hBD-3 expression detectable in this experimental set-up confirming that TLR9 and bacterial DNA respectively are not involved in hBD-3 induction (Fig.13A) in human macrophages.

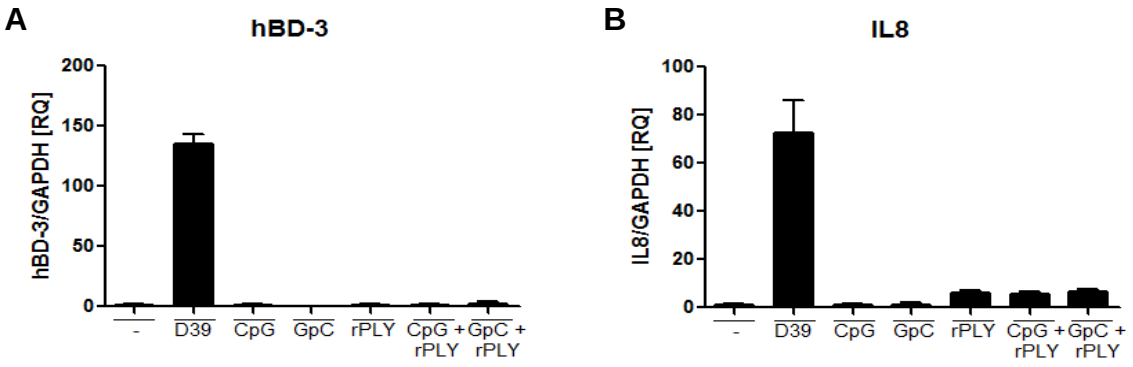

FIGURE 13: Expression of hBD-3 is not dependent on TLR9. Expression of hBD-3 in THP-1 cells uninfected or infected with D39 (MOI 50), rPLY (0.5µg/ml), CpG (2.5µg/ml) and CpG + rPLY for 8h. GpC is the negative control to CpG and was used in a concentration of 2.5µg/ml. **A:** Expression of hBD-3 was induced when cells were infected with D39 but not when CpG, GpC or rPLY were used, either alone or in combination. **B:** IL8 expression levels in stimulated THP-1 cells. IL8 expression was increased when cells were infected with D39 and to a lesser extent when rPLY was added to the cells. As was expected CpG and GpC do not induce an IL8 response.

3.1.5 EXPRESSION OF hBD-3 IS DEPENDENT ON TLR4, MyD88 AND PI3K

For induction of hBD-3, PLY is a necessary factor and since there are reports indicating that PLY is detected by TLR4 [117] involvement of this receptor in hBD-3 signaling was analyzed. Additionally, MyD88, one of the most important adapter molecules in immunological TLR-signaling in general is utilized by TLR4 to convey signals into the cell [109]. To confirm TLR4 and MyD88 involvement in hBD-3 induction, stable THP-1 cell lines with a lentiviral knock-down of either TLR4 or MyD88 were established. Knock-down rates were 60% for TLR4 and 80% for MyD88 (Fig.14A and Fig.14B). When TLR4 was knocked-down, expression of hBD-3 was reduced ~180-fold (Fig.14C). Similarly when MyD88 expression was abolished, hBD-3 induction was reduced ~200-fold (Fig.14D). These results clearly show that TLR4 as well as MyD88 are important for induction of hBD-3 in human macrophages.

The phosphatidylinositide 3-kinases (PI3Ks) are a family of related intracellular signal transducer enzymes capable of phosphorylating the 3rd position hydroxyl

group of the inositol ring of phosphatidylinositol [416]. Some isoforms regulate different aspects of immune responses thus, the PI3Ks were inhibited and it was analyzed if this has an effect on hBD-3 induction. Inhibition was done with the inhibitor Ly294002. Expression of hBD-3 was considerably decreased when cells were pretreated with Ly294002 compared to untreated cells (Fig.14E). Consequently, these results show that PI3Ks are needed for the induction of hBD-3 in THP-1 cells.

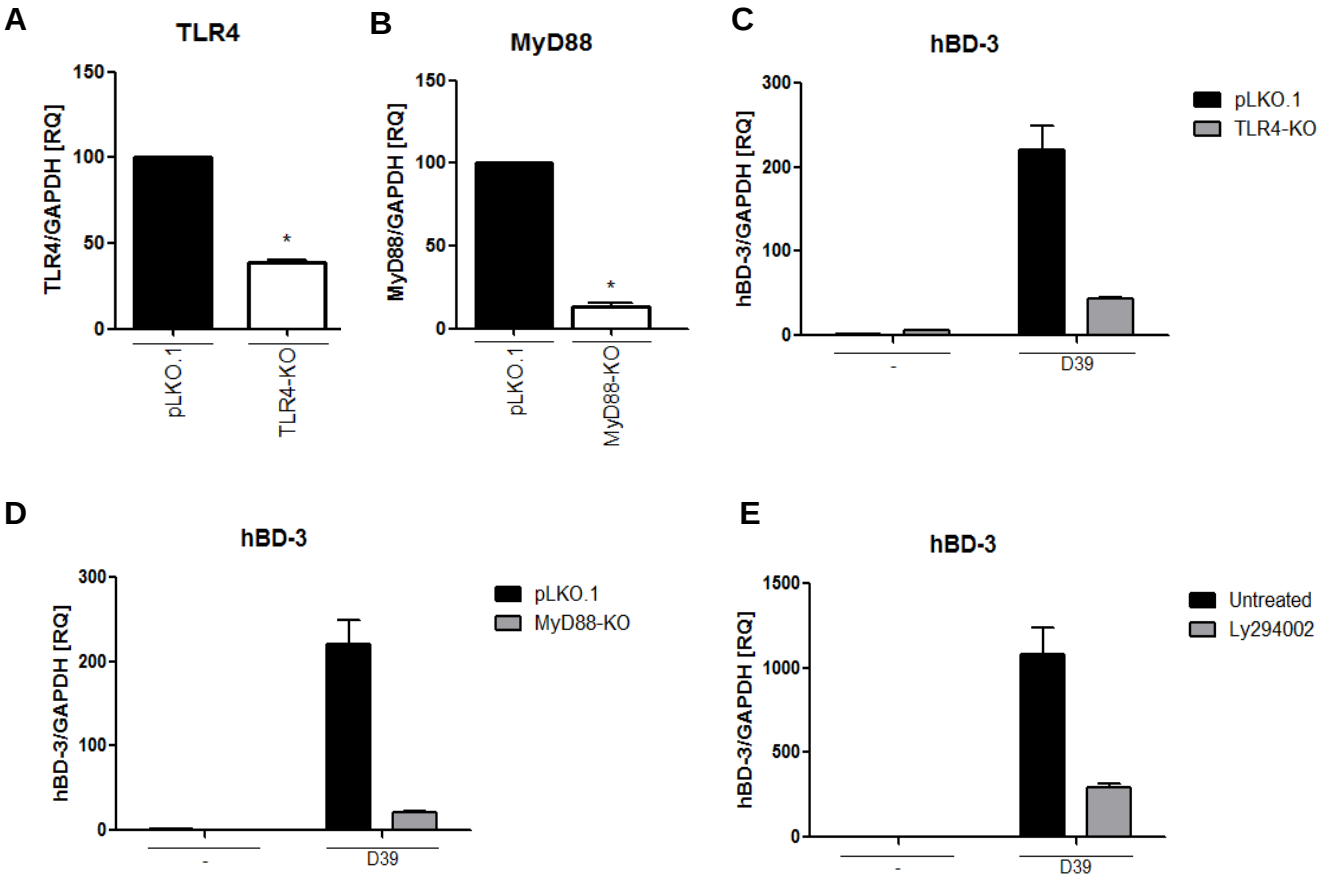

FIGURE 14: Expression of hBD-3 is dependent on TLR4, MyD88 and PI3Ks. A and B: Lentiviral-transduced THP-1 cells were used. Cells were either transfected with shRNA specific for TLR4, MyD88 or with a scrambled control. The vector backbone is pLKO.1. **C and D:** TLR4 and MyD88 knock-down cells were stimulated with D39 (MOI 50) for 8h. When TLR4 or MyD88 were knocked-down, expression of hBD-3 strongly decreased. **E:** Cells were pretreated with PBS or the PI3K-inhibitor Ly294002 (10µM) 3h before infection with D39 (MOI 50). Pretreated cells showed a decrease in hBD-3 expression compared to untreated cells. Expression levels were obtained by q-RT-PCR. All data sets were normalized to GAPDH and graphs show the mRNA expression of hBD-3 relative to the uninfected control. **A and B**: Data are shown as mean +/- SEM of three independent experiments. Significance is indicated by asterisks,*=$p<0.05$. Statistical test used: two-tailed Mann-Whitney-Test. **C-E:** Data are representative of at least three independent experiments.

3.1.6 KLF4 IS A NEGATIVE REGULATOR OF hBD-3

An important transcription factor in immune signaling is KLF4. This protein is induced in human macrophages by LPS, TNFα and INFγ [168]. Furthermore there are reports showing that TLR2 and TLR4 are involved in KLF4 induction [232,233]. Since it was previously shown that KLF4 down-regulates PI3Ks [417], hBD-3 expression in KLF4 knock-down cell lines was determined. Even without a significant knock-down of KLF4 of around 40% (Fig.15A), there is highly increased expression of hBD-3 in cells stimulated with D39 (Fig.15B). Furthermore, the hBD-3 expression is already increased ~30-fold in the uninfected cells confirming that KLF4 is a negative regulator of hBD-3 in human macrophages possibly via its effects on PI3K. Another important transcription factor, NF-κB was also tested for involvement in hBD-3 induction. THP-1 cells were pretreated with the IKK-NBD peptide, a selective inhibitor for NF-κB activation but showed no significant alteration in hBD-3 expression (Fig.15C).

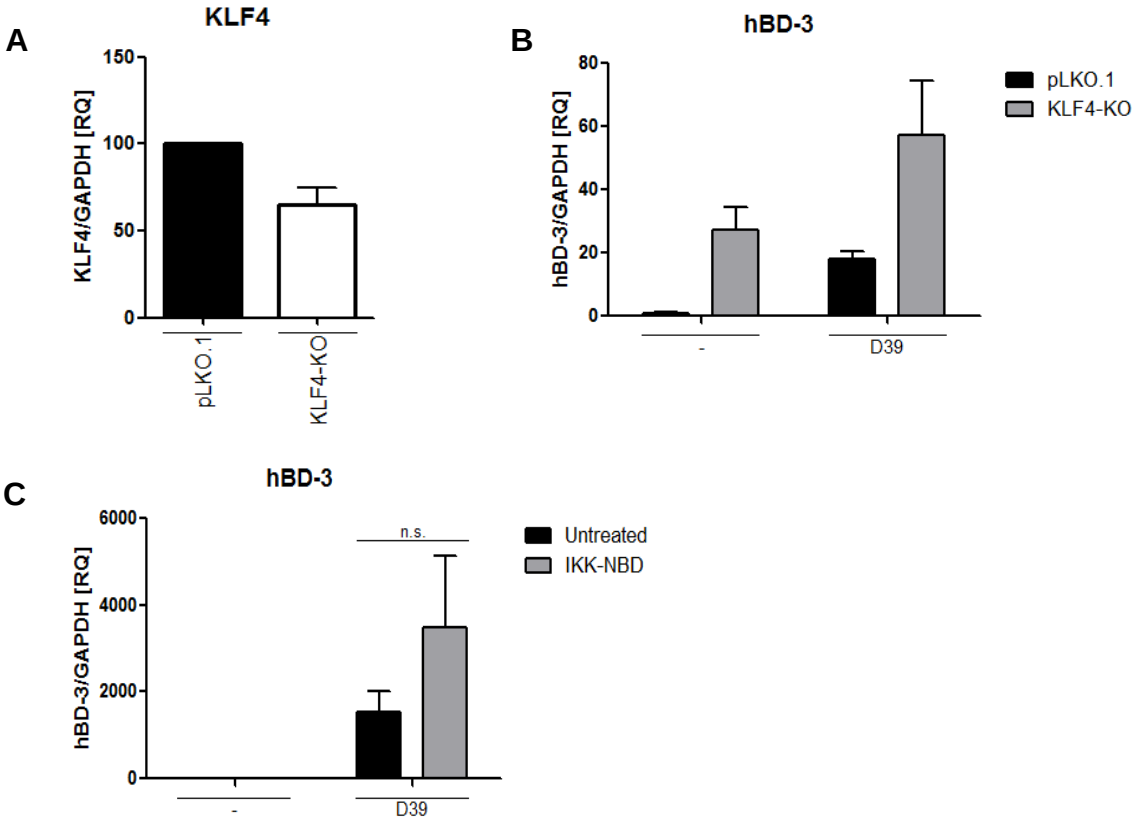

FIGURE 15: KLF4 is a negative regulator of hBD-3. A: Lentiviral-transduced THP-1 cells were used. Cells were either transfected with shRNA specific for KLF4 or with a scrambled control. The vector backbone is pLKO.1. **B:** KLF4 knock-down cells were infected with D39 (MOI 50) for 8h. When KLF4 was knocked-down expression of hBD-3 highly increased, even in the untreated cells, suggesting that KLF4 is a negative regulator of hBD-3. **C:** Cells were pretreated with PBS or the NF-κB inhibitor IKK-NBD (1μM) 3h before infection with D39 (MOI 50). Pretreated cells did not show a decrease in hBD-3 expression. Expression levels were obtained by q-RT-PCR. All data sets were normalized to GAPDH and graphs show the mRNA expression of hBD-3 relative to the uninfected control. **A and C:** Data are shown as mean +/- SEM of three independent experiments. Statistical test used: two-tailed Mann-Whitney-Test. **B:** Data are representative of at least three independent experiments.

3.1.7 hBD-3 IS BACTERICIDAL AGAINST UNENCAPSULATED BACTERIA

The antimicrobial activity of hBD-3 has been shown in several publications [332,339] thus in this study it was tested if recombinant hBD-3 would be able to kill encapsulated and unencapsulated *S. pneumoniae* strains D39 and R6x. hBD-3 was clearly antimicrobial against unencapsulated R6x (Fig.16A) whereas there was no effect on the encapsulated D39 (Fig.16B).

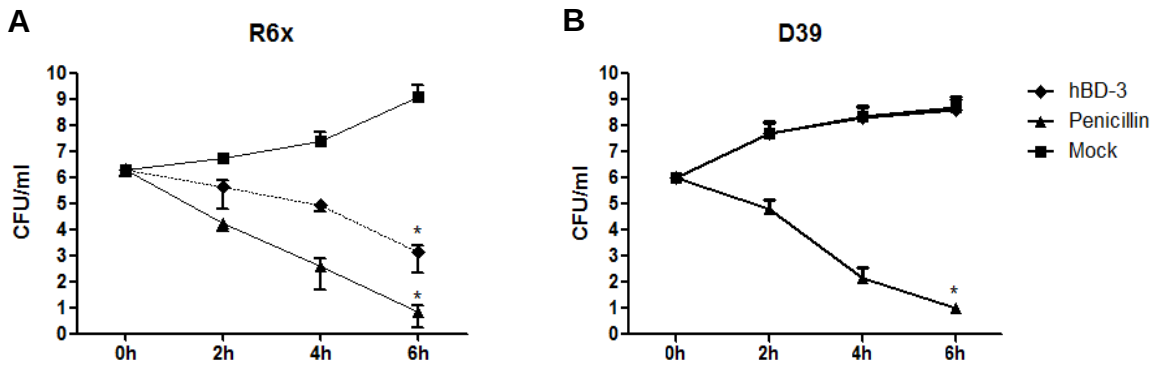

FIGURE 16: hBD-3 is bactericidal against unencapsulated pneumococci but has no effect on the encapsulated wt strain. Growth-curve of encapsulated *S. pneumoniae* strain D39 and unencapsulated *S. pneumoniae* strain R6x. Concentrations used were 1µg/ml of Penicillin G and 7µg/ml of hBD-3. **A:** Bacteria that were treated with recombinant hBD-3 showed a significant reduction of growth. **B:** On encapsulated D39 hBD-3 had no effect. Penicillin G achieved a significant reduction of bacterial number. Data are shown as mean +/- SEM of three independent experiments. Significance is indicated by asterisks,*=p<0.05. Statistical test used: two-tailed Mann-Whitney-Test.

3.1.8 hBD-3 HAS AN EFFECT ON TNFα PRODUCTION IN *S. PNEUMONIAE* INFECTED CELLS

To further investigate whether hBD-3 might have an additional function to antimicrobial activity; cytokines produced by THP-1 cells infected with *S. pneumoniae* and stimulated with recombinant hBD-3 were analyzed. Before stimulation of cells with rhBD-3, an LDH assay was done to determine cytotoxic effects rhBD-3 could have on THP-1 cells. The LDH assay confirmed the used concentrations of rhBD-3 not to be cytotoxic (Fig.17A).

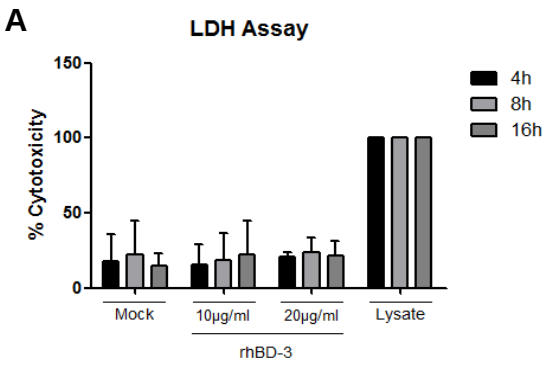

FIGURE 17: hBD-3 has no cytotoxic effect on THP-1 cells. THP-1 cells were stimulated with 10µg/ml or 20µg/ml rhBD-3. LDH release was measured in cell free supernatant after 4h, 8h and 16h of stimulation. As positive control cells were lysed by 1% triton x-100. **A:** There was no cytotoxic effect of hBD-3. Data are shown as mean +/- SEM of two independent experiments.

Therefore cells were infected with D39 alone and with D39 and rhBD-3 in different concentrations. After 8h of infection IL1β and TNFα were measured in the supernatant by ELISA. There was no alteration of IL1β production by adding hBD-3 to infected cells (Fig.18A) but there was a decrease in TNFα production when infected cells were also treated with rhBD-3 (Fig.18B).

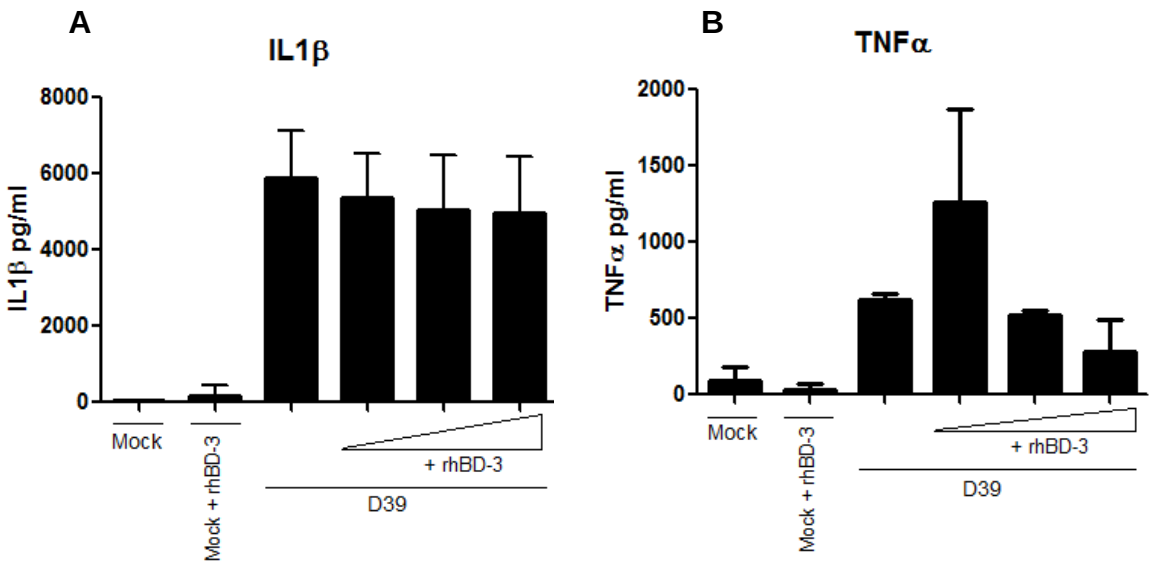

FIGURE 18: Recombinant hBD-3 decreases TNFα production in D39 stimulated THP-1 cells. ELISA of supernatant of D39 (MOI 50) and rhBD-3 (5µg/ml; 10µg/ml; 20µg/ml) stimulated cells. Cells were stimulated for 8h. **A:** IL1β production in D39 infected THP-1 cells was not altered by addition of rhBD-3. **B:** TNFα production of D39 infected THP-1 cells increases with 5µg/ml of rhBD-3 and decreases dose dependently with the higher concentrations. Interestingly even the basal expression of TNFα was decreased in uninfected but hBD-3 treated cells showing that rhBD-3 has an immunomodulating effect on macrophages by down-regulating TNFα production. Data are shown as mean +/- SEM of three independent experiments.

3.2 HUMAN BETA DEFENSIN 2

3.2.1 hBD-2 EXPRESSION PATTERNS

In contrast to hBD-3, the hBD-2 protein has been shown to be bacteriostatic against various bacteria [252,255,257]. Although reports state that it has no effect on gram-positive bacteria it might have immune modulatory functions when it comes to pneumococcal pneumonia. To determine if hBD-2 is expressed in *S. pneumoniae* infection, HuLus were stimulated with *S. pneumoniae* strain D39 (1x10^6 CFU/ml) for 24h. Although there is no statistical significance, hBD-2 expression clearly increases in infected human lung tissue (Fig.19A). Since in HuLus there are various cell types present, this study focused on macrophages as they are an important part of the innate immune system bridging innate to adaptive immunity. For analysis of expression patterns of hBD-2 in human macrophages, PBMCs were infected with *S. pneumoniae* and mRNA levels were analyzed. In PBMCs hBD-2 was expressed in a dose dependent manner when cells were infected with different MOIs of *S. pneumoniae* D39 (Fig.19B).

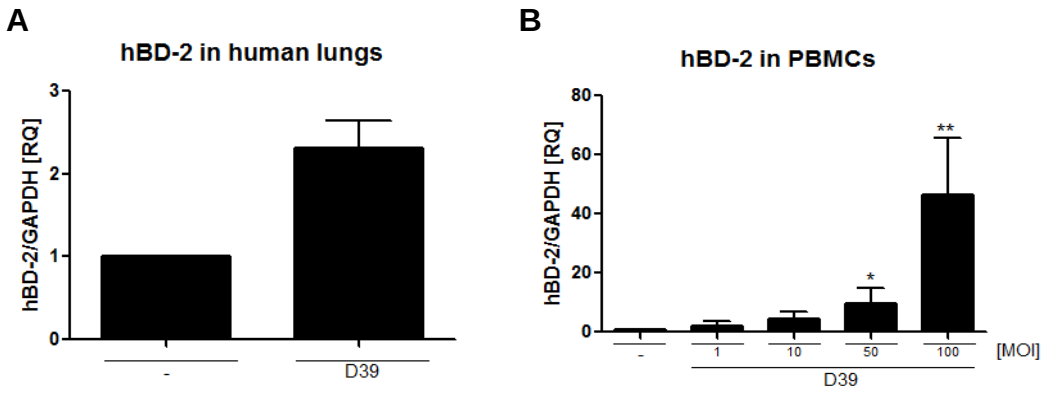

FIGURE 19: Expression of hBD-2 in human lung tissue and macrophages. **A:** Expression of hBD-2 is induced by *S. pneumoniae* D39 in HuLus after 24h of infection. Lungs were infected with 1x10^6 CFU/ml D39 for 24h. **B:** Expression of hBD-2 was induced in human PBMCs after 8h of infection with D39 in a dose-dependent manner. MOIs used range from 1-100. Cells were infected in RPMI 1640 w/o FCS. Expression levels were obtained by q-RT-PCR. All data sets were normalized to GAPDH. Graphs show the mRNA expression of hBD-2 relative to the uninfected control. Data are shown as mean +/- SEM of three independent experiments. Significance is indicated by asterisks,*=p<0.05, **=p<0.01. Statistical test used: Kruskal-Wallis Test followed by a Dunn's post-hoc test.

Since hBD-2 and hBD-3 are both small, antimicrobial peptides and are located in close proximity to each other on chromosome 8, a similar expression pattern would be expected. To determine if expression patterns of hBD-2 are really similar to these of hBD-3, PBMCs were infected with D39ΔPLY in addition to D39. Interestingly hBD-2 is more strongly induced when the bacteria are deficient for PLY (Fig.20A), which is in clear contrast to the expression patterns of hBD-3. To confirm that this result is not due to chance or specific for the donors, the macrophage cells lines THP-1 and U937 were similarly infected with D39ΔPLY. In both cell lines, hBD-2 expression is increased when cells were infected with D39ΔPLY compared to infection with wt D39 (Fig.20B and Fig.20C). Furthermore expression of hBD-2 is dose and time dependent in THP-1 cells (Fig.20D and Fig.20E).

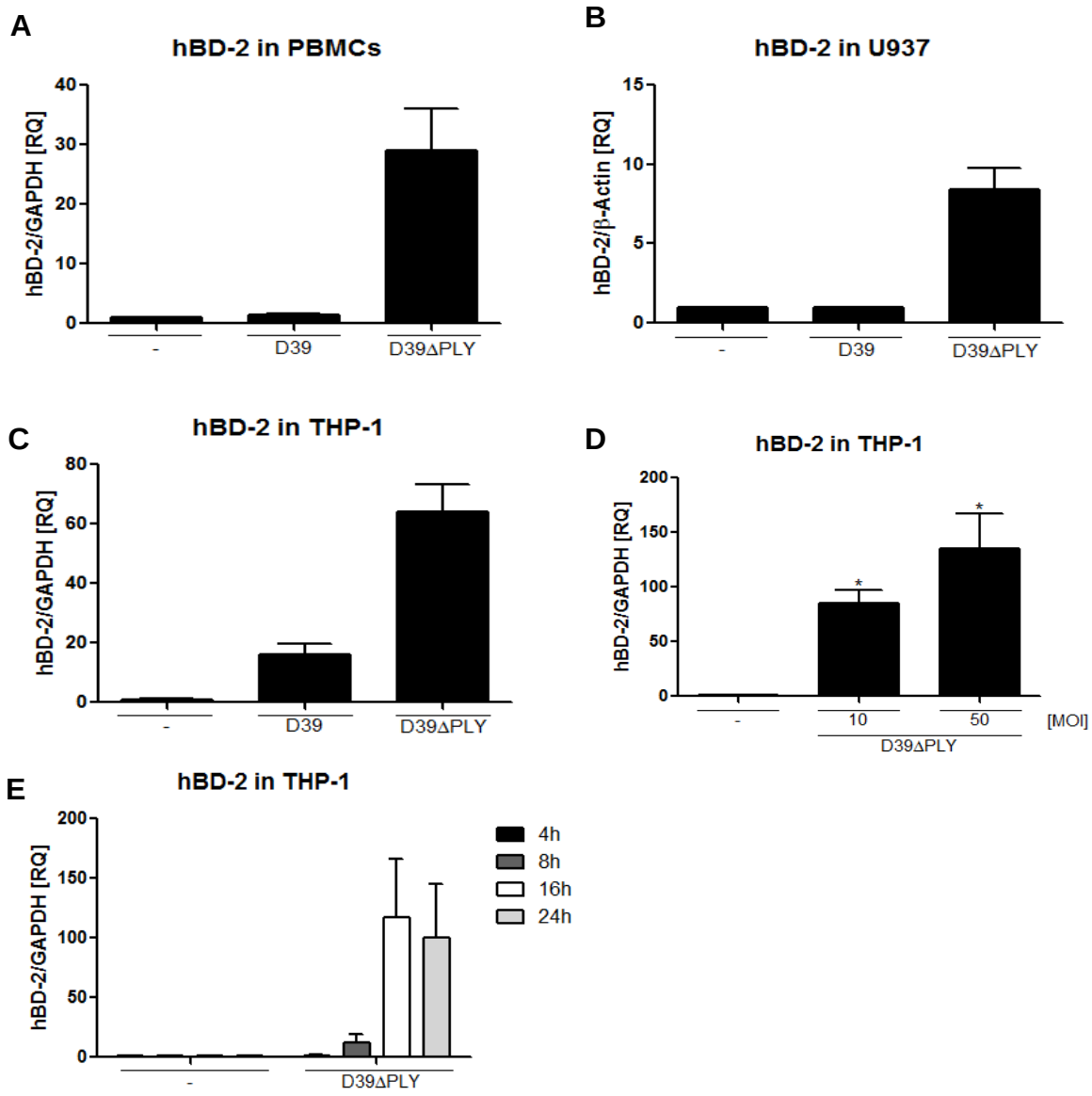

FIGURE 20: Expression of hBD-2 is increased in the absence of PLY. All cells were infected with MOI 50, THP-1 cells were additionally infected with MOI 10 (**D**). Infection was done for 8h (**B**) and 16h (**A, C and D**) in RPMI 1640 w/o FCS. **A:** Infection of PBMCs with D39 and D39ΔPLY. hBD-2 induction is increased in cells infected with D39ΔPLY. **B:** In U937 cells expression of hBD-2 is increased when cells were infected with D39ΔPLY compared to infection with D39. **C and D:** THP-1 cells infected with D39 and D39ΔPLY showed the same expression patterns as PBMCs and U937 cells. hBD-2 increased when cells were infected with D39ΔPLY for 8h (**C**) and 16h (**D**). Furthermore, induction of hBD-2 is dose dependent (**D**). **E:** There is a time dependency of hBD-2 induction in THP-1 cells infected with D39ΔPLY, with the highest expression levels after 16h. Expression levels were obtained by q-RT-PCR. All data sets were normalized to GAPDH and β-Actin respectively. Graphs show the mRNA expression of hBD-2 relative to the uninfected control. Data are shown as mean +/- SEM of three independent experiments (**D**) or are representative of at least three independent experiments (**A, B, C, E**). Significance is indicated by asterisks,*=p<0.05, **=p<0.01. Statistical test used: Kruskal-Wallis Test followed by a Dunn's post-hoc test.

For analysis of hBD-2 expression on protein level, hBD-2 was measured in cell free supernatant with an hBD-2 ELISA kit. Concentrations of hBD-2 were expected to be similarly low as concentrations of hBD-3, therefore samples were concentrated 25x. hBD-2 is measurable in THP-1 cells infected with D39ΔPLY MOI 100 for 24h (Fig.21A), confirming that induction is not limited to the mRNA level.

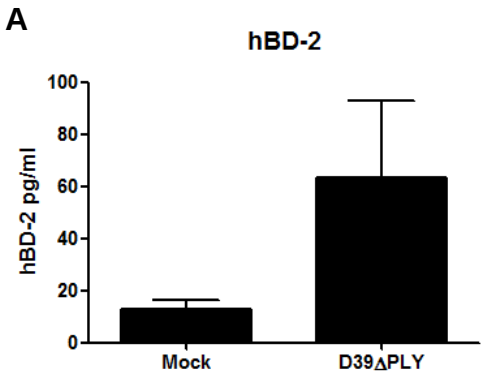

FIGURE 21: hBD-2 is expressed on protein level. A: THP-1 cells were infected with D39ΔPLY (MOI 100) for 24h. There is an induction of hBD-2 protein measurable in cell free supernatant. Data are shown as mean +/- SEM of two independent experiments.

3.2.2 INDUCTION OF hBD-2 IS NEGATIVELY REGULATED BY PNEUMOLYSIN

To further determine the involvement of PLY in hBD-2 induction, THP-1 cells were infected with D39, D39ΔPLY and rPLY; alone and in combination. Since the general expression pattern showed that wt D39 induced less hBD-2 than D39ΔPLY it was expected that rPLY added to D39ΔPLY will reduce the expression levels considerably. As shown in Fig.22A rPLY does indeed reduce expression levels of hBD-2 in THP-1 cells infected with D39ΔPLY. In line with my expectations, rPLY alone did not induce hBD-2. Since PLY seems to be a negative regulator of hBD-2 induction, cells were stimulated with hK D39. As dead bacteria are not able to release PLY, induction of hBD-2 was expected. In accordance with the expectations and results obtained so far, hK D39 did induce hBD-2 expression and the expression level was decreased when cells were stimulated with rPLY in addition to hK D39 (Fig.22B), although this reduction is not as strong as in viable bacteria. To verify that reduced expression levels in rPLY treated cells are not due to excessive cell death, IL8 was measured as

control. IL8 mRNA levels were increased in all samples simulated with *S. pneumoniae* D39 and D39ΔPLY (Fig.10B and Fig.10D).

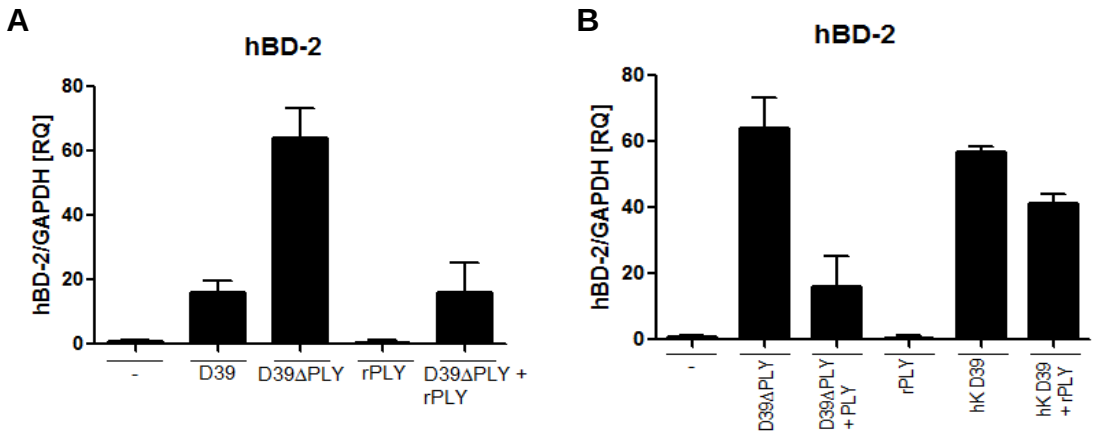

FIGURE 22: PLY-dependent expression of hBD-2 in THP-1 cells. THP-1 cells were left uninfected or infected with D39, D39ΔPLY, rPLY, D39ΔPLY + rPLY, heat-killed (hK) D39 and hK D39 + rPLY for 8h. **A:** Expression of hBD-2 in THP-1 cells infected with D39 or D39ΔPLY (MOI 50). Expression levels obtained by stimulation with D39ΔPLY could be decreased to levels induced by D39 by adding 0.5µg/ml rPLY. **B:** hK D39 (MOI 200) as well as D39ΔPLY did induce hBD-2. When rPLY was added to D39ΔPLY expression levels were decreased. When rPLY was added to hK D39 reduction of expression levels was less pronounced. Expression levels were obtained by q-RT-PCR. All data sets were normalized to GAPDH and graphs show the mRNA expression of hBD-2 relative to the uninfected control. Data are representative of at least three independent experiments.

3.2.3 INDUCTION OF hBD-2 IS DEPENDENT ON TLR2 AND MyD88

The signaling pathways leading to induction of hBD-2 in human macrophages are still poorly understood, thus this study aims to shed some light on the receptors and adapter molecules involved in these signaling cascades. TLR2 was shown to be the receptor for hBD-2 related signaling and MyD88 is an important adapter molecule connected to TLR2 signaling pathways. For analysis of MyD88 involvement, the MyD88 knock-down THP-1 cells as well as the pLKO.1 control cells were infected with D39ΔPLY. The cell lines used were the same as for analysis of MyD88 involvement in hBD-3 induction and the knock-down rate was 80% (Fig.14B). MyD88 is clearly important for hBD-2

induction as mRNA expression levels were decreased around 30-fold in the knock-down cell line compared to the control cell line (Fig.23A).

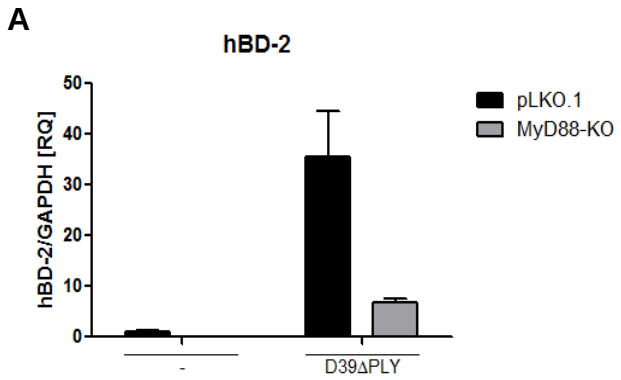

FIGURE 23: Expression of hBD-2 is dependent on MyD88. Stimulation was done with D39ΔPLY (MOI 50) for 8h. **A:** A lentiviral transduced knock-down THP-1 cell line was used. In MyD88 knock-down cells, expression of hBD-2 was decreased severely compared to the control cell line pLKO.1. Expression levels were obtained by q-RT-PCR. All data sets were normalized to GAPDH and graphs show the mRNA expression of hBD-2 relative to the uninfected control. Data are representative of at least three independent experiments.

The signaling pathway leading to induction of hBD-2 is obviously very different from that of hBD-3 although both peptides have a lot in common. Since up to now there is no clear knowledge on the pathways involved in hBD-2 induction in human macrophages this study aims to clarify certain aspects of the signaling that follows *S. pneumoniae* infection. Recognition of pathogens by the innate immune systems relies to a great extent on PAMPs. These PAMPs are recognized by TLRs. Since hBD-2 was induced by the PLY-deficient strain rather than the wt strain, TLR4 is most likely not involved in hBD-2 induction. More likely receptors would be TLR9 and TLR2. Macrophages are professional phagocytic cells of the immune system; therefore D39ΔPLY might be detected by TLR9 in the phagolysosome. TLR9 involvement was verified by stimulation of THP-1 cells with the CpG oligonucleotide mimicking bacterial DNA. The expression of hBD-2 was not increased by CpG (Fig.24A) showing that TLR9 is not responsible for hBD-2 induction in human macrophages. IL8 was induced in the bacteria stimulated samples (Fig.13B).

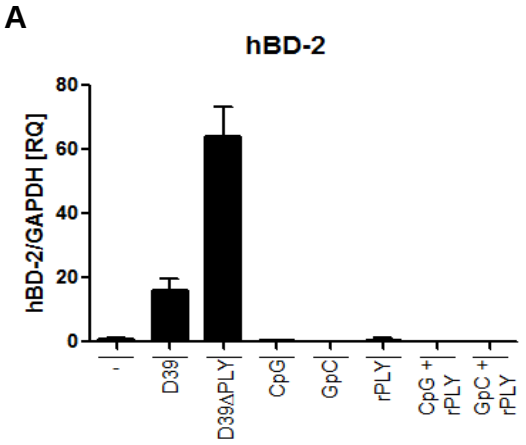

FIGURE 24: Expression of hBD-2 is not dependent on TLR9. Expression of hBD-2 in THP-1 cells uninfected or infected with D39 (MOI 50), rPLY (0.5µg/ml), CpG (2.5µg/ml) and CpG + rPLY for 8h. GpC is the negative control to CpG and was used in a concentration of 2.5µg/ml. **A:** Expression of hBD-2 was induced when cells were infected with D39ΔPLY and to a lesser extent with D39 but not when CpG, GpC or rPLY were used, either alone or in combination. All data sets were normalized to GAPDH and graphs show the mRNA expression of hBD-2 relative to the uninfected control. Data are representative of at least three independent experiments.

TLR2 recognizes cell wall components such as lipoteichoic acid. In this study the TLR2/6 antagonist MALP-2 was used to stimulate THP-1 cells in addition to the bacterial strain D39ΔPLY. Induction of hBD-2 was achieved by D39ΔPLY and MALP-2 (Fig.25A) whereas MALP-2 lead to a higher induction of hBD-2 mRNA than the bacteria, confirming an important role of TLR2 in the signaling cascade leading to hBD-2 induction in human macrophages. Interestingly, when rPLY was added to MALP-2, expression levels decreased comparable to when rPLY was added to D39ΔPLY. In addition to hBD-2, IL8 mRNA levels were measured in stimulated cells. As is shown in Fig.25B IL8 levels were high in all stimulated samples leading to the conclusion that PLY is responsible for the decrease of hBD-2 levels (The graph is identical to Fig.11B; only D39ΔPLY is shown instead of D39). Following the experiments with MALP-2 a knock-down cell line for TLR2 was created. In TLR2 knock-down cells hBD-2 induction was severely decreased compared to induction of hBD-2 in the control cell line pLKO.1 (Fig.25C). Induction was not completely abolished because the knock-down of TLR2 was not complete but around 60% (Fig.25D). Nevertheless, this

set of experiments clearly shows that TLR2 is responsible for induction of hBD-2 in human macrophages stimulated with *S. pneumoniae*.

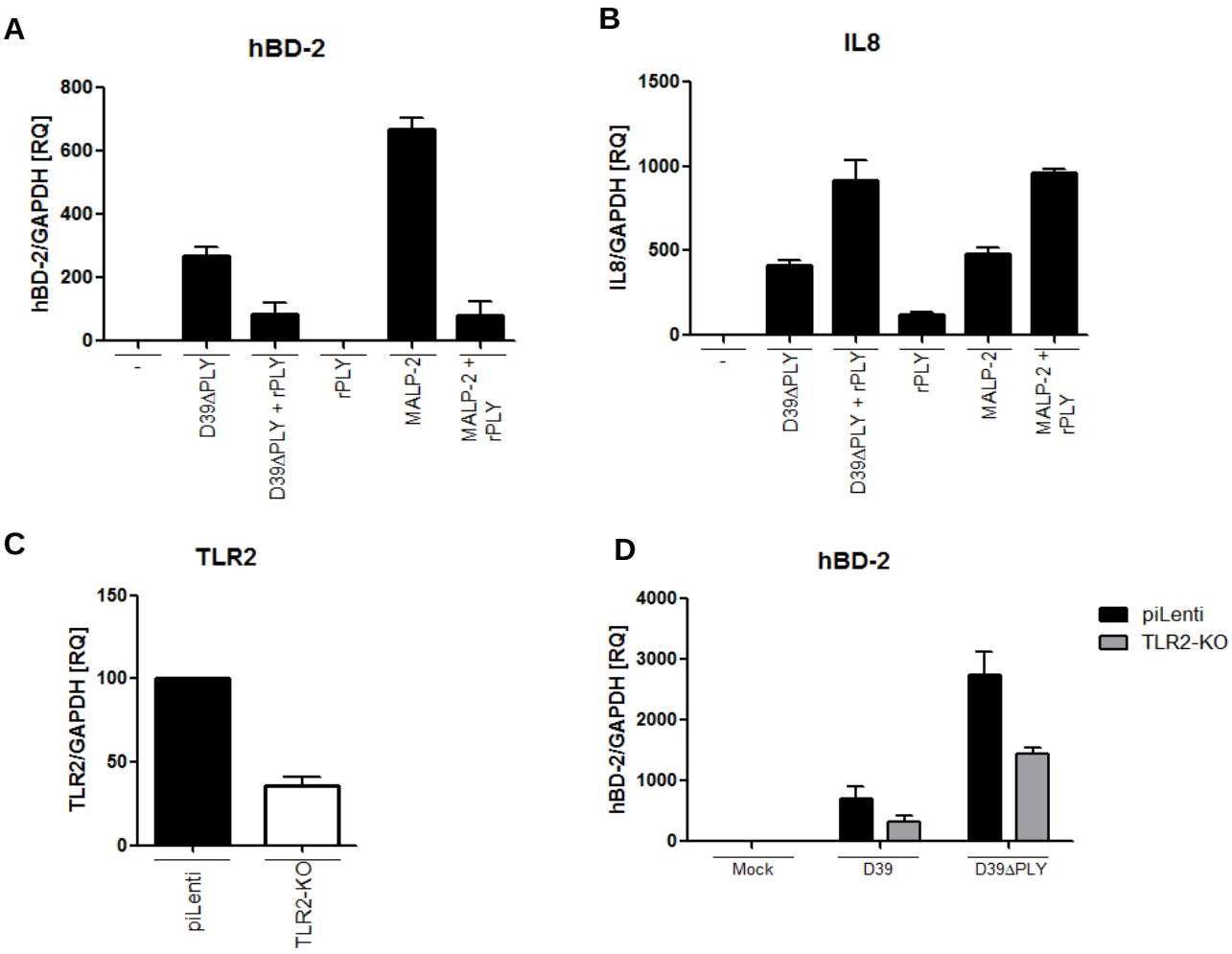

FIGURE 25: TLR2 is implemented in pneumococci-induced hBD-2 expression in macrophages. Expression of hBD-2 in THP-1 cells uninfected or infected with D39 or D39ΔPLY (MOI 50), rPLY (0.5µg/ml), MALP-2 (50ng/ml) and MALP-2 + rPLY for 8h. **A:** Expression of hBD-2 was induced by stimulation of cells with D39ΔPLY as well as by stimulation with the TLR2/6 agonist MALP-2. In both cases addition of rPLY reduced the hBD-2 expression levels in THP-1 cells. **B:** IL8 control for stimulation of the THP-1 cells. D39ΔPLY as well as MALP-2 and rPLY alone and in combination led to an induction of IL8 mRNA in THP-1 cells. **C:** Knock-down of TLR2 was 60%. **D:** Expression of hBD-2 was clearly decreased when TLR2 knock-down cells were stimulated with D39 or D39ΔPLY compared to the control cell line piLenti, confirming TLR2 to be important in hBD-2 induction. Expression levels were obtained by q-RT-PCR. All data sets were normalized to GAPDH and graphs show the mRNA expression of hBD-2 relative to the uninfected control. **A and B:** Data are representative of at least three independent experiments. **C:** Data are shown as mean +/- SEM of two independent experiments. **D:** Data are representative of two independent experiments.

3.2.4 INDUCTION OF hBD-2 IS INCREASED WHEN PHAGOCYTOSIS IS BLOCKED

Although TLR9 is not involved in hBD-2 induction, phagocytosis might still be important for detection of bacteria since macrophages are professional phagocytes. To verify if phagocytosis has an influence on hBD-2 expression levels, THP-1 cells were treated with CytD prior to infection with *S. pneumoniae*. For stimulation of the cells the strains D39 and D39ΔCPS were used. mRNA levels of hBD-2 were measured in all samples. When it comes to induction of hBD-2, phagocytosis is not important as expression levels increase when phagocytosis is blocked (Fig.26A) which is in clear contrast to hBD-3 where phagocytosis does play a role in induction of the protein. For corresponding expression levels of IL8 please refer to Fig.12B.

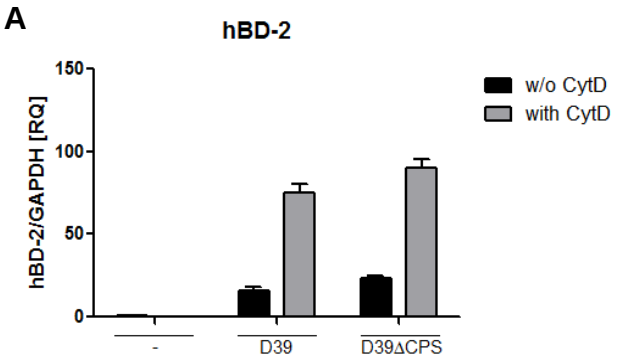

FIGURE 26: Phagocytosis-related expression of hBD-2. A: Expression of hBD-2 in THP-1 cells with or without prior CytD treatment. Cells were infected with D39 and D39ΔCPS (MOI 50) for 8h. One set of cells was pretreated with CytD at a concentration of 1mM 30min before infection to inhibit phagocytosis. When phagocytosis was inhibited expression levels of hBD-2 were higher than in non-treated cells. Expression levels were obtained by q-RT-PCR. All data sets are normalized to GAPDH and graphs show the mRNA expression of hBD-2 relative to the uninfected control. Data are representative of at least three independent experiments.

3.2.5 EXPRESSION OF hBD-2 IS DEPENDENT ON p38 MAPK AND ERK

MAP kinases are a group of signal transducer molecules that are important for expression of inflammatory cytokines in macrophages and are activated by TLRs [418,419]. Therefore involvement of p38, JNK and ERK in hBD-2 induction were tested in this study. THP-1 cells were pretreated with inhibitors specific for ERK (U0126), p38 (SB202190) or JNK (SP600125) for 3h before infection. The inhibitor was left on the cells for the whole infection period. hBD-2 was induced by D39ΔPLY in untreated cells but not in cells where ERK or p38 were inhibited. Expression of hBD-2 was reduced 20-fold when p38 was inhibited and 25-fold when there was inhibition of ERK (Fig.27B). The JNK inhibitor had no significant effect on hBD-2 expression levels (Fig.27B).

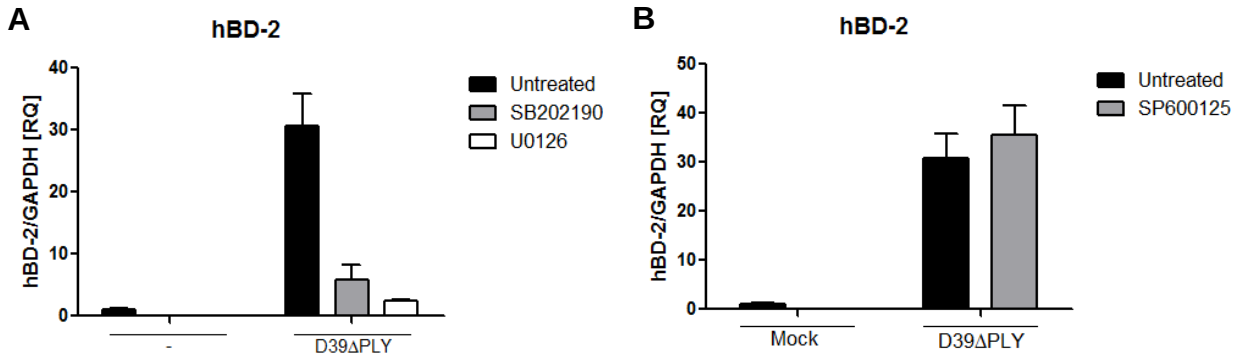

FIGURE 27: Expression of hBD-2 is dependent on ERK and p38. Stimulation was done with D39ΔPLY (MOI 50) for 8h. Cells were pretreated with the p38 inhibitor SB202190 (10µM), the ERK inhibitor U0126 (10µM) or the JNK inhibitor SP600125 (10µM) 3h before infection. **A:** Pretreated cells showed a decrease in hBD-2 expression compared to untreated cells. **B:** Pretreated cells did not show a difference in hBD-2 expression when compared to untreated cells. Expression levels were obtained by q-RT-PCR. All data sets were normalized to GAPDH and graphs show the mRNA expression of hBD-2 relative to the uninfected control. Data are representative of at least three independent experiments.

3.2.6 NF-κB IS NOT IMPLEMENTED IN hBD-2 INDUCTION

With NF-κB being the most important transcription factor in TLR signaling, its role in hBD-2 induction was determined. Therefore, THP-1 cells were pretreated with the IKK-NBD peptide, a specific inhibitor of NF-κB activation. Results demonstrate that NF-κB is not implemented in hBD-2 induction in *S. pneumoniae* infected macrophages (Fig.28A).

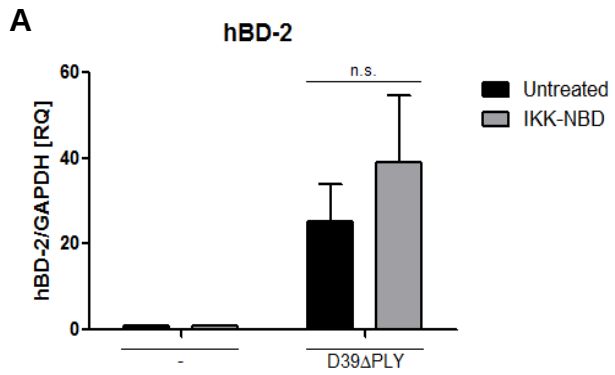

FIGURE 28: NF-κB does not play a role in hBD-2 induction in human macrophages. Stimulation was done with D39ΔPLY (MOI 50) for 8h. **A:** Cells were pretreated with the NF-κB inhibitor IKK-NBD (1μM) 3h before infection. Pretreated cells did not show a difference in hBD-2 expression when compared to untreated cells. Expression levels were obtained by q-RT-PCR. All data sets were normalized to GAPDH and graphs show the mRNA expression of hBD-2 relative to the uninfected control. Data are shown as mean +/- SEM of three independent experiments. Statistical test used: two-tailed Mann-Whitney-Test.

3.2.7 hBD-2 DOES NOT HAVE A BACTERIOSTATIC EFFECT AGAINST *S. PNEUMONIAE*

hBD-2 belongs to the group of antimicrobial peptides and is closely related to hBD-3. However, reports on its antimicrobial activity vary. It has been reported to be bactericidal [333,334], bacteriostatic [316,420] or non effective against gram-positive bacteria [336]. Nevertheless a growth curve was done in the framework of this study to confirm that there is no effect of this protein against the encapsulated and the unencapsulated strains. The data obtained clearly show that hBD-2 did not have a bactericidal or bacteriostatic effect against unencapsulated R6x (Fig.29A) or encapsulated D39 (Fig.29B).

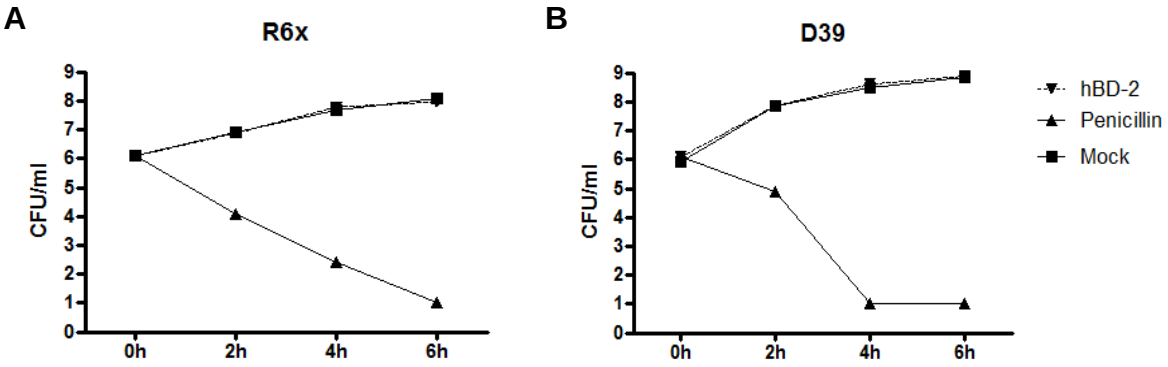

FIGURE 29: hBD-2 has no bactericidal or bacteriostatic effect against encapsulated and unencapsulated pneumococci. Growth-curve of encapsulated *S. pneumoniae* strain D39 and unencapsulated *S. pneumoniae* strain R6x. Concentrations used were 1µg/ml of Penicillin G and 7µg/ml of hBD-2. At time-point zero 10^6 cfu/ml were transferred to fresh THY medium and left in the incubator at 37°C. Every two hours a sample was taken and plated onto blood agar plates. Colony number was determined after 20h of growth at 37°C and 5% CO_2. **A:** Only bacteria that were treated with penicillin G showed a growth reduction, hBD-2 had no effect **B:** On encapsulated D39 hBD-2 had no effect. Penicillin G achieved a clear reduction of bacterial number. Data are shown as mean +/- SEM of two independent experiments.

3.2.8 hBD-2 HAS AN EFFECT ON TNFα PRODUCTION IN *S. PNEUMONIAE* INFECTED CELLS

Since there is neither a bacteriostatic nor a bactericidal effect of hBD-2 against *S. pneumoniae*, I decided to check the immune modulatory functions of the protein. There are reports stating that the beta defensins act as chemokines via CCR6 and CCR2 but up till now the modulating effect of hBD-2 on cytokine production is not well understood. Thus the ability to alter cytokine expression in macrophages was investigated. hBD-2 has no cytotoxic effect on macrophages as was determined by LDH-assay (Fig.30A).

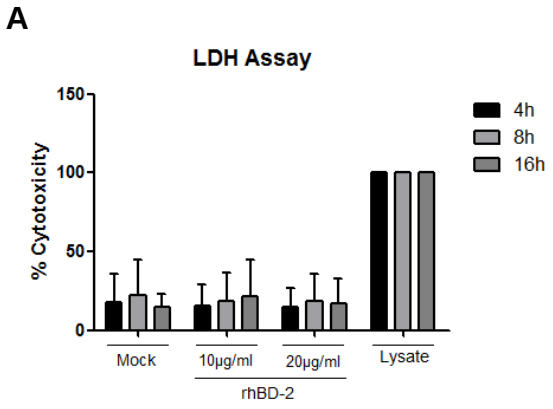

FIGURE 30: hBD-2 has no cytotoxic effect on THP-1 cells. THP-1 cells were stimulated with 10µg/ml or 20µg/ml rhBD-2. LDH release was measured in cell free supernatant after 4h, 8h and 16h of stimulation. As positive control cells were lysed by 1% triton x-100. **A:** There was no cytotoxic effect of hBD-2. Data are shown as mean +/- SEM of two independent experiments.

For analysis of cytokines cell free supernatants of THP-1 cells infected with D39 and different concentrations of rhBD-2 were collected and analyzed by ELISA. There was no difference in IL1β secretion (Fig.31A) but the amounts of TNFα were considerably lower in cells treated with rhBD-2 compared to non-treated cells (Fig.31B). Although the reduction in cytokine production was not significant, these results clearly hint at an immune modulatory effect of hBD-2 and should be further investigated.

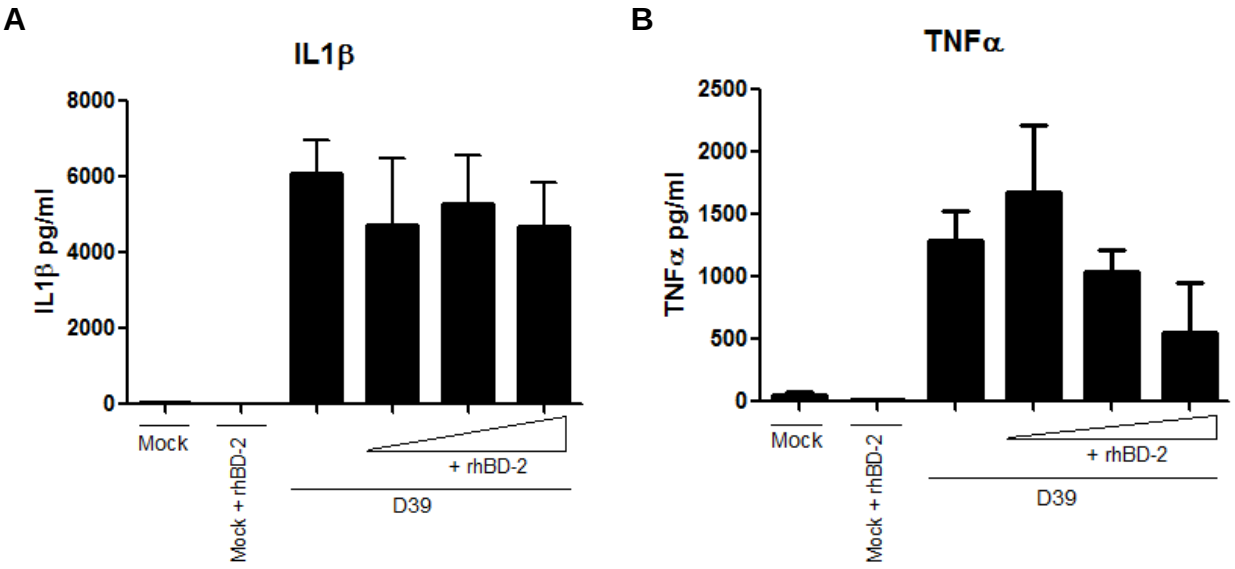

FIGURE 31: Recombinant hBD-2 decreases TNFα but not IL1β production in D39 stimulated THP-1 cells. ELISA of supernatant of D39 (MOI 50) and rhBD-2 (5µg/ml; 10µg/ml; 20µg/ml) stimulated cells. Cells were stimulated for 8h. **A:** IL1β production in D39 infected THP-1 cells was not altered by addition of hBD-2. **B:** TNFα production of D39 infected THP-1 cells decreased with higher concentrations of rhBD-2 and even the basal expression of TNFα was decreased in uninfected but hBD-2 treated cells showing that rhBD-2 has an immunomodulating effect on macrophages by down-regulating TNFα production. Data are shown as mean +/- SEM of three independent experiments.

4. DISCUSSION

According to various previous studies, human β-defensins have an antimicrobial effect on a multitude of pathogens [264,341,343,421] and in addition are able to modulate immune responses thus if the mechanisms leading to induction of these endogenous peptides would be fully understood, exogenous regulation of the β-defensins in pneumococcal pneumonia patients could improve the outcome of the disease. Hence, the expression patterns, the signaling pathways as well as bacterial factors that lead to induction of hBD-2 and hBD-3 in human macrophages, challenged with the causative agent of CAP, *S. pneumoniae,* were analyzed. Furthermore, this study exploits the functions of both defensins in *S. pneumoniae* infected macrophages. Results obtained by this study show that hBD-2 and hBD-3 are induced in human macrophages upon stimulation with *S. pneumoniae* and for the first time it was shown that both molecules are differentially regulated via distinct signaling pathways. While induction of hBD-3 is achieved via TLR4, MyD88 and PI3K in dependence of the bacterial exotoxin PLY, induction of hBD-2 involves TLR2, MyD88 and p38 MAPK / ERK signaling and is negatively regulated by PLY. The capsule and bacterial DNA are not involved in this differential activation of the β-defensins. Moreover, both defensins modulate the immune response of infected macrophages by regulation of TNFα production. An increase of TNFα was observed at low β-defensin concentration whereas a decrease was measurable at higher concentrations, indicating a bifunctional role of these peptides during infection and inflammation.

4.1 EXPRESSION PATTERNS OF hBD-2 AND hBD-3

Since the discovery of human endogenous antimicrobial peptides, extensive studies have been conducted to investigate cells expressing these peptides. It has been found that hBD-2 and hBD-3 are expressed by human keratinocytes, endothelial and epithelial cells, some muscle cells as well as neutrophils and macrophages stimulated with pathogenic micro-organisms [308,316,321,352,355,390,391,422]. In accordance with the results obtained by Scharf *et al.* in human pulmonary cells [352], this study shows expression of hBD-2 and hBD-3 in *S. pneumoniae* stimulated human lung tissue. The expression of hBD-3 and hBD-2 increased up to 40-fold compared to the uninfected control, indicating an important role of these peptides in *S. pneumoniae* infected lungs. This is in line with current literature as several others found β-defensins to be important in lung associated diseases [420,423–425]. In this experimental set-up the β-defensin producing cell type could not be determined and since the induction and part of the signaling cascade in human lung epithelial cells has already been shown [352] this study focused on macrophages, the main effector cells of innate immunity and therefore the first line of defense against invading pathogens. For analysis of β-defensin expression in human macrophages, peripheral blood mononuclear cells (PBMCs) were isolated from blood donors. This study is the first to show induction of hBD-2 and hBD-3 in human PBMCs by *S. pneumoniae* in a dose-dependent manner. For the following experiments the immortalized cell line THP-1 was used as a model cell line for human macrophages as the expression patterns of both β-defensins were identical in PBMCs, THP-1 and U937 cells leading to the assumption that the identified expression patterns can be found in all macrophages independent of origin. Interestingly, hBD-3 expression was more elevated than hBD-2 expression in all D39 infected samples. This was due to the PLY-dependent induction of hBD-3, which will be discussed in more detail below.

4.2 BACTERIAL FACTORS IMPLEMENTED IN hBD-2 AND hBD-3 INDUCTION

S. pneumoniae utilizes various virulence factors to invade the host and escape the host's immune system. One of the most important virulence factors is the bacterial capsule. The pneumococcal capsule is a layer of polysaccharides and peptidoglycan of varying thickness on top of the cell wall of the pathogen [34]. It aids invasion of the lower respiratory tract by preventing phagocytosis and by masking bacterial cell wall antigens, thereby shielding the pathogen from the immune system [7,42]. The capsule has been shown to play a role in immune recognition and response by several other studies [426–428] and de Vos *et al.* found that the capsule impairs recognition of *S. pneumoniae's* TLR ligands [429]. In contrast to these findings, the capsule is neither necessary for, nor preventive of hBD-2 and -3 induction in human macrophages (PBMCs and THP-1) as was confirmed in this study by stimulation of cells with the wt D39 as well as the unencapsulated strains D39ΔCPS and R6x. This result is not due to immune evasion mechanisms of *S. pneumoniae* and a subsequent unresponsiveness of cells in general because all stimulated macrophages up-regulated IL8 expression, a clear indicator for recognition of microorganisms. Hence, the capsule is not a defining factor for the induction of either β-defensin.

Since the capsule is not involved in induction of either β-defensin, the bacterial exotoxin PLY was investigated with regards to hBD-2 and -3 expression. PLY is released during bacterial autolysis [430] and in higher doses leads to cell damage and cell death [48,49]. In lower doses it is able to impair the respiratory burst of phagocytic cells and activate cytokine and chemokine production as well as the complement system [431–433]. This study revealed a clear PLY-dependency in hBD-3 induction. The wt strain induced increased amounts of hBD-3 compared to only a slight increase of expression as response to the PLY-deficient strain D39ΔPLY. In contrast to hBD-3, hBD-2 is strongly induced by D39ΔPLY and only to a lesser extent by wt D39. To further confirm PLY to be responsible for the differential regulation of both β-defensins, rPLY was used for stimulation of cells in addition to the PLY-deficient strain. When rPLY was added to D39ΔPLY, expression of hBD-3 increased and expression of hBD-2 decreased to levels matching expression induced by D39 alone, thereby in both

cases reconstituting the phenotype. While the absence of PLY clearly enhances hBD-2 expression in human macrophages, Kim *et al.* found that PLY is required for the induction of hBD-2 in human airway epithelial cells [434] and although they too confirmed p38 MAPK as important signaling transducer (see section 4.3.1 below), their results regarding hBD-2 being dependent on PLY are in clear contrast to the results obtained in this study. These differences probably stem from the different cells used, as macrophages are professional cells of the immune system and are able to apply various defense mechanisms, whereas epithelial cells are barrier cells, relying on immune cells to clear infections. Both cell types differ in their receptor configuration and consequently in their ability to recognize different stimuli. It has been shown by Thorley *et al.* [435] that human lung epithelial cells and macrophages respond in a distinct manner to LPS stimulation. After receptor activation macrophages utilized p38 MAPK and ERK signaling to induce IL1β and TNFα whereas this signaling cascade was not observed in the epithelial cells. These differences in signaling could also explain why PLY might be important for hBD-2 induction in epithelial cells but not in macrophages. As this study focused on macrophages only further investigations regarding the differences in signaling and induction of antimicrobial peptides in both cell types should be conducted. Moreover, it would be interesting to analyze β-defensin induction in co-culture experiments or in an *in vivo* mouse model as this would be closer to physiological conditions.

Additionally, PLY itself is not sufficient to induce hBD-3 but hBD-2 was induced when cells were stimulated with heat-killed (hK) D39 as was expected since these bacteria are no longer able to undergo autolysis and therefore cannot release PLY which counter-regulates hBD-2 induction. Consequently the hBD-2 induction decreased when bacteria were supplemented with rPLY. For induction of hBD-3 on the other hand viable bacteria were needed and stimulation of cells with dead bacteria did not induce hBD-3, not even when PLY was added. This leads to the assumption that hBD-3 induction requires a co-factor only present in viable bacteria in addition to PLY. To this end, it was not possible to identify the said factor but since it is connected to the viability of cells it could be one of the recently discovered vita PAMPs [436] or another bacterial factor released during bacterial autolysis. These autolysis-related PAMPs or bacterial factors as

well as the special viability patterns would be an interesting approach for future investigations.

To sum up this paragraph, this study is the first to describe a PLY-dependent regulation of hBD-3 as well as PLY negatively regulating hBD-2 induction in human macrophages upon stimulation with *S. pneumoniae*.

4.3 SIGNALING PATHWAYS

4.3.1 hBD-2

The signaling pathways leading to induction of hBD-2 in human macrophages are still poorly understood, thus this study aims to shed some light on the receptors and adapter molecules involved in these signaling cascades. As macrophages are professional phagocytic cells of the immune system, this study investigated for the first time if phagocytosis is required for hBD-2 induction. THP-1 cells were treated with CytD, a potent inhibitor of actin polymerization and therefore also of phagocytosis. The results revealed phagocytosis to be inhibitory in hBD-2 induction as there were increased amounts of hBD-2 mRNA when cells were rendered unable to phagocytose. This is in line with the finding that TLR9 is not implemented in hBD-2 induction. TLR9 is an internal receptor and will only be activated by internalized/phagocytosed bacterial ligands [108]. Since blockage of phagocytosis increased induction of hBD-2 it was hypothesized that activation of an extracellular receptor is a prerequisite for hBD-2 expression.

For analysis of receptors and the corresponding adapter molecule involved, knock-down THP-1 cell lines for TLR2, TLR4 and MyD88 were created and infected with *S. pneumoniae*. Furthermore, MALP-2 was used as a specific agonist for TLR2/6. TLR2/6 recognizes diacylated lipopeptides that are predominantly found in gram-positive bacteria [437]. The results demonstrate that hBD-2 induction in macrophages is dependent on TLR2, but not TLR4, which is in line with several other publications showing TLR2-dependency of hBD-2 induction in keratinocytes and other epithelial cells [327,350,438,439]. Although others also found TLR2 responsible for hBD-2 induction, this study is the first to suggest TLR2 signaling crucial for induction of hBD-2 in macrophages stimulated with *S. pneumoniae*.

TLR2 signaling always involves the adapter protein MyD88. Thus, it was expected to play a significant role in the induction of hBD-2 in human macrophages. In the present study it was found that MyD88 is indeed implemented in hBD-2 expression as was also shown by others in keratinocytes and other epithelial cells [350,440].

The aforementioned studies stimulated epithelial cells with the fungus *Malassezia furfur* and *Haemophilus influenza* (a gram-negative bacterium) and this study used *S. pneumoniae* to stimulate human macrophages which leads to the conclusion that the signaling pathways resulting in induction of hBD-2 are similar across cell types and independent of the stimulating pathogen as long as TLR2 is activated.

For determining the role of kinases downstream of TLR2, cells were treated with specific inhibitors for JNK, ERK and p38 MAPK. Results indicate that p38 MAPK and ERK but not JNK are necessary for elevation of hBD-2 expression in *S. pneumoniae* stimulated macrophages. While the involvement of MAPK in hBD-2 induction in general is a well established fact [323,324,358,441,442], the role of ERK remains controversial. Some studies identified ERK to be important in regulation of hBD-2 expression [323,440,443,444] whereas others found ERK not to be involved [329,442,445]. This discrepancy is probably due the use of different stimuli and different tissues. Gan *et al.* [444] postulate that the induction of hBD-2 release could be determined by different signaling molecules and at the same time seems to be dependent on the stimulus used. Furthermore, Krisanaprakornkit *et al.* [442] found signaling cascades leading to hBD-2 induction in epithelial cells to differ depending on whether a bacterial or a chemical stimulus was used. All cited studies focused on epithelial cells with gram-negative bacteria or chemicals as stimuli while this study analyzed macrophages infected with the gram-positive bacterium *S. pneumoniae*. Thus the obtained data showing that ERK inhibition lead to a considerable down-regulation of hBD-2 and thereby hinting at the involvement of this MAPK in hBD-2 induction could be specific for macrophage signaling as response to gram-positive pathogens. As in this study only *S. pneumoniae* was used as stimulus, it would be interesting to determine if this assumption holds true for other gram-positive bacteria associated with lung diseases. In conclusion, further investigations are certainly needed to determine ERKs role in induction and regulation of hBD-2 in different cell types and varying infectious settings. In contrast to ERK, many studies agreed on p38 MAPK to be necessary for hBD-2 induction [329,440,442–446]. In accordance with these publications this study also demonstrates an important role of p38 MAPK in hBD-2 induction as

expression of the peptide was severely decreased when a p38 MAPK inhibitor was applied.

Regarding transcription factors, this study analyzed if NF-κB was implemented in hBD-2 induction and found it not to play a role. This result is supported by Krisanaprakornkrit *et al.* [442] who explicitly found hBD-2 induction being independent of NF-κB. However, there are several other studies who found NF-κB and/or AP-1 to be crucial for expression of hBD-2 [358,444,447,448] one of them being Scharf *et al.* who found NF-κB to be necessary for hBD-2 induction in epithelial cells [352]. An explanation for this discrepancy of results might lie in the copy number and sequence variation. As β-defensin genes are not only copy number variable but also show a high density of sequence variations in promoter regions [287], transcription factor binding sites may vary in different cells from different donors. All in all, further investigations on transcription factors, ideally with regards to promoter sequence variations, are needed to completely understand the pathways leading to hBD-2 induction, not only in epithelial cells but also in leukocytes such as macrophages.

For a graphic representation of signaling leading to induction of hBD-2 in human macrophages infected with *S. pneumoniae* as identified in this study please refer to Fig.32.

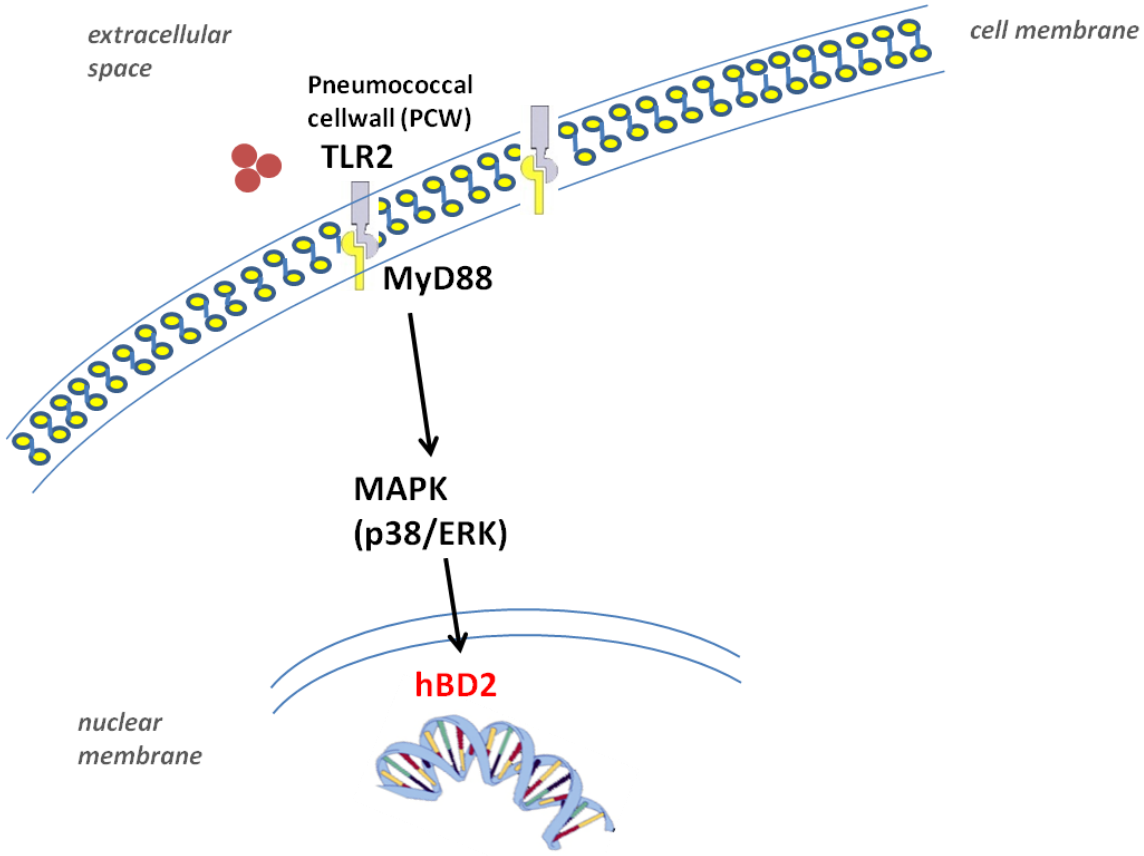

FIGURE 32: Proposed signaling pathway leading to hBD-2 induction in *S. pneumoniae* infected macrophages. Recognition of the bacteria via TLR2 is followed by MyD88 recruitment and subsequent activation of p38 MAPK and ERK. These kinases activate one or more transcription factors which up-regulate hBD-2 expression in macrophages. The template for this drawing was kindly provided by Toni Herta.

4.3.2 hBD-3

In contrast to hBD-2, hBD-3 is induced via TLR4 (and not TLR2) signaling. Since the results demonstrate that induction of hBD-3 in human macrophages requires PLY, involvement of TLR4 would be expected as PLY was shown to be detected by this receptor [117,372]. However, TLR4 involvement in hBD-3 induction remains controversial. Earlier studies in epithelial cells and keratinocytes found hBD-3 to be induced via TLR2 [350–352] while others also found TLR4 to be important [353,354]. Results of this study are supportive of the latter publications since stimulation of a TLR4 knock-down cell line confirmed this receptor to play a major role in hBD-3 induction in macrophages. These different observations regarding the receptors might be due to differences in cells and/or differences in microbial stimuli used. There are many factors that contribute to the infectious environment and little variations of cell culture and/or stimulus are probably sufficient to change the reactions of the analyzed cells.

As MyD88 is the most important adapter molecule downstream of TLR4 [449,450], its role in hBD-3 induction was investigated in a MyD88 knock-down cell line. Knock-down of MyD88 lead to decreased expression of hBD-3 in *S. pneumoniae* stimulated macrophages. Although Baroni *et al.* were able to detect up-regulation of MyD88 and hBD-3 in human keratinocytes stimulated with *Malassezia furfur* [350], this study confirms for the first time that MyD88 is necessary for hBD-3 induction in macrophages.

Furthermore, results revealed PI3K as important signaling molecule in hBD-3 induction. Our data is in accordance with Kawsar *et al.* who found that hBD-3 is induced via PI3K and not via the JAK/STAT signaling cascade in oral epithelial cells [357]. Moreover, it was shown that PI3K is indeed activated following TLR4 ligand binding [169,451] supporting the finding of this study. Nevertheless, there are other publications implicating a role of JNK [352,390] and ERK [351,452,453] in hBD-3 induction in epithelial cells and keratinocytes. As in hBD-2, there are no studies regarding macrophages thus the differences observed could be cell type specific. Moreover, PI3K itself was found to activate different MAPKs, including ERK [451,454]. Hence, induction of hBD-3 could either be a synergistically effect of PI3K and ERK or redundancy in signaling

pathways. Again, studies confirming involvement of ERK or JNK used epithelial cells or keratinocytes as well as a broad variety of stimuli with only one other study infecting cells (epithelial) with *S. pneumoniae* [352]. In conclusion, the results obtained by this study strongly point towards cell type specific signaling cascades in hBD-3 induction.

As activation of transcription factors is a prerequisite for induction of inflammatory genes, the role of two important transcription factors, KLF4 and NF-κB, was determined in the framework of this study. While inhibition of NF-κB did not alter hBD-3 expression, knock-down of KLF4 lead to a highly increased induction, measurable already in the uninfected cells. The results for NF-κB are in accordance with other publications that also found hBD-3 induction to be NF-κB independent [349,358,455]. As of yet there are no publications regarding the role of KLF4 in hBD-3 induction, thus this study is the first to identify KLF4 as negative regulator for hBD-3 expression. Since KLF4 was found to mediate pro-inflammatory responses in macrophages [168], this down-regulation of hBD-3 hints to an anti-inflammatory role of this β-defensin. The inhibition of hBD-3 expression in macrophages by KLF4 could be achieved by at least two mechanisms, either by the inhibitory domain of KLF4 directly at the hBD-3 promoter or via blockage of PI3K signaling. The inhibition of PI3K by KLF4 has been shown previously by Zheng *et al.* [417].

In summary, data and literature suggest a cell type-dependent and multiple pathways involving regulation of hBD-3 [357,456–458]. Data obtained on signaling leading to hBD-3 induction in human macrophages stimulated with *S. pneumoniae* have been summarized in Fig.33.

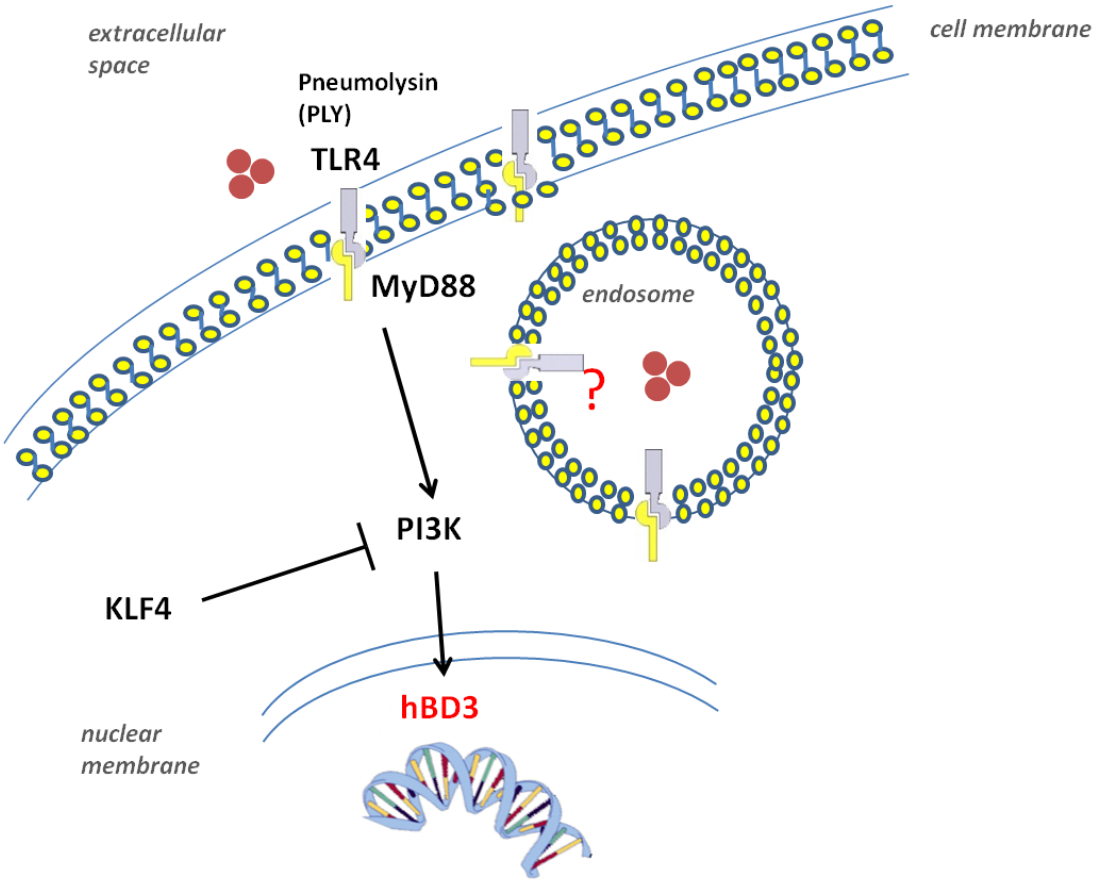

FIGURE 33: Proposed signaling pathway leading to hBD-3 induction in *S. pneumoniae* infected macrophages. Recognition of the bacteria via TLR4 is followed by MyD88 recruitment and subsequent activation of PI3K. This kinase activates one or more transcription factors which up-regulate hBD-3 expression in macrophages. The transcription factor KLF4 negatively regulates hBD-3 expression, most probably via down-regulation of PI3K. The template for this drawing was kindly provided by Toni Herta.

In addition to the signaling pathways, phagocytosis and its effect on hBD-3 induction in macrophages were investigated. As TLR4 is an external receptor, it was hypothesized that hBD-3 induction increases when phagocytosis is blocked. This would be similar to the results obtained for hBD-2. But contrary to hBD-2, hBD-3 induction decreases in CytD treated cells, clearly revealing that phagocytosis of bacteria is of utmost importance for hBD-3 induction. This data for the first time confirms phagocytosis to be involved in hBD-3 expression. As was stated earlier (section 4.2), a second bacterial stimulus is needed for hBD-3 induction. This stimulus is connected to viability of bacteria and is probably recognized by an internal receptor. TLR9 is an internal receptor for bacterial DNA and was tested in this study but was found not to be involved in hBD-3 induction. According to recent publications, TLR4 can be internalized into phagolysosomes in an infectious environment [459–461]. This would be one explanation of the severely decreased hBD-3 expression in macrophages unable to phagocytose. However, it was not tested in this study if the receptor is internalized in *S. pneumoniae* infected macrophages. Another explanation would be the involvement of another receptor. Since PLY is required for hBD-3 induction, not only receptors of the phagolysosome but also cytosolic receptors would be possible detectors of *S. pneumoniae* PAMPs as PLY contributes to lysosomal/phagolysosomal membrane permeabilization [462]. Thus, it would be interesting to determine the role of NOD-like receptors and the inflammasome in hBD-3 induction. Furthermore, involvement of TLR10 should be investigated in the future as TLR10 is an receptor located in the endolysosome able to heterodimerize with TLR2 [463]. Therefore, it possibly detects bacterial cell wall components such as peptidoglycan degraded by lysozyme in the phagolysosome [464]. However, further studies have to be conducted to confirm or discard a role of TLR10 in hBD-3 induction in macrophages as up until now there are only putative ligands for this receptor and it is not even clear it TLR10 could detect bacterial cell wall components.

4.4 FUNCTIONS OF hBD-2 AND hBD-3 IN *S. PNEUMONIAE* INFECTED MACROPHAGES

4.4.1 ANTIMICROBIAL FUNCTIONS

One of the main features of human β-defensins is their antimicrobial activity against many different microorganisms. There are various publications not only on the killing capacity but also on the mode of action of both β-defensins [252–258,268,269,465]. Although hBD-2 and -3 have been shown to eliminate different pathogens, there are mechanisms of evasion utilized by *S. pneumoniae* to escape β-defensin killing. In currently available studies β-defensin efficacy against individual *S. pneumoniae* strains was tested but since there are 90 different serotypes the results for individual ones may not be applicable to all of them. Hence, the effect of hBD-2 and -3 on *S. pneumoniae* serotype 2 was tested in this study. Contrary to other publications, hBD-3 proved to be antimicrobial only against unencapsulated *S. pneumoniae* and hBD-2 was neither bactericidal nor bacteriostatic against the strains tested. This could be due to the proposed mechanism of bacterial killing which is by forming pores in the bacterial membrane. hBD-2 and -3 are positively charged, very small peptides. They are directed to the negatively charged membrane of bacteria by their positive charge [421]. The capsule of *S. pneumoniae* might prevent hBD-2 and -3 attachment and pore-formation, thereby inhibiting the antimicrobial activity. Another explanation is the previously found shedding of capsular polysaccharides by *S. pneumoniae* [407]. In this model the anionic polysaccharides function as decoy for cationic β-defensins and thus protect the bacteria from the toxic effects of the antimicrobials. If this is the case in this experimental setting was not tested but would be an interesting approach for future projects. There are others who found hBD-2 not effective against the gram-positive *Staphylococcus aureus* [336] but the reasons for the ineffectiveness of hBD-2 remain unclear.

4.4.2 IMMUNE MODULATING FUNCTIONS

Apart from their antimicrobial effect, β-defensins have been shown to exhibit several immune modulating functions. They act as chemoattractants for other leukocytes via the chemokine receptors CCR6 and CCR2 [276] and are able to modulate cytokine production [359]. In this study their immune modulating effect on *S. pneumoniae* infected macrophages was tested. Interestingly, there was an effect of hBD-2 and -3 on the cytokine production of the macrophages. Application of different concentrations of rhBD-2 and -3 respectively in addition to stimulation with *S. pneumoniae* enhanced TNFα secretion when low concentrations were applied and decreased TNFα production in a dose-dependent manner when higher concentrations of the proteins were used. However, this effect was not statistically significant, but IL1β production on the other hand was not altered by application of rhBD-2 and -3. Both cytokines are deemed pro-inflammatory but their induction and effect on the innate immune system differs. IL1β is predominantly induced in damaged cells [466] in a PLY-dependent manner [467]. The cell damage leads to the activation of the NLRP3 inflammsome and thereby production of mature IL1β. IL1β is associated with necrosis and pyroptosis [468] both connected to tissue damage and increased inflammatory processes [469,470]. Although pyroptosis is effective against intracellular bacteria [471,472] and seems to play a role in *S. pneumoniae* clearance as well [473], pyroptosis is inferior to apoptosis in pneumococcal pneumonia which might be due to enhanced tissue damage promoting disease progression. Nevertheless clearance of bacteria is not possible without an inflammatory reaction and subsequent activation of immune cells from the innate and adaptive immune system.

In contrast to IL1β, TNFα is associated with apoptosis and necroptosis of cells [474], both mechanisms are important for bacterial clearance without excessive tissue damage [475]. Furthermore, TNFα-deficient mice are dying earlier than wild-type mice infected with S. *pneumoniae* [476] which corresponds to decreased neutrophil numbers in the blood and enhanced bacteremia in mice treated with an TNFα inhibitor [477]. These data indicate that local TNFα production is protective in pneumococcal pneumonia, whereas an infection that is not cleared or contained sufficiently leads to detrimental effects caused by

bacteria entering the blood stream and subsequent systemic production of TNFα. Hence, a fine balancing of this cytokine is necessary to effectively clear an infection without unnecessary damage to host cells. In this study it was found that low concentrations of hBD-2 and -3 in addition to the bacterial stimulus increased and higher concentrations (10 and 20µg/ml) decreased TNFα production. However, concentrations applied for analyzing modulation of TNFα production are higher than the protein concentrations measured in supernatants of infected cells. One explanation for this discrepancy might be that cells adjust their hBD-2 and -3 expression according to the pathogens they encounter. Since *S. pneumoniae* responds only weakly to the antimicrobial effects of hBD-2 and -3, the chemoattractant functions of these peptides might be more relevant in this infectious context. This would explain the low amount of β-defensins in supernatants as for chemotaxis of other leukocytes the required concentrations are much lower than for bacterial killing. Another explanation would be that the local concentrations accumulating around the cells are much higher than the absolute concentrations measured in the supernatants since the β-defensins are attracted to the bacterial membrane and thus are not necessarily detectable in cell free supernatant. An interesting approach for further studies would be to determine hBD-2 and -3 concentrations in co-culture experiments, ideally with epithelial cells, or in an *in vivo* mouse model as these experimental set-ups allow for analysis of β-defensin induction in settings closer to physiological conditions.

Nevertheless, the immune modulating effect detected in this work suggests a bifunctional role of hBD-2 and -3 in macrophages infected with *S. pneumoniae*. Reasons for this bidirectional effect of the β-defensins could lie in their multiple functions in infectious environments. When detection of pathogens occurs, hBD-2 and -3 are secreted to directly antagonize the invading microorganisms, by pore-formation in the bacterial membrane. Peptide concentration around macrophages stays low as long as there are viable bacteria that attract the β-defensins. This low concentration probably acts as co-stimulus to enhance TNFα production and thereby facilitating containment and eventual clearance of the bacteria. Consequently a rise of hBD-2 and -3 concentrations around cells could be indicative for decreased bacterial numbers and therefore a resolving infection where TNFα needs to be down-regulated to control inflammation and

prevent excessive tissue damage. Following that train of thought, hBD-2 and -3 serve a bifunctional role in infection and inflammation by enhancing TNFα production in early stages of infection and by dampening this response in the resolution phase of inflammation. This bifunctional role would also serve as explanation for the differing reports on hBD-2 and -3 being pro- or anti-inflammatory as it clearly matters what phase of infection is investigated and which bacterial stimulus is applied [318,360,361,457,478,479].

In pneumococcal pneumonia the invading *S. pneumoniae* are capable of evading elimination by hBD-2 and -3 by various mechanisms, thus, both peptides accumulate around the macrophages causing down-regulation of TNFα in a highly infectious environment. It could be speculated that virulent *S. pneumoniae* have found an immunological niche in a non-apoptotic environment, where inflammation and excessive neutrophil recruitment to the airway is rather beneficial in terms of bacterial invasiveness. Furthermore, the secreted but ineffective β-defensins decrease TNFα production and thereby further facilitate dissemination of disease. If this assumption is true, hBD-2 and -3 would not be considered as helpful endogenous molecules in pneumococcal pneumonia. In conclusion, it is of utmost importance to further investigate the effect hBD-2 and -3 have on macrophages and other immune cells in specific infectious settings. While apparently detrimental in pneumococcal pneumonia the effects of the β-defensins might be beneficial in other circumstances [480] as they could probably be applied to patients threatened by an overwhelming inflammatory reaction to prevent sepsis and septic shock.

4.5 CONCLUDING REMARKS

This study identified the bacterial exotoxin PLY as negative regulator for hBD-2 induction in human macrophages while hBD-3 was induced in a PLY-dependent manner in stimulated cells. Another virulence factor, the bacterial capsule, had no effect on β-defensin induction, rendering the β-defensin response most probably serotype unspecific. Interestingly, both peptides are induced via distinct signaling pathways and while phagocytosis is required for hBD-3 expression, it is rather impedimental for hBD-2 induction. Although peptide expression is regulated differentially, hBD-2 and -3 exhibit the same immune modulating functions, clearly indicating an important role for the β-defensins in the course of infection. The results on TNFα modulation suggest that both β-defensins have pro- and anti-inflammatory properties depending on the phase of infection and inflammation. Moreover, these peptides are likely to be important for prevention of septic shock and in the resolution of inflammatory processes [361]. However, if the assumption that both β-defensins down-regulate TNFα in pneumococcal pneumonia is true, the expression of both peptides would be disadvantageous since *S. pneumoniae* developed various mechanisms to escape elimination by these peptides and the subsequent down-regulation of TNFα further facilitates disease progression. Thus, in pneumonia patients rightly timed inhibition of hBD-2 and -3 expression could probably improve the outcome of the disease. Furthermore, application of β-defensins to patients threatened by an overwhelming inflammatory response might be protective of sepsis and septic shock. For a better understanding of the multifunctional roles of the β-defensins further investigations are needed. Not only to understand the signaling that leads to induction of hBD-2 and -3 in different cells of the immune system but also to broaden the knowledge of the impact these peptides have on cytokine and chemokine production in different infectious environments. This better understanding might help to develop novel strategies to treat not only pneumonia but also other infectious diseases by either enhancing or weakening the β-defensin response.

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APPENDIX

LIST OF ABBREVIATIONS

A20	see TNFAIP3
AB	antibody
AKT	protein kinase B
AP-1	activator protein 1
APC	antigen-presenting cell
ASK1	apoptotic signal kinase-1
ATP	adenosine triphosphate
BCAP	B-cell adapter for PI3K
BSA	bovine serum albumin
BTK	Bruton's tyrosine kinase
°C	centigrade
Ca	calcium
C. albicans	*Candida albicans*
CAP	community-acquired pneumonia
CCR	chemokine receptor
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
C/EBPβ	CCAAT-enhancer-binding protein beta
C. elegans	*Caenorhabditis elegans*
CFU	colony-forming unit
CLR	c-type lectin receptor
COX2	cyclooxygenase-2
CpG	cytidine-phosphateguanosine DNA sequence 2'-deoxyribocytidine-phosphate-guanosine
CPS	capsule
CXCR	chemokine receptor
CytD	cytochalasin D
D39	wild type serotype 2 strain of *S. pneumoniae*
D39ΔCPS	capsule-deficient D39
D39ΔPLY	pneumolysin-deficient D39
DAMP	damage-associated molecular pattern
DBD	DNA-binding domain
DC	dendritic cell
D. discoideum	*Dictyostelium discoideum*
DLK	dual leucine zipper kinase
D. melanogaster	*Drosophila melanogaster*
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ds	double stranded

E. coli	*Escherichia coli*
e.g.	for example (*exempli gratia*)
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERK	extracellular-signal regulated kinase
FCS	fetal calf serum
g	gravitational force (9.81m/s)
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
h	hour(s)
hBD	human beta defensin
HEK cells	human embryonic kidney cells
HNP	human neutrophil peptide
HRP	horseradish peroxidase
HuLu	human lung tissue
i.e.	that means (*id est*)
Ig	immunoglobulin
IκB	inhibitor of kappa B
IKK	IκB kinase
IL	interleukin
INF	interferon
IRAK	IL1 receptor-associated kinase
IRF	interferon regulatory factor
JNK	c-Jun N-terminal kinase
kb	kilo bases
kDa	kilo Dalton
KLF4	krüppel-like factor 4
LB	lysogeny broth
LD_{90}	lethal dose for killing 90% of organisms
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
LRR	leucine-rich repeat motif
LytA	pneumococcal autolysin (N-acetylmuramoyl-l-alanine amidase)
MAC	membrane attack complex
MALP-2	macrophage-activating lipopeptide-2
MAMP	microbial-associated molecular pattern
MAPK	mitogen-activated protein kinase
MAPKK	MAP-kinase kinase
MAPKKK	MAP-kinase kinase kinase
MD2	lymphocyte antigen 96
MEK	MAPKK
MEKK	MAPKKK
Mg	magnesium

MHC	major histocompatibility complex
MIC	mininmal inhibitory concentration
min	minute
MKK	MAPKK
MKP	MAP-kinase phosphatase
ml	milliliter
MLK	mitogen lineage kinase
MOI	multiplicity of infection
Mos	proto-oncogene serine/threonine protein kinase
mRNA	messenger ribonucleic acid
MyD88	myeloid differentiation primary response 88
NEMO	NF-κB essential modulator
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NK cells	natural killer cells
NLR	NOD-like receptor
NLRP3	NOD-like receptor family, pyrin domain containing 3
NOS	nitric oxide synthase
n.s.	not significant
p38	one of the mitogen-activated protein kinases
PAMP	pathogen-associated molecular pattern
PAGE	polyacrylamid-gel-electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PDK	phosphoinositide-dependent kinase
PEG	polyethylene glycol
Pen/Strep	Penicillin/Streptomycin
p.i.	post infection
PI	phosphatidylinositol
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP	PI3-phosphate
PIP2	PI3,4-bisphosphate
PIP3	PI3,4,5-trisphosphate
PLY	pneumolysin
PMA	phorbol 12-myristate 13-acetate
PRRs	pattern recognition receptors
Psa	pneumococcal surface antigen
Psp	pneumococcal surface protein
PTEN	phosphatase and tensin homolog
q-RT-PCR	quantitative real-time polymerase-chain-reaction
R6x	capsule-deficient derivate of D39
Raf	proto-oncogene serine/threonine-protein kinase
rhBD	recombinant human beta defensin
RLR	RIG-I-like receptor

ROS	reactive oxygen species
rPLY	recombinant pneumolysin
RT	room temperature
SARM	sterile alpha and HEAT/Armadillo motif
SDS	sodium dodecyl sulfate
SH2	Src hology 2 domain
SHIP	SH2-containing inositol phosphatase
SIGIRR	single immunoglobulin IL1R-related molecule
S. pneumoniae	*Streptococcus pneumoniae*
Src	proto-oncogene tyrosine-protein kinase
ss	single stranded
SSD	signal sensing domain
TAB	TAK binding protein
TAD	trans-activating domain
TAK1	TGFβ activated kinase-1
TGFβ	transforming growth factor beta
Th	T helper cell
THP-1	human monocytic cell line, derived from an acute monocytic leukemia patient
TIR	Toll/IL1R homology domain
TIRAP	TIR domain-containing adapter protein
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
TNFAIP3	tumor necrosis factor alpha-induced protein 3
Tpl2	Tumor progression locus 2 or MAPKKK 8
TRAF6	TNF receptor-associated factor 6
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adapter inducing IFN-β
Tris	tris-hydroxylmethyl-aminomethylamin
U937	human monocytic cell line, derived from a histiocytic lymphoma patient
UV	ultraviolet
v/v	volume per volume
WHO	World Health Organization
wt	wild type
w/v	weight per volume

VECTOR MAPS

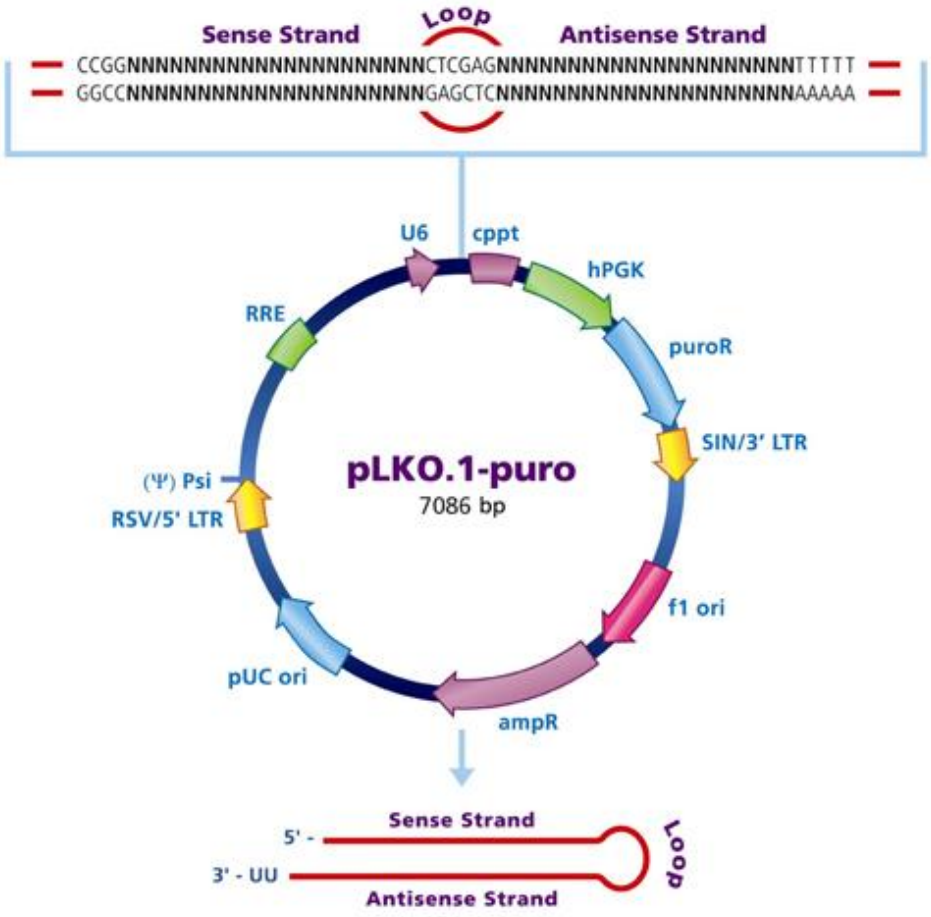

FIGURE 34: pLKO.1 vector map (Source: www.sigmaaldrich.com)

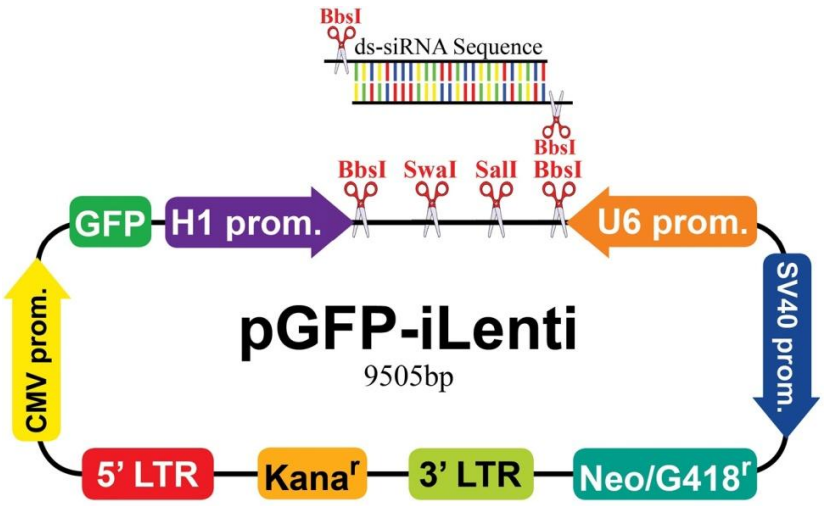

FIGURE 35: pGFP-iLenti vector map (Source: https://www.abmgood.com/pGFP-iLenti-Vector-Map.html)

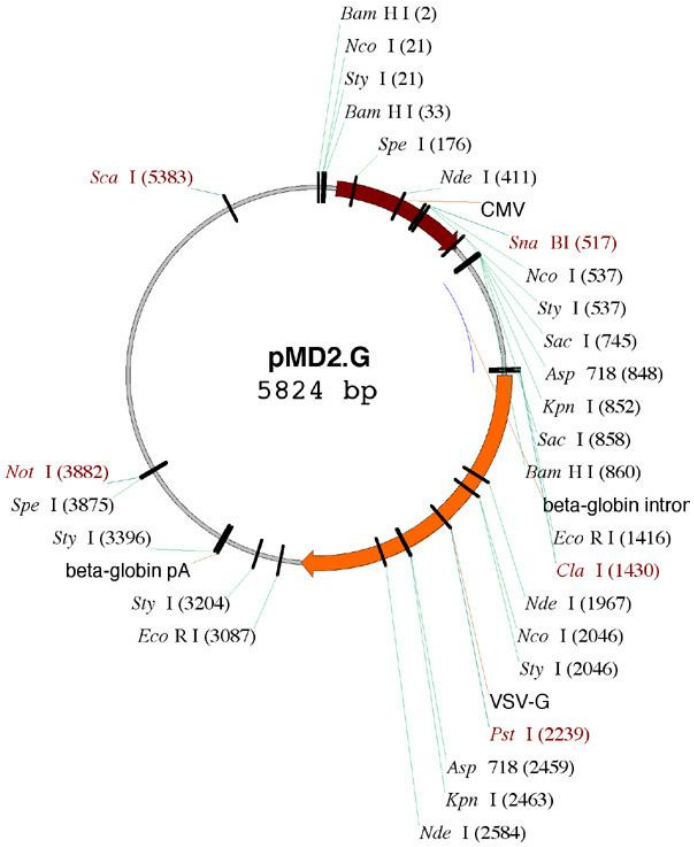

FIGURE 36: pMD.G vector map (Source: https://www.addgene.org/12259/)

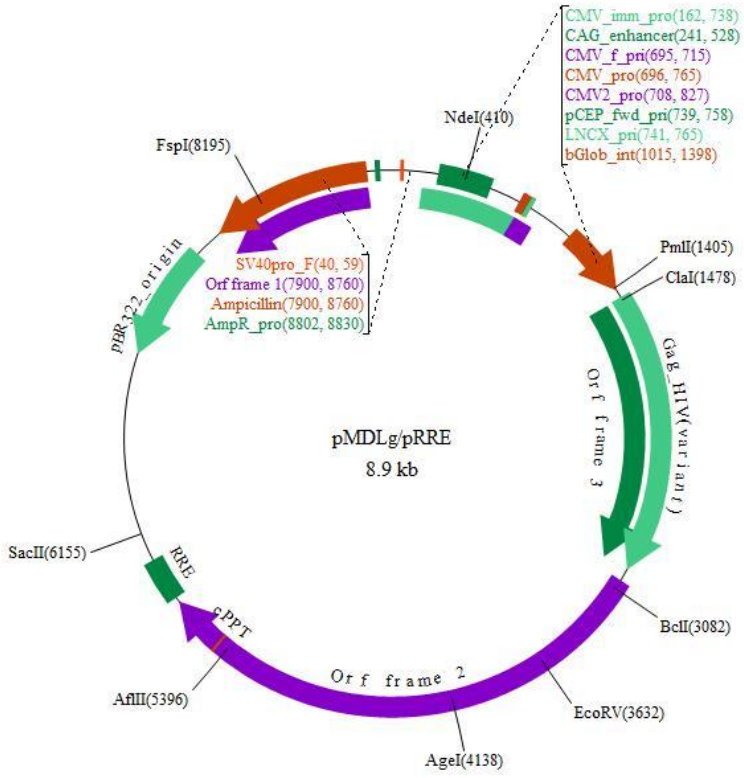

FIGURE 37: pMDLg/pRRE vector map (Source: http://www.biovisualtech.com/bvplasmid/pRSV-Rev.htm)

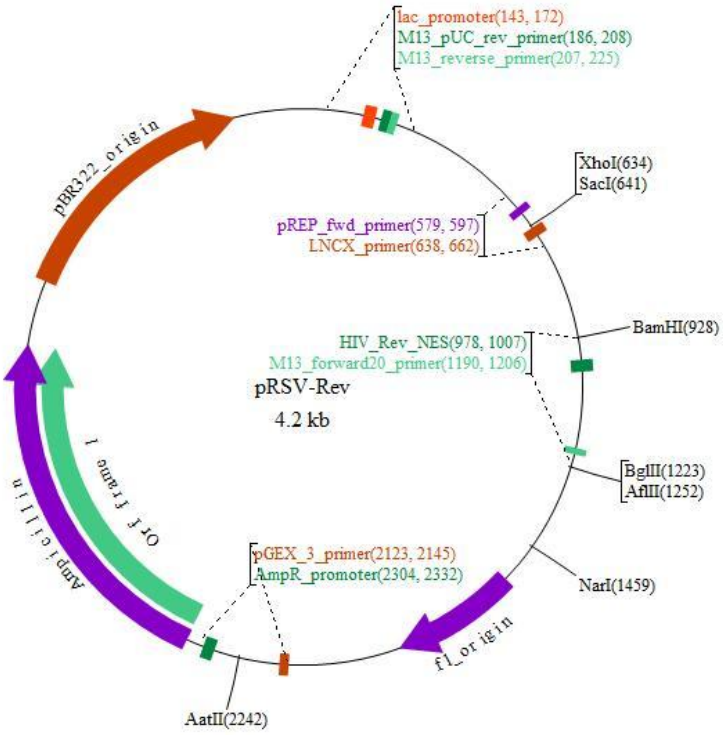

FIGURE 38: pRSV-Rev vector map (Source: http://www.biovisualtech.com/bvplasmid/pRSV-Rev.htm)

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CURRICULUM VITAE

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