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ROLE OF ANTIMICROBIAL PEPTIDES IN TUBERCULOSIS AND RESPIRATORY TRACT INFECTIONS: CLINICAL AND MECHANISTIC STUDIES

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Role of antimicrobial peptides in tuberculosis and respiratory tract infections: clinical and mechanistic studies
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my
beloved family

ABSTRACT

Antimicrobial peptides (AMPs) are effector molecules of the innate immune system in multicellular organisms. They are mainly expressed in epithelial cells and immune cells, providing the first line of defense against a wide range of pathogens. AMPs are able to kill pathogens and show additional important functions such as chemotaxis, angiogenesis, and wound healing. These peptides are constitutively expressed; however, their expression can also be induced or suppressed by different stimuli in a cell and tissue specific manner. The overall aim of the present thesis was to elucidate the role of AMPs in tuberculosis and respiratory tract infections by clinical and mechanistic studies.

Vitamin D_3 ($vitD_3$) is known as a potent inducer of AMPs. Low levels of serum $vitD_3$ are associated with an increased risk of respiratory tract infections (RTIs). One of our objectives was to elucidate whether supplementation with $vitD_3$ could reduce infectious symptoms and antibiotic consumption in patients with antibody deficiency or frequent RTIs. Patients (n=140) were included with symptoms of respiratory tract infections for more than 42 days over a 12-month period. They were randomized and received either $vitD_3$ (4000 IU) or placebo daily for one year. The primary endpoint was an infectious score based on five parameters: symptoms from the respiratory tract, sinuses, and ears, malaise, and antibiotic consumption. Secondary endpoints were serum 25-hydroxyvitamin D_3 [$25(OH)D_3$] levels, microbiological outcomes, and the levels of the antimicrobial peptides LL-37 and Human Neutrophil Peptides (HNP) 1-3 in nasal fluids. We observed that $vitD_3$ supplementation reduced infectious score, antibiotic consumption, and increased serum $25(OH)D_3$ concentrations in the patients compared to placebo control group. However, no major changes were observed for LL-37 and HNP 1-3.

To control the global spread of tuberculosis (TB) and multi-drug resistance TB, development of new anti-tuberculosis drugs and alternative treatment strategies are urgently required. PBA is a potent inducer of AMPs, and together with 1,25-dihydroxyvitamin D_3, it synergistically induces the expression of LL-37 in lung epithelial cell line. Thus, we aimed to estimate a therapeutic dose of PBA alone, or in combination with $vitD_3$ for the induction of LL-37 expression in immune cells, and enhanced antimycobacterial activity in monocyte-derived macrophages (MDMs). Healthy volunteers were enrolled in an 8-days open trial (n=15). The expression of the *CAMP* (cathelicidin antimicrobial peptide) gene encoding LL-37 was measured in immune cells both in mRNA and peptide levels. MDM-mediated killing of *Mycobacterium tuberculosis* (*Mtb*) (H37Rv) was performed. From this trial, we demonstrated that 500 mg PBA twice daily with 5000 IU $vitD_3$ once daily was the optimal dose for the induction of LL-37 in MDMs and lymphocytes, and the enhancement of intracellular killing of *Mtb* by MDMs. Using these findings, we further investigated if oral adjunctive therapy with 5000 IU $vitD_3$ and/or 2x500 mg PBA along with standard anti-TB therapy would lead to an enhanced recovery in sputum smear-positive pulmonary TB patients. Adult TB patients (n=288) were enrolled in a randomized, double-blind, placebo-controlled trial. Primary endpoints were the proportion of patients with a negative sputum

culture at week 4, and the reduction in clinical symptoms at week 8. Secondary endpoints included sputum smear conversion time, radiological findings, concentrations of $25(OH)D_3$ in plasma, expression of the antimicrobial peptide LL-37 in immune cells and intracellular killing of *Mtb* by MDMs. We found that the adjunct therapy with PBA and $vitD_3$ treatment significantly reduced the sputum culture conversion time together with better clinical recovery in pulmonary tuberculosis patients. Additionally we observed that PBA and $vitD_3$ treatment enhanced the expression of LL-37 in immune cells and increased intracellular killing of *Mtb* by MDMs.

Next, we explored the potential mechanisms of PBA and LL-37-induced intracellular killing of *Mtb* in macrophages by autophagy. We observed that *Mtb* infection of MDMs downregulated the expression of LL-37 and certain autophagy-related genes (Beclin1 and ATG5) at both mRNA and protein levels. We also found that PBA and/or 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] were able to overcome the *Mtb*-induced suppression of LL-37 expression. In addition, autophagy process was activated by stimulation of MDMs with PBA and promoted co-localization of LL-37 and LC3-II (a marker of autophagy) in autophagosomes. When LL-37 expression was silenced, PBA treatment failed to induce autophagy in *Mtb*-infected THP-1 cells. On the other hand, when LL-37 knockdown cells were supplemented with synthetic LL-37, autophagy was restored. Additionally, we found that LL-37-induced autophagy was mediated via the P2RX7 receptor and intracellular free Ca^{2+}, the AMPK and the PI3K pathways. These results suggest that PBA induces autophagy in LL-37-dependent manner and promotes intracellular killing of *Mtb* in human MDMs.

In summary, $vitD_3$ supplementation could beneficial for the patients with antibody deficiency or frequent RTIs. PBA and $vitD_3$ supplementation improves the clinical outcomes through the increased expression of LL-37, indicating that this supplementation might be an alternative strategy to treat pulmonary TB patients. The enhanced expression of LL-37 activates the cellular host defense mechanism autophagy, and subsequent killing of *Mtb* in human macrophages.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals I-IV:

I. Bergman, P.*, Norlin, A.C.*, Hansen, S., **Rekha, R.S**., Agerberth, B., Bjorkhem-Bergman, L., Ekstrom, L., Lindh, J.D., and Andersson, J. (2012). Vitamin D_3 supplementation in patients with frequent respiratory tract infections: a randomised and double-blind intervention study. **BMJ Open 2**.
* equal contribution

II. Mily, A.*, **Rekha, R.S**.*, Kamal, S.M., Akhtar, E., Sarker, P., Rahim, Z., Gudmundsson, G.H., Agerberth, B., and Raqib, R. (2013). Oral intake of phenylbutyrate with or without vitamin D_3 upregulates the cathelicidin LL-37 in human macrophages: a dose finding study for treatment of tuberculosis. **BMC Pulm Med** 13, 23.
*These authors contributed equally to this work as first authors

III. Mily, A.*, **Rekha, R.S**.*, Kamal, S.M.M., Arifuzzaman, A.S.M., Rahim, Z., Khan, L., Haq, M.A., Zaman, K., Bergman, P., Brighenti, S., Gudmundsson, G.H., Agerberth, B., and Raqib, R. (2015). Significant effects of oral phenylbutyrate and vitamin D_3 adjunctive therapy in pulmonary tuberculosis: A randomized controlled trial. **PLoS One** 10, e0138340.
*These authors contributed equally to this work as first authors

IV. **Rekha, R.S**., Muvva, S.S.V.J., Wan, M., Raqib, R., Bergman, P., Brighenti, S., Gudmundsson, G.H., and Agerberth, B. (2015). Phenylbutyrate induces LL-37-dependent autophagy and intracellular killing of Mycobacterium tuberculosis in human macrophages. **Autophagy** 11(9), 1688-1699.

LIST OF RELATED SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. **Rekha, R.S.**, Kamal, S.M., Andersen, P., Rahim, Z., Hoq, M.I., Ara, G., Andersson, J., Sack, D., and Raqib, R. (2011). Validation of the ALS assay in adult patients with culture confirmed pulmonary tuberculosis. **PLoS One** 6, e16425.

II. Cederlund, A., Olliver, M., **Rekha, R.S.**, Lindh, M., Lindbom, L., Normark, S., Henriques-Normark, B., Andersson, J., Agerberth, B., and Bergman, P. (2011). Impaired release of antimicrobial peptides into nasal fluid of hyper-IgE and CVID patients. **PLoS One** *6*, e29316.

III. Sarker, P., Ahmed, S., Tiash, S., **Rekha, R.S.**, Stromberg, R., Andersson, J., Bergman, P., Gudmundsson, G.H., Agerberth, B., and Raqib, R. (2011). Phenylbutyrate counteracts Shigella mediated downregulation of cathelicidin in rabbit lung and intestinal epithelia: a potential therapeutic strategy. **PLoS One** 6, e20637.

IV. Raqib, R., Sarker, P., Mily, A., Alam, N.H., Arifuzzaman, A.S., **Rekha, R.S.**, Andersson, J., Gudmundsson, G.H., Cravioto, A., and Agerberth, B. (2012). Efficacy of sodium butyrate adjunct therapy in shigellosis: a randomized, double-blind, placebo-controlled clinical trial. **BMC Infect Dis** 12, 111.

V. Ashenafi, S., Aderaye, G., Zewdie, M., Raqib, R., Bekele, A., Magalhaes, I., Lema, B., Habtamu, M., **Rekha, R.S.**, Aseffa, G., Maeurer, M., Aseffa, A., Svensson, M., Andersson, J. and Brighenti, S. (2013). BCG-specific IgG-secreting peripheral plasmablasts as a potential biomarker of active tuberculosis in HIV negative and HIV positive patients. **Thorax** 68, 269-276.

CONTENTS

LIST OF ABBREVIATIONS

AMPK	Adenosine monophosphate-activated protein kinase
AMP	Antimicrobial peptide
ATG	Autophagy related
CAMP	Cathelicidin antimicrobial peptide
CVID	Common variable immunodeficiency
DCs	Dendritic cells
HBD	Human β-defensin
HNP	Human Neutrophil Peptide
LC3/MAP1LC3	Microtubule-associated protein 1 light chain 3
MDM	Monocyte-derived macrophage
MDR-TB	Multi-drug resistance-TB
MHC	Major histocompatibility complex
Mtb	*Mycobacterium tuberculosis*
PBA	Phenylbutyrate
PIDs	Primary immunodeficiencies
PI3K	Phosphatidylinositol 3-kinase
PMN	Polymorphonuclear neutrophil
P2RX7	Purinergic receptor P2X, ligand gated ion channel 7
RTIs	Respiratory tract infections
TB	Tuberculosis
TLR	Toll-like receptor
VDR	Vitamin D receptor
$VitD_3$	Vitamin D_3
$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
$25(OH)D_3$	25-hydroxyvitamin D_3

1 INTRODUCTION

We are surrounded by an enormous number of pathogens, and they are the causes of different types of diseases. Our immune system consists of a variety of cells and molecules, mediating defense against almost all kinds of pathogens. The immune system comprises two lines of defense e.g., innate and adaptive immunity. While the innate immunity acts immediately when recognizing a pathogen, adaptive immunity takes longer time to respond and becomes fully matured after exposure to microbes and microbial components.

The present thesis elucidates the novel role of antimicrobial peptides (AMPs) in tuberculosis and respiratory tract infections. Antimicrobial peptides are included in the first-line immune defense and known as endogenous antibiotics, produced by different types of immune cells, mucosal cells, and epithelial cells to fight against invading pathogens. The respiratory system consists of specific organs that are used for the process of respiration in an organism. Since the respiratory system plays a major role for oxygen exchange, any dysfunction and dysregulation of this system affects the whole body. Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (*Mtb*) and mainly affects the lung, one of the most important organs of the respiratory system. Thus, it is important to reveal the potential therapeutic effects of antimicrobial peptides in tuberculosis and respiratory tract infections by clinical and experimental studies.

1.1 ANTIMICROBIAL PEPTIDES, EFFECTOR MOLECULES OF THE INNATE IMMUNE SYSTEM

1.1.1 Historical background

Antimicrobial peptides (AMPs) are peptides consisting of 12-50 amino acids residues with a broad spectrum of antimicrobial activity. These peptides are capable to kill a diverse range of microorganisms including bacteria, viruses, and fungi. AMPs are evolutionary conserved and present in almost all living organisms including human, mammals, vertebrates, invertebrates, plants, insects, and even in some microorganisms (Zasloff, 2002). The innate defense system is one of the most primordial strategies against unwanted microbes and for the regulation of normal flora that evolved long time before the adaptive immune system (Lehrer, 2004). In the middle of the 20th century, oxygen-dependent killing mechanisms of granulocytes were studied by various research groups and it was revealed that neutrophil granules contain basic proteins that were able to kill microbes (Boman et al., 1972; Klebanoff, 1975). In 1981, the first AMPs was discovered by Hans G Boman and coworkers in the silk moth, *Hyalophora cecropia* (Steiner et al., 1981). They isolated and characterized two AMPs that were capable of killing bacteria efficiently, and they named the peptides ‘‘Cecropins’’ based on the origin of the species. In 1983, mammalian AMPs were discovered in rabbit macrophages (Selsted et al., 1983) and two years later in human polymorphonuclear neutrophils (PMNs) (Selsted et al., 1985). Additional discoveries in this research field were the characterization of magainins in the skin of frog *Xenopus laevis* by Michael Zasloff (Zasloff, 1987); and the isolation and characterization of the cathelicidin

LL-37 in human PMNs (Gudmundsson et al., 1996). To date, more than 2000 AMPs have been identified or predicted from gene sequence analyses and this information can be found in an antimicrobial peptide database (Wang et al., 2009).

1.1.2 Structure of AMPs

Antimicrobial peptides (AMPs) are a heterogeneous group of molecules. They show high diversity in primary, secondary and tertiary structure, but share some common features, generating an optimal strategy to kill microbes. They can fold into α-helical, β- sheet, and loop structures together with extended helices rich in certain amino acids (Zasloff, 2002). They are usually generated by proteolytic cleavage from larger precursor proteins with or without antimicrobial activity (Brogden, 2005). AMPs consist of a high proportion of cationic amino acid residues combined with hydrophobic residues, forming a cationic amphipathic secondary structure (Yeaman and Yount, 2003). The main secondary structures of AMPs are antiparallel β-sheets (rich in disulfide bonds such as defensins and protegrins), α-helical folded peptides (cathelicidins and magainins), and peptides enriched with specific amino acids such as proline, glycine, arginine or phenylalanine, for example PR-39 (Agerberth et al., 1991; Tossi and Sandri, 2002). The amphipathic properties of AMPs increase their solubility in aqueous solutions, e.g., blood, urine or saliva, which improve their bioavailability (Yeaman and Yount, 2003).

1.1.3 Mechanisms of action

Antimicrobial peptides (AMPs) are positively charged and via electrostatic interaction they bind to the negatively charged constituents of microbial membranes, including phospholipids, lipopolysaccharides, lipoteichoic acid and peptidoglycan (Brogden, 2005). Furthermore, the amphipathic nature of the peptides, with one hydrophobic and one cationic side, allows them to be incorporated into the lipid bilayer of the bacterial membrane (Tossi et al., 2000). This interaction facilitates the destruction of microbial membrane integrity, and subsequently the microbes are killed by lysis.

Several biochemical and immunological methods, including microscopy, model membranes, fluorescent dyes, nuclear magnetic resonance (NMR) and circular dichroism have been applied to study the mechanism of action of AMPs. They utilize different strategies to disrupt the microbial membrane, and several mechanistic models have been developed to elucidate the bacterial membrane disruption by AMPs. For some peptides (α-helical peptides) this process is rapid, making it challenging to characterize the steps prior to the microbial killing. It has been demonstrated that some α-helical peptides kill the microbes within a minute (Boman, 1995). In contrary, most of the peptides such as magainin 2 (Zasloff, 1987) and PR-39 (Boman et al., 1993) require at least 15-90 minutes to kill the microbes. The most established models are the barrel-stave pore, toroidal pore and carpet models (Brogden, 2005) (**Figure 1**).

In the barrel-stave pore model, peptides (generally α-helical peptides) form a bundle in the membrane with the hydrophobic surfaces oriented towards the lipid core and the hydrophilic interphase directed inwards from the interior region of the pore (Lee et al., 2004; Oren and Shai, 1998). For example, alamethicin forms a multimeric helical bundle like the staves of a barrel (Yang et al., 2001b).

In the toroidal pore model, peptides are inserted and bend towards the lipid monolayer continuously through the pore in a manner that causes the lipid head groups to be lined towards the water core. These pores are induced by α-helical peptides such as LL-37 (Henzler Wildman et al., 2003) and magainin 2 (Matsuzaki et al., 1998) and are often larger than the barrel-stave pore.

In the carpet model, peptides are accumulated on the bacterial surface and form a carpet-like structure, lying parallel with the membrane and disrupt the membrane in a detergent-like manner (Oren and Shai, 1998). Cecropin (Gazit et al., 1995) and ovispirin (Yamaguchi et al., 2001), aggregates parallel to the membrane surface, coating the lipid bilayer like a carpet.

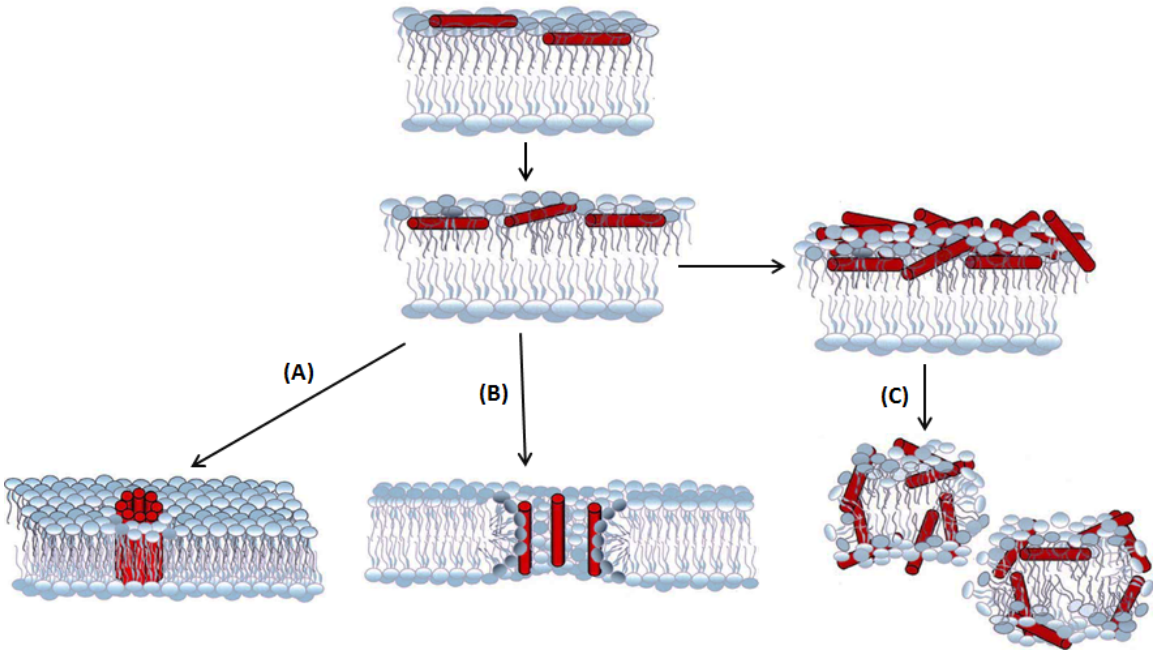

Figure 1: **Different models of mechanisms of action of membrane-active AMPs with microbial cell membrane.** Peptides (cylinders) initially bind and accumulate in an orientation parallel to the membrane surface. (**A**) Barrel-Stave pore model. (**B**) Toroidal pore model. (**C**) Carpet model. Adapted with modification (Sato and Feix, 2006).

In addition, apart from membrane disruption some peptides target different intracellular components or processes to kill microorganisms (Brogden, 2005). After being translocated into cytoplasm, the porcine cathelicidin PR-39 affects the cytoplasmic membrane formation, inhibits synthesis of cell wall/nucleic acids/proteins resulting in microbial death (Boman et al., 1993; Gennaro et al., 2002). The histidine-rich peptides Histatins enter into the cytoplasm of fungi, causing ATP loss, production of reactive oxygen species, and disruption of the cell cycle (Kavanagh and Dowd, 2004). Due to the presence of neutral zwitterions and cholesterol in the eukaryotic membrane, AMPs have a weak attraction to the eukaryotic cells than microbes (Zasloff, 2002). However, AMPs are cytotoxic to eukaryotic cells, albeit at higher concentrations than the bactericidal concentration (Johansson et al., 1998).

Antimicrobial peptides (AMPs) are multifunctional and often termed as host defense peptides. In addition to their antimicrobial activity, several AMPs contribute in immunomodulatory activities such as chemotaxis, angiogenesis, alteration, and activation of cytokine/chemokine responses of both innate and adaptive immune cells (Koczulla et al., 2003; Yang et al., 2001a; Zanetti, 2004). AMPs, therefore, form an intimate connection between innate and adaptive immune system. In addition, AMPs play an important role in the maintenance of epithelial membrane integrity (Otte et al., 2009), neutralization of LPS (Larrick et al., 1995; Larrick et al., 1991), regulation of the normal flora (Salzman et al., 2010), and wound repair (Heilborn et al., 2003; Shaykhiev et al., 2005).

1.1.4 Cathelicidins

The cathelicidins are one of the major families of AMPs in mammals (Zanetti, 2004). However, cathelicidins are also found in fish (Maier et al., 2008), birds (Lynn et al., 2004) and snakes (Wang et al., 2008). Cathelicidins are translated from their corresponding genes as prepro-proteins (128-143 amino acid residues), consisting of an N-terminal signal peptide (29-30 residues), connected to a conserved cathelin domain (99-114 residues), and a highly variable C-terminal domain (12-100 residues) (Zanetti et al., 1995). After the release of the signal peptide, the pro-protein (precursor protein) is stored inside the cells. To generate active mature peptide, the C-terminal domain is cleaved off from the cathelin domain extracellularly (Zanetti, 2004). The cathelicidins are evolutionary divergent both in sequence and in length. They can be α-helical (such as LL- 37, rabbit CAP-18), can form β-hairpins (such as pig protegrin 1-5) or can have extended helices due to abundance of certain amino acid residues (such as porcine PR-39) (Zanetti, 2004).

1.1.4.1 LL-37

LL-37 is the only cathelicidin peptide found in human and is encoded by the *CAMP* (cathelicidin antimicrobial peptide) gene consisting of four exons. From the spliced transcript, a prepro-protein is translated and after cleavage of the signal peptide the inactive pro-protein hCAP-18 (human cationic antimicrobial protein 18 kDa) is produced. Exons 1-3 encode the cathelin pro-domain and the signal peptide, whereas exon 4 encodes the AMP

LL-37 (Gudmundsson et al., 1996). To generate the active LL-37 peptide, hCAP-18 has been shown to be cleaved by proteinase 3 secreted from neutrophils (Sorensen et al., 2001), by gastricsin present in seminal plasma (Sorensen et al., 2003b) or by kallikrein from keratinocytes (Yamasaki et al., 2006). The peptide contains 37 amino acid residues with two leucine at the N-terminal end, resulting in the name LL-37 (**Figure 2**). LL-37 is an arginine- and lysine-rich cationic peptide folded into an amphipathic α-helical structure at physiological pH (Li et al., 2006). The orthologue of LL-37 in mouse is mCRAMP (Gallo et al., 1997), in rat rCRAMP (Termen et al., 2003), and in rabbit CAP-18 (Larrick et al., 1991). The orthologues of LL-37 are also α-helical in structure.

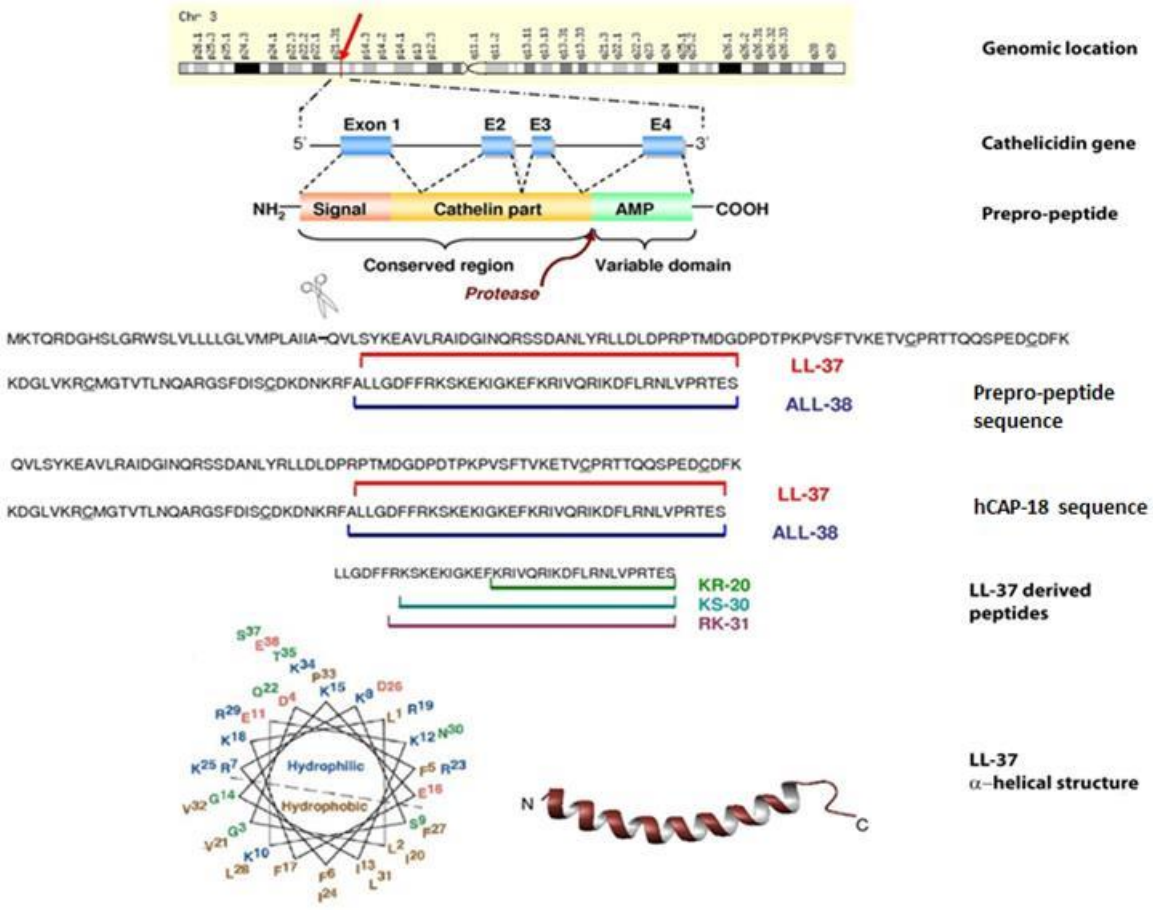

Figure 2: Schematic representation of cathelicidin gene and LL-37 derived peptides. The genes consist of 4 exons. The first three exons encode the signal peptide and the cathelin domain, while the fourth exon encodes the mature active peptide and the processing site. Adapted with modification (Seil et al., 2010).

The expression of LL-37 can be either constitutive or inducible by different stimuli. LL-37 is constitutively expressed in different cell types such as epithelial cells, monocytes,

macrophages, mast cells, natural killer cells, B cells, γδ T cells and neutrophils (Agerberth et al., 2000; Di Nardo et al., 2003; Frohm Nilsson et al., 1999; Gudmundsson et al., 1996). The expression of LL-37 can also be controlled by both host and pathogenic factors in a tissue and cell specific manner. Induced expression of LL-37 is observed in inflamed and non-inflamed colonic mucosa of patients with inflammatory bowel disease (Schauber et al., 2006b) and in psoriatic lesions (Frohm et al., 1997; Ong et al., 2002), in wounds (Dorschner et al., 2001), hypoxia (Peyssonnaux et al., 2008), and by growth factors (Sorensen et al., 2003a). Induction of LL-37 has also been noted in bacterial and parasitic infections such as by *Staphylococcus aureus* in keratinocytes (Midorikawa et al., 2003), *Helicobacter pylori* in gastric epithelial cells (Hase et al., 2003), uropathogenic *Escherichia coli* in epithelial cells of the urinary tract (Chromek et al., 2006) and *Entamoeba histolytica* in colonic epithelial cells (Cobo et al., 2012). In contrary, glucocorticoids (Simmaco et al., 1997) and microbial virulence factors from *Neisseria* (Bergman et al., 2005) and *Shigella* (Islam et al., 2001) have been shown to down-regulate the expression of LL-37.

LL-37 exhibits a broad range of antimicrobial activity. It is active against gram negative bacteria such as *Pseudomonas aeruginosa, Salmonella typhimurium, Proteus mirabilis* (Turner et al., 1998), *Escherichia coli* (Chromek et al., 2006), *Neisseria gonorrhoeae* (Bergman et al., 2005), *Neisseria meningitidis* (Bergman et al., 2006) as well as gram positive bacteria such as *Staphylococcus aureus*, *Staphylococcus epidermidis, Listeria monocytogenes* and Group A *Streptococcus* (GAS) (Dorschner et al., 2001; Turner et al., 1998). In addition, LL-37 has also been found to be active against fungi and viruses (Howell et al., 2004; Turner et al., 1998; Yasin et al., 2000). Interestingly, Johansson *et al.* have found that antibacterial activity of LL-37 positively correlates with the secondary structure of LL-37. Probably higher helix content increases its antibacterial effects, which depends on the ionic environment and pH (Johansson et al., 1998).

In addition to antimicrobial activity, LL-37 exhibits numerous immunomodulatory and tissue homeostatic functions. It has chemotactic activity to PMNs, monocytes and T cells via binding to formyl peptide receptor like-1 (FPRL-1) receptor (Agerberth et al., 2000; De et al., 2000). LL-37 shows chemotactic activity to mast cells (Niyonsaba et al., 2002). LL-37 is also an agonist of the P2RX7 (purinergic receptor P2X ligand-gated ion channel 7) receptor (Elssner et al., 2004). Recently it has been reported that P2RX7 receptor also regulates the internalization of LL-37 by human macrophages, promoting intracellular bacterial killing (Tang et al., 2015). Additionally, LL-37 induces chemokine and cytokine productions in epithelial cells and stimulates degranulation of mast cells (Niyonsaba et al., 2001; Tjabringa et al., 2003). Our group has demonstrated that LL-37 can stimulate the synthesis of the pro-inflammatory lipid mediator leukotriene B4 (LTB4) in PMNs through binding to the FPR2/ALX receptor (Wan et al., 2011). LL-37 also mediates the process of wound healing of the skin and the airway epithelia through the induction of re-epithelialization and cell proliferation (Heilborn et al., 2003; Shaykhiev et al., 2005). In addition, LL-37 acts as a suppressor of immune responses by binding to endotoxins such as LPS, lipoteichoic acid, and lipoarabinomannan (Kandler et al., 2006; Larrick et al., 1995;

Scott et al., 2002). Intestinal epithelial barrier integrity is also maintained and regenerated by LL-37 (Otte et al., 2009). An angiogenic role has been observed for LL-37. LL-37 increases the proliferation and formation of vessel-like structures in endothelial cells (Koczulla et al., 2003). Most of the functions of LL-37 are associated with host defense, enhancing the probabilities of avoiding diseases.

There are limited data available on the *in vivo* situation of the antimicrobial functions of LL-37. One example is the chronic congenital neutropenia known as morbus Kostmann characterized by recurrent infections and chronic periodontal disease, these patients lack LL-37 in PMNs and in saliva (Putsep et al., 2002). In the *Cnlp-/-* mouse, in which the gene encoding the cathelicidin mCRAMP has been knocked out, exhibits an increased susceptibility to infection by Group A *Streptococcus* (Nizet et al., 2001), *Pseudomonas aeruginosa* (Huang et al., 2007), *Vaccinia virus* (Howell et al., 2004) and *Herpes simplex virus* (Howell et al., 2006).

1.1.5 Defensins

The defensins constitute another main family of AMPs present in mammals, plant, and fungi. These peptides have a broad and potent antimicrobial activity against gram positive and gram negative bacteria as well as fungi and viruses (Ganz, 2003). The defensins are processed from prepro-proteins into 18-45 amino acid residue long peptides containing six conserved cysteine residues. Three disulfide bonds are formed between the cysteine residues, stabilizing a cationic amphipathic β-sheet conformation (Ganz, 2003). Defensins are subdivided into α, β- and θ-defensins based on the size, distribution of cysteine residues and the pairing of the disulfide bonds (Selsted and Ouellette, 2005). Humans only express α and β-defensins (Ganz, 2003).

1.1.5.1 α- defensins

There are six α-defensins expressed in human. Human neutrophil peptides (HNP) 1-4 are mostly present in primary (azurophilic) granules of PMNs (Wilde et al., 1989). In addition to neutrophils, monocytes, NK cells, B cells and γδTcells also express HNP-1 to -4 (Agerberth et al., 2000). Paneth cells present at the base of the small intestinal crypts constitutively express human defensins-5 and -6 (HD-5 and -6) (Jones and Bevins, 1992, 1993). HD-5 has also been detected in female genital tract (Svinarich et al., 1997). HNPs have broad antimicrobial activity against gram-positive and gram-negative bacteria. Enteric α-defensins are performed as key effectors in the defense against enteric pathogens and regulate the intestinal microflora (Chu et al., 2012; Salzman et al., 2003; Salzman et al., 2010). Thus, they are the essential component in the maintenance of the gut microflora (Bevins and Salzman, 2011). Additionally, human HD-6 is capable of making nanonets, inhibiting the motility of invading pathogens (Chu et al., 2012). Similar to cathelicidins, α-defensins are produced as prepro-protein followed by removal of pre- and pro-sequences to generate active peptides (Selsted and Ouellette, 2005).

1.1.5.2 β- defensins

More than 30 β-defensins genes have been identified in human genome, but only four of them, human β-defensins (HBD) 1-4 peptides have so far been characterized (Schutte et al., 2002). β-defensins are mainly present in different epithelial cells, where their expressions are either constitutive or induced by cytokines or bacterial components (Ali et al., 2001; Selsted and Ouellette, 2005). HBD-1 was originally isolated from human blood filtrate (Bensch et al., 1995) and HBD-2 and -3 were isolated from psoriatic lesions (Harder et al., 1997, 2001). HBD-4 was discovered, based on the screening of human genome sequences (Garcia et al., 2001). Synthetic HBD-4 exhibits antimicrobial activity against *Pseudomonas aeruginosa* and *Staphylococcus carnosus* (Garcia et al., 2001). In contrast to α-defensins, β-defensins have a very short pro-sequence, separating the signal and mature peptide regions (Selsted and Ouellette, 2005). HBD-1 displays effective antimicrobial activity against *S. aureus* and in addition to induce PMNs to form neutrophil extracellular traps (NETs) (Kraemer et al., 2011). HBD-2 and -3 inhibit HIV-1 replication in oral epithelial cells (Quinones-Mateu et al., 2003). HBD-3 shows also anti-viral activity against vaccinia virus (Howell et al., 2007), and prevent cervical epithelial cells from herpes simplex virus (HSV) infection (Hazrati et al., 2006). In addition, HBD-2 chemoattracts dendritic cells (DCs) and memory T cells through the Chemokine receptor 6 (CCR6) (Yang et al., 1999). HBD-2 also stimulates chemotaxis, proliferation, capillary-like tube formation and enhances wound healing of human umbilical vein endothelial cells (Baroni et al., 2009).

1.1.6 Induction of AMPs by exogenous compounds

The expression of AMPs (cathelicidins and defensins) can be induced by different exogenous compounds. Nicotinamide (vitamin B_3) (Kyme et al., 2012), vitamin D (Liu et al., 2006), histone deacetylase inhibitors (HDACis), such as the colonic fermentation product butyrate (Schauber et al., 2003), phenylbutyrate (PBA) (Steinmann et al., 2009), entinostat (Nylen et al., 2014), trichostatin (Schauber et al., 2004) are examples of such inducers or activators.

Histone acetylation has been anticipated for the induction of *CAMP* transcript by relaxing the chromatin structure, facilitating binding of transcription factors at the promoter region (Kida et al., 2006; Schauber et al., 2004). We have reported that the effect of butyrate on LL-37 expression is also mediated via p38 mitogen-activated protein kinase (MAPK) and mitogen-activated protein kinase kinase-1 and -2 (MEK1/2) pathways (Schauber et al., 2003). The inducing effect of butyrate is connected to an inhibition of NF-κB signaling and a recruitment of the transcription factors such as activator protein 1 (AP-1), PU.1, vitamin D receptor (VDR) (Schwab et al., 2007), steroid receptor coactivator 3 (SRC3) (Schauber et al., 2008) and cAMP-response element-binding protein (CREB) to the *CAMP* gene promoter (Chakraborty et al., 2009; Kida et al., 2006; Termen et al., 2008). Butyrate also induces HBD-2 in monocytes (Schauber et al., 2006a).

Phenylbutyrate (PBA), an analogue of butyrate induces the expression of the *CAMP* gene in different epithelial cell lines and also in macrophages (Mily et al., 2013; Steinmann et al., 2009). MAPK signaling cascade is involved in this induce expression of *CAMP* by PBA. PBA enhances histone acetylation and subsequently promote the expression of additional genes, encoding different regulatory factors, which induce *CAMP* gene expression. HBD-1 was found to be induced by PBA in lung epithelial cells, but opposite result was found in monocytes.

Several studies have demonstrated that the hormonal form of vitD_3,1,25-dihydroxy vitamin D_3 [1,25$(OH)_2D_3$] upregulate the *CAMP* gene expression in keratinocytes (Gombart et al., 2005), monocyte-derived macrophages (Mily et al., 2013), lung/colonic epithelial cells (Gombart, 2009), neutrophils (Wang et al., 2004), and bone-marrow derived macrophages (Gombart et al., 2005). The expression of HBD-2 is also up-regulated by vitamin D in keratinocytes and oral/ lung epithelial cells (Wang et al., 2004). The inducing effect of vitamin D is mediated by VDR that binds to a consensus vitamin D response element (VDRE). This induction is associated with a recruitment of PU.1 to the *CAMP* gene promoter (Gombart et al., 2005; Wang et al., 2004). Since VDRE is absent in the *Cnlp* gene, vitamin D_3 treatment does not induce mCRAMP expression in murine cells (Gombart et al., 2005). Activation of toll-like receptor (TLRs) upregulates VDR and the vitamin D1 hydroxylase genes, leading to the induction of LL-37 with subsequent killing of intracellular *Mtb* in human macrophages (Liu et al., 2006). Furthermore, the secondary bile acid lithocholic acid, a ligand to VDR has been shown to recruit PU.1 and VDR to the *CAMP* gene promoter with subsequent upregulation of LL-37 expression (Termen et al., 2008). A synergistic effect of PBA and vitamin D_3 was observed on *CAMP* gene expression in lung epithelial cells (Steinmann et al., 2009) and monocyte-derived macrophages (MDMs), which was correlated with increased antimicrobial activity of MDMs (Mily et al., 2013).

1.2 CELLS OF INNATE AND ADAPTIVE IMMUNE SYSTEM

1.2.1 Cells of innate immune system

1.2.1.1 Epithelial cells

The epithelial cells are present at the host-microbe interface of the skin and all mucosal surfaces. The epithelial cells prevent pathogen entry, while functioning as a gatekeeper for molecules transported in and out of the body. The epithelial cells act as orchestrators of the barrier defenses. These cells exhibit considerable contributions in immune defenses, and in the regulation of the microbiota through the secretion of antimicrobial components, including antimicrobial peptides and mucins (Bevins and Salzman, 2011; Linden et al., 2008; Zasloff, 2002).

1.2.1.2 Granulocytes

The most abundant leucocytes in peripheral blood are polymorphonuclear neutrophils (PMNs), constituting 40-70 % of all leukocytes (Naranbhai et al., 2015). Eosinophils and basophils are also granulocytes. The granules of PMNs contain compounds that are required for successful elimination of microbes including AMPs, proteases and enzymes, generating reactive oxygen species (Borregaard et al., 2007). The characteristics of neutrophilic granulocytes are a multi-lobed nucleus. The PMNs are recruited to a site of infection by chemotactic signals from the epithelia and resident macrophages.

1.2.1.3 Macrophages

Macrophages are one of the main cell-types of phagocytes in the innate immune system. Resident macrophages are constantly present in connective tissues, the liver, the lung and the skin and are often the first immune cells that encounter infecting microbes. The circulating form of macrophages is known as monocytes. Macrophages are covered with different types of pattern recognition receptors, sensing microbes or cell debris (Lavelle et al., 2010). Macrophages act as professional phagocytes, engulfing and digesting cell debris or pathogens in their phagolysosome. These cells also coordinate the innate and adaptive immune responses by antigen presentation, stimulation of lymphocytes and other immune cells through the release of cytokines and additional immune mediators (Medzhitov, 2007).

Additional cells also contribute to the innate immune defense against infection. These include the cytotoxic natural killer cells, acting on virally infected or tumor cells and mast cells contributing to inflammatory responses against parasites. Dendritic cells (DCs) act like macrophages, process antigen materials and present them through major histocompatibility complex (MHC) molecules on the cell surface. These cells then migrate to the lymph nodes and present the antigens to the T- and B-cells of the adaptive immune system (Medzhitov, 2007).

1.2.2 Cells of adaptive immune system

The adaptive immune system is also known as the acquired immune system. When cells of the adaptive immune system encounter a pathogen it takes several days to be fully activated. Hence, the adaptive immune system is dependent on the innate immune system to recognize and hold the line against invading microbes early in the infection (Medzhitov, 2007). Cells of the adaptive immune system are mainly B- and T-cells that are dependent on antigen-presenting cells such as macrophages and dendritic cells for their activation and selective expansion (Medzhitov, 2007).

1.2.2.1 T-lymphocytes

T-lymphocytes play a central role in cell mediate immunity. They can be generally divided into T-helper cells ($CD4^+$) and cytotoxic T-cells ($CD8^+$). Other important T- lymphocytes are Th17 cells and regulatory T-cells. The T-cell receptor identifies an antigen when it is associated with a MHC molecule on the surface of an antigen presenting cell. MHC class I

molecules are found on all nucleated cells and MHC class II molecules are found on all antigen presenting cells. $CD4^+$ cells have the capacity to recognize MHC class II-associated antigens, for example, foreign antigens presented by dendritic cells. $CD8^+$ cells provides defense against intracellular pathogens and different types of virus infections. They eliminate target cells that present foreign antigens through MHC class-I molecules on the cell surface (Broere et al., 2011).

1.2.2.2 B-lymphocytes

B-lymphocytes are the antibody producing cells and also act as antigen presenting cells. Mature B-cells first express IgD (immunoglobulinD) and IgM on their surface. Depending on the pathogenic activation, B-cells switch to a specific Ig isotype and produce either IgM, IgA, IgG, or IgE. Some B-cells become memory cells, resting in the bone marrow, while other B cells turn into plasma cells, which produce and secrete large amounts of specific antibodies. Memory B cells can eliminate a re-infected pathogen by producing pathogen-specific antibodies within a short period of time (Delves and Roitt, 2000).

Along with all immune cells, cytokines also play an important role in the immune system. Cytokines are proteins or glycoproteins, which have potent biological effects on many cell types. They are released by several cells types in the immune system, and in general, the function of cytokines is to coordinate the action of different cell types, participating in the immune and inflammatory processes.

1.3 IMMUNODEFICIENCY

Immunodeficiency is a condition, where the immune system is unable to make a proper immune reaction against infectious pathogens or tumor cells. This situation results in an increased susceptibility to infections. Generally, primary immunodeficiencies (PIDs) are genetic disorders. The PIDs affect both the adaptive and innate immune system, leading to a dysregulation of the immune response (Geha et al., 2007). On the other hand, secondary immunodeficiency is acquired as a result of immunosuppressant drug treatment or diseases such as HIV/AIDS (Chinen and Shearer, 2010).

Primary immunodeficiencies (PIDs) are considered by a heterogeneous group of disorders characterized by poor or absent function in one or more components of the immune system. PIDs are classified as disorders of adaptive immunity (e.g., T-cell or B-cell or combined disorders) or innate immunity (e.g., phagocyte and complement disorders) including syndromes such as severe combined immunodeficiency (SCID), common variable immunodeficiency (CVID), selective IgA-deficiency and IgG-deficiency. The PID patients are indicated by increased susceptibility to respiratory tract infections (RTIs), but also by autoimmune diseases (Wood, 2010). The patients are commonly treated with IgG preparations that generally reduce the occurrence of RTIs (Wood, 2010). CVID is the most common clinically significant PID and this disease might be caused by a number of different

mutations and unknown genetic disorders, resulting in low titers of IgG, IgA and/or IgM. For example, in X-linked agammaglobulinaemia (XLA), a mutation in the Bruton's tyrosine kinase (Btk)-gene leads to inhibition of the maturation of B-cells, and therefore, lack of all immunoglobulin classes (Stewart et al., 2001). The majority of the PIDs patients are diagnosed under the age of one year, although milder forms may not be recognized until adulthood.

1.4 RESPIRATORY TRACT INFECTIONS

Respiratory tract infections (RTIs) are defined as any infections in the respiratory system consisting of sinuses, throat, airways and lungs (**Figure 3**). RTIs are usually caused by viruses, bacteria, or fungi. RTIs can be divided into upper respiratory tract infections and lower respiratory tract infections.

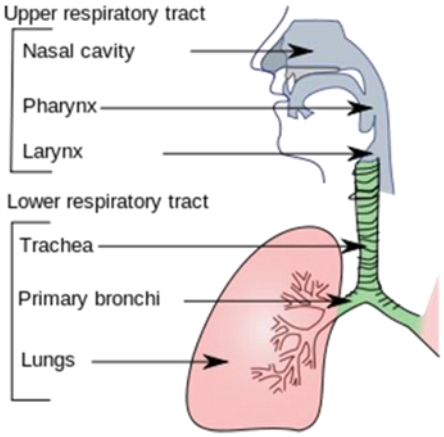

Figure 3: The main features of the human respiratory system. Adapted from (NationalCancerInstitute, 2010).

1.4.1 Upper respiratory tract infections

Upper respiratory tract infections are usually acute and involve the nose, sinuses, pharynx or larynx, and the throat. These infections commonly include influenza, tonsillitis (infection of the tonsils and tissues at the back of the throat), pharyngitis, laryngitis (infection of the larynx/voice box), sinusitis (infection of the sinuses), otitis media, and common colds. Cough and fever is the most common symptom of an upper RTI. Other symptoms include headaches, a stuffy or runny nose, a sore throat, sneezing and muscle aches.

1.4.2 Lower respiratory tract infections

Lower respiratory tract infections can be either acute or chronic and affects the airways and lungs. These infections include common flu (which can affect either the upper or lower respiratory tract), bronchitis (infection of the airways), pneumonia (infection of the lungs), bronchiolitis (infection of the small airways, affecting small children under the age of two), tuberculosis (persistent bacterial infection of the lungs, mainly caused by *Mycobacterium tuberculosis*).

1.5 TUBERCULOSIS (TB)

Tuberculosis is a major public health problem worldwide, caused by *Mycobacterium tuberculosis (Mtb)*. After the human immunodeficiency virus (HIV), TB ranks as the second leading cause of death from an infectious disease in the world. In the past 150 years, several medical advances have been discovered to facilitate prevention and control of TB. In 1882, Robert Koch discovered *Mycobacterium tuberculosis* as the etiological agent of TB. In 1921, the Bacillus Calmette Guerin (BCG) vaccine was introduced, and over 4 billion doses have been administrated worldwide. In 1944, first anti-TB drug were available on the market, as streptomycin was the only drug used to treat TB patients (Daniel, 2006). During the 20th century, TB mortality rates started to decrease in most developed countries probably due to a better socioeconomic status including better-quality of nutrition and living environments (Lienhardt et al., 2012).

1.5.1 TB Epidemiology

According to a recent WHO report the estimation was that, there were 9.0 million new TB cases and 1.5 million TB deaths (1.1 million HIV-negative patients and 0.4 million HIV-positive patients) (WHO, 2014). TB is known as a disease of poor people, more than 80% of all TB cases in the world are found in 22 low- and middle-income countries; 13 in Africa and 9 in Asia. Due to the dreadful effect of HIV, people in sub-Saharan Africa has been extremely affected and accounts for around 80% of all TB/HIV co-infected cases (Lawn and Zumla, 2011). In 2013, globally approximately 5% of all new TB cases and 20.5% of previously treated TB cases are identified as multi-drug resistance-TB (MDR-TB) (WHO, 2014 Update).

Bangladesh is ranked as number 7 out of the 22 highest TB-burden countries in the world. MDR-TB is an emerging threat for the treatment of tuberculosis. In Bangladesh, according to the WHO report, the prevalence of MDR-TB among all newly diagnosed cases is estimated to 2.2%, and 15% among previously treated cases.

1.5.2 TB infection in humans

TB is a contagious and an airborne disease. A few numbers of bacteria (less than 10) are enough to cause an infection. *Mtb* is an aerobic rod-shaped bacillus that is transmitted from a

person with active disease by small droplets of the infection through coughing or sneezing (Gordon et al., 2009). Lung is the primary site of infection, although infection and disease can be developed in any organ in the body (Lawn and Zumla, 2011). However, following the exposure to *Mtb* approximately 10% of the exposed persons will ever develop active disease, while the majority of infected persons contain the bacteria in a latent or sub-clinical state (Pieters, 2008). The outcome of the infection is dependent on the balance between the host immune system and the pathogen. The possibility to develop active TB is highest during the first 1-5 years after exposure, while the latent infection can persist for a long time.

The risk factors for developing active TB are malnutrition, overcrowded living conditions, poverty, immunosuppressive treatments including TNF-α inhibitors, diabetes, cancer, age, alcohol abuse and smoking (Brassard et al., 2011; Faurholt-Jepsen et al., 2012; Keane et al., 2001). Immunocompromised individuals such as HIV-infected patients are more susceptible to develop active TB compared to immunocompetent individuals. TB/HIV co-infection is one of the major factors for TB epidemic in worldwide (Pawlowski et al., 2012).

1.5.3 Clinical symptoms and diagnosis of TB

Human TB is a multifaceted disease with various clinical features. *Mtb* primarily infects the lung and cause pulmonary TB, while infection of other organs such as lymph nodes or pleura or bone is called extra-pulmonary TB. Clinical symptoms of active pulmonary TB include persistent cough at least for few weeks, hemoptysis, weight loss, fever, malaise and night sweats.

Diagnosis of TB is usually based on a combination of clinical, radiological, microbiological and histopathological features of a patient (Sia and Wieland, 2011). Clinical examination includes several parameters such as medical history of the patient, cough, fever, weight loss, etc. Radiological features are based on the findings of chest x-ray. Microbiological methods are sputum-smear microscopy and sputum culture. Sputum-Smear microscopy (detection of acid-fast stained bacilli in sputum samples) is the most widely used and cost-effective diagnostic method. However, about 50% of culture-confirmed pulmonary TB patients are sputum smear negative, and thus microscopy is insufficient to provide a correct diagnosis (Siddiqi et al., 2003). Culture of *Mtb* from clinical specimen is considered as the gold standard to confirm TB diagnosis, but it is time-consuming as it takes 4-8 weeks to grow the bacteria in culture medium. Besides these methods, *Mtb* specific PCR, tuberculin skin tests, IFN-γ release assays (Quantiferon or T-SPOT.TB), histopathological examination of biopsies or cell samples and ALS (antibody in lymphocyte supernatant) assays (Lawn and Zumla, 2011; Raqib et al., 2003) are utilize for TB diagnosis.

1.5.4 Immunology of TB

Since only 10% of the exposed individuals develop active TB, the immune system plays a pivotal role in controlling TB disease (North and Jung, 2004). *Mtb* infection can induce both innate and adaptive immune responses in humans.

1.5.4.1 The innate immune response in TB

Upon exposure to mycobacteria or mycobacterial components, the innate immune system is activated. Primarily, in macrophages and additional leukocytes utilize several pattern recognition receptors to recognize *Mtb*, which include toll-like receptors (TLRs), mannose receptors (MRs), complement receptors (CRs), class A scavenger receptor, dectin 1 (C-type lectin), dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DCSIGN), nucleotide oligomerization domain (NOD)-like receptors (Ernst, 1998; Ilangumaran et al., 1995; Kang et al., 2005; Schlesinger, 1993). When these receptors are activated, they promote the secretion of innate immune mediators that are involved in phagocytosis of mycobacteria. This activation also induces the signaling pathways that trigger production of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-12 (IL-12) (Carvalho et al., 2011; Court et al., 2010).

Toll-like receptors (TLRs) play an important role in the recognition of a wide range of microbes by antigen-presenting cells (APCs) such as macrophages and dendritic cells. In TB, a mycobacterial component is recognized by TLR2, and the role of TLR2 has been described as central in many TB cases (Hertz et al., 2001; Thoma-Uszynski et al., 2000). TLR4 and TLR9 also play an important role in the innate responses against *Mtb* (Bafica et al., 2005; Chen et al., 2010; Kleinnijenhuis et al., 2011). It has been reported that polymorphisms in TLR2 and TLR9 are associated with increased susceptibility to TB in humans, confirming the important role of TLRs in host defense against mycobacteria (Velez et al., 2010). TLR signaling is also very important in the vitD_3 activation pathway. It has been shown that activation of TLR2/1 enhances VDR and 25- hydroxyvitaminD_3-1α-hydroxylase, which convert the pro-form of vitD_3 to the active form. The activation of vitamin D pathway in a TLR-dependent manner leads to the synthesis of LL-37 (Liu and Modlin, 2008). Notably, *Mtb* has also developed strategies to interfere with TLR activation, resulting in inflammation (Barth et al., 2013).

Mtb interact with mannose receptors via mannose-capped lipoarabinomannan (ManLAM) facilitating phagocytosis by macrophages. *Mtb* ManLAM blocks phagosome-lysosome fusion and thereby enhances survival of *Mtb* in human macrophages.

Macrophages are known as the primary home for mycobacteria. Alveolar macrophages engulf mycobacteria after entering into the host via the nasal route. Besides the alveolar macrophages, pulmonary DCs, and neutrophils are activated at the site of infection in the lung after inhalation of *Mtb* (van Crevel et al., 2002). Activated macrophages are producing antimicrobial peptides (Liu and Modlin, 2008) as well as nitric oxide (Scanga et al., 2001), which provides the first line of defense to restrict intracellular bacterial replication. *Mtb* needs to come out from macrophages to infect other cells. Different pathways of host cell death play different roles during *Mtb* infections in terms of host defense and microbal survival (Lamkanfi and Dixit, 2010; Persson et al., 2008). *Mtb* has developed mechanisms to limit macrophage apoptosis (Danelishvili et al., 2010; Velmurugan et al., 2007), which is a programmed cell death, depending on the induction of caspases. Conversely, *Mtb* promote

host cell necrosis, which is a passive form of cell death. As a result, necrosis prevents cross-presentation of *Mtb*-antigens by DCs that could hamper and delay T cell activation (Molloy et al., 1994) and subsequent immune activation.

1.5.4.2 The adaptive immune response in TB

Mtb is an intracellular pathogen. Therefore, cell-mediated immune responses are very crucial for host protection. However, humoral or antibody-mediated immune responses may also contribute to the immune protection to TB (Glatman-Freedman, 2006). A slow induction of the adaptive immune response allows *Mtb* infection to become established in the host before effective bacterial elimination can occur (Cooper, 2009).

Development of effective T cell responses depends on the bacterial antigen presentation by DCs and macrophages (Chackerian et al., 2002). TLRs and other pattern recognition receptors are expressed on DCs, recognizing *Mtb* products and triggers functional maturation of DCs and leads to initiation of antigen-specific adaptive immune responses. DCs are also involved in the uptake of *Mtb*-infected cells or bacterial products in the lung and carry them to the regional lymph nodes to initiate cross-presentation and activation of specific T cell responses (Bhatt et al., 2004).

Upon antigen presentation, $CD4^+$ and $CD8^+$ T cells become activated and express cytolytic effector molecules, and also regulate the inflammatory environment to limit tissue damage (Green et al., 2013; Serbina et al., 2001). Activated $CD4^+$ T cells produce the key cytokine IFN-γ and these activate macrophages, leading to the production of antibacterial components to kill the bacteria, which is the major effector mechanism of cell-mediated immunity against TB (Ottenhoff et al., 1998). A defective $CD4^+$ T-cell function increases the susceptibility to *Mtb* infection, which indicates a central role of $CD4^+$ T cell in protection against TB (Caruso et al., 1999). $CD8^+$ cytolytic T cells (CTLs) are able to kill *Mtb*-infected macrophages by expressing granule-associated effector molecules such as granulysin, Fas-L, and perforin (Canaday et al., 2001). It has been demonstrated that granulysin alters the integrity of the mycobacterial cell wall and in combination with perforin, reduces the viability of *Mtb* (Stenger et al., 1998).

1.5.5 TB vaccine

Bacillus Calmette-Guerin (BCG) is the first and only existing vaccine against TB. BCG is made from a live attenuated *M. bovis* strain. In 1908, Albert Calmette and Camille Guerin at the Institute of Pasteur developed this vaccine. They generated an attenuated form of *M. bovis* strain which was then utilized for the development of the first TB vaccine (Daniel, 2005; Sakula, 1983). BCG is the most comprehensively used vaccine with more than 4 billion doses administered worldwide (Dietrich et al., 2003). Depends on the different populations and geographic locations BCG provides various levels of protection against TB (average 35-65%) (Fine, 1995). Several factors are believed to influence the discrepancy in BCG-induced protection, e.g., genetic and nutritional differences in populations, environmental factors such as sunlight contact, temperature variations, cross-reactivity between BCG and

other environmental mycobacterial strains, and batch differences during BCG preparation (Agger and Andersen, 2002; Behr, 2002; Brandt et al., 2002).

1.5.6 Anti-TB treatment

The global situation of TB changed significantly after the introduction of the first antimycobacterial drug. Anti-TB drugs promptly decrease bacterial loads in the lung and reduce the chance of transmission when the drugs regimen is followed correctly. During the year 1944, TB patients were treated only with streptomycin. Professor Selman A. Waksman was awarded the Nobel Prize in Physiology or Medicine in 1952 for his discovery of streptomycin. Nowadays, TB is treated with a combination therapy with first- and/or second-line of anti-TB drugs for at least 6 months. Treatment of the first-line anti-TB drugs includes rifampicin, isoniazid, pyrazinamide and ethambutol for 2 months, followed by rifampicin and isoniazid for additional 4 months (Ramachandran and Swaminathan, 2012). This prolonged multidrug treatment often inhibits the development of drug resistance, but it creates lots of compliance problems among the patients. However, in recent times, increasing numbers of MDR-TB, extensively drug-resistant (XDR) and totally drug-resistant cases are threatening for the TB control worldwide (Gandhi et al., 2010; Zumla et al., 2012).

Before the effective chemotherapy was accessible, different approaches were taken to treat TB. One approach was that the TB patients were isolated in sanatoria and were placed out under the sun for fresh air. The sunlight treatment often promoted clinical recovery among these patients. In 1903, Niels Ryberg Finsen was awarded the Nobel Prize in Medicine and Physiology for the treatment of TB patients with sunlight. Now, it has been discovered that the sunlight stimulates the production of vitD_3 in the skin. Vitamin D_3 has recently been shown to enhance autophagy and the expression of cathelicidin LL-37 in activated macrophages, with subsequent killing of *Mtb* inside the infected macrophages (Liu et al., 2006; Yuk et al., 2009). Thus sunlight and vitD_3 promotes the recovery of TB patients.

Regardless of several medical advances, it is urgent to increase and improve research on human TB to discover novel therapeutic interventions including better drugs, new diagnostic methods, and more efficient vaccines.

1.6 AUTOPHAGY

Autophagy is a vital intracellular process that controls the recycling system of the cells, and delivers cytoplasmic constituents to the lysosome. It has an extensive diversity of physiological and pathophysiological roles to maintain the balance between the synthesis and degradation of the cellular components. Recent studies have explored that autophagy has a great variety of cellular function such as adaptation to starve condition, clearance of intracellular misfolded protein and organelle, elimination of intracellular microorganisms,

anti-aging, and cell death. In addition, autophagy is involved in cell survival, tumor suppression, and antigen presentation (Mizushima, 2005).

1.6.1 Different types of autophagy

There are three main types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy.

1.6.1.1 Macroautophagy

Macroautophagy is a lysosome mediated process, occurring primarily to eradicate damaged cell organelles as well as intra-cellular pathogens or unused proteins (Levine et al., 2011). The term "autophagy" usually designates for macroautophagy. This process involves the formation of a double-membrane vehicle known as autophagosomes (Mizushima et al., 2002). In the canonical autophagy process such as during starvation, the autophagy-related gene (ATG) 6 (known as Beclin-1), class 3 phosphatidylinositol-3-kinase, and ubiquitin-like conjugation reactions induce the formation of the autophagosome. The autophagosome finally fused with lysosomes, and the contents of the autolysosome are degraded via acidic lysosomal hydrolases (Mizushima, 2005). Additional ATG proteins such as ATG4, ATG12, ATG5, and ATG16 are also involved in the regulation of this macroautophagy pathway.

1.6.1.2 Microautophagy

Microautophagy pathway is mediated by direct lysosomal (in mammals) or vacuolar (in fungi and plant) engulfment of the cytoplasmic materials (Mijaljica et al., 2011). Engulfment of cytoplasmic cargo into the boundary membrane of autophagic tubes is mediated by invagination and vesicle scission into the lysosomal lumen. The main functions of microautophagy are to maintenance organellar size, membrane homeostasis, and cell survival during nitrogen restriction. Additionally, microautophagy is coordinated and complemented with macroautophagy and chaperone-mediated autophagy.

1.6.1.3 Chaperone-mediated autophagy

Chaperone-mediated autophagy (CMA) is a complex and specific pathway. It plays an important role in protein quality control in cells by selectively delivering cytoplasmic misfolded proteins together with a CMA targeting motif to lysosomes for degradation (Kaushik and Cuervo, 2008). Proteins that contain the recognition site for heat shock cognate protein 70 (hsc70) complex bind to this chaperone, and form the CMA-substrate/chaperone complex. This complex is recognized and bind to the CMA receptor (known as lysosome associated membrane protein 2A; LAMP2A) that is present on lysosome membranes. The substrate protein becomes unfolded and is translocated across the lysosome membrane by assistance of the lysosomal hsc70 chaperone and is finally degraded into the lysosome.

1.6.2 Autophagy machinery

In a normal healthy cell, the autophagy process is suppressed by the mTORC1 (mammalian target of rapamycin complex 1) kinase which phosphorylates and inactivates two autophagy-

related proteins, unc-51-like kinase 1/2 (ULK1/2) complex and autophagy related 13 (ATG13) (Dorsey et al., 2009; He and Klionsky, 2009). Once the ULK1/2 is activated, nucleation of the vesicle is initiated; as a result phagophore is formed (Chang and Neufeld, 2010). In the vesicle nucleation process class III phosphatidylinositol 3 kinase (PI3K) complex is activated to generate phosphatidylinositol-3 phosphate. PI3K contains the VPS34 (vacuolar protein sorting 34), and the activation of VPS34 depends on the formation of a complex that includes VPS15, Beclin 1, AMBRA1 (activating molecule in Beclin1 regulated autophagy protein 1), ATG14 or UVRAG (ultraviolet irradiation resistance-associated gene) and BIF1 (Bax-interacting factor 1) (He and Levine, 2010). The inhibitor of apoptosis BCL-2 (B-cell lymphoma 2) can act as an inhibitor of autophagy by binding to Beclin 1 and AMBRA1. The vesicle elongation process is mediated by the action of two ubiquitin-like conjugation systems. In the first pathway, phosphatidylethanolamine (PE) is conjugated to microtubule-associated protein 1 light chain 3 (LC3) by the protease ATG4, the E1-like enzyme ATG7 and the E2-like enzyme ATG3 (Satoo et al., 2009; Sugawara et al., 2005). This lipid conjugation process allows LC3 to be attached to the phagophore membrane; hence LC3-II is known as the lipidated form of LC3. LC3 plays an important role in phagophore elongation and in recognition of the molecules to be degraded by the autophagy process (Chen and Klionsky, 2011). In particular, the conversion of LC3 from processed LC3-I to the lipidated LC3-II is the main readout used in the analysis of autophagy (Klionsky et al., 2012). In the second pathway, ATG12 becomes covalently bound to ATG5 by the action of the E1-like enzyme ATG7 and the E2-like enzyme ATG10. The complex of ATG5, ATG12 and ATG16-like1 (ATG16L1) can potentially act as an E3-like ligase on LC3 (Chen and Klionsky, 2011). One of the interaction partners of LC3 is sequestosome 1 (SQSTM1, also called p62 protein), which is a cargo receptor, identifying ubiquitinated protein, organelles or even intracellular bacteria (Shvets et al., 2008). Once the autophagosome is formed, ATG proteins are released and recycled. The autophagosome then fuses with a lysosome, creating the autolysosome. The acidic pH and the lysosomal enzymes, degrade the cargo of the autolysosome and its inner membrane (**Figure 4**).

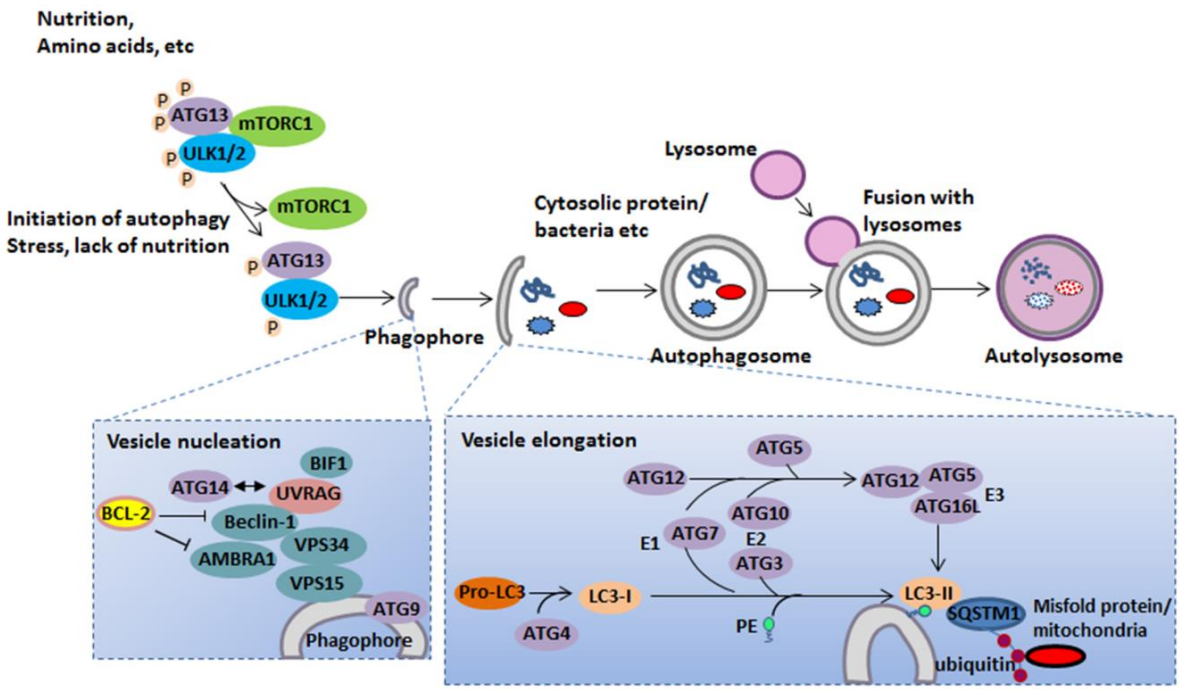

Figure 4: Schematic representation of mammalian autophagy core machinery. Adapted with modification (Fullgrabe et al., 2014).

1.6.3 Role of autophagy in different diseases

Autophagy is known as a cell survival mechanism under conditions of stress, maintaining cellular integrity by regenerating metabolic precursors and clearing subcellular debris. Besides this function autophagy has a huge influence on different diseases (Levine and Kroemer, 2008; Mizushima et al., 2008). In cancer, autophagy exerts a multifactorial influence on the initiation and progression of cancer, as well as on the effectiveness of therapeutic interventions. It has been reported that in 40 to 75% cases of human breast, ovarian, and prostate tumors have mono allelic disruption of Beclin1(Gao et al., 1995; Liang et al., 1999; Rubinsztein et al., 2012). In mice, homozygous deletion of Beclin-1 results in embryonic lethality, and mono allelic loss of Beclin1 (*Becn1*+/−) results in spontaneous tumorigenesis (Qu et al., 2003; Yue et al., 2003). A different picture appears when autophagy is inhibited the growth of pancreatic cancer cells are halted (Yang et al., 2011). This contradiction suggests that autophagy may facilitate chemotherapeutic or radiation-induced cytotoxicity in apoptosis-resistant tumor cells through autophagy-associated cell-death pathways (White, 2012). In neurodegenerative disorders such as Alzheimer's disease, the beta-amyloid peptide (Aβ) is accumulated when autophagosome–lysosome fusion is impaired (Jellinger, 2010).

A number of human pathogens are degraded *in vitro* by autophagy, including bacteria (e.g., group A *Streptococcus*, *Mycobacterium tuberculosis*, *Shigella flexneri*), viruses such as

herpes simplex virus type 1 (HSV-1) and chikungunya virus, and parasites such as *Toxoplasma gondii* (Deretic and Levine, 2009; Levine et al., 2011; Rubinsztein et al., 2012). Recent studies have shown the mechanisms by which intracellular bacteria and viruses are targeted to autophagosomes for degradation (Johansen and Lamark, 2011; Orvedahl et al., 2011; Watson et al., 2012). In mice when ATG7 expression is deleted, they become more susceptible to *Mtb* infection (Bonilla et al., 2013). Notably, it has been shown that vitamin D inhibits the replication of human immunodeficiency virus (HIV) and *Mtb* in human macrophages through an autophagy dependent mechanism. Additionally, the antimycobacterial action of some anti-TB drugs is associated with induction of autophagy (Campbell and Spector, 2011; Kim et al., 2012). In chronic obstructive pulmonary disease (COPD), the expression of LC3-II and autophagosome formation are increased in lung tissue (Ryter and Choi, 2015). In a recent preclinical study of cystic fibrosis, it is suggested that in autophagy pathway is impaired, with subsequent failure to clear the aggregated proteins (Luciani et al., 2010). Impaired autophagy in human idiopathic pulmonary fibrosis has been noted recently (Patel et al., 2012). Furthermore, increased number of autophagosomes have been observed in bronchial-biopsy specimens from patients with asthma (Poon et al., 2012) and ATG5 expression is elevated in nasal-biopsy specimens from children with asthma (Martin et al., 2012).

In heart diseases including cardiomyopathies, cardiac hypertrophy, heart failure, and ischemia modulations of autophagy have been reported (Kirshenbaum, 2012). In patients with genetic X-linked deficiency in lysosome associated membrane protein 2 (LAMP2), which promotes the fusion of autophagosome–lysosome, causes cardiomyopathy known as Danon's disease (Nishino et al., 2000). Chromosomal deletion of gene encoding autophagy proteins affects the storage of triglycerides in lipid droplets in the liver (Singh et al., 2009). Mutations of SQSTM1/p62 have been linked to bone metabolism disorder known as Paget's disease (Laurin et al., 2002). During exercise, autophagy is increased. In contrast, it has been shown that expression of the autophagy related genes ATG5, ATG7, and Beclin1 is decreased with increasing of age (Lipinski et al., 2010).

2 AIM OF THE THESIS

The general aim of the present thesis is to elucidate the role of antimicrobial peptides in tuberculosis and respiratory tract infections, and to dissect the underlying mechanisms. The specific aims are:

- To examine if supplementation with vitamin D_3 can reduce infectious symptoms and antibiotic consumption among patients with antibody deficiency or frequent RTIs (**Paper I**).

- To find out the optimal dose of phenylbutyrate (PBA) in healthy volunteers that can be used in the clinic to treat pulmonary tuberculosis (TB) patients (**Paper II**).

- To investigate if oral adjunctive therapy with PBA and/or vitD_3 along with standard anti-TB therapy will lead to improve clinical recovery in pulmonary TB patients (**Paper III**).

- To evaluate whether PBA can induce LL-37-dependent autophagy in human macrophages and THP-1 cells, and to dissect the underlying mechanisms (**Paper IV**).

3 METHODS AND MATERIALS

This section of the thesis is a summary of the methods and materials that are used in study I to IV. For more details the individual papers are referred (**Paper I-IV**).

3.1 STUDY DESIGN AND PARTICIPANTS

The clinical studies in the present thesis have been conducted at Karolinska University Hospital, Huddinge (**Paper I**) and at International Centre for Diarrheal Disease Research, Bangladesh (icddr,b) (**Paper II** & **III**).

Study I (**Paper I**) was a double-blind, randomized and placebo-controlled study of $vitD_3$ supplementation to patients with increased susceptibility to respiratory tract infections. Patients (n=140) were enrolled at the Immunodeficiency Unit, Karolinska University Hospital, Huddinge, Sweden. Inclusion criteria was an increased susceptibility to respiratory tract infection; i.e > 42 days with symptoms from the respiratory tract infection over period of one year prior to inclusion and the age range was 18–75 years. Patients received $vitD_3$ (4000 IU) or placebo daily for one year. The study was registered at www.clinicaltrials.gov (NCT01131858, EudraCT nr: 2009-011758-16) before inclusion of the first patient.

Study II (**Paper II**) was conducted to determine the optimal dose of phenylbutyrate (PBA) that would be used in a clinical trial of pulmonary TB patients (study III). Healthy volunteers (age range 18 to 55 yrs; n=15) were invited to participate and enrolled in an eight-day open trial. They were divided into five groups, each group consisting of three participants. Group-I received 250 mg PBA twice daily together with 5000 IU $vitD_3$, Group-II received 500 mg PBA twice daily plus 5000 IU $vitD_3$, Group-III received 1000 mg PBA twice daily together with 5000 IU $vitD_3$, Group-IV received 500 mg PBA twice daily, and Group-V received 5000 IU $vitD_3$. These doses were given to the participants for 4 consecutive days.

Study III (**Paper III**) was a randomized, double-blind and placebo-controlled 4-arm intervention trial of pulmonary TB patients treated with adjunct therapy and PBA and/or $vitD_3$ for two months. Patients (n=288) were enrolled from the National Institute of the Diseases of the Chest and Hospital (NIDCH) in Dhaka, Bangladesh, after providing written informed consent. Male and female patients (age range 18 to 60 yrs) with newly diagnosed sputum smear-positive TB were enrolled. Patients were randomized to the following adjunct treatment and received oral doses for 2 months of either: (1) placebo PBA and placebo $vitD_3$ or (2) 500 mg PBA and placebo $vitD_3$ or (3) placebo PBA and 5000 IU of $vitD_3$ (Cholecalciferol) or (4) 500 mg of PBA and 5000 IU of $vitD_3$. PBA was given twice daily where as $vitD_3$ was given once daily. The trial was registered at www.clinicaltrials.gov (NCT01580007).

3.2 OUTCOMES

In study I (**Paper I**), the primary outcome was the infectious score, based on an infection diary. The following information's were recorded in the diary to measure the primary outcome: symptoms from the respiratory tract, and the ears, sinuses infection, malaise due to infection, and use of antibiotics. The patients were trained to mark their daily symptoms in a diary known as ''infection diary'' (**Paper I, Figure S1**). They sent back their filled-out diary to the study site by regular mail every month. Secondary outcomes were the levels of serum $25(OH)D_3$ (at baseline, 3, 6, 9, and 12 months), total numbers of bacterial cultures, microbiological findings, and levels of the antimicrobial peptides LL-37 and HNP1-3 in nasal fluid (at baseline, 6 and 12 months). Additionally, single nucleotide polymorphisms (SNPs) analyses were performed for VDR (Taq1 and Foq1), CYP27B1, CYP24A1, CYP2R1, and vitamin D binding protein.

In study II (**Paper II**), the main outcomes were the level of LL-37 expression in immune cells (at baseline, day 4 and 8) and antimycobacterial activity in monocyte-derived macrophages (MDMs) (at baseline, day 4 and 8).

In study III (**Paper III**), primary outcomes were assessment of both microbiological and clinical parameters. The microbiological outcome was measured as the proportion of TB patients who became culture negative at week 4. The major clinical endpoints were cough remission, reduction in lung involvement in the chest x-ray, normalization of fever and weight gain at week 8. Secondary outcome were time to sputum smear conversion, radiological findings, concentrations of $25(OH)D_3$ in plasma, the expression of the LL-37 in immune cells and intracellular killing of *Mtb* by MDMs *ex vivo*. In addition to the outcomes measurement, TB scoring system was also used to monitor the therapeutic effects of PBA and vitamin D_3. Clinical parameters with or without chest x-ray findings were used to measured TB score (**Paper III, Table S1**).

3.3 EXPERIMENTAL METHODOLOGIES

3.3.1 Blood cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood (**Paper II & III**) and buffy coat (**Paper IV**) by Ficoll-Hypaque density gradient centrifugation, and plasma was separated. Human monocyte-derived macrophages (MDMs) were prepared by culturing peripheral blood monocytes in the presence of 10% autologous serum (**Paper II & III**) or 50 ng/mL human macrophage colony-stimulating factor (M-CSF) (**Paper IV**). Non-adherent cells were collected for further analysis (**Paper II & III**). The viability of MDMs was determined with Trypan blue staining.

Additionally, the human monocytic THP-1 cell line was utilized and treated with 10 ng/mL phorbol myristate acetate (PMA) to be differentiated into macrophage-like cells (**Paper IV**).

3.3.2 Cell treatments

Human MDMs and THP-1 cells were treated with PBA, and/or $1,25(OH)_2D_3$, or synthetic LL-37 or rapamycin. To block the P2RX7 receptor, primary MDMs were pre-treated with KN62 (1-[N,O-bis(5-Isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine) an inhibitor of P2RX7receptor. To inhibit different signaling pathways, primary MDMs were pretreated with the intracellular calcium-specific chelator 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid-acetoxymethyl ester (BAPTA-AM), the ATP-competitive inhibitor of AMP-activated protein kinase (compound C) or the 3-methyl adenine (3-MA) an inhibitor of phosphatidylinositol 3-kinase pathway (**Paper IV**).

3.3.3 Peptide and protein extraction in nasal fluid sample

Peptides and proteins from nasal fluid samples were extracted with an equal volume of 60% acetonitrile in 1% trifluoroacetic acid and lyophilized. Extracts were enriched for polypeptides by using reversed-phase columns (**Paper I**).

3.3.4 Serum/Plasma 25-hydroxyvitamin D_3 measurment

Levels of 25-hydroxyvitamin D_3 in serum were determined by using DiaSorin immunochemical method (**Paper I**). In plasma, 25-hydroxyvitamin D_3 was measured either by ELISA (**Paper II**) or by an electrochemiluminescence immunoassay (**Paper III**).

3.3.5 ELISA of LL-37

Levels of LL-37 peptide in the intracellular fluid of MDMs and lymphocytes (**Paper II & III**), extra cellular fluid of PBMCs (**Paper II & III**) and MDMs (**Paper IV**) were measured by ELISA.

3.3.6 Plasma/Serum Biomarkers

Levels of calcium (**Paper I, II & III**), albumin (**Paper I, II & III**), creatinine (**Paper I, II & III**), phosphate (**Paper I**), and C-Reactive Protein (CRP) (**Paper III**) were assessed in plasma by an automated clinical chemistry analyzer. Serum glutamate-pyruvate transaminase (SGPT) was measured by a Beckman-Coulter laboratory analyzer (**Paper II**).

3.3.7 Quantitative real time PCR

RNA was extracted from macrophages and lymphocytes, and cDNA was synthesized. The *CAMP* gene encoding LL-37 transcript relative to the housekeeping 18S rRNA was measured in cDNA samples by quantitative real-time PCR using a CFX96 Real-Time quantitative-PCR Detection Systems (**Paper II, III & IV**). Transcripts of the autophagy markers ATG5 and Beclin-1 relative to the housekeeping 18S rRNA were measured by real-time quantitative - PCR (**Paper IV)**. The results were analyzed using the relative standard method (Bergman et al., 2005).

3.3.8 Macrophage mediated killing of *Mycobacterium tuberculosis*

The virulent *Mtb* strain H37Rv was cultured in Middlebrook 7H9 broth. MDMs were infected with this virulent strain of *Mtb* with MOI 25. After 2 hours of exposure, MDMs were washed to remove the extracellular bacteria, and the infected MDMs were cultured for three additional days. After that, the cells were lysed, and the lysates were cultured on Middle Brook 7H11 agar medium for 21–28 days to count the viable bacteria by colony forming units (CFU) (**Paper II & III**).

In study IV (**Paper IV**), MDMs were infected with the virulent strain of *Mtb* (MOI 1:5) for 4 hours and then treated with PBA and/or $1,25(OH)_2D_3$ or rapamycin or LL-37 for three days. After that, the intracellular bacteria were harvested and cells were lysed. The lysates were then cultured on Middle Brook 7H11 agar media and after 21 to 28 days of culture at 37ºC bacterial viability was calculated by the CFU count.

3.3.9 *Mtb* microscopy, culture and drug susceptibility test in sputum sample

Acid-fast bacilli (AFB) in patient's sputum samples were detected with Ziehl-Neelsen staining and direct smear microscopy. Sputum samples were cultured for 6 to 8 weeks to assess *Mtb* growth in the sputum, and calculated by the CFU count. Drug susceptibility tests (DST) were performed for rifampicin, isoniazid, ethambutol and streptomycin by the minimum inhibitory concentration method (**Paper III**).

3.3.10 Western blot

Cellular extracts, cell culture supernatants and nasal fluid samples were used for Western blot analysis. Expression of LL-37 (**Paper I & IV**), different markers of autophagy such as LC3, ATG5, Beclin-1 and p62 (**Paper IV**) and α-defensins (HNP1-3) (**Paper I**) were measured by Western blot analysis.

3.3.11 Fluorescence Microscopy

Fluorescence microscopy technique was utilized to visualize the cellular localization of LL-37, LC3 and p62 protein in *Mtb* infected and treated MDMs and THP-1 cells (**Paper IV**). Cells were imaged with an Olympus confocal microscope. Quantification of autophagy was performed based on the percentage of the cells with LC3-II positive punctate dots by ImageJ software.

3.3.12 Generation of *CAMP* gene knockdown THP-1 cell line

Transient transfection method was utilized to knockdown the *CAMP* gene expression in the THP-1 cell line. Cells were transfected with either pLKO.1-based *CAMP* short hairpin RNA (shRNA) constructs or a control vector without any insert using the transfection reagent Turbofect.

3.3.13 Genotyping

All the patients of study I (**Paper I**) were genotyped for 6 single nucleotide polymorphisms by using a TaqMan@ genotyping assay.

3.4 ETHICAL CONSIDERATION

Written informed consent was obtained from the participants of all clinical studies (**Paper I, II & III**). Study I (**Paper I**) was approved by the local ethics committee in Stockholm (DNR 2009/1678-31/4 and 2010/498-32). Study II & III (**Paper II & III**) have been approved by the Research Review Committee and Ethical Review Committee at icddr,b, Bangladesh (PR-09068).

3.5 STATISTICAL ANALYSIS

Statistical analyses were conducted using the software R 2.11.1 (R Development Core Team), GraphPad Prism (version 5.0, GraphPad Software, La Jolla, CA, USA), different versions of SPSS Statistics (IBM SPSS 20.0 and PASW 22.0) (SPSS Inc. Chicago, USA) and Stata 13 (StataCorp, Texas, USA).

Depending on the data distribution student's t -test or Mann-Whitney U test were used to compare between two groups. One-way ANOVA was used for comparing more than two groups.

Effects of supplementation on outcomes were evaluated by multi-variable adjusted models (linear regression, logistic regression, analysis of covariance). Potential covariates were selected if they were significantly associated with the outcomes or if they were considered as biologically relevant covariates. $P \leq 0.05$ was considered as statistically significant.

4 RESULTS AND DISCUSSION

This section summarizes the main results that are also discussed. For further details about the results and discussion, the readers are referred to the individual papers (**Paper I-IV**).

4.1 VITAMIN D_3 SUPPLEMENTATION IN PATIENTS WITH FREQUENT RESPIRATORY TRACT INFECTIONS CORRELATES WITH IMPROVED CLINICAL OUTCOME (PAPER-I)

4.1.1 Effect of vitamin D_3 supplementation on infectious score

Treatment with $vitD_3$ for one year was significantly associated with a reduced total infectious score in patients with frequent respiratory tract infections (RTIs). The unadjusted relative score in the $vitD_3$ supplemented group was 0.754 (p=0.024) and after adjustment for potential confounding factors (age, gender, smoking, type of immune deficiency and significant comorbidities), the relative score was 0.771 (p=0.04). In the temporal analysis, the effect of $vitD_3$ supplementation on infectious score had a tendency to reduce over time (**Figure 5A**). We observed a significant reduction of infectious score (47 points per patient) in the treatment group (p=0.023). When we compared for individual parameters of infectious scores, significant reduction of antibiotic use was observed in $vitD_3$ supplemented group. The rest of the four parameters of the primary endpoints were favored in the supplemented group. In average, the placebo group was on antibiotic treatment for 33 days, whereas the $vitD_3$ group was treated with antibiotic for only 16 days (**Figure 5B**). These findings suggest that $vitD_3$ supplementation may prevent respiratory tract infections and reduce antibiotic consumption in patients with an increased susceptibility to RTIs.

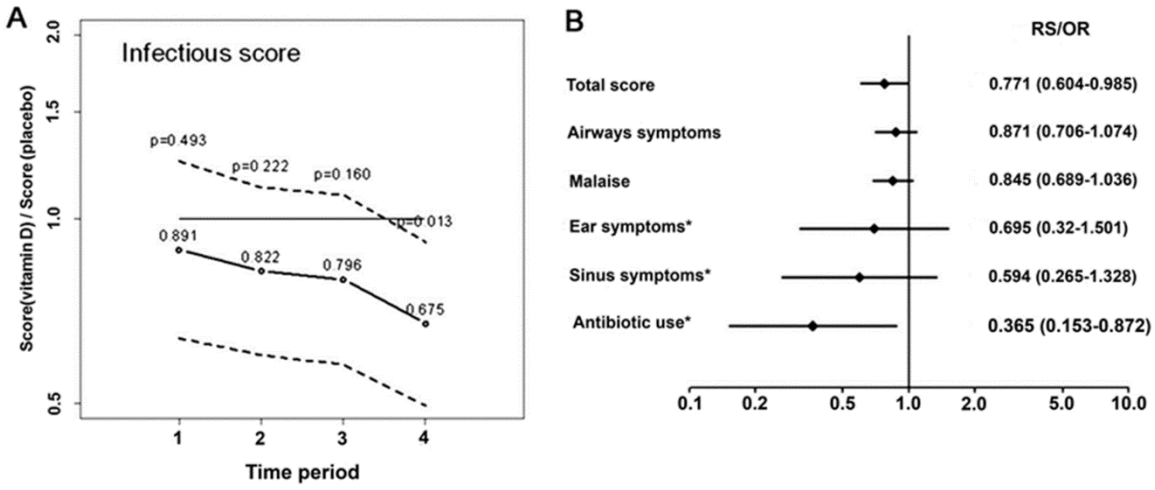

Figure 5: Primary endpoint-infectious score. The adjusted total relative infectious score (**A**) is expressed in every quarter (3-month periods). The adjusted one-year scores (total score, airways, malaise, ear, sinus and antibiotics) are presented in a Forest-plot (**B**) together with 95% confidence intervals. Effects of the total score, airway symptoms and malaise due

to infection are presented as relative scores and the effects of ear symptoms, sinus symptoms, antibiotics use are presented as odds ratio (indicated with asterisks).

4.1.2 Effect of vitamin D_3 supplementation on serum 25-hydroxyvitamin D_3, microbiological findings and the levels of antimicrobial peptides in nasal fluid

There was no significant difference in serum $25(OH)D_3$ level between the treatment group (51.5 nmol/L) and the placebo group (46.9 nmol/L) at baseline. After three months of intervention, a significantly higher level of $vitD_3$ (133 nmol/L) was observed in $vitD_3$ supplemented group compared to the placebo group (67 nmol/L). This enhanced level of serum $25(OH)D_3$ was maintained at steady state level throughout the study period (one year).

During the study period, 173 microbiological samples were collected from the $vitD_3$ group and 301 samples from the placebo group (p=0.01). Patients were instructed to leave samples upon need, e.g., when they had symptoms of infection. The number of samples with at least one positive microbiological finding was higher in the placebo group (p=0.052). The treatment group did not differ from the placebo group in the detection of traditional respiratory pathogens, such as *Haemophilus influenza*, *Moraxella catharralis*, and *Streptococcus pneumoniae*. However, there were significantly fewer numbers of *Staphylococcus aureus* (p=0.019), and fungi (*Candida spp.* and *Aspergillus spp*. p=0.028) were found in the $vitD_3$ group after one year of supplementation.

In nasal fluid, LL-37 and α-definsin (HNP1-3) levels were analyzed. No statistically significant differences were observed between two groups. Surprisingly, after one year, a non-significant trend of higher levels of AMPs was observed in placebo treated patients compared to $vitD_3$ treated patients. This unexpected finding could be explained by the fact that patients given placebo had a higher frequency of primary pathogens present in their nasal fluid. Our group has previously shown that primary pathogens, including *H. influenza*, *S pneumoniae* and *M. catharralis* are associated with higher levels of AMPs in nasal fluid (Cederlund et al., 2011). Interestingly, no primary pathogens were detected in the nasal swabs from the $vitD_3$ supplemented patients, which corresponded to lower levels of AMPs.

Vitamin D_3 acts as an immune-modulator of both the innate and adaptive immune system. For example,$vitD_3$ exhibits broad anti-inflammatory effects (Hewison, 2012) and is able to modulates Th1/Th17-response to a Th2/Treg response (Daniel et al., 2008; Lemire et al., 1995). It was already mentioned that $1,25(OH)_2D_3$ induces AMPs in immune cells (Liu et al., 2006). However, in this study we could not identify any significant differences of LL-37 or HNP1-3 in the two groups during the study period. We expected a reduction in bacterial load of *H. influenza, M. catharralis* and *S. pneumoniae* in the patients treated with $vitD_3$. However, the frequency of these bacteria was not reduced; instead, we found a reduction of *S. aureus* and fungal *species* that often colonize in the airways. A possible explanation may be that $vitD_3$ has specific effects on immunity against *S. aureus* and other pathogens (Wang et

al., 2004). For example HBD-2 and HBD-3 are highly active against *S. aureus* (Harder et al., 1997). Another study showed that low levels of vitD_3 were associated with an increased risk of colonization by *S. aureus* (Olsen et al., 2012). Furthermore, vitD_3 has been shown to affect the immunity against *C. albicans* by modulating cytokine production in humans (Khoo et al., 2011).

From previous clinical trials it has been suggested that the dose of vitD_3, the dosing interval, time of the year for the intervention and the study length could be important issues in the prevention of infections. In our study we used a relatively high dose of vitD_3 (4000 IU) in a daily schedule for one year. It should be noted that some randomized trials have used lower doses of vitD_3 (400–2000 IU/day) and the outcome in these trials have failed to find a beneficial effect of vitD_3 against infections (Laaksi et al., 2010; Li-Ng et al., 2009). A recent meta-analysis suggested that the dosing interval also appears to be a key factor and intervention studies using daily doses of vitD_3 showed a better therapeutic effect than studies where participants were given large doses of vitD_3 at intervals of one to three months (Bergman et al., 2013).

To our knowledge this is the first study, where vitD_3 supplementation covered all four seasons. This is important in Sweden, since there is a known variation in serum 25(OH)D_3 levels during different seasons of the year (Lindh et al., 2011). In addition, several studies showed no preventive effect of vitD_3 supplementation on infections, when supplementation was given only during the winter season when vitD_3 levels were normally low or due to shorter periods of time (6–12 weeks) (Laaksi et al., 2010; Martineau et al., 2011; Nursyam et al., 2006; Urashima et al., 2010). Murdoch et al., showed no effect of vitD_3 supplementation on respiratory tract infections of healthy participants with a normal mean vitD_3 level at baseline (72 nmol/L) (Murdoch et al., 2012), which add further evidence to the fact that participants with normal vitD_3 level probably will have no additional benefit of vitamin D_3 supplementation. However, in our study the enrolled patients had insufficient levels of vitD_3 at the beginning of the study (baseline levels were 50 nmol/L). Thus, our results suggest that supplementation of vitD_3 is beneficial for individuals with frequent symptoms of infection and with insufficient serum-levels of vitamin D_3.

One of the limitations of our study was that the primary endpoint relied only on patient-reported information. Another limitation was that the patients were heterogeneous concerning the immune-deficient disorder as well as concomitant diseases. We tried to adjust for these factors in the multivariate analysis of the primary endpoint, but, unfortunately, the sample sizes in each subgroup were too small to draw firm conclusions. Thus, the results from our study are most relevant for the studied group of patients but cannot be extrapolated to the general population.

4.2 CATHELICIDIN INDUCTION BY PHENYLBUTYRATE AND VITAMIN D_3 TREATMENT CORRELATES WITH IMPROVED CLINICAL FEATURES OF TUBERCULOSIS PATIENTS (PAPER II-III)

Healthy volunteers were supplemented with different doses of phenylbutyrate (PBA) with/without $vitD_3$, to find out the optimal dose of PBA (**Paper II**) that could be used for the treatment of pulmonary TB patients (**Paper III**). The efficacy of PBA and/or $vitD_3$ as an adjunct therapy together with standard therapy (antibiotics) was then evaluated in adult pulmonary TB patients in a double-blind, randomized and placebo-controlled clinical trial (**Paper III**).

4.2.1 Effect of phenylbutyrate and/or vitamin D_3 treatment on the expression of LL-37 in immune cells with or without *Mycobacterium tuberculosis* infection (Paper II-III)

By utilizing quantitative RT-PCR, *CAMP* mRNA expression was measured in macrophages (**Paper II & III**) and lymphocytes (**Paper II**). ELISA was used to measure the level of LL-37 peptide in macrophages, non-adherent lymphocytes, and PBMC culture supernatant (**Paper II & III**).

Healthy volunteers were enrolled in an 8-days open trial (n=15) and divided into five different groups (**Paper II**). They received oral treatment of 250 mg PBA twice daily with 5000 IU $vitD_3$ (**Group-I**), 500 mg PBA twice daily with 5000 IU $vitD_3$ (**Group-II**), 1000 mg PBA twice daily with 5000 IU $vitD_3$ (**Group-III**), 500 mg PBA twice daily (**Group-IV**), and 5000 IU $vitD_3$ (**Group-V**), for 4 consecutive days. Besides this, they were followed for another four days after the treatment to monitor possible side effects. Oral intake of PBA and $vitD_3$ significantly increased *CAMP* mRNA ($p= 0.011$) and LL-37 peptide ($p=0.05$) expression in MDMs from individuals in Group-II at day-4 compared to day-0. When comparing between groups, Group-II exhibited significantly higher levels of *CAMP* mRNA on day-4 compared to Group-III ($p=0.03$) and Group-V ($p=0.035$). Furthermore, MDMs from Group-II showed higher levels of LL-37 peptide compared to Group-IV ($p=0.01$). Additionally, lymphocytes from Group-II expressed a significantly higher levels of LL-37 peptide at day-4 compared to Group-I ($p=0.03$) and Group-IV ($p = 0.05$).

Adult pulmonary TB patients (n=288) were enrolled in a randomized, double-blind and placebo-controlled four-arm intervention trial with adjunct therapy supplemented with PBA and/or $vitD_3$ for 2 months conducted in Bangladesh (**Paper III**). The dose of 500 mg PBA twice daily was selected from the pilot study (**Paper II**). After enrollment, patients were randomized to the following adjunct treatment arms and received oral doses of either: (1) placebo PBA and placebo $vitD_3$ (**Placebo-group**) or (2) 500 mg PBA twice daily and placebo $vitD_3$ (**PBA-group**) or (3) placebo PBA and 5000 IU of $vitD_3$ (Cholecalciferol) once daily (**$vitD_3$-group**) or (4) 500 mg PBA twice daily combined with 5000 IU $vitD_3$ once daily (**PBA+$vitD_3$-group**). LL-37 peptide concentrations in MDMs were significantly enhanced in the PBA group compared to placebo at week 12 ($p=0.034$). In addition, LL-37 peptide concentrations in lymphocytes from the PBA, $vitD_3$, and PBA+$vitD_3$-groups were

significantly higher at week 4, 8 and 12 compared to the placebo-group (p=0.009, p=0.053, and p=0.022, respectively). In PBMC, LL-37 peptide levels increased significantly in the three intervention groups compared to placebo at week 4 (p<0.003 for all groups) and at week 8 (p<0.030 for all groups), while enhanced level of LL-37 peptide at week 12 was found only in the vitD_3-group compared to placebo, p=0.030). *CAMP* mRNA expression in MDMs was increased in the PBA+vitD_3-group (p=0.036) at week 4, in the PBA-group at week 8 (p=0.057) and in the vitD_3-group at week 12 (p=0.003) compared to placebo.

From the healthy volunteer's trial, the dose of **Group-II** (500 mg PBA twice daily together with 5000 IU vitD_3 once daily) appeared better in inducing both LL-37 peptide and *CAMP* mRNA expression in MDMs, and the level of LL-37 peptide in lymphocytes was higher compared to the other four groups. In the TB clinical trial, PBA and/or vitD_3 treatment upregulated LL-37 peptide in lymphocytes, PBMC and macrophages at different time point of the study compared to placebo. A number of cell types including T-lymphocytes are known to express LL-37 (Agerberth et al., 2000; Kin et al., 2011). Our finding thus stresses the significance of PBA therapy in TB infection, since T cells play a major role in the host defense against tuberculosis (Ottenhoff and Kaufmann, 2012). Several studies have reported multiple possible mechanisms by which PBA induced LL-37 expression in different cell types. Mitogen-activated protein kinase (MAPK) signaling via JNK and ERK1/2 pathways have been shown to be involved in PBA mediated induction of the *CAMP* gene in a lung epithelial cell line (Steinmann et al., 2009). Recently, it was demonstrated that PBA and vitD_3 synergistically upregulated the *CAMP* gene expression that was mediated via p38 or ERK1/2 signaling in monocyte-derived macrophages (Coussens et al., 2015). Furthermore, VDR expression was also induced in MDMs by the treatment of PBA (Coussens et al., 2015). Our research group has also reported that VDR knockdown prevented *CAMP* gene induction by PBA treatment in lung epithelial cells (Kulkarni et al., 2015).

4.2.2 Effect of phenylbutyrate and vitamin D_3 treatment on intracellular killing of *Mycobacterium tuberculosis* in macrophages *ex vivo* (Paper II-III)

MDM-mediated killing of *Mtb* (H37Rv) was performed by conventional culture method at day-0, 4 and 8 (**Paper II**). MDMs in Group-II showed significantly higher intracellular killing of *Mtb* at day-4 (p=0.027) compared to day-0. MDMs in Group-I and -V also exhibited significant enhancement in intracellular killing of *Mtb* at day-4 (p<0.001 and p=0.019, respectively) and at day-8 (p<0.001 and p=0.051, respectively) compared to day-0.

MDM-mediated killing of *Mtb* (H37Rv) at week 0, 4, 8, and 12 were also performed (**Paper III**). MDMs in PBA-group demonstrated significantly earlier decline in viable *Mtb* CFU compared to the placebo-group (95% CI of time (days) to zero count, 65.3–74.7 vs. 76.5–82.3 respectively, log rank 11.38, p=0.01) (**Figure 6**).

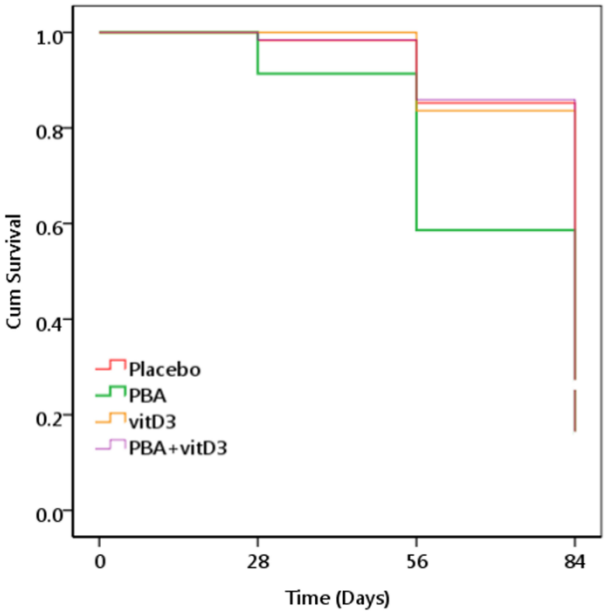

Figure 6. Kaplan-Meier survival graph for monocyte-derived macrophage (MDM)-mediated killing of *Mycobacterium tuberculosis (Mtb)*. Data are expressed as viability of *Mtb* in 'relative CFU counts'. A 'relative CFU count' was calculated by normalizing the data in each time point with the inoculated *Mtb* CFU. A cut-off of 0.1 was considered as zero.

Thus, both of our studies suggest that MDMs from PBA and/or vitD_3 treated patients enhance the killing of *Mtb*. Similar to our findings, a previous study reported that the killing capacity of macrophages was directly correlated to *CAMP* gene expression in macrophages and the levels of 25-hydroxyvitamin D_3 (Liu et al., 2007). It has been reported that vitamin D_3 dependent TLR 2/1 activation of macrophages up-regulates the expression of the VDR and vitamin D-hydroxylase genes, which subsequently lead to the production of LL-37 expression. This enhanced expression of LL-37 correlates with intracellular killing of *Mtb* (Liu et al., 2006; Liu et al., 2007). Two other studies have demonstrated that LL-37 is a key mediator of vitD_3-induced autophagy that is a possible mechanism of LL-37 dependent killing of *Mtb* (Campbell and Spector, 2012; Yuk et al., 2009). In this thesis, we have also revealed that PBA induces autophagy in human macrophages via LL-37 and control the intracellular growth of *Mtb* (**Paper IV**). A recent study has reported that PBA and vitD_3 supplementation increases *Mtb* killing in human macrophage (Coussens et al., 2015).

4.2.3 Clinical outcomes in pulmonary TB patients after treatment with PBA and/or vitamin D_3 (Paper III)

Sputum culture conversion is usually the method of choice as an early microbiological endpoint in phase 2 clinical trials of TB patients. We found that the odds of sputum culture being negative at week 4 was 3.42 times higher in patients of the PBA+vitD_3-group (95% Confidence interval (CI), 1.64-7.15, p=0.001), 2.20 times higher in patients of vitD_3-group

(95% CI, 1.07-4.51, p=0.032), and 1.22 times higher in patients of the PBA-group (95% CI, 0.59-2.53, p=0. 635) compared to patients of the placebo-group (**Figure 7**).

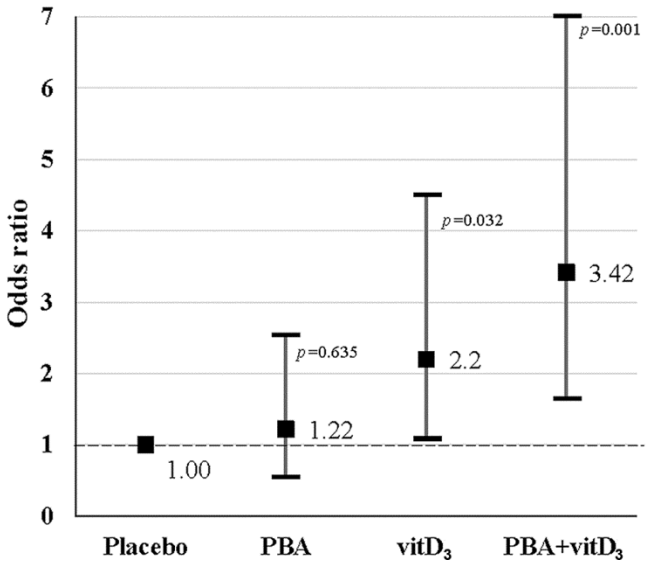

Figure 7: Sputum culture conversion at week 4. Results are shown as adjusted odds ratio (adjusted for age and sex) with 95% confidence intervals.

In addition, a TB scoring system was utilized to monitor the effect of the intervention. Patients of the PBA-group showed a marked decline in TB score at week 2, 4 and 8 compared to patients of the placebo-group (p=0.032, p=0.006 and p=0.026, respectively). However, at week 10 patients in all three groups exhibited significantly lower score than patients in the placebo-group (PBA, $vitD_3$ and PBA+$vitD_3$ vs. placebo (p=0.003, p=0.042, and p=0.036, respectively). At week 12, TB score remained significantly lower in patients of the PBA and PBA+$vitD_3$ (p=0.001 and p=0.009) group compared to the placebo. However, no significant change was observed in patients supplemented with only $vitD_3$. Chest x-ray evaluation was assessed and scored at week 0, 8, 12, and 24, following the National TB program guidelines in Bangladesh. Hence, for these four-time points TB score that included chest x-ray was determined separately. Patients in the PBA-group showed significantly lower TB score compared to patients in the placebo-group at week 8 (p=0.042) and at week 12 (p=0.003) when Chest x-ray results were included.

In this study, we have demonstrated novel functions of PBA to fight against *Mtb* infection. It has been reported that AMPs play an important role against *Mtb* infection (Liu et al., 2006; Liu et al., 2007). Indeed, several synthetic AMPs are under development as potential therapeutic drugs for treatment of wide variety of diseases (Fjell et al., 2012). Several studies have focused on the mechanisms behind how AMPs interfere with *Mtb* survival in macrophages. Is it a direct or indirect killing mechanism? *In vitro* studies from Yuk *et al*.

have demonstrated that $1,25(OH)_2D_3$ boosts innate immunity by increasing the expression of AMPs, including cathelicidin, and induces autophagy in *Mtb* infected cells restricting the intracellular growth of *Mtb* in macrophages (Yuk et al., 2009). Moreover, $1,25(OH)_2D_3$ is able to suppress the pro-inflammatory cytokine response, while the anti-inflammatory cytokine response is enhanced in macrophages (Xu et al., 2014). Here we speculate that induced LL-37 expression by PBA and/or $vitD_3$ may enhance the anti-inflammatory response, which restrict *Mtb* survival. This anti-inflammatory property of LL-37 may be beneficial with respect to minimizing the excessive tissue damage and inflammation that occur during the active stage of tuberculosis. This is probably reflected by reduced clinical scores. In line with this, our results have revealed that PBA and/or $vitD_3$ treatment significantly reduced the sputum culture conversion time together with better clinical recovery in pulmonary tuberculosis patients.

4.3 PHENYLBUTYRATE TREATMENT ACTIVATES HOST DEFENSE MECHANISMS AND CONTROL INTRACELLULAR GROWTH OF *MYCOBACTERIUM TUBERCULOSIS* IN HUMAN MACROPHAGES (PAPER IV)

We investigated the effects of PBA and LL-37 on *Mtb* growth in macrophages and the underlying mechanisms (**Paper IV)**.

4.3.1 Effects of phenylbutyrate treatment on the expression of LL-37 is associated with the control of *Mtb* growth in human macrophages

Mtb is a facultative intracellular pathogen that primarily infects and multiplies within macrophages. To survive inside the macrophages, *Mtb* apply different strategies to escape host defense mechanisms. Here we found that the virulent strain of *Mtb* (H37Rv) suppressed the expression of *CAMP* mRNA ($p<0.001$) and LL-37 peptide ($p=0.041$) in human MDMs. Notably, treatment of MDMs with PBA counteracted this suppressive effect of *Mtb*, and instead upregulated both *CAMP* mRNA ($p=0.023$) and LL-37 peptide ($p=0.031$) expression. Active $vitD_3$ [$1,25(OH)_2D_3$] also counteracted the effect of *Mtb*. Furthermore, the combination of PBA and $1,25(OH)_2D_3$ exhibited a synergistic effect on *CAMP* mRNA ($p<0.001$) (**Figure 8A**) and LL-37 peptide expression ($p=0.033$) (**Figure 1C, Paper IV**) in *Mtb*-infected MDMs.

A significant correlation between LL-37 expression, and reduced bacterial load of *Mtb* in MDMs (40 to 60% growth inhibition, $p=0.002$) was observed after PBA treatment. Similar effects were observed when infected MDMs were treated with $1,25(OH)_2D_3$, synthetic LL-37 and rapamycin (positive control). The most prominent effect on inhibition of *Mtb* growth was observed when MDMs were treated with PBA together with $1,25(OH)_2D_3$ (50 to 80% growth inhibition, $p<0.001$) (**Figure 8B**).

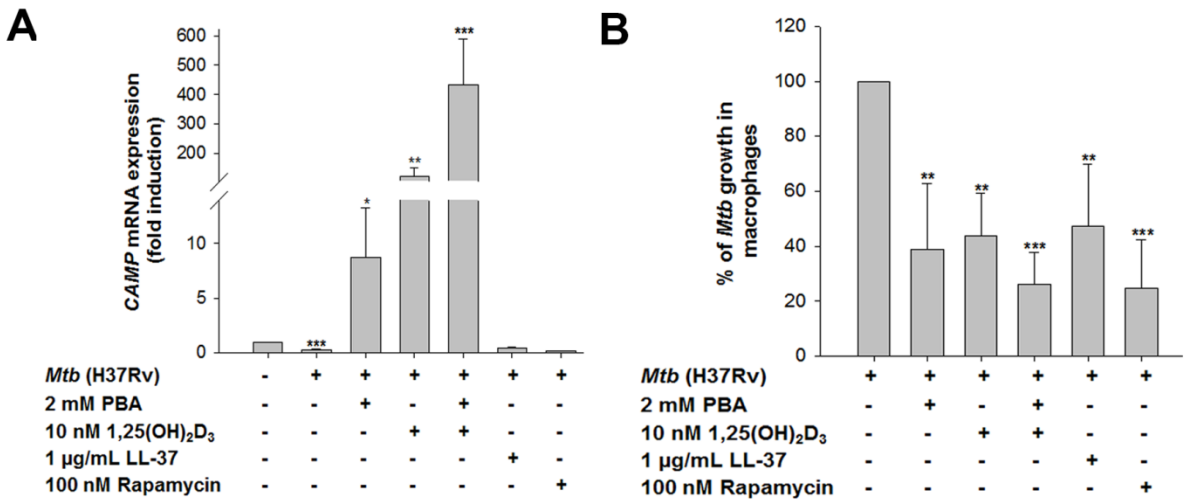

Figure 8. Phenylbutyrate (PBA)-induced LL-37 expression was correlated with the *Mtb* killing in human monocyte-derived macrophages (MDMs). (**A**) *CAMP* mRNA expression (normalized to 18S rRNA expression). (**B**) Intracellular bacterial viability based on the number of CFUs.

4.3.2 Effect of phenylbutyrate treatment on the activation of LL-37 dependent autophagy

Autophagy process is involved in the control of intracellular *Mtb* growth. Therefore, we continued to explore if PBA is capable to activate autophagy. Three essential proteins involved in autophagosome initiation, elongation and maturation processes are Beclin 1, ATG5 and LC3-II. We found that Beclin 1and ATG5 expression were significantly downregulated in *Mtb*-infected MDMs. However, PBA, alone or in combination with $1,25(OH)_2D_3$, was able to overcome this effect of *Mtb*, and Beclin1 and ATG5 expression were upregulated both on mRNA and protein levels. When we investigated the cellular localization of LC3-I, we detected a faint staining in the cytoplasm of uninfected and *Mtb*-infected MDMs. However, we detected and visualized puncta structures with the LC3 antibody. These structures are known as LC3-II (lipidated form of LC3) surrounding the autophagosomes after treatment with PBA and/or $1,25(OH)_2D_3$. Notably, LL-37 peptide was also upregulated in PBA-treated cells and further found to be co-localized with the LC3 puncta structures, indicating the presence of the peptide in the autophagosomes (**Figure 9A**). The combined action of PBA and $1,25(OH)_2D_3$ resulted in the highest percentage of cells with LC3-positive puncta structures (**Figure 9A**). Interestingly, synthetic LL-37 peptide turned out to be as efficient as PBA, $1,25(OH)_2D_3$, and rapamycin (positive control) to induce autophagosome formation in MDMs (**Figure 9A**). In Western blot analysis, a similar effect on LC3 was observed (**Figure 9B**).

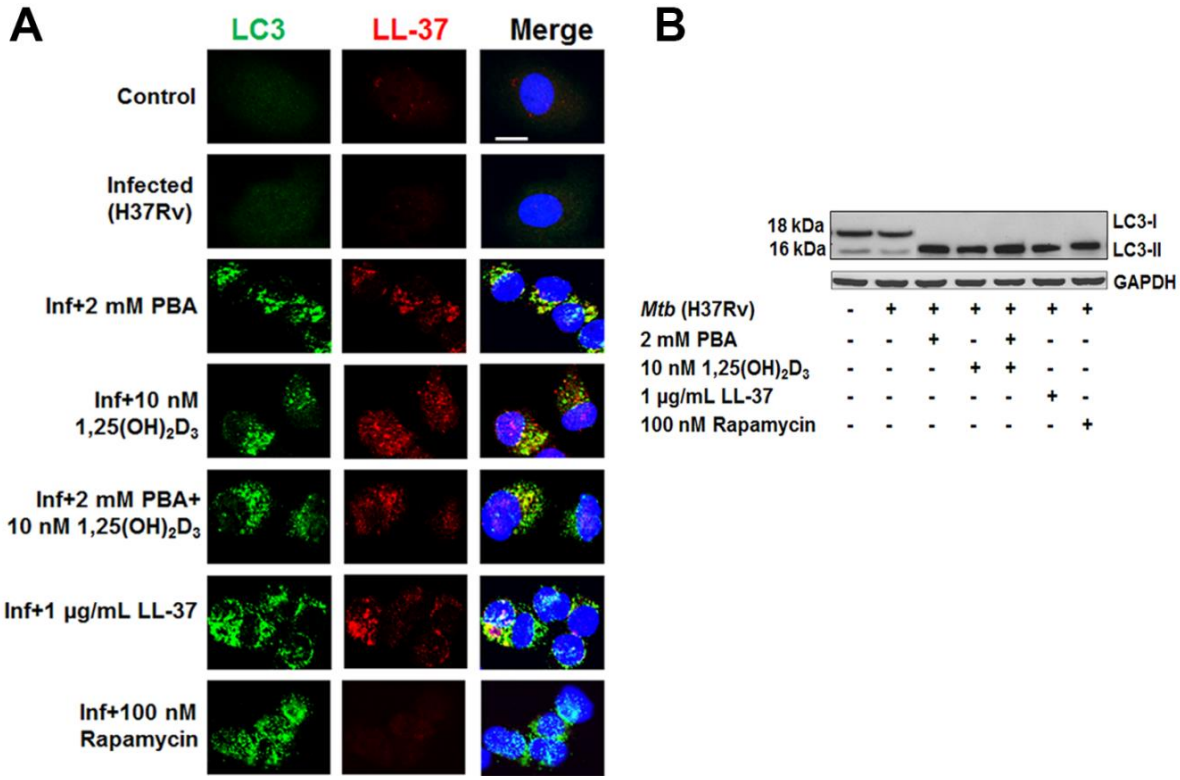

Figure 9. Phenylbutyrate (PBA)-induced autophagy in human macrophage. (**A**) Co-staining of LC3 (green), LL-37 (red) and nuclei (blue) in *Mtb* infected MDMs upon different treatments. Scale bar: 10 µm. (**B**) A representative Western blot shows the conversion of LC3-I to LC3-II and the house keeping protein GAPDH (glyceraldehyde 3-phosphate dehydrogenase).

To evaluate the role of LL-37 in autophagy, we silenced LL-37 expression by knocking down the *CAMP* gene in the THP-1 cell line. In *CAMP* short hairpin RNA (shRNA)-transfected THP-1 cells, PBA and $1,25(OH)_2D_3$ treatment failed to induce autophagy, while LC3-II was clearly detected in nonspecific (NS) shRNA-transfected cells (**Figure 10**). Autophagy was restored not only when *CAMP* shRNA-transfected THP-1 cells were stimulated with synthetic LL-37 peptide, but also upon treatment with rapamycin, which activated autophagy in an mTOR-dependent manner (**Figure 10**). Consistent with the microscopy analysis, similar findings were observed in Western blot analysis for LC3 and mRNA expression of Beclin1, and ATG5. All together, these results support our hypothesis that LL-37 is essential for PBA-mediated activation of autophagy.

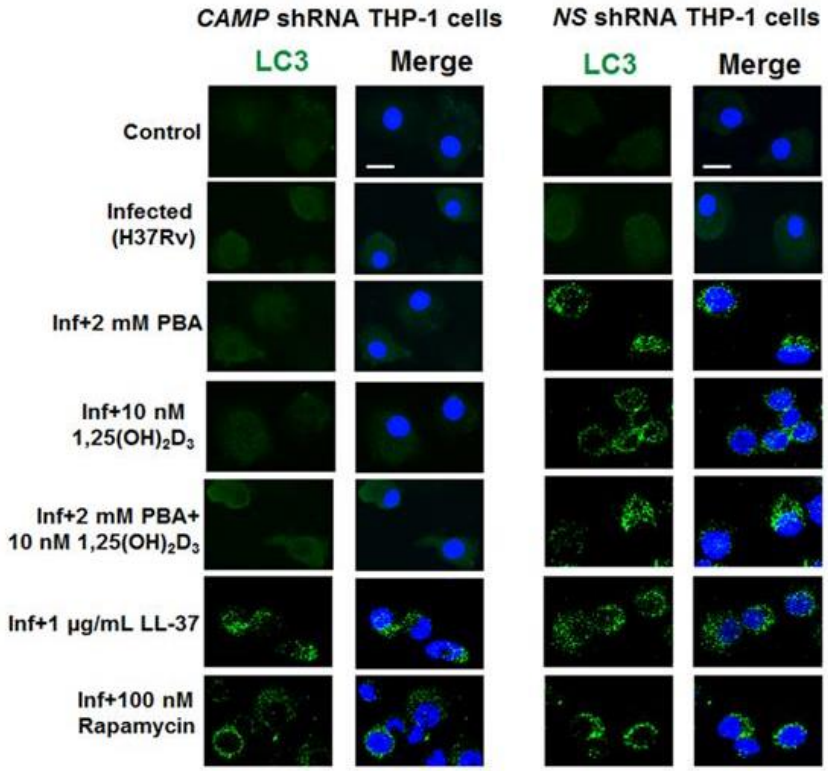

Figure 10. LL-37 is essential for phenylbutyrate-induced autophagy. Cells were stained for LC3 (green) and nuclei (blue) upon different stimulations. Scale bar: 10 µm.

4.3.3 LL-37 needs to be secreted and taken up by macrophages via P2RX7 receptor to activate autophagy

Here we revealed that PBA and $1,25(OH)_2D_3$ treated macrophages secreted a soluble factor in the supernatant that significantly activated autophagy in freshly prepared macrophages. To examine if LL-37 was the main mediator of autophagy, the supernatant fraction was pre-incubated with a neutralizing monoclonal antibody against LL-37 prior to stimulation of the macrophages. Interestingly, neutralization of LL-37 in the culture supernatants prevented the activation of autophagy in macrophages, while autophagy was maintained in cells treated with the supernatant that was pre-incubated with an isotype control antibodies. Accordingly, this result demonstrated that LL-37 activate autophagy in an autocrine or paracrine fashion.

Next, we aimed to explore which receptor that was responsible for LL-37-mediated activation of autophagy in human macrophages. We found that MDMs pretreated with KN62, a potent inhibitor of the P2RX7 (purinergic receptor P2X ligand-gated ion channel 7) receptor, leads to the suppression of LL-37-mediated autophagy in human MDMs. LC3-II was detected in LL-37 and rapamycin-treated MDMs, while pretreatment with KN62 inhibited the activation of autophagy in the presence of LL-37, resulting in a faint spread staining of LC3 in the cytoplasm (**Figure 11**). These results indicate the involvement of P2RX7 receptor in LL-37 mediated induction of autophagy in human MDMs.

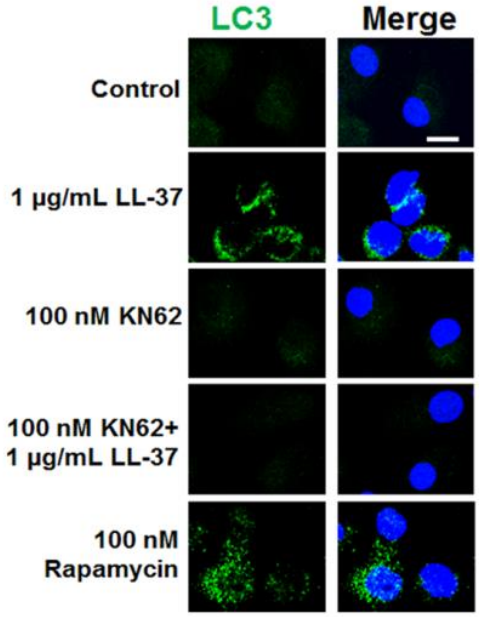

Figure 11. Activation of autophagy by LL-37 in human macrophages is mediated via the P2RX7 receptor. Cells were stained for LC3 (green) and nuclei (blue) upon different treatment of MDMs. Scale bar: 10 µm.

4.3.4 LL-37-mediated activation of autophagy in human macrophages depends on intracellular free Ca^{2+}, AMP-activated protein kinase and Phosphatidylinositol 3-kinase pathways

It has been reported that enhanced intracellular free Ca^{2+}, AMP-activated protein kinase (AMPK) and the phosphatidylinositol 3-kinase (PI3K) pathways play important roles in the activation of autophagy (Heras-Sandoval et al., 2014; Hoyer-Hansen and Jaattela, 2007). In order to investigate if these downstream mediators or signaling pathways were responsible for LL-37-mediated autophagy in human macrophages, MDMs were pre-treated with selected inhibitors of these different intracellular signaling pathways after treatment of LL-37. Pretreatment of MDMs with the respective inhibitors of intracellular Ca^{2+} (BAPTA-AM) or AMPK (compound C) or PI3K (3-methyladenine) inhibited the conversion of LC3-I to LC3-II and consequently the LC3 puncta formation. Our results suggest that all three signaling pathways were involved in LL-37-mediated activation of autophagy in human macrophages that are the downstream signal of P2RX7 receptor.

Autophagy, the cell survival mechanism has been identified as an important cellular defense mechanism of macrophages against *Mtb* infection. Our results from this study suggests that PBA and $vitD_3$ induce the innate immune effector molecule LL-37, which is responsible for the activation of autophagy in *Mtb* infected macrophages, with subsequent killing of *Mtb*. It has been reported that LL-37 is the main mediator of $vitD_3$ induced autophagy in *Mtb*

infected human macrophages (Yuk et al., 2009). We have now shown that PBA, a histone deacetylase inhibitor, activates autophagy in *Mtb* infected macrophages (**Figure 12**). Other HDAC inhibitors, such as butyrate and suberoylanilide hydroxamic acid activate autophagy in several human cancer cell lines (Shao et al., 2004). Here, we have demonstrated that PBA and LL-37 have the capacity to activate different sequential steps of autophagy. In *Mtb* infected macrophages PBA and LL-37 treatment upregulates the expression of Beclin1, ATG5 as well as convert LC3-I to LC3-II (**Figure 12**). Additionally, PBA and LL-37 treatment facilitates the degradation of SQSTM1/p62 protein, a marker of autophagy flux (Bjorkoy et al., 2009).

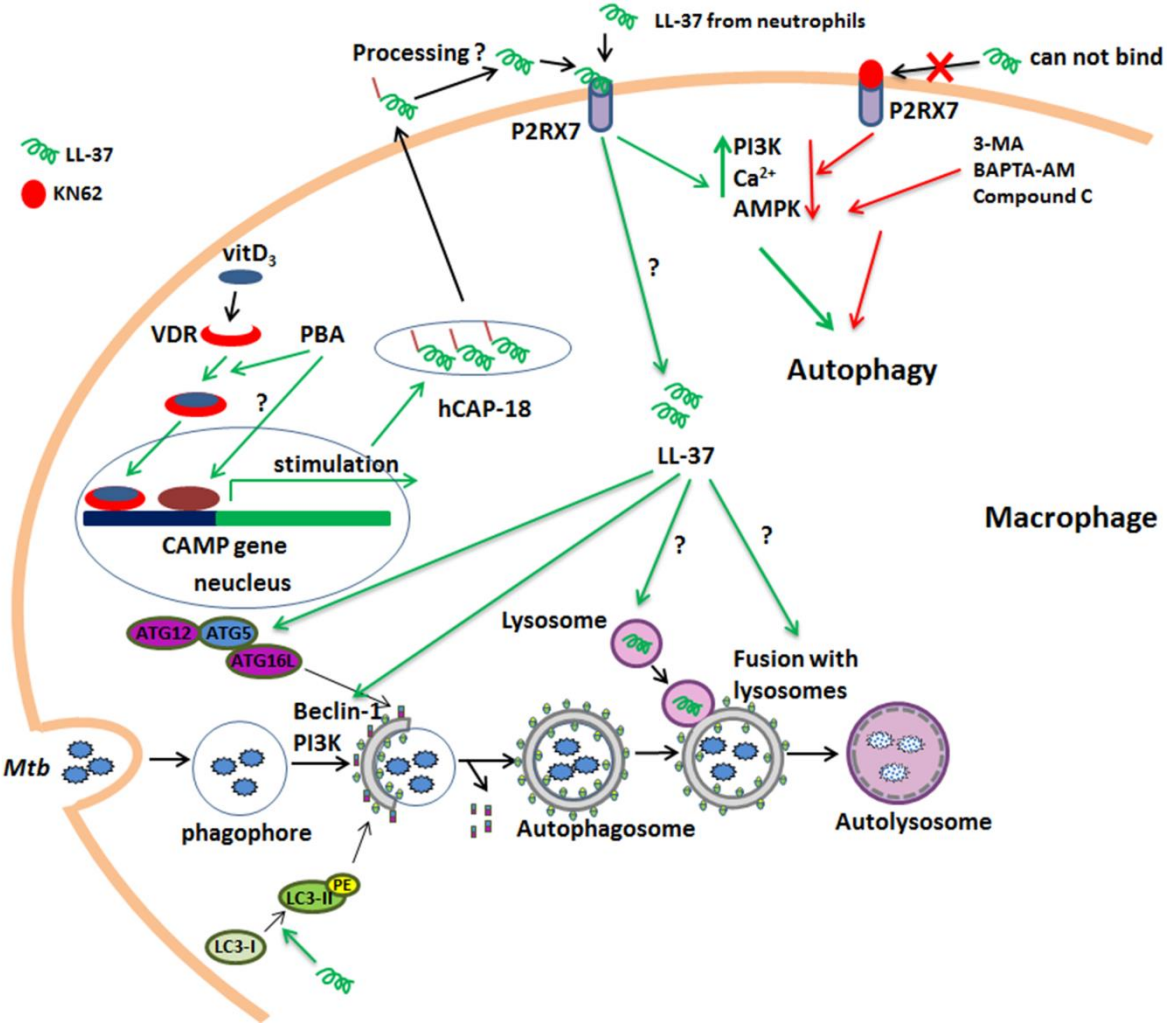

Figure 12: A schematic presentation of activation of autophagy by PBA in human macrophages. Green arrows indicate stimulation of the process and red arrows indicate downregulation of the process.

Furthermore, we have found that LL-37 needs to be secreted and taken up by the macrophages to activate autophagy. These findings may explain the *in vivo* situation, where active LL-37 peptide may originate from other immune cells, such as neutrophils (Sorensen et al., 2001) and exhibit a double function in the elimination of *Mtb* by activating autophagy and enhancing the phagocytic capacity of macrophages (Wan et al., 2014). In our study we also established that the cell surface receptor P2RX7 was involved in LL-37-mediated activation of autophagy in human MDMs. Interestingly ATP is a ligand for the P2RX7 receptor and is able to activate autophagy that associated with rapid killing of intracellular *Mtb* in human macrophages (Biswas et al., 2008). In addition, we have demonstrated that intracellular free Ca^{2+}, AMPK and PI3K pathways are essential for LL-37-mediated autophagy in human MDMs. These results suggest that all three signaling pathways are involved in LL-37-mediated induction of autophagy in human macrophages that are downstream signaling molecules of P2RX7 receptor (**Figure 12**). It has been reported that intracellular Ca^{2+} signaling is a crucial component in mTOR-dependent activation of autophagy. Buffering cytosolic Ca^{2+} with the intracellular Ca^{2+} chelator BAPTA has impeded rapamycin-induced autophagy (Decuypere et al., 2013). Autophagy activation is promoted by AMPK, which is a key energy sensor molecule and regulates cellular metabolism in order to maintain energy homeostasis. During starvation, AMPK promotes autophagy by directly activating Ulk1 (mammalian autophagy-initiating kinase) through phosphorylation of Ser 317 and Ser 777. Conversely, during nutrient sufficiency, mTOR activity prevents Ulk1 activation by phosphorylating Ulk1 Ser 757 and disrupting the interaction between Ulk1 and AMPK. This harmonized phosphorylation is important for Ulk1 in autophagy activation (Kim et al., 2011). Activation of PI3K is essential for the initiation of autophagy via recruitment of several ATG proteins at the phagophore (Pattingre et al., 2008; Zeng et al., 2006). In our study we have utilized the PI3K inhibitor 3-methyladenine that target both class I and class III PI3K (Knight and Shokat, 2007; Kong and Yamori, 2008). This treatment suppresses autophagy by inhibiting the class III PI3K with subsequent blockage of the production of phosphatidylinositol 3-phosphate (Petiot et al., 2000). All together we have demonstrated a novel role of PBA as an inducer of autophagy, which is LL-37-dependent and enhance intracellular killing of *Mtb* in human macrophages. Further studies are required to explore more detailed mechanisms of LL-37 mediated activation of autophagy.

5 CONCLUSIONS

- Vitamin D_3 supplementation improves clinical outcomes (reduces infectious score) and antibiotic consumption (approximately 60%) in patients with frequent respiratory tract infections. Therefore, vitD_3 supplementation could provide a novel strategy to reduce antibiotic intake of these patients, and thus indirectly prevent the emerging problem of bacterial resistance (**Paper I**).

- The oral dose of 500 mg PBA twice daily together with 5000 IU vitD_3 once daily is the optimal dose to induce LL-37 expression in specific immune cells and to enhance intracellular *Mtb* killing in macrophages (**Paper II**). This study has demontrated the potential dose of PBA and vitD_3 that was subsequently used in a clinical trial of active pulmonary TB.

- The oral intake of a combination of PBA and vitD_3 significantly reduces sputum culture conversion time and improves clinical outcome in pulmonary tuberculosis patients. Our results support a novel host-directed therapy that might be used to fight against tuberculosis, by enhancing host antimicrobial activity, and modulating immune responses (**Paper III).**

- Phenylbutyrate and/or 1,25$(OH)_2D_3$ treatment activates LL-37-dependent host defense mechanism autophagy, and subsequently promotes intracellular killing of *Mtb* in human macrophages (**Paper IV**).

6 FUTURE RESEARCH

- The beneficial effect of PBA and vitD_3 supplementation could be tested in multidrug-resistant pulmonary TB, and another lung diseases.

- LL-37 is an essential component of the autophagy process. However, additional studies are necessary to precisely delineate how LL-37 upregulates the expression of autophagy related genes and proteins.

- Additional studies are needed to find out how LL-37 access into the lysosomes and to evaluate the underlying mechanisms.

- Epigenetic changes of immune related genes in relation to PBA and/or VitD_3 treatment would be investigated.

- Further studies are required to find out which transcription factors are recruited to the *CAMP* gene promoter that induce autophagy related genes in human macrophages upon stimulation with PBA or LL-37.

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