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STUDIES OF EFFECTOR FUNCTIONS IN *MYCOBACTERIUM TUBERCULOSIS*-INFECTED MACROPHAGES WITH IMPLICATIONS FOR HOST-DIRECTED THERAPIES

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Cover Photo: LC3 puncta aggregates in *Mycobacterium tuberculosis* infected macrophages treated with PBA+VitD_3 and isoniazid.

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Studies of effector functions in *Mycobacterium tuberculosis*-infected macrophages with implications for host-directed therapies

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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This thesis is dedicated to the field of Medicine

ABSTRACT

Tuberculosis infection remains a global health problem and the emergence of multidrug-resistant TB (MDR-TB) adds further challenges in the battle to stop the spread of infection. Macrophages are the primary host cells to be infected with *Mycobacterium tuberculosis* (Mtb), and are therefore key cells in the initial control of TB infection. However, to survive and persist in activated macrophages, Mtb has developed strategies to counteract antimicrobial immunity. This thesis work aimed to explore macrophage responses in Mtb-infected cells, the nature of innate effector mechanisms and how these can be enhanced. For this purpose, we used an *in vitro* macrophage infection model as well as an organotypic lung tissue model system.

The thesis work is based on four papers, Study I and III, involved studies on the effects of the immunomodulatory compounds, vitamin D_3 ($VitD_3$) and phenylbutyrate (PBA), on the induction of antimicrobial peptides, primarily human cathelicidin LL-37, and the ability to kill intracellular Mtb. In Study I, we observed that Mtb can down-regulate the expression of LL-37 in infected macrophages, but this effect was effectively counteracted by treatment with $VitD_3$ and/or PBA. PBA and/or $VitD_3$ prevented intracellular Mtb growth via induction of LL-37 as well as activation of autophagy in Mtb-infected macrophages. In Study III, we further demonstrated that PBA+$VitD_3$ enhanced intracellular Mtb killing of both MDR-TB and drug-susceptible strains and this effect was dependent on LL-37.

In Study IV, we explored the role of $VitD_3$ in polarization of macrophages in comparison to activation with conventional stimuli such as GM-CSF (polarization of inflammatory M1 macrophages) or MCSF (polarization of anti-inflammatory M2 macrophages). The findings from Study IV, suggested that polarization with $VitD_3$ enhanced the ability of Mtb-infected macrophages to control intracellular Mtb growth in comparison to M1 and M2 subsets. Enhanced Mtb growth control was associated with elevated levels of pro-inflammatory cytokines and LL-37, but reduced Mtb-induced expression of the immunosuppressive enzyme IDO (indoleamine 2,3-dioxygenase).

Study II, aimed to investigate the expression and function of matrix metalloproteinases (MMPs) in early TB granuloma formation using the organotypic lung tissue model that enabled studies on macrophage-Mtb interactions in a more physiological environment in tissue. In this study, we found that pre-treatment of macrophages with the global MMP inhibitor, marimastat, resulted in effective inhibition of TB granuloma formation, which was associated with reduced Mtb growth detected in the lung tissue model. Thus, reduced degradation of extracellular matrix proteins, could prevent Mtb multiplication and spread of the infection.

The main conclusion from this thesis work is that immunomodulatory compounds with the ability to boost or block innate effector mechanisms in Mtb-infected macrophages may be used as adjunct host-directed therapies that could support standard anti-TB drugs to enhance clinical recovery from TB.

LIST OF SCIENTIFIC PAPERS

I. Rokeya Sultana Rekha, **SSV Jagadeeswara Rao Muvva**, Min Wan, Rubhana Raqib, Peter Bergman, Susanna Brighenti, Gudmuundur H Gudmundsson and Birgitta Agerberth. Phenylbutyrate induces LL-37-dependent autophagy and intracellular killing of *Mycobacterium tuberculosis* in human macrophages. ***Autophagy.*** 2015 Sep; 11(9): 1688–1699.

II. Venkata R.Parasa, **Jagadeeswara Rao Muvva**, Jeronimo F. Rose, Clara Braian, Susanna Brighenti and Maria Lerm. Inhibition of Tissue Matrix Metalloproteinases Interferes with *Mycobacterium tuberculosis*-Induced Granuloma Formation and Reduces Bacterial Load in a Human Lung Tissue Model. ***Frontiers in Microbiology***. 2017; 8: 2370.

III. **Jagadeeswara Rao Muvva**, Sultan Ahmed, Rokeya Sultana Rekha, Thomas schon, Ramona Groenheit, Peter Bergman, Susanna Brighenti and Birgitta Agerberth. Vitamin D- and Phenylbutyrate-mediated growth inhibition of multidrug-resistant *Mycobacterium tuberculosis* in human macrophages is dependent on the antimicrobial peptide LL-37. ***Manuscript.***

IV. **Jagadeeswara Rao Muvva**, Venkata Ramanarao Parasa, Maria Lerm, Mattias Svensson and Susanna Brighenti.Polarization of human macrophages with vitamin D promotes control of *Mycobacterium tuberculosis* infection. ***Manuscript***.

ADDITIONAL PUBLICATIONS

Min Wan, Xiao Tang, Rokeya Sultana Rekha, **S. S. V. Jagadeeswara Rao Muvv**a, Susanna Brighenti, Birgitta Agerberth and Jesper Z. Haeggstrom. Prostaglandin E_2 suppresses hCAP18/LL-37 expression in human macrophages *via* EP2/EP4: implications for treatment of *Mycobacterium tuberculosis* infection. *FASEB Journal*. 2018 May; 32(5):2827-2840.

Tadepally Lakshmikanth Frank Heuts, **S. S. V. Jagadeeswara Rao Muvva**, Robert P. A. Wallin, Anna-Karin Persson, Cyril Fauriat, Steven E. Applequist, Hans-Gustaf Ljunggren, Petter Hoglund, Klas Karre, Mattias Svensson and Julius G. Juarez. In vivo engineering of mobilized stem cell grafts with the immunomodulatory drug FTY720 for allogeneic transplantation. *European Journal of Immunology*. 2016 July; 46(7):1758-69.

CONTENTS

LIST OF ABBREVIATIONS

3MA	3-Methyladenine
AMPK	5' Adenosine Monophosphate-activated Protein Kinase
Arg1	Arginase1
ATG5	Autophagy Related Gene 5
ART	Anti Retroviral Therapy
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetra acetic acid
BECN1	Beclin1
CAMP	Cathelicidin Antimicrobial Peptide Gene
CCR7	C-C Chemokine Receptor Type 7
DEFB1	Human β Defencin 1 Gene
DUOX2	Dual Oxidase 2
GFP	Green Fluorescent Protein
GMCSF	Granlocyte Monocyte Colony Stimulating Factor
HDAC inhibitor	Histone Deacetylase Inhibitor
IDO	Indoleamine-Pyrrole 2,3-Dioxygenase
IFNγ	Interferon Gamma
IL-1β	Interleukin -1 Beta
IL-10	Interleukin-10
IL-4	Interleukin-4
IL-12	Interleukin-12
IL1Ra	Interleukin-1 Receptor Antagonist
IL-6	Interleukin-6
IL-8	Interleukin-8
INH	Isoniazid
iNOS	Inducible Nitric Oxide Synthase
IP10	Inducible Protein 10
KN62	4-[(2S)-2-[(5-isoquinolinyl sulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl]phenyl isoquinoline sulfonic acid ester

LAM	Lipoarabinomannan
L-NMMA	N^G-Methyl-L-arginine Acetate Salt
LC3I	5' Adenosine Monophosphate-activated Protein Kinase
LC3II	5' Adenosine Monophosphate-activated Protein Kinase
LL-37	Cathelicidin Antimicrobial Peptide
LPS	Lipopolysaccharide
MCP-1/CCL2	Monocyte Chemoattractant Protein 1/Chemokine (C-C motif) Ligand 2
MCSF	Monocyte Colony Stimulating Factor
MET	Macrophage Extracellular Trap
MIC	Minimum Inhibitory Concentration
MMP	Matrix Metallo Proteinases
Mtb	Mycobacterium tuberculosis
NAC	N- Acetyl Cysteine
NET	Neutrophil Extracellular Trap
NLRP	Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain
NO	Nitric Oxide
NOD	Nucleotide-binding oligomerization domain
NOS2A	Nitric Oxide Synthase2 Gene
oxATP	oxidized Adenosine 5′-Triphosphate
P2RX7	P2X Purinoceptor 7
PBA	Sodium Phenyl Butyric Acid
PI3K	Phosphatidylinositol 3-kinase
RANTES/CCL5	Regulated on Activation, Normal T cell expressed and secreted/ chemokine (C-C motif) ligand 5
RIF	Rifampicin
RNI	Reactive Nitrogen Intermediates
ROS	Reactive Oxygen Species
shRNA	Short hair pin RNA

siRNA	Small interference RNA
TB	Tuberculosis
TB-IRIS	Tuberculosis- Immune Reconstitution Inflammatory Syndrome
THP-1	Human Monocytic Cell Line
TIMP	Tissue inhibitor of Metalloproteinases
TLR2	Toll like receptor 2
VDR	VitaminD receptor
$VitD_3$	Active vitamin D($1,25(OH)_2D_3$)

1 BACKGROUND ON TB INFECTION

Tuberculosis is a human disease caused by *Mycobacterium tuberculosis* (Mtb) and accounts for more than 1.5 million deaths every year and still remains one of the major killers among infectious diseases (Lozano, Naghavi et al. 2012). The incidence of TB is increasing due to diverse factors including rapid globalization (Pareek, Greenaway et al. 2016), HIV co-infection (Moir, Chun et al. 2011) and other comorbidities such as diabetes (Jeon and Murray 2008) and rheumatoid arthritis (Kisacik, Pamuk et al. 2016) but also because of the emergence of multi-drug resistant TB (MDR-TB). This also includes extensively drug resistant TB strains (XDR-TB) in different parts of the world, which is a matter of concern as no definitive cure is currently available. So, it is a dire necessity to understand the pathogenesis of the disease in the human host and based on this knowledge, explore new alternative treatment strategies, such as host-directed therapies that could support conventional standard antibiotic treatment. Standard chemotherapy used to treat drug-susceptible TB, normally requires 6-8 months of treatment with multiple drugs. Instead, treatment of MDR-TB is considerably more complicated, costly and longer, usually from 9 month up to 2 years, also including second line-drugs that are less efficient in Mtb killing and also associated with unwanted side-effects (Lange, Chesov et al. 2018).

1.1 PATHOGENESIS OF TB INFECTION

TB is a chronic infectious disease primarily affecting the lungs. Mtb mainly enters the host via the airways, where bronchial epithelial cells and alveolar macrophages are the primary cells that encounter the bacteria. Mtb is an intracellular bacterium that multiplies in macrophages and eventually exit the host in infectious aerosols that contribute to the spread of TB infection. After gaining entry into the host, Mtb needs to pass through a mechanical barrier in the form of airway epithelium and also functional barriers of innate and adaptive immunity, to establish a successful infection (Cambier, Falkow et al. 2014). Hence, the host immune response to Mtb infection is multifaceted, involving a spectrum of innate and adaptive immune responses. Innate immune response to Mtb are executed by neutrophils, macrophages, dendritic cells (DCs) and NK cells, whereas T and B cells are key cells of the adaptive immune response(Ulrichs and Kaufmann 2006). Mtb can efficiently avoid, circumvent or manipulate host defense mechanisms to survive in the host. Accordingly, Mtb has evolved efficient strategies to escape from innate as well as adaptive immune responses (Cambier, Falkow et al. 2014).

A productive Mtb infection is established in the macrophages harbors live bacteria for extended periods of time. The first-line of host defense includes production of toxic nitric oxide (NO) via inducible nitric oxide synthase (iNOS) (Rich, Torres et al. 1997) and antimicrobial peptides, primarily human cathelicidin, LL-37 (Rivas-Santiago, Hernandez-Pando et al. 2008). Macrophages also eliminate intracellular bacteria by different cellular mechanisms such as apoptosis (Keane, Balcewicz-Sablinska et al. 1997), phagocytosis or by activation of autophagy (Gutierrez, Master et al. 2004) (Figure 1). Here, autophagy, which is a homeostatic cellular process to degrade intracellular debris or altered cells, has recently been described as one of the most important mechanisms to control intracellular growth of Mtb. However, complete eradication of Mtb is rare, as virulent mycobacteria have developed sophisticated strategies to persist in macrophages even in the presence of inflammation. Shortly after Mtb has successfully invaded its host, Mtb-infected macrophages and other immune cells initiate a granulomatous inflammatory reaction, which is characterized by the formation of granulomas. The TB granuloma is composed of aggregates of immune cells located at the site of Mtb infection in the lung that is the hallmark of human TB.

1.2 THE TB GRANULOMA

Granulomas are dynamic, ball-like collections of Mtb-infected macrophages that cluster together with other immune cells such as T and B cells, neutrophils and DCs, probably in an attempt to contain the Mtb infection (Figure 1) (Andersson, Samarina et al. 2007). However, early in the TB infection granulomas may also have a function to seed the infection to newly derived monocytes that migrate to the site of infection (Davis and Ramakrishnan 2009). Mtb-infected macrophages in the lungs attract monocytes from the blood, which migrate into the tissue and differentiate into macrophages that participate in the immune responses against Mtb. These monocytes/macrophages may also become infected with Mtb and propagate the infection. DCs are normally not infected with Mtb, but instead take up bacteria and debris from dead Mtb-infected cells to process these for antigen-presentation and triggering of Mtb-specific T cell responses. Effective TB control is dependent on Th1 CD4+ T cell and cytolytic CD8+ T cell responses. This includes production of IFNγ, TNF-α (Newport, Huxley et al. 1996, Keane, Gershon et al. 2001) as well as cytotoxic and antimicrobial effector molecules such as perforin and granulysin (Stenger, Hanson et al. 1998, Bruns, Meinken et al. 2009). Effector T cells contribute to the protective response in the granulomas and localize in the peripheral rim surrounding Mtb-infected macrophages that constitutes the core of the granuloma (Brighenti and Andersson 2012).

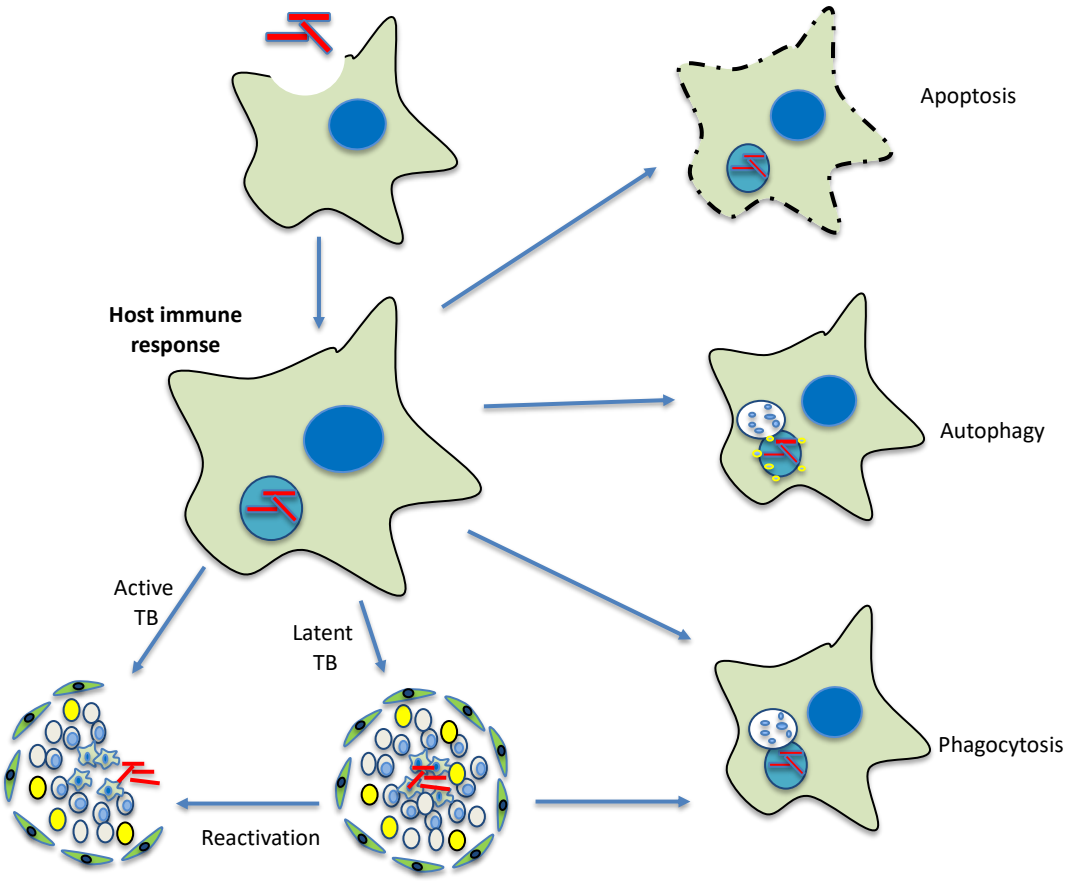

Figure 1. Mtb-infected macrophages can eradicate or control the infection by induction of autophagy or phagocytosis, or by undergoing apoptosis. T cells executing granule-mediated cytotoxicity (i.e. perforin and granulysin) are efficient to kill mycobacteria that can otherwise spread the infection to adjacent cells. Mtb-infected macrophages can also attract new monocytes and other immune cells to form dynamic cellular structures called granulomas that contain the infection at the disease site(s) (Brighenti and Andersson 2012). Most individuals can control the infection in a latent state, but if the immunological balance is tipped in favor of the bacteria, some progress to active, contagious TB disease (Saunders and Britton 2007). Picture modified from (Andersson, Samarina et al. 2007).

The macrophages involved in the granuloma formation are rich in cytoplasm and are termed as epithelioid cells. Upon chronic activation, macrophages in turn fuse together to form multi-nucleated giant cells, another typical hallmark of human tuberculosis (Brighenti and Andersson 2012). These giant cells destroy the surrounding tissue using lytic enzymes. However the functional role of these giant cells and the dynamic changes in the granuloma is yet to be determined. At the site of Mtb infection, macrophages further differentiate into pro-inflammatory M1 or anti-inflammatory M2 depending on the environmental cytokine milieu (Italiani and Boraschi 2014). One hypothesis is that the ratio between M1 and M2 macrophage responses are the main players in determining the fate of infection (Flynn, Chan et al. 2011).

The persistent bacterial infection in the host is maintained as a latent infection or manifests as active disease based on several host as well as bacterial virulence factors. Infection can become

latent or progress into active disease, although the bacterial and/or immunological factors that determine this fate are largely unknown (Saunders and Britton 2007).

The dissemination of Mtb infection depends on the integrity and size of granulomas. Larger, necrotic and poorly organized granulomas, spread the infection extensively. Granulomas are hypoxic and it has been shown that in hypoxic environment neutrophils secrete MMP8 and MMP9 upon Mtb infection (Ong, Fox et al. 2018), which in turn disturbs the architecture of the granuloma resulting in dissemination of infection. Additionally, Mtb infection also induces the secretion of MMPs by different cell types at the site of infection (Parasa, Muvva et al. 2017). Depending on the host response and severity of infection granulomas in human TB are classified based on morphology into fibrotic, necrotic and caseous granulomas (Kim, Wainwright et al. 2010). Necrotic granulomas easily rupture into the airways, releasing live mycobacteria that can spread the infection to another host.

1.3 MACROPHAGE POLARIZATION IN TB INFECTION

As described above, macrophages play a central role in the regulation of TB infection, as the primary host immune cell that become infected with Mtb. Macrophages are versatile cells that can be polarized to express a spectrum of functional programs in response to different stimuli. Montovani et.al. has classified the macrophages into classically activated M1 macrophages (pro-inflammatory) or alternatively activated M2 macrophages (anti-inflammatory and tissue repair) based on their functional responses to different exogenous and environmental stimuli (Mantovani, Sica et al. 2004) (Figure 2a). Adding more complexity to this paradigm, the M2 macrophages can display a number of functional changes and thus be further divided into M2a (inflammation and allergy), M2b (immunoregulation) and M2c (tissue repair). RNA sequencing of M1/M2 markers have been described in both humans and non-human primates (Beyer, Mallmann et al. 2012, Gonzalez, Novak et al. 2015).

However, the differences in the phenotypic and functional responses in these macrophages are not black and white, but should be considered as a continuum where there is overlap with certain phenotypes and functions (Figure 2b). M1 and M2 macrophages may be considered as the extremes on a broad scale of macrophage activation. While the nomenclature of polarized macrophages are quite variable in the literature, the importance is to stay focused on the function of the macrophage, regardless of the terminology (Murray, Allen et al. 2014).

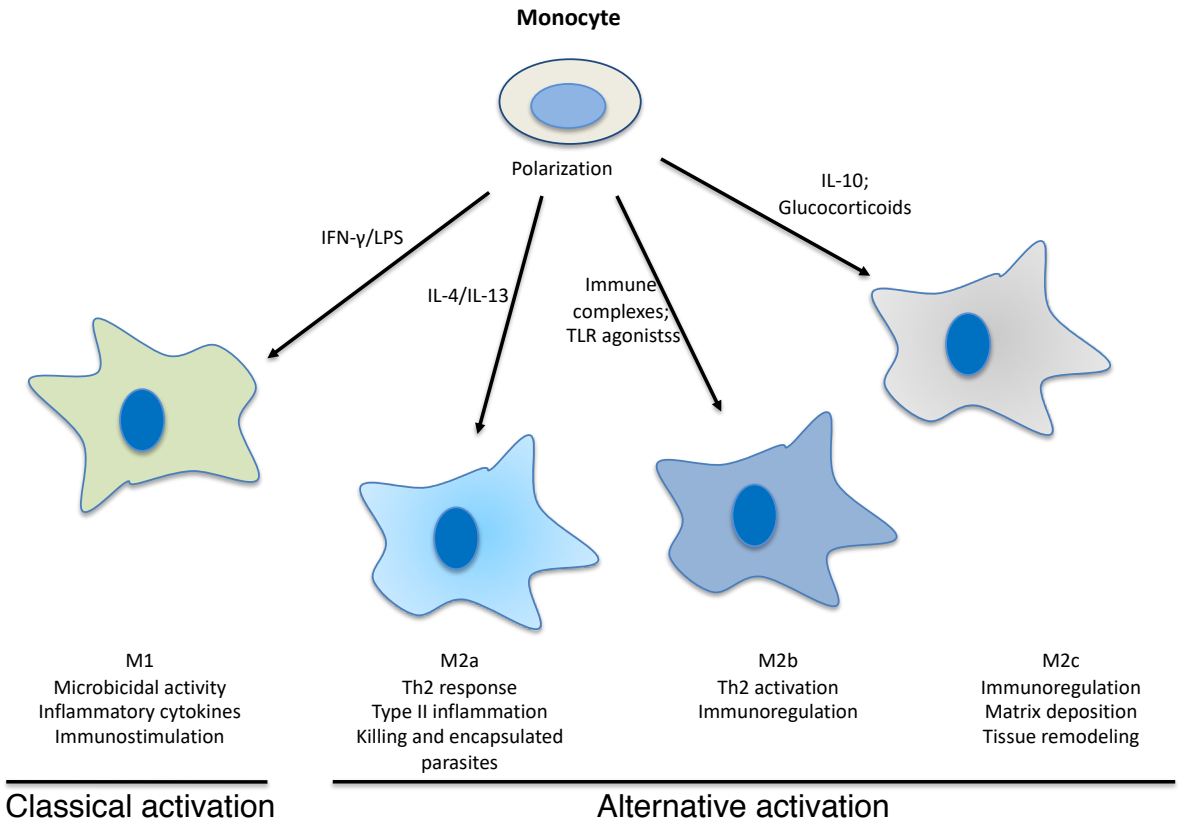

Figure 2. Monocytes can be polarized to pro-inflammatory M1 or anti-inflammatory M2a, M2b and M2c with different functional responses depending on the exogenous stimuli. Picture modified from (Nagela Ghabdan Zanluqui, and et al. 2015).

M1 macrophages are believed to be important in the clearance of intracellular mycobacteria, either via direct antimicrobial functions or by the induction of Th1 responses that is required to control TB. Macrophages can exert M1-like functional responses when stimulated with IFNγ, TNFα and TLR ligands to produce pro-inflammatory cytokines and effector molecules such as TNFα, IL-1β, IL-6, CCL3, CCL4 and iNOS (Bashir, Sharma et al. 2016). Proof of the classical role of IFNγ and TNFα in TB disease has been studied in knock-out mice, that died rapidly after mycobacterial infection, primarily due to the lack of NO-production in infected macrophages (Bloom and Modlin 2016). But excess pro-inflammatory responses can result in serious immunopathology, which is counteracted by M2-like alternatively activated macrophages known to differentiate in a microenvironment rich in IL-4 and IL-13 (Gordon 2003). These M2 macrophages may induce a Th2 or regulatory T cell response by secreting anti-inflammatory cytokines like IL-10 and TGFβ, which is believed to be less protective in human TB (Bashir, Sharma et al. 2016).

M2 macrophages may typically express several surface receptors that promote phagocytosis and antigen presentation and aid in resolving inflammation by transmitting inhibitory signals

including mannose receptor CD206, scavenger receptor CD163, inhibitory CD200R, IL-1RA, chemokines CCL18 and CCL22 (Mosser and Edwards 2008, Koning, van Eijk et al. 2010). M2 macrophages also express the intracellular enzyme arginase that is known to down-regulate NO synthesis and NO-mediated cytotoxicity, by competing with iNOS for the cellular substrate L-arginine. Arginase-mediated L-arginine depletion profoundly suppresses T cell immune responses and support enhanced collagen synthesis and fibrosis as well as tissue remodeling, which are not associated with protective immunity in TB (Munder 2009). Accordingly, mice with arginase-deficient macrophages, demonstrated decreased bacterial load upon Mtb infection, which correlated with increased NO production (El Kasmi, Qualls et al. 2008). So, while there is a tight regulation of the ratio of M1 and M2 macrophages in healthy tissue, this ratio may be disturbed at the site of Mtb infection, leading to either confinement or progression of TB disease (Mattila, Ojo et al. 2013, Marino, Cilfone et al. 2015). Recently it has been reported that Mtb induce macrophage polarization through micro RNAs (Ahluwalia, Pandey et al. 2017).

During initial inflammation there is an increase in the M1/M2 ratio with an elevated Th1 response and once TB infection is controlled there is a Th2 response with more M2 macrophages secreting IL-10 to down-regulate the inflammation and maintain tissue integrity. However, skewing the immune response towards excess Th2, including activation of antibody-producing B cells, may be a strategy used by virulent mycobacteria to subvert protective immunity (Ashenafi, Aderaye et al. 2013, Ashenafi, Aderaye et al. 2014). Several immunomodulatory compounds are being studied that could modify the host response in TB disease by polarizing macrophages towards specific M2-type cells, which can kill the bacteria by enhancing the LL-37 secretion, inducing autophagy and simultaneously handle tissue remodeling by secretion of IL-10 (Patel, Rajasingh et al. 2016).

1.4 ROLE OF MATRIX METALLOPROTEINASES IN THE TB GRANULOMA

Macrophages are the corner stone in TB granuloma formation, although the mechanisms that drive this process are still mostly unknown. Matrix metalloproteinases (MMPs) is a family of zinc-containing proteases that are involved in matrix and tissue destruction (Elkington and Friedland 2006, Volkman, Pozos et al. 2010, Ong, Elkington et al. 2014) and remodeling and thus play a role in the pulmonary pathology of TB and other diseases (Elkington, Nuttall et al. 2005, Elkington and Friedland 2006, Volkman, Pozos et al. 2010). MMP1 has been demonstrated to be up-regulated in both animal models of TB and in patients with clinical TB

using gene expression analysis and also protein expression in bronchoalveolar lavage fluid and sputum samples (Elkington, Shiomi et al. 2011, Ugarte-Gil, Elkington et al. 2013). Levels of MMP1, 2, 3, 7, 10 and 12 are elevated in TB patients with diabetes that correlates with disease severity and inflammation, which is resolved after anti-TB treatment or metformin treatment (Kumar, Moideen et al. 2018). Likewise, the levels of MMP1 in patient´s sputum drop during medical treatment of TB (Ugarte-Gil, Elkington et al. 2013). MMP9 is secreted by epithelial cells, macrophages and giant cells in humans (Zhu, Price et al. 2007) and is also involved in epithelial cell migration, and recruitment of macrophages and other inflammatory cells to the site of Mtb infection (Chakrabarti and Patel 2005, Elkington and Friedland 2006, Ramakrishnan 2013). High levels of circulating MMP1 and MMP9 have been shown to correlate with TB disease severity (Elkington and Friedland 2006, Ugarte-Gil, Elkington et al. 2013). Moreover, MMP's can be inhibited by a group of enzymes, tissue inhibitors of metalloproteinases (TIMPs). These TIMPs play a key role in the fibrosis of the granuloma. However, the exact stage of TB disease at which the MMPs and TIMPs act and how these are regulated in human pulmonary TB is still unclear (Ong, Elkington et al. 2014).

1.5 IMMUNE EVASION MECHANISMS OF MTB

Mtb has existed in humans for centuries and it is a highly successful pathogen that has developed several virulence factors to secure its survival in the human host. One such factor is the ESAT-6 protein, a key component of the type VII secretion system, ESX-1. Virulent mycobacteria expressing ESAT-6 can translocate from the phagosome into the cytoplasm and replicate in the cytoplasm thereby escaping phagosomal degradation (van der Wel, Hava et al. 2007). The ESX-1 virulence factor is also implicated in the impairment of autophagy-mediated clearance of intracellular bacteria (Romagnoli, Etna et al. 2012). ESAT-6-expressing mycobacteria has also been shown to inhibit initial granuloma formation in TB (Parasa, Rahman et al. 2014). Some evidence suggest that mycobacterial virulence factors can interfere with M1 polarization and skew the host response to an M2 phenotype which are poorly bactericidal, in order to facilitate their intracellular replication and persistence (Freeman, Post et al. 2006).

1.6 ANTIMICROBIAL EFFECTOR MECHANISMS IN TB INFECTION

Macrophages can employ different effector mechanisms to eradicate intracellular mycobacteria, such as direct killing by producing NO, ROS, antimicrobial peptides, or activation of

autophagy (Figure 3) but also by the formation macrophage extracellular traps (Metushi and Uetrecht) and cytokine release.

1.6.1 NO and ROS production:

Polymorphisms in the NOS2 (NO) and NOX2 (Weiss and Schaible) genes increases the susceptibility to TB infection indicating that reactive nitrogen or oxygen intermediates may play a role in TB pathogenesis (Ehrt and Schnappinger 2009, Bustamante, Arias et al. 2011). Although, NO-mediated killing of Mtb has been shown to be efficient for TB control in the mouse model (Scanga, Mohan et al. 2001), less is known about the clinical importance of NO in humans. NO is detectable in the exhaled air and urine of TB and TB/HIV patients and also in close contacts. But the exact role of NO in the risk of contracting TB disease or in the severity of the disease is not clear (Idh, Westman et al. 2008). Interestingly, some groups have tested the 'peanut therapy for TB' aiming to increase the availability of the NO-substrate arginine in the host cells. This peanut therapy was previously shown to improve the clinical outcome of TB in HIV-positive patients (Schon, Idh et al. 2011). iNOS expression in the alveolar macrophages and NO in the exhaled air increased in patients with active TB and decreased with anti-TB treatment (Wang, Liu et al. 1998). Similar to NO, the role of reactive oxygen species (ROS) in human TB control have remained obscure. Several studies have addressed the role of neutrophil generated ROS in the induction of apoptosis, amd a recent study revealed that TLR4-mediated NOX2 stimulation generated ROS, aiding in the fusion of phagosome as well as bacterial control (Lv, He et al. 2017).

1.6.2 Antimicrobial peptides:

LL-37 is an antimicrobial peptide that was discovered and isolated in humans from bone marrow cells (Gudmundsson, Agerberth et al. 1996). It can increase the phagocytosis of gram+ve and gram-ve bacteria, enhancing bacterial clearance (Wan, van der Does et al. 2014). LL-37 has an important role in the innate defense mechanisms at mucosal surfaces by interacting with the bacteria using ionic strength and killing through osmotic lysis (Nizet and Gallo 2003, Tsai, Yang et al. 2011). Previous studies have demonstrated that LL-37 efficiently reduce intracellular survival of mycobacteria (Sonawane, Santos et al. 2011). Macrophages have been shown to engulf LL-37 present in NETs and use the peptide for killing of intracellular mycobacteria (Stephan, Batinica et al. 2016). Interestingly, high levels of LL-37 in serum or plasma of patients with active TB may correlate with TB disease progression (Majewski, Agier et al. 2017). Instead, LL-37 expression has been reported to be low at the site

of Mtb infection in tissue (Rahman, Rehn et al. 2015, Ashenafi, Mazurek et al. 2018). This suggests that high LL-37 levels in the peripheral circulation could be used as a biomarker for active TB, while low levels of LL-37 at the site of Mtb infection is suggestive of low protective TB immunity.

It has previously been shown that vitamin D_3 ($VitD_3$) can enhance intracellular killing of Mtb in human monocytes and macrophages via induction of LL-37 (Liu, Stenger et al. 2006, Liu, Stenger et al. 2007). Later on, it was demonstrated that $VitD_3$ had a role in LL-37-dependent activation of autophagy in Mtb-infected macrophages, which resulted in increased clearance of intracellular mycobacteria (Liu, Stenger et al. 2007, Yuk, Shin et al. 2009). LL-37-mediated activation of autophagy have been shown to occur through the P2X7 receptor pathway (Rekha, Rao Muvva et al. 2015). Additionally, apart from its bactericidal properties, LL-37 promotes macrophage functional differentiation (van der Does, Beekhuizen et al. 2010). MCSF generated macrophages acquired a pro-inflammatory signature with low expression of CD163 and IL-10, in the presence of LL-37 (van der Does, Beekhuizen et al. 2010). LL-37 can also act as a chemoattractant for other immune cells (De, Chen et al. 2000).

Another antimicrobial peptide, human β-defensin 2, has been implicated in the control of TB infection (Reuschl, Edwards et al. 2017). Interestingly, it has been reported that human β-defensin 2 controls cutaneous TB (Zhao, Mu et al. 2016).

1.6.3 Autophagy:

Autophagy is a highly conserved lysosomal degradation process, where cellular long-lived proteins and damaged organelles are engulfed into a double-membrane structure called autophagosome. Later on, the autophagosome fuses with a lysosome to form the autolysosome, in which the contents are digested and released back into the cytoplasm for further utilization by the cells (Shintani and Klionsky 2004). Moreover, autophagy has been implicated in innate and adaptive immunity (Levine and Deretic 2007) as it can manage and promote killing of several intracellular pathogens like Shigella (Ogawa, Yoshimori et al. 2005), Salmonella (Birmingham, Smith et al. 2006) and Mtb (Gutierrez, Master et al. 2004). Mycobacteria secretes different virulence factors that inhibit several genes involved in autophagy signaling, from the initiation of the autophagosome like Atg5, and Beclin-1, to the fusion with the lysosome (Rekha, Rao Muvva et al. 2015) (Figure 3). Guitierrez *et.al*, have shown that induction of autophagy by rapamycin in Mtb-infected macrophages increases the maturation of

the autophagosome thereby enhancing the intracellular killing of mycobacteria (Gutierrez, Master et al. 2004).

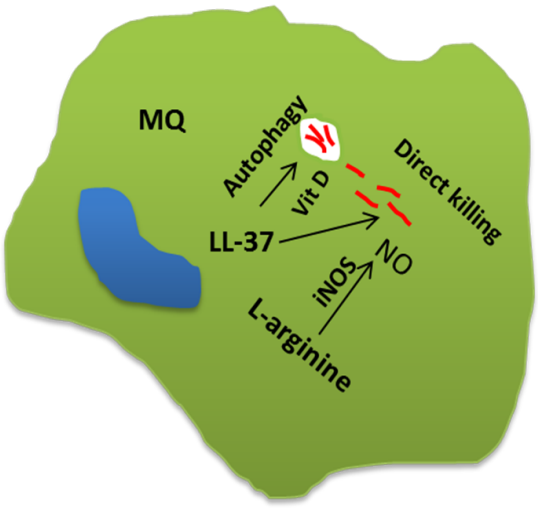

Figure 3. Intracellular bacterial clearance can be employed by the macrophage using different mechanisms such as direct killing by LL-37, LL-37 mediated autophagy and by Nitric oxide production.

Several studies indicate that TNFα and IFNγ (Singh, Davis et al. 2006) induce autophagy in immune cells, but Mtb have the capacity to inhibit TNFα-mediated autophagy by inhibition of the NF-kb pathway (Djavaheri-Mergny, Amelotti et al. 2006). Likewise, IFNγ-mediated activation of autophagy is inhibited by the Th2 cytokines, IL-4 and IL-13, secreted by Mtb-infected macrophages in an autocrine manner (Manca, Reed et al. 2004, Freeman, Post et al. 2006, Sun, Lim et al. 2006). These studies suggest that autophagy can be activated by Th1 cytokines and inhibited by Th2 cytokines.

1.6.4 Macrophage extracellular traps (MET's):

Macrophage extra cellular traps (MET's) were first reported in mouse macrophages against *Staphylococcus aureus* (Chow, von Kockritz-Blickwede et al. 2010). Macrophage extracellular traps, similar to neutrophil extracellular traps (NET), contain cellular or mitochondrial DNA along with antimicrobial peptides. However, macrophage traps are not as common as neutrophil extracellular traps. Mtb induces the formation of MET's, especially when the bacteria form clumps and are difficult to phagocytose. Mycobacterial virulence factors such as ESX-1 have been shown to be involved in inhibiting the formation of MET's (Kalsum, Braian et al. 2017).

1.6.5 Cytokines:

Macrophages can produce cytokines like IL-6, IL-1β, TNFα, IL-12 and IL-10 upon activation including infection with Mtb, and these cytokines likely play an important role in disease pathogenesis. The definitive role of **IL-6** in human TB is not clearly understood. However, it has been shown that IL-6 inhibits IFNγ signaling in human macrophages, thereby inhibiting autophagy that facilitates bacterial growth (Nagabhushanam, Solache et al. 2003, Dutta, Kathania et al. 2012). Moreover, an association between SNP's (single-nucleotide polymorphism) in IL-6 and TNFα has been observed in pulmonary TB patients compared with latent TB (Wu, Wang et al. 2018). **IL-10** is an anti-inflammatory cytokine that acts directly on the macrophage and promotes a switch to the M2 phenotype, which are less bactericidal and more involved in tissue remodeling (Redford, Murray et al. 2011). Furthermore, IL-10 may counteract the effects of TNFα and IL-12 (Abdalla, Lambert et al. 2016). **IL-12** is crucial in the activation of dendritic cells and also stimulates CD4+ T cells to produce IFN-γ during Mtb infection. Both humans and mice deficient in IL-12 signaling pathways are more susceptible to TB infections (Cooper, Solache et al. 2007). It has also been observed that the Mtb cell wall component LAM (lipoarabinomannan) inhibits IL-12 production thereby inhibiting IFNγ secretion (Cooper, Solache et al. 2007). **IL-1β** is an important pro-inflammatory cytokine produced by innate immune cells during Mtb infection. IL-1β synthesis is stimulated by TLR4 and NOD2 mediated pathways. The TLR4 pathway signals via NFkB, which results in production of pro-IL-1β (Greten, Arkan et al. 2007). Pro-IL-1β is further cleaved by the NLRP3 inflammasome inducing caspase-1 to synthesize active IL-1β or alternatively, the NLRP3 pathway induces IL-1β secretion directly. Mycobacterial virulence factors may inhibit the activation of inflammasome that results in declining IL-1β production (Master, Rampini et al. 2008). It has been reported that a genetic variation, resulting in enhanced NLRP3 inflammasome activity, promote control of Mtb infection in human macrophages mediated via IL-1β (Eklund, Welin et al. 2014). $VitD_3$ treatment of macrophages and lung epithelial cells, have also been shown to enhance IL-1β secretion, facilitating in enhanced Mtb killing (Eklund, Persson et al. 2013). **TNFα** is produced by a number of cell types, including macrophages, lymphocytes, neutrophils, mast cells and endothelial cells. TNFα play a critical role in TB granuloma formation by enhancing the recruitment of macrophages and T cells to participate in granuloma formation and also in reducing bacterial replication (Clay, Volkman et al. 2008, Egen, Rothfuchs et al. 2008). In an IFNγ dependent manner, TNFα promotes the fusion of

lysosomes with phagosomes and further initiates autophagy (Andrade, Wessendarp et al. 2006, Jia, Cheng et al. 2006). Low TNFα levels result in reduced TB disease control and poorly organized granuloma formation (Chakravarty, Zhu et al. 2008). But high, excessive levels of TNFα may also be destructive and correlate with extensive tissue damage and inflammation. The effects of TNFα in human TB, was discovered from rheumatoid arthritis patients using TNFα antagonists (Askling, Fored et al. 2005). It was observed that latent TB infection was re-activated in patients who received treatment with TNFα antagonists. Low TNFα impairs CD8+ effector memory T cells, highlighting the importance of TNFα in eliciting adaptive immune response against Mtb infection (Bruns, Meinken et al. 2009).

1.7 NEGATIVE REGULATORS OF PROTECTIVE TB IMMUNITY

Protective immune responses in TB can be suppressed by different regulatory immune cell subsets and inhibitory pathways. The main cell types participating in this negative regulation of protective immunity are regulatory T cells (Treg), regulatory B cells, tolerogenic DCs, and M2 or regulatory macrophages as well as myeloid suppressor cells (Brighenti and Joosten 2018). Mtb has developed hostile strategies that can induce, expand and in other ways enhance the activity of these different regulatory subsets. For example, the inhibitory enzyme, IDO (indoleamine 2,3-dioxygenase), is involved in tryptophan catabolism, which can slow the growth of microbes as well as T cells. IDO has been shown to be highly expressed in *in vitro* infected macrophages, and it was recently reported that IDO is increased in the macrophage rich areas of pulmonary TB in non-human primates (Gautam, Foreman et al. 2018). Tolerogenic DCs can also express high levels of IDO (Harden and Egilmez 2012), or secrete different immunosuppressive cytokines such as IL-10 or IL-13 to dampen the immune response upon Mtb infection (Kim, Kwon et al. 2017). Furthermore, naturally occurring CD4+CD25+ or CD4+FoxP3+ Treg cells are the most well studied subset in the context of negative immune regulation in TB. FoxP3+ Treg cells are increased at the site of Mtb infection in pulmonary TB (Sharma, Saha et al. 2009) and CD4+CD25+ Treg cells dampen IFNγ production from effector T cells in active TB (Ribeiro-Rodrigues, Resende Co et al. 2006). CD4+CD25+FoxP3+ Treg cells have also been shown to contribute to immune dysfunction and bacterial persistence in patients with XDR-TB (Davids, Pooran et al. 2018). In addition, activated T cells can be induced to secrete IL-10 and thereby decreasing T cell proliferation and switching the macrophage phenotype to an anti-inflammatory M2 in Mtb infection (Moreira-Teixeira, Redford et al. 2017). Ultimately, alternatively activated M2 macrophages and tolerogenic DCs

could promote Treg cells ad their function, and correspondingly, Treg cells could suppress effector responses of other immune cells at the site of Mtb infection.

1.8 DRUG RESISTANCE IN TB

Multidrug resistance is a common problem among TB patients and according to the WHO Global TB report in 2018, 558 000 patients had MDR-TB or XDR-TB and 230 000 deaths occurred due to drug-resistant Mtb in 2017. Socioeconomic factors such as poverty, overcrowding conditions, and malnourishment, non-compliance to treatment and recurrence of the disease, all contribute to drug resistance. In olden days, multidrug resistance incidence was higher among prisoners due to poor living conditions (Davies 2001). The emergence of resistance is caused by mutations in the bacterial genome that occurs naturally in the bacterial population. Thus, drug-resistance can be acquired during inadequate treatment by selection of drug-resistant bacilli that occur with a certain frequency. Or drug-resistance can spread via transmission of MDR-TB strains, which is called primary resistance. Notably, the chance of TB recurrence is high, even after successful completion of treatment. Accordingly, multidrug resistance has been reported to be substantially higher among previously treated TB cases compared to newly diagnosed TB cases (Manjelievskaia, Erck et al. 2016, Lange, Chesov et al. 2018).

Multidrug resistance is generally defined as Mtb bacteria resistant to two of the first line drugs used in TB treatment, isoniazid (INH) and rifampicin (RIF). Other classifications, according to resistance are XDR-TB, where the bacteria are found to be resistant to INH, RIF and to one of the injectable second line drugs such as amikacin, kanamycin and fluoroquinolones. The mechanism of action for different drugs is illustrated in figure 4.

Isoniazid is a bactericidal drug that acts on actively dividing bacteria. It is effective in dramatically reducing the bacterial load up to 95% within two days of starting the therapy. The drug is produced in the form of prodrug that is activated by the enzyme mycobacterial catalase-peroxidase, katG (Unissa, Subbian et al. 2016). The activated drug acts primarily on the metabolically active bacteria by inhibiting mycolic acid synthesis. Bacteria develop INH resistance, mainly through mutations in the *katG* gene that encodes the catalase enzyme. This is the most frequently observed mutation among clinical isolate samples (Somoskovi, Parsons et al. 2001). The other common mutation includes the *inhA* gene that encodes NADH-dependent enoyl-acyl carrier protein (ACP)-reductase required for mycolic acid synthesis. Moreover, mutations in promoter and coding regions of the *inhA* gene were shown to result in resistance

not only to INH, but also cross-resistance to ethionamide. Additionally, mutations in NDH (NADH dehydrogenase), *ahpC* and *kasA* genes may lead to INH resistance (Palomino and Martin 2014).

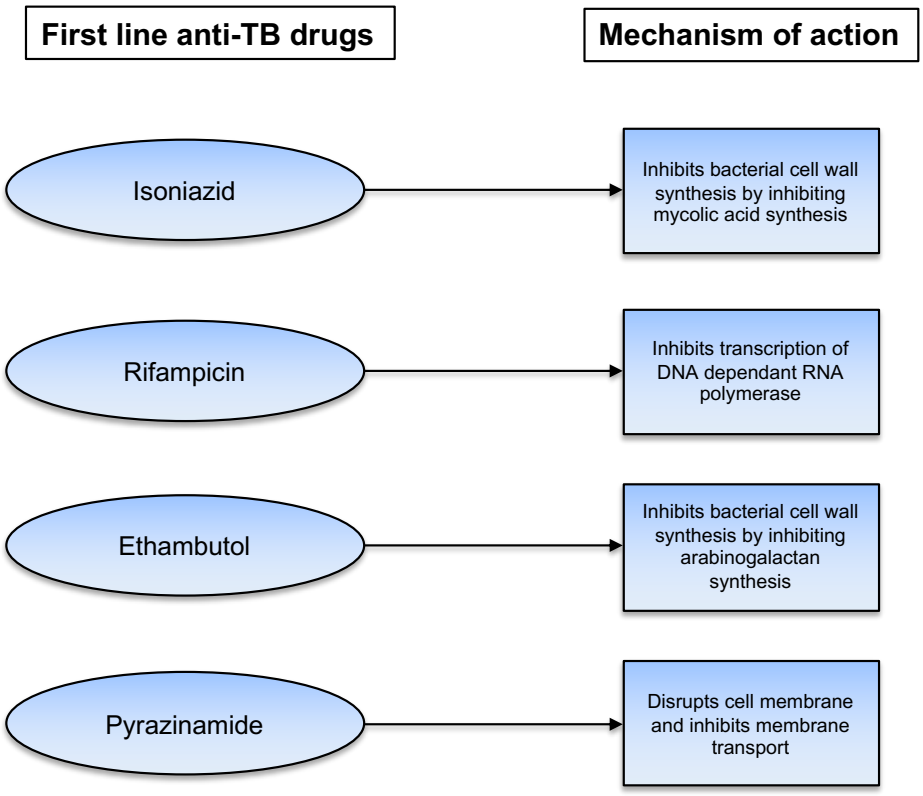

Figure 4. First line anti-TB drugs and their mechanism of action.

Rifampicin acts both on active and non-active or slow metabolizing bacteria. The bactericidal effect of the drug is attributed to its inhibitory action on transcription by binding to the enzyme DNA-dependent RNA polymerase (Somoskovi, Parsons et al. 2001). Bacteria develop resistance to RIF due to mutations in the hot spot region in the RNA polymerase gene. The clinical relevance of RIF resistant Mtb strains is highlighted by the fact that mycobacteria resistant to RIF are typically resistant to other anti-TB drugs. Thus, resistance to RIF is suggested to be a surrogate marker for multidrug resistance (Palomino and Martin 2014).

Ethambutol works as bacteriostatic drug and interferes with cell wall synthesis by inhibiting arabinogalactan, a component of the mycobacterial cell wall. Arabinogalactan synthesis requires arbinosyl transferase, an enzyme encoded by the *embCAB* gene in mycobacteria. Resistance to this drug involves mutations in the *embB* gene. (Palomino and Martin 2014).

Pyrazinamide is, similar to INH, also a prodrug. It is converted to the active form by the enzyme pyrazinamidase encoded by the *pncA* gene. It acts on slow growing mycobacteria and

exerts its function by inhibiting cell membrane transport. Resistance to this drug has been shown to result mainly from mutations in the *pncA* gene (Palomino and Martin 2014).

Apart from the bactericidal or bacteriostatic actions of standard anti-TB drugs, these antibiotics also modulate host immune responses such as phagocytosis, chemotaxis, antigen presentation, cytokine secretion and autophagy (Giuliani, Porcelli et al. 1998, Minic, Bojic et al. 2009). RIF is one of the first-line drugs that enhance the expression of CD1b on host cells, thereby increasing lipid antigen presentation (Giuliani, Porcelli et al. 1998). Pyrazinamide and INH have been shown to induce host autophagy (Kim, Lee et al. 2012) but these antibiotics can also reduce the release of essential pro-inflammatory cytokines and chemokines such as IL-1β, IL-6, TNFα, IL-2, and MCP-1 (Mendez, Traslavina et al. 2009, Kim, Lee et al. 2012, Manca, Koo et al. 2013).

The above mentioned four drugs fall under first-line drugs for treatment of active TB, indicating that the drugs that are available for effective treatment are limited and bacteria has developed resistance mechanisms to circumvent their actions. Thus, there is a dire need to develop new drugs or treatment strategies to prevent the emergence of drug-resistant bacteria and to reduce the duration of treatment. But using compounds with direct anti-bacterial effects will eventually always result in drug-resistance. To overcome this problem, a possible method would be to boost protective host immunity that could support the standard drugs to fight intracellular as well as invading bacteria. This way, the bacterial infection would be targeted from two directions: 1.) direct killing effects by the drugs, and 2.) indirect killing effects of compounds that enhance antimicrobial host responses.

1.9 IMMUNOMODULATORY COMPOUNDS-HOST-DIRECTED THERAPIES IN TB

Treatment of TB involves intake of several drugs daily for six months or more depending on drug-susceptibility testing and the site of Mtb infection. MDR-TB or XDR-TB may extend the time for treatment considerably, for up to two years. In spite of these long treatment regimens, the successful cure rates of MDR-TB are low, around 54% (WHO Global TB report 2018). Multiple strategies have been considered or developed that could enhance host immune responses to efficiently handle TB infection. Basically, host-directed therapies can modulate TB immunity in three different ways, 1.) to increase effective elimination of Mtb bacteria, 2.) to limit excess inflammation and collateral tissue damage associated with TB and 3.) to interfere with host molecules that are required for bacterial replication and persistence (Kaufmann, Dorhoi et al. 2018). Targeting different aspects such as induction of autophagy, granuloma

formation, modulation of T cell responses, exogenous monoclonal antibodies against bacterial antigens and improved drug delivery, may become the future in developing personalized therapies for patients with different forms of TB (Figure 5). Inexpensive alternatives as host-directed therapies may represent immunomodulatory compounds such as vitamin D_3 ($VitD_3$), phenylbutyrate (PBA) or peanut (arginine) therapy, which have been shown to potentiate innate immunity in the host.

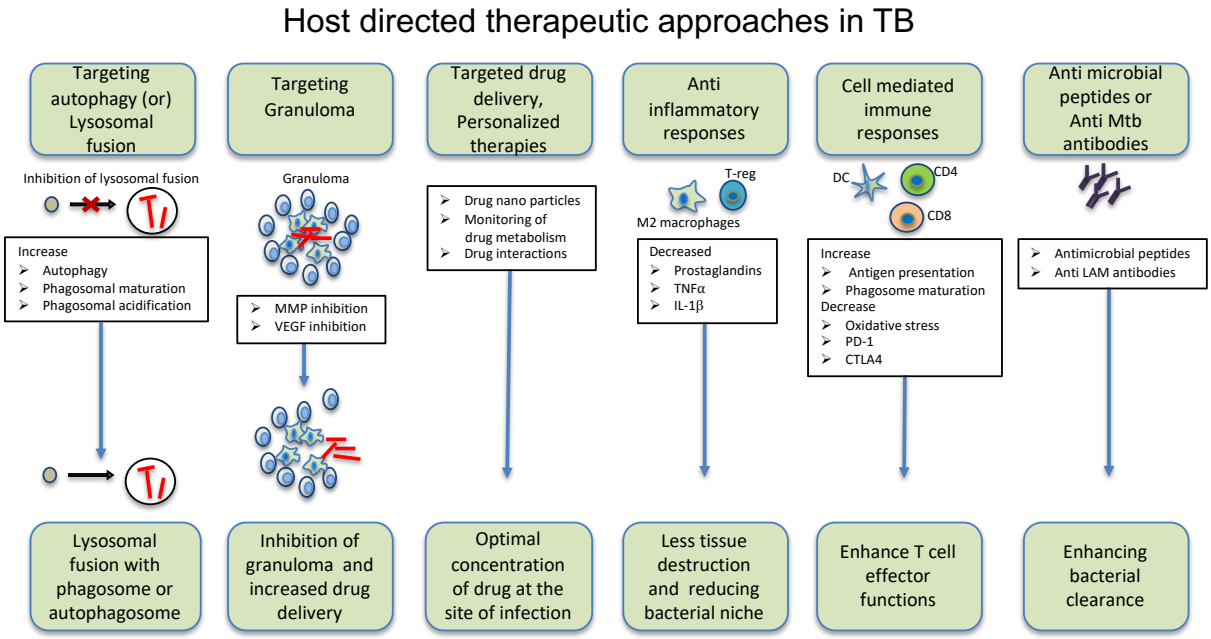

Figure 5. Approaches for host directed therapies in TB.

1.9.1 Vitamin D:

Apart from its well-known effects on bone health and calcium homeostasis, $VitD_3$ is famous for its immunomodulatory properties that have been investigated in a number of chronic diseases (Adams and Hewison 2008). $VitD_3$ is a fat-soluble vitamin synthesized in the skin upon sunlight exposure. $VitD_3$ exists as an inactive proform ($25(OH)D_3$) that is converted to an active form ($1,25(OH)_2D_3$) by hydroxylation. It is metabolized to $25(OH)D_3$ in the liver followed by further hydroxylation by 1-α hydroxylase to metabolically active $vitD_3$, mainly in the kidney, but also in tissues and immune cells (Nair and Maseeh 2012, Bikle 2014).

$VitD_3$ is important for several physiological processes such as calcium and phosphorus absorption, cell proliferation and differentiation, insulin synthesis, angiogenesis, immunomodulation etc. These functions are mediated via the $VitD_3$ receptor (VDR), which is an intracellular receptor located in almost all the tissues in the human body (Nair and Maseeh

2012). Active $VitD_3$ exerts its effects by binding to the VDR, generating a transcription factor complex that can bind to $VitD_3$ response elements present in the DNA that could modulate the expression of several genes (Wang, Nestel et al. 2004, Gombart, Borregaard et al. 2005, Martineau, Wilkinson et al. 2007).

Circulating levels of $25(OH)D_3$ in peripheral blood below 50 nmol/l is described as $VitD_3$ deficiency and is frequently observed in different populations, in different parts of the world, also in countries with a lot of sunshine. The major contributing factors for this deficiency include insufficient exposure to sunlight, inadequate diet, application of high percentage sunblock lotions, other chronic conditions such as liver or kidney diseases, or a natural protection due to a high melanin content in the skin (dark skin color) (Nair and Maseeh 2012).

Interestingly, polymorphisms in the VDR alone did not correlate with TB disease, but the genotype TT/Tt in combination with $VitD_3$ deficiency was associated to development of disease and the genotype ff combined with undetectable levels of $VitD_3$ had a strong correlation to disease (Wilkinson, Llewelyn et al. 2000).

From the era of sanatoria to the present days, research on $VitD_3$ as an immunomodulatory compound that could enhance clinical recovery from TB disease, has been extensively studied. Several *in vitro* and *in vivo* studies described the role of $VitD_3$ in control of TB infection (Brighenti, Bergman et al. 2018). With addition of $VitD_3$, intracellular mycobacterial growth reduction was reported in human monocytes and macrophages (Rook, Steele et al. 1986, Crowle, Ross et al. 1987). Later on, it was found that $VitD_3$ induces antimicrobial activity in human Mtb-infected macrophages via the induction of LL-37, which reduced intracellular Mtb growth substantially (Liu, Stenger et al. 2007). $VitD_3$ deficiency is commonly observed in TB disease, although the cause and effect relationship is unclear. But maintaining sufficient levels of $VitD_3$, is likely beneficial for promoting host immunity and clinical recovery from TB. Supplementation of $VitD_3$ to standard anti-TB treatment have been shown to reduce the time to sputum conversion and improve TB treatment outcome including improvement in clinical symptoms and radiological features (Afzal, Rathore et al. 2018). However, several other clinical trials fail to show convincing effects of $VitD_3$ supplementation (Brighenti, Bergman et al. 2018). Importantly, $VitD_3$ can only be expected to result in significant effects if given to patients or individuals that are $VitD_3$ deficient, as it would be difficult to supplement someone who already has optimal levels of $VitD_3$ (Martineau, Jolliffe et al. 2017). Moreover, the effects of $VitD_3$ is most likely dependent on

the treatment regimen (no bolus dosing, but high doses should be administered frequently (Hollis and Wagner 2013).

$VitD_3$ is also implicated in tissue remodeling during Mtb infection, as $VitD_3$ treatment of PBMCs (Coussens, Timms et al. 2009) or in TB patients (Ong, Elkington et al. 2014) resulted in decreased MMP7. Moreover, people with deficient $VitD_3$ levels may be more susceptible or have a higher risk of contracting TB when exposed to people with active TB infection (Buonsenso, Sali et al. 2018). Several research groups have shown the ability of $VitD_3$ to induce antimicrobial peptides; LL-37 and defensins by human macrophages and thereby efficiently clearing intracellular bacteria. $VitD_3$ may also aid in overcoming several bacterial virulence mechanisms to survive in the host such as inhibition of autophagy, inhibition of fusion of lysosome and phagosome and decreased LL-37 production (Yuk, Shin et al. 2009, Rekha, Rao Muvva et al. 2015). Epigenetic changes such as methylation of $VitD_3$ genes enhance the risk of TB disease and poor prognosis (Wang, Kong et al. 2018).

1.9.2 Phenylbutyrate:

PBA is a registered drug for urea cycle disorders as a scavenger for ammonia (Batshaw, MacArthur et al. 2001). PBA is a short chain fatty acid and a histone deacetylase (HDAC) inhibitor. This means that PBA could unwind the chromatin and facilitate binding of transcription factors to the VDR response elements in the DNA to allow the transcription of target genes such as LL-37. PBA has been demonstrated to be a potent inducer of LL-37 in macrophages and lung epithelial cells (Steinmann, Halldorsson et al. 2009). PBA restricts the phagocytic receptor expression and uptake of Mtb in macrophages and further act as a bacteriostatic agent against Mtb in broth cultures (Coussens, Wilkinson et al. 2015). Together with $VitD_3$, PBA has a synergistic effect on the induction of LL-37 in Mtb-infected macrophages and thereby enhance intracellular killing of Mtb bacteria (Coussens, Wilkinson et al. 2015, Rekha, Rao Muvva et al. 2015).

Thus, adjunctive immunotherapy using $VitD_3$ and PBA is an attractive approach to enhance the efficacy of standard anti-TB treatment, to shorten the duration of treatment, to reduce side-effects induced by the standard drugs and to increase treatment compliance to prevent MDR-TB. It will be interesting to study whether adjuvant therapy along with standard chemotherapy can elicit a better host response towards the infection and achieve a better clinical outcome (Mily, Rekha et al. 2015, Bekele, Gebreselassie et al. 2018).

2 AIMS, MATERIALS AND METHODS

2.1 AIMS

The specific aims of this thesis were:

Study I: To study the role of novel inducers of antimicrobial peptides, PBA and $VitD_3$, on the activation of autophagy in Mtb-infected human macrophages.

Study II: To investigate the contribution of matrix metalloproteinases (MMPs) in Mtb growth and early granuloma formation in a novel organotypic 3D-lung tissue model.

Study III: To explore the effect of PBA+$VitD_3$ on intracellular growth of MDR-TB and the induction of antimicrobial effector responses in human macrophages alone or in combination with first-line anti-TB drugs.

Study IV: To study the phenotype and ability of human macrophages polarized with $VitD_3$ to control intracellular Mtb growth compared with polarization of conventional M1 and M2 macrophage subsets.

2.2 Background on disease models of TB infection

Even after decades of research in TB, pathogenesis in humans is not clearly understood. Despite the advancements in research infrastructure and technology, there are many questions still unanswered in the field, and one of the main reasons behind this challenge is the lack of proper disease model(s) to study human TB. Most studies on TB are conducted in mice, which fundamentally does not represent human disease (Gupta and Katoch 2005). Mice do not form well-organized or necrotic TB granulomas, there are no multinucleated giant cells and the effector mechanisms used to control TB are different from humans, ie. mainly dependent on iNOS/NO, while a murine homologue for granulysin is missing. Other widely used models in the TB research include guinea pig (Fok, Ho et al. 1976, Kashino, Napolitano et al. 2008), rabbits (Manabe, Dannenberg et al. 2003), rats (Elwood, Wilson et al. 2007), and zebra fish (Ramakrishnan 2013), but all these models display a disease that is profoundly different from human TB. The only animal model, which closely reflects the human disease, is the non-human primate (Flynn, Capuano et al. 2003), but this model involves high running costs, big infrastructure and also ethical considerations. On the other hand, experiments with single-cell cultures from humans, most often involve cells derived from blood as this is the most easy accessible clinical sample (Brighenti and Andersson 2012). This enables simplified *in vitro* infection models, but cannot provide the complex physiological environment corresponding to the site of Mtb infection in human tissue. Recently, Dr. Mattias Svensson and his group have developed an organotypic lung tissue model that mimics human lung tissue (Nguyen Hoang, Chen et al. 2012), which has proven useful to study early TB granuloma formation (Parasa, Rahman et al. 2014). This model uses epithelial and fibroblast cell lines that after air-exposure and cell stratification, can be cultured together with human primary monocytes and differentiated macrophages, in a bed of bovine collagen. In this model, macrophage responses including early granuloma formation can be studied in the presence and absence of different Mtb strains (see description below).

2.3 Materials and methods

The project outline of this thesis is based on studies of innate immune responses in human macrophages infected with different strains of Mtb (Figure 6). Monocyte-derived macrophages (MDMs) were obtained from healthy donor buffy coat blood and infected with the 1.) laboratory strain H37Rv, or 2.) MDR-TB or drug-susceptible Mtb strains isolated from patients with active TB. Study I and III involves studies on the responses of Mtb-infected macrophages after treatment with PBA, $VitD_3$ or PBA+$VitD_3$ *in vitro*. Study II explores the response of Mtb-infected macrophages in an organotypic 3D-lung tissue

model, while Study IV focus on macrophage polarization and Mtb growth after stimulation with different growth factors *in vitro*. In Study II and IV, lung tissue biopsies obtained from patients with active pulmonary TB were also used to confirm mRNA or protein expression determined *in vitro*.

mRNA expression in cells or lung tissue was quantified using quantitative real-time PCR. Protein expression and distribution was studied by staining Mtb-infected macrophages or tissues with fluorescent dyes or conjugated antibodies that were assessed using *in situ* computerized image analysis or fluorescence microscopy. Intracellular Mtb bacteria, was monitored by flow cytometry or confocal microscopy while Mtb growth was determined using colony forming unit (CFU) counts but also the MGIT assay.

In summary, the following techniques were used to study the responses of Mtb-infected macrophages *in vitro*:

- Quantitative real-time PCR (qPCR): Study I-IV
- Immunofluorescence and confocal microscopy: Study I, II and III
- Colony forming units: Study I, III and IV
- Immunohistochemistry and *in situ* image analysis: Study II
- Western blot: Study I
- Enzyme Linked Immunosorbent Assay (ELISA): Study I
- Luciferase assay: Study II
- Cytometric bead array: Study II
- Mycobacteria Growth Inhibitor Tube (MGIT) assay: Study III
- Small interfering (si) RNA silencing of primary cells: Study III
- Flow cytometry: Study IV

All work with virulent Mtb including H37Rv and clinical Mtb isolates was performed at a biosafety level 3 (BSL-3) laboratory at the Public Health Agency of Sweden (FOHM) (Study I, III and IV) or at a BSL-3 laboratory at Linköping University (Study II). Mtb-infected cells or tissues were chemically inactivated before transfer to a BSL-2 laboratory for further analysis.

A brief description of materials and methods is given below, while a detailed methodological description is given in the 'Materials and methods' sections of the respective studies.

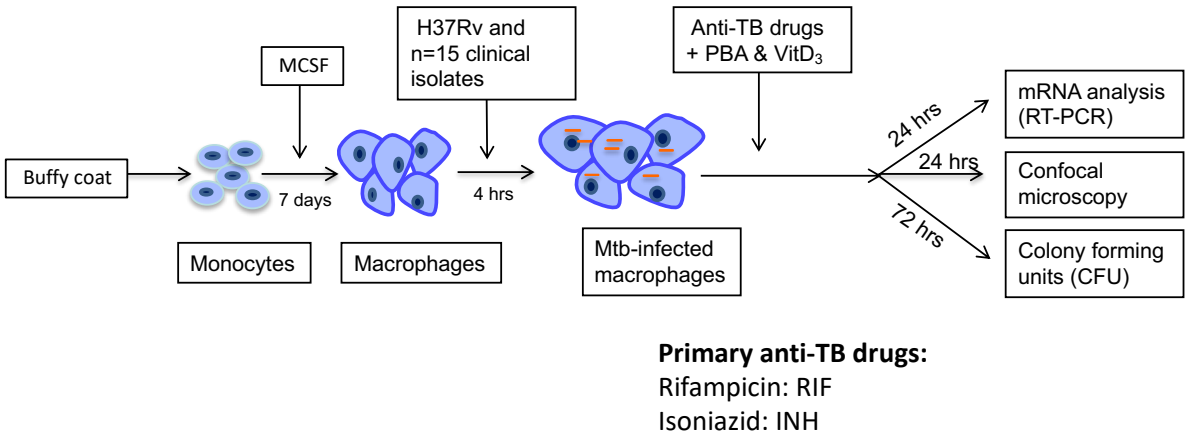

Figure 6. *In vitro* macrophage infection model: Macrophages derived from monocytes were infected with Mtb and treated with PBA and $VitD_3$ in the presence or absence of anti TB drugs.

2.3.1 Cell cultures:

Blood mononuclear cells were isolated from peripheral blood samples (Study II) or buffy coat blood (Study I, III and IV) obtained from healthy donors using ficoll-hypaque density gradient centrifugation. Monocytes were retrieved using beads coated with human CD14 antibodies (Study II) or using plastic adherence (Study I, III and IV). Monocytes were differentiated into macrophages in the presence of RPMI containing 50 ng/ml of MCSF (Study I, III and IV) or 50 ng/mL of GMCSF (Study IV) or pooled human serum (Study II). In Study IV, macrophages differentiated until day 6, were polarized for 18 hours using, 10 nm of $1,25(OH)_2D_3$, 50 ng/mL of IFN-γ and 10 ng/mL of LPS or 20 ng/mL of IL-4.

In addition, the human monocytic cell line THP-1 was used to generate macrophage like cells using 10 ng/ml of PMA in Study I. For the setup of the organotypic lung tissue model, the human lung fibroblast cell line MRC5 and the human bronchial epithelial cell line 16HBE were used (Study II).

2.3.2 Bacterial cultures:

The standard laboratory strain of virulent Mtb, H37Rv, was used in most experiments, either as wild type or expressing GFP (green-fluorescent protein) (Mtb-GFP) or luciferase (Mtb-lux). Mtb-GFP enabled visualization of bacteria with confocal microscopy (Study II) or flow cytometry (Study IV), while Mtb-lux enabled assessment of bacterial replication (Study II). For Study III, clinical isolates obtained from patients with MDR-TB or drug-susceptible TB, were kindly provided by our collaborators at FOHM. MDR-TB strains were resistant against one or several first line and/or second line anti-TB drugs as described (Study III). Macrophages were infected at an MOI 5 or 10.

2.3.3 Human organotypic lung tissue model:

A protocol for productive mycobacterial infection of human primary macrophages cultured in a 3D lung tissue model was previously set up by our group and collaborators (Braian, Svensson et al. 2015). The model is composed of lung fibroblasts (MRC-5, a human lung fibroblast cell line) and epithelial cells (16HBE, a human bronchial epithelial cell line), which are cultured on a collagen matrix and air-lifted to initiate stratification of the epithelial cell layer. Before air-exposure, primary MDMs derived from healthy blood donors and differentiated with pooled human serum, were infected with Mtb-GFP and cultured together with uninfected monocytes in the model for 7 days. PKH26 red dye-labeled monocytes and Mtb-GFP could be visualized and quantified using confocal microscopy. At day 7, the models were harvested and fixed with 4% paraformaldehyde for 30min, while culture supernatants were collected and stored at −80°C for cytokine and chemokine analysis.

2.3.4 Inhibitors:

Before infection with Mtb, macrophages were treated for 2 hours or 16-20 hours with inhibitors (underlined) of different receptors and pathways: KN62 (inhibition of P2X7R, Study I and III), oxATP (inhibition of P2X7R, Study III), BAPTA-AM (intracellular calcium-specific chelator, Study I), Compound C (ATP-competitive inhibitor of AMP-activated protein kinase, Study I), 3-MA (inhibition of the PtdIns3K pathway, Study I), Marimastat (inhibition of MMPs, Study II), L-NMMA (inhibition of iNOS, Study III) and NAC (scavenger of free radicals including ROS enzymes, Study III).

2.3.5 Knockdown/silencing of CAMP:

THP-1 cells were knocked down for the expression of the CAMP gene using short hairpin RNA (shRNA) by a transient transfection method (Study I). Silencing of macrophages for CAMP gene expression in primary macrophages was done using small interference RNA (siRNA) from the silencer select siRNA kit according to the manufacturer's instructions (Study III).

2.3.6 Quantitative PCR:

mRNA was extracted from macrophages (Study I-IV) or the organotypic lung tissue model (Study II) or patient lung tissue (Study II and IV) using the ribopure kit from ambion and synthesis of cDNA was done by superscript vilo according to manufacturer´s instructions. Thin cryosections (2x50 microns) were obtained from the lung tissue model or patient tissue that were used for the mRNA isolation. Gene transcripts for the described target genes relative to the house-keeping gene 18s rRNA was measured by RT-PCR. Gene expression

was calculated as fold change against untreated Mtb-infected cells (Study I and III), or uninfected tissues (Study II) or uninfected cells (Study IV).

2.3.7 ELISA and western blot:

Release of the LL-37 peptide in the supernatants of Mtb-infected macrophages was measured using an LL-37 specific ELISA (Study I). Protein levels of LL-37, ATG5, BECLIN 1 and p62 in Mtb-infected macrophage cell extracts and cell culture supernatants were detected by western blot analysis (Study I).

2.3.8 Microscopy:

2.3.8.1 Immunohistochemistry and light microscopy:

In Study II, organotypic lung tissue models or patient lung biopsies were snap frozen in OCT using liquid nitrogen and were stored at -80°C. 8 µm thick sections were cut in a cryostat and fixed for 15 min with 4% formalin. Tissues were stained with primary antibodies and HRP-conjugated secondary antibodies as described in Study II. Stained proteins in the tissue were visualized after development of peroxidase activity detected as a brown color. Protein expression *in situ* was quantified using computerized image analyses (Leica Qwin software) and presented as the % positively stained area in the total cell area.

2.3.8.2 Fluorescent staining and confocal microscopy:

In Study II, granuloma formation in the organotypic lung tissue model was assessed by visualizing PKH26-stained macrophage clusters (red) together with GFP-labelled H37Rv bacteria (green) (Study II). Quantification of the granulomas were performed by determining the MFI (mean fluorescence intensity) in selected regions of interest with and without bacteria, respectively. Clustering of monocytes/macrophages at the site of infection was expressed as the ratio of the MFIs determined in ROIs with and without bacteria (Zeiss LSM700 Confocal system and Nikon software).

In Study I, MDMs were seeded in so called chamber slides (chambered microscope slide) that enabled us to grow the cells in the presence or absence of PBA+VitD_3 before the grid was removed to continue with fluorescent staining and examination with confocal microscopy. Co-localization of the autophagy marker LC3-II with LL-37 was assessed in THP-1 cells or Mtb-infected macrophages including quantification of LC3 puncta (Olympus confocal microscope and the image J software).

2.3.9 Flow cytometry:

2.3.9.1 Cytokine bead array (CBA):

In study II, cytokines and chemokines in supernatants from uninfected or Mtb-infected lung tissue models were quantified using a commercial cytokine bead array kit from BD. Data was acquired on a Gallios flow cytometer and was analyzed with the kaluza analysis software.

2.3.9.2 Cell surface markers:

In Study IV, detection and quantification of different molecules and receptors expressed on the surface of uninfected and Mtb-infected macrophages was performed by extracellular staining using fluorescent conjugated anti-human antibodies. CD68, which is mainly an intracellular protein expressed in macrophages, was stained intracellularly using BD cytofix/permeabilization kit. Data was acquired on a BD LSR Fortessa flow cytometer and was analyzed with the FlowJo v.9 analysis software. Uninfected macrophages were separated from Mtb-infected macrophages based on expression of Mtb-GFP that was visualized in the FITC channel.

2.3.10 Bacterial growth:

2.3.10.1 Luciferase assay:

In Study II, bacterial replication was measured as fold change from day one compared to day seven by detecting the luciferase activity of Mtb-lux, both in cell culture supernatants (extracellular Mtb-lux) and cellular extracts (intracellular Mtb-lux) from Mtb-infected macrophages.

2.3.10.2 MGIT assay:

In Study IV, extracellular bacterial growth rates of clinical Mtb isolates and H37Rv were monitored in MGIT tubes and expressed as fluorescence units. The **MGIT 960** instrument is a fully automated mycobacterial detecting system that exploits the fluorescence of an oxygen sensor to detect growth of mycobacteria in culture.

2.3.10.3 Colony forming units (CFU):

In Study I, III and IV, intracellular bacterial growth was determined using CFU counts. Mtb-infected macrophages were lysed with water containing SDS. Serial dilutions were made from the extract and plated on 7H9 middle brooks agar plates. Growth of intracellular bacteria was measured by counting the colony forming units after 21 to 28 days. CFU counts is the golden standard for detection and quantification of Mtb growth.

2.4 Statistical analyses

A normality test was performed on all data sets using D' Agostino's K-squared test and Kolmogorov-Smirnov test. Normally distributed data was analyzed using parametric tests whereas small data sets or data that did not pass the normality test(s) was analyzed using non-parametric tests to determine the level of significance.

Comparison between two groups was done using:

- Parametric analyses: unpaired or paired t-test
- Non-parametric analyses: Mann-Whitney U test or Wilcoxon signed-rank test

Comparison between multiple groups was done using:

- Parametric analyses: one-way ANOVA or Kruskal-Wallis test and Dunn´s post-test
- Non-parametric analyses: Friedman's test

Comparisons between multiple groups influenced by two independent variables were done using 2-way ANOVA. Correlation analyses was done using Spearman´s correlation test.

Data was presented as mean and standard deviation or median and interquartile range (IQR). Statistical analyses was done using Graphpad prism, version 6.0 (Graphpad prism software, San Diego, CA).

2.5 Ethical considerations

Study I, III and IV:

Buffy coats were obtained from healthy donors recruited at the Karolinska University Hospital blood bank, Stockholm, Sweden (EPN dnr: 2010/603-31/4). Blood samples were also obtained from healthy donors at Linköping University Hospital, but ethical permit was not required for collection of peripheral blood from these healthy anonymous donors.

Study II and IV:

Lung tissue biopsies from pathological and non-pathological regions were obtained from Russian TB patients. In the study, patients were recruited after signed and informed consent and approval from the local and Swedish ethical boards in Russia and Stockholm (EPN dnr: 238/02).

3 RESULTS AND DISCUSSION

All original tables, figures and graphs discussed in this section can be found in Paper I-IV included at the end of this thesis.

3.1 STUDY I

3.1.1 Background

Before the discovery of effective anti-TB drugs, heliotherapy or open-air treatment was often offered to TB patients and clinical improvement was frequently reported (McCarthy 2001). The enhanced clinical recovery may be attributed to the sunshine hormone, $VitD_3$, and its metabolites to induce microbial immune defense pathways. While $VitD_3$ has a key function in the regulation of bone health, the role of $VitD_3$ in controlling human infections such as TB is not clear. The hand-full of randomized clinical trials that have tested the effects of $VitD_3$ in TB show conflicting results (Brighenti, Bergman et al. 2018), but have mostly failed to demonstrate positive effects on clinical or microbiological outcomes (Wejse, Gomes et al. 2009, Martineau, Timms et al. 2011, Ganmaa, Munkhzul et al. 2017). Contrary, *in vitro* studies have provided convincing evidence that $VitD_3$ can enhance killing of intracellular Mtb in macrophages via induction of LL-37 (Liu, Stenger et al. 2006, Liu, Stenger et al. 2007). These differences between *in vivo* and *in vitro* studies might be multifactorial such as ethnicity, polymorphism in genes encoding $VitD_3$ metabolism or life style (Brighenti, Bergman et al. 2018). Other potent inducers of LL-37 have been shown to involve short-chain fatty acids including butyrate and PBA (Raqib, Sarker et al. 2006, Gudmundsson, Bergman et al. 2010). Previous studies found that short-chain fatty acids such as butyrate could improve clinical and pathological features of shigellosis in a rabbit model (Rabbani, Albert et al. 1999, Raqib, Sarker et al. 2006, Gudmundsson, Bergman et al. 2010). Later on, it was also discovered that PBA could induce LL-37 in lung epithelial cells and importantly, there was a synergistic effect between PBA and $VitD_3$ on LL-37 expression (Steinmann, Halldorsson et al. 2009).

Study I and III of this thesis work, was part of collaboration in an international consortium that aimed to explore the antimicrobial effects of PBA and $VitD_3$ in the induction of mucosal immunity. Now, we were interested to explore the effects of PBA on intracellular killing of Mtb and if PBA could be used together with $VitD_3$ to enhance potential anti-mycobacterial effects in Mtb-infected macrophages.

3.1.2 Mtb-mediated down-regulation of LL-37 expression in human primary macrophages can be counteracted by PBA+VitD$_3$

In Study I, we tested the effects of PBA, VitD$_3$ or the combination of PBA+VitD$_3$ on human Mtb-infected macrophages. Initially, we observed that mRNA expression of the CAMP gene encoding LL-37, was down-regulated human macrophages after *in vitro* infection with virulent Mtb (H37Rv) (Figure 7). In addition, a decrease in protein levels of the LL-37 peptide was detected in Mtb-infected cells. Down-regulation of LL-37 at mucosal sites has also been demonstrated in other infections. It was recently reported that chronic periodontitis causing bacteria, *Porphyromonas gingivais*, could down-regulate LL-37 production in gingival epithelial cells (Tada, Shimizu et al. 2017). Similarly, LL-37 was down-regulated in the rectal epithelium of patients infected with the intracellular bacteria Shigella (Islam, Bandholtz et al. 2001) and it was also shown that the rabbit homologue of cathelicidin, CAP-18 (Cathelidin Antimicrobial Peptide), was down-regulated in the mucosal epithelia in the lung and intestine of rabbits with shigellosis (Sarker, Ahmed et al. 2011). These studies suggested that down-regulation of LL-37 expression at the site of infection may be an immune evasion mechanism used by different bacteria.

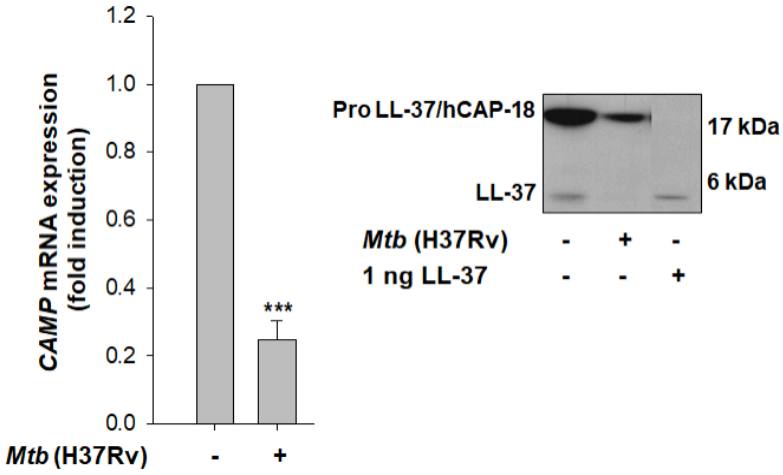

Figure 7. Mtb infection down regulates the production of LL-37 in macrophages.

Interestingly, butyrate was shown to up-regulate LL-37 expression in colonic epithelial cells *in vitro* (Schauber, Svanholm et al. 2003). In *shigellosis*, mucosal CAP-18 expression could be restored after oral treatment with PBA (Sarker, Ahmed et al. 2011) or butyrate (Raqib, Sarker et al. 2006), which resulted in reduced clinical illness and reduced inflammation. Likewise, adjunct butyrate treatment to patients with shigellosis resulted in an early reduction of inflammation and enhanced LL-37 expression in the rectal epithelia (Raqib, Sarker et al. 2012). In Study I, we showed that a combination of PBA and VitD$_3$ up-regulated the expression of LL-37 at substantially higher levels when compared to treatment with either

PBA or $VitD_3$ alone. A corresponding decrease of intracellular Mtb growth was detected in the presence of PBA or $VitD_3$, while Mtb growth was further reduced upon combined treatment with PBA and $VitD_3$. Therefore, treatment of Mtb-infected macrophages with PBA+$VitD_3$ could prevent down-regulation of LL-37 and instead resulted in a potent expression of LL-37 that was associated with reduced Mtb growth in infected macrophages. Our group has previously found that $VitD_3$ deficient MDR-TB patients expressed low levels of LL-37 *in situ* in granulomatous tissue obtained from Mtb-infected lungs (Rahman, Rehn et al. 2015). Moreover, plasma $25(OH)D_3$ levels correlated with local LL-37 expression in granulomatous lesions in TB infected lymph nodes (Ashenafi, Mazurek et al. 2018), which suggest that $VitD_3$ status could be important for antimicrobial activity at the site of Mtb infection.

3.1.3 Activation of autophagy in Mtb-infected macrophages by PBA and $VitD_3$ treatment is dependent on LL-37

Autophagy is a physiological process known to be important for the elimination of intracellular pathogens such as Mtb (Gutierrez, Master et al. 2004). It has been shown that MDR-TB strains such as the Beijing genotype strains, are resistant to killing by autophagy by inhibiting the autophagosome and lysosome fusion (Haque, Boonhok et al. 2015). In addition, microRNA 155 induced by virulent Mtb can down-regulate autophagy by decreasing the expression of the autophagy protein Atg3 and the number of autophagosomes in dendritic cells (Etna, Sinigaglia et al. 2018). These studies suggest that Mtb has evolved strategies to hijack autophagy in different cell types to promote intracellular survival. In line with these findings, it had previously been determined that $VitD_3$ could kill intracellular Mtb by activation of autophagy in infected macrophages (Yuk, Shin et al. 2009). To investigate if autophagy contributed to reduced Mtb growth upon treatment with PBA and $VitD_3$, we studied the activation of autophagy using the autophagy related genes *Beclin 1, Atg5* and *LC3II*. While Beclin 1 initiates the formation of the autophagosome, Atg5 is involved in the elongation of the autophagosome and LC3II is a marker of the mature autophagosome (Figure 8). mRNA expression and protein levels of both Beclin 1 and Atg5, were significantly down-regulated in Mtb-infected macrophages. Interestingly, this reduction was effectively counteracted by PBA and $VitD_3$ treatment. Likewise, evidence of elevated autophagic activity in PBA and/or $VitD_3$ treated Mtb-infected cells was visualized by detection of enhanced intracellular LC3 expression that co-localized with LL-37. This was also supported by an enhanced conversion of LC3I (cytosol) to LC3II (autophagosome membrane) as detected by western blot.

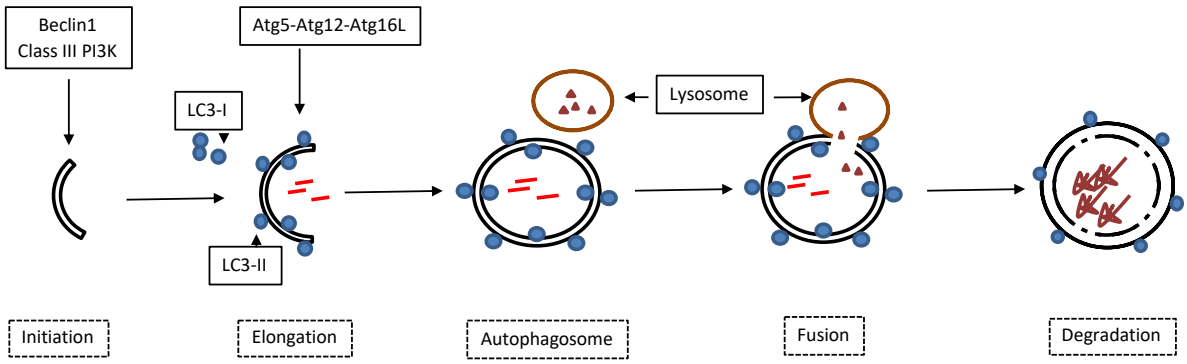

Figure 8. Mtb clearance by autophagy mechanism: Multiple steps and genes involved in the autophagy process.

To dissect the role of LL-37 in the activation of autophagy, we knocked down the CAMP gene encoding LL-37, in the macrophage cell line THP-1 by using shRNA and treated the cells with PBA and/or $VitD_3$. Interestingly, shRNA transfected THP-1 cells expressed significantly lower mRNA levels of *Beclin 1* and *Atg5* compared to mock shRNA-transfected cells, and also failed to induce the LC3 protein in Mtb-infected macrophages. These results suggested that PBA- and $VitD_3$-induced autophagy in Mtb-infected macrophages was dependent on the up-regulation of LL-37.

3.1.4 LL-37 activates macrophage autophagy in an autocrine or paracrine manner via the P2X7 receptor

To understand how LL-37 contributed to the induction of autophagy in Mtb-infected cells, a neutralizing antibody for LL-37 was added to the Mtb-infected cells in the presence or absence of PBA and/or $VitD_3$. This experiment revealed that autophagy was not activated in the cell cultures depleted of LL-37. Next, we explored the receptors involved in this mechanism. It was earlier demonstrated that LL-37 could bind to and activate the P2X7 receptor (P2X7R) on monocytes, leading to release of pro-inflammatory IL-1β (Elssner, Duncan et al. 2004). The P2X7R is a purinergic receptor involved in many cellular processes of macrophages involving cytokine secretion and inflammation (Elssner, Duncan et al. 2004). So, we blocked P2X7R by pretreating the macrophages with KN62, which revealed that autophagy was not activated. This suggested that LL-37, secreted by PBA and/or $VitD_3$ treated and Mtb-infected macrophages, needs to be taken up by the macrophages via P2X7R to activate autophagy (Tang, Basavarajappa et al. 2015). This result could also explain how LL-37 can be produced by other immune cells such as neutrophils that can lead to activation of autophagy in macrophages (Sorensen, Follin et al. 2001).

It is known that intracellular Ca^{2+}, PI3 kinase and AMP kinase pathways are involved in the activation of autophagy (Heras-Sandoval, Perez-Rojas et al. 2014, Hoyer-Hansen and Jaattela

2007). To further elucidate the down-stream signaling of the P2X7R, we blocked all the above pathways using BAPTA-AM, Compound C and 3-MA, respectively, by pre-treatment of macrophages before Mtb infection and stimulation with PBA and/or $VitD_3$. Intriguingly, we found that all these three signaling pathways were involved in the activation of autophagy induced via LL-37. Altogether, the results from Study I demonstrated that Mtb can down-regulate LL-37 expression and also prevent the activation of autophagy in infected macrophages, and thereby avoid intracellular killing. Treatment with PBA and/or $VitD_3$ can circumvent these blockades and induce LL-37 expression as well as autophagy in Mtb-infected cells (Manjelievskaia, Erck et al.) (Figure 9).

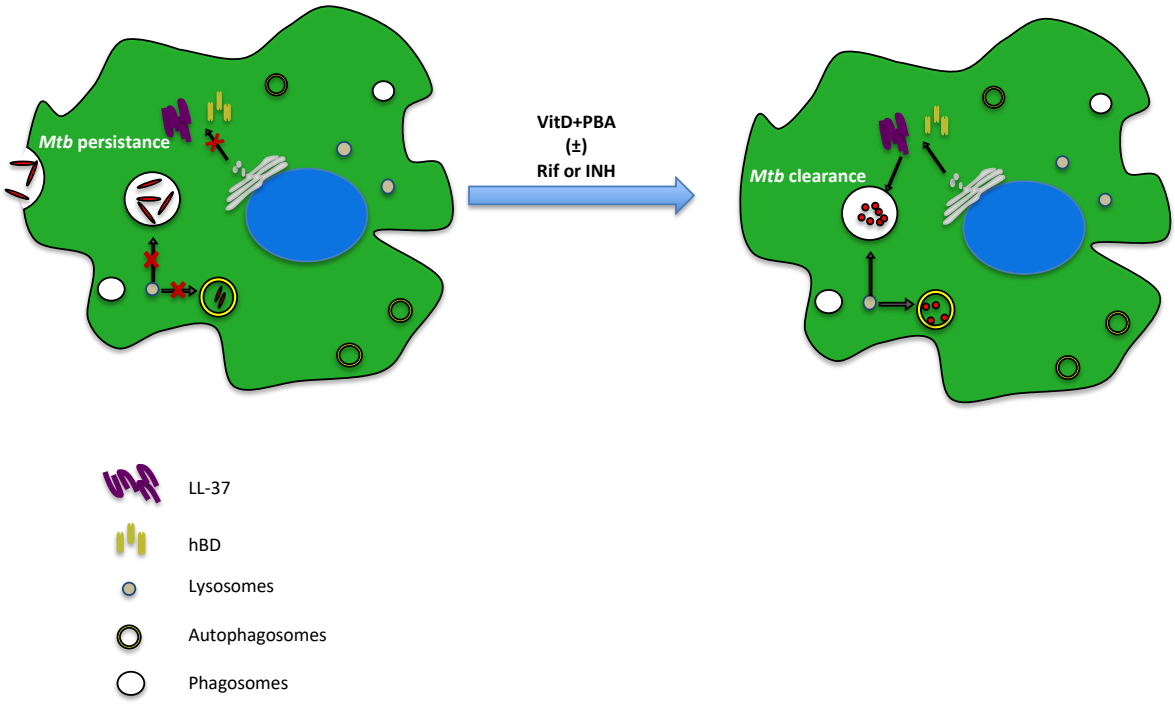

Figure 9. Infected macrophage response to PBA and $VitD_3$: Mtb infected macrophage treated with PBA and $VitD_3$ in the presence or absence of RIF or INH enhances intracellular Mtb clearance by LL-37 and autophagy.

3.2 STUDY III

3.2.1 Background

Simultaneously as we worked with Study I, our research constellation had conducted two clinical trials to investigate the effects of adjunct treatment with PBA and $VitD_3$ in patients with pulmonary TB. One study was conducted in Dhaka Bangladesh (Mily, Rekha et al. 2015) and the other one was conducted in Addis Ababa, Ethiopia (Bekele, Gebreselassie et al. 2018), following slightly different study protocols. The results showed significant effects on reduction of clinical TB symptoms as well as reduced sputum-microscopy conversion. But as PBA and $VitD_3$ had to be administered together with standard anti-TB drugs used for treatment of drug-susceptible TB, we developed an interest to study the effects of

PBA+VitD$_3$ on 1.) MDR-TB and also 2.) the interaction of PBA+VitD$_3$ with first-line anti-TB drugs, RIF and INH. Therefore, we proceeded with Study III, aiming to explore the effects of PBA+VitD$_3$ on a panel of MDR-TB strains with different resistance patterns to various anti-TB drugs. Since we had observed potent synergistic or additive effects on LL-37 expression in human macrophages induced by different concentrations of PBA and/or VitD$_3$ (Steinmann, Halldorsson et al. 2009, Rekha, Rao Muvva et al. 2015), we decided to use combination treatment of PBA+VitD$_3$ at a fixed concentration to enable the work in this study. In addition, we decided to focus on the two most important anti-TB drugs included in first-line chemotherapy, RIF and INH. Two concentrations of the drugs were chosen based on the wild-type distribution of the MIC (minimal inhibitory concentration) values determined for extracellular growth inhibition of a number of Mtb isolates (Schon, Jureen et al. 2009). Thus, we used a high but physiological concentration as well as a lower, sub-inhibitory concentration of RIF and INH, respectively, to be able to study potential effects on drug interactions with PBA+VitD$_3$.

We had access to unique clinical isolates, three drug-susceptible strains and twelve drug-resistant strains from our collaboration with FOHM (Table 1). Initially, we cultured the isolates in LJ medium and observed that two strains were growing very slowly and some exhibited different colony morphology, reflecting the variability of naturally occurring Mtb isolates. Next, we started the cumbersome work to infect primary MDMs with the different Mtb strains to test the growth inhibitory effects of PBA+VitD$_3$ in the presence or absence of RIF or INH. Typically, we could set up the different conditions with MDMs obtained from two donors and divide the experiment in mRNA analysis and CFU counts.

Table 1. *M. tuberculosis* strains	**No. of isolates**
Laboratory strain H37Rv (golden standard)	
Drug-susceptible	3
RIF mono-resistant	1
INH mono-resistant	3
RIF+INH resistant (MDR-TB)	2
Other MDR-TB (RIF+EMB+PZA and ETH or OFX)	3
XDR-TB	3

3.2.2 PBA+VitD$_3$ reduced intracellular growth of MDR-TB as well as drug-susceptible Mtb strains and possessed additive effects together with RIF and INH

Our first observation was that PBA+VitD$_3$ had a very potent effect on killing of both MDR-TB as well as drug-susceptible Mtb strains (median 39.8%). There was an additive effect on Mtb killing using a combination of PBA+VitD$_3$ and RIF and particularly INH (Figure 10). In fact, this additive effect was relatively stronger on MDR-TB growth than on growth of drug-susceptible strains, which suggested that PBA+VitD$_3$ could compensate for the loss of effect of the antibiotics (due to drug resistance) on MDR-TB growth. Contrary, PBA+VitD$_3$ treatment of MDMs infected with the drug-susceptible Mtb strains did not enhance the effects of high-dose RIF and INH to a similar extent, probably because the standard drugs alone were very efficient in Mtb killing. One problem in the clinical PBA+VitD$_3$ trials (Mily, Rekha et al. 2015, Bekele, Gebreselassie et al. 2018), was that these compounds had to be given together with already very effective antibiotics. Therefore, in these trials it was only possible to study the PBA+VitD$_3$-mediated effects on top of the standard drugs, providing a risk that any true effects of PBA+VitD$_3$ could be masked. The initial 8-week intensive-phase standard treatment with RIF and INH results in rapidly reduced bacterial loads in the lung and bacterial clearance from sputum in more than 80% of patients with drug-susceptible TB (Parikh, Nataraj et al. 2012, Nahid, Dorman et al. 2016). In contrast, according to the WHO, the treatment success rates for MDR-TB is considerably lower, around 54% (WHO, Global TB report 2018). Therefore, our findings of an additive effect between PBA+VitD$_3$ and especially high-dose INH on intracellular killing of MDR-TB strains, suggested that PBA+VitD$_3$ supplementation may have a superior effect as adjunct treatment to support less efficient second-line drugs used to treat MDR-TB.

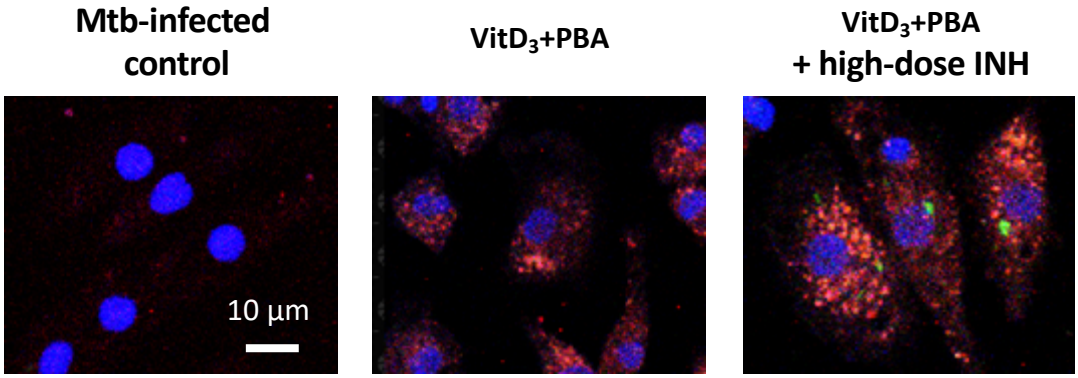

Figure 10. Enhanced LC3 punta in H37Rv infected macrophages with high dose INH and PBA+VitD$_3$.

In addition to their bactericidal or bacteriostatic actions, RIF (Tentori, Graziani et al. 1998, Liang, Zhou et al. 2017) and INH (Kim, Lee et al. 2012) may possess immunomodulatory

functions such as enhanced expression of antigen-presenting molecules or induction of autophagy. Confocal microscopy revealed that high-dose RIF or INH significantly increased the levels of LC3 puncta, which was further enhanced in the presence of PBA+$VitD_3$. It was previously reported that INH or pyrazinamide can induce autophagy and ROS, resulting in reduced intracellular load of *Mycobacterium marinum* (Kim, Lee et al. 2012). Furthermore, it was shown that autophagy is essential for an optimal effect of the drugs on bacterial killing (Kim, Lee et al. 2012). These results hold promise for using PBA+$VitD_3$ as adjunct therapy to support the new nine-month short-course treatment regimen for MDR-TB (seven drugs supplemented with high-dose INH) as recommended by the WHO (Moodley, Godec et al. 2016).

3.2.3 PBA+$VitD_3$-mediated inhibition of intracellular growth of MDR-TB or H37Rv is dependent on LL-37

The second major observation in Study III was that PBA+$VitD_3$ alone or in combination with low or high doses of RIF or INH up-regulated mRNA expression, primarily of LL-37 but also of human β-defensin-1, in MDMs infected with MDR-TB or the drug-susceptible strains. This increase in mRNA expression was slightly more pronounced in the INH-treated groups. Basal mRNA expression of LL-37 and β-defensin-1 was also up-regulated by RIF or INH alone, but to a much lower extent compared with PBA+$VitD_3$ treatment. We also observed an up-regulated mRNA expression of the enzymes involved in NO and ROS production (iNOS and DUOX2), but DUOX2 mRNA expression was not specific to PBA+$VitD_3$ treatment. Treatment of H37Ra-infected MDMs with active $VitD_3$ were previously shown to result in enhanced mRNA and protein levels of iNOS, but primarily in the presence of IFNγ (Lee, Yang et al. 2009). It has also been reported that INH can induce ROS activity, which is involved in *Mycobacterium marinum* clearance in a drosophila model (Kim, Lee et al. 2012). But inhibition of iNOS or ROS activity using L-NMMA or NAC, receptively, did not prevent PBA+$VitD_3$-mediated reduction of intracellular Mtb growth, which suggested that PBA+$VitD_3$ is not dependent on these effectors for bacterial killing.

Although ROS is not considered as one of the major pathways involved in TB control, Mtb can induce the production of ROS in human macrophages that has been shown to enhance bacterial clearance (Lv, He et al. 2017). The iNOS pathway has been shown to play an important role in control of Mtb infection in the mouse model of TB (Scanga, Mohan et al. 2001), while the effect of NO in humans is more controversial. Our group and others have demonstrated expression of iNOS and the NO metabolite nitrotyrosine in macrophages located in Mtb-infected tissues from patients (Schon, Elmberger et al. 2004, Rahman, Gudetta

et al. 2009, Rahman, Rehn et al. 2015). But it is difficult to induce expression of iNOS and NO in human cells *in vitro*, which may be dependent on the source and stimuli of human macrophages (Nicholson, Bonecini-Almeida Mda et al. 1996, Jagannath, Actor et al. 1998, Thomas and Mattila 2014). Clinical studies using nutritional supplementation with the iNOS substrate arginine, have demonstrated that enhanced NO may contribute to some protective effects in clinical TB (Schon, Elias et al. 2003, Schon, Idh et al. 2011).

To date, there is no specific chemical inhibitor of LL-37. Instead, similar to Study I, we attempted to inhibit the uptake of LL-37 by blocking P2X7R using KN62 or oxATP. This receptor has been shown to regulate the internalization of LL-37 into human macrophages (Tang, Basavarajappa et al. 2015). Blocking P2X7R resulted in slightly elevated Mtb growth in some of the tested conditions, but these results were not very clear. It is possible that inhibition of LL-37 uptake inhibit some intracellular effects mediated by exogenous LL-37, while the endogenous production and function of LL-37 is not prevented by P2X7R blockade. We then decided to use a more specific method for inhibition of LL-37 function, by silencing of the *CAMP* gene in primary MDMs using siRNA. Gene silencing using siRNA in primary macrophages may be difficult due to their active nuclease machinery, but we managed to optimize a protocol for *LL-37* gene silencing that maintained cell viability also after Mtb infection. siRNA transfection abrogated PBA+$VitD_3$-induced mRNA expression of LL-37 to a great extent, which resulted in a corresponding enhancement of intracellular growth of both MDR-TB and H37Rv after treatment with PBA+$VitD_3$. Consistent with this finding, we observed that autophagy was inhibited when we knocked down the expression of LL-37 in THP-1 cells in Study I (Rekha, Rao Muvva et al. 2015). Accordingly, lack of LL-37 expression seems to abolish the activity of PBA+$VitD_3$ to induce autophagy (Study I) and to execute intracellular killing of Mtb (Study I and III) in human macrophages.

3.2.4 The role of bacterial fitness in MDR-TB

The former dogma suggest that drug-resistance mutations provide a fitness cost for Mtb, which would reduce the ability of MDR-TB strains to spread successfully in humans (Muller, Borrell et al. 2013). Accordingly, previous findings from our group demonstrated that the susceptibility of MDR-TB strains to antimicrobial peptides was significantly higher among slow-growing MDR-TB strains with a reduced fitness phenotype (Toro, Hoffner et al. 2006). But more recent studies have shown that MDR-TB strains can acquire compensatory mutations that will restore or enhance bacterial fitness during evolution (Maisnier-Patin and Andersson 2004, Muller, Borrell et al. 2013). As such, the MDR-TB strains tested in Study III, had similar or faster growth rates compared with H37Rv, which could indicate well-

preserved fitness. Genome sequencing data also illustrated that some of the MDR-TB strains possessed compensatory mutations in the bacterial RNA polymerase (*rpoA* and *rpoC*) or in the alkyl hydroperoxide reductase (*ahpC*) subunit. It has been shown that some clinical Mtb strains possess reduced susceptibility to reactive nitrogen species (Barberger-Gateau, Jutand et al.), which promotes survival in activated macrophages (Idh, Andersson et al. 2017). Furthermore, a significant association was observed between reduced susceptibility to NO and resistance against first-line anti-TB drugs, in particular INH (Idh, Mekonnen et al. 2012). Another study reported that reduced susceptibility of mycobacteria to RNI *in vitro*, correlated with the virulence of these strains in guinea pigs (O'Brien, Carmichael et al. 1994). Similar mechanisms may promote transmissibility of hypervirulent MDR-TB strains such as the Beijing genotype family (Li, Jiao et al. 2016). An increasing prevalence of the Beijing strains in many parts of the world suggest that these possess an enhanced bacterial pathogenicity, which is reinforced by the ability of hypervirulent MDR-TB to modulate protective immunity (Manca, Reed et al. 2004, Reed, Domenech et al. 2004, Hanekom, Gey van Pittius et al. 2011). Altogether, these studies suggest that is important that novel immunotherapeutic drugs such as PBA+VitD_3 maintain effectivity against Mtb strains with enhanced fitness phenotypes.

3.3 STUDY II

3.3.1 Background

A strength of the experimental setup used in this thesis, was that primary MDMs obtained from a number of donors, were used to study macrophage responses and intracellular Mtb growth (Figure 11). However, a limitation of this *in vitro* Mtb infection model, is its simplified nature that enable reproducible results from several donors, but do not take into account the tissue microenvironment at the site of infection *in vivo*. A relevant option was to study the role and interaction of macrophages and mycobacteria in an organotypic lung tissue model that was previously established in our laboratory by co-supervisor Dr. Mattias Svensson (Nguyen Hoang, Chen et al. 2012). This model offers the advantage to study immune cells in a physiological milieu *in vitro*.

Our group collaborated with Prof. Maria Lerm and her postdoc from Linköping University, to optimize this model for infection with Mtb that would allow studies of host-bacteria interactions and early granuloma formation at the site of infection (Parasa, Rahman et al. 2014) (Figure 12). Briefly, this culture system contains tissue specific epithelial cells and

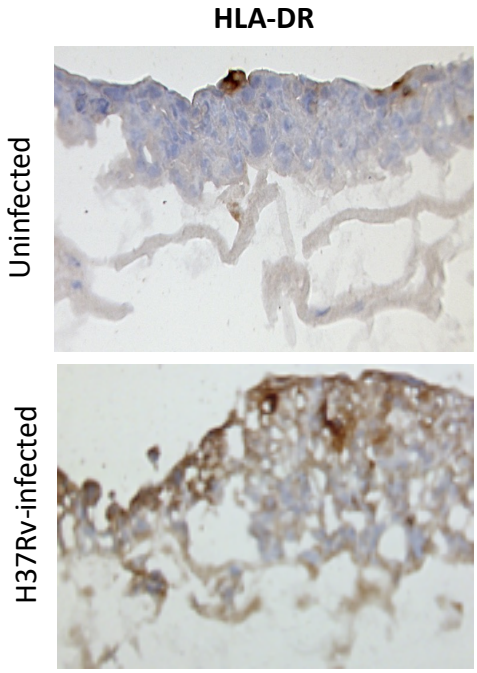

Figure 11. H37Rv infected macrophages expressing HLADR in organotypic lung tissue model.

fibroblasts that form structures recapitulating normal human lung tissue (Nguyen Hoang, Chen et al. 2012). Furthermore, this system allows implantation of Mtb-infected MDMs that are cultured together with uninfected red-labelled monocytes in the lung tissue model, to support cell migration and granuloma formation (Braian, Svensson et al. 2015). Our previous results showed that infected macrophages cluster to form granuloma-like structures similar to what is seen in human TB lung tissue and this process is dependent on mycobacterial virulence factors such as ESAT-6 (protein secreted via the type VII secretion machinery) (Parasa, Rahman et al. 2014, Davis and Ramakrishnan 2009).

3.3.2 Early granuloma formation and Mtb growth is inhibited by the global MMP inhibitor marimastat

Granuloma formation is a characteristic hallmark of human TB. This process involves the recruitment of uninfected immune cells to the site of Mtb replication and is dependent on tissue remodeling. MMPs belong to a group of proteases that have been suggested to contribute to remodeling but also to pathology and tissue destruction in TB (Elkington and Friedland 2006). In Study II, we used the lung tissue model to investigate the role of MMPs in TB granuloma formation and Mtb growth. Our hypothesis was that Mtb infection would induce the expression of a number of MMPs that could facilitate monocyte clustering and spread of Mtb, resulting in granuloma formation and enhanced bacterial growth. MMPs digest the extracellular matrix between the cells and thus facilitate granuloma formation.

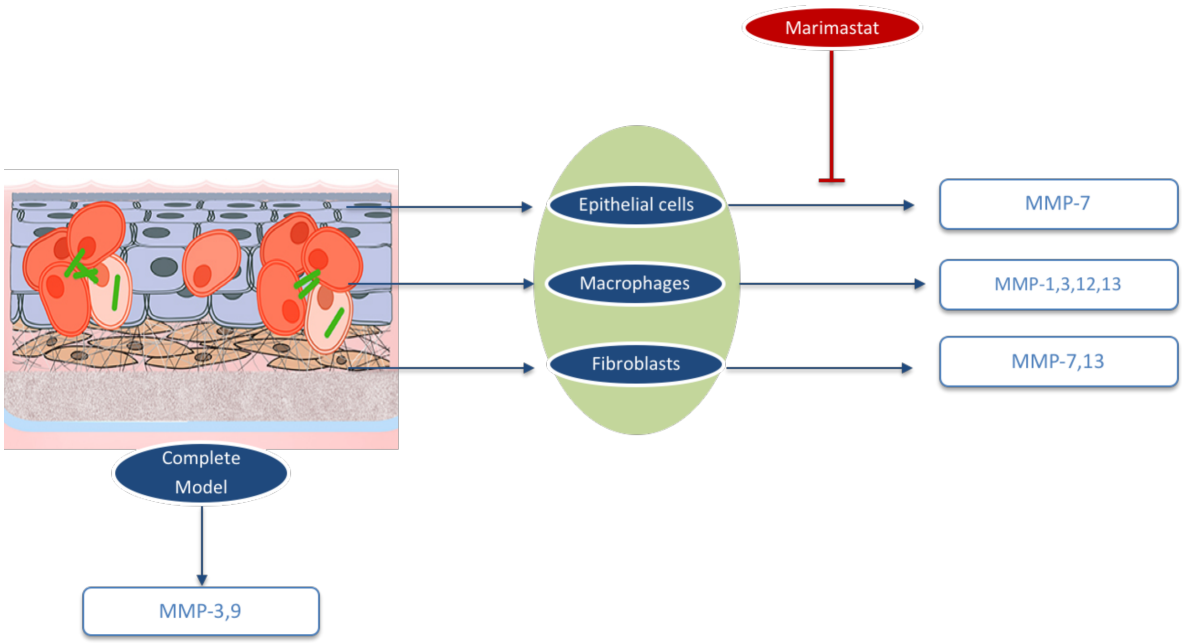

Figure 12. Organotypic lung tissue model expressing MMPs upon Mtb infection**:** Infected lung tissue expressed MMPs from epithelial, macrophages and fibroblasts. Marimastat a global inhibitor of MMPs inhibited the expression of MMPs.

Indeed, pre-treatment of macrophages with the global MMP inhibitor marimastat, before culture in the lung tissue model, effectively prevented monocyte clustering, which abrogated granuloma formation as well as intracellular Mtb growth as compared with the untreated control. While the dogma is that TB granulomas are formed in an attempt to wall off the infection and contain the bacteria in a sealed structure, it has previously been determined that Mtb instead can exploit early granuloma formation to seed the infection locally in the tissue (Davis and Ramakrishnan 2009, Cronan, Beerman et al. 2016). This is a feature of virulent bacteria, as mycobacteria lacking virulence factors that are part of the type VII secretion system, fail to promote granuloma formation (Parasa, Rahman et al. 2014, Davis and Ramakrishnan 2009).

3.3.3 The effect of marimastat is not dependent on pro-inflammatory chemokines and cytokines secreted by Mtb-infected lung tissue models

Culture supernatants from Mtb-infected or uninfected lung tissue models were assessed for a panel of pro-inflammatory cytokines and chemokines known to be involved in macrophage activation and resulted in detection of IL-6, IP-10, IL-8, MCP and RANTES. IL-6 is involved in the TB disease progression was significantly up-regulated in Mtb infected lung tissue model. It has been reported that IL-6 inhibits IFNγ responses and activation of autophagy, and thereby increase bacterial survival (Dutta, Kathania et al. 2012). In the lung tissue model supernatants, we also found significantly elevated levels of chemokines such as IP-10, which

is well studied as a biomarker in human TB, and MCP-1 that has been associated with leucocyte recruitment and disease severity as well as susceptibility in TB (Hasan, Cliff et al. 2009, Feng, Flores-Villanueva et al. 2012). Treatment of the lung models with marimastat, did not affect cytokine or chemokine expression from Mtb-infected lung tissue model and ultimately early granuloma formation was not dependent on the presence of these cytokines and chemokines.

3.3.4 Mtb infection resulted in expression of several MMPs both in lung tissue models and in lung tissue from patients with non-cavitary TB

It has been shown that Mtb can disturb the balance between MMPs and tissue inhibitors of MMPs (TIMPs) to facilitate granuloma formation (Kubler, Luna et al. 2015, Ordonez, Tasneen et al. 2016) and cavitation (Ordonez, Pokkali et al. 2018). To investigate MMP and TIMP expression in Mtb-infected lung tissue, we isolated mRNA from whole lung tissue models or from different cell subsets isolated from the lung tissue models (macrophages, epithelial cells or fibroblasts) and monitored mRNA expression of MMPs and TIMPs. MMP expression *in situ* was thereafter determined using image analysis. Altogether, these analyses demonstrated an up-regulation of MMP-1, MMP-3, MMP-9 and MMP-12 in Mtb-infected compared with uninfected lung tissue models. Monocytes/macrophages seemed to be the major MMP-producing subset. Contrary, no significant differences were observed in the expression of TIMP 1, 2 and 3 indicating that overall MMP and TIMP expression was altered with Mtb infection. We further confirmed these findings in lung tissue biopsies obtained from patients with non-cavitary forms of TB. Non-cavitary TB was chosen since macrophages were the main producers of MMPs, and macrophage numbers are severely depleted in patients with cavitary forms of TB (Andersson, Samarina et al. 2007). Quantification of MMP protein expression in the lung tissues biopsies, revealed a strong up-regulation of MMP-9 but also a relatively enhanced expression of MMP-1, MMP-3 and MMP-12 in granulomatous TB lesions as compared to the distal lung parenchyma. In addition, mRNA analysis indicated a relatively increased expression of TIMP-1 in the TB lesions. Increased levels of MMP-1, 2, 3, 8 and 9 as well as TIMP-1 and 2 in sputum samples, have been suggested to be associated with enhanced disease severity in TB patients (Ugarte-Gil, Elkington et al. 2013).

From these results, we concluded that granuloma formation and intracellular Mtb growth was effectively abrogated upon inhibition of MMP activity. Using quantitative mRNA and protein analysis we also demonstrated that Mtb-infected lung tissue models expressed a distinct set of MMPs and TIMPs that was comparable to MMP/TIMP expression in granulomatous lesions

from Mtb-infected human lung tissue. In mice, it was recently shown that inhibition of MMPs can prevent the leaky blood vessels and also maintain the integrity of the lung tissue, thereby increasing delivery and efficacy of RIF and INH at the site of infection (Xu, Wang et al. 2018). Moreover, it has been reported that antiretroviral therapy (ART) can lead to an enhanced expression of MMPs, which are associated with impaired lung function in TB immune reconstitution inflammatory syndrome, TB-IRIS (Ravimohan, Tamuhla et al. 2016). Overall these studies suggest that targeting Mtb-induced tissue remodeling can be developed for use as adjunct therapy in TB to enhance the efficacy of standard drug regimens.

3.4 STUDY IV

3.4.1 Background

The last Study IV in this thesis work aimed to examine the role of macrophage polarization in TB infection. Since Study I and III were designed to explore the antimicrobial effects of PBA+$VitD_3$, we decided to continue to study the effects of particularly $VitD_3$, on macrophage polarization. *In vivo*, macrophages can adopt a variety of functional phenotypes depending on subtle and continuous changes in the tissue microenvironment (Mantovani, Sica et al. 2004, Mantovani, Biswas et al. 2013, Italiani and Boraschi 2014). To define the complexity and plasticity of macrophages, the concept of M1/M2 polarization of macrophage functions has been founded as a simplified conceptual framework describing a non-linear continuum of diverse functional states (Mosser and Edwards 2008). Similar to Th1 and Th2 cells, classically activated M1 cells are inflammatory while alternatively activated M2 cells are considered wound healing or tissue remodeling macrophages (Mantovani, Sica et al. 2004, Mantovani, Biswas et al. 2013, Italiani and Boraschi 2014). Classically activated M1 macrophages are regarded as key effector cells in the elimination of microbes and cancer cells, while M2 macrophages contribute to the production of extracellular matrix components such as MMPs involved in tissue remodeling (Mantovani, Biswas et al. 2013).

In Study IV, we wished to investigate the ability of $VitD_3$-polarized macrophages to control intracellular Mtb infection compared with polarization of conventional M1 and M2 macrophage subsets, and the phenotypic alterations associated with immune polarization *in vitro*. Also in this study, we took advantage of our *in vitro* macrophage infection model, and here we applied a protocol for macrophage polarization with different stimuli as described in in figure 13.

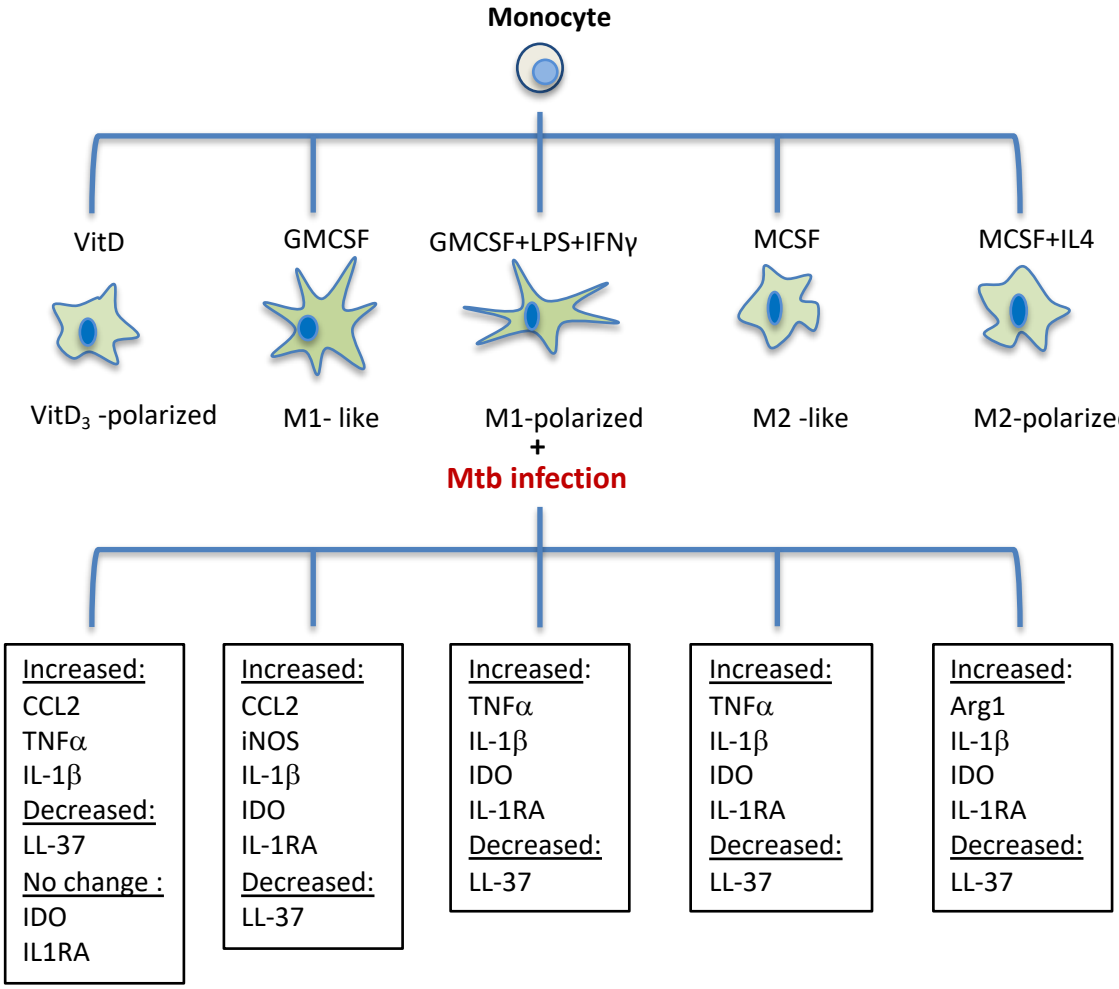

Figure 13. Cytokine and chemokine expression of polarized macrophages upon H37Rv infection.

3.4.2 *In vitro* polarized macrophages express typical M1 and M2 phenotypic markers but adopt a mixed M1/M2 phenotype post-Mtb infection

Initially, we wanted to confirm that uninfected MDMs activated with classical M1 or M2 stimuli could express phenotype markers typically associated with M1 or M2 polarization and how these phenotypes differed to $VitD_3$-polarized macrophages. An array of different markers used to identify and characterize M1 or M2 macrophages have been described (Macrophage Polarization Mini-review, Bio-rad). We tested different markers using multicolor flow cytometry, and ultimately decided on one M1 and one M2 panel that showed reproducible and reliable results. While $VitD_3$-polarized cells maintained a phenotype similar to unstimulated MDMs, we could determine that M1 markers such as CCR7, CD64, CD86 and TLR2 or M2 markers such as CD163, CD200R and CD206, were proportionately higher on M1- or M2-polarized cell subsets, respectively. M1/M2 polarization of uninfected MDMs was also associated with typical morphological changes including a more dendritic shape of

M1 cells and more rounded appearance of M2 cells. Interestingly, Mtb infection resulted in an up-regulation of most M1 and M2 markers on the different macrophage subsets, generating a mixed M1/M2 profile. Mtb-infected $VitD_3$-polarized macrophages had a significantly up-regulated expression of HLADR, CD86, CD80 and CD206. Both M1 and M2 subsets exhibited an up-regulated CD86 expression, while the M1 subsets also had a significantly up-regulated expression of the M2 marker CD163. A mixed M1/M2 activation profile has previously been shown in several chronic conditions such as melanoma (Bardi, Smith et al. 2018) and multiple sclerosis (Vogel, Vereyken et al. 2013), providing examples where macrophages adopt a mixed phenotype *in vivo*. As an alternative to mixed M1/M2 responses, there is evidence from *in vitro* studies that macrophages also can switch their phenotype from M1 to M2 or *vice versa* depending on sequential micro-environmental challenges in the tissue (Italiani and Boraschi 2014).

3.4.3 Enhanced Mtb growth in both M1 and M2 subsets at 72 h post-Mtb infection compared with $VitD_3$-polarized macrophages despite lower bacterial uptake in M1 cells at 4 h

To study Mtb uptake as well as productive infection and Mtb growth, we infected the different macrophage subsets at 4 h, 24 h and 72 h. Mtb uptake (4 h) and productive infection (24 and 72 h) was monitored using flow cytometric analyses of Mtb-GFP expression in the macrophages, whereas intracellular Mtb growth was assessed using CFU counts after 4 and 72 h post-Mtb infection. Mtb uptake was remarkably lower in M1-like and M1-polarized subsets compared to all other subsets, but especially compared with the M2 subsets (18% versus 82%). Also intracellular Mtb growth was considerably lower in the M1 subsets compared to the M2 subsets at 4 h post-Mtb infection. But productive infection and Mtb growth inside the M1-like and M1-polarized macrophages progressed rapidly overtime, and at 72 h post-infection, Mtb growth was similar in the M1 and M2 subsets. Notably, while M1 cells lost their capacity to control Mtb growth, M2 cells stabilized and reduced Mtb survival to levels comparable to the M1 subsets. Instead, $VitD_3$-polarized cells very efficiently controlled Mtb infection over time. Despite a higher uptake of Mtb bacilli in $VitD_3$-polarized compared to M1 cells (33% versus 18%), Mtb growth of both H37Rv and an MDR-TB isolate was maintained at stable, low levels at 24 and 72 h post-Mtb infection. In addition, Mtb growth was around 40% lower in $VitD_3$-polarized macrophages compared with the unstimulated M0 control. A previous study reported that macrophages treated with a low-dose of $VitD_3$ during differentiation, were less infected with dengue virus when compared to untreated macrophages (Arboleda Alzate, Rodenhuis-Zybert et al. 2017). Our experiments suggested that $VitD_3$-pretreatment can enhance the ability of macrophages to control Mtb

infection more efficiently compared to M1- or M2-polarized cells. Thus, sufficient $VitD_3$ status may be crucial to support macrophage polarization *in vivo*.

Normally, M1 macrophages have been found to be better equipped with microbial killing capacities (Flannagan, Cosio et al. 2009). It was previously demonstrated that M1 macrophages are infected with BCG at a lower proportion as compared to M2 macrophages, and similar levels of BCG infection were maintained even at 6 days post-infection. (Verreck, de Boer et al. 2004). Our results of reduced infectivity of M1 compared to M2 macrophages at 4 h concur with their findings, but at 3 days post-infection we observed progressive multiplication of bacteria, which could be a trait of intracellular survival or persistence in virulent mycobacteria. Similarly, it has been shown in a chlamydia infection model that despite a lower infectivity of M1 macrophages, bacteria persist in the M1 subset while permissive growth is evident in M2 macrophages (Gracey, Lin et al. 2013). These findings support the notion that several pathogens have developed strategies to persist for prolonged periods of time in the host and may allow low, but persistent growth in activated macrophages despite induction of antimicrobial responses.

3.4.4 Mtb-infected $VitD_3$-polarized macrophages are more pro-inflammatory and express higher mRNA levels of LL-37 compared with M1 and M2 subsets

To obtain a better view on the function of the different macrophage subsets, we quantified host cell mRNA expression of pro-inflammatory, antimicrobial as well as inhibitory effector molecules before and after Mtb infection. $VitD_3$-polarized macrophages specifically up-regulated TNFα, IL-1β, CCL2, and LL-37, whereas immunosuppressive molecules such as IL-1RA, IDO and Arg-1 were expressed at relatively lower levels after Mtb infection. $VitD_3$-polarized cells also expressed relatively high levels of IL-10 and LC3b compared with the M1 and M2 subsets, but this expression was not changed with Mtb infection. Impaired TNFα and IL-1β production, may worsen disease progression in TB patients (Waitt, Banda et al. 2015), while CCL2 and TNFα enhance recruitment of immune cells to the site of Mtb infection (Hasan, Cliff et al. 2009). As we observed in Study I, Mtb infection resulted in a potent down-regulation of LL-37, however, LL-37 expression was still substantially higher in Mtb-infected $VitD_3$-polarized cells compared to M1 and M2 subsets. As mentioned above, TB patients expressed low levels of LL-37 in TB granulomas (Rahman, Rehn et al. 2015), while $VitD_3$ supplementation enhanced the production of LL-37 in blood cells (Mily, Rekha et al. 2015). Contrary to $VitD_3$-polarized cells, Mtb-infected M1 and M2 subsets exhibited high levels of IL-1RA and IDO but low

levels of LL-37. M2-polarized macrophages also expressed high IL-10 levels, but this was not changed with Mtb infection. As expected, the ratio between iNOS/Arg-1 expression was higher in M1- compared with M2-polarized macrophages (Flynn, Chan et al. 2011).

The immunosuppressive enzyme IDO, effectively diminish activation of Th1 cells by degradation of the essential amino acid tryptophan that may result in growth arrest of T cells as well as decreased activity of antigen presenting cells (APCs) (Harden and Egilmez 2012). As such, enhanced IDO activity in PBMCs from individuals vaccinated with a novel TB vaccine candidate was inversely correlated to $CD4^{+}IFN\gamma^{+}$ T cell responses (Tanner, Kakalacheva et al. 2014). Increased IDO expression has been detected in the macrophage rich areas of TB granulomas (Mehra, Alvarez et al. 2013) and high levels of IDO has also been found in patients with TB pleuritis (Suzuki, Miwa et al. 2013). Interestingly, inhibition of IDO in Mtb-infected non-human primates enhanced T cell proliferation and recruitment of effector T cells to the granulomas, which resulted in enhanced bacterial control (Gautam, Foreman et al. 2018). It has been reported that $VitD_3$ possess anti-inflammatory properties, which may protect the host from extensive tissue damage and inflammation (Harishankar, Anbalagan et al. 2016). Thus, $VitD_3$ may enhance innate antimicrobial responses but simultaneously contribute to responses that dampens inflammation but prevents immunosuppression. Further *in vitro* and *in vivo* studies will enhance our understanding of the role of $VitD_3$ on macrophage polarization and protective TB immunity.

4 CONCLUSIONS

Altogether, the key conclusions from this thesis work are listed below:

- PBA and/or $VitD_3$ can overcome Mtb-mediated suppression of LL-37 in human macrophages and instead induce LL-37 and LL-37-dependent activation of autophagy in Mtb-infected cells.

- PBA+$VitD_3$ treatment of macrophages infected with MDR-TB or drug-susceptible strains results in potent Mtb growth inhibition, especially in combination with high-dose INH, that is dependent on the expression of LL-37 but not iNOS/RNI or ROS.

- Marimastat, a global inhibitor of MMP activity, inhibits monocyte aggregation and subsequent TB granuloma formation, which reduce the intercellular spread of Mtb and the possibilities for the bacteria to propagate the infection.

- $VitD_3$-polarized macrophages express high levels of LL-37 but low levels of immunosuppressive IDO, which is associated with potent Mtb growth inhibition in Mtb-infected macrophages compared with conventional M1 and M2 subsets.

5 SUMMARY

As the emergence of drug-resistance is a common problem in many types of infections, it is crucial to find new alternative treatment strategies, also for TB including MDR-TB. Instead of developing new antibacterial drugs that the bacteria could develop resistance to, we should invest more research in finding compounds that could enhance protective innate or adaptive immune responses. This requires enhanced knowledge about the nature of protective TB immunity, to define what immune mechanisms are important for TB control and how these could be induced. In addition, we need to increase our understanding for the interactions between potential host-directed therapies and conventional antibiotics.

The current thesis demonstrate that Mtb can modulate macrophage responses to become less effective in intracellular bacterial killing. Imperative antimycobacterial effector mechanisms involved in growth inhibition of Mtb in human macrophages involves the antimicrobial peptide LL-37 and activation of autophagy. PBA+$VitD_3$-induced LL-37-dependent activation of autophagy, is mediated by the P2X7 receptor involving intracellular free calcium, and activation of the AMPK and PI3 kinase pathways. Potent induction of LL-37 expression was also found to be imperative in intracellular growth inhibition of MDR-TB as well as drug-susceptible Mtb strains. Instead, induction of RNI or ROS production in Mtb-infected macrophages seemed to be less important to control Mtb growth mediated by PBA+$VitD_3$.

Importantly, treatment of Mtb-infected cells with a combination of PBA and $VitD_3$ resulted in an additive effect on the induction of LL-37 and autophagy, which correlated with enhanced Mtb killing. Additive or synergistic effects between PBA and $VitD_3$ may be explained by the HDACi activity of PBA, which loosens up the chromatin and promotes binding of the intracellular $VitD_3$-VDR transcription complex that could result in enhanced transcription of $VitD_3$-target genes such as LL-37 and markers of autophagy. Moreover, our results support an additive effect also upon treatment of Mtb-infected cells with a combination of PBA+$VitD_3$ and high-dose INH. These results are promising for clinical implementation of PBA+$VitD_3$ as adjunctive treatment to strengthen the effect of short-course MDR-TB treatment along with high-dose INH as well as for difficult-to-treat XDR-TB patients when treatment options are scarce.

Our results also show that the lung tissue model provides a unique and novel system to study human TB infection in a physiologically relevant milieu. New insights into how macrophage polarization and bacterial virulence factors contribute to granuloma formation and control of Mtb infection may be key to develop new treatment strategies in TB. Mtb infection may

promote a mixed M1/M2 macrophage activation phenotype that may reduce the ability to control Mtb growth at the local site of infection. Our results also demonstrate that expression of MMPs are induced in the lung tissue model with a similar pattern to MMP expression observed in human lung tissue biopsies from active TB patients. Inhibition of MMP activity could block early TB granuloma formation and bacterial growth. This represents another potential mechanism that could be implemented as host-directed TB therapy, targeting the TB granuloma, aiming to inhibit tissue-degradation and bacterial spread.

6 FUTURE PERSPECTIVES

It is clear that antibiotic treatment could be improved by promising adjunct therapies that could boost protective immunity to enhance cure, reduce disease severity and side effects, while preventing the emergence of MDR-TB. Effective host-directed therapies should have the ability to target multiple immune pathways to induce specific antimicrobial effector functions and simultaneously prevent pathological inflammation. Attractive candidates are newly discovered small molecules or repurposed drugs (inexpensive alternatives such as PBA or $VitD_3$) that could promote synergistic or overlapping effects with first- or second line antibiotics.

Future challenges involve the design of randomized clinical trials to test new and old host-directed therapy concepts as adjunct treatment for TB, as is difficult to show potential effects of such therapies on top of the effects of conventional anti-TB drugs. Ultimately, personalized treatment options for TB may be tailored and open up the possibilities for using host-direct therapies particularly in patient with a poor prognosis such as MDR- or XDR-TB, cavitary TB, TB/HIV co-infected patients, TB patients with diabetes or TB patients with severe nutritional deficiencies i.e. with $VitD_3$. Furthermore, the prophylactic potential of host-directed therapies to prevent progression of active TB from latent TB, may be more relevant, but are currently logistically complex to investigate.

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