Mycobacterium tuberculosis and HIV coinfection

Effects on innate immunity and strategies to boost the immune response

Anna-Maria Andersson
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Cover: Confocal microscopy images of a macrophage that have ingested *Mycobacterium tuberculosis* (green) and remnants of apoptotic neutrophils (yellow).

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ABSTRACT

Tuberculosis (TB) still remains a big threat today, being the leading cause of death by a single infectious agent. The TB epidemic is fueled by HIV along with the increasing drug-resistance which prolongs the already long treatment duration and decreases the success rate for curing TB. In most cases an infection results in latency but HIV patients have a 20-30 times higher risk of developing active TB. There are around 36.9 million people living with HIV globally, with the highest burden in Africa. Although there are effective treatments against the disease, there is no cure for AIDS and the availability of the lifelong treatment is limited in low-income countries were the burden is highest. HIV infection causes an immunodeficiency characterized by the progressive loss of CD4 T cells which increases the risk of opportunistic infections, and infection by *Mycobacterium tuberculosis* (Mtb), the causative agent of TB. Mtb spreads through aerosols from one person with active tuberculosis to a healthy person. Upon inhalation the bacteria are phagocytosed by alveolar macrophages that secrete cytokines and chemokines to recruit more cells, such as dendritic cells, macrophages and lymphocytes, leading to the formation of a granuloma. During a single TB infection the bacteria are usually contained within the granuloma, but HIV can disrupt the stable granuloma, causing a rupture and dissemination of Mtb. This inflammatory site is also beneficial to HIV since it promotes replication of the virus within infected cells. HIV and Mtb are two successful intracellular pathogens able to avoid immune defense mechanisms both of the innate and adaptive immunity in order to persist and replicate. Their virulence factors can manipulate or inhibit cell signaling, phagosome maturation, autophagy, ROS production, apoptosis and antigen presentation, to promote survival. Boosting of immune defenses with host-directed therapies (HDT) has been proposed as a treatment strategy against TB, either alone or adjunctive to the current regimen.

In this thesis, ways to boost the innate immune responses in Mtb and HIV coinfected macrophages were investigated, along with studies of the effect of HIV on Mtb antigen presentation in coinfected dendritic cells. The initial hypothesis was that autophagy induction through inhibition of mammalian target of rapamycin (mTOR) could suppress Mtb growth in HIV coinfected macrophages. However, during a low grade infection, autophagy induction increased Mtb replication due to a decreased autophagic flux and acidification of Mtb phagosomes. A general autophagic flux was induced, although not localized to the Mtb phagosomes, thus not inducing a xenophagy (autophagy of intracellular pathogens). Other ways of inducing autophagy or boosting the response in coinfected macrophages might be more beneficial and therefore the effect of efferocytosis was investigated. Uptake of apoptotic neutrophils by coinfected macrophages did not induce autophagy but enhanced the control of Mtb by other means. Upon efferocytosis, the macrophages acquired active myeloperoxidase (MPO) from the neutrophils that suppressed Mtb growth. The coinfected macrophages also produced more ROS after efferocytosis. The inhibition of Mtb growth could thus be mediated by MPO and the increased ROS production either directly or indirectly.
The possibility to boost the innate immunity could prove to be important during an HIV coinfection, when the adaptive immunity is deficient. In addition to the well-known decline in CD4 T cells during the course of HIV progression, we found that HIV infection of dendritic cells inhibited antigen presentation by suppressing the expression of HLA-DR and co-stimulatory molecules on coinfected dendritic cells. Furthermore, HIV reduced secretion of pro-inflammatory cytokines and suppressed antigen processing through inhibition of autophagy. This impaired antigen presentation in coinfected dendritic cells resulted in a decreased activation and response of Mtb-specific CD4 T cells.

In conclusion, this thesis shows how HIV can manipulate antigen presentation in Mtb coinfected dendritic cells and subsequently inhibit the adaptive immune response. It also contributes to insights on how efferocytosis of apoptotic neutrophils can boost the innate immune responses during coinfection. Lastly, autophagy induction through mTOR inhibition does not enhance protection against TB. Induction of autophagy should therefore be handled with care, particularly during HIV coinfection.

Både HIV och tuberkelbakterien är framgångsrika patogener som kan överleva genom att manipulera olika processer och signalvägar i immunceller. Exempel på försvarsmekanismer i infekterade immunceller är fagosomal mognad och autofagi som båda resulterar i en sänkning av pH inuti vakuolen där patogenen befinner sig (fagosomen). Dessutom kan immuncellen initiera en kontrollerad celldöd (apoptos) och dessa döda celler kan sedan tas om hand av andra immunceller, i en process som kallas efferocytos. Aktivering av det adaptiva immunförsvarset genom antigenpresentation är ytterligare en försvarsmechanism för att eliminera patogener. Försök att stimulera dessa immunreager skulle kunna förbättra avdödandet av tuberkelbakterien, och kan vara ett bra behandlingsalternativ, särskilt i kombination med nuvarande antibiotikabehandling. 

I denna avhandling har syftet varit att undersöka olika sätt att stimulera det medfödda immunförsvarset och utreda hur HIV kan påverka immunceller för att minska immunsvaret mot tuberkelbakterien. Autofagstimulering studerades som ett sätt att förbättra eliminering av tuberkelbakterien i HIV/Mtb dubbelinfekterade makrofager. Generell autofagstimulering ökade inte eliminationen av tuberkelbakterien utan resulterade i ökad tillväxt av bakterien på grund av hämmad surgörning i fagosomen. Vi undersökte även om autofagstimulering kan åstadkommas på andra mer förmånliga sätt, såsom vid efferocytos. Apoptotiska immunceller (neutrofila granulocyter) som togs upp av HIV/Mtb dubbelinfekterade makrofager hämmade tillväxten av tuberkelbakterien, men utan att stimulera autofagi. Dessa celler producerade
istället mer syreradikaler med hjälp av ett enzym från granulocyterna och stimulerade därmed avdödning av tuberkelbakterien i HIV/Mtb dubbelinfekterade makrofager. Denna möjlighet till förstärkning av det medfödda immunförsvar mot tuberkelbakterien kan vara särskilt viktig vid samtidig HIV infektion när det adaptiva immunförsvarvet är bristfälligt.

Utöver den välkända progressiva förlusten av T celler, upptäcktes även att HIV kan manipulera andra immunceller (dendritiska celler) och därmed minska det adaptiva immunförsvar mot tuberkelbakterien. Dendritiska cellers uppgift är att presentera antigen (del av patogen som kan stimulera ett immunsvar) för celler tillhörande det adaptiva immunförsvarvat (T celler). HIV hämmade denna antigenpresentation genom att dämpa autofagiprocessen, i den dendritiska cellen. Detta resulterade i ett minskat T cell svar mot tuberkelbakterien.

Sammanfattningsvis så innehåller denna avhandling information om hur HIV kan hämma antigenpresentation i infekterade dendritiska celler, och därmed dämpa det adaptiva immunförsvar mot tuberkelbakterien. Avhandlingen bidrar också till insikter i hur det medfödda immunförsvarvet kan förstärkas för att öka skyddet mot tuberkelbakterien och varför stimulering av autofagi bör undvikas vid HIV/Mtb dubbelinfektion.
LIST OF PAPERS

Paper I


Paper II


Paper III

Andersson AM, Larsson M, Stendahl O, Blomgran R. The enhanced control of *Mycobacterium tuberculosis* in HIV coinfected macrophages by apoptotic neutrophils is myeloperoxidase dependent. Submitted
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABAH</td>
<td>4-Aminobenzoic acid hydrazide</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>ART</td>
<td>Antiretroviral therapy</td>
</tr>
<tr>
<td>Atg</td>
<td>Autophagy related</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacille Calmette-Guérin</td>
</tr>
<tr>
<td>CFP-10</td>
<td>10 kDa culture filtrate protein</td>
</tr>
<tr>
<td>cGAS</td>
<td>Cyclic GMP-AMP synthase</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptor</td>
</tr>
<tr>
<td>CR</td>
<td>Complement receptor</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin</td>
</tr>
<tr>
<td>EEA1</td>
<td>Early endosome antigen 1</td>
</tr>
<tr>
<td>Eis</td>
<td>Enhanced intracellular survival</td>
</tr>
<tr>
<td>ESAT-6</td>
<td>6 kDa early secreted antigenic target</td>
</tr>
<tr>
<td>ESX-1</td>
<td>ESAT-6 secretion system-1</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>fMLP</td>
<td>N-formyl-methionyl-leucyl-phenylalanine</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IRIS</td>
<td>Immune reconstitution inflammatory syndrome</td>
</tr>
<tr>
<td>LAM</td>
<td>Lipoarabinomannan</td>
</tr>
<tr>
<td>LAMP</td>
<td>Lysosomal-associated membrane proteins</td>
</tr>
<tr>
<td>LC3</td>
<td>Microtubule-associated protein light chain 3</td>
</tr>
<tr>
<td>LM</td>
<td>Lipomannan</td>
</tr>
<tr>
<td>LpdC</td>
<td>Lipoamide dehydrogenase C</td>
</tr>
<tr>
<td>ManLAM</td>
<td>Mannose-capped lipoarabinomannan</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MDR-TB</td>
<td>Multi-drug resistant TB</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>Mincle</td>
<td>Macrophage-inducible C-type lectin</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>MR</td>
<td>Mannose receptor</td>
</tr>
<tr>
<td>Mtb</td>
<td><em>Mycobacterium tuberculosis</em></td>
</tr>
<tr>
<td>MTBC</td>
<td><em>Mycobacterium tuberculosis</em> complex</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response protein 88</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>Ndk</td>
<td>Nucleoside diphosphate kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
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<tr>
<td>Nef</td>
<td>Negative regulatory factor</td>
</tr>
<tr>
<td>NETs</td>
<td>Neutrophil extracellular traps</td>
</tr>
<tr>
<td>NFKB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide oligomerization domain-like receptor</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non-nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide oligomerization domain</td>
</tr>
<tr>
<td>NOX</td>
<td>Nicotinamide adenine dinucleotide phosphate oxidase</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside reverse transcriptase inhibitors</td>
</tr>
<tr>
<td>PDIM</td>
<td>Phthiocerol dimycocerosates</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PI3P</td>
<td>Phosphatidylinositol-3-phosphate</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>POA</td>
<td>Pyrazinoic acid</td>
</tr>
<tr>
<td>PPD</td>
<td>Purified protein derivative</td>
</tr>
<tr>
<td>PR</td>
<td>Protease</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>RD1</td>
<td>Region of difference 1</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SR</td>
<td>Scavenging receptor</td>
</tr>
<tr>
<td>SQSTM1</td>
<td>Sequestosome 1</td>
</tr>
<tr>
<td>T7SS</td>
<td>Type VII secretion systems</td>
</tr>
<tr>
<td>TACO</td>
<td>Tryptophan-aspartate containing coat protein</td>
</tr>
<tr>
<td>Tat</td>
<td>Trans-activator of transcription</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TDM</td>
<td>Trehalose dimycolate</td>
</tr>
<tr>
<td>Th cell</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRAIL-R</td>
<td>TNF-related apoptosis-inducing ligand receptor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>ULK1</td>
<td>Unc-51-like kinase 1</td>
</tr>
<tr>
<td>Vif</td>
<td>Virion infectivity factor</td>
</tr>
<tr>
<td>Vpr</td>
<td>Viral protein R</td>
</tr>
<tr>
<td>Vpu</td>
<td>Viral protein unique</td>
</tr>
<tr>
<td>XDR-TB</td>
<td>Extensively drug-resistant tuberculosis</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>i</td>
</tr>
<tr>
<td>POPULÄRVETENSKAPLIG SAMMANFATTNING</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF PAPERS</td>
<td>v</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>vi</td>
</tr>
<tr>
<td>BACKGROUND</td>
<td>1</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>1</td>
</tr>
<tr>
<td>Epidemiology</td>
<td>1</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>1</td>
</tr>
<tr>
<td>Virulence factors</td>
<td>2</td>
</tr>
<tr>
<td>Pathogenesis</td>
<td>2</td>
</tr>
<tr>
<td>Symptoms and diagnosis</td>
<td>4</td>
</tr>
<tr>
<td>Treatment strategies</td>
<td>5</td>
</tr>
<tr>
<td>Pathogenesis</td>
<td>6</td>
</tr>
<tr>
<td>The HIV particle</td>
<td>7</td>
</tr>
<tr>
<td>HIV life cycle</td>
<td>8</td>
</tr>
<tr>
<td>Treatment and prevention</td>
<td>8</td>
</tr>
<tr>
<td>HIV and Mycobacterium tuberculosis coinfection</td>
<td>9</td>
</tr>
<tr>
<td>Innate immune cells</td>
<td>11</td>
</tr>
<tr>
<td>Macrophages</td>
<td>11</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>11</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>12</td>
</tr>
<tr>
<td>Sensing of Mtb/HIV and signaling responses</td>
<td>12</td>
</tr>
<tr>
<td>Phagocytosis and phagosome maturation</td>
<td>14</td>
</tr>
<tr>
<td>Autophagy</td>
<td>15</td>
</tr>
<tr>
<td>Reactive oxygen species</td>
<td>17</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>19</td>
</tr>
<tr>
<td>Activation of adaptive immunity</td>
<td>20</td>
</tr>
<tr>
<td>Antigen presentation</td>
<td>20</td>
</tr>
<tr>
<td>Effects of Mtb and HIV on antigen presentation</td>
<td>21</td>
</tr>
<tr>
<td>T cell responses</td>
<td>22</td>
</tr>
<tr>
<td>AIMS</td>
<td>25</td>
</tr>
</tbody>
</table>
RESULTS .......................................................................................................................... 27

Paper I .................................................................................................................................. 27

*Autophagy induction targeting mTORC1 enhances Mycobacterium tuberculosis replication in HIV co-infected human macrophages* .......................................................................................................................... 27

Paper II ................................................................................................................................ 29

*HIV interferes with Mycobacterium tuberculosis antigen presentation in human dendritic cells* .... 29

Paper III .................................................................................................................................. 31

*The enhanced control of Mycobacterium tuberculosis in HIV coinfected macrophages by apoptotic neutrophils is myeloperoxidase dependent* .................................................................................................................. 31

DISCUSSION ......................................................................................................................... 33

GENERAL CONCLUSIONS ................................................................................................. 37

REFERENCES ....................................................................................................................... 39

ACKNOWLEDGEMENTS ....................................................................................................... 57
**BACKGROUND**

**Tuberculosis**

Tuberculosis (TB) is an ancient disease that has caused several epidemics throughout history, with signs of the disease found in Egyptian mummies. The genus *Mycobacterium* is believed to have existed for about 150 million years, with *Mycobacterium tuberculosis* (Mtb) originating around 15 000 years ago. In 1882, the German microbiologist Robert Koch was the first to discover the causative agent of TB, which was later named *Mycobacterium tuberculosis*. Although being an old disease it took a long time before the realization that it was a transmittable disease which was caused by a bacteria. With these important findings the spread of TB could be prevented and was halted further by the discovery of a vaccine that was started being used in children in 1921. Albert Calmette and Camille Guérin successfully attenuated *Mycobacterium bovis* to create this vaccine called bacille Calmette-Guérin (BCG). Later came the discovery of treatments for tuberculosis with the antibiotics streptomycin in 1944 and isoniazid in 1952. (1). However, TB remains a big threat fueled by the HIV epidemic and the growing drug resistance problem.

**Epidemiology**

According to the latest TB report from the World Health Organization (WHO), TB is the top ten cause of death worldwide and the leading cause of death by a single infectious agent. 1.7 billion people are estimated to be infected with TB, of which only around 5-10% will develop active disease. The biggest risk factor for developing active TB is HIV (20-30 times higher risk), but other examples are undernutrition, diabetes, smoking and alcohol consumption. In 2017 an estimate of 10 million people fell ill with TB and 1.3 million people died of the disease with additionally 300 000 deaths among HIV coinfected people. The following countries had the highest incidence rates: India, China, Indonesia, the Philippines, Pakistan, Nigeria, Bangladesh and South Africa, which together accounted for two thirds of the new TB cases in 2017. Furthermore the multi-drug resistant TB (MDR-TB) burden is highest in India, China and the Russian Federation and around 8.5% of MDR-TB cases had extensively drug-resistant TB (XDR-TB) in 2017 (2).

**Mycobacterium tuberculosis**

There are several species of *Mycobacterium* of which most are environmental bacteria. Human tuberculosis is mainly caused by members of the *Mycobacterium tuberculosis* complex (MTBC) but in immune-compromised individuals also non-tuberculous mycobacteria can cause disease. MTBC can be divided into human-adapted MTBC and animal-adapted MTBC. The human-adapted MTBC consists of *M. tuberculosis sensu stricto* (with the phylogenetic linages L1, L2, L3, L4, L7) and *M. africanum* (with linages L5 and L6). Examples of
some members of animal-adapted MTBC are *M. bovis*, *M. caprae*, *M. microti* and *M. pinnipedii* (3).

*Mtb* is a slow-growing bacterium with a generation time of around 24h, forming colonies on agar after around 3-4 weeks. *Mtb* is a rod-shaped non-motile intracellular bacteria about 1-4µm in length. It has a robust and thick cell wall that shields it from the environment and limits drug permeability. Due to its unique cell wall, *Mtb* is classified as an acid fast bacterium, meaning that it resists decolorization by acidic alcohol and can be visualized through Ziehl–Neelsen acid-fast staining (4). The complex cell envelope consists of a bilayer phospholipid plasma membrane surrounded by a layer of peptidoglycans that attaches the arabinogalactans that are linked with mycolic acid. The envelope is surrounded by a capsule mainly containing polysaccharides. The mycolic acids are responsible for the low permeability of the cell envelope and are essential for *Mtb* survival (5).

**Virulence factors**

Lipomannan (LM), lipoarabinomannan (LAM) and mannose-capped lipoarabinomannan (manLAM) are lipoglycans within the cell wall of *Mtb* which are important for virulence. ManLAM can bind to mannose receptors on macrophages and dendritic cells and DC-SIGN on dendritic cells and can promote the uptake of *Mtb* but also inhibit a pro-inflammatory response and phagosomal maturation (5). Furthermore, the cell wall lipid phthiocol dimycocerosates (PDIM) has been shown to contribute to phagosomal escape of *Mtb* (6). Apart from components of the cell wall, *Mtb* also secrete some virulence factors. The genome of mycobacteria encodes for five type VII secretion systems (T7SS) of which the ESAT-6 secretion system-1 (ESX-1) is the most important, secreting the two major virulence factors; 6 kDa early secreted antigenic target (ESAT-6) and 10 kDa culture filtrate protein (CFP-10) that together form a heterodimer. ESX-1 is encoded by the genetic locus region of difference (RD1) which is deleted in the vaccine strain *Mycobacterium bovis* bacille Calmette-Guérin (BCG) and other attenuated strains of *Mtb*. The secretion of ESAT-6 is essential for virulence of *Mtb* and has been associated with phagosomal escape, cytolysis, necrosis and apoptosis (7). ESX-1 is also important for blocking phagosomal acidification and impairing autophagic flux. Other effectors that also have been associated with blocking of phagosomal maturation are PtpA, SapM and EsxH (8).

**Pathogenesis**

*Mtb* spreads through aerosols from cough of an individual with active tuberculosis to a new host that gets infected upon inhalation. *Mtb* first encounter alveolar macrophages that recognize the bacteria through different surface receptor such as scavenging receptors (SR), complement receptors (CR) and C-type lectin receptors (CLRs). These receptors mediate the uptake of the bacteria and initiate signaling pathways leading to production of cytokines and chemokines which promotes recruitment of more cells (9,10). The alveolar macrophages are not well equipped to control *Mtb* alone but by recruiting more macrophages, and other cells
such as dendritic cells (DCs) and lymphocytes to the site of infection the bacteria can be contained through the formation of a granuloma (9). The granuloma is an organized structure of cells which is the hallmark of tuberculosis. Typically, macrophages are localized in the middle together with some dendritic cells and neutrophils, which are all surrounded by lymphocytes (mainly T cells). The environment inside the granuloma is deprived of both oxygen and nutrients. The granulomas are very heterogeneous even within the same individual, but can be divided into three types; solid granulomas which contain Mtb (latency), necrotic granulomas that are found during early stages of active disease, and caseous granulomas found during severe tuberculosis when the bacteria disseminates (11,12). Solid granulomas are well organized and surrounded by a fibrotic wall, within which the Mtb burden is low. In necrotic granulomas the infected cells in the center of the granuloma have become necrotic forming a necrotic zone called caseum. Upon progression into caseous granulomas, the center becomes liquefied and the oxygen and nutrient supplies are reestablished promoting growth of Mtb (11).

Most of the macrophages involved in granuloma formation are the differentiated epithelioiid macrophages. Furthermore, another characteristic cell type often found in granulomas are multinucleated giant cells (MGCs), which are formed when several macrophages fuse together. The MGCs are not able to phagocytose bacteria, but still has the capacity to present antigens (13). Mtb also induce the differentiation of macrophages into foamy macrophages, which accumulate lipids within intracellular lipid bodies or droplets. These cells also phagocytose poorly and lack bactericidal activities, instead it is believed that they provide Mtb with nutrients and allow the bacteria to persist in a dormant state (14). Although macrophages are abundantly found in granulomas, neutrophils are also recruited as the first line of defense. They get activated by LAM of Mtb and respond by producing oxygen radicals and secreting chemokines to recruit more leukocytes and to organize the granuloma. In addition to macrophages and neutrophils, dendritic cells are also found in granulomas and although these cells are less effective at phagocytosing and killing Mtb than macrophages, they control the infection through activation of lymphocytes. After ingestion of Mtb, the dendritic cells travel from the granuloma to the lymph nodes where they present antigens to T cells to mount a T-cell response. T cells are very important for the organization of the granuloma as well as for the control of the bacteria. The T cells can activate the macrophages through the secretion of cytokines (for example IFN-γ), making them better at controlling the infection (15).

There is still a question of whether or not the granuloma favors the host or the bacteria. On one hand Mtb is contained by the granuloma and the bacterial replication is suppressed, but on the other hand the bacteria have adapted to this environment and are able to persist for decades and can eventually cause a rupture leading to bacterial dissemination. In most cases the infection results in asymptomatic latency and the granuloma remains intact, however in 10% of the cases the granuloma gets compromised, leading to active tuberculosis (9,16). HIV patients have a higher risk of developing active disease due to the immunosuppression (2). It is believed that the lack of oxygen and nutrients in solid granulomas forces Mtb into dormancy
and that this non-replicating state is especially difficult to treat since most antibiotics target the replication machinery and thus a long treatment period is required (17).

Figure 1. **Solid granuloma.** The hallmark of tuberculosis is the granuloma, an organized structure of cells within which Mtb is contained. The innate immune cell types within the granuloma are macrophages, epithelioid macrophages, foamy macrophages, multinucleated giant cells, neutrophils and dendritic cells. These cells are surrounded by lymphocytes and an outer fibrotic wall.

**Symptoms and diagnosis**

TB can be asymptomatic, with more symptoms as the disease progresses. The initial symptoms can be fever, night sweats, fatigue and weight loss. Later the patient can develop a cough and mild hemoptysis, and in severe cases shortness of breath and chest pain (18). In order to treat the disease, a proper diagnosis has to be made to detect it. The diagnosis options for low-income countries where the TB burden is highest are limited. These countries mainly rely on the cheap and quick sputum smear microscopy, making it difficult to detect early stages of TB. However, additional diagnostic methods have been developed. WHO has recommended the rapid molecular test called Xpert MTB/RIF assay which gives the results within two hours in addition to detecting rifampicin resistance and being more accurate than sputum smear microscopy for diagnosis. The use of Xpert MTB/RIF has expanded greatly since 2010 when it was first recommended and it has great advantages to other tests such as the reference standard culture-based methods, which take up to 12 weeks for results (2). Another test is the tuberculin skin test (TST) that is based on the injection of purified protein derivative (PPD) into the skin which causes a hypersensitivity reaction within 48-72 hours in people with a mycobacterium-specific immunity. This test is used for diagnosis of latent TB, but with limited specificity since it cannot distinguish Mtb from other mycobacteria such as BCG (19). The TST could give a false negative result due to poor sensitivity in immune-compromised
individuals for example in HIV infected patients. Another more sensitive and specific test for latency, based on an individual’s immune reaction is the interferon-γ release assay (IGRA), which measures the interferon-γ levels upon Mtb antigen (ESAT-6 and CFP-10) stimulation of mononuclear cells of the peripheral blood (20).

Treatment strategies

The treatment of TB includes a combination of the four first-line drugs isoniazid, rifampicin, ethambutol and pyrazinamide during two months followed by a four months treatment with only isoniazid and rifampicin (2,21). This treatment is successful for at least 85% of the cases with drug-susceptible TB. Drug resistant TB, including multidrug-resistant (MDR) or extensively drug-resistant (XDR) TB, requires a longer treatment period with more toxic drugs and for MDR-TB the success rate is only about 55% (2).

The effective TB drugs isoniazid and ethambutol target the cell wall of Mtb, more specifically the synthesis of the mycolic acids and arabinogalactan, respectively (22). Rifampicin blocks mRNA synthesis and subsequent protein synthesis by inhibiting the β-subunit of the DNA-dependent RNA polymerase of Mtb. Resistance is caused by mutations in the β-subunit, making it impossible for rifampicin to bind to it. Pyrazinamide is an important drug in the regimen since it kills semi-dormant Mtb that resides in an acidic environment, where other TB drugs cannot act. Pyrazinamide passively diffuses into the bacteria, where it is converted into its active form pyrazinoic acid (POA) by an Mtb enzyme that if mutated causes resistance. The POA is expelled out of the bacteria and can bind protons in the acidic environment that it can bring into the bacteria, causing acidification inside of Mtb, leading to inhibition of vital enzymes and disruption of membrane function (23).

There is currently only one existing vaccine, called BCG, but it only protects against severe childhood TB and not against pulmonary TB in adults (2). A new and improved vaccine is therefore warranted. Furthermore, the increasing drug resistance seems to be progressing faster than the development of new antibiotics and this has made researchers focus on how to improve the host immune response. Host-directed therapies (HDTs) might be a promising strategy to fight off the infection, either alone or as adjunctive treatment to the current regimen. HDTs can for example include drugs that either boost the immune defenses against TB, reduce the inflammatory response and tissue damage, or interfere with host mechanisms that are exploited by Mtb. Boosting of macrophage responses may include inducing production of free radicals, antimicrobial peptides, cytokines/chemokines, or inducing processes such as apoptosis, phagosomal maturation or autophagy (24). Examples of HDTs that have shown positive results in vitro or in mouse or zebrafish models are drugs that target the granuloma to increase antibiotic access, drugs that induce autophagy, decrease inflammation, or enhance T cell responses, or antibodies that target Mtb specifically. However, clinical studies using HDT agents such as vitamin D, the corticosteroid prednisolone or IFN-γ have shown controversial results (25) and the pursuit for effective HDTs continues. In the pipeline right now is a phase 2 trial (NCT02968927) investigating efficacy of adjunctive therapy with the TB HDT candidates Everolimus (mTOR inhibitor), Auranofin (antirheumatic
drug), Vitamin D3 and the novel anti-inflammatory compound CC-11050. Furthermore, the adjunctive potential of ibuprofen (NCT02781909), N-acetyl cysteine (NCT03702738) and Pravastatin (NCT03456102) are also started to be investigated in clinical trials for treatment of TB.

**HIV**

*Pathogenesis*

Human immunodeficiency virus (HIV) is the cause of acquired immune deficiency syndrome (AIDS) which was first recognized in the 1980s. The first cases of the disease appeared in 1981 but the causative virus was not discovered until 1983 (26,27). AIDS is a severe disease that if left untreated leads to death, often by an opportunistic infection or *Mycobacterium tuberculosis* infection (28). HIV can be found in several body fluids of an infected individual, for example in blood, semen, vaginal and rectal secretions and in breast milk. The most common route of HIV transmission is through sexual intercourse but the virus can also be transmitted from mother to infant, or through blood upon needle sharing (29). During acute HIV infection, the risk of transmission is high due to the very high replication of the virus, peaking at around 2-4 weeks after infection. Patients can be experiencing unspecific symptoms such as headache, fever and rashes. This is followed by the chronic stage which can persist for years and be asymptomatic. It is characterized by lower virus replication and antibody production along with a gradual decline of CD4 T cells. The last stage, AIDS is characterized by a further reduction in CD4 T cells together with high viral load, causing an immunodeficiency that increases the risk of opportunistic infections. Diagnosis and monitoring of the disease is based on measurement of HIV antibodies, p24 antigen, viral load in plasma and CD4 count (28).

CD4 T cells are the primary target cells for HIV, but the virus can also infect other CD4-positive cells such as macrophages, dendritic cells and astrocytes (30). In addition to binding of CD4, the virus also binds to a chemokine co-receptor, either CCR5 or CXCR4, depending on the strain of HIV. R5 (M-tropic) virus is present during the whole course of the infection and typically binds to CCR5 on monocytes/macrophages and T cells, but cannot infect T cell lines since they only have CXCR4. X4 (T-tropic) virus, which is typically more prominent during the later stages of infection and is associated with disease progression, normally binds to CXCR4 on T cells and T cell lines. Whether or not this X4 virus can infect macrophages (which also express CXCR4) is controversial. Dual tropic virus (R5X4), as the name implies, can bind to both co-receptors. The virus can also switch from R5 to R5X4 or X4 as the disease progresses. T cells can be infected by any strain of HIV since they express both CCR5 and CXCR4. While CCR5 is mainly expressed on memory T cells, CXCR4 expression is more widespread but is mainly found on naïve T cells (31).

Immature dendritic cells at the mucosa, called Langerhans´ cells, are believed to be the first cells to encounter HIV. They are important for the transfer of the virus to T cells, by migrating to the lymph nodes (31). Upon HIV transmission at the genital mucosa, the virus can either
be internalized by Langerhans’ cells or it can directly infect intraepithelial CD4 T cells. The free virus or the infected cells can then make their way into the stroma where they either migrate to the lymph node or blood circulation, or make contact with dendritic cells, T cells and macrophages. The virus can either directly infect dendritic cells, become surface-bound to a C-type lectin receptor such as DC-SIGN or upon DC-SIGN binding become internalized into the endocytic compartment of these cells. In any case, the resulting accumulation of virus from dendritic cells efficiently passes to CD4 T cells across an infectious synapse. The interaction between the dendritic cell and the CD4 T cell also results in activation of the CD4 T cell, promoting gene transcription and HIV replication (32).

**The HIV particle**

The HIV virus particle is around 100 nm in diameter and consists of an envelope (env) with an associated matrix which surrounds the capsid that protects the inner core. The inner core contains two copies of single-stranded RNA genome, polymerase and viral enzymes (30,33). HIV belongs to the genus Lentivirus, within the family of Retroviridae meaning that the virus is a retrovirus which viral RNA is transcribed into DNA by the action of a reverse transcriptase (30). This transcription from RNA to DNA was first discovered in 1970 simultaneously and independently by Howard Temin and David Baltimore (34), having an important impact on future understanding of the HIV life cycle along with treatment development.

![Figure 2. The HIV particle.](image)

The glycoproteins gp120 and gp41 are attached to the lipid bilayer creating the envelope of the HIV particle. Underneath the envelope is the matrix protein p17 which surrounds the capsid that contains enzymes and the genome consisting of two copies of single-stranded RNA molecules.
**HIV life cycle**

HIV enters the target cell by binding its glycoprotein gp120 of the envelope to the cell surface receptor CD4. Upon binding, the gp120 becomes rearranged, allowing subsequent binding to the chemokine co-receptors CCR5 or CXCR4. This is followed by the insert of the transmembrane glycoprotein gp41 of the envelope into the plasma membrane, creating a pore where the viral envelope and the plasma membrane fuses, allowing the viral capsid to enter the cytoplasm (33,35,36). Once the virion is uncoated, its RNA genome is reversed transcribed into double-stranded complementary DNA (cDNA) by viral reverse transcriptase (RT) and is transported into the nucleus by the help of viral protein R (Vpr). Once in the nucleus the cDNA gets incorporated into the host genome by the viral enzyme integrase (IN). In this stage the virus can remain latent, but upon activation of the host cell and induction of cell division, also the viral genome will be transcribed. The HIV genome carries nine genes that encodes for 19 proteins that can be divided into three classes; major structural proteins (encoded by Gag, Pol and Env), regulatory proteins (encoded by Tat (trans-activator of transcription) and Rev) and accessory proteins (encoded by Vif (virion infectivity factor), Vpr, Vpu (viral protein unique) and Nef (negative regulatory factor)). The mRNA translated proteins, along with newly transcribed copies of genomic RNA, all migrate to the cell surface for assembly of new virus particles. These viral particles bud off the plasma membrane after viral glycoproteins gp120 and gp41 have been incorporated, providing the lipid envelope. The final step is maturation of the virus particle, exerted by viral proteases (PR) that cleaves HIV Gag and Gag-Pol polyproteins into matrix protein (MA), capsid (CA), nucleocapsid (NC), PR, RT and IN, making the virus ready to infect new cells (33,36). It takes around 24 hours from that the HIV binds to the CD4 receptor until new virus particles are produced and released from the cell. However in long living cells like macrophages or memory T cells the virus can remain latent for years and only upon activation of these cells, infectious HIV can be produced (30). In tissues such as brain, lungs and liver, macrophages are the main reservoirs for HIV (31). Infected T helper (Th) cells on the other hand, have a shorter life span and are either lysed by the virus or by cytotoxic T lymphocytes (CTL), leading to the decrease of these cells and subsequent immunodeficiency (30).

**Treatment and prevention**

The first drug against HIV called zidovudine (ZDV) or azidothymidine (AZT) started being used already in 1987 and although effective, this monotherapy soon resulted in HIV resistance. The improved prognosis of the disease was only accomplished later with a treatment strategy that included several efficient drugs used in combination (27). Although effective antiretroviral therapy (ART) is available against HIV, the treatment is not a cure and cannot kill the virus but only suppresses it and therefore requires life-long treatment. ART can however increase the number of CD4 T cells in the blood and reduce the viral load, thereby greatly reducing the risk
of transmission and the patients can have a rather normal life on ART. ART targets several steps of the HIV life cycle in order to prevent viral replication (37). The standard regimes consist of 3 or more drugs in combination, including two nucleoside reverse transcriptase inhibitors (NRTI) together with a non-nucleoside reverse transcriptase inhibitor (NNRTI), protease inhibitor or integrase inhibitor, all disrupting different parts of the HIV life cycle. WHO are now, as first line regimen, recommending using two NRTI (Tenofovir and Lamivudine) together with the integrase inhibitor Dolutegravir as preferred to the previously used NNRTI Efavirenz (38). Within three months of treatment most patients have a great decrease in plasma viral load down to below the detection limit (37).

HIV infection is most common in Africa where 25.7 million people out of globally 36.9 million people are living with HIV (in 2017). Furthermore, the access to ART is limited especially in developing countries and in 2017 only 59% of the people globally living with HIV (21.7 million) received the treatment. This is a major issue since without ART, the infection easily transmits to healthy people, causing 1.8 million people to be newly infected and 0.9 people to die of the disease in 2017 (39). Therefore, prevention strategies has been employed in order to decrease the risk of transmission. Some examples are the use of male and female condoms, testing for HIV, voluntary medical male circumcision, antiretroviral medicines as pre-exposure prophylaxis (PrEP) for HIV-negative partners and post-exposure prophylaxis (PEP) within 72h of exposure to prevent infection. Prevention of mother to child transmission during pregnancy, labour, delivery or breastfeeding is achieved through antiretroviral drugs provided to both the mother and the baby (40).

Although effective treatment and prevention strategies, HIV remains a big threat due to the life-long treatment and the limited availability of ART and thus the best hope for eradicating the disease would be the development of a vaccine.

**HIV and Mycobacterium tuberculosis coinfection**

The HIV-caused immunosuppression creates a high risk for coinfections, most commonly with Mtb, either through reactivating latent TB or acquiring a new infection. The reduction in CD4 T cells caused by the virus likely contributes to the susceptibility to TB since these T cells are important in the control of TB. It is well-known that HIV impairs the control of Mtb infection and that active TB increases the risk of death in HIV infected individuals (41–43). An HIV co-infection affects the TB granuloma formation and appearance, seen by a reduction in lymphocytes and epithelioid macrophages along with larger areas of necrosis and higher Mtb burden (44). However, it’s not only HIV that can promote replication of Mtb, but Mtb can also induce HIV replication and thus the coinfection accelerates the progression of both diseases. This Mtb-induced HIV replication was shown to be dependent on a simultaneous pro-inflammatory response (45), showing that activated cells support HIV replication. Further studies have confirmed an increase in cytokines in the pleural fluid compared to plasma of co-infected individuals and also discovered a higher viral replication in macrophages and lymphocytes in these Mtb located sites (46–48). HIV has also been shown to preferentially
replicate in Mtb specific CD4 T cells, leading to the depletion of these cells (49). In summary, the pro-inflammatory environment in Mtb located sites increases the replication of HIV, leading to a reduction in CD4 T cells with subsequent disruption of the granuloma and reduced control of Mtb, resulting in dissemination of both diseases.

Upon ART treatment the number of central and naïve CD4 T cells increase, along with Mtb specific T cells (50). However this treatment may not always be beneficial during coinfection and can lead to exacerbation of TB, resulting in the immune reconstitution inflammatory syndrome (IRIS). IRIS can be divided into two groups; paradoxical TB IRIS (patients receive TB treatment before ART) or unmasking TB IRIS (patients with latent or undiagnosed TB that becomes active upon receiving ART). The TB symptoms are believed to be enhanced by the dysregulated recovering immune responses with exaggerated inflammation and sustained Th1 responses against Mtb antigens, following ART (51,52). The granulomas can get disrupted by this, since they are dependent on a balance of pro- and anti-inflammatory cytokines for optimal control of Mtb.

In addition to infecting and decreasing CD4 T cell numbers, HIV can also infect macrophages and affect their function, reducing their phagocytic capacity by the action of Nef and inhibiting phagosomal maturation through Vpr (53–55). Thus, macrophages are a reservoir for both HIV and Mtb and the manipulation of these cells by either one of the pathogens can promote each other’s replication during coinfection (56). The HIV protein Nef can for example inhibit apoptosis of Mtb infected macrophages (57), preventing engulfment of apoptotic bodies by surrounding macrophages that could kill the bacteria and present antigens to T cells to promote a T cell response (described later). Furthermore coinfection with Mtb leads to the activation of macrophages, resulting in production of pro-inflammatory cytokines and chemokines that are sustained by HIV inhibition of IL-10, causing a failure of immunoregulation (58,59). The cytokines and chemokines recruit and activate T cells, providing another niche for HIV to replicate within (59). Pro-inflammatory cytokines such as IL-6 and TNF-α promote HIV replication (60), through activation of the transcription factor NF-κB that promotes transcription of the HIV integrated genome, leading to replication of HIV (59). Furthermore Mtb can increase the expression of CXCR4 in alveolar macrophages, favoring entry and replication of X4 viruses (61), as well as increase CXCR4 and CCR5 on CD4 T cells (62). In addition, HIV can promote Mtb replication by disrupting macrophages’ microbicidal activity against Mtb, as well as decreasing antigen presentation (described later).

The following chapters will focus on the role of different immune defense mechanisms and how these are manipulated by HIV and Mtb, showing how the pathogens can evade the immune response and increase pathogenesis, making the coinfection so fatal.
Innate immune cells

Macrophages

Macrophages play a big role during Mtb pathogenesis since they are the primary cells to be infected in the lungs. Mtb is engulfed by alveolar macrophages which have good phagocytic ability but are weakly bactericidal with limited oxidative burst and poor antigen presenting capacity, creating a niche for the bacteria (63). Alveolar macrophages relocalize from the airways to the lung interstitium upon Mtb infection and thereafter initiate dissemination to monocyte-derived macrophages and neutrophils (64). The main functions of alveolar macrophages are to maintain steady-state in the alveolar microenvironment by removing debris and dead cells, but they can also initiate an inflammatory response upon infection (65).

Macrophages, which are differentiated from circulating monocytes, have many different mechanisms for defending against Mtb, including phagosome acidification, autophagy, ROS and cytokine production. Macrophages are heterogeneous and can be polarized into the two main groups M1 or M2 macrophages depending on the environment. The classically activated M1 macrophages are induced by LPS, IFN-γ and TNF-α. They produce pro-inflammatory cytokines and are important during host response against intracellular bacteria such as Mtb. The alternatively activated M2 macrophages are induced by IL-4, IL-13, IL-10 and TGF-β. They are on the other hand immunosuppressive and poor antigen presenters (66). Alveolar macrophages cannot be classified into M1 or M2 since they exhibit characteristics of both phenotypes and exert both pro- and anti-inflammatory actions (65). Mtb can manipulate the polarization of macrophages in order to survive and replicate inside the cells. By inducing the production of IL-10 from Mtb infected macrophages, M2 polarization is promoted causing increased susceptibility to Mtb (67). Furthermore, Mtb can also induce the differentiation of macrophages into foamy macrophages, through ESAT-6 (68). These cells could serve as a reservoir for dormant Mtb (14), exhibiting reduced antigen processing capacity and increased secretion of TGF-β (66).

Dendritic cells

Dendritic cells (DCs) are important for Mtb antigen presentation and activation of the adaptive immunity, by migrating to the lymph nodes. Upon infection or uptake of antigens DCs go through a maturation process. Immature DCs are specialized in antigen capture and express high CCR1, CCR5 and CCR6, while expressing low CCR7 (lymph node homing molecule), CD40, CD54, CD80, CD83, CD86 and CD58. When the DCs have phagocytosed a pathogen/antigen they migrate to the draining lymph node while they undergo a maturation process. Their functions are now specialized for antigen processing and presentation and the capacity to capture antigens is downregulated. These mature DCs have a high surface expression of MHC II, along with the opposite low/high expression of the already mentioned markers (69). Mtb is able to modulate DC functions by for example impairing antigen
processing and preventing DC maturation (70) or on the contrary by promoting maturation and MHC expression before antigen processing and loading occurs (71).

**Neutrophils**

Neutrophils are important cells of the innate immunity since they are the first line of defense against pathogens. They are also the first cells to infiltrate the lungs upon Mtb infection (66). Their main functions are phagocytosis and killing of invading bacteria through several mechanisms such as degranulation, production of reactive oxygen species (ROS) and formation of neutrophil extracellular traps (NETs) (72). The neutrophils secrete ROS, elastase, collagenase and myeloperoxidase, all of which can damage both the bacteria and the host cells (73). Their role during TB is complex and they can contribute to both defense and tissue damage (66). However, depletion of neutrophils in whole blood leads to impaired growth restriction of Mtb due to lack of human neutrophil peptides (74), indicating the importance of these cells and their antimicrobial peptides in the defense against TB. Mtb infection of neutrophils leads to cell death, either by apoptosis (75,76) or necrosis (77). Apoptosis of neutrophils promotes their uptake by surrounding phagocytes in a process called efferocytosis, leading to inhibition of Mtb replication (78,79), while macrophage uptake of Mtb-induced necrotic neutrophils results in Mtb growth (77). ESAT-6 induced necrosis can also result in formation of neutrophil extracellular traps (NETs) with extruded DNA and granular proteins in a myeloperoxidase-dependent process (80,81). NETs function as a tool for host defense by trapping the pathogen and participating in the killing (73). Mtb-induced NETs can be phagocytosed by macrophages and activate a pro-inflammatory response (82).

**Innate immune defense mechanisms and counterstrategies by the pathogens**

**Sensing of Mtb/HIV and signaling responses**

Recognition of bacterial or viral pathogen-associated molecular patterns (PAMPs) induce activation of membrane-bound or cytosolic pattern recognition receptors (PRRs) (83). Surface receptors on cells that encounter pathogens can mediate signaling and uptake. The main families of surface receptors to interact with Mtb are Toll-like receptors (TLRs), c-type lectin receptors (CLRs) and scavenger receptors. The complement receptor 3 can also interact with Mtb and upon pathogen opsonization mediate its uptake (84). CLRs are a class of PRRs that upon recognition of Mtb mediate uptake and cytokine signaling. Examples of some CLRs are the mannose receptor (MR), Dectin-1, and DC-SIGN. The MR is one of the main Mtb receptors of macrophages, and facilitates uptake of Mtb, while DC-SIGN is abundantly expressed on DCs and is important for uptake of Mtb (83). The mycobacteria cell wall component ManLAM can upon binding to DC-SIGN cause impaired DC maturation and production of IL-10, thereby promoting immunosuppression (85). MR binding of LAM or ManLAM also induces an anti-inflammatory response along with inhibition of phagosome maturation (84). Dectin-1 induces
production of TNF-α, IL-6, IL-1β, and IL-23 in DCs upon Mtb recognition which leads to Th17 generation (86). Yet another CLR is Macrophage-inducible C-type lectin (Mincle), a receptor essential for the recognition of Mtb’s glycolipid trehalose dimycolate (TDM) which activates macrophages and promotes Th1 and Th17 polarization of T cells through DCs (87).

Other common PRRs are TLRs and Nucleotide oligomerization domain (NOD)-like receptors (NLRs), which upon activation induce production of pro-inflammatory cytokines such as TNF, IL-1β and IL-12 that are important for elimination or control of pathogens. Both TLRs and NLRs activate multiple signaling pathways including NF-κB and mitogen-activated protein kinases (MAPKs). TLR signaling can also lead to the assembly of the inflammasome (a multiprotein complex including NLR proteins) which activates caspase-1, which in turn cleaves pro-IL-1β and pro-IL-18 into their active and secreted forms (88).

Mtb possess many agonists for TLRs, including TLR4 and TLR9, but mainly TLR2 (84). Mycobacterial cell wall lipids induce production of pro-inflammatory cytokines such as TNF in a TLR2 dependent manner (89). Many members of the TLR family are dependent on the adaptor protein myeloid differentiation primary response protein 88 (MyD88) for downstream signaling and a lack of this protein makes the host susceptible to Mtb (90). The role of TLRs in the protection against TB is complex and not all TLR signaling is beneficial for the host. Prolonged TLR2 signaling in macrophages by Mtb can downregulate some immune functions such as antigen presentation (91), however knockout of TLR2 results in susceptibility to Mtb (92). Furthermore Mtb can also manipulate the TLR2 signaling through its lipoprotein LprG, that induces TLR2 and causes inhibition of antigen processing in macrophages (93).

Secreted TNF and IL-12 upon TLR activation stimulate IFN-γ production mainly from natural killer (NK) cells and T cells (84). Both IFN-γ and TNF-α are important pro-inflammatory cytokines involved in controlling Mtb infection. They can either act in concert, or separately, activating macrophages to increase their antimicrobial activities. Too much TNF can however result in tissue damage and disease progression, and thus an optimal amount is needed for protection (94). During HIV coinfection, addition of TNF-α or IFN-γ increased the growth of Mtb in human macrophages, while they in HIV uninfected cells could control Mtb growth (95). INF-γ can promote killing of Mtb through nitric oxide production, autophagy induction and phagosomal maturation, as seen in mouse macrophages (96–98), while IFN-γ stimulation of human macrophages can result in extracellular trap formation and necrosis, promoting Mtb growth in an ESX-1 dependent manner (99). Thus, the role of INF-γ and TNF-α seems to differ depending on dose and cell type, but are generally believed to be important during Mtb infection.

The PRRs interferon inducible protein 16 (IFI16) and cyclic GMP-AMP synthase (cGAS) can recognize cytosolic DNA from Mtb or reversed transcribed HIV DNA if it is not successfully masked by the viral capsid in a complex with CypA (100,101). Upon DNA recognition, both PRRs activate STING that recruits the signaling cofactors TBK1 and IKK-α/β to activate the transcription factors IRF3 and NF-κB. NF-κB induces production of pro-inflammatory cytokines and chemokines. IRF3 can be suppressed by the viral proteins Vpu, Vif and Vpr but its
activation induces the expression of HIV restriction factors and type I IFN. Furthermore the produced IFN-α/β can bind to its receptor on HIV infected cells, leading to activation of the STAT1-STAT2 complex and IRF9, that induces the expression of anti-HIV IFN-stimulated genes (ISGs) encoding IFI16 and cGAS. Furthermore, HIV can also be recognized by endosomal TLR7 and TLR8 which can activate the NLRP3 inflammasome leading to the release of IL-1β. This cytokine is a potent inducer of other pro-inflammatory cytokines that can activate and recruit innate immune cells (100). Although IFNs can be harmful to HIV, other cytokines can have a positive effect on viral replication, by activating infected cells. Upon HIV infection, the glycoprotein gp120 induces TNF-α secretion by macrophages, mediated by PI-3K and MAPK activation (102). TNF-α has been shown to promote HIV transcription through induction of NF-κB (103). Several HIV proteins (Vpr, Tat and Nef) can mimic TNF signaling in HIV infected cells and thereby also promote HIV replication (104,105).

Phagocytosis and phagosome maturation

Mt� can bind to a number of receptors, which will influence and determine the ability of the macrophages to control the infection. By interaction with certain receptors, Mt� can promote its own survival even before entering the phagosome (84). Upon binding to surface receptors, phagocytosis is initiated, leading to the generation of a phagosome where the pathogen resides. Once in the phagosome, the bacteria will experience a gradual decrease of pH due to a number of fusion events with early to late endosomes. This chain of events is called phagosome maturation, and results in the fusion of the late phagosome with a lysosome, creating a phagolysosome wherein most bacteria are being degraded (106). The process starts with the recruitment of Rab5 from early endosomes. This small GTPase interacts with Vps15 (of the Class III PI3K complex) which assists in the recruitment of Vps34 (phosphatidylinositol 3-kinase (PI3K)) that generates phosphatidylinositol-3-phosphate (PI3P) on the early phagosomal membrane. PI3P is a docking site for the early endosome antigen 1 (EEA1) which together with Rab5 are necessary for the maturation process to proceed. In the late phagosome, recognized by the presence of Rab7 (replacing Rab5), the pH has dropped considerably (pH 5.5) due to the acquisition of several V-ATPases. The phagosome is now also enriched with lysosomal-associated membrane proteins (LAMP). The phagolysosome is characterized by even lower pH (pH 4.5), a greater number of V-ATPases in the membrane and an accumulation of active hydrolases and antimicrobial peptides (106–108). This environment is bactericidal to most pathogens, including Mt� although this bacteria is more resistant to acidic compartments than most other microbes. Still in order to persist inside the phagosome, Mt� is able to block these fusion events and reside in an early phagosome (109).

Mt� has several effectors and virulence factors able to obstruct phagosome maturation. Among them are nucleoside diphosphate kinase (Ndk) and the tyrosine phosphatase PtpA that interferes with Rab conversion (106). ManLAM can by binding to the mannose receptor impair phagosome maturation, through activation of the protein tyrosine phosphatase (SHP-1) which inhibits the activity of PI3K (110,111). The secreted SapM is able to inhibit the recruitment of EEA1, by removing PI3P through dephosphorylation (106). Furthermore, the
host protein tryptophan-aspartate containing coat protein (TACO) is recruited to phagosomes and is normally released upon fusion with lysosomes, but the mycobacterial lipoamide dehydrogenase C (LpdC) can bind and retain this protein on the phagosomes and thereby block the phagosome maturation (112,113). The TACO can however be downregulated by Vitamin D3 together with retinoic acid (114), which may lead to control of Mtb. Yet another way of which Mtb prevents phagosome maturation is through the ubiquitination and degradation of V-ATPase, which prevents acidification (115). HIV is also able to block phagosome maturation, by the action of the HIV protein Vpr (55), but at the same time the virus also utilizes the endocytic pathway in macrophages in order to replicate inside the cells. The budding of HIV virions is directed into the lumen of late endosomes, and these vesicles are then moved to the cell surface for virus release (116,117). Thus, although the endocytic pathway is important for protection against pathogens, HIV can utilize it and both Mtb and HIV can block the phagosome maturation, in order to persist inside cells. This block can however be overcome by pro-inflammatory cytokine (INF-γ and LPS) activation of macrophages (97).

Phagosomal escape is another essential part of Mtb virulence. By translocating to the cytosol Mtb makes itself exposed to cytosolic sensor pathways such as the NLRP3 inflammasome, the cGAS-STING pathway and autophagy. The phagosomal rupture can either be complete and the bacteria then translocate to the cytosol, or it can be a partial rupture and the bacteria then only gain access to the cytosol and can release bacterial effectors to inhibit host defenses (118). The phagosomal rupture is dependent on Mtb's ESX-1 secretion system and is enhanced by restriction of phagosomal acidification (119). Furthermore, the Mtb cell wall lipid phthicerol dimycocerosates (PDIM) contribute to the phagosomal escape, causing necrosis of the host cell which favors spreading to other cells (6). The phagosomal escape can enable replication in the cytosol unless the bacteria is targeted by other intracellular surveillance pathways, such as autophagy.

**Autophagy**

There are different types of autophagy, such as microautophagy and chaperone-mediated autophagy but herein the focus will be on the main one, called macroautophagy (further referred to as autophagy). The discovery of autophagy started with the discovery of lysosomes in 1955 by Christian de Duve who in 1963 coined the term autophagy (120,121). Since then there have been several advances in methods and the knowledge about autophagy has grown extensively (121).

The characteristics of autophagy; the formation of the double-membrane vacuole called the autophagosome, was first detected during starvation conditions (122). Indeed, autophagy is a process targeting pathogens (called xenophagy) or old organelles for degradation as a part of the innate immune defense or for energy supply respectively. Upon initiation, a phagophore (isolation membrane) is formed that elongates to enwrap portions of the cytoplasm and upon closure creates an autophagosome containing its cargo. This
autophagosome then fuses with a lysosome, creating an autolysosome (autophagolysosome) where the content is degraded and the amino acids can be recycled by the cell (123–125).

After observing autophagy in yeast, a number of proteins required for autophagy were discovered (126,127), which in mammalian nomenclature are called autophagy related (Atg) proteins (128). This breakthrough led to the understanding of how the autophagosome is formed. Upon autophagy stimulation, the mammalian target of rapamycin complex 1 (mTORC1) is inhibited leading to the activation of the Unc-51-like kinase 1 (ULK1) complex that in turn is recruited to the PI3K complex where it phosphorylates Beclin 1 (Atg6 homologue), and initiates the phagophore formation (129,130). For elongation of the phagophore, Atg conjugation systems are necessary. In the Atg12 conjugation system, Atg12 gets conjugated with Atg5 and forms a complex together with Atg16L, that localizes to the phagophore and dissociates upon completion of the autophagosome. The Atg12 conjugation system is necessary for the Atg8/LC3 conjugation system, where the microtubule-associated protein light chain 3 (LC3) is processed into the cytosolic form LC3-I (125,129,131). LC3-I gets conjugated to phosphatidylethanolamine, forming LC3-II that is attached to both the inner and outer membranes of the autophagosomes (129,132,133). By measuring the amount of LC3-II, which represents the amount of autophagosomes, it is possible to monitor autophagy. However, over the process of autophagy when autophagosomes fuse with lysosomes the content is degraded leading to reduction of LC3-II expression (133,134). To study this autophagic flux, inhibitors that block this degradation such as Bafilomycin A1 (135,136) can be used when monitoring LC3-II levels (133,134).

Figure 3. Autophagy in homeostasis and during Mtb infection. Upon mTOR inhibition autophagy is induced, leading to the formation of a phagophore that encloses the target within an autophagosome. When the autophagosome fuses with a lysosome, an autolysosome is formed, wherein the target is degraded, in a process called autophagic flux. Mtb can inhibit the fusion with lysosomes and thereby evade autophagic flux and degradation.
Another autophagy protein that has been used to study the process is sequestosome (SQSTM1/p62), which binds directly to LC3, and is necessary for the degradation of ubiquitinated proteins during autophagy (137). SQSTM1 can also target intracellular bacteria to the autophagy pathway (138), and deliver ubiquitinated proteins with microbicidal properties to autolysosomes to facilitate killing of the bacteria (139). Thus, autophagy is an important part of the innate immune response and has been shown to contribute to the killing of a number of intracellular bacteria, including *Mycobacterium tuberculosis* (139). However, both Mtb and HIV has several ways to manipulate autophagy, causing an induction of the process, but at the same time blocking the later steps, the autophagic flux, in order to avoid killing. In HIV, Nef is the protein involved in this autophagic block through interaction with Beclin 1 (140), while the ESX-1 secretion system is essential for Mtb (141). Additionally the Mtb secreted protein enhanced intracellular survival (Eis) can also inhibit autophagy through induction of IL-10 expression that activates the mTOR pathway (142). Several autophagy inducers can reverse the block in autophagic flux exerted by the pathogens, and thereby promote killing. Some examples are Vitamin D, IFN-γ and the commonly used autophagy inducer rapamycin (141,143,144). However, virulent Mtb upregulate macrophage IL-6 production which can inhibit IFN-γ induced autophagy, and prevent killing (145). Many studies suggest a beneficial role of autophagy induction during Mtb infection and there are attempts of developing adjunctive treatments or vaccines that modulate autophagy. In this regard, rapamycin which is an immunosuppressive drug has surprisingly been shown to enhance BCG efficiency in mice at low concentrations (146). Moreover it has been shown in mice that inhalable particles of rapamycin decreased Mtb burden in the lungs, but the bacteria were only eliminated upon combination with TB drugs (147). Clinical studies are however needed to investigate if rapamycin could be an option for TB treatment. Additionally, several *in vitro* and *ex vivo* studies using D vitamin has shown a beneficial effect on host response against Mtb (144,148–151). However the effect of vitamin D supplementation on Mtb clearance in clinical studies has been controversial and only shown some promise (152–154). Thus, the search for effective host directive therapies continues.

**Reactive oxygen species**

The innate immune defense in phagocytic cells is not only limited to a decrease in pH but also includes reactive oxygen species (ROS) production. ROS has several sources, and can be produced from the mitochondria, from nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) located on cellular membranes or from the endoplasmic reticulum (ER) where it facilitates protein folding. To avoid damage of DNA, lipids and proteins from the highly reactive ROS, the cells have several antioxidants (glutathione and thioredoxin) and redox proteins (catalase, SOD) to regulate ROS. However, if ROS production is greater than the scavenging abilities, it leads to oxidative stress which can damage the host cells, but also kill pathogens. NOXs are important for killing of pathogens as well as for cell signaling and they are present in the membrane of phagosomes of phagocytic cells such as macrophages and neutrophils. Upon phagocytosis, an oxidative burst is induced and ROS is pumped into the phagosome by NOX2 which damages the engulfed pathogen (155,156). The oxidative
burst starts with the transfer of electrons by the NOX2 from cytosolic NADPH to phagosomal oxygen forming superoxide anions (O$_2^-$), which quickly dismutates to hydrogen peroxide (H$_2$O$_2$) which in turn transforms into toxic hydroxyl radicals (OH), all of which are part of the ROS family. The dismutation to hydrogen peroxide can either be spontaneous or mediated by superoxide dismutase (SOD). More toxic ROS can be produced by superoxide reacting with nitric oxide forming peroxynitrite, or peroxidase catalyzation of hydrogen peroxide into hypochlorous acid (HOCl) (157). HOCl is the most effective antimicrobial oxidant of neutrophils, generated by myeloperoxidase (MPO) which is released upon degranulation. During ROS production, protons are consumed, raising the pH in the phagosome and reducing it in the cytoplasm, but this is then balanced by proton transfer (155). NOX2 is also important for antigen presentation in DCs. The complex gets assembled onto the phagosome where it prevents the degradation of antigen by increasing the pH through a continuous production of low levels of ROS (158). Furthermore, ROS is important for cell signaling and can act as a second messenger to mediate apoptosis, proliferation, cytokine release and gene regulation. ROS can also modulate redox-sensitive transcription factors such as NFκB (155). Furthermore NOX2-derived ROS can be important for granuloma formation and NET formation (159).

The bactericidal effect of ROS on Mtb is controversial and Mtb has several ways to counteract redox stress. Some examples are the mycolic acids in the cell wall which form a barrier to protect against ROS, in addition to the secretion of the antioxidant enzymes SOD (SodA and SodC) and KatG, the latter accounting for catalase, peroxidase and peroxinitrate activity. KatG is required for ROS protection, but it also activates the efficient prodrug isoniazid to its active form which inhibits the synthesis of mycolic acid (160). KatG converts hydrogen peroxide into water and oxygen, and a loss of this catalase results in hypersensitivity to ROS, but resistance to isoniazid (161). Although the bacterium has ways to protect itself against exogenous ROS, Mtb (including MDR/XDR strains) has been shown to be more sensitive to endogenous ROS produced within the bacteria (162). Another study suggested that the toxicity of exogenous ROS on Mtb is concentration dependent; with low concentrations of H$_2$O$_2$ not affecting survival but resulting in induction of responsive genes to initiate DNA repair, while high concentrations of H$_2$O$_2$ was lethal to Mtb (163). Phagosomal ROS is generated through NOX2 upon Mtb infection. NOX2 is also important for apoptosis induction through TNF-α signaling. By inhibiting the activity of NOX2, Mtb is protecting itself against both ROS and apoptosis, another important defense mechanism (164). The effects of ROS may not be directly bactericidal to Mtb, but the radicals can mediate cell signaling events that are important for host defense (159).

In contrast to Mtb, HIV benefits from oxidative stress. HIV patients have a depletion of glutathione in plasma, lymphocytes and monocytes (165) along with reduced levels of SOD (166). At the same time, monocytes from HIV patients have increased ROS production, which correlated with viral load (167). HIV induces oxidative stress, with a number of its proteins (Tat, gp41, Vpr) involved in deregulating different pathways to increase ROS production (168). Tat can for example induce oxidative stress by activating NOX2 through the PI3K/Akt signaling pathways, leading to activation of NFκB (169). Furthermore, ROS induces HIV transcription through NFκB activation (170), while antioxidants reduce the transcription of the virus (171).
In summary, HIV induces ROS production that in turn promotes HIV replication, thereby creating a viscous circle.

![Figure 4. ROS production in a neutrophil.](image)

**Figure 4. ROS production in a neutrophil.** Upon phagocytosis, ROS is produced inside the phagosome, starting with the production of superoxide anions ($O_2^-$) by the NADPH oxidase (NOX). This radical then dismutates to hydrogen peroxide ($H_2O_2$) that is converted into hypochlorous acid (HOCl) by myeloperoxidase (MPO). MPO is transported into the phagosome by the fusion of azurophil granules in the cytosol.

**Apoptosis**

Apoptosis (programmed cell death) is characterized by controlled breakdown of a cell, morphologically seen as cell shrinking, membrane blebbing and chromatin condensation. This results in the formation of apoptotic bodies that can be engulfed by surrounding phagocytes, in a process called efferocytosis (172). Efferocytosis of apoptotic cells is considered to be anti-inflammatory, through the induction of transforming growth factor (TGF)-β secretion (173), but during Mtb infection a pro-inflammatory response upon efferocytosis has been observed (174). It is also a defense mechanism during infection, killing the cell wherein the pathogens reside and replicate and delivering it to a non-infected cell. Efferocytosis of Mtb-infected macrophages by uninfected ones has been shown to decrease growth of Mtb through lysosome fusion (175).

Apoptosis is activated through an intrinsic or extrinsic pathway and is dependent on caspases. DNA damage or oxidative stress activates the intrinsic pathway leading to activation of the pro-apoptotic proteins Bax and Bak which creates a channel in the mitochondria for release of cytochrome c into the cytosol. This leads to the activation of pro-caspase-9 which initiates a proteolytic cascade that activates the executioner caspases-3, -6 and -7, leading to
apoptosis. Smac/Diablo are other pro-apoptotic proteins released from the mitochondria. The extrinsic pathway is induced by external stimuli (such as TNF-α and Fas ligand (FasL)) binding to death receptors such as the TNF receptor, Fas and TRAIL-R (TNF-related apoptosis-inducing ligand receptor). This leads to the formation of a death-inducing signaling complex that recruits the initiator caspases-8 or -10 that in turn activates the executioner caspases (172,176).

Another type of cell death is necrosis which is characterized by uncontrolled cell swelling and loss of cell membrane integrity, leading to the release of the cell content into the surroundings (172). Apoptosis is generally believed to promote killing of mycobacteria and promote antigen presentation, mounting a T-cell response, while necrosis is considered to promote spreading of the bacteria along with inflammation and tissue damage (177). Thus, it is not surprising that virulent mycobacteria inhibit apoptosis and promote necrosis (178). The attenuated Mtb strain H37Ra was shown to induce more apoptosis than the virulent H37Rv (179). Virulent Mtb has several ways to inhibit apoptosis including; the induction of IL-10 which decreases TNF-α mediated apoptosis (180), decreased Fas receptor expression (181), and upregulation of the expression of the antiapoptotic Mcl-1 protein (182). Apoptosis of Mtb infected macrophages can also be inhibited by HIV Nef by reducing TNF-α production (57). HIV can also manipulate apoptosis through regulation of pro- and anti-apoptotic proteins and FasL, TRAIL and TNF-α expression. The viral proteins gp120, Tat, Vpu, Nef, Vpr and protease inhibit apoptosis early during infection to promote viral replication but later when the infection is established they induce apoptosis. Apoptosis is mainly induced in immunocompetent cells such as CD4 T cells and uninfected by-stander cells, while it is inhibited in latently infected cells (viral reservoirs) (183). This contributes to the depletion of CD4 T cells along with progression of the disease.

**Activation of adaptive immunity**

**Antigen presentation**

Macrophages and dendritic cells are the main antigen presenting cells (APCs) during Mtb infection. These cells get infected or pick up antigens to be presented to naïve T cells through the major histocompatibility complex (MHC), and thereby mount a T cell response. Dendritic cells are especially important in priming of T cell responses, while macrophages mainly present antigens to effector T cells (184). Typically intracellular antigens are presented to CD8 T cells by MHC I and exogenous antigens are presented to CD4 T cells by MHC II. However, through cross-presentation exogenous antigens can be presented by MHC I and if cytosolic proteins are degraded through pathways such as autophagy, they can be presented by MHC II. MHC I molecules are ubiquitously expressed by all nucleated cells, while MHC II are expressed mainly by APCs. During MHC I antigen presentation, the antigens are degraded by proteasomes and the resulting peptides of 8-9 amino acids are loaded onto the MHC I in the endoplasmic reticulum (ER) before transport of the complex to the cell surface and presentation to CD8 T cells. The MHC II is assembled in the ER, and is then transported to a
late endosomal compartment, where it can bind a specific peptide derived from a protein degraded in the endosomal pathway. The complex is then transported to the plasma membrane where it presents the antigen to CD4 T cells (185). The T cell receptor (TCR) on CD8/CD4 T cells interacts with the MHC I/II and its antigen, creating the first signal, determining the antigen specificity of the response. A second signal is required for T cell activation and is obtained through binding of the co-stimulatory molecules CD80 and CD86 to the primary costimulatory receptor CD28 on T cells. This is followed by the third signal in shape of cytokine secretion from the APC, inducing proliferation of T cells and polarizing the CD4 T cell response. The type of cytokines secreted depends on the stimuli that primed the APC through PRR, and will determine which type of CD4 T cell response will be mounted (186). There are also several co-stimulatory molecules on DCs that can either promote an activation or inhibition response. Examples of co-stimulatory molecules on DCs are: CD80/B7-1, CD86/B7-2, CD83, CD40 and PDL-1. CD40 on DCs binding to CD40L on T cells results in the activation of both cells while CD80/CD86 binding of CTLA-4 on T cells results in inhibition of T cell activation. Binding of CD80/CD86 with CD28 leads to T cell proliferation and increased secretion of IL-2 and is essential for preventing T cell apoptosis (187,188).

Effects of Mtb and HIV on antigen presentation

Both Mtb and HIV can inhibit antigen presentation in order to persist inside the host. HIV can down-modulate the surface expression of CD80 and CD86 on APCs through Nef (189), and inhibit up-regulation of CD40L on T cells and CD80 on APCs through gp120, thus inhibiting T cell activation (190). HIV also causes a reduction in IL-12 and an increase in IL-10 production (191), which reduces induction of Th1 responses. HIV infection in dendritic cells promotes upregulation of inhibitory molecules such as PD-1 and CTLA-4 on T cells, which suppresses T cells and decrease their proliferation (188). Mtb also has strategies to evade T cell activation. As mentioned earlier, Mtb can inhibit phagosomal maturation and autophagic flux, thereby preventing antigen processing and sequentially antigen presentation. Mtb can also export their antigens from infected dendritic cells in order to reduce their presentation and activation of T cells. Although these exported antigens can be presented by uninfected cells the protective T cell response remains limited (192). Furthermore, the Mtb lipoprotein LpqH can inhibit MHC II expression in macrophages through TLR2 signaling and interfere with the peptide loading to MHC II and the subsequent trafficking to the cell surface. The inhibition of antigen presentation promotes Mtb survival in APCs and enables persistence without the recognition of CD4 T cells (91,193). Mtb can also disrupt presentation of Mtb antigens in DCs by for example promoting DC maturation and MHC expression before antigen processing and loading occurs (71). Conversely, mycobacteria-induced TLR2 and TLR4 signaling promote antigen presentation, through stimulation of DC maturation including increased expression of MHC class II along with the co-stimulatory molecules CD80 and CD86. This mycobacteria-induced maturation also leads to the activation of Th1 and Th17 cells (194,195).

The activation of CD8 T cells during Mtb infection has mainly been associated with cross-presentation since Mtb primarily is confined within endosomal compartments, but through
phagosomal rupture antigens can gain access to the cytosol where they can be processed by the proteasome and loaded onto MHC I (196). Mycobacterial antigens from apoptotic cells can also be presented through cross-presentation. The released apoptotic vesicles from the infected cells can be taken up and processed by DCs which in turn can present the antigens to CD8 T cells through MHC class I. By enhancing apoptosis of Mtb infected cells a higher CD8 T cells response can be mounted (197). However, as mentioned earlier virulent mycobacteria inhibit apoptosis and induce necrosis (178) and can thereby prevent cross-presentation to CD8 T cells. By a mechanism similar to the cross-presentation of antigens from apoptotic cells, Mtb lipids could also be accessed by this route, and be presented by CD1 on DCs to Natural killer T cells (NKT cells) which upon activation produce large amounts of IFN-γ (196). However, this lipid presentation can also be inhibited by Mtb by similar mechanisms already described for antigen presentation.

T cell responses

The adaptive immune responses against Mtb, mainly consisting of antigen specific T cell responses, are not detected until several weeks after infection but plays an important part in the outcome of the disease (184). CD4 T cells are essential in the immune response against TB for controlling the infection. Naïve CD4 T cells can differentiate into different subsets depending on the cytokines produced by the APC during antigen presentation and T cell activation. The CD4 T cell subset T helper type 1 (Th1) cells have typically been considered as one of the most important during Mtb infection, but in recent years the importance of Th17 cells has also been emphasized. Th17 cell polarization is induced by IL-6, IL-23, IL-1β, IL-21 and TGF-β which activates STAT3 that induces the expression of the transcription factor ROR-γt, leading to the production of IL-17, IL-22, IL-26 and GM-CSF. These cytokines mediate pleiotropic activities such as induction of pro-inflammatory genes and antimicrobial peptides along with stimulation of granulopoiesis and neutrophil recruitment and activation. Th17 cells have mainly been associated with clearance of extracellular pathogens, but they are also involved in protection against some intracellular pathogens. Their role during TB infection is still controversial, but some studies suggest a protective role in combination with Th1 responses. Th1 cell polarization is induced by IL-12 or INF-γ and upon activation, these cells mainly secrete IFN-γ. When IL-12 binds to its receptor on T cells, STAT4 is activated which induces the transcription factor T-bet which in turn binds to many Th1 specific genes and induces their expression. Th1 cells with the production of INF-γ are important in the activation of macrophages for antimicrobial defense and for promotion of MHC II expression on macrophages (198). This cytokine can act on both infected and uninfected macrophages and thus when evaluating antigen presentation the MHC expression on APCs may be a poor indication since not all cells contain antigens. Additionally, antigens also needs to be sufficiently processed to be presented and therefore functional studies (of T cell activation) are preferred in order to properly evaluate antigen presentation (199).

HIV infection is characterized by the progressive loss of CD4 T cells, causing an immunodeficiency that leaves the host more vulnerable to other infections. It has also been
shown that during a co-infection, HIV preferentially infects and causes depletion of Mtb-specific CD4 T cells (49). Furthermore, PBMCs from coinfected individuals were shown to proliferate less, release less IFN-γ and express less IL-2 and IL-12 when stimulated with heat-killed Mtb, compared to PBMCs from TB only infected individuals (200). Apart from HIV’s manipulation of the immune response, Mtb can by manipulating DCs accelerate the progression of HIV. Mtb can promote trans infection, when DCs do not become infected with HIV but deliver the virus directly to T cells across an infectious synapse, leading to infection of the T cell. Furthermore Mtb can reduce the processing of HIV and inhibit antigen presentation of HIV through MHC II, leaving the virus undetected by CD4 T cells (201).

Another subset of CD4 T cells are regulatory T cells (Tregs), which are important in the control of the immune response and in preventing autoimmunity. However during TB the Tregs dampen the immunity and function of effector T cells and may promote disease progression (202). During active disease an increased number of Tregs have been found in both blood and at the site of infection. These cells suppressed the Mtb-specific immunity by reducing IFN-γ and IL-10 production (202).

CD8 T cells (CD8 cytotoxic T lymphocytes (CTL)) can upon activation produce IFN-γ, lyse the infected antigen presenting cell, and mediate an antimicrobial activity through secreted granulysin and pore-forming perforin, thereby contributing to the reduction in Mtb burden (203). CD8 T cells also play a major role during virus infections, since viral antigens are mainly presented by MHC I molecules. CD8 T cells have been shown to be able to control HIV during early infection but later fail to prevent replication of the virus (204). This was due to the viral Nef protein, which blocks MHC I expression on the surface of the infected cell in a PI3K-dependent manner, and thus inhibits CTL-mediated lysis of the HIV infected CD4 T cells (205,206). Nef is an important HIV protein, contributing to pathogenicity, promoting virus replication and is known to also downregulate CD4 of infected cells (207,208) thereby preventing superinfection (209). HIV also avoids T cell responses by upregulating Fas on CD8 T cells, leading to apoptosis of these cells upon interaction with FasL on APCs (210). Furthermore, HIV causes an up-regulation of PD-1 on CD4 and CD8 T cells which impairs their proliferation and response (211).
AIMS

The overall aim was to investigate how Mtb and HIV can impair the protective immune system, and how it can be boosted in order to enhance the innate immune response against Mtb during HIV coinfection.

Paper I

The aim was to investigate if autophagy induction could impair Mtb replication in HIV coinfected macrophages. More specifically if the autophagy induction would cause an increased acidification in Mtb phagosomes when the macrophages were coinfected with Mtb and HIV.

Paper II

The aim was to investigate how HIV affects processing and presentation of Mtb antigen in coinfected dendritic cells, focusing on the expression of MHC II and co-stimulatory molecules as well as the Mtb-specific CD4 T cell response.

Paper III

The aim was to investigate how apoptotic neutrophils could augment the antimicrobial response against Mtb in HIV coinfected macrophages through efferocytosis, focusing on the role of ROS and myeloperoxidase.
RESULTS

Paper I

*Autophagy induction targeting mTORC1 enhances Mycobacterium tuberculosis replication in HIV co-infected human macrophages*

In this paper the effect of autophagy induction on Mtb was studied in HIV coinfected macrophages. Initially an increased LC3 formation and co-localization to Mtb was observed in HIV coinfected cells compared to Mtb single infected cells. Unexpectedly, when inducing autophagy with the well-known inducer rapamycin an increased Mtb replication was detected in both Mtb single and HIV coinfected cells, while autophagy inhibition with 3-Methyladenine (3-MA) had no effect. The autophagy inducer and inhibitor had no direct effect on Mtb (in the absence of macrophages), confirming that they only target processes inside the macrophages. The increased Mtb replication after autophagy induction, lead us to try yet another autophagy inducer, Torin1, which also inhibits the mTORC1 pathway. Previous studies have shown that this inducer is more efficient than rapamycin (212) which we confirmed by western blot. With increasing concentrations of Torin1 and rapamycin an increased autophagic flux was observed, leading to degradation of the autophagy proteins LC3 and SQSTM1 (also called p62). To confirm an inhibition in the mTORC1 pathway, the phosphorylated downstream targets S6 and 4EBP1 was also detected through western blot, showing a reduction with increasing concentrations of the inhibitors. These effects were especially prominent with Torin1, which lead us to investigate its effect on Mtb replication. Using a low MOI (MOI=1) of Mtb, a concentration dependent increase in replication upon Torin1 treatment was observed, although abolished when cells were infected with an MOI of 5 or 10. Thus, during a low grade infection the induction of autophagy can promote Mtb replication in single and coinfected macrophages.

The reason why the bacteria replicated could be due to inhibition of flux. Therefore acidification of the Mtb phagosomes was studied. Using the probe LysoTracker to visualize acidic compartments in the cells, co-localization to Mtb phagosomes was studied through microscopy. Yeast particles were used as a positive control and bafilomycin was added as a negative control, since this compound inhibits the V-ATPase and thereby prevents autophagic flux and autophagosome-lysosome fusion (135,136). When single and coinfected macrophages were treated with Torin1, a decreased co-localization between LysoTracker and Mtb phagosomes was detected. A decrease in acidification can lead to an increased replication of Mtb, as seen when treating infected macrophages with bafilomycin. Mtb can also inhibit phagosome maturation and autophagic flux in order to survive in the cells (213). Previous studies have shown that this block can be overcome by inducing autophagy, which increases the flux in the cells (143,144,214). Our study demonstrates that the autophagic flux is cellular (seen by western blot) and not specifically localized to the Mtb phagosomes (as seen by microscopy). Hence, we hypothesize that Mtb, especially during HIV coinfection, benefits from decreased phagosomal acidification and increased cellular flux, providing nutrients to the pathogen, which leads to increased replication. Further evidence for this was
the build-up of SQSTM1 in Mtb phagosomes upon Torin1 treatment and the increase in autophagosomes (LC3+ Mtb phagosomes) without an increase in autophagolysosomes (LC3+LysoTracker+ Mtb phagosomes). These findings stresses the importance of microscopy studies of the Mtb phagosomes as preferred to looking at the cell as a whole by western blot.

In summary, autophagy induction through mTORC1 inhibition during a low grade Mtb infection causes a cellular autophagic flux that is not localized to the Mtb phagosomes. This autophagy induction causes a disruption in the growth equilibrium of Mtb, leading to increased replication caused by the decreased phagosomal acidification, especially in HIV coinfected macrophages. Therefore autophagy induction through mTORC1 inhibition does not seem to be a good treatment option during Mtb and HIV coinfection.

Figure 5: Schematic summary of Paper I. Autophagy in macrophages infected with yeast leads to degradation through autophagic flux, while Mtb can inhibit this acidification. Upon autophagy induction with Torin1 in Mtb and HIV co-infected cells, the acidification is further reduced leading to Mtb replication.
**Paper II**

**HIV interferes with Mycobacterium tuberculosis antigen presentation in human dendritic cells**

In this paper antigen presentation by Mtb and HIV coinfected dendritic cells and their subsequent activation of Mtb antigen-specific (Ag-specific) CD4 T cells were studied, focusing on the role of HIV. During antigen presentation, three signals are required in order to mount a T cell response; the binding of MHC II to CD4, the subsequent binding of co-stimulatory molecules with receptors on T cells, and cytokine secretion from APCs (186). Therefore we studied these signals before measuring the T cell response.

Surface expression of MHC class II (HLA-DR), co-stimulatory molecules (CD40, CD80, CD83 and CD86) and CCR7 was analyzed through flow cytometry in coinfected DCs. There were no significant differences in the percentage of marker positive DCs, but the total surface expression on DCs revealed an increase of co-stimulatory molecules and HLA-DR in Mtb single infected cells, with exception of CCR7. The Mtb-induced increase was inhibited in HIV coinfected DCs, with a reduction in the expression of CD40, CD80, CD86, HLA-DR and slightly of CD83. As the third signal of antigen presentation includes the secretion of cytokines (186), the production of both pro- and anti-inflammatory cytokines were measured. Mtb induced the production of IL-6, IL-1β and TNF-α from dendritic cells, while HIV coinfected cells had reduced levels of these cytokines in comparison to Mtb single infected cells. Production of IL-12p70, IFN-α/β, IFN-γ and IFN-γ inducible protein 10 (IP-10) which is a surrogate cytokine for type I IFNs, were low in both single and coinfected cells. The pathogens did not induce significant levels of IL-10 either.

These initial experiments were performed to get an indication of how HIV interferes with antigen presentation, but in order to confirm its impact on T cell activation, functional studies are needed. Mtb Ag-specific CD4 T cells were therefore generated and co-cultured with Mtb and HIV coinfected autologous dendritic cells. The readout for T cell activation was production of IFN-γ. Two populations of CD4 T cells were generated, which were specific to different Mtb antigens, namely PPD and Ag85B. Both types of Mtb Ag-specific CD4 T cells exhibited a decrease in IFN-γ production after stimulation with HIV coinfected DCs compared to Mtb single infected cells. Also, PD stimulation as replacement for Mtb displayed the same reduction of IFN-γ production upon HIV infection of DCs. To reveal if HIV reduced antigen presentation and subsequent T cell activation through decreasing antigen processing, DCs were pulsed with Ag85B peptides and co-cultured with the Mtb Ag-specific T cells. HIV did not affect the IFN-γ production of the T cells, which indicates that HIV affects the antigen presentation by disrupting antigen processing. An important antigen processing pathway is autophagy (215). To investigate if this pathway was affected by the pathogen, the levels of LC3 and SQSTM1 was quantified through western blot. Bafilomycin was used to measure autophagic flux, where a high flux would be recognized by increased accumulation of the autophagic protein upon bafilomycin treatment. Mtb infected DCs displayed an increase in autophagosome formation, and blocked acidification. HIV co-infected DCs however showed...
a decreased autophagosome formation in addition to decreased accumulation of autophagy proteins. These findings indicate that HIV infection could lead to disruption in the autophagy-dependent processing of Mtb antigens. This suppression of autophagy was however not achieved through inhibition of the cGAS-STING pathway, which has been shown to induce autophagy upon DNA sensing (216,217).

In summary, HIV reduces Mtb antigen presentation and subsequent Mtb Ag-specific CD4 T cell activation and response by decreasing the Mtb-induced expression of HLA-DR and co-stimulatory molecules on DCs, and through suppression of antigen processing by autophagy. Impaired Mtb antigen presentation would allow the bacteria to remain undetected by the adaptive immunity, accelerating the pathogenesis of the Mtb/HIV coinfection.

Figure 6: Schematic summary of Paper II. HIV inhibits antigen presentation by suppressing autophagy of Mtb, decreasing expression of HLA-DR and co-stimulatory molecules on coinfected DCs and decreasing the release of cytokines. This leads to inhibited activation of Mtb specific T cells, with decreased release of IFN-γ.
Paper III

The enhanced control of Mycobacterium tuberculosis in HIV coinfected macrophages by apoptotic neutrophils is myeloperoxidase dependent.

In this paper the effect of efferocytosis of apoptotic neutrophils by Mtb and HIV coinfected macrophages was investigated. Previous studies have shown a beneficial role of efferocytosis in combating mycobacteria infection (78,175,218), and our group has previously shown that apoptotic neutrophils can induce a pro-inflammatory response and enhanced control of Mtb in infected macrophages (174). The aim was to explore this further, and see if apoptotic neutrophils also could inhibit Mtb growth during coinfection. Therefore human macrophages were infected with HIV prior to Mtb infection and stimulation with apoptotic neutrophils. After 5 days the growth of Mtb was significantly decreased after efferocytosis, both in single and HIV coinfected macrophages. To confirm uptake of apoptotic neutrophils, we performed kinetics experiments. A time-dependent uptake was observed, with 50% of macrophages having ingested apoptotic neutrophils after 1h of stimulation, increasing to almost 100% after 24h. Moreover, there was a time-dependent increase of Mtb co-localization with apoptotic neutrophils. Additionally, flow cytometry experiments revealed an increase in phagocytosis of Mtb by macrophages containing apoptotic neutrophils.

To investigate if Mtb growth inhibition was caused by autophagy induction triggered by the apoptotic neutrophils, western blot was performed. Studying the levels of SQSTM1 and LC3 II, no increased autophagic flux upon apoptotic neutrophil stimulation could be observed. Furthermore, the results point towards a decrease of autophagosome formation upon stimulation with apoptotic neutrophils. Since Paper I showed that western blot analysis is not reliable for studying the environment in the phagosome, also microscopy studies were performed, using LysoTracker as a marker for acidic compartments. LysoTracker co-localized with apoptotic neutrophils, but to a lesser degree with Mtb, indicating a decreased flux/acidification in the Mtb phagosomes. Although flow cytometry data showed a general increase in LysoTracker signal in cells containing apoptotic neutrophils, the microscopy results clearly show that this acidification is concentrated to apoptotic neutrophils and did not localize to Mtb. Therefore, other mechanisms by which the apoptotic neutrophils exert their effects must be involved.

Since a previous study has shown that the granules from neutrophils can be utilized by macrophages for antimicrobial defense (78), we studied the putative role of neutrophil granules and ROS. The most abundant granule protein of neutrophils is myeloperoxidase (MPO) (219). Immunostaining showed that MPO was present in macrophages that had ingested apoptotic neutrophils. With increasing uptake of apoptotic neutrophils, the presence of MPO was also increased until 6h, then a decrease of the protein was observed. The distribution of MPO was dispersed within the macrophage and very little co-localized to Mtb. Using luminol-enhanced chemiluminescence we observed that PMA stimulated ROS production in apoptotic neutrophils. The irreversible MPO inhibitor ABAH (4-Aminobenzoic acid hydrazide) inhibited chemiluminescence in a dose-dependent manner, proving that MPO and the NADPH oxidase (NOX2) are active in apoptotic neutrophils. ROS production was also
measured in macrophages after efferocytosis. Apoptotic neutrophils increased the ROS production in infected or PMA stimulated macrophages. Furthermore, the inhibited Mtb growth caused by apoptotic neutrophils was dependent on MPO, since apoptotic neutrophils treated with ABAH abolished the suppressed bacterial growth in coinfected macrophages.

In summary, this paper shows that efferocytosis of apoptotic neutrophils enhance the control of Mtb growth in Mtb and HIV coinfected macrophages. This effect was not dependent on autophagy or increased phagosome acidification, but on MPO and ROS from apoptotic neutrophils.

**Figure 7: Schematic summary of Paper III.** Uptake of apoptotic neutrophils by Mtb and HIV coinfected macrophages leads to increased ROS production. Upon efferocytosis of apoptotic neutrophils MPO spreads out in the macrophage and mediates inhibition of Mtb growth.
DISCUSSION

Since a common scenario during a TB and HIV coinfection is a preceding HIV infection, which makes the patient more susceptible to secondary infections, we established an HIV infection in macrophages prior to Mtb infection. Macrophages are the main cells to be infected by Mtb, and we have therefore primarily used them for our in vitro experiments to study if their antimicrobial defense could be enhanced by different means. Dendritic cells on the other hand are superior cells for antigen presentation, and we used these cells to study how HIV can manipulate this process, in addition to Mtb Ag-specific CD4 T cells. The focus of this thesis is on the innate immune reactions, but we have also studied the link to the adaptive immunity.

Autophagy is believed to be an important antimicrobial defense mechanism affecting both Mtb viability and antigen processing (215). Therefore we investigated (i) the antitycobacterial capacity in HIV coinfected macrophages (Paper I), (ii) if HIV affected autophagy and antigen processing in Mtb co-infected dendritic cells (Paper II) and (iii) if autophagy could be enhanced by efferocytosis of apoptotic neutrophils (Paper III).

In coinfected dendritic cells, HIV is able to inhibit autophagy and flux and thereby decrease Mtb antigen processing (Paper II), making autophagy an important part in activating the adaptive immunity. However, in macrophages we surprisingly found that during a low grade infection, induction of autophagy through inhibition of mTOR results in an increased replication of Mtb (Paper I). This was however not observed with higher MOIs. We also detected a general increase in autophagic flux with increased acidification which was not localized to the Mtb phagosomes, showing how resistant and competent this pathogen is in escaping and battling defense mechanisms of the cells. We hypothesized that other ways of inducing autophagy than through mTOR inhibition might be more beneficial and we therefore investigated if efferocytosis of apoptotic neutrophils could induce this process in infected macrophages (Paper III). We did not detect an increased autophagic flux upon uptake of apoptotic neutrophils, but enhanced antimicrobial activity mediated through MPO and ROS.

Cells have a basal level of autophagy to maintain homeostasis and if this process is inhibited, it can lead to cell death, which is another innate defense mechanism against Mtb (220,221). When investigating autophagy during infection it is important to differentiate between basal autophagy and xenophagy (autophagy of intracellular pathogens) (221). Therefore, microscopy studies in addition to western blot analysis is preferred to detect phagosomal flux. Chandra et al (222) has also used this approach to study the difference in autophagy in macrophages infected with either H37Ra or H37Rv. They showed that both the virulent and the nonvirulent strain induced similar levels of overall autophagic flux (western blot). Microscopic examination however showed that H37Rv inhibited maturation of the autophagosomes that harbored the bacteria (xenophagosomes), but allowed maturation of other autophagosomes. This selective inhibition was caused by the virulence factors PhoP and ESAT-6, and could therefore not be accomplished by the nonvirulent H37Ra (222). This and our study (Paper I) stress the importance of studying the actual autophagosomes wherein the bacteria reside, in order to determine any effects of autophagy on the bacteria. To conclude,
virulent Mtb inhibits maturation of xenophagosomes but not autophagosomes, thereby promoting Mtb survival and preventing cell death (221). Killing of Mtb will therefore depend on the induction of xenophagosome maturation and not only the induction of general autophagy. Rapamycin stimulation of dendritic cells infected with virulent Mtb was able to override the inhibition of autophagic flux (xenophagic flux), and thereby increase cytokine release to stimulate a Th1 response. The autophagy induction did however not decrease Mtb viability, but merely promoted Mtb infected DCs to stimulate a Th1 response (141). Although other studies have shown that autophagy induction with rapamycin decreases the viability of Mtb (143,147), the T cell response with production of IFN-γ might be more efficient in promoting Mtb killing. Along with inducing autophagy, IFN-γ also increases other antimicrobial activities in macrophages (223). Furthermore, Mtb-specific T cells have been shown to restore the inhibited autophagic flux and increase Mtb killing, although independently of IFN-γ (224). Thus, a T cell response may be crucial for control of Mtb. The stimulation of such a response is however inhibited in HIV coinfected DCs (Paper II), decreasing the immunity against Mtb. HIV inhibits the Mtb specific T cell response through inhibition of antigen presentation at the level of autophagy inhibition and reduction in cytokine secretion and expression of HLA-DR and co-stimulatory molecules. By inducing autophagy the processing of antigens might be increased, which can promote a protective T cell response. How autophagy is induced might however be important. For example by inducing autophagy with Earle's balanced salts solution (EBSS) in BCG infected DCs, cytokine production was promoted and expression of HLA-DR was increased, while treatment with rapamycin had the opposite effect (225). Since rapamycin is a rather poor inducer of autophagy in mammalian cells with its effect depending on cell type (212), we additionally used Torin1 in our experiments (Paper I). Torin1 is an ATP-competitive inhibitor that suppresses both mTORC1 and mTORC2 and is more potent and selective than rapamycin (212). Despite of this we found that also Torin1 increased the replication of Mtb, indicating that inhibition of mTOR might not be a beneficial way of inducing autophagy during HIV coinfection.

We further explored the possibility of autophagy induction in macrophages by efferocytosis of apoptotic neutrophils (Paper III), but did not find such an effect. Still, efferocytosis has been shown to be able to promote a T cell response. Dendritic cells that phagocytose apoptotic macrophages containing Mtb antigens could through cross-presentation activate CD8 T cells for IFN-γ production (226,227). The efferocytosis, promoted MHC-I upregulation along with increased expression of CD40, CD80 and CD86 and increased cytokine production (226). Furthermore Mtb infected neutrophils provided antigens to DCs upon efferocytosis and facilitated activation of CD4 T cells (228), while Mtb-inhibited neutrophil apoptosis lead to delayed activation of T cell responses (229). These findings suggest a beneficial role of efferocytosis for activation of the adaptive immunity during Mtb infection. However during HIV coinfection the activation of the adaptive immunity is impaired (Paper II), and the well-known decrease in CD4 T cells creates a severe immunodeficiency. Therefore we instead investigated if efferocytosis of apoptotic neutrophils could boost the innate immune response against Mtb in macrophages (Paper III).
Most studies investigating efferocytosis have focused on the antibacterial effect of uptake of infected apoptotic cells by uninfected cells. However since our group has previously found that uninfected apoptotic neutrophils promote an antibacterial response in Mtb infected macrophages (79), we explored this further in a HIV coinfection model (Paper III). We found that efferocytosis of apoptotic neutrophils promoted ROS production in infected macrophages. The role of ROS in defense against Mtb is controversial and the bacteria has several ways to counteract redox stress. However we hypothesize that neutrophils which are able to mount a strong oxidative burst can aid macrophages in their ROS production upon uptake of apoptotic neutrophils and their granules. This increased ROS production might override the bacterial defense and cause decreased replication. We found not only increased ROS production but also MPO after efferocytosis, which indicates a role for neutrophils which harbor abundant amounts of MPO in their granules. Neutrophils are the biggest cell source of MPO since 5% of their dry weight consist of it, while monocytes and macrophages have considerably less (219). MPO is responsible for the production of the radical HOCl which is not produced in the absence of the enzyme (230). Although this radical and other oxidative metabolites can be bactericidal for some pathogens, we do not exclude the possibility of other ROS-mediated effects in addition to a direct effect of ROS or MPO on Mtb. ROS can promote cell signaling and production of cytokines (155,230), and since we have previously seen an increased IL-1β production upon efferocytosis of apoptotic neutrophils (79) it might be mediated by ROS. We used ABAH to specifically inhibit MPO in neutrophils and thereby confirmed that decreased Mtb growth was caused by this enzyme, derived from the neutrophils. Although MPO was able to boost the immunity against Mtb in macrophages (Paper III), the enzyme has been shown to have a suppressive effect on the adaptive immunity (231). MPO released in lymph nodes by neutrophils inhibited DC activation and caused a decreased T cell response (231). Upon infection however, neutrophil-derived MPO mediates a bactericidal effect in macrophages (Paper III), which are the primary host cells of Mtb.

Boosting of the innate immunity might therefore be a good strategy to compensate for the adaptive immunity, which is normally delayed during Mtb infection (184), and severely deficient during HIV coinfection. Along with our observed impaired CD4 T cell response during coinfection (Paper II), the CD8 T cell response has also been shown to be affected. TB and HIV coinfected patients display an absence of co-stimulatory molecules and cytokine secretion in CD8 T cells, along with diminished levels of intracellular IFN-γ, perforin and granzyme B in HIV specific CD8 T cells (232). Thus, HIV does not only impair the CD4 T cell response against Mtb, but also during coinfection reduces the CD8 T cell response against the virus. The HIV-mediated T cell suppression by secretion of IDO and upregulation of PD-1 and CTLA-4 on T cells, further decrease the responses against both Mtb and HIV, and contributes to disease progression (52,211).

Previous work have shown that HIV preferentially infects and depletes Mtb Ag-specific T cells and thereby decrease the immune response against TB (49). We found that HIV also interfered with the response of Mtb Ag-specific T cells upon antigen presentation by HIV coinfected dendritic cells (Paper II). In order to create Mtb Ag-specific T cells we used the Mtb antigens Ag85B and PPD. The Ag85 complex (Ag85A, Ag85B, Ag85C) is one of the major
secretory antigens of Mtb, with Ag85B as the most abundant one, which is able to induce a Th1 response with production of IFN-γ (233,234). After confirming the specificity of the T cells, we observed an increased IFN-γ production upon Mtb infection and antigen presentation by dendritic cells. However, HIV inhibited Mtb antigen presentation and IFN-γ production from the Mtb Ag-specific CD4 T cells. Furthermore, our group has recently shown that HIV coinfected dendritic cells induce increased expression of co-inhibitory molecules on Mtb Ag-specific T cells, along with increased production of IL-10 and TGF-β. These suppressive Mtb Ag-specific T cells failed to control Mtb in macrophages (235). Without the activation of T cells and their production of IFN-γ, the enhancement of macrophage immunity against Mtb is impaired, and need to be activated in other ways during an HIV coinfection. A boosting of macrophages through cooperation with neutrophils (Paper III) could therefore compensate for the deficient adaptive immune response against Mtb during HIV infection.
GENERAL CONCLUSIONS

In conclusion this thesis demonstrates that autophagy induction through mTOR inhibition by Torin1 caused an increased replication of Mtb along with decreased acidification of Mtb phagosomes in HIV coinfected macrophages. In contrast to the belief that autophagy is an important defense mechanism against Mtb, mTOR inhibition might play a detrimental role during an HIV coinfection scenario.

Furthermore HIV can inhibit autophagy and antigen processing and prevent Mtb Ag-specific T cell responses through decreasing cytokine production and expression of HLA-DR and costimulatory molecules on coinfected dendritic cells. This inhibited antigen presentation and T cell activation explains the impaired immune defense against Mtb during an HIV coinfection.

Boosting the innate immunity was attempted by adding apoptotic neutrophils to coinfected macrophages. The macrophages became more capable of controlling Mtb infection since they acquired MPO. MPO or ROS could either have a direct bactericidal effect on Mtb or mediate it through cell signaling and cytokine production in the infected macrophages.

Stimulation of an increased uptake of apoptotic neutrophils or MPO could arm the macrophages in the battle against Mtb, and possibly compensate for the inhibited T cell response during HIV coinfection.
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Papers

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