Immunological and antimicrobial strategies against tuberculosis

Tenland, Erik

2019

Document Version:
Publisher's PDF, also known as Version of record

Link to publication

Citation for published version (APA):
Tenland, E. (2019). Immunological and antimicrobial strategies against tuberculosis Lund: Lund University, Faculty of Medicine
Immunological and antimicrobial strategies against tuberculosis

Erik Tenland

DOCTORAL DISSERTATION
by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at Belfragesalen, BMC D15, Sölvegatan 19, Lund. February 22nd
at 09:00

Faculty opponent
Professor Martin E Rottenberg,
Karolinska Institute, Stockholm, Sweden
Tuberculosis (TB) is the leading cause of death in the world from a single infectious agent, with approximately 1.5 million deaths every year. It is caused by Mycobacterium tuberculosis, a bacteria with the ability to manipulate our immune system and even survive inside phagocytes such as macrophages. The only approved vaccine, BCG, was developed a century ago and only protects well against severe forms of tuberculosis in children.

In the first part of this thesis, we study early immune responses during mucosal BCG vaccination in the lungs, to investigate if this route of administration may be beneficial. We use primary human airway epithelial cells from to study the effects on BCG on these cells. In Paper I, we report that airway epithelial cells produce the pro-inflammatory cytokines IL-6 and IL-8 that helps recruit neutrophils. In addition, we study the two cytokines IL-17A and IFN-γ, each being a key effector molecule in CD T cell memory responses. We find that IFN-γ reduce neutrophil recruitment while it is maintained with IL-17A. An IL-17A driven recall response may therefore gather more neutrophils, which may be beneficial for the eradication of TB-bacteria, but may also be potential risk for inflammation-induced lung pathology. In Paper II we report that BCG bacteria also induce antiinflammatory signals by manipulating small GTPases in epithelial cells. Most effect are dependent on signaling through CXCR1 and/or CXCR2, which make them potential targets for vaccine boosters.

In the second part of the thesis, we study antimicrobial peptides as potential drug candidates against tuberculosis. Today, the treatment for tuberculosis takes at least six months and involves four different antibiotics. For drug-resistant strains, the treatment takes two years. This puts enormous strain on both the health care system and the patient and highlights the dire need for new therapeuic candidates. In Paper III, we report that the novel peptide NZX has effect against strains of Mycobacterium tuberculosis, the causative agent for tuberculosis. It works in vitro against both laboratory strains and clinical isolates. Further, the peptide showed no toxicity towards human cells and was able to reduce bacterial burden in a mouse model of tuberculosis.

In Paper IV, we demonstrate that nanoparticles can be used as carriers for peptide antibiotocs such as NZX. They are easily synthesised and efficiently engulfed by macrophages and monocytes, which shows potential ofr treating intracellular bacteria. The particles did not reduce the efficacy of NZX in vitro or in vivo.

Together, these papers demonstrate both immunological and antimicrobial strategies against tuberculosis with the purpose of either developing new vaccine strategies or antibiotics. Both are critical topics in the struggle against this ancient disease.
Immunological and antimicrobial strategies against tuberculosis

Erik Tenland
Cover photo: Mouse macrophage infected with *Mycobacterium tuberculosis.*
Photo by Matthias Mörgelin

Copyright Erik Tenland

Faculty of Medicine
Department of Laboratory Medicine

ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2019
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table of Contents</td>
<td>6</td>
</tr>
<tr>
<td>List of Publications</td>
<td>8</td>
</tr>
<tr>
<td>Papers included in this thesis</td>
<td>8</td>
</tr>
<tr>
<td>Peer-reviewed papers outside this thesis</td>
<td>9</td>
</tr>
<tr>
<td>Populärvetenskaplig sammanfattning</td>
<td>10</td>
</tr>
<tr>
<td>List of abbreviations</td>
<td>12</td>
</tr>
<tr>
<td>Introduction</td>
<td>14</td>
</tr>
<tr>
<td>Brief history of tuberculosis</td>
<td>14</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>15</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis complex</td>
<td>16</td>
</tr>
<tr>
<td>The mycobacterial cell wall</td>
<td>16</td>
</tr>
<tr>
<td>Pathogenesis</td>
<td>18</td>
</tr>
<tr>
<td>Transmission</td>
<td>18</td>
</tr>
<tr>
<td>First encounter with the host</td>
<td>18</td>
</tr>
<tr>
<td>Surviving phagocytosis</td>
<td>21</td>
</tr>
<tr>
<td>Activation of the adaptive immune response</td>
<td>22</td>
</tr>
<tr>
<td>The adaptive immune response</td>
<td>23</td>
</tr>
<tr>
<td>Granulomas</td>
<td>26</td>
</tr>
<tr>
<td>Clinical manifestations</td>
<td>28</td>
</tr>
<tr>
<td>Diagnosis</td>
<td>29</td>
</tr>
<tr>
<td>Treatment</td>
<td>30</td>
</tr>
<tr>
<td>Multi-drug resistant tuberculosis</td>
<td>31</td>
</tr>
<tr>
<td>Extensively drug resistant tuberculosis</td>
<td>32</td>
</tr>
<tr>
<td>Vaccine strategies</td>
<td>33</td>
</tr>
<tr>
<td>The BCG vaccine</td>
<td>33</td>
</tr>
<tr>
<td>Vaccine development</td>
<td>33</td>
</tr>
<tr>
<td>Antimicrobial peptides</td>
<td>36</td>
</tr>
<tr>
<td>AMPs in tuberculosis</td>
<td>36</td>
</tr>
<tr>
<td>NZX – The new defensin</td>
<td>39</td>
</tr>
<tr>
<td>Nanoparticles</td>
<td>40</td>
</tr>
</tbody>
</table>
## Aims of the thesis

Methods ................................................................................................................................. 44

| Cells ................................................................................................................................. 44 |
| Bacteria .......................................................................................................................... 44 |
| Major molecular techniques ......................................................................................... 45 |
| Western Blot .................................................................................................................... 45 |
| Enzyme-linked immunosorbent assay ............................................................................ 45 |
| Flow cytometry ................................................................................................................ 45 |
| Fluorescence microscopy ............................................................................................... 46 |
| Minimum inhibitory concentration .............................................................................. 46 |
| Cytotoxicity assays ........................................................................................................ 46 |
| Animal models ............................................................................................................... 47 |
| Statistics ......................................................................................................................... 48 |
| Ethical approval ............................................................................................................. 48 |

Results ................................................................................................................................ 49

| Paper I ............................................................................................................................. 49 |
| Paper II ......................................................................................................................... 49 |
| Paper III ....................................................................................................................... 50 |
| Paper IV ....................................................................................................................... 51 |

Discussion .......................................................................................................................... 52

| Immunological strategies .............................................................................................. 52 |
| Antimicrobial strategies ............................................................................................... 54 |

Conclusions ......................................................................................................................... 57

Acknowledgements ............................................................................................................. 58

References ........................................................................................................................... 60

“The idea is to go from numbers to information to understanding”

- Hans Rosling
List of Publications

Papers included in this thesis

This thesis is based on the following papers, which will be referred to by their roman numerals in the text.


Peer-reviewed papers outside this thesis


“A modest wish: That our doings and dealings will be of little more significance to life than a man’s dinner jacket is to his digestion. Yet not a little of what we describe as our achievement is, in fact, no more than a garment in which, on festive occasion, we seek to hide our nakedness”

- Dag Hammarskjöld 1960
**Populärvetenskaplig sammanfattning**

Tuberkulos, eller lungsot som det länge kallades i Sverige, är en lungsjukdom som orsakas av bakterien *Mycobacterium tuberculosis*. Sjukdomen tros ha funnits redan hos de första människorna som vandrade ut ur Afrika för över 70 000 år sedan. Den har sedan följt med på människans stora vandringar och på så sätt spritt sig över hela världen. Forskare har kunnat hitta spår av bakterier i såväl egyptiska som peruanska mumier.


Världshälsoorganisationen uppskattar att cirka tio miljoner människor får tuberkulos varje år och 1.5 miljoner dör i sjukdomen. Varje dag dör alltså fler än 4000 personer, vilket placerar tuberkulos i toppen på listan av dödligaste infektionssjukdomarna i världen. Behandlingen som finns idag är dyr och tar minst sex månader, vilket sätter enorm press på såväl sjukvården som patienten. Felaktig antibiotikaanvändning har även lett till uppkomsten av resistenta bakterier, mot vilka behandlingen blir mer komplicerad och tar ännu längre tid. Inte heller vaccinet räcker till för att stoppa tuberkulos. Det skyddar väl mot de livsfarliga former av tuberkulos som kan drabba barn, men har däremot bristande förmåga att skydda mot sjukdomen hos vuxna.

I de arbeten som presenteras i denna avhandling studerar vi hur vaccinet eventuellt kan förbättras genom att, istället för en injektion i armen, tas via lungorna. Dessutom utvärderar vi en ny antibiotika och dess potential som behandling mot tuberkulos.

Tuberkulosbakterien är väldigt väl anpassad till att leva i människan som är bakteriens enda naturliga värd. Bakterien kan gömma sig inuti värdens celler, där
den skyddas från många av de strategier som immunförsvaret vanligtvis använder mot inkärtande bakterier.


I den andra artikeln (Paper II) ser vi hur BCG-vaccinet påverkar epitelcellerna ytterligare. Förutom de inflammatoriska signalerna vi sett tidigare, ser vi att BCG-vaccinet även genererar ett anti-inflammatoriskt svar. Vid vaccination är det viktigt att ett tillräckligt starkt immunsvar skapas så att det immunologiska minne som skapas är starkt nog för att stå emot när den riktiga infektionen sker. Vi fann att det anti-inflammatoriska svaret berodde på en specifik receptor på cellens yta. Denna receptor kan vara en intressant att blockera för att öka det immunologiska svaret vid vaccination.

Utvecklingen av antibiotika mot tuberkulos är den huvudsakliga anledningen till att sjukdomen kommit under kontroll i länder med tillräckligt utvecklad sjukvård. Dock har utvecklingen stannat av och endast två antibiotika har godkänts för användning mot tuberkulos de senaste 40 åren. Dessa två används idag nästan uteslutande för behandling av resistenta bakterier, där den vanliga antibiotikan inte längre hjälper. Antibiotikaresistensen bland tuberkulosbakterier ökar i världen och i kombination med de långa behandlingstiderna gör det att det finns ett väldigt stort behov av nya mediciner.

I den tredje artikeln (Paper III) undersöker vi, för första gången, hur det lilla proteinet NZX har antibakteriell funktion mot tuberkulosbakterier. Vi ser också att den inte är farlig för mänskliga celler och att den fungerar som behandling i tuberkulosinfekterade möss. Vi föreslår därför NZX som en mycket intressant ny kandidat för behandling av tuberkulos.

I vår sista artikel (Paper IV) testar vi om små så kallade nanopartiklar kan fungera som bärare av protein-antibiotika (NZX) vid tuberkulosbehandling. Vi rapporterar att partiklarna inte försämrar den antimikrobiella effekten hos NZX. Partiklarna tas dessutom lätt upp i cellerna, vilket skulle kunna öka förmågan för NZX att nå fram till bakterier som gömmer sig inuti celler.
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEC</td>
<td>Airway epithelial cell</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>AMP</td>
<td>Antimicrobial peptide</td>
</tr>
<tr>
<td>BCG</td>
<td><em>Mycobacterium bovis</em> Bacilli Calmette-Guérin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFP-10</td>
<td>Culture filtrate protein 10</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DOT</td>
<td>Directly observed therapy</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMB</td>
<td>Ethambutol</td>
</tr>
<tr>
<td>ESAT-6</td>
<td>Early secreted antigen 6</td>
</tr>
<tr>
<td>FQ</td>
<td>Fluoroquinolone</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HβD2</td>
<td>Human beta-defensin 2</td>
</tr>
<tr>
<td>hCAP18</td>
<td>Human cationic antimicrobial peptide 18</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HNP</td>
<td>Human neutrophil peptide</td>
</tr>
<tr>
<td>IGRA</td>
<td>Interferon gamma release assay</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL17R</td>
<td>Interleukin 17 receptor</td>
</tr>
<tr>
<td>INH</td>
<td>Isoniazid</td>
</tr>
<tr>
<td>LAM</td>
<td>Lipoarabinomannan</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTBI</td>
<td>Latent tuberculosis infection</td>
</tr>
<tr>
<td>MAIT</td>
<td>Mucosal associated invariant T cell</td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosa-associated lymphoid tissue</td>
</tr>
<tr>
<td>MDR-TB</td>
<td>Multidrug-resistant tuberculosis</td>
</tr>
<tr>
<td>MGIT</td>
<td>Mycobacteria growth indicator tube</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MMP-1</td>
<td>Matrix metalloproteinase 1</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>MSP</td>
<td>Mesoporous silica nanoparticle</td>
</tr>
<tr>
<td>Mtb</td>
<td><em>Mycobacterium tuberculosis</em></td>
</tr>
<tr>
<td>NET</td>
<td>Neutrophil extracellular trap</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS2</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>PDIM</td>
<td>Phtiocerol dimycocerosate</td>
</tr>
<tr>
<td>PYR</td>
<td>Pyrazinamide</td>
</tr>
<tr>
<td>Rac1</td>
<td>Ras-related C3 botulinum substrate toxin 1</td>
</tr>
<tr>
<td>RD1</td>
<td>Region of difference 1</td>
</tr>
<tr>
<td>RIF</td>
<td>Rifampicin</td>
</tr>
<tr>
<td>RR-TB</td>
<td>Rifampicin-resistant tuberculosis</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TDM</td>
<td>Trihalose dimycolate</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TST</td>
<td>Tuberculin skin test</td>
</tr>
<tr>
<td>T7S</td>
<td>Type VII secretion system</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organisation</td>
</tr>
<tr>
<td>XDR-TB</td>
<td>Extensively drug-resistant tuberculosis</td>
</tr>
</tbody>
</table>
Introduction

Brief history of tuberculosis

Tuberculosis (TB) is an ancient disease and most probably developed around the time of the first humans. As a result, the disease has followed the human population for thousands of years, and spread through all corners of the earth during the great human migrations out of Africa (1). Researchers have found signs of TB disease and mycobacterial DNA in mummies from Egypt as well as from Peru (2, 3).

Tuberculosis has been called many names throughout the years, one of them being “the White Plague” (4). The name is in contrast to the Black Plague, or Black Death, that was caused by *Yersinia pestis* during the Middle Ages and responsible for quickly killing one third of the population in Europe (5). The White plague, on the other hand, was a chronic disease that took a long time to develop. The patients became pale, coughed, fatigued and lost weight. This pattern of slow deterioration gave rise to another famous name for tuberculosis, “consumption”. This name described the look of the infected patients that appeared to be slowly consumed by the disease.

Consumption was a mystery and for a time in the 19th century the disease was even romanticized. In a time when science offered little help for those affected, some viewed consumption as a path to redemption, a beautiful death (6). Several famous cultural works from the time depicts tuberculosis. One example is Victor Hugo’s *Les Misérables*, where Fantine, facing the hopelessness of her circumstances, succumbs to death by consumption, her only way out. In *La traviata* by Verdi, the main character Violetta, who also suffers from consumption is depicted as particularly beautiful due to the paleness and upon her death described as a beautiful ghost (6). Towards the end of the 19th century science finally caught up, when Robert Koch discovered the causative agent of consumption, *Mycobacterium tuberculosis*. The discovery put an end to the mystification of the disease and his discoveries awarded him the Nobel Prize in Medicine and Physiology in 1905 (6, 7). Immediately, efforts to create a functional vaccine started. Edward Jenner had already created the first vaccine ever in 1796 against smallpox (8). It would prove much harder, however, to develop a vaccine against
tuberculosis. Robert Koch himself, using a crude mixture of mycobacterial components known as tuberculin, made one of the first attempts. It unfortunately failed to protect people against tuberculosis. However, it was discovered that tuberculin induced a local immune reaction in people infected with *Mycobacterium tuberculosis* (Mt(b), and the tuberculin skin test has since been used as a clinical test for exposure to mycobacteria (9).

In the 1920s, Albert Calmette and Camille Guérin finally developed the first vaccine against tuberculosis at their lab at the Pasteur Institute in Paris. The vaccine was based on an attenuated strain of *Mycobacterium bovis*, which could no longer cause tuberculosis disease. The *Mycobacterium bovis* Bacilli Calmette-Guérin (BCG) vaccine is still, a hundred years later, the only vaccine approved against tuberculosis, and have been given to more than 3 billion people over the years, making it the most widely used vaccine in the world (7, 10, 11).

Treatment for tuberculosis has also changed substantially over the years. The conventional regimens in the early 20th century were fresh air, rest and high altitude, preferably at a sanatorium. In 1947, the big breakthrough came when Streptomycin, the first anti-tuberculosis drug, was discovered (12). Other important agents such as Rifampicin and Isoniazid were introduced in the 1950s and 1960s and both became the part of the standard drug regimen that is still used today. No new drugs were introduced until 40 years later, when Bedaquiline and Delanamid became available for treatment against drug-resistant tuberculosis in 2012 (12).

Today, approximately 10 million people develop active tuberculosis every year and approximately 1.5 million die from the disease (13). Tuberculosis remains a major health concern and there is a dire need for new treatments and vaccine strategies if we are to end the reign of this ancient disease.

**Mycobacterium tuberculosis**

*Mycobacterium tuberculosis* is a rod-shaped, slow-growing bacterium which belong to the taxa Actinomyces (14). The name “mycobacteria” stems from the Greek word *mükês*, which means fungus. It does not mean, however, that mycobacteria are related to fungi. Rather, the name originates from the hydrophobic nature of mycobacteria which cause their colonies to look similar to mold (15). The two most well-known species of mycobacterium are *Mycobacterium tuberculosis* and *Mycobacterium leprae*, responsible for causing tuberculosis and leprosy respectively, both considered diseases that have been present in human population since ancient times (16).
**Mycobacterium tuberculosis complex**

*Mycobacterium tuberculosis*, as its name implies, is the most common cause of tuberculosis and considered to be the etiological agent. However, Mtb is not the only mycobacterium able to cause tuberculosis. *Mycobacterium canettii*, *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium bovis*, the vaccine strain *Mycobacterium bovis* BCG as well as Mtb, all belong to a group of closely related bacteria, called the *Mycobacterium tuberculosis* complex (17). All of the members are able to cause tuberculosis in humans, but differ in their prevalence and pathology. BCG is a special case, since it is an attenuated strain of *M. bovis* and cause tuberculosis disease only in very rare cases, mostly in immunocompromised individuals (18).

It was thought for a long time that *M. bovis* predated Mtb and was the origin of the Mtb species by infection from animals to humans (17). Zoonosis is a common origin of many diseases in humans and *M. bovis* is also less limited in its host range. Recent genome analysis, however, points in the opposite direction and rather supports Mtb to be the origin of the *M. bovis* by infection from humans to animals (17).

**The mycobacterial cell wall**

The reason for the hydrophobic and characteristic appearance of mycobacteria in culture is due to its complex and lipid-rich cell wall (15, 16). The cell wall is the major factor that sets mycobacteria apart from other prokaryotes (19). Mycobacteria stain poorly with Gram-staining, which have made Gram-classification difficult and often controversial (20, 21).

A schematic representation of the mycobacterial cell wall is displayed in Figure 1. Outside of the plasma membrane, the innermost layer of the mycobacterial cell wall consists of peptidoglycans, similar to those found in other bacteria (22). The second layer is a large network of arabinose and galactose, known as arabinogalactan. The arabinogalactans are bound to long fatty acid chains, the mycolic acids (19). These acids are a major part of the mycobacterial cell wall and responsible for much of its thickness and hydrophobicity, since they each consist of two long fatty acid branches.

The outmost layer of the cell wall, sometimes called the outer membrane, isn’t anchored to the rest by covalent bonds, but rather use hydrophobic forces to associate with the mycolic acids. (15). Among the most important molecules of the outer layer is a glycolipid known as trihalose dimycolate (TDM). TDMs are highly immunogenic and sometimes used experimentally to boost immune response during studies of vaccination (23, 24). Phthiocerol dimycocerosate (PDIM) is also
part of the outer layer. While the role of PDIM is not completely clear, studies suggest that loss of PDIM expression leads to attenuation of Mtb (25). Also, in vitro culture of Mtb can lead to the spontaneous loss of PDIM expression, affecting their ability to infect animal models (26). The outer layer also contains mycosides. The expression of these peptidoglycolipids varies in mycobacteria and affect their colony appearance, which can be used to distinguish between species (15). Due to it forming a thick protective layer in some mycobacteria, the mycosides are sometimes referred to as the mycobacterial capsule (15).

Anchored in the plasma membrane, the lipoarabinomannan (LAM) is a lipopoly saccharide that project outwards through all the layers of the cell wall (15). Slow-growing, pathogenic mycobacteria have mannose-capped LAM residue, while the LAM of fast-growing strains, such as M. smegmatis is capped with phosphoinositol. This highlights the slight differences in cell wall compositions between species of mycobacteria (16).

The exact conformation of the mycobacterial cell wall is not completely understood and there are several models discussed by researchers in the field. The cell wall, and especially the composition of the outer layer is more dynamic than previously thought. One example of this is that the outer layer is often very thin when bacteria are grown in vitro, but rather pronounced in vivo (19).

Figure 1: Structure of the mycobacerial cell wall
The mycobacterial cell wall consists of several layers. The innermost layer is the cell membrane to which a layer of peptidoglycan is anchored. Attached to the peptidoglycans are the arabinogalactan which in turn connects to the mycolic acids. Glycolipids, such as TDMs and PDIMs make up the outer layer. LAM is anchored in the cell membrane and extends through all layers. Reprinted from “Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi”, Brown et al 2015, with permission from Springer Nature. Reference (27)
Pathogenesis

Transmission

Tuberculosis is transmitted through the inhalation of aerosolized droplets containing live bacteria. These droplets are produced by coughing or sneezing by an individual with active pulmonary tuberculosis disease (28). Much of our knowledge about transmission dates back to the 1950s, when Riley et al. performed studies with guinea pigs in a hospital ward where tuberculosis patients were treated. It was found that outgoing air from the ward resulted in disease in 3 out of 156 animals on average every month (29). With no other sources of transmission, it was concluded that tuberculosis spreads through aerosolized droplets. However, the studies also showed that the infectivity of tuberculosis patients may be lower than previously believed and exposure time was put forward as an important factor for the risk of infection. Modern studies support this, since the risk of disease is higher in household contacts than casual contacts, although the overall risk of infection is only a few percent (30). Some argue that tuberculosis is mainly a socio-economic disease of the poor, that could most efficiently be dealt with by improving living conditions and decreasing crowding as these measures would greatly decrease the risk of transmission (31).

"The risk of infection becomes particularly high if healthy people have to sleep with the sick in the same rooms, and especially, as still unfortunately happens with the poorer section of the population, in one and the same bed"

– Robert Koch, Nobel acceptance speech, 1905.

First encounter with the host

After being inhaled, the droplets containing *Mycobacterium tuberculosis* (MtB) reach the lower respiratory tract and the alveoli, where it encounters the host cells for the first time. A key event in the early stages of MtB infection occurs when the bacteria encounters and is engulfed by patrolling alveolar macrophages, which carries them across the basal membrane into the lung interstitium (28). Classically, macrophages have been given a lot of attention in tuberculosis research, as they have been believed to be the entry mechanism for MtB into the host lung tissue. Indeed, eliminating alveolar macrophages prior to lethal infection with MtB resulted in improved protection and reduced mycobacterial growth (32). However, macrophages may pay more than one role in tuberculosis. One example
of this was shown when specific elimination of activated macrophages at a later stage of infection resulted in less control of infection and poorer prognosis (33).

*Epithelial cells*

Nowadays, other cell types are given more attention and their roles in tuberculosis have been investigated more thoroughly, one of them being the epithelial cells. For every macrophage in the alveoli, there are approximately ten alveolar epithelial cells (34). Increasing evidence point towards alveolar epithelial cells as active players in the response and outcome of tuberculosis (35). Epithelial cells can recognize Mtb through pattern recognition receptors such as toll-like receptor 2 (TLR-2), TLR-4, TLR-6 and TLR-8 (36). This leads to production of pro-inflammatory cytokines such as interleukin 6 (IL-6) and chemokines such as IL-8, which helps the recruitment of phagocytes such as neutrophils (37). Epithelial cells have also been reported to produce antimicrobial peptides (AMPs), such as LL-37 and human beta defensin 2 (HβD2) when encountering mycobacteria (38, 39).

While these mechanisms may all contribute to better control of Mtb infection, other findings support that epithelial cells could contribute to tuberculosis disease progression. Virulent mycobacteria can grow inside epithelial cells, cause cell lysis and infect neighbouring cells (40). Mtb actually replicates much better inside alveolar epithelial cells (50-fold) than inside mouse or human macrophages (41). Mycobacteria have been shown to bypass epithelial pro-inflammatory signalling by NF-kB in favour of a more anti-inflammatory IL-10 and IL-22 response (42). Mtb, but not BCG have been shown to be toxic to epithelial cells and macrophages and this effect is enhanced after the bacteria have been passaged inside the cells (43). In addition, epithelial cells have been reported to produce intestinal collagenases such as MMP-1 in response to infection, which could contribute to tissue destruction in tuberculosis (44).

*Non-classical lymphocytes*

Epithelial cells harbouring intracellular Mtb activate a subset of tissue immune cells known as mucosal-associated invariant T cells (MAIT). This activation is dependent of the non-classical MHC class I receptor MR1 (45). These CD8+ T cells are present in uninfected lungs and enriched in the lungs of patients with active tuberculosis. IFN-γ production by MAIT cells correlates with more limited Mtb infection (46). Other cell types that could play a role in limiting early infection include innate lymphoid cells such as invariant natural killer T cells (iNKT cells), which produce GM-CSF in response to mycobacterial infection (47) and the γδT cells, which display several effector functions and diversity after activation with Mtb (48).
Neutrophils

Neutrophils are not very well studied in their role in tuberculosis. Much of this is due to the difficulty of studying these cells in vitro, since neutrophils are short-lived, non-dividing and reactive (49). Neutrophil numbers have been found to correlate with better resistance to Mtb infection in people in the vicinity of individuals with active tuberculosis. This resistance was explained by the possibility of the neutrophils to produce AMPs such as HNP 1-3 and LL-37 (50). Rats that had been pre-treated with LPS in the lungs to recruit neutrophils, were more resistant to airway infection with Mtb (51). This did not occur if the neutrophils had been depleted. When administered at later stages of infection, LPS treatment showed no effect on the outcome. Similarly, depletion of neutrophils during infection in mice resulted in higher bacillary load, smaller granulomas and slower onset of adaptive immunity (52). Neutrophils may also be a source of chemokines, since BCG infection can trigger TLR-2 and TLR-4 dependent production of IL-8 (53).

While these data argues for neutrophils having an overall beneficial role in tuberculosis, other studies have pointed out the need for caution when assessing their role. In patients with active tuberculosis, neutrophils are the most numerous phagocytic cells present, and have been shown to harbour dividing intracellular Mtb (54). Indeed, many researchers in the field argue that accumulation of neutrophils is a sign of progression to active disease due to the loss of Mtb containment (28). Thus, the overall role of neutrophils, like epithelial cells, cannot simply be seen as either beneficial or harmful during tuberculosis. It probably depends on the timing and interplay with other cells and mechanisms during the infection.

Neutrophil extracellular traps

In 2004, Neutrophil Extracellular Traps (NETs) were described for the first time as a mechanism for killing extracellular bacteria (55). NETs are DNA fibers associated with granular proteins, which are released from neutrophils as a response to stimuli and have been shown to trap and kill bacteria (55). While NETs can be induced by Mtb, it only traps the bacteria but does not kill it (56). Although extracellular mechanisms have previously been largely overlooked by researchers due the intracellular nature of Mtb, new theories point out that the bacteria may spend a much larger portion of its lifecycle being extracellular (57). Therefore, new extracellular mechanism may be of interest. In the 15 years that have passed since the discovery of NETs, it has become clear that the mechanism may involve more than just a way to kill bacteria (58). One key property of NETs is their pro-inflammatory nature. While this may be beneficial to mount an appropriate immune response, extensive inflammation may cause severe tissue destruction and contribute to disease progression.
Surviving phagocytosis

Being engulfed by a macrophage through phagocytosis marks the end of the life for most types of bacteria. For Mtb, however, this may only mark the beginning of the disease, as entry point into the host.

Phagocytosis is a mechanism by which foreign particles and pathogens become engulfed and destroyed by professional phagocytes. One may expect professional phagocytes to offer one of the worst intracellular niches for bacteria to survive. Despite this, several bacteria such as Mtb, have developed mechanisms to survive in one of the most hostile intracellular environment the host have to offer, namely the phagosome.

The mycobacteria phagosome
Intracellular pathogens use many different strategies to evade destruction by phagocytosis, such as evading phagocytosis altogether, getting engulfed by less bactericidal cells or deploying mechanisms to survive the hostile environment of the phagolysosome (59). In the case of mycobacteria, the key to surviving phagocytosis is the inhibition of phagosome maturation. Some of the important strategies are visualized in Figure 2. Early studies found that untreated Mtb-infected macrophages had lower activity of lysosome enzymes than infected macrophages after treatment (60). Follow-up studies revealed that live Mtb could reduce phagosome-lysosome fusion, indicating an active mechanism of action (61). Infected human alveolar macrophages from tuberculosis patients are still able to engulf foreign material and successfully fuse those phagosomes with lysosomes. However, the fusion isn’t seen in the compartments containing bacteria, pointing towards Mtb actively preventing the lysosome fusion of its own phagosome (62). Mycobacteria-containing phagosomes are arrested within the early endosomal network where they are still accessible to trafficking by other endosomes (63, 64). Mycobacteria manipulate accumulation of small GTPases from the Rab family to prevent endosome maturation. Rab5, associated with early endosomes, is enriched, while Rab7, associated with late endosomes is inhibited (65). Mycobacteria inhibit the acidification of its phagosome, not letting it fall below pH 6.2. It does this by preventing the accumulation of proton pumps in the phagosome membrane (66).
Activation of the adaptive immune response

Most pathogens trigger an adaptive immune response about one week after infection. However, during an infection with Mtb, it takes between 5-6 weeks for adaptive immunity to develop, detected through a type IV hypersensitivity reaction to the tuberculin skin test (68). Why this delay in onset of the adaptive immune response occurs have puzzled researchers in the field throughout the years. While several plausible explanations exist, the truth may be in the combination of several factors.

Delayed dissemination to lymph nodes

In one study, researchers tested if the delayed adaptive immune response in the lungs was due to slower priming of naïve T cells or the trafficking of effector cells (69). They found that the activation of T cells depended on the production of Mtb antigens by bacteria in the lymph node, which in turn was delayed due to lack of shuttling from the lungs. When comparing the immune response between the “TB-resistant” C57/B6 mouse model and the more sensitive CH3 mouse model, earlier dissemination of Mtb to the lymph nodes and quicker onset of the adaptive
immune response were all found to be hallmarks for C57/B6 (70). In addition, recent evidence suggests that the dissemination of the bacteria to the lymph node may involve interplay between several cell types and not only be restricted to the classical shuttling of antigens by professional antigen-presenting cells such as dendritic cells (DCs) (71).

**Cells involved in dissemination**

In a mouse model with aerosol infection, the dissemination of Mtb through the lymphatic system to the draining lymph node occurred 9-11 days post infection (70). However, migration of infected DCs from the lungs to the lymph node was detected only after 14 days, which was after the activation of T cells had occurred. Migrating lung DCs were therefore probably not the first initiators or the reason for Mtb presence in the lymph nodes (71). The study used GFP-expressing bacteria and found that Mtb did infect DCs to a large extent, but was also present in other phagocytes such as neutrophils. The Mtb infected DCs were relatively inefficient at T cell activation. This was attributed to inhibited MHC class II-dependent antigen presentation, but not due to reduction of MHC class II receptors on the cell surface (71). In a chimeric mouse model, inflammatory CCR2+ monocytes were found to be crucial for the shuttling of bacteria to the lymph node and activation of CD4+ T cells. However, proper activation didn't occur without the presence of DCs (72). Even neutrophils have been shown to shuttle live bacteria to lymph nodes at early stages of infection, showing more pronounced transport than both DCs and macrophages (73).

Taken together, the onset of adaptive immunity may require DCs for proper activation, while also needing the help of professional phagocytes to shuttle live Mtb bacteria to the lymph nodes. Both processes appear delayed during Mtb infection, possibly as a result of inhibition by the bacteria. Another interesting theory of the delayed dissemination, is the slow growth of bacteria combined with the fact that alveolar macrophages and dendritic cells in the lungs show reduced tendency to migrate due to the immunologically permissive environment of the airways (74).

**The adaptive immune response**

The study of the adaptive immune response in tuberculosis is vast, and has been one of the major topics in the fields for the past one hundred years. At its core lies the question of what gives immunological protection against tuberculosis. While the question may seem simple, the issue stems from the fact that Mtb has developed alongside humans since our very beginning (1), becoming highly adapted to life in the human host and an expert at dealing with the mechanisms of
our immune system. Therefore, the border between which immune responses are for the benefit of the host and which are actually to the benefit of the pathogen becomes blurred. Answering these questions holds the key to development of host-targeted therapies and arguably most importantly for vaccine development.

**Interferon-γ**

Traditionally, tuberculosis immunology has been focused on the role of IFN-γ and cells that produce this cytokine. This is not surprising, since correlative data heavily points towards IFN-γ producing CD4+ T cells playing a major role in protective immunity against tuberculosis (74). When macrophages receive signal from IFN-γ, it triggers maturation and acidification of its phagosomes (75) as well as the production of reactive nitrogen intermediate such as nitric oxide (NO). Both mechanisms limit the intracellular viability of Mtb (76, 77). NO is produced by the enzyme inducible nitric oxide synthase (NOS2) and mice lacking this enzyme suffer from accelerated tuberculosis disease progression and susceptibility (78). In addition, NO can prevent immune-driven pathology during tuberculosis by inhibiting the processing of the pro-inflammatory cytokine IL-1 (79).

Patients with active tuberculosis generate a Mtb specific, IFN-γ producing T cell response, that can be detected in blood (80). This response is also the basis of the interferon gamma release assay (IGRA), a key test for exposure to Mtb. In a study comparing sensitive CH3 mice with resistant C57BL/6 mice after intravenous infection with Mtb the immune response in the lung was the delayed and the IFN-γ production (81). Genetic predisposition to mycobacterial disease can be seen in children and adults with mutations in the IFGR1/IFGR2, encoding the subunits of the IFN-γ receptor. Other genes where mutations cause similar predisposition include: STAT1, an important relay for IFN-γ signalling and IL12B and its receptor IL12RB1, involved in IL-12 signalling, an important induction of IFN-γ production. These mutations not only cause predisposition to tuberculosis, but also BCG induced disease (82). Mice lacking IFN-γ are very susceptible to mycobacterial infection and suffer from rapid disseminated disease (83, 84).

Infection with human immunodeficiency virus (HIV) leads to the reduction of CD4+ T cells and if not treated, the development of acquired immunodeficiency syndrome (AIDS) (85). Tuberculosis is classified as an opportunistic infection during AIDS and one of the main causes of death in patients with HIV (86). In patients with AIDS, tuberculosis is more prone to disseminated/miliary disease, negative tuberculin skin tests and has worse prognosis (87). The risks involved in co-infection with HIV are further illustrated by the risk of reactivated disease from latent tuberculosis. HIV patients with positive tuberculin skin tests have an annual risk of 10% to develop active tuberculosis disease, while for HIV negative, the risk is only 5-10% over the persons lifetime (88).
Understandingly, these findings regarding IFN-γ producing CD4+ T cells and tuberculosis have shaped the research to focus largely on these factors. However, accumulating evidence points towards a more complex picture with many other factors whose potential for protective immunity has just begun to be discovered.

CD8+ T cells play a big role during intracellular infections, especially those caused by viruses. CD8+ effector cells such as cytotoxic T lymphocytes can recognize foreign antigens presented on the surface of infected cells. The lymphocyte can then induce apoptosis in the affected cell (89). Targeted depletion in mice of either CD4+ or CD8+ cells makes them more sensitive to Mtb infection (90). A study in mice where CD4 had been knocked out the mice had lower levels of IFN-γ during early infection (91). However, at later stages the IFN-γ levels were the same, much owing to production by CD8+ cells. This indicates that a CD4-dependent, yet IFN-γ-independent mechanism is responsible for tuberculosis control or that IFN-γ is especially important during early infection. In a follow-up study, CD4+ cells were depleted in a mouse model of latent tuberculosis. This resulted in dramatic increase in bacterial numbers, pathology and decreased survival. IFN-γ levels, however, were unaffected and so was NOS2 activity, indicating once again an IFN-γ independent mechanism by CD4+ cells to control infection (92).

**Interleukin 17**

In recent years, IL-17 has become an interesting cytokine in the protection against tuberculosis. The IL-17 response is dependent on the expression of the cytokine IL-23, but it is not required for early control of mycobacterial growth (93). However, in the later stages of infection, mice deficient in IL-23 have smaller B cell follicles in the lungs, which leads to hampered immunity against tuberculosis. The absence of IL-23 leads to less expression of CXCL13, a chemokine responsible for organization of B cell follicles. Expression of IL-17 or IL-22 increases the expression of CXCL13, and lack of the IL-17 receptor; IL-17RA causes smaller B cell follicles early during infection, while lack of IL-22 causes smaller follicles at an intermediate time. This points towards both IL-17 and IL-22 signalling being at least partly responsible for proper B cell follicle formation. IL-23 however, is suggested to be critical, as the B cell follicles are small throughout the infection (93). Finally, IL-17 is important for the recruitment of neutrophils, and lack of IL-17 signalling in the lungs lead to impaired neutrophil recruitment (94).
Granulomas

One of the most characteristic hallmarks of tuberculosis is the formation of round immunological structures called granulomas. The name tuberculosis stems from the word “tubercle” meaning “round” or “node”, a reference to the presence of granuloma structures found in patients (95, 96).

Granuloma structure and protection

When the immune system fails to eradicate an inflammatory stimulus, granuloma formation is believed to be a mechanism to sequester the irritant from the rest of the host by walling it off (97). In the case of tuberculosis, infected macrophages, unable to destroy intracellular mycobacteria due to the mechanisms described previously, aggregate and trigger the onset of granuloma formation (98). Figure 3 shows a schematic representation of a tuberculosis granuloma with its cellular constituents. In the granuloma, macrophages become activated and commonly fuse together to form multinucleated giant cells or differentiate into foam cells, the latter being characterized by the accumulation of lipids. The consequences of these changes are not fully understood (99, 100). Later stages of granulomas are also characterized by the transformation of macrophages into so called epithelioid cells, which can link themselves together in tight formations (101). Numerous other cell types can be found within the granuloma, ranging from other professional phagocytes such as dendritic cells and neutrophils to, at a later stage, cells of the adaptive immune system such as T and B cells (95, 98). Tissue fibroblasts will produce extracellular matrix proteins and create a fibrous cuff around the granuloma. When this cuff becomes calcified the granuloma becomes visible on X-ray, which is why this method has been used extensively for diagnosis of tuberculosis (102).

Granulomas as a walled off compartment to protect the host from hyper-inflammation may seem as a successful strategy for the host. In support of this, healed tuberculosis granulomas have been found in people dying from other causes, without the individuals ever showing clinical symptoms of tuberculosis during their lifetime (103). In further support of granulomas as a key strategy in host protection, is the fact that highly tuberculosis susceptible mice with defects in inflammatory pathways such as IFN-γ and IL-12, fail to establish proper granulomas (84, 104). However, granuloma formation has also been described as an important event in disease progression and crucial for making a host able to transmit tuberculosis (95).

Granulomas in pathology

One of the hallmarks of a tuberculosis granuloma is its necrotic centre, classically called “caseum” due to its appearance being described as cheese-like.
(caseous=cheesy) by pathologists (95, 100). The necrotic centre arises when cells in the middle of the granuloma die as a result of hypoxia and stress. When the host cell die, Mtb become extracellular and proliferate in the granuloma centre (98). Surrounding cells gets activated by the pro-inflammatory signals from necrotic cell debris and live bacteria. The resulting inflammation destabilizes the granuloma structure. If the granuloma is ruptured, its content, including large numbers of live mycobacteria, are expelled into the airways, forming a lung cavity. The resulting inflammation in the lungs triggers the patient to cough, enabling the transmission of bacteria to a new host and thus completing the lifecycle of Mtb (105).

Figure 3: Structure and cellular constituents of a tuberculosis granuloma
In the center of the granuloma are necrotic cells and extracellular mycobacteria. Activated macrophages sometimes differentiate into foam cells or fuse together into multi-nucleated giant cells. At later stages, macrophages may differentiate into epitheloid cells, which link together with tight junctions. Other cell types such as neutrophils, dendritic cells and, at later stages, lymphocytes are also present. Reprinted from “Revisiting the role of the granuloma in tuberculosis”, Ramakrishnan, L. 2012, with permission from Springer Nature. Reference (95)
Clinical manifestations

The clinical manifestations of tuberculosis disease can be broadly divided depending on the outcome of the initial infection. The amount of people that have been infected by Mtb have been estimated at a staggering one third of the whole world population (106). However, 90 % of immunocompetent people will never develop active tuberculosis disease after infection (107). Instead, the bacteria are contained by the immune system in a state named latent tuberculosis infection (LTBI). Of the remaining 10 %, half will develop active tuberculosis disease within two years and the other half at some point during their life (107, 108). This results in approximately 10 million new cases and 1.5 million deaths from tuberculosis every year, ranking the disease as the leading cause of death from a single infectious agent (13). Figure 4 shows the current incidence rate of active tuberculosis disease in the world. In 2017, the countries with the highest TB incidence rate were sub-Saharan countries such as South Africa and Asian countries such as India and Indonesia (13).

While tuberculosis is most commonly presented in the lung, the bacteria can spread through the bloodstream and manifest outside of the lungs where it can cause extra-pulmonary tuberculosis in any organ (109, 110). Symptoms of active
pulmonary tuberculosis are classically described as fever, night sweats, weight loss, loss of appetite, fatigue and cough. Severe pulmonary disease may also include haemoptysis, chest pain and shortness of breath (109, 111). The symptoms of extra-pulmonary tuberculosis vary greatly and depend on the specific organ involved (107). The clinical presentation caused by resistant strains do not usually differ from drug susceptible strains (109).

**Diagnosis**

*Active disease*

Although the knowledge of tuberculosis pathology has increased tremendously over the years, the diagnosis of active tuberculosis disease remains complicated and resource intense. Diagnosis of tuberculosis is based on the combination of patient symptoms, physical evaluation at the clinic, laboratory analysis and history of exposure to contacts with active disease (111).

The gold standard and most sensitive method is the culture of bacteria in liquid or solid medium (107). The most common are automated liquid culture systems such as those using Mycobacteria growth indicator tubes (MGIT) (109). While cultures also provide material for subsequent drug sensitivity testing, the slow growth of mycobacteria means it can take six to eight weeks to get a positive culture. For a contagious disease such as tuberculosis, this is not optimal. In addition, culture of mycobacteria requires biosafety level 3 laboratories, which requires large resources and staff experience and the method is prone to cross-contamination (112).

A more rapid and cheap alternative to culture is Ziehl-Neelsen (ZN) staining of sputum smears and detection by microscopy (113). This method is an adaptation of the acid-fast staining method for detection described by Robert Koch in 1882. The method has become standard in many low-income countries, but suffers from several drawbacks. ZN staining requires high bacterial levels in the sputum, which is indeed often the case in many immunocompetent individuals with active pulmonary disease. Children, immunocompromised individuals, and patients with extra-pulmonary disease, however, often have very low bacterial numbers in the sputum and can therefore not be detected with ZN staining (9).

More modern methods include the Gene-Xpert MTB/RIF assay, which uses PCR to amplify mycobacterial DNA. More specifically, it amplifies the rpoB gene, which is commonly involved in resistance to Rifampicin, which allows for the rapid detection of mycobacteria as well as early assessment of rifampicin resistance (114). Due to its automated procedure and use of cartridges, the method,
while expensive and in need for evaluation, has been endorsed by the WHO for use in tuberculosis diagnostics (13).

*Latent TB infection (LTBI)*

Latent tuberculosis infection is defined as persistent and specific immune response to *Mycobacterium tuberculosis*, while at the same time not displaying any clinical symptoms of active disease (13). While clinical diagnosis of LTBI may have a binary criterion, it has become increasingly evident that LTBI includes a spectrum of conditions, ranging from the patients with successful elimination of bacteria after onset of adaptive immune response, to patients in early stages of active disease (9).

The only two tests available to diagnose LTBI are the tuberculin skin test (TST) and the Interferon-gamma release assay (IGRA) (10). Both tests measure the memory T cell response to mycobacterial antigens, thereby indicating exposure to mycobacteria. The TST, as mentioned before, originates from Robert Koch and involves a mixture of mycobacterial antigens injected into the skin. If memory T cells recognize the antigens, a type IV hypersensitivity reaction will occur after two to three days. This causes a raised and hardened area around the injection, whose diameter can be measured. One limitation of the TST is that the mixture includes antigens present in other mycobacteria, including BCG, thus causing reaction in BCG-vaccinated individuals and individuals exposed to environmental mycobacteria (115). In addition, TST also have poor sensitivity in immunocompromised individuals (110).

The IGRA is a more specific test where blood from patients is incubated with a mixture of antigens only present in Mtb and not BCG. Memory T cells in the blood that recognize the antigens will react by producing IFN-γ, which can be quantified. Logistically, IGRA has the advantage over TST, since patients are not required to revisit the clinic. However, IGRA is a more expensive test that requires more from a laboratory standpoint (115).

While both TST and IGRA offer diagnostic value by measuring exposure to mycobacteria, they are both poor predictors of clinical outcomes such as reactivation (10, 115). Therefore, better diagnostic methods are required to distinguish between subgroups among patients with LTBI.

**Treatment**

The standard treatment for drug-susceptible tuberculosis disease have remained largely the same for many years and involves a combination of four drugs;
Rifampicin (RIF), Isoniazid (INH), Ethambutol (EMB) and Pyrazinamide (PYR) (116). Treatment takes six month and is divided into an intense treatment phase of two month with all four antibiotics, followed by a continuation phase of four month with only RIF and INH. The global success rate for standard TB treatment in patients with drug susceptible TB is 82 % (13). When combined with Directly Observed Therapy (DOT), where the patients take their drugs at the clinic, a procedure recommended by the WHO, the success rate is further increased (12, 116).

As with most infectious diseases, treatment of tuberculosis is becoming increasingly difficult due to the emergence of antibiotic resistance among bacteria. The WHO classifies resistance in two main categories; multi-drug resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis (XDR-TB) (10).

**Multi-drug resistant tuberculosis**

MDR-TB is defined as tuberculosis disease caused by strains of mycobacteria that are resistant to both RIF and INH. Due to the increased use of the Gene-Xpert MTB/RIF assay described previously, the WHO have also included a subgroup of MDR-TB known as rifampicin resistant tuberculosis (RR-TB), where the INH resistance is still unknown (10). Still, treatment guidelines for MDR-TB and RR-TB are the same (117).

The recommended treatment for MDR-TB have changed a lot in recent years and most recent guidelines from the World Health Organization were published in 2016 (117). Previously, the standard treatment of MDR-TB involved an intensive treatment phase for 6-8 months with five antibiotics. At least one of the drugs should be a fluoroquinolone (FQ) and one injectable aminoglycoside. The intensive phase was followed by another 12 months treatment without the aminoglycoside (118). The new guidelines include a shorter, 12-month total treatment regimen for MDR-TB if the strain have no other resistances in addition to RIF and INH (117). This regimen is based on superior results for patients with MDR-TB compared to the longer standard MDR-TB treatment. The first clinical studies were performed in Bangladesh and have later been repeated with similar results in other cohorts (119-121). MDR-TB, as is displayed in Figure 5, is most prevalent in east-European countries and China. The global treatment success rate for MDR-TB was 55 % in 2017 (13).
Extensively drug resistant tuberculosis

Resistance to either FQ or injectable antibiotics lowers the success rate of MDR-TB treatment by an average of 10-15%, with FQ resistance being the most impactful (122). If an MDR-TB strain is resistant to FQs and any of the injectable antibiotics, it is classified as XDR-TB.

Treating XDR-TB is very challenging and there is not one recommended regimen for all patients. Rather, the WHO provide a list of drugs and advice a personalized treatment regimen, supported by confirmed drug sensitivity testing for selected compounds (117). Many of these compounds already work poorly against mycobacteria, and therefore require high dosages to have an effect, which often results in adverse effects. Due to these factors, the global success rate for treating XDR-TB is only 34% (13), and highlights the huge demands of new compounds for the treatment of drug resistant tuberculosis. In the past 40 years, only two new compounds, Bedaquiline and Delamanid, have been approved, and they are exclusively used as treatment for XDR-TB (117).
Vaccine strategies

The BCG vaccine

Albert Calmette and Camille Guérin developed the live *Mycobacterium bovis* bacilli Calmette-Guérin (BCG) vaccine in the early 1900s by repeated passaging of a culture of *M bovis* until it was no longer able to cause tuberculosis in animal models (7). In the 1920s, they vaccinated more than 25,000 children in families where at least one member had active tuberculosis. In contrast to unvaccinated children, where 25% died within their first year, the death of children in the vaccinated group was less than 1% (7, 123).

BCG vaccine was originally given orally. This was, according to Calmette and Guérin crucial for its protective effect. However, safety considerations lead many countries to instead use intradermal injection, which is also how the vaccine is given today (7). Almost a hundred years later, BCG remains the only approved vaccine against tuberculosis, with excellent safety records and administered to more than 3 billion children since its development (10, 11).

While all BCG vaccines originate from the same strain which was distributed around the world, hundred years of passage have given rise to several BCG lineages, with slight genetic differences (124). BCG provides excellent protection against disseminated and military tuberculosis in children, preventing approximately 40,000 cases every year (125). These manifestations of tuberculosis, which is common in children, are very dangerous and often leads to death. However, the ability of the vaccine to protect against adult pulmonary tuberculosis is poor. In the second half of the 20th century, several studies were undertaken to assess the efficacy of the vaccine and the results varied between 0-80% protection (126). While the results varied greatly, the studies highlighted the need of a more efficient vaccine especially for adult pulmonary tuberculosis.

Vaccine development

One of the biggest challenges for vaccine development against tuberculosis is the lack of knowledge about what gives efficient protective immunity against the disease. We are still not sure which aspects of BCG vaccination is critical for the protection it currently gives or which aspects are missing for the protection to become long lasting, efficient against pulmonary tuberculosis and work in adults (127, 128). There are currently around a dozen vaccines in clinical efficacy trials, but so far, BCG remains the only approved and recommended vaccine for tuberculosis (129).
Supplementing BCG

One of the main strategies to approach the question of protective immunity is the hypothesis that BCG lost several important genes when it went through its attenuation from *M. bovis*. Thus, while able to protect against certain forms of tuberculosis, its poor efficacy against other forms could be explained by what is lacking in BCG compared to virulent strains. In support of this theory is a study reporting that up to 25% of known T cell epitopes from Mtb were missing in BCG vaccine strains (130).

One of the most important attenuating steps for BCG was the loss of a large portion of the genome known as Region of difference 1 (RD1), which is present in virulent strains of *M. tuberculosis* and *M. bovis* (131). RD1 encodes the genes for the type VII secretion system (T7S) ESX-1, as well as many of its associated secreted proteins, such as Early secreted antigen 6 (ESAT-6) and Culture filtrate protein 10 (CFP-10). The ability to secrete ESAT-6 and CFP-10 have been found to be key for virulence of many tuberculosis-causing bacteria (132).

In a study where RD1 was reintroduced to BCG and used for vaccination, it resulted in superior ESAT-6 dependent protection to Mtb infection compared to BCG alone. However, the question of safety was a major concern, since the recombinant strain appeared more virulent in immunosuppressed animals (133). Similar protection was later achieved in vaccine models with rhesus macaques, vaccinated with BCG and supplemented with ESAT-6 (134). The only vaccine that has so far to completed a clinical efficacy trial is the MVA85A (127, 135). Similar to previous strategies, MVA85A is based on the expression of one of the major T cell antigens, 85A, found in Mtb. Instead of recombinant expression in BCG, a viral vector produces the antigen, which was administrated to children that had already received BCG. However, the results of the study were disappointing, as it didn't improve protection against tuberculosis compared to only BCG (135).

Boosting specific responses

Another common approach to tuberculosis vaccine, is to look at the immunological pattern during Mtb infection and try to identify which responses are important for disease control. One could then try to use vaccines or vaccine supplements i.e. boosters that specifically enhances these responses (128). However, as was previously touched upon in the section on adaptive immunity, we still don’t know which type of immune responses are efficient at containing or eliminating Mtb infection (127).

The CD4+ T cell response with IFN-γ has been given a lot of focus, due to its protective properties discussed previously. Vaccine strategies aimed to boost CD4+ or CD8+ T cell responses have dominated the vaccine pipeline for many years (129). Adjuvants boosting the CD4+ responses have been tested, as well as
recombinant BCG bacteria, that is able to escape the phagosome to increase MHC class I presentation and thereby boosting of CD8+ T cell response (127). Although these cellular immune responses in tuberculosis control are important, neither strategy has been successful in clinical trials (129). Similarly, another strategy aimed to boost the Th17 responses. While it was successful in reducing bacterial numbers in a mouse model, it has not yet been tested in humans (127).

The role of B cells and the humoral response with antibodies have long been considered to have little effect on immune protection against tuberculosis. Recent data, however, suggests that B cells and antibodies may actually be efficient in controlling Mtb infection, even when the bacteria is intracellular (136). Therefore, researchers have started to explore vaccine strategies with enhanced humoral responses (129).

**Mucosal administration**

A third vaccine strategy, that has been given increasing attention, is changing the route of administration. As mentioned previously, BCG vaccine is given as an intradermal injection, but was originally developed as an oral solution (7).

The total surface area of the adult lung is huge, somewhere between 50 to 70 m² (137). Most of it is made up by the mucosal surfaces of the alveoli, in which the gas exchange between the air and the blood occurs. Studies have revealed that mucosal immunology is quite different from immune responses seen elsewhere in the body. Foreign antigens are common at mucosal surfaces and the mucosal immune system must balance its responses not to risk causing unnecessary inflammation and tissue damage. Further, the mucosal immune system also utilizes inducible secondary lymphoid structures such as mucosal associated lymphoid tissue (MALT) for antigen presentation in addition to primary lymphoid tissues such as lymph nodes (137, 138). For these reasons, it is hypothesized that the mucosal immune response to vaccine differs greatly to the immune response of injections. Thus, changing route of administration to airway challenge could potentially result in a better immune protection against the disease.

Studies of BCG vaccination through the airways have been conducted in mice, showing enhanced protection against tuberculosis infection compared to subcutaneous injection (139, 140). The MVA85A vaccine has also been tested with mucosal administration in a study using rhesus macaques (141). Even though the study didn’t address efficacy, it addressed several safety concerns and studied immunological responses, both important information for future vaccine studies (141). Finally, another study in rhesus macaques used mucosal administration of the vaccine AERAS-402, but it failed to protect the animals from Mtb infection (142).
In Paper I and II of this thesis, we study early immunological responses to mucosal vaccination with BCG in the airways. We describe what responses are to be expected, should such a strategy be used, and discuss how they may be beneficial or not to the host.

Antimicrobial peptides

Antimicrobial peptides (AMPs) are small proteins, consisting of short amino acid sequences between 20-60 residues, which exhibit antimicrobial activity (143, 144). AMPs are considered as part of the innate immune system and have been described as one of the most ancient defence mechanisms in nature, present in animals as well as in plants and fungi (143). Today, there are over 2500 peptide sequences that are listed as AMPs (145). The two major classes of AMPs found in mammals are the Cathelicidins and the Defensins (143), the former being a linear alpha-helical peptide, while the latter also contains beta-strands. AMPs are most commonly amphipathic and positively charged, which is believed to enable them to interact with the high negative charge of bacterial membranes (143, 144). Although sequence similarity is sometimes low between AMPs, they often have similar structural folds (146). Although one single mechanism of action cannot account for all AMPs, the vast majority acts by causing disruption of the bacterial cell wall after interaction through electrostatic forces (146). Resistance to AMPs is often found to be due to changes in the charge of the outer membrane of bacteria, thus preventing the binding of the peptides (143). The complex multi-layered cell wall of mycobacteria is one of the main reasons for its resistance to many conventional antibiotics. Thus, drugs such as AMPs, which targets the cell wall and potentially disrupts its integrity, are interesting drug candidates with potential of synergistic effects with conventional antibiotics (143).

AMPs in tuberculosis

Since the discovery of AMPs, the role of peptides during disease and their potential as therapeutic agents have been studied extensively for different diseases including tuberculosis. While many cells in the human body have the ability to produce AMPs, the main sources are the cells of the immune system, especially neutrophils that carry many AMPs in their granules (147). As mentioned previously, neutrophil numbers and AMP production have been found to correlate with better resistance to MtB infection in people having been in contact with persons with active tuberculosis, in turn explained by their ability to produce AMPs (50). However, in patients with active pulmonary tuberculosis, higher
systemic levels of defensins and cathelicidin were detected and the levels were correlated with higher bacterial burden and increased risk of cavitary disease (148). Thus, the exact role of AMPs in tuberculosis could be a dual one. Below, the families of the main types of AMPs that have been studied in tuberculosis so far will be listed, divided into families.

**Cathelicidins**

Humans express only a single protein from the family of Cathelicidins, the human cationic antimicrobial peptide 18 (hCAP18) (144, 149). If hCAP18 is cleaved by serine proteases, such as Elastase, one of the cleavage products is LL-37, a small peptide found in its C-terminal region (150). LL-37 is a small helical peptide with high positive charge. It is expressed by a variety of different cell types, including leukocytes and epithelial cells (149). Expression is increased during Mtb infection, especially in epithelial cells (38). While being the only member of the Cathelicidin family, LL-37 and its equivalent in mice, CRAMP, are by far the most studied antimicrobial peptides in tuberculosis (143, 146). As mentioned, while LL-37 has been linked to decreased susceptibility to Mtb infection (50), high levels are found in patients with active pulmonary tuberculosis. LL-37 levels are further correlated to higher bacterial burden and cavitary disease (148).

In a study using LL-37, CRAMP and three synthetic variants, all five peptides were found to be able to inhibit growth of Mtb *in vitro*, with LL-37 being the best with a minimum inhibitory concentration (MIC) of 1.1 μM (5 μg/ml) (151). In a mouse model of chronic Mtb infection, all peptides reduced bacterial numbers compared to untreated controls. However, LL-37 did not reduce lung pathology, which some of the other peptides did. Also, when tested in a mouse model infected with MDR-TB, only the synthetic peptides showed significant reduction of bacterial numbers. The study did not assess cytotoxicity (151). Another study compared the anti-tuberculosis effect of different peptide enantiomers and found the natural (L)-LL-37 to be inactive against both Mtb H37Rv strain and against a MDR-TB strain (152). The synthetic (D)-LL-37 had a high MIC for H37Rv at 44 μM (200 μg/ml) and no effect of MDR-TB. Further, starting at approximately 10 μM (45 μg/ml), (L)-LL-37 caused 50 % haemolysis in human red blood cells, highlighting the risk of cytotoxic adverse effects.

It has become increasingly evident that, while LL-37 may show antimicrobial activity against several pathogens including Mtb, one of the major problems is its cytotoxic effects at higher concentrations (153). Several studies have shown that LL-37 can cause cytotoxicity in human red blood cells, immune cells, astrocytes and microglial cells (154-156). While the concentrations required for cytotoxic effects may differ between studies, a review from 2013 states that concentrations higher than 13 μM (60 μg/ml) are considered cytotoxic (153). Since antimicrobial activity often requires concentrations close to this concentration, LL-37 may be a very
challenging candidate for treatment of tuberculosis due to the risk of harmful adverse effects.

Still, LL-37 has also been shown to have immune-modulatory functions and was able to reduce inflammatory signalling from Mtb-infected macrophages without decreasing the antimicrobial activities (157). Since significant effect was seen already at 0.2 µM (1 µg/ml), LL-37 may have potential at lower concentrations as treatment to reduce tissue damage caused by inflammation.

**Defensins**

The human genome encodes more than a dozen AMPs from the family of defensins (144, 158). The defensin family consists of cysteine-rich peptides with high positive charge. The human neutrophil peptides 1-3 (HNP 1-3) and human β-defensin 2 (HBD-2) are the most studied defensins in tuberculosis (144). As their names imply, human defensins are primarily produced by neutrophils, but have also been found in other cell types (143, 158). Interestingly, HBD-2 expression is induced in alveolar epithelial cells when infected with Mtb (159). However, similar to LL-37, the role of human defensins in tuberculosis protection is unclear. Higher levels of defensins may protect against infection (50), but are also common in patients with active pulmonary tuberculosis and correlated with higher disease severity (148).

In a study on infected macrophages, it was demonstrated that apoptotic bodies from neutrophils, containing HNPs, were taken up and co-localized with the mycobacteria in the early endosome network of the macrophage, which limited intracellular growth (160). This is an interesting example of interplay between cell types of the innate immune system in antibacterial defence.

The potential of human defensins as treatment for tuberculosis were assessed already in 1996, where an in vitro study showed a 99 % growth inhibition of Mtb with HNP-1 at a concentration of 15 µM (50 µg/ml) after 48 hours (161). This was confirmed by other studies where MIC for Mtb was described as much lower at 0.75 µM (2.5 µg/ml) and able to inhibit intracellular growth in vitro (162). HNP-1 was also shown to reduce Mtb numbers in a mouse model after subcutaneous administration (163). HBD-2 can be induced in alveolar epithelial cells by treating the cells with L-isoleucine. In a mouse model of Mtb infection, treatment with L-isoleucine induced the production of mouse β-defensins and correlated with decreased bacteria numbers and less tissue damage (164).

While the issue of cytotoxicity is also relevant for defensins, it is much more unclear than for LL-37. Early after their discovery, HNPs were reported to lyse various human cell lines at concentrations as low as 0.3 µM (1 µg/ml) after 14 hours (165). However, it was later found that cells could be protected from lysis by the presence of serum (166). In one of the studies with Mtb, it was performed
with a low (2%) concentration of serum, only minor cytotoxic effect was seen at up to 12 µM (40 µg/ml) (162). Thus, many researchers argue that defensins do not cause cytotoxic effects at therapeutic levels, but rather are a family of AMPs with high potential for becoming therapeutic agents for different diseases, including tuberculosis (143, 144, 158).

Other peptides
Although the peptides from the cathelicidin and defensin families are the most studied other peptides, especially synthetic candidates, have also been tested. Below is a brief summary of some of the most interesting studies.

Among naturally occurring peptides, PR-39, a proline-arginine rich peptide with positive charge isolated from pig intestines, successfully inhibited *in vitro* growth of H37Rv and clinical MDR-TB strains, but have not been tested *in vivo* (167). Ecumicin is an AMP isolated from another species of bacteria belonging to the same taxa as mycobacteria (168). Ecumicin inhibited growth of *in vitro* and *in vivo*.

The synthetic peptides named Innate defence regulators (IDRs), offer an interesting alternative strategy to protect against Mtb infection (169). Instead of acting on the bacteria, these peptides modulate the immune response, more specifically by inducing the expression of chemokines. Mtb-infected mice treated with IDRs showed significantly lowered bacterial numbers in the lung, both when tested with H37Rv and a MDR-TB strain (169). Another study aimed to use a bioinformatics approach to find new candidate peptides for treatment of Mtb. Although the peptides were slightly cytotoxic, they had MIC values of around 4 µM and the authors argued this was proof of concept for their approach (170). Finally, in a screening study of small positively charged synthetic peptides, several candidates were found to have *in vitro* activity against Mtb, while at the same time not being toxic to THP-1 monocytes (171).

NZX – The new defensin

In two of the papers in the thesis (Paper III and IV), we study the antimycobacterial activity of a novel AMP known as NZX. NZX is a derivative of Plectasin, a fungal defensin isolated from *Pseudoplectania nigrella* in 2005 (172).

Plectasin was shown to have antimicrobial activity against several gram-positive bacteria and was therefore selected for a mutational screening study designed to find variants with better activity against *Streptococcus pneumonia* and *Staphylococcus aureus* (173). The best candidate from the screening NZ2114, as
well as the original Plectasin peptide have both been used in several studies since then, especially on gram-positive bacteria.

NZ2114 have been successfully used in vivo to treat pneumococcal meningitis as well as endocarditis caused by Methicillin-resistant *S. aureus* (MRSA) (174, 175). Further, Plectasin was able to treat intracellular *S. aureus* in infected THP-1 monocytes (176). In a second round of mutational screening using NZ2114 as base, one of the most promising resulting candidates was NZX. However, no results using this third generation Plectasin peptide have been published so far. Having shown great potential and a wide range of activity against gram-positive bacteria, Plectasin peptides were interesting candidates to study antibacterial activity against mycobacteria.

In Paper III we report, for the first time the potential of the Plectasin peptide NZX as novel treatment for tuberculosis.

**Nanoparticles**

Exposure to proteolytic enzymes such as those secreted by immune cells during inflammation is one major concern for the success of AMPs as treatment (177). An increasingly popular strategy to protect drugs during delivery is to load them into small particles (178). In addition to protection, the particles may also improve delivery to airways purely by being bigger and therefore having a more aerodynamically favoured size. Macrophages tend to actively pick up small particles, which may improve treatment against intracellular pathogens such as *Mtb* (179).

Several different types of particles exists and have mostly been investigated as delivery systems for drugs during cancer treatment (180). Figure 6 depicts the particles subclasses developed so far, where liposomes are the most studied subclass (181). A handful of particles from the liposomes, micelle and polymeric nanoparticle classes are approved for the use in treatment of several cancer forms, such as breast cancer, lung cancer, ovarian cancer and Kaposi’s sarcoma (181).
Nanoparticles have also been investigated as carries for the conventional anti-tuberculosis drugs (178). The main findings are listed in Table 1. In Paper IV, we study the potential of mesoporous silica nanoparticles (MSPs) as carriers for peptide antibiotics in tuberculosis treatment.

**Table 1 Nanoparticle formulations with TB drugs**

<table>
<thead>
<tr>
<th>Particle</th>
<th>Drugs</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly-butylcyanoacrylate</td>
<td>Streptomycin, Moxifloxacin, RIF, INH, 3 to 10-fold lower MIC in vitro</td>
<td>(182, 183)</td>
<td></td>
</tr>
<tr>
<td>Gelatin nanoparticles</td>
<td>RIF</td>
<td>Sustained release in vivo after injection</td>
<td>(184)</td>
</tr>
<tr>
<td>Poly-lactic-co-glycolic acid (PLGA)</td>
<td>RIF, INH, PZA</td>
<td>Prolonged release after aerosolized administration and higher intra-cellular levels in macrophages</td>
<td>(185, 186)</td>
</tr>
<tr>
<td>Solid lipid particles</td>
<td>RIF, INH, PZA</td>
<td>Sustained release after aerosolized administration</td>
<td>(187)</td>
</tr>
<tr>
<td>Mesoporous silica nanoparticles</td>
<td>INH</td>
<td>Efficient intracellular delivery</td>
<td>(188)</td>
</tr>
</tbody>
</table>
Aims of the thesis

• To study early events of innate immune reaction to mucosal stimulation by mycobacteria (Paper I and II)
• To investigate antimicrobial peptides to find compounds with activity against Mycobacterium tuberculosis (Paper III)
• To evaluate mesoporous silica nanoparticles as potential carriers for peptide antibiotics in tuberculosis (Paper IV)

Don't assume that what we currently think is out there is the whole story. Go after the dark matter, in whatever field you choose to explore”
- Nathan Wolfe
Methods

Cells

For the majority of in vitro work on cells, we use primary human cells. In Paper I we purify human venous neutrophils from heparinized whole blood from healthy donors using Polymorphprep density gradient. Similarly, Paper III & IV use primary human blood monocytes, separated by Lymphoprep density gradient and magnet associated cell sorting on CD14+ cells. This is followed by differentiation in vitro into macrophages by the addition of GM-CSF for one week. In Paper I & II also includes experiments with primary human bronchial epithelial cells. These cells are harvested from organ donor with no history of lung disease, but where no recipient was found. Briefly, surrounding tissue and mucus are removed and the bronchi are subjected to protease treatment. HBECs are recovered by intraluminal rinsing and filtration. In Paper I, Primary Human Umbilical Vein Endothelial cell were purchased and cultured in vitro for use in the transwell model system for neutrophil recruitment.

In addition to primary cells, Paper IV includes studies on nanoparticle uptake using the THP-1 monocyte cell line and Paper III utilizes the THP-1 XBlue™ reporter gene monocyte cell line for the study of NF-κB.

Bacteria

The most common bacteria model used in Paper I-IV is a BCG Montreal strain expressing the pSMT1 plasmid construct described by Snewin et al in 1999 (189). The plasmid contains the luxAB operon, encoding for a luciferase from Vibrio harveyi as well as a hygromycin resistance gene for selection. The presence of luciferase enables quantification of bacterial numbers by the addition of an aldehyde substrate, which generates bioluminescence. In Paper III & IV, M. tuberculosis H37Rv (ATCC 27294) strain is used for in vitro experiments. For in vivo work in mice, an H37Rv strain, with known expression of PDIM (a kind gift from Christophe Guilhot, Institut de Pharmacologie et de Biologie Structurale
(IPBS), Toulouse, France) is used. Further, in Paper III, two antibiotic susceptible clinical strain of Mtb and one clinical MDR-TB strain are used for in vitro studies.

Major molecular techniques

Western Blot

In Western Blot, proteins are first separated based on size by gel electrophoresis and transferred to a membrane. Primary antibodies bind to the specific protein of interest and secondary antibodies, conjugated with a peroxidase enzyme binds to the primary antibodies. After addition of substrate, the peroxidase reaction produces luminescent light, which can be detected and quantified where the signal strength correlates with the amount of protein. Western Blot Paper I & II to detect levels of intracellular proteins such as Forkhead Box O3a (FOXO3a) and Ras-related C3 botulinum substrate toxin 1 (Rac1).

Enzyme-linked immunosorbent assay

For the detection of extracellular proteins, we used Enzyme-linked immunosorbent assay (ELISA). The principle of sandwich ELISA, the method used for detection of cytokines and chemokines in Paper I & II, is based on the detection of the protein of interest by binding it to specific antibodies in 96-well plates. Primary antibodies, bound to the bottom of each well, capture the protein of interest. Secondary antibodies are added on top, forming a “sandwich”. The secondary antibody is conjugated to an enzyme, which upon substrate addition forms a coloured product. After the reaction is stopped, the amount of product can be quantified in a spectrophotometer.

Flow cytometry

In flow cytometry, cell suspensions are passed through an instrument, where the flow of cells passes through a laser. The refraction of light as the cell passes the laser is detected and can be used for cell counting, specifically exciting and measuring the light from fluorochromes and even assist in sorting of cells based on these properties. In Paper I we use flow cytometry to measure receptor expression of surface receptors by fluorochrome-conjugated antibodies and in Paper IV we use the method to measure uptake of fluorochrome-conjugated nanoparticles. In
addition, the Annexin V analysis of apoptosis used in Paper I & II are performed with flow cytometry.

**Fluorescence microscopy**

Fluorescence microscopy can be used to study expression of specific target proteins or structures by labelling with fluorochromes. In Paper I, we label DNA with DAPI to visualize NETs from neutrophils stimulated with BCG. Paper II studies the expression of Rac1 and actin on lung sections from BCG-infected mice. In Paper III, we visualize neutrophils, Mtb and cell nuclei in lung sections from peptide-treated mice infected with Mtb. Finally, in Paper IV, uptake of fluorochrome-conjugated nanoparticles in primary macrophages were studied with a confocal fluorescence microscope.

**Minimum inhibitory concentration**

The purpose of minimum inhibitory concentration (MIC) assays is to determine the concentration of antibiotics required to prevent bacterial growth. In our screening studies for compounds with antimycobacterial activity in Paper III we used the luminescence of our BCG bacteria to measure growth inhibition over 24 hours. To determine MIC values for 90 and 99 % inhibition, MGIT-based method where growth of treated bacteria was compared to diluted controls. If 1:10 or 1:100 controls showed growth before undiluted treated Mtb, the treated tubes were considered 90 % or 99 % inhibited respectively.

The intracellular growth assays used in Paper III & IV used primary macrophages infected with Mtb and BCG respectively. For the Mtb-infected cells, the bacteria and cells were stained with fluorochromes after one week and counted in with an automated imaging system. BCG-infected macrophages were lysed and intracellular bacteria were quantified using luminescence.

**Cytotoxicity assays**

There are numerous techniques for studying toxicity in cells. In Paper I & II we use Annexin V, a kit for the detection of phosphatidylserine on the outer layer of the plasma membrane, an early sign of apoptosis.

In Paper III & IV we use three different assays for cytotoxicity; MTT, ATPlite™ and PrestoBlue. In an MTT assay, a dye is added which forms purple formazan crystals when reacting with reducing agents from the cell metabolism. The crystals
are then dissolved and the amount of colour quantified in a spectrophotometer. In ATPlite™ a luciferase and its substrate D-Luciferin is added to the cells and produces luminescence. The amount of light correlates with the energy levels of the cells since it requires ATP. Similar to MTT, in PrestoBlue the chemical resazurin is added to the cells and reacts with reducing agents formed by the cells metabolism. This reaction changes colour of resazurin from blue to pink and the amount of colour is quantified in a spectrophotometer.

In Paper IV, we also use fluorescence labelling of human macrophages followed by cell count by an automated imaging technique. Paper III also includes analysis of inflammatory response by utilizing the reporter monocyte cell line THP-1 XBlue™ for the detection of NF-κB activation.

Animal models

All experiments and maintaining of animal models for Paper III & IV were performed in biosafety level 3 facilities at Imperial College London, London, United Kingdom according to institutional protocols. A BALB/c mouse model of acute infection was used to study peptide and peptide-nanoparticle formulations treatment of tuberculosis. Briefly, mice are infected by intranasal administration of approximately $10^4$ CFU. Two days later, one group of three mice is sacrificed and the lungs plated to determine the actual lung infectious dose. Infection is allowed to establish for three weeks and mice are divided into treatment groups of five individuals in each. Treatment is given daily for five consecutive days, followed by sacrificing and collection of lungs. For every mouse, one lobe was saved for immunohistochemistry and electron microscopy, while the rest were plated for CFU determination.

In Paper II, a similar model with BCG in BALB/c mice is used. Mice were maintained in the biosafety level 2 animal facilities at the Department of Microbiology, Immunology, and Glycobiology at Lund University, Lund, Sweden according to institutional protocols. Briefly, mice were infected with $5 \times 10^4$ CFU trough intranasal administration. After five weeks, mice were sacrificed and one lobe was saved for immunohistochemistry, while the rest were plated for CFU determination.
Statistics

Prism 6f for Mac OS X was used for statistical analysis. For analysis with two groups, we used student’s t-test or Mann-Whitney and for analysis of data with three or more groups we used ANOVA and post-hoc analysis with Tukey’s or Dunett’s correction for multiple analysis. Generally, experiments were performed at least three times to account for experimental variance. In Paper III & IV all experiments using primary macrophages were repeated at least two times, with different donors every time to account for donor bias. Due to the scarcity of the material, experiments on primary human bronchial epithelial cells in Paper I & II could most often only be repeated once, but each with cells from a different donor. In the mice experiments in Paper III & IV, we used five mice in each group and retrieved lungs for both CFU count and staining from each mouse.

Ethical approval

The Swedish Research Ethical Committee in Lund (FEK 413/2008) approved the isolation of the primary airway epithelial cells used in Paper I & II. These were acquired from lung explants of healthy donors with irreversible brain damage and no history of lung disease; the lungs were intended for transplantation but could instead be used in this study as no matched recipients were available at that moment. Written consent was obtained from the donors’ closest relatives. The mouse model study in Paper II was approved by the Animal Experiment Ethics Committee at the Lund District Court in Sweden (M7–15). For Paper III & IV, the animal studies were approved (PPL 70/7160 and 70/ 8653) by the Local Animal Welfare and Ethical Review Board (London, UK). The Local Ethical Review Board (Dnr 2011/403) and (2014/35) approved the donation of blood from human volunteers for the in vitro studies in all papers (Lund, Sweden). No personal data was collected from the volunteers and, the blood was sometimes pooled for the isolation of the monocytes. For the intracellular assays in Paper III, human donor blood was purchased from the blood bank of Linköping University hospitals and blood donors gave written informed consent for research use of the blood.
Results

Paper I

Primary human airway epithelial cells from bronchial tissue produced the pro-inflammatory cytokines CXCL-8 (IL-8) and IL-6 when stimulated with BCG (Figure 1, Paper I). The production, especially for IL-6 was increased in the presence of IL-17A or IFN-γ. In the presence of a combination of both IL-17A and IFN-γ, the expression of CXCL-8 was lowered while the expression of IL-6 increased.

IL-17 receptor expression was studied on primary epithelial cells and neutrophils. The main subunit IL-17RA were expressed on both cell types, while IL-17RE, a subunit for the recognition of IL-17C, was only found on epithelial cells (Figure 2, Paper I). Upon stimulation with BCG for 3 hours, neutrophil IL-17RA expression decreased. IL-17RA expression of epithelial cells was not changed significantly after three days stimulation, while IL-17RE decreased (Table 1, Paper I).

Addition of IFN-γ or IL-17A and IFN-γ in combination during mycobacteria stimulation of airway epithelial cells decreased the recruitment of neutrophils in a transwell model.

Finally, neutrophils encountering BCG produce neutrophil extracellular traps (NETs) already after one hour. This production is not affected by the presence of IL-17A, IL-17C or IFN-γ

Paper II

Intranasal infection of mice increased Rac1 expression in lung epithelium (Figure 1 A&B, Paper II). Primary human airway epithelial cells from bronchial tissue also displayed an increased Rac1 expression during BCG infection. Blocking of the G-protein coupled chemokine receptors CXCR1 and CXCR2 during infection decreased Rac1 expression (Figure 1 C&D, Paper II).
BCG infection triggered actin expression in epithelial cells, which could be abrogated by the blocking of CXCR2 or a Rac1 inhibitor. Increased actin expression was also found in vivo during BCG infection (Figure 2, **Paper II**).

We investigated expression of intracellular signalling pathways in infected epithelial cells and found that infection triggered the MAPK pathway while also modulating the p53 and STAT pathways (Figure 3 and 4, **Paper II**).

Further investigation of intracellular pathways during BCG infection revealed no activation of NF-κB or c-Jun unless CXCR1 or CXCR2 were blocked. In addition BCG infection did trigger secretion of IL-10 and IL-6. IL-10 secretion was further increased with CXCR1 blocking, while blocking of CXCR2 lowered the secretion substantially. IL-6 secretion, on the other hand, was increased when either receptor was blocked.

**Paper III**

We evaluated six peptides for their anti-mycobacterial activity against BCG and toxicity on primary macrophages. LL-37 and three of the small cationic peptides have previously been shown to have anti-mycobacterial activity (151, 171). In our study the defensin NZX, a Plectasin variant, was the best candidate and chosen for continuation for further studies (Figure 1, **Paper III**).

*In vitro*, a minimum of 1.6 µM NZX was required to eliminate BCG. At 3.2 or 1.6 µM, complete killing occurred after 8 or 11 days respectively (Figure 2A, **Paper III**). In a growth kinetics model, a single dose NZX inhibited growth of virulent *Mycobacterium tuberculosis* by up to 99% at 3.2 µM (Figure 2B, **Paper III**). Minimum inhibitory concentrations for the lab strain H37Rv, two clinical strains of *Mtb* and one clinical strain of MDR-Mtb, all were found to have values between 3.2 and 6.3 µM (Table 2, **Paper III**).

NZX displayed no cytotoxicity towards human primary macrophages, whether they were infected with *Mycobacterium tuberculosis* or uninfected (3A-C, **Paper III**). In addition, NZX didn't trigger activation of NF-κB in monocytes (Figure 3D, **Paper III**).

We studied enzymatic breakdown of the peptide using Cathepsin G, α-Thrombin and Human neutrophil elastase (HNE). Of the enzymes tested, only HNE showed degradation of NZX, with about 20% degradation after 6 hours (Figure 3E, **Paper III**).

NZX inhibited intracellular growth of *Mycobacterium tuberculosis* inside primary macrophages (Figure 4A, **Paper III**). In addition, gold-labelled NZX could be
detected on intracellular bacteria in mouse macrophages after treatment in a murine infection model (Figure 4B, Paper III).

Treatment with NZX in an acute model of murine tuberculosis significantly decreased CFU numbers in the lungs (Figure 5A & B, Paper III). Finally, immunohistochemistry showed decreased bacterial numbers, neutrophil infiltration and tissue destruction in NZX-treated mice compared to control (Figure 5C, Paper III).

Paper IV

Mesoporous silica nanoparticles (MSPs) were synthesized to have a diameter of approximately 200 nm, which was assessed with transmission electron microscopy (Figure 1A, Paper IV).

When evaluating the adsorption of NZX onto the MSPs, the maximum loading capacity were determined to be 15 % weight (Figure 1B, Paper IV). Release of NZX from particles were slightly better in simulated lung fluid (SLF) as compared to PBS (Figure 1C & D, Paper IV). NZX, released from MSPs, retained its ability to inhibit mycobacterial growth in vitro (Figure 2A, Paper IV).

Particles were taken up by primary human macrophages and THP-1 monocytes (Figure 2B & C, Paper IV). Uptake of MSPs into primary human macrophages was further visualized by transmission electron microscopy (TEM) and confocal microscopy. Uptake was evident in both experiments already after 2 hours (Figure 3, Paper IV). TEM was further used to follow the intracellular breakdown of particles over 72 hours, at which point particles seem to have lost their structural integrity completely.

MSP induced toxicity in a dose-dependent manner in primary macrophages, both uninfected as well as H37Rv infected (Figure 4A-C, Paper IV).

In an in vitro NZX loaded MSPs induced intracellular growth inhibition of BCG in primary macrophages (Figure 4D, Paper IV)

Finally, in an in vivo model of acute tuberculosis in mice, treatment with NZX loaded in MSPs retained the same antibacterial effect as seen with free peptide (Figure 5, Paper IV)
Discussion

Immunological strategies

During mucosal vaccination, the first line of defence is the barrier of epithelial cells that line the airways. If the mucosal route would be used for vaccination, there needs to be a better understanding of the early reaction by cells of the innate immune system in order to discuss the potential benefits or risks.

*Innate immune responses to mucosal vaccination*

In **Paper I**, our general approach was to study the innate immune responses during mucosal infection in regard to cytokine signalling and neutrophil recruitment. BCG was used as model organism and tested with and without presence of addition of IL-17A- or IFN-γ. The presence of these cytokines was meant to mimic how an immunological memory may regulate the behaviour and mechanisms of early host responses to mycobacterial infection.

We report that primary human airway epithelial cells (AECs) produce IL-8 and IL-6 upon BCG stimulation. Production increased, especially for IL-6, in the presence of IL-17A or IFN-γ, which indicate them having a regulatory role in epithelial IL-6 and IL-8 production during mycobacterial infection. Thus, during an immunological memory response with either IL-17A- or IFN-γ-producing CD4+ T cells, epithelial cells may secrete higher levels of IL-6 and IL-8.

IL-6 is a major inflammatory cytokine and an important factor in promoting the switching from innate to adaptive immune responses (190). IL-6 also promotes the maintenance of IL-17A producing T cells, at least in part by trans-signalling (191). Trans-signalling is a mechanism whereby soluble IL-6 receptors are shed from cells such as neutrophils and able to transduce signals by binding other cells and its ligand (190). The local production of IL-6 upon infection in our experiments could therefore help shift toward adaptive immune responses, especially during memory responses.

As previously mentioned, IL-8 is a major chemokine for attracting neutrophils (192), and airway epithelial cell signalling has been shown to increase neutrophil diapedesis during mycobacterial infection (37). In our transmigration model, however, there was no increase in neutrophil influx in the presence of IL-17A or
IFN-γ. Thus, although the IL-8 production is increased during these conditions, other regulatory signals are most probably present that changes the level of neutrophil recruitment. It has been reported that IFN-γ signalling lowers neutrophil accumulation in lungs of Mtb-infected mice (193). Similarly, neutrophil recruitment was reduced in the presence of IFN-γ in our study. IL-17A, a cytokine known to favour phagocytes (194), did not reduce neutrophil recruitment.

IL-17 cytokines bind to receptor dimers of IL-17RA in combination with IL17-RA, -RB, RC or RE on the cell surfaces (195).

We found expression of IL-17RA on both epithelial cells and neutrophils, and neutrophils were found to down-regulate the receptor upon infection with BCG. As this receptor subunit is required for all IL-17 signalling, a reduction may render neutrophils less responsive during infection. Epithelial cells, on the other hand, did not change the expression of IL-17RA, but rather down-regulated IL-17RE. This subunit is required for the binding of IL-17C, a cytokine reported to be involved in maintaining the inflammatory responses during mucosal infections (195). The down-regulation of IL-17 receptors on epithelial cells and neutrophils, supports previous findings showing that BCG infection can promote anti-inflammatory responses (42).

A pro-inflammatory environment may be beneficial for the generation of a protective memory immune response. As seen IL-17A, but not IFN-γ, induce high levels of IL-6 while also sustaining the recruitment of neutrophils. While our data did not indicate induction of apoptosis in neutrophils, as have been reported for macrophages (196), it did show that BCG infection lead to the generation of NETs. Although NETs have been reported to lack ability to kill Mtb, a study showed that Mtb-induced NETs could lead to the activation of macrophages (56, 197).

The pro-inflammatory nature of NETs may seem like a good driving force for the generation of protective immunity. However, the role of NETs in health and disease are still unclear and must therefore be approached with caution. Several reports show evidence towards NETs playing a role in autoimmune diseases such as small vessel vasculitis and systemic lupus erythematosus, with clearance being highlighted as an important factor (198, 199).

**BCG infection regulate inflammatory responses**

In Paper II, we further investigated the role of epithelial cells and the early events induced by BCG infection. We found that BCG infection increased epithelial Rac1 expression. Blocking the G-protein coupled receptor CXCR2 could inhibit the increased Rac1 expression. Rac1 belongs to the Rho family of small GTPases, which are key signal transducing factors, linking G-protein coupled receptor signalling to cytoskeletal rearrangements (200). In our study, we saw increased
expression of actin in cells infected with BCG, which could be prevented by inhibition of CXCR2 or a Rac1 inhibitor. In our animal infection model, Rac1 and actin were both up-regulated in mice five weeks after infection with BCG. Both BCG and Mtb have the ability to invade airway epithelial cells and for Mtb, this is at least in part dependent on actin rearrangements (201, 202).

If the sustained increase in actin in our data depends on cellular imprint and/or BCG persistence remains unclear as only very few bacteria was detected in the lungs after five weeks. BCG have previously been shown to persist in mice in part by changes in their metabolism (203).

BCG infection did not affect epithelial cell survival, which supports the findings that Mtb, but not BCG can induce cell death in epithelial cells (43). In fact, our study showed that BCG utilizes CXCR receptors to keep NF-κB and c-Jun (AP-1) levels low, thereby preventing inflammatory responses, which is highlighted by the lack on inflammatory signals. The lack of activation of NF-κB has in favour of a more anti-inflammatory environment been reported in an earlier study (42). Taken together, our study highlights how BCG infection suppresses inflammatory responses in epithelial cells. This is an important finding for the development of a mucosal BCG-vaccine, and suggests inhibition of G-protein coupled receptors as potential targets to stimulate a more rigorous response in epithelial cells.

Antimicrobial strategies

NZX – a new defensin against tuberculosis

In Paper III, we report for the first time how NZX, a third generation Plectasin peptide is active against tuberculosis. During screening of six different peptides, NZX were the best candidate with pronounced antibacterial activity and low toxicity. NZ2114, a second-generation plectasin peptide, showed similar results as NZX but slightly less efficient and more toxic. The synthetic WKWLKKWIK peptides have previously been reported to inhibit growth of Mtb with up to 90% at 1 µM (171). In our study, even at concentration a hundred times higher, growth of BCG was only inhibited by up to 40 % for the best peptide. At these concentrations, the WKWLKKWIK peptides showed high levels of cytotoxicity in our study, which support previous work where toxicity was detected at concentrations >25 µM (171). The results for LL-37 were similar and supports the theory that LL-37 may have broad antimicrobial activity, but at the high concentrations required for this, it also show high levels of toxicity (153).

We choose to continue with NZX and investigated its effect on Mtb. For H37Rv and the three clinical strains of Mtb, one of them an MDR-TB strain, were all
similar around 6.3 µM. This may indicate that resistance to rifampicin and isoniazid does not confer resistance to NZX, however more studies are needed. NZX was not toxic to primary human macrophages, or able to generate activation of inflammation through NF-κB, which further highlights the potential for defensin peptides in therapy that have been suggested by others (158).

NZX was successful at lowering lung bacterial numbers in lungs of mice in an acute model of tuberculosis infection after five days of treatment, the same levels achieved by rifampicin treatment. NZX-treated mice also showed less lung pathology, lower bacterial numbers and less neutrophil influx when studied with immunohistochemistry.

Finally, NZX were shown to inhibit growth of intracellular Mtb in vitro and macrophages isolated from mice treated with gold-labelled NZX were found to have intracellular bacteria that was associated with NZX. This indicate an ability of NZX to inhibit both intra- and extracellular bacteria, a promising feature for a potential TB drug, as bacteria may reside in different compartments during different stages of the disease (67). Taken together, NZX is a promising novel candidate for drug development against tuberculosis.

Nanoparticles as carriers for peptide antibiotics

In Paper IV, we study the use of mesoporous silica particles (MSPs) as carriers for a peptide antibiotic against tuberculosis. MSPs with a radius of 200 nm were synthesized and able to adsorb NZX. The efficacy of NZX at inhibiting BCG growth was not affected by the adsorption and release from MSPs. In addition, NZX-loaded MSPs also had similar MIC values to free peptide against BCG.

The particles were taken up efficiently by both THP-1 monocytes and primary macrophages. Similarly, Clemens et al. showed that macrophages were efficient at taking up RIF-loaded MSPs (188). In our study, primary macrophages were more efficient at engulfing particles than THP-1 cells. This finding is supported by previous studies where primary macrophages showed superior uptake of fluorescent beads compared to THP-1 monocytes (204). As mentioned, macrophages have been shown to remain able to engulf particles even after infection with Mtb (62). Mycobacteria-containing phagosomes are still accessible to trafficking by endosomes (63, 64). In our study, NZX-loaded MSPs were able to lower growth of intracellular BCG in primary macrophages that may indicate an ability to reach the mycobacterial phagosome. The NZX-MSP formulation was also as successful as free peptide and rifampicin in lowering lung bacterial numbers in a mouse model of acute Mtb infection.

One of the main advantages of MSPs compared to most other particles is the amount of flexibility available (205). Further fine-tuning of pore- and particle size could be used to further optimize delivery. In addition, functional groups, such as
pH-sensitive nanovalves could be tested to trigger release only after arriving to the endosome compartment (206).

It is common that nanoparticles show some level of toxicity toward human cells. In the case of our MSPs we saw dose-dependent toxicity with primary macrophages. However, toxicity was not very high and required concentrations above the MIC to be pronounced. Should toxicity prove problematic, the flexibility of MSPs also offers possibility to change surface charge, size functional groups and more, which could help reducing the cytotoxic effects (205). Still, the risk of toxicity could limit the maximum MSP concentrations used for therapeutics and require the peptide antibiotic to be very potent.

In summary, MSPs are interesting candidates for delivery of peptide antibiotics. With NZX as model peptide, we found that interaction with particles did not change the efficacy of peptide, which remained as potent as free peptide. MSPs may also improve the delivery of peptide to intracellular compartments.
Conclusions

- During mucosal vaccination with BCG, airway epithelial cells produce pro-inflammatory signals which help recruit neutrophils to the site of infection. Inflammatory signals can be enhanced by the addition of IL-17A and IFN-γ, but the latter lowers the recruitment of neutrophils. Skewing the memory response towards IL-17A production may thus increase neutrophil influx during recall responses. However, while the presence of neutrophils may be beneficial for protective immune response, pro-inflammatory mechanisms such as NETs may also pose a risk for pathology (Paper I).

- BCG also manipulates cellular responses in epithelial cells that promote a more anti-inflammatory environment. This is in part through the engagement of G-protein coupled receptor signalling, which could be an interesting target for future boosters for BCG mucosal vaccination (Paper II).

- NZX is an antimicrobial peptide, which may be a good candidate for drug development against tuberculosis. It is non-toxic to human cells while potent against both clinical and laboratory strains of Mtb (Paper III).

- NZX can be loaded on mesoporous silica nanoparticles while still retaining the same potency against mycobacteria in vitro and in vivo. Formulations of peptide antibiotics with MSPs could possibly improve intracellular delivery (Paper IV).
Acknowledgements

First of all, a huge thanks to my main supervisor, Gabriela Godaly. It feels surreal that the journey I started all those years ago finally has come to this, our work presented in my thesis. I want to thank you for the way you have guided me through this whole experience. We have both challenged each other and I believe it has formed a great bond between us. I respect you tremendously as a researcher and I’m very grateful for your welcoming attitude whenever I need to discuss anything. One of your great strengths is the way you have always made us, your students, feel prioritized even if multiple people demand your attention. You are a true inspiration with such a great mind-set that I have tried my best to learn and keep in mind for my future endeavours. Finally, thank you for becoming my friend.

To my co-supervisors, Erik Sturegård & Artur Schmidtchen, for all the scientific discussions and input throughout the years. Thank you for helping me view my research from other angles which have opened my mind to new possibilities. To my colleagues Nader, Anna & Komal. Anna, thank you for being my work buddy and co-writer for “The Lab”. I will fondly remember the hours spend together in the lab and lunch/fika breaks. Nader, I’ve learned so much through you and our discussions. You always joined me for lunch walks, even in the cold weather, and I’m so glad you allowed me to show you some of the great things Sweden has to offer. We learned much about each other, our roots, our political views, our cultures and not the least how similar we are. Komal, in the short time you have been working with us, I feel we have already come very close. Thank you for supporting me every day during my thesis writing. You are a brilliant scientist and we both share a deep interest in science and you have helped me trigger my curiosity many times.

All my mentors and role models during my years in Lund. Thomas Hellmark, Magnus Hillman, Mattias Collin, Rolf Lood & Catarina Rippe. Thank you all for helping me shape myself as a scientist. You have all been people I’ve appreciated for your scientific knowledge, your great spirit and your friendship. Special thanks to Birgitta “Gittan” Gullstrand. You have been like an extra parent to me. The way you care for the people around you is so beautiful to see and I’m very grateful for all those moment where you have taken time from your
busy schedule just to hear how I’m doing. You are a true inspiration in how to show care and respect for everyone you meet.

To Svanborg lab. First, Catharina Svanborg, thank you for all the support and encouragement that you have shown me. To current and previous members of the lab with a special thanks to Ines, Aftab, Manoj, Daniel, Nina, Caterina, Karoly and Gustav for all your help and friendship.

Thank you to my family. To my loving parents, Torsten & Maria. Thanks you for all your support and unconditional love throughout my whole life. All those long phone calls and encouraging words will not be forgotten. Thank you for all your help. To my sisters, Kajsa & Tove and their beautiful families, thank you for all the time I get to spend with you all. I truly value our bonds as siblings, and the love we have for each other.

To my wonderful wife Anna. Words cannot describe how important you are in my life. I’m so grateful for the way you’ve stood by my side throughout my whole journey, patiently listening to all my worries. I’m so fortunate to have you by my side, now and in the future.

Thank you to my friends; Adam, Linnéa, Tim, Eva, Christian, Rickard, Henrik, Johan, Otto, Simon, Elias, Lisa & Arvin. I’m truly blessed by having such great friends and all the support you have shown me throughout the years. Hanging out with you at lunches, new years celebrations, holidays and parties have enriched my life outside of my studies.

My friends from the biomedicine program and PhD studies; Johan, Nathalie, Erik, Frida, Emma, Eleni, Torgny, Sofia, Karl & Ludwig, we have all spent much time in the lecture halls together and had each others backs during this whole experience. I truly value the ability to discuss with all of you the challenges of the path we all started. I think we have a special bond that I hope will let us keep in touch in the future as well. Special thanks to Jonatan & Henrik, with whom I have enjoyed many evenings of after work with board games and discussions about or PhD education. I’m the last of the three of us to finish, and I hope we continue hanging out even after.

Finally, a big thanks to our collaborators. Brian Robertson, Mika Lindén, Matthias Mörgelin & Maria Lerm for all the help in making our studies a reality and valuable input throughout the process. Also, a big thank to our funders; the FORMAMP project, Swedish Heart & Lung foundation & Kungliga Fysiografiska sällskapet for making our research possible.
References


70. Chackerian AA, Alt JM, Perera TV, Dascher CC, Behar SM. Dissemination of Mycobacterium tuberculosis is influenced by host factors and precedes the initiation of T-cell immunity. Infection and immunity. 2002;70(8):4501-9.

91. Caruso AM, Serbina N, Klein E, Triebold K, Bloom BR, Flynn JL. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. Journal of immunology. 1999;162(9):5407-16.


166. Lichtenstein AK, Ganz T, Nguyen TM, Selsted ME, Lehrer RI. Mechanism of target cytolysis by peptide defensins. Target cell metabolic activities,