Innate immune responses to *Mycobacterium tuberculosis* infection

How extracellular traps and trained
immunity can restrict bacterial growth

Clara Braian

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can restrict bacterial growth

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ABSTRACT

Tuberculosis (TB) is an infectious disease caused by the bacterium *Mycobacterium tuberculosis*, and the cause of 1.5 million deaths in 2018. During a pulmonary TB infection, the bacterium reaches the lungs and is phagocytosed by cells of the innate immune system, primarily macrophages. The macrophages are either able to eradicate the bacteria or the bacteria start to replicate, and the following immune response leads to the formation of a large cluster of different cell types called a granuloma. In the granuloma the mycobacteria are contained in a latent infection, or they can start to replicate causing rupture of the granuloma and spread of the disease. Neutrophils are also innate immune cells that are rapidly recruited to the site of infection. They are phagocytes, but they also exert extracellular effector mechanisms by their release of microbicidal granule proteins, reactive oxygen species and neutrophil extracellular traps. *M. tuberculosis* has co-evolved and adapted to the human host making it ingenious at exploiting the human immune response, promoting its survival and replication in human host cells. The human immune system has also evolved mechanisms to limit *M. tuberculosis*-replication and spread. This thesis covers work on the innate immune response to TB and how neutrophils and macrophages respond to a mycobacterial infection and can control *M. tuberculosis*-replication.

Neutrophils and macrophages can respond to *M. tuberculosis* by releasing extracellular traps. We demonstrated that neutrophil extracellular traps contain the danger signal heat-shock protein 72 when induced by mycobacteria, which subsequently mediate a proinflammatory activation of adjacent macrophages. Macrophages can also release extracellular traps, and we observed the release of macrophage extracellular traps in response to *M. tuberculosis* that grow in cord-structures. We further demonstrated that the induction of extracellular traps also required the mycobacterial virulence factor ESAT-6.

Trained immunity is an epigenetically regulated memory of the innate immune system that results in a heightened response to a later encounter of the same or different pathogen. β -glucans are structural components of microbial cell walls and known inducers of trained immunity. We studied the effects of β -glucan from a bacterial source (curdlan from *Alcaligenes faecalis*), from yeast (WGP dispersible from *Saccharomyces cerevisiae*) and from the supernatant of a

multicellular fungi (*Alternaria*) in search of functional changes in human macrophages which enhanced their anti-mycobacterial capacity. *M. tuberculosis* growth reduction was observed in WGP dispersible-trained macrophages when co-cultured with neutrophils. We also discovered that the interferon-gamma (IFN γ) signaling pathway, which is important for mycobacterial control, is hypomethylated in the WGP dispersible-trained macrophages. Since hypomethylation of genes typically is associated with gene activation, this suggests a more active IFN γ signaling in response to β -glucan innate immune training.

Most of our studies were performed using *in vitro* culturing of primary human macrophages and neutrophils. However, an *in vitro* 3D tissue model is a valuable tool when studying complex events that occur during a TB infection that involves both multiple cell types and requires knowledge of the spatial movement of cells. In this thesis we also describe an *in vitro* lung tissue model, which we could use to observe the clustering of monocytes around mycobacteria and quantify the size and number of macrophage clusters.

In conclusion, this thesis comprises work on innate immune functions during tuberculosis infection. We describe extracellular trap formation in macrophages and neutrophils in response to *M. tuberculosis*. We also explore trained immunity and how β -glucan training can enhance mycobacterial growth restriction.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Tuberkulos (TB) är en infektionssjukdom som orsakade 1,5 miljoner dödsfall år 2018. Man smittas av TB via inandning av aerosoler som bildas när en sjuk person hostar eller nyser. Ett av de vanligaste symptomen vid TB är svår hosta, vilket medför att sjukdomen sprider sig vidare till andra i omgivningen. Det finns ett godkänt vaccin som ges till många spädbarn runtom i världen, men som endast ges till riskgrupper i Sverige. Detta för att det är inte ger ett bra skydd mot TB. Det finns även antibiotika som verkar mot TB, om man inte blir smittad med en antibiotika-resistent bakteriestam, men behandlingen tar upp till ett halvår och har många bieffekter. Dessutom tror man att uppemot en fjärdedel av jordens befolkning bär på latent TB, det vill säga TB som varken är smittsam eller orsakar symptom, men som kan bryta ut till aktiv sjukdom och sprida vidare infektionen till nya människor.

TB orsakas av bakterien *Mycobacterium tuberculosis* som vid smitta transporteras ner i lungorna och träffar på celler som tillhör det medfödda immunförsvaret, som till exempel makrofager. Makrofagerna äter upp mykobakterierna och skickar ut signaler, så kallade cytokiner, till andra celler som kommer och hjälper till, däribland neutrofiler. I bästa fall lyckas makrofagen döda bakterien men ofta kan mykobakterier överleva inne i makrofagerna, för att sedan föröka sig och sprida sig vidare och på så sätt orsaka sjukdom. Mykobakterier har en unik cellvägg som fungerar som ett skydd mot yttre påverkan och gör dem svåra att avdöda. Detta skyddar dem både från att dödas av kroppens celler men skyddar även till viss del mot antibiotika.

I den här avhandlingen har vi studerat funktioner hos det medfödda immunförsvaret vid infektion med *M. tuberculosis*. Bland annat har vi undersökt hur makrofager och neutrofiler aktiveras av mykobakterier, samspelet mellan de två celltyperna, samt hur man kan förstärka cellernas förmåga att avdöda bakterier.

Om man stimulerar neutrofiler med mykobakterier kan de försvara sig genom att frisätta molekyler som är toxiska för bakterier, men som mykobakterierna inte påverkas så mycket av på grund av deras cellvägg. Som en extra försvarsmekanism kan neutrofilerna även begå självmord och i processen skicka ut strängar av DNA som kan fänga in bakterier och förhindra att de sprids i vävnaden. Dessa DNA-nät kallas NETs, och innehåller toxiska molekyler men

även signalmolekyler som kan överföras till makrofager. I vårt första arbete kunde vi visa att makrofager aktiverades av signalerna som överfördes via NETs och började då utsöndra mer proinflammatoriska cytokiner. I vårt andra arbete visade vi att även makrofager kan kasta ut DNA i strängar (kallas METs) när de stimulerades av mykobakterier som växer i större repliknande strukturer. Vi visar också på en koppling mellan virulens hos bakterierna och makrofagernas frisättning av METs.

I vårt tredje arbete beskriver vi en lungvävnadsmodell som kan användas för att studera tuberkulosinfektion. Vi visar att man kan använda den för att studera hur celler förflyttar sig i vävnaden och hopar sig runt mykobakterierna.

I vårt fjärde arbete studerade vi ett koncept som kallas 'tränat medfött immunförsvar'. Det medfödda immunförsvaret har ett 'minne' och kan tränas till att bättre försvara oss mot tuberkulos. Vi har undersökt betaglukaner som är molekyler som finns som byggstenar i olika mikroorganismer och har studerat hur cellerna som tränats med dessa blir bättre på att avdöda mykobakterier. Vi har även tittat på de tränade cellernas DNA för att hitta epigenetiska förändringar som förklarar vad som ändras i cellerna när de blir tränade.

Kunskap om vad som händer när celler blir infekterade av mykobakterier, och hur de kan stimuleras eller tränas för att bättre avdöda bakterierna, är högst relevant i sökandet efter nya metoder för att förebygga och behandla TB.

LIST OF PAPERS

Paper I

Braian C, Hoge V, Stendahl O. *Mycobacterium tuberculosis*-induced neutrophil extracellular traps activate human macrophages. *Journal of Innate Immunity*. 2013;5(6):591-602.

Paper II

Kalsum S, **Braian C**, Koeken VACM, Raffetseder J, Lindroth M, van Crevel R, Lerm M. The Cording Phenotype of *Mycobacterium tuberculosis* Induces the Formation of Extracellular Traps in Human Macrophages. *Frontiers in Cellular and Infection Microbiology*. 2017 Jun 26;7:278.

Paper III

Braian C, Svensson M, Brighenti S, Lerm M, Parasa VR. A 3D Human Lung Tissue Model for Functional Studies on *Mycobacterium tuberculosis* Infection. *Journal of Visual Experiments*. 2015 Oct 5;(104):53084.

Paper IV

Braian C, Das J, Lerm M. Exploring β -glucan immune training of primary human macrophages and their control of virulent *Mycobacterium tuberculosis* infection. Manuscript.

ABBREVIATIONS

3D	three dimensional
AIM	apoptosis inhibitor of macrophages
BCG	<i>Bacille Calmette-Guérin</i>
CFP-10	10 kDa culture filtrate protein
CLR	C-type lectin receptor
CR	complement receptor
CR3	complement receptor 3
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin
ESAT-6	6 kDa early secretory antigenic target
ESX-1	ESAT-6 secretion system 1
Hsp72	heat shock protein 72
IFN γ	interferon-gamma
IGRA	interferon-gamma release assay
LPS	lipopolysaccharide
MAP kinase	mitogen-activated protein kinase
MARCO	macrophage receptor with collagenous structure
MDP	muramyl dipeptide
METs	macrophage extracellular traps
MPO	myeloperoxidase
MyD88	myeloid differentiation primary response protein 88
NE	neutrophil elastase
NETs	neutrophil extracellular traps
NK cell	natural killer cell

NLR	NOD-like receptor
NO	nitric oxide
NOD-like receptor	nucleotide-binding oligomerization domain-like receptor
PBMC	blood mononuclear cells
PDIM	phthiocerol dimycocerosates
PMA	phorbol myristate acetate
PPD	purified protein derivative
RD1	region of difference 1
ROS	reactive oxygen species
SEM	scanning electron microscopy
SR	scavenger receptor
SRA	scavenger receptor A
TB	tuberculosis
TDM	trehalose dimycolate
TLR	Toll-like receptor
TST	tuberculin skin test

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BACKGROUND

Tuberculosis – an ancient and deadly disease

History

Tuberculosis (TB) is an ancient disease caused by the bacterium *Mycobacterium tuberculosis* (*M. tuberculosis*). An early progenitor of *M. tuberculosis* has genetically been traced back to East Africa to around three million years ago, and has been suggested to have infected early hominids at that time (1, 2). All modern members of the genetically related group *M. tuberculosis* complex are thought to have a common African ancestor about 35,000-15,000 years ago (1). Since then, traces of TB have been found in both Peruvian and Egyptian mummies as well as being frequently described throughout recorded history under various names, for example Phthisis and consumption.

One of the first scientific descriptions of TB was a book in 1819 by the French medical doctor Laennec, in which he explained the pathology of TB and described the physical signs of pulmonary TB (1). But still in the 19th century the cause of TB was unknown, and it was only by the middle of the 1800's that it was convincingly demonstrated to be an infectious disease. Before then it was often considered to be an inheritable disease. TB was a huge problem in Europe and North America at this time, due to the poor socioeconomic conditions and people crowding up in cities to find work during the industrial revolution (3). Up to 25% of deaths in Europe during the 19th century were caused by TB (4). It was also in the mid-19th century that sanatoriums started being used to care for TB patients (5). Sanatoria were not a cure, but they served a purpose in isolating TB patients from those whom they might infect, as well as providing rest, fresh air, and a nutritious diet (1).

A milestone in the history of TB came in 1883, when the German doctor Robert Koch identified the bacillus *M. tuberculosis* as the causative agent of TB (1). This finding later awarded him the Noble Prize in Medicine or Physiology in 1905 for his elucidation of the etiology of TB. Koch also isolated tuberculin, a substance from tubercle bacilli, and attempted to use this as a TB treatment. This was not successful, but some years later, in 1907, the Austrian pediatrician, Clemens Freiherr von Pirquet published a study of tuberculin reactions in which he showed that a tuberculin skin test could be used as a marker for latent TB in children without TB symptoms.

This was further standardized and refined by Charles Mantoux and Florence Seibert as the purified protein derivative (PPD) that is used for the tuberculin skin test (TST) that we still use today.

In the early 20th century, long before the discovery of antituberculosis drugs, TB rates began to fall across Europe, as living standards started to improve (4, 6). The next milestone in TB history came with the discovery of the TB vaccine by the French scientists Albert Calmette and Camille Guérin in 1921 (1). By continuously passaging *Mycobacterium bovis* for many years, they managed to attain an attenuated strain that could be used as a vaccine, *Bacille Calmette-Guérin* (BCG). This was a breakthrough in TB prevention, but it would then take more than 20 years before the first TB drug were discovered. Paraamino salicylic acid (PAS) by Jörgen Lehman in 1943, and thiosemicarbazone by Gerhard Domagk in 1945 were among the first drugs, but these were only bacteriostatic. The real breakthrough came with the discovery of streptomycin by Albert Schatz, Elizabeth Bugie, and Selman Waksman in 1944. This was the first antibiotic and effective bactericidal agent against *M. tuberculosis*. In the following years more drugs were added to the repertoire with the discovery of isoniazid in 1952 and rifamycins in 1957. These TB antibiotics are still today part of the standard treatment of TB.

Improved living standards along with antituberculosis drugs drove TB prevalence to low levels in high- and middle-income countries in the world. However, in low-income countries TB was still a very large part of everyday life. In the 1990's the spotlight was again turned towards TB, as the Global Burden of Disease report placed TB as one of the top ten causes of morbidity and mortality in the world (7). Another huge emerging problem at this time was the HIV/AIDS epidemic. In many countries in sub-Saharan Africa, the spread of HIV lead to a three to five times increase in TB incidence rates (4). When it comes to risk factors for developing TB, HIV is the greatest risk factor, followed by diabetes and undernutrition. This rise in TB incidence led to World Health Organization (WHO) launching a new control strategy for TB, the Directly Observed Treatment and Short-course drug therapy (DOTS) in 1994 (4, 7).

In the last few decades further improvements in TB diagnostics have aimed at making diagnosis faster but also allowing drug susceptibility testing (4). For example, automated liquid culture systems for diagnosis and drug susceptibility testing and nucleic acid amplification tests. In 2006, the WHO launched the Stop TB strategy, a global plan to lower TB rates which also included the HIV-associated TB epidemic and drug resistant TB. These global efforts have led

to a decline in TB incidence. With better treatment options for HIV/AIDS, TB drug resistance is now the growing challenge. The current global TB strategy initiated by the WHO in 2015 is the End TB Strategy further aiming at reducing TB incidence and the socioeconomic impact of TB.

Epidemiology

TB is an infectious disease that mainly affects the lungs (pulmonary TB) but can also affect other sites of the human body (extrapulmonary TB). The clinical symptoms of pulmonary TB are chronic cough (including coughing up blood), sputum production, loss of appetite, fever, weight loss, and night sweats (4). Extra-pulmonary TB can for example present as miliary TB or TB meningitis. This is more common in children under the age of 5 or in HIV-positive individuals, and occurs through dissemination of bacteria from the lungs via the lymphatic system.

About a quarter of the world's population is infected with *M. tuberculosis* and thus at risk of developing TB disease (8). These are mostly carriers of a latent infection. According to the WHO, ten million people were diagnosed with TB during 2018, and 1.5 million died from TB. One third of these deaths were caused by drug resistant *M. tuberculosis*. TB remains a disease of poverty, mainly inflicting low-income countries in the world.

M. tuberculosis is typically spread via aerosols from someone with active pulmonary TB. The small droplets containing bacteria are then inhaled by another person. The respiratory mucosa lines the airways and comprises the first line of defense against the bacteria. It consists of epithelial cells, lymphocytes and tissue resident phagocytes, the alveolar macrophages. The bacteria are primarily phagocytosed by alveolar macrophages (9), but dendritic cells and epithelial cell can also phagocytose *M. tuberculosis* (10). Other inflammatory cells are then recruited to the lungs during the early infection. From this initial point of infection there are different clinical outcomes: early clearance, latent disease, or active disease (**Figure 1**).

Early clearance

Early clearance of the *M. tuberculosis* infection has been described as the eradication of bacteria before the adaptive immune response develops (11, 12). The evidence for this has been cases

where persons, despite being heavily exposed during a long period of time, remained negative when tested for latent TB, using tests like the tuberculin skin test (TST) or the Interferon- γ -release assay (IGRA) (described later) (13). This indicates that some intrinsic abilities in the hosts' innate immune system make them able to resist infection, but the exact mechanisms are not known, and may vary from case to case (14, 15).

Latent TB

Latent TB is the most common form of TB as most who get infected with TB do not develop active disease, and instead remain asymptomatic (8, 16). The host is able to contain the bacteria within macrophages and also wall off the infection within pathological structures known as granulomas (described later) (17), as part of an acquired cell mediated immunity (18). The bacteria can remain in a dormant state, but latent TB may also be a dynamic process with a balance between bacterial replication and host control. Latent TB can progress into active TB and this reactivation of latent TB may occur when the host's immune system is otherwise compromised due to illness, environmental factors, or old age. Latent TB is clinically diagnosed with an IGRA that measures T cell responses to *M. tuberculosis* antigens.

Active TB

Active disease can develop from a primary infection, or from reactivation of a latent infection, and occurs when bacterial replication is not inhibited by the cellular immune response, usually when the integrity of the granuloma is lost (4). This leads to spread of bacteria in the lungs, tissue damage, and this is when the clinical symptoms of active TB commence.

Figure 1. *M. tuberculosis* enter the lungs and are phagocytosed by alveolar macrophages. From this initial point of infection there are different clinical outcomes: early clearance, latent disease, or active disease. Early clearance entails that the bacterium is cleared by the innate immune cells before commencement of an adaptive immune response. During latent TB, the infection is contained within a granuloma and the individual is mainly asymptomatic. Active disease develops when the integrity of the granuloma is lost which leads to the spread of bacteria.

The bacterium

M. tuberculosis is an intracellular pathogen with humans being its main host, however it can infect several other animal species (4). *M. tuberculosis* is part of the *M. tuberculosis* complex of organisms, a range of mycobacteria able to cause human disease. The *M. tuberculosis* complex consists of *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium microti* and *Mycobacterium canetti*.

M. tuberculosis is the bacterium causing TB. It does not classify under the regular Gram classification of bacteria as it has very lipid-rich cell wall that is impermeable to basic dyes (4). Rather, a special Ziehl-Neelsen or Hallberg staining is used for staining *M. tuberculosis* and detection in sputum by direct microscopy. *M. tuberculosis* is classified as an aerobic, acid-fast, nonmotile, non-spore-forming bacillus. *M. tuberculosis* is a very slow-growing bacterium, with a replication rate of 15-20 hours which complicates both diagnosis and treatment of TB, and allows it to remain in a latent, persistent state in the human host.

Cell envelope and virulence

The *M. tuberculosis* cell envelope (**Figure 2**) consists of a cell membrane, a lipid bilayer, which is anchored to the thick cell wall (19, 20). The cell wall consists of an inner peptidoglycan layer covalently linked to arabinogalactans. These in turn are linked to long mycolic acids, which are branched fatty acids making up 60% of the cell wall. These fatty acids give mycobacterium their waxy, impermeable features. Also situated in the cell wall are the glycolipids lipomannan (LM), lipoarabinomannan (LAM) and mannosylated LAMs (ManLAM) which are important virulence factors and essential for the bacterium's interactions and survival inside host cells. The outermost capsule of mycobacteria consists mainly of polysaccharides, with α -glucan being the most abundant (21). Virulence factors can also be found in the capsule (22), for example secreted proteins like the 6 kDa early secretory antigenic target (ESAT-6) (23), or glycolipids, like phthiocerol dimycocerosates (PDIM) (24). The capsule is the primary contact zone between the bacterium and host cells, but since many routinely culture *M. tuberculosis* in liquid cultures with detergent in the media, the capsule is lost.

Figure 2: Schematic illustration of the *M. tuberculosis* cell envelope. (LM) Lipomannan, (LAM) Lipoarabinomannan, (ManLAM) Mannose-capped lipoarabinomannan, (AM) Arabinomannan, (PDIM) Phthiocerol dimycocerosate, (PGL) Phenolic glycolipid, (TDM) Trehalose dimycolate.

Mycobacteria use specialized secretion systems for the transport of secretory proteins across the mycobacterial cell envelope (25). The most important secretion system is the ESAT-6 secretion system 1 (ESX-1), a type VII secretion system, used for secreting ESAT-6 and 10 kDa culture filtrate protein (CFP-10). These virulence factors have been demonstrated to be important for the escape of *M. tuberculosis* from the phagosome, but also for necrosis and apoptosis of host cells (23, 26). ESX-1 is encoded by the genetic locus region of difference (RD1), which is deleted in the vaccine strain *Mycobacterium bovis* BCG.

The surface glycolipid trehalose dimycolate (TDM), also known as cord factor, was early on associated with the virulence of *M. tuberculosis* (27). TDM is present in all mycobacteria, not only in virulent species, which disputes its role as a virulence factor (28). It is constantly produced by the bacteria and released into the surrounding environment. However, TDM has been shown to be critical for the survival of *M. tuberculosis* in murine macrophages (29) and it

has been observed to play a role for the development of caseating granulomas, also in mice (30).

Corded cell aggregation (**Figure 3**), a type of biofilm formation, has also been described as a virulence factor of *M. tuberculosis* (31-33). Interestingly, the avirulent strain of *M. tuberculosis*, H37Ra, does not form cords (33). Culturing mycobacteria in detergent-free liquid media favors the phenotype of cording, in which the bacteria form multicellular coiled structures or pellicles. In 1947, Dubos and Middlebrook introduced the use of the detergent polysorbate-80 (Tween-80) to growth culture media, in order to culture homogeneous planktonic mycobacteria. This is still today a standard ingredient in culture media, but it is known to affect the virulence of the bacteria as it dissolves the *M. tuberculosis* capsule and disperses secreted virulence factors sequestered in the capsule (23, 34). Aggregated forms of mycobacteria are thought to be prevalent in pulmonary cavities of patients with active TB where extracellular bacterial growth is predominant (30). Cording may both be able to shield the bacteria from the surrounding environment, protect from phagocytes (35), as well as prevent antibiotics to penetrate and be able to act on single cells inside the cord or biofilm (36). We have also shown that cording *M. tuberculosis* induce extracellular trap formation in human macrophages (37).

Figure 3. *M. tuberculosis* can grow in a multicellular coiled structure called cords.

Diagnosis

The standard methods for diagnosis of active pulmonary TB, have earlier been sputum microscopy and culture techniques, as well as chest X-ray. These are still frequently used and are the primary options available for low-income countries. Sputum microscopy is a relatively fast and cheap method for diagnosis, but the number of bacteria in sputum can be very few, and difficult to detect, especially in early stages of TB, or in children (38). Culture techniques can take up to 12 weeks for results as *M. tuberculosis* are very slow growing bacteria, which delays

proper diagnosis. Rapid molecular tests like Xpert MTB/RIF assay are now the new standard for TB diagnosis in middle- and high-income countries, and with the endorsement from WHO it is also becoming more widely available in resource-poor countries (8). In addition to giving a fast, accurate result it can also detect rifampicin resistance.

The prevalence of latent TB is estimated to be high in many parts of the world and is a reservoir for *M. tuberculosis* which can lead to active TB. Individuals with latent TB do not present with clinical symptoms but can be diagnosed by measuring the specific cellular immune response to TB antigens (7). The tuberculin skin test (TST) was the first test developed, and it measures the reaction to an intradermal injection of TB antigens called PPD (4). The hypersensitivity reaction to PPD in people with mycobacterium-specific immunity can be measured after 48-72 hours, but the test lacks specificity to *M. tuberculosis* and can give a false-positive result if the person was exposed to nontuberculous mycobacteria or BCG (4). In the beginning of the 21st century the IGRAs were developed. They measure the release of IFN γ from blood mononuclear cells (PBMCs) in response to TB antigens such as ESAT-6 and CFP-10. The IGRA tests are as sensitive as the TST but with added specificity to *M. tuberculosis* and are now the gold standard for diagnosis of latent TB in resource-rich settings, where TB contact tracing is performed.

Therapy

The complexity of TB treatment is due to the fact that *M. tuberculosis* is a slow growing bacterium that also can enter a phase of dormancy. The dormant *M. tuberculosis* are thought to be drug-refractory (39). Within the human lung there may therefore exist different populations of *M. tuberculosis*: 1) actively growing extracellular bacteria in well-aerated cavities, 2) slow or intermittently growing bacteria in unstable parts of lesions, 3) bacteria in microaerobic compartments either intracellularly in macrophages or in inflammatory lesions, or 4) completely dormant bacteria surviving in anaerobic conditions. In order to reach and effectively eradicate the mycobacteria in different phases, and in different compartments of the lung, the standard regimen for drug susceptible TB is first a combination of the 4 drugs isoniazid, rifampicin, pyrazinamide and ethambutol for 2 months, followed by isoniazid and rifampicin for another 4 months. With this regimen, called the “short-course”, the success rate for treating drug susceptible TB is around 85% (40). Patient noncompliance is a problem due to the length of treatment but also due to the side-effects, which can lead to treatment failure and the

emergence of drug resistance. Drug resistant TB requires up to 24 months of combination therapy with different TB drugs that are more toxic (39). Drug-resistant TB also holds poorer prognosis, with only around 50% survival rate with the WHO recommended treatment regimen (40). However, there are promising results with new antibiotic regimens being tried for MDR/XDR-TB treatment. For example, with the new antibiotics bedaquiline and pretomanid, used in combination with linezolid, a relapse-free cure of 87% was achieved in one study. Delamanid is another promising new drug used for treatment of MDR/XDR-TB.

Vaccine

A vaccine that can confer life-long and effective protection against TB is crucial in order to reach the goal of eradicating TB (41). There is only one licensed vaccine available for prevention of TB, and it is the BCG vaccine that first was given to an infant in 1921 (4). It is a live vaccine consisting of the attenuated *M. bovis* *Bacille Calmette-Guérin* and it is the most widely used vaccine in the world today. It is administered to newborn children in endemic regions where it gives significant protection against severe childhood TB, like TB meningitis and miliary TB (42). However, the preventive effects in adults is variable and is thought to wane over time. Also, revaccination does not improve protective efficacy for TB (43). Vaccine development has proven difficult due to our insufficient knowledge of what factors correlate with protective immunity in TB (12). Most vaccines boost the humoral immunity against the pathogen, but the intracellular nature of *M. tuberculosis* requires a strong cellular immune response. However, there are many promising new vaccines in the pipeline and the BCG vaccine still holds promise due to its heterologous effects and induction of trained immunity, which is an epigenetically based reprogramming of innate immune cells which can protect against severe infections unrelated to the immune-training agent. These beneficial effects of BCG will be described more in later chapters.

Pathogenesis and granuloma formation

After phagocytosis of *M. tuberculosis* by alveolar macrophages in the lung, the bacterium interferes with key antimicrobial mechanisms, and can also start replicating intracellularly (44). The macrophage produces a range of cytokines and chemokines that will attract additional

innate immune cells like monocytes, neutrophils and dendritic cells (DCs). This comprises a localized inflammatory response, and in some cases the infection is stopped here resulting in early clearance of the pathogen (11, 45). For example, TNF α signaling is important for restricting growth of *M. tuberculosis* within macrophages (46). If infection persists, the recruitment of immune cells will lead to a macroscopic cluster of cells termed granuloma (**Figure 4**). Early granuloma formation has been observed to be dependent on *M. tuberculosis* virulence as an ESAT-6 deficient strain did not induce granuloma formation (47). The granuloma is considered both host-beneficial, in that it prevents spread of the bacteria in the lungs (48). But it is also considered as being pathogen-beneficial, as it creates a niche in which *M. tuberculosis* can replicate and hide away from the adaptive immune response.

Figure 4. *M. tuberculosis* infection can lead to the formation of a macroscopic cluster of cells called granuloma. The innate immune cells within the granuloma are macrophages, neutrophils and dendritic cells. Other variants of macrophages can also be found like epithelioid macrophages, foamy macrophages and multinucleated giant cells. The innate immune cells are surrounded by lymphocytes and an outer fibrotic wall.

Infected DCs migrate to the lymph nodes and present *M. tuberculosis* antigens to naïve T cells, initiating the adaptive immune response to TB. After a primary infection in humans, it takes 3-8 weeks before an adaptive immune response becomes detectable (49). The T cells will differentiate into antigen-specific T cells which return to the site of infection and help limit mycobacterial growth via IFN γ secretion (44). IFN γ from CD4 $^{+}$ T cells induces bactericidal mechanisms in macrophages, such as induction of autophagy, phagosomal maturation and production of antimicrobial peptides (50, 51).

As the granuloma formation progresses, some macrophages will differentiate into specialized cells like foamy macrophages, epithelioid cells and multinucleated giant cells (52). These cells, along with recruited natural killer (NK) cells, neutrophils, DCs, B cells and T cells can form a structured granuloma that encloses the infected macrophages in the core. A fibrous layer may separate the mostly immobile macrophages from the more dynamic lymphocytes that also appear to be able to traffic through the granuloma (53). The previous dogma that granuloma are static structures in the lung has been revised after studies in zebrafish have shown trafficking of infected cells from granulomas (48), where infected macrophages have been shown to leave the early granuloma and migrate to other parts of the lung where they initiate new granuloma (44, 54). Moreover, studies in macaques have demonstrated that bacteria can be cleared from the granuloma, and that in the same individual there can be bacterial lesions that progress and others that are healed (55, 56). These findings also add evidence to the phenomena of “early clearance”.

The intracellular growth of bacteria will kill the cell and the necrotic debris created in the center of the granuloma is termed “caseum” (44). In active TB the collapse of a granuloma will lead to the release of viable *M. tuberculosis* to the airways, which can lead to transmission of the disease by coughing (52). The granuloma has in this case benefited and facilitated the spread of bacteria by providing a niche where it can hide and replicate until reaching large numbers, which can be released from the ruptured granuloma and infect a new host.

Tuberculosis and innate immune cells

Neutrophils

Neutrophils are the most abundant leukocytes in human blood, and quick to respond during an infection. Neutrophils migrate from the vasculature into the local tissue where they can exert their function. They are among the first cells on site upon *M. tuberculosis* infection, in response to chemotactic signaling from infected macrophages (57-59). Also, a transient rise in neutrophil blood count has been observed in household contacts after exposure (60). They are phagocytes but can also exert extracellular effector mechanisms by the release microbicidal granule proteins, reactive oxygen species (ROS) and extracellular traps, in response to infection. Neutrophils are short-lived cells and after apoptosis ensues, they are cleared by efferocytosis by macrophages. *M. tuberculosis* infection has been shown to increase the rate of neutrophil apoptosis, and the apoptotic cells further triggered a proinflammatory response in macrophages (61-63), which was mediated by the release of heat shock protein 72 (Hsp72) (63, 64).

Neutrophils are not able to kill virulent *M. tuberculosis* (65). However, studies in mice have shown that neutrophils are important for early granuloma formation and containment of the *M. tuberculosis* infection, even though they do not contribute to restricting mycobacterial growth (66). Additionally, infected neutrophils interact with DCs and promote their acquisition of bacteria, which in turn is necessary for a prompt delivery of *M. tuberculosis* antigens to the lymph nodes and initiation of the adaptive immune response (67). Apoptotic neutrophils also play a role during *M. tuberculosis* infection, and macrophage acquisition of antimicrobial peptides from ingested apoptotic neutrophils has been shown to contribute to more efficient killing of intracellular mycobacteria (68). Likewise Andersson et al. demonstrated that macrophages acquired MPO and increased their ROS production upon efferocytosis of apoptotic neutrophils, which in turn led to increased mycobacterial growth restriction in macrophages coinfecting with HIV (69). However, neutrophil activation may also lead to excessive inflammation and tissue damage as they release elastase and other proteases, as well as ROS, upon degranulation (70). During *M. tuberculosis* infection, neutrophils have been shown to be drivers of active TB, associated with increased pathology and mycobacterial growth (71, 72). So, the role of neutrophils can be double-edged: they appear to have important effector functions against TB during early stages of infection but can also cause exacerbating disease in late stages.

Neutrophil extracellular traps

Neutrophils possess an impressive arsenal of extracellular effector mechanisms with their ability to release toxic granule proteins and ROS. The release of neutrophil extracellular traps (NETs) is another defense mechanism where they release extracellular DNA to capture and eliminate pathogens (73). However, NETs are also implicated in a range of pathophysiological processes when the generation and clearance of NETs are poorly controlled, for example thrombosis (74), atherosclerosis (75), and a range of autoinflammatory disorders (76-79). In TB, it has been shown that plasma levels of NETs corresponds to disease activity *in vivo* (80). The viral pulmonary infection SARS-CoV-2 has also been associated with elevated markers of NETs in patients' sera (81). Moreover, NET-release was triggered *in vitro* when neutrophils were incubated with sera from individuals infected with SARS-CoV-2.

Extracellular trap formation is a conserved cellular mechanism that may have evolved as a defense strategy to protect amoeba from pathogenic bacteria, including mycobacteria (82, 83). It has also been suggested that the casting of extracellular traps is a result of frustrated phagocytosis when the pathogen is too large to ingest, for example in response to bacterial clumps or yeast hyphae (84, 85).

The distinct cell death pathway which results in the release of NETs is called NETosis (86). The release of NETs can be triggered in response to both microbial stimuli through signaling via Toll-like receptors (TLRs), Fc-receptors, chemokine and cytokine receptors (87), but also in response to chemical stimuli, such as phorbol myristate acetate (PMA). The mechanism by which PMA induces NET formation involves the interaction of PMA with protein kinase C, which triggers the release of calcium from intracellular stores (88). This results in the activation of the Raf-MEK-ERK pathway and further downstream the assembly of NADPH oxidase at the phagosomal membrane, which in turn generates ROS (86). Neutrophil elastase (NE) and myeloperoxidase (MPO) are transported to the nucleus where they process histones, which leads to the decondensation of chromatin (89). The nucleus swells and the nuclear envelope disintegrates (86). Granules and mitochondria also break down and granule proteins attach to the DNA strands. Finally, the cell membrane ruptures, and the NETs are released into the extracellular space.

The antimicrobial activity of NETs comes from the bound granule enzymes like NE, MPO and cathepsin G, and also other antimicrobial peptides like cathelicidins and defensins (90). The negatively charged DNA backbone of NETs, as well as histones, also have antimicrobial activity (91, 92). However, mycobacteria were not killed by NETs *in vitro* (93), nor *in vivo* in a guinea pig model (94).

Another indirect function of NETs is binding and preventing the spread of bacteria (84). Moreover, since neutrophils respond to mycobacteria by producing NETs, the extracellular traps may be important for the crosstalk between innate immune cells during an infection. We and others have shown that neutrophils' proinflammatory activation of macrophages can in part be mediated by the phagocytosis of NETs (95, 96). Others have shown that cathelicidin from mycobacteria-induced NETs, was internalized by macrophages and increased the antimicrobial activity against the BCG strain of mycobacteria (97).

Macrophages

Macrophages are the main phagocytes, and central for TB immunity, as they are the primary host cell for *M. tuberculosis*. However, there are different subsets of human macrophages based on their functional phenotype and anatomical location. Furthermore, transcriptome-studies of macrophages have revealed a spectrum of macrophage polarization states which are reversible and highly plastic (98, 99). Previously, macrophage polarization was simplistically divided into two subtypes M1 or M2 (100). M1 are the classically activated macrophages, which have microbicidal and inflammatory properties and can be induced by LPS (lipopolysaccharide) or other microbial products, IFN γ and TNF α . The alternatively activated M2 macrophages are induced by IL-4, IL-10, IL-13 and TGF- β . They have more regulatory functions and are not microbicidal. M2 macrophages are also poor antigen presenters.

Alveolar macrophages

When mycobacteria enter the lung they first encounter the alveolar macrophages (9), which are the tissue-resident macrophages of the lungs and mostly considered to belong to the M2 phenotype (101). These cells originate from fetal monocytes that reach the alveoli at birth and differentiate into alveolar macrophages (102). The population of alveolar macrophages is then thought to be maintained *in situ*. However, it is not known whether adult bone-marrow derived

macrophages regularly contribute to the pool of alveolar macrophages, or if instead the alveolar macrophage pool is populated only once, or maybe in several waves, during early childhood, but then later remains constant (103).

Alveolar macrophages are the most abundant innate immune cells in the alveoli and are responsible for maintaining homeostasis, as well as regulating the response to epithelial damage or infection (104). Alveolar macrophages are mainly anti-inflammatory, which helps moderate the immune response to the steady onslaught of chemical and biological stimuli reaching the airways. In mouse studies, they have been shown to be metabolically distinct from interstitial lung macrophages (105). Alveolar macrophages are upregulated for fatty acid uptake and β -oxidation, while interstitial macrophages are highly glycolytically active. Consequently, the alveolar macrophage is an advantageous niche for mycobacteria in which they have access to fatty acids and cholesterol from the host cell, and this leads to increased replication of the bacteria. During the first 10 days after infection in mice, mycobacteria have been shown to reside exclusively in alveolar macrophages (106), and these then facilitate the dissemination of the bacteria from the airways to the lung interstitium (107). Within 10-14 days post *M. tuberculosis* infection, recruited neutrophils and macrophages replace the alveolar macrophages in being the main phagocytic cells carrying bacteria (101).

Macrophage recognition and phagocytosis of mycobacteria

Recruited monocytes enter the lung tissue in response to signaling from infected alveolar macrophages. These monocytes differentiate into DCs and macrophages, which recognize and internalize *M. tuberculosis* using a range of surface and intracellular receptors. Numerous macrophage receptors have been shown to interact with *M. tuberculosis*, and the main families of these surface receptors are c-type lectin receptors (CLRs), Toll-like receptors (TLRs), scavenger receptors, and complement receptors (108).

C-type lectin receptors

CLRs are a family of receptors that recognize carbohydrate structures on pathogens (109). Both soluble and membrane-bound receptors can be found in the CLR family. However, of the transmembrane receptors, the mannose receptor dendritic cell-specific intercellular adhesion

molecule-3 grabbing nonintegrin (DC-SIGN) and dectin-1 are the most important for infection with *M. tuberculosis*.

The mannose receptor is a CLR that is highly expressed on alveolar macrophages and binds to mannose in the mycobacterial cell wall moieties LAM and ManLAM on virulent *M. tuberculosis* (110). Recognition and uptake of mycobacteria by the mannose receptor leads to the production of anti-inflammatory cytokines. In addition, the intracellular fate of *M. tuberculosis* is affected as entry into the host cell by this route leads to inhibition of phagolysosomal fusion, which promotes survival of the bacteria (111).

DC-SIGN is a CLR that plays an important role in *M. tuberculosis*-interactions with DCs, but the receptor is also expressed on macrophages. Similarly to the mannose receptor, DC-SIGN binds ManLAM in the mycobacterial cell wall of virulent *M. tuberculosis* (108). Interestingly, it has also been demonstrated that α -glucan in the mycobacterial capsule can bind DC-SIGN (112). *M. tuberculosis* interactions with DC-SIGN promotes an anti-inflammatory response in DCs and induces IL-10 production.

Dectin-1 is primarily known as the macrophage receptor for fungal β -glucan (113). But since the mycobacterial cell wall does not contain β -glucan the *M. tuberculosis* ligand for dectin-1 is unknown (114), and as of date no one has shown whether mycobacterial α -glucan can bind dectin-1. Avirulent or attenuated mycobacteria, but not virulent strains, have been demonstrated to activate human macrophages via dectin-1 in a TLR2 dependent manner leading to increased production of TNF α (115). However in airway epithelial cells, dectin-1 conjugated with TLR2 upregulates cytokine expression upon virulent *M. tuberculosis* stimulation (116). Another study demonstrated that virulent *M. tuberculosis* can induce IL-17A responses via dectin-1 conjugated with TLR4 (117).

Dectin-2 is another C-type lectin which more recently has been observed to bind mycobacterial ManLAM (118). Dectin-2 appears to be specialized in binding lipoglycans, triggering a Th17 immune response.

Toll-like receptors

The Toll-like receptors are expressed on cell membranes or the membranes of endocytic vesicles (108). In mammals there are 12 different TLRs, while mainly TLR2, TLR4, TLR8 and

TLR9 are involved in the recognition of *M. tuberculosis*. TLR2 and 4 are extracellular receptors on the plasma membrane, while TLR8 and 9 are located intracellularly, on vesicle membranes, and mostly involved in recognition of nucleic acids (119). TLR activation leads to an upregulation of proinflammatory cytokines via the myeloid differentiation primary response protein 88 (MyD88)-dependent pathway which leads to activation of mitogen-activated protein (MAP) kinases, the translocation of NFκB to the nucleus and the subsequent transcription of multiple cytokines such as TNFα, IL-1β and IL-12 (120). During *M. tuberculosis* infection, secreted TNFα and IL-12 from macrophages activate IFNγ secretion from T-cells and NK-cells, which in turn will enhance microbial effector mechanisms in the macrophage.

TLR2 forms heterodimers with either TLR1 or TLR6, and these can then recognize mycobacterial cell wall glycolipids and glycoproteins (108). TLR2 is critical for controlling *M. tuberculosis* infection and it has been observed that TLR2-deficient mice succumb faster to low-dose aerosol infections (121). TLR2 activation is also known to induce apoptosis (122), autophagy (123) as well as proinflammatory cytokine production via NFκB (120).

Similarly to TLR2, TLR4 also induces proinflammatory signaling via MyD88 and NFκB (120). LPS from Gram-negative bacteria is the most well-known ligand for TLR4, however the receptor also recognizes cell wall lipids, glycoproteins and secreted proteins from mycobacteria. TLR4 stimulation can also activate a MyD88-independent pathway which induces IFN-β secretion. Nevertheless, the importance of TLR4 in mycobacterial infections is not clear and TLR4 deficient mice are not more susceptible to TB (124, 125).

TLR8 and TLR9 are intracellular receptors located on endosomal membranes (119, 126), and signaling via these receptors leads to IL-12 and IFNγ secretion. TLR8 recognizes mycobacterial RNA while mycobacterial DNA is the ligand for TLR9. There are also studies indicating crosstalk and synergistic effects between TLRs, for example with TLR9 and TLR2, where double knockout mice do not secrete IL-12 and succumb faster during *M. tuberculosis* infection (127). Moreover, there is an interest in using TLR8 agonists as vaccine adjuvants in order to increase IL-12 and IFNγ production, thus increasing the Th1 response (119, 128).

Complement receptors

The most important complement receptor in mycobacterial infections is the complement receptor 3 (CR3) that, despite its name, mediates both complement opsonized as well as non-

opsonized phagocytosis of *M. tuberculosis* (129). CR3 has been shown to mediate approximately 80% of serum-opsonized monocyte phagocytosis of virulent *M. tuberculosis* (130), however phagocytosis by this route has no significant effect on the induction of antimicrobial effector mechanism or intracellular survival of bacteria (131). The receptor has a binding site for β -glucan and CR3 has been reported to be the major neutrophil receptor for β -glucan (132).

Scavenger and Fc γ receptors

Scavenger receptors (SRs) represent a broad family of cell surface and secreted receptors that can recognize both self and non-self lipoproteins (120). The cell surface receptors CD36, macrophage receptor with collagenous structure (MARCO) and scavenger receptor A (SRA) are involved in *M. tuberculosis* recognition, as well as the secreted SR named apoptosis inhibitor of macrophages (AIM). However, these receptors probably play a less important role in TB pathogenesis. The Fc γ receptor mediates uptake of IgG opsonized mycobacteria leading to the generation of reactive oxygen species, phagolysosomal fusion and increased killing of mycobacteria (133).

Nucleotide-binding oligomerization domain-like receptors (NOD-like receptors)

M. tuberculosis is an intracellular pathogen that resides in the phagosomes of macrophages, but can also escape into the cytoplasm (134), there activating intracellular receptors like the nucleotide-binding oligomerization domain-like receptors (NOD-like receptors or NLRs) (120). Stimulation of NLRs leads to an increased expression of IL-1 β and other cytokines of the IL-1 cytokine family, which all play an important role in the host defense against mycobacteria (135-139). NLRP3 is part of a multiprotein complex termed the inflammasome that also consists of the effector protein caspase-1, which cleaves pro-IL-1 β to its active form IL-1 β . Gain of function polymorphisms in NLRP3, or its adaptor protein CARD8, leads to enhanced control of mycobacterial growth in macrophages through increased IL-1 β production (139). Likewise, polymorphisms in NLRP3 and CARD8, leading to a more susceptible inflammasome, is associated with poor treatment outcome and increased risk of extra pulmonary TB in Ethiopia (140). These observations highlight the importance of inflammasome function and IL-1 β in mycobacterial control.

NOD1 and NOD2 recognize peptidoglycan on bacterial cell walls, leading to NF κ B dependent production of proinflammatory cytokines (120). NOD2 senses muramyl dipeptide (MDP), the

minimal bioactive peptidoglycan motif of peptidoglycan, which is also present in the mycobacterial cell wall. Silencing of NOD2 in human macrophages leads to an increased growth of *M. tuberculosis* (141). Similarly, MDP treatment of human alveolar macrophages improved their control of *M. tuberculosis* infection through increased cytokine production and autophagy (142). NOD2 stimulation using MDP or the BCG vaccine can also induce an innate immune memory phenotype in human macrophages by activation of NF κ B and epigenetic rewiring of macrophages (143). More about trained immunity will follow in a later chapter.

Survival inside macrophages

M. tuberculosis interactions with innate immune cells initiates a cascade of intracellular events, some of which lead to signaling to bystander cells and upregulation of effector mechanism, another is the direct phagocytosis of the bacterium into an intracellular compartment called the phagosome (144). The bacteria-containing phagosome subsequently fuses with vesicles known as lysosomes. This maturation of the phagosome leads to a change to more acidic pH, as well as the acquisition of proteases, peptidases and lipases which aid in killing of phagocytosed pathogens. Mycobacteria are also killed in the mature phagosome, and the acidification of the phagosome, through activation of lysosomal hydrolases, is important for mycobacterial control (145). However, *M. tuberculosis* are more resistant to this bactericidal environment than most other microbes. In addition, *M. tuberculosis* can block the maturation process and reside in an early phagosome, and it has been suggested that mycobacterial cell wall lipids or glycolipids like manLAM and TDIM may contribute this phagosomal arrest (111, 146). The intracellular fate of mycobacteria is also affected by the route of entry into the macrophage. Entry via the Fc γ receptor hastens phagosome maturation compared to CR-mediated uptake. Also, phagocytosis mediated by the mannose receptor leads to delayed maturation. However, the mycobacterial induced phagosomal arrest can be overcome by IFN γ (147). Another way that *M. tuberculosis* avoids degradation inside a mature phagosome is by escaping into the cytoplasm, where it can gain access to nutrients and further replicate (134). This ability is dependent on the virulence factors CFP-10 and ESAT-6. Once inside the cytoplasm the host cell has other surveillance mechanisms to inhibit the bacterium by targeting it for autophagy (148). Autophagy is a mechanism for degradation of old organelles by enclosing them in a double-membrane vesicle called the autophagosome, which then fuses with lysosomes. However, autophagy can also be used to capture and degrade intracellular pathogens, making

it an important strategy for eliminating mycobacteria (50, 148). However, *M. tuberculosis* has been shown to inhibit autophagosome-lysosome fusion in DCs, and this was dependent on the ESX-1 secretion system (149). IFN γ and vitamin D can increase autophagy and vitamin D supplementation has been suggested as an adjunctive TB treatment (150, 151).

Macrophage cell death in TB

A natural turn-over of cells occurs constantly in the human body, and regulated cell death is required for tissue development and homeostasis. Apoptosis is a tightly regulated cell death pathway where the contents of the cells are packaged in membrane-bound vesicles called apoptotic bodies. These apoptotic bodies are then engulfed by other phagocytes in an anti-inflammatory process called efferocytosis. Apoptosis is considered a host-protective response in TB as it serves as a defense mechanism for the cell when threatened by an intracellular pathogen which uses its resources for survival and replication (148). By sacrificing itself the cell can deliver the pathogen packaged in an apoptotic body to a non-infected cell, and efferocytosis of *M. tuberculosis*-infected macrophages has been shown to restrict bacterial growth through the delivery of bacteria to lysosomes (152). Apoptosis can be induced by FasL or TNF α (148). Virulent *M. tuberculosis* are able to evade apoptosis by downregulating Fas on macrophages, interfering with death signals downstream of the TNF α receptor-1, as well as neutralizing TNF α by shedding of soluble TNF α receptor-2 (153). By inhibiting apoptosis, the bacteria instead promote another cell death pathway known as necrosis (154). Necrosis is characterized by loss of membrane integrity and uncontrolled rupture of the cell, leading to inflammation and tissue damage. Necrosis is thought to facilitate the spread of bacteria inside the host.

Macrophage extracellular traps (METs)

Neutrophil extracellular traps (NETs) were described in an earlier chapter. Macrophages and monocytes can also produce extracellular traps in response to mycobacteria and other pathogens, and these are subsequently called macrophage extracellular traps (METs) (35, 37, 85, 155, 156). The common cell death pathway for cells, which ends up in the release of extracellular traps, has been termed ETosis (148). Monocyte and macrophage extracellular traps have been less studied compared to NETs (157). And while neutrophils are very reactive cells, quick to degranulate or release NETs, ETs release in monocytes and macrophages occurs more

seldom. Still, ETosis in macrophages has been suggested to be a distinct cell death pathway, different from both apoptosis and necrosis, and characterized by an initial disintegration of the nuclear and granular membranes before rupture of the plasma membrane with the following release of DNA strands (158). Otherwise, there have been contradicting reports on the METs release being dependent on cytoskeleton remodeling and ROS, but also the origin of the DNA present in METs is unclear with both markers of mitochondrial and nuclear DNA being present at the same time (159). Macrophages can be primed by IFN γ to release METs during *M. tuberculosis* infection (85), but also several other microorganisms can induce MET-formation (158).

Trained innate immunity

The previous dogma that the innate immune response is a nonspecific immune response which lacks memory has been challenged by the concept of trained immunity, also called innate immune memory (160). The innate immune system builds up a memory, which entails the training of innate immune cells after their first exposure to a pathogen (**Figure 5**). This results in a heightened, but nonspecific response, which also is T and B cell independent, when they are infected again with the same or a different pathogen. The most well-studied inducers of trained immunity are the BCG vaccine and β -glucan, which will be described more in detail later in this chapter.

Figure 5. Trained immunity leads to a functional reprogramming of innate immune cells after a first stimulation, leading to a heightened response to a second stimulation from an unrelated pathogen.

The epigenetic basis of trained immunity

The trained immunity phenomenon relies on epigenetic reprogramming, which in turn results in an upregulated transcriptomic profile of genes involved in the antimicrobial program of innate immune cells. However, studies have revealed that BCG and β -glucan also have the

ability to reprogram hematopoietic progenitors of the myeloid lineage in the bone-marrow (161-163), which can account for the prolonged generation of trained cells in the circulation. The epigenetic reprogramming involves histone modifications which leads to chromatin reconfiguration and accessibility to genes (164, 165), as well as DNA methylation (166) and modulation of the expression of long noncoding RNA (167) and microRNA (168). When specific promoters and enhancers of genes associated with metabolic and immune pathways are modified, the trained macrophages can produce increased levels of cytokines when confronted with microbial compounds (164, 169), for example TNF α , IL-1 β and IL-6.

Immunometabolic regulation of trained immunity

Metabolites are known to modulate the activity of chromatin-modifying enzymes (170), and one of the hallmarks of trained immunity is the shift from oxidative phosphorylation toward aerobic glycolysis in immune cells trained with β -glucan from *Candida albicans* (165, 171). This shift in metabolism supplies the cell with energy needed for activation. The process is mediated by the binding of β -glucan to the Dectin-1 receptor, followed by down-stream signaling via the AKT-mTOR-HIF1 α pathway. The enhanced metabolic activity in the cells leads to the generation of metabolites with immunomodulatory functions, such as itaconate, fumarate, mevalonate or succinate (172-175). For example, accumulation of fumarate causes inhibition of demethylases which in turn leads to increased trimethylation of histones (172). The increased glycolysis in cells results in increased lactate production, which accumulates in cell culture medium and can be analyzed.

Beta-glucans in trained immunity

β -glucans are polysaccharides that serve as energy stores and structural components in cell walls of plants, algae, fungi, and bacteria (176). They share a common structure consisting of a β -(1,3)-glucan backbone, which then can differ in length and branching structure depending on species and isolation procedure. β -glucans have long been studied as immunomodulators with both antitumoral and anti-infective activity (177). After introducing the concept of trained immunity in 2011 (160), Netea and his group went on to show that *C. albicans* β -glucan could induce a trained immunity phenotype in human monocytes, which lead to enhanced cytokine

production upon re-stimulation, and protected them from infection with *C. albicans* (164). They also showed that β -glucan training required the Dectin-1 receptor and the Raf-1 pathway and was associated with stable changes in histone methylation at H3K4. Additionally, they introduced the *in vitro* experimental scheme in which they stimulated monocytes with β -glucan, followed by a wash-out period of a week before re-stimulation. In contrast to earlier studies of the immunomodulatory effects of β -glucan, this immune training scheme ensures that it is a functional reprogramming leading to a memory in the cells which confers the enhanced protection against re-infection. Many studies of the beneficial effects of β -glucan immune training both *in vitro* and *in vivo* have followed after the first published study by Quintin et al. in 2012 (164), but to large extent the same training scheme is used and also the same source of β -glucan.

BCG in trained immunity

The anti-TB vaccine BCG, and other vaccines containing attenuated live microorganisms, have long been observed to lead to beneficial heterologous effects against other childhood infections (178-180). In this context, the BCG-vaccine has been shown to affect the overall mortality in children by decreasing the incidence of pneumonia and sepsis and other non-TB related infections. The first documented evidence of this phenomenon originates from the early 1900's in Sweden, when the physician Carl Näslund found that BCG-vaccinated newborns had a three times lower mortality rate than unvaccinated infants (181, 182). More recently it has been hypothesized that trained immunity may be responsible for this BCG-mediated survival-advantage. Early experimental models demonstrating that BCG vaccination in mice protect against secondary infection with *C. albicans* or *Schistosoma mansoni* have added evidence to the heterologous effects of vaccines (160, 183, 184). These effects were shown to be partially T cell-independent and involved activated tissue macrophages.

Once the innate trained immunity dogma was introduced in 2011 (160), Kleinnijenhuis et al. went on to demonstrate that BCG could induce a trained immunity phenotype via epigenetic reprogramming of monocytes that protected them from reinfection with *M. tuberculosis*, but also *S. aureus* and *C. albicans* (143). This study was performed in severe combined immunodeficiency (SCID) mice that lack an adaptive immune response. In the same study they studied the human BCG response in adults vaccinated with BCG. They isolated PBMCs and

upon re-stimulation with the *S. aureus* and *C. albicans* the cells responded with increased IFN γ , TNF α and IL-1 β secretion, and this heightened cytokine response lasted up to 3 months after BCG vaccination. More recently others have observed that the systemic administration of BCG or β -glucan in mice affects the differentiation of myeloid cells in the bone marrow, and this resulted in an increased release of monocytes with the enhanced capacity to kill pathogens and secrete cytokines (161, 163). These findings help explain how a BCG vaccination can induce a more long-lasting trained immunity response. Cirovic et al. also demonstrated that human *in vivo* intradermal BCG vaccination could induce a similar effect at the level of myeloid cell development in the bone marrow (162). They found that BCG vaccination was associated with the rewiring of the transcriptional programs of bone marrow hematopoietic stem and progenitor cells, affecting their development and function, and resulting in increased responsiveness to unrelated bacterial and fungal stimuli in *ex vivo* PBMCs, but also increased neutrophil counts.

Models for studying *M. tuberculosis* infection

One limitation when studying *M. tuberculosis* infection is finding good model systems. Many things are important to consider when choosing the correct model, such as how well it mimics the human *in vivo* mechanism to be studied. But also, availability, cost and animal ethics are things to be considered.

In vitro *M. tuberculosis* infection models

The simplest infection models are based on *in vitro* monocultures of cells. Mouse cells have a more robust nitric oxide (NO) production compared to human cells, and studies have shown that the antimycobacterial activity in murine macrophages depends on NO (185). The role of NO in the antimycobacterial activity in human cells is less clear. Human monocultures are often used, mainly studying the phagocytes; monocytes, macrophages, dendritic cells or neutrophils (186). Other human cell culture systems consist of cocultures, usually phagocytes together with T lymphocytes or NK cells. But also, *M. tuberculosis* infection-models using PBMC or whole blood are common. PBMC can cluster upon *M. tuberculosis* infection, and can thus be used to study early granuloma formation and signaling between cells (187).

In order to study the cellular dynamics of the TB granuloma, three-dimensional (3D) cell culture or tissue models are required (187). These *in vitro* model systems allow for the study of more human cell types and a better understanding of host-*M. tuberculosis* interactions in the tissue microenvironment. A collagen matrix can be used and both cell lines and primary cells, which adds to the potential of the model. 3D tissue models can be used to study early cellular dynamics and clustering of cells after a *M. tuberculosis* infection. However, for longer experiments and more complex granuloma studies, animal models are required.

In vivo *M. tuberculosis* infection models

For *in vivo* studies of TB granuloma formation, there are several nonhuman models. The mouse model is the most common, but often insufficient as mice do not exhibit the same pathological characteristics as humans, such as necrosis, caseation and fibrosis (188). However, there are some strains that have been shown to develop necrotic granuloma like the C3HeB/FeJ strain

(189) and also humanized mice have been shown to develop granuloma that are CD4+-dependent and where TNF α is important for *M. tuberculosis* restriction (190). The rabbit and guinea pig models more closely resemble human TB infection, but are less common than human cellular models, and mice models, due to a limitation in the variety of reagents available (187). Primate models are very relevant to study since both necrotic granulomas and latent TB infection can be studied. However, cost and ethics are the main drawbacks.

The zebrafish model is maybe the simplest *in vivo* model, which also allows for live imaging of granuloma formation using *M. marinum* for infection. *M. marinum* possess the ESX-1 secretion system making it an interesting model to study the virulence factor ESAT-6 (191). The zebrafish model has been used for studying trafficking of the bacteria in and out of the granuloma and dissemination of the infection (48, 54, 192, 193).

The overall aim of the work compiled in this thesis was to understand the innate immune functions, the partnership between innate immune cells, trained immunity and how these different concepts are regulated and can be enhanced during human TB infection.

Paper I

This study explored how human neutrophils respond to *M. tuberculosis* by releasing extracellular traps (NETs) and how the pathogen-induced NETs mediate a proinflammatory activation of macrophages.

Paper II

This study aimed at investigating how the cording phenotype of *M. tuberculosis* interacts with human macrophages to induce extracellular trap formation.

Paper III

The aim of this project was to demonstrate how an *in vitro* three-dimensional lung tissue model can be used to study innate immune dynamics during *M. tuberculosis* infection.

Paper IV

The aim of this study was to explore how the macrophages' antimycobacterial activity could be enhanced by inducing trained immunity with different β -glucans as stimuli.

RESULTS AND DISCUSSION

PAPER I: *Mycobacterium tuberculosis*-induced neutrophil extracellular traps (NETs) activate human macrophages.

In this study we wanted to explore the role of neutrophil extracellular traps (NETs) in response *M. tuberculosis*. Neutrophils can release NETs upon activation, which contain both cytosolic and granule proteins (73). NETs can assist in the defense against different bacteria by capturing and killing them. Furthermore, *M. tuberculosis* has been shown to induce NET-formation but these could not kill the mycobacteria (93). Earlier studies have highlighted the role of neutrophil granule proteins, that were acquired by macrophages during phagocytosis of apoptotic neutrophils, in enhancing the macrophage's antimicrobial activity against intracellular pathogens (68). Also, previous work, performed in our group, demonstrated that pathogen-induced apoptotic neutrophils mediated a proinflammatory activation of macrophages that involved the release of heat-shock protein 72 (Hsp72) (61-63).

We initially demonstrated that γ -irradiated *M. tuberculosis* could induce NET-formation, however these exhibited a different time-kinetics (**Paper I, figure 2 a**) and appearance (**Paper I, figure 1**) compared to chemically activated NETs induced by phorbol myristate acetate (PMA). The NET-formation in both *M. tuberculosis*- and PMA-stimulated cells was abrogated when pre-incubating the cells with a NADPH-oxidase inhibitor (**Paper I, figure 2 b-d**), indicating that the process is dependent on reactive oxygen species (ROS). Additionally, the *M. tuberculosis*-induced NETs could be inhibited by adding a neutrophil elastase inhibitor. The translocation of neutrophil elastase to the nucleus has been shown to drive NET-formation by degrading histones, leading to chromatin decondensation (89). We also investigated whether the *M. tuberculosis*-induced NETs were dependent on phagocytosis of the bacteria by inhibiting actin-polymerization using cytochalasin D (CytD) (**Paper I, figure 3**). CytD treatment did in fact inhibit NET-formation in *M. tuberculosis*-stimulated neutrophils, however it did not impede PMA-induced NET-formation. We further studied the interaction between *M. tuberculosis*-induced NETs and macrophages in cocultures. Using microscopy, we could observe macrophages interacting with the NETs but also phagocytosis of NETs (**Paper I, figure 4**). By measuring cytokines in culture supernatants, we found that macrophages stimulated with *M. tuberculosis*-induced NETs secreted significantly higher amounts of IL-6, TNF α , IL-1 β and

IL-10 than when stimulated with PMA-induced NETs (**Paper I, figure 5**). By adding the NADPH-oxidase inhibitor DPI and thus inhibiting NET-formation, the release of IL-6 and IL-10 was reduced. However, the neutrophil elastase inhibitor did not have the same effect, although we previously observed that the elastase inhibitor also inhibited *M. tuberculosis*-induced NETs. Since Hsp72 from apoptotic neutrophils had previously been demonstrated to mediate a proinflammatory activation of macrophages (63), we performed antibody staining of Hsp72 in NETs. *M. tuberculosis*-induced NETs contained Hsp72 sequestered in the NETs, in contrast to PMA-induced NETs (**Paper I, figure 6**). By adding PMA-induced NETs along with recombinant Hsp72, we could again observe an increase in cytokine release, indicating that the proinflammatory activation of macrophages was in part mediated by Hsp72 (**Paper I, figure 7**).

Since we published our study the research on NETs has exploded and there are now a multitude of studies on how NETs are involved on both infectious and non-infectious inflammatory diseases such as: air way inflammation, atherosclerosis, Alzheimer's and stroke (194). Apart from being beneficial to some extent during infections by immobilizing microbes, preventing their spread, and potentially killing them with antimicrobial molecules, excessive NET formation also drives tissue damage. Additionally, in viral pulmonary infections like SARS-CoV-2, excessive NET-formation has been observed in patient's lungs and drugs that target NETs have been proposed as possible therapeutic options (195).

The role of NETs in TB is still controversial, however a correlation between plasma NET levels in patients with TB and disease severity has been observed (80). Moreover, many suggest that NETs have a role in minimizing the spread of an infection (196). We were in this project able to demonstrate that NETs can contain different danger-signals, depending on what stimulated their release. In our case the *M. tuberculosis*-activated NETs contained Hsp72, which in turn activated adjacent macrophages. This suggests that NETs, during a *M. tuberculosis* infection, could carry and sequester macrophage-activating signals.

PAPER II: *The cording phenotype of Mycobacterium tuberculosis induces the formation of extracellular traps in human macrophages.*

The chance discovery of extracellular DNA strands in a culture of *M. tuberculosis*-infected macrophages led us to further investigate extracellular trap formation in macrophages. Common laboratory practice is to culture *M. tuberculosis* in culture medium containing the detergent Tween-80, to acquire well-dispersed and homogenous cultures of bacteria. However, physical perturbation along with Tween-80 has also been shown to dissolve the *M. tuberculosis* capsule (34). Reducing the amount of Tween-80, led to cord-formation of *M. tuberculosis* (**Paper II, figure 1**). Cord-formation is a known mycobacterial virulence factor, where the bacteria form large coiled structures (31-33), and cording bacteria have been observed in pulmonary cavities of patients with active TB (30). Cord-formation may play a role in protecting the bacteria from phagocytes (35), as well as shielding the bacteria from the surrounding environment preventing antibiotics to act on bacteria inside the cord or biofilm (36).

In our study, the human macrophages infected with the cording phenotype of *M. tuberculosis* responded by releasing macrophage extracellular traps (METs) (**Paper II, figure 2**). We further characterized the METs using scanning electron microscopy (SEM) (**Paper II, figure 3**) and confocal microscopy, staining for DNA and citrullinated histone 4 (**Paper II, figure 4**). Macrophages and monocytes have in other studies also been observed to release extracellular traps in response to microbes, including mycobacteria (35, 85, 197). In contrast to NETs, MET-formation is not dependent on the NADPH-oxidase (**Paper II, figure 5**), which has been confirmed by others (159). It is not clear what the physiological relevance of METs is, and it does not occur at the same rate or as often as NET-formation. One concept is that METs are formed in response to bacterial aggregates that are too large to ingest by the phagocyte, as a plan B when the regular mode of eliminating the pathogen fails (35, 159). We likewise found that the aggregated cord-form of *M. tuberculosis* induced more METs-release compared to well-dispersed, single cell bacteria (**Paper II, figure 2**). However, in a study on *Mycobacteria massiliense*, the METs not only failed in killing the bacteria, they instead facilitated their growth (159).

The relationship between cording and virulence of *M. tuberculosis* is still debated, namely whether cording is just an indicator for the presence of a specific virulence factor like ESAT-6 or TDM, or if it is the physical aggregation itself that enhances the virulence. Raffetseder et al.

discovered that ESAT-6 was retained in the *M. tuberculosis* capsule but could be lost when the bacteria were cultured in media containing detergent, which dissolved the lipid-rich capsule (23). We likewise observed a link between the same culture conditions and the ability of *M. tuberculosis* to form cords (**Paper II, figure 1**). Additionally, we studied whether an ESAT-6-deficient strain of *M. tuberculosis* also could induce MET-formation and found that the mutant strain was not capable of inducing METs (**Paper II, figure 7**). Still, the ESAT-6-deficient *M. tuberculosis* grew extracellularly in aggregates that resembled cords, similar to the wild-type *M. tuberculosis*. A recent study observed virulent *M. tuberculosis* growing in cords intracellularly in primary human lymphatic endothelial cells (35). The same phenomenon was discovered in lymph nodes of patients with TB. They concluded that cording was a size-dependent mechanism used by the bacterium to avoid recognition by cytosolic sensors, and that cording took place in the cytosol of cells after escape from the phagosome. However, a difference was observed between infected endothelial cells and macrophages. While the endothelial cells were permissive to intracellular growth of *M. tuberculosis* in the cytosol, necrotic cell death was triggered when *M. tuberculosis* escaped into the cytosol of macrophages. These observations help explain the MET-formation we observed when macrophages encountered virulent cording *M. tuberculosis*. We hypothesize that the virulence factor ESAT-6 is retained in the capsule of *M. tuberculosis* that are grown without detergent and are prone to grow in cords. ESAT-6 enables the escape of *M. tuberculosis* into the cytosol of the macrophage and cell death is triggered which also involves the release of METs. The mycobacterium can then continue to grow in cords also extracellularly using nutrients from dying cells as energy source.

PAPER III: *A 3D human lung tissue model for functional studies on Mycobacterium tuberculosis infection.*

One part of this project was to compile a detailed protocol of how to construct and apply a human lung tissue model that we developed in order to study early granuloma formation during *M. tuberculosis* infection. The other part was to make a video demonstration of crucial parts of the method, described in the written protocol, that would be available online as an aid to anyone interested in starting up the lung tissue model in their lab.

Monocultures of human macrophages or other peripheral blood cells have often been used when studying human *M. tuberculosis* infection. An *in vitro* human tissue model has the advantage of being able to better reflect the *in vivo* situation where several cell types, spatially organized, are involved. While animal models are sometimes useful, they often poorly reflect human TB infection. The lung tissue model we described in this paper was originally developed to study dendritic cell functions (198). It was adapted for studies of macrophage *M. tuberculosis* infection where *M. tuberculosis* infected macrophages could be implanted in the tissue allowing for the study of clustering of immune cells in response to the infection (47).

Two tissue-specific human cell lines, epithelial cells (16HBE) and fibroblasts (MRC-5), are used in the lung tissue model, and later primary human monocytes and macrophages are added (**Paper III, figure 1**). First the fibroblasts are cultured in a collagen matrix on top of a porous membrane in a trans-well insert. The membrane supports the cells and allows exchange of nutrients from the cell culture medium. After approximately one week the fibroblasts have multiplied, and contraction of the collagen-fibroblast layer can be observed. *M. tuberculosis*-infected macrophages are then added to the model along with uninfected monocytes. The infected macrophages migrate into the fibroblast-collagen matrix along with the monocytes. The monocytes later differentiate into macrophages in the lung tissue. Following the addition of immune cells, epithelial cells are added to the models. These cells multiply, forming structures resembling normal human lung tissue. The culture medium is subsequently reduced, air-exposing the epithelial cells, which stratify and start secreting mucus. After additional days, the lung tissue models are fixed in paraformaldehyde and mounted for microscopy viewing. Confocal microscopy acquiring images in optical Z-stacks, and the availability of fluorescently labeled *M. tuberculosis* and human cells, enabled us to study the spatial localization of bacteria and cells.

The human lung tissue model allowed us to observe monocytes clustering around *M. tuberculosis* (**Paper III, figure 2**). We could also quantify the size and number of macrophage-cell clusters after infection with mycobacteria, and observed that *M. tuberculosis* infection led to fewer, but larger cell clusters, compared to uninfected controls (**Paper III, figure 3**). The human lung tissue model provides an ideal three-dimensional system for studying migration of cells, cell-interactions, and other complex mechanisms that occur during *M. tuberculosis* infection. The lung tissue model has subsequently been used to study matrix metalloproteinases and their role in tissue remodeling during early granuloma formation (199).

Our future plan for the lung tissue model is to be able to incorporate primary human neutrophils, in order to study macrophage-neutrophil interactions and NET-formation. Neutrophils are yet another innate immune cell that are important in early stages of a *M. tuberculosis* infection (57-59), however due to their short life-span they are challenging to work with in longer *in vitro* experiments. We have attempted to add neutrophils to the model, but optimizations when it comes to timing and logistics are still needed. In order to study temporal features of an *M. tuberculosis* infection, we would also like to perform live-cell imaging of the lung tissue model.

PAPER IV: Exploring β -glucan immune training of primary human macrophages and their control of virulent *Mycobacterium tuberculosis* infection.

In this project we wanted to explore the concept of innate immune training and establish protocols for culturing, stimulating, and analyzing human immune-trained macrophages infected with *M. tuberculosis*. The use of β -glucan from *Candida albicans* as an immune training agent was first published by Quintin et al. in 2012 (164) and since then numerous publications have followed. β -glucans are polysaccharides that occur naturally in cell walls of bacteria, fungi, yeast and plants, where they serve as structural components and energy stores (176). The immunomodulatory role of β -glucans has a long history, and fungi have been used in traditional Chinese medicine for centuries (200). β -glucans from different sources vary in their length and branching structure, which is thought to influence their immunogenic properties. These structural differences also affect their binding to receptors on immune cells which trigger subsequent immune responses.

We selected β -glucans from different sources with the aim of studying their immune-training effect on human monocytes. The β -glucans used for the study were from a bacterial source (curdian from *Alcaligenes faecalis*), from yeast (WGP dispersible from *Saccharomyces cerevisiae*) and from the supernatant of a multicellular fungi (*Alternaria*). The supernatant from *Alternaria* was known to be rich in β -glucan and had also been shown to improve the antimycobacterial response in mice (personal communication). Monocytes, isolated from human blood, were cultured for 24 hours with the β -glucan, followed by a wash-out period during which the cells differentiated into macrophages. We then infected the macrophages with *M. tuberculosis* and followed the growth of bacteria using live-cell imaging. We could not observe any significant improvement in the macrophage's mycobacterial control after β -glucan training (**Paper IV, figure 2A-C**). However, there was a trend pointing to a beneficial effect of the immune-training and more experiments are needed before final conclusions can be drawn. We also investigated if β -glucan training affected macrophage phagocytosis of *M. tuberculosis*, but we found no differences in the phagocytosis rate (**Paper IV, figure 2D**).

In vivo TB infection involves a multitude of different cell types and various immune cells have been shown to play a role in the early events during infection (44). Neutrophils migrate to the infection site where they can participate in phagocytosis, however they also exert other effector mechanisms by the release of ROS, microbicidal granule proteins and extracellular traps (60).

By adding neutrophils to the *M. tuberculosis*-infected macrophages, we wanted to study if this could enhance the antimycobacterial capacity of β -glucan-trained macrophages (**Paper IV, figure 6**). Indeed, we could observe a reduction in mycobacterial growth when neutrophils were added, and in WGP dispersible-trained macrophages there was a significant reduction in *M. tuberculosis* numbers after co-incubation with neutrophils. Other studies have shown that macrophages can acquire antimicrobial granule proteins from neutrophils which can assist in the elimination of intracellular pathogens (68), but also that efferocytosis of neutrophils enhance macrophage's mycobacterial control by increased ROS-production in a MPO-dependent manner (69).

Innate immune training is a result of epigenetic reprogramming of cells after the first training stimulation, which then leads to an upregulated transcriptomic profile of genes involved in antimicrobial pathways. The epigenetic reprogramming involves histone modifications which leads to chromatin reconfiguration and accessibility to genes (164, 165), as well as DNA methylation (166). In our study, we investigated DNA methylation changes after β -glucan training of macrophages. We found an upregulation of several pathways involved in inflammation and signaling (**Paper IV, figure S4-6**), however with a large variability between donors. When doing a Venn analysis of overlapping pathways, the IFN γ signaling pathway was consistently hypomethylated, i.e. upregulated, in all donors trained with WGP dispersible (**Paper IV, figure 5**). IFN γ is known to be an important factor in the control of a *M. tuberculosis* infection (201), and our group has previously demonstrated that IFN γ gene expression correlates with increased mycobacterial control in BCG-vaccinated subjects (166). Other gene expression pathways that were consistently upregulated in response to β -glucan training were associated with Alzheimer's and Huntington's disease. Despite the pathways being named after neurological diseases, many genes involved in these pathways may also be relevant to macrophage's mycobacterial defense as they regulate tissue remodeling, as well as cell adhesion, motility and signaling.

This study gave us promising indications pointing to that β -glucans could be beneficial in boosting macrophages' antimycobacterial capacity. Both the fact that key signaling pathways are upregulated in response to β -glucan training, but also the functional improvement in mycobacterial control that we could observe after *in vitro* infection of the trained macrophages. However, more work is needed to strengthen the findings presented in our manuscript. We are planning to run more infection

experiments and in addition perform cytokine measurements on saved supernatants from trained macrophages.

GENERAL CONCLUSIONS

This thesis was written during the year 2020 when the COVID-19 pandemic plagued the world and people once again were forced into the awareness of the impact an infectious disease can have if we lack both a vaccine and effective treatment options. This was also the case for TB if we go back a hundred years. Extensive research has led us to where we are today with both a TB vaccine and antibiotics. However, even with these tools we have not been able to eradicate TB, and in 2018 the WHO reported 10 million new TB cases and 1.5 million deaths (8). Hence, there is still a need for more research on TB in order to understand the complexity of a *M. tuberculosis* infection, leading to better options for prevention and treatment.

M. tuberculosis has co-evolved and adapted to the human host making it ingenious at exploiting the human immune response, promoting its survival and replication in human host cells. The human immune system has also evolved mechanisms to limit *M. tuberculosis*-replication and spread. This thesis covers work on the innate immune response to TB and how neutrophils and macrophages respond to a mycobacterial infection and can control *M. tuberculosis*-replication.

Neutrophils and macrophages can respond to *M. tuberculosis* by releasing extracellular traps. Neutrophils are quick to respond to *M. tuberculosis* by releasing their cargo of antimicrobial peptides and reactive oxygen species, but also by releasing strands of extracellular DNA. These NETs can bind antimicrobial peptides and other danger signals which subsequently can be used by adjacent macrophages (**Paper I**). Macrophages' primary response to *M. tuberculosis* is to phagocytose them and kill them intracellularly. However, *M. tuberculosis* can avoid killing and degradation inside the macrophage by interfering with the phagosomal maturation process (144). The acquisition of granule proteins from neutrophils may increase the macrophage's ability to control and kill the intracellular bacteria (68, 69). *M. tuberculosis* have a unique lipid-rich cell wall which favors aggregation of the bacteria, and massive growth and aggregation of extracellular bacteria has been observed in patients with cavitary TB (30). Macrophages are unable to phagocytose large cords or aggregates of *M. tuberculosis* and may instead respond by the release of extracellular traps (**Paper II**).

Recent discoveries regarding the innate immune memory has helped us to understand that the diverse response to *M. tuberculosis*, and risk of acquiring TB, not only has genetic aspects but

also epigenetic components that could be harnessed in order to provide better prevention and treatment options. The TB vaccine has been shown to mediate some of its protective effects by stimulating an innate immune memory that protects against *M. tuberculosis* infection (143, 162), and β -glucan from *C. albicans* has been established as an immune training agent able to induce a trained immunity phenotype in macrophages (164). We contributed to the growing knowledge base by demonstrating that other β -glucans also can mediate immune training effects on macrophages (**Paper IV**). Specifically, the β -glucan WGP dispersible from *S. cerevisiae* increased to antimycobacterial ability of macrophages in cocultures with neutrophils. This yet again highlights the fact that multiple innate immune cells in the human host, contribute to controlling an *M. tuberculosis* infection. Another finding we made was that the IFN γ signaling pathway is upregulated in the WGP dispersible trained macrophages. This also points to the importance of crosstalk between cells and that an increased responsiveness to IFN γ signaling from T cells would be a beneficial trait in the trained immune cells. IFN γ signaling from primarily T cells activate macrophages by increasing their ability to restrict to growth of intracellular *M. tuberculosis* (202).

We have made preliminary observations of BCG immune training effects on macrophages, and this is something we have plans to continue investigating. We would also like to study the IFN γ -priming effects on the antimicrobial capacity of macrophages trained with β -glucan or BCG. We have observed MET-formation in response to cording mycobacteria (**Paper II**). However, if MET-formation occurs in immune trained macrophages, and if this can be host-beneficial resulting in an extracellular scaffold that contains the bacteria leading to recruitment of uninfected macrophages? Or on the other hand, if NETs can act in a host-beneficial way to stimulate immune trained macrophages to better control *M. tuberculosis*? These questions and many more remain to be answered.

The complex events that occur during a TB infection involve both multiple cell types and requires knowledge of the spatial movement of cells. An *in vitro* infection model with a single cell type is valuable when studying specific cellular mechanisms, and cocultures of multiple innate immune cells can also add insight when studying the interplay and signaling between cells during infection. However, for a better understanding of the movement of cells, a 3D tissue model is a valuable tool. Using our *in vitro* lung tissue model, we could observe the clustering of monocytes around mycobacteria and quantify the size and number of macrophage clusters (**Paper III**). Further studies using the *in vitro* model to study the interplay between innate

immune cells during *M. tuberculosis* infection and the trained immunity response would be highly relevant.

Thesis Conclusions

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Papers

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