Mycobacterial regulation of macrophage responses to infection

Induction and functional role of type I interferon

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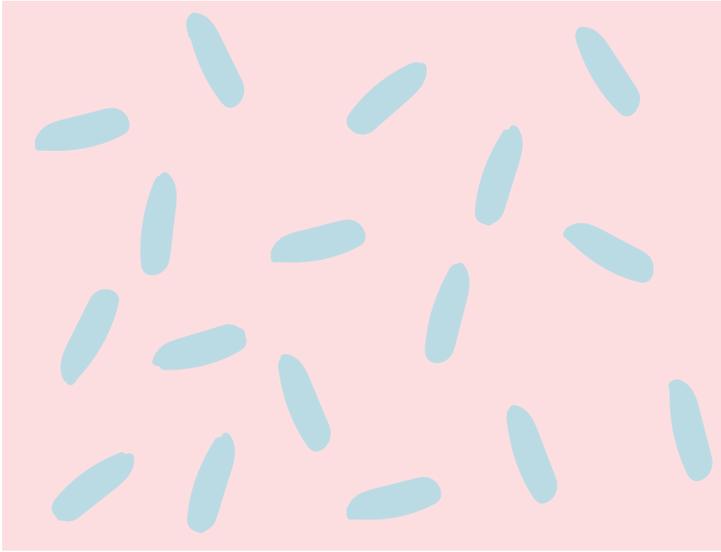
List of papers

I. Julia Lienard, **Esther Nobs**, Victoria Lovins, Elin Movert, Christine Valfridsson and Fredric Carlsson (2020). The *Mycobacterium marinum* ESX-1 system mediates phagosomal permeabilization and type I interferon production via separable mechanisms. *Proc Natl Acad Sci U S A*. 117(2):1160-6.

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Induction and functional role of type I interferon

Esther Nobs

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Science at Lund University to be publicly defended on 4th of June at 09.00 in the Blue Hall, Department of Biology, Sölvegatan 37, Lund, Sweden.

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Abstract:

Infection represents a complex interplay between invading microorganisms and the immune system. The immune system dynamically responds to the presence of pathogens, employing various defence mechanisms to neutralize and eliminate invaders. However, pathogens have evolved strategies to evade detection and elimination, leading to infections. This thesis focuses on mycobacterial regulation of macrophages, a key interplay in the pathogenesis of tuberculosis. The macrophage aims to neutralize intruding mycobacteria through the process of phagocytosis, however, *Mycobacterium tuberculosis* evades the phagosome, allowing it to gain access to the cytosol of the macrophage. From here it manipulates macrophage functions and other immune responses. The specialized protein secretion system ESX-1 is required for full virulence of mycobacteria and is involved in evasion strategies such as phagosomal escape and induction of type I interferons. The role of type I interferons in mycobacterial infection remains incompletely understood, although evidence strongly suggests a host detrimental role. The work presented in this thesis brings light on these key events during mycobacterial infection and contributes with new insights regarding the onset and functional role of the type I interferon response during infection.

Key words: infection, macrophage, mycobacteria, ESX-1, membrane permeabilization, type I interferon

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Induction and functional role of type I interferon

Esther Nobs

LUND
UNIVERSITY

Upp och vakna
Titta solen
Måste fråga
Varför då?

Måste titta
Måste fråga
Varför det
och Varför då?

Barnboken Varför då?
av Anna-Clara Tidholm

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Abstract

Infection represents a complex interplay between invading microorganisms and the immune system. The immune system dynamically responds to the presence of pathogens, employing various defence mechanisms to neutralize and eliminate invaders. However, pathogens have evolved strategies to evade detection and elimination, leading to infections. This thesis focuses on mycobacterial regulation of macrophages, a key interplay in the pathogenesis of tuberculosis. The macrophage aims to neutralize intruding mycobacteria through the process of phagocytosis, however, *Mycobacterium tuberculosis* evades the phagosome, allowing it to gain access to the cytosol of the macrophage. From here it manipulates macrophage functions and other immune responses. The specialized protein secretion system ESX-1 is required for full virulence of mycobacteria and is involved in evasion strategies such as phagosomal escape and induction of type I interferons. The role of type I interferons in mycobacterial infection remains incompletely understood, although evidence strongly suggests a host detrimental role. The work presented in this thesis brings light on these key events during mycobacterial infection and contributes with new insights regarding the onset and functional role of the type I interferon response during infection.

Populärvetenskaplig sammanfattning

Mycobacterium tuberculosis är en av världens mest framgångsrika sjukdomsorsakande bakterier. Denna plåga har gäckat människan i tiotusentals år, och är fortfarande en av de 20 vanligaste dödsorsakerna i världen med nästan 1,5 miljoner dödsoffer varje år.

Tuberkulossmitta sprids genom hostningar, där små partiklar med bakterier flyger genom luften från den sjuka individen och andas in av en person i omgivningen. I lungorna stöter bakterierna på celler från vårt immunsystem, så kallade makrofager, vars uppgift är att äta upp, eller fagocytera, främmande substanser så som bakterier. Vid fagocytering hamnar bakterierna i en fagosom, en sluten blåsa som liknande vår magsäck bryter ner dess innehåll genom att syror tillsätts. Men, häpnadsväckande nog, så har *Mycobacterium tuberculosis* funnit ett sätt att hindra produktionen av syror, och därmed även nedbrytningen. I stället tar bakterien sönder fagosomen och får tillgång till makrofagens insida, den så kallade cytosolen. På så vis kan bakterien undvika avdödning, och därtill manipulera makrofagen att bete sig till bakteriens fördel. Makrofagen blir därmed bakteriens kompanjon, liknande ett Stockholms-syndrom i miniatyr.

I vår forskningsgrupp studerar vi mötet mellan bakterie och makrofag och försöker förstå hur bakterien lyckas ta sönder fagosomen och fly till cytosolen. För att lyckas med detta behöver bakterien ett fungerande proteinutsöndringssystem vid namn ESX-1. Tidigare trodde man att utsöndring av faktorn ESAT-6 skapade porer i fagosomen, som resulterade i att den gick sönder. Vi kan dock visa att så sannolikt inte är fallet, vilket är en betydelsefull insikt för fältet då denna teori behöver revideras. Våra resultat tyder i stället på att fagosomens membran går sönder på grund lipidperoxidering, vilket är en process där fria syreradikaler förstör fettkedjor, och för framtida arbete är det av intresse att utreda hur bakterien driver denna process.

I samband med att fagosomen, och andra interna membran, går sönder så läcker DNA ut i cytosolen där makrofagens försvarssystem reagerar och börjar producera signalsubstansen typ I interferon. Under en virusinfektion så hjälper typ I interferon oss att röja infektionen, men i fallet med *Mycobacterium tuberculosis*, och dess kusin *Mycobacterium marinum* vilken vi använder som modelsystem, så verkar typ I interferon snarare gagna bakterien. Detta är ytterligare en fråga vi försöker få svar på, hur det kommer sig att makrofagens eget försvarssystem inte gagnar oss, utan bakterien. Vi har förstått att en bidragande faktor verkar vara att typ I interferon resulterar i produktion av två skyddsproteiner, vilka hindrar makrofagen från att dö som resultat av de membran som går sönder i samband med att bakterien tar sig ut från fagosomen. Detta blir en skyddande feedbackloop för bakterien, då den kan ta sönder membran och fly till cytosolen, men samtidigt förhindra att makrofagen inte dör på grund av skadorna. Enligt våra resultat skulle det möjligen faktiskt vara bättre

om makrofagen dog, och därmed blottade bakterien för andra medlemmar av immunförsvaret.

Trots omfattande forskning så är insatser mot *Mycobacterium tuberculosis* och resulterande sjukdom inte tillräckliga. Det nuvarande vaccinet är över 100 år gammalt och de mest framgångsrika läkemedlen har nästan 50 år på nacken. Det är tydligt att ett mer framgångsrikt vaccin och/eller läkemedel behövs för att få bukt på denna uråldriga plåga. Jag hoppas att min avhandling har bidragit med några insikter som hjälper oss att uppnå detta mål.

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Paper I

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Author's contribution to the papers

Paper I

I took part in performing experiments and in analysis and interpretation of the data. I took part in figure design and reviewed and provided input to the manuscript.

Paper II

I took major part in designing and performing experiments, in the analysis and interpretation of the data, creation of figures and writing the manuscript.

Paper III

I took major part in designing and performing experiments, in the analysis and interpretation of the data, creation of figures, writing the manuscript and in the submission/publication process.

Abbreviations

AIM2	Absent in melanoma 2
APC	Antigen presenting cell
BCG	Bacille Calmette-Guérin
CCR2	C-C chemokine receptor 2
CFU	Colony forming unit
CFP-10	10 kDa culture filtrate protein (EsxB)
cGAMP	cyclic GMP-AMP
cGAS	cGAMP synthase
DAI	DNA-dependent activator of IFN-regulatory factors
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
Ecc	ESX-conserved component
Esp	ESX-1 secretion-associated protein
ESAT-6	6-kDa early secretory antigenic target (EsxA)
EsxA	(also known as ESAT-6)
EsxB	(also known as CFP-10)
ESX-1	ESAT-6 secretion system 1
ETC	Electron transport chain
IFN	Interferon
IFNAR	Type I IFN receptor
IL	Interleukin
IL-1R(A)	IL-1 receptor (agonist)
IRF	IFN-regulatory factor
ISG	IFN-stimulated gene
ISGF3	ISG factor 3
ISRE	IFN-stimulated response elements
JAK1	Janus kinase 1
MCP	Monocyte chemoattractant protein

MDA5	Melanoma differentiation-associated gene 5
MyD-88	Myeloid differentiation primary response 88
NK cell	Natural killer cell
NLR	NOD-like receptor
NLRP3	NLR family pyrin domain containing 3
NOD	Nucleotide-binding oligomerization domain
NOD1/2	NOD-containing protein 1/2
NOS	Nitric oxide synthase
Nox2	NADPH oxidase 2
RD1	Region of difference 1
RIG-I	Retinoic acid-inducible gene I
RNI	Reactive nitrogen intermediates
ROS	Reactive oxygen species
SERPIN	Serine protease inhibitor
STAT	Signal transducer and activator of transcription
STING	Stimulator of IFN-genes
TB	Tuberculosis
TBK1	Tank binding kinase 1
TLR	Toll-like receptor
TNFα	Tumour necrosis factor α
TNFR	Tumour necrosis factor receptor
TYK2	Tyrosine kinase 2
PAMP	Pathogen-associated molecular pattern
PDIM	Phthiocerol dimycocerosate
PE	Pro-Glu
PPE	Pro-Pro-Glu
PGL	Phenolic glycolipids
PRR	Pathogen recognition receptor
WT	Wild type

Preface

Infection is a complex interplay between invading microorganisms and the host's defence mechanisms. Whether caused by bacteria, viruses, fungi or parasites, infections often initiate a battle of survival where pathogens strive for resources and shelter, while the immune system assembles its defences to eliminate the threat.

The process of infection begins with the entry of pathogens into the host through various routes such as inhalation, ingestion or direct contact with the skin. Once inside, pathogens may adhere to host cells, evade detection by the immune system and proliferate, leading to tissue damage and the manifestation of symptoms specific for that particular infection. However, infections are not one-sided encounters. The host's immune system responds dynamically to the presence of pathogens, deploying a myriad of defence mechanisms aimed at neutralizing and eliminating the invader. These defences include physical barriers, such as the skin and mucous membranes, as well as a complex array of immune cells and molecules that recognize, confront and eliminate foreign substances.

Despite the remarkable response of the immune system, pathogens have evolved elegant strategies to subvert or evade detection by the immune system, allowing establishment of infection. This ongoing evolutionary arms race between pathogen and host presents a challenge for medical science and underscores the importance of continued research to understand the mechanisms of infection.
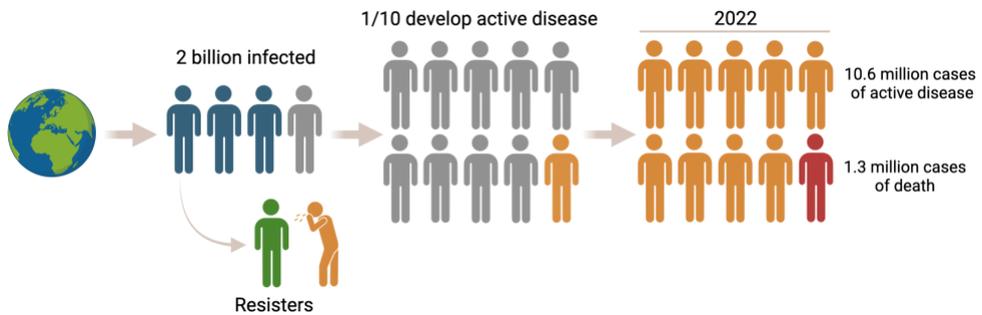
Tuberculosis

The lung disease tuberculosis (TB) is caused by the bacterium *Mycobacterium tuberculosis*. The ancestor of *M. tuberculosis* arose some 70'000 years ago making it the oldest known human pathogen, and it has caused more human deaths than any other pathogen in history (1–3). In fact, *M. tuberculosis* is still the pathogen that causes most deaths world-wide, making it one of the top twenty causes of death globally (4). One quarter of the world's population is estimated to be infected with the bacteria, resulting in 1.3 million deaths each year. The densest TB-areas of the world are currently South-East Asia (46% of all cases), Africa (23% of all cases) and the Western Pacific (18% of all cases), whereas in some parts of the world, such as Sweden, it is almost like an ancient memory (4). However, with the global mobility of today's society, in combination with an alarming rate of increased cases of antibiotic resistant strains, the geographical patterns of TB-disease could inevitably change.

Out of 10 individuals infected with *M. tuberculosis*, one will develop active disease in their lifetime (**Fig 1**). This is most likely to occur during the first year after infection but may happen many years afterwards (4,5). Naturally, the high level of TB disease draws attention, however, it is rather striking that out of 10 infections only one leads to active disease. This indicates that 9 out of 10 infected individuals either live with latent infection, or that some individuals can clear the infection. Why some infected individuals, or individuals who are heavily exposed to TB contamination, do not develop disease is poorly understood (6). Several historical case-contact studies suggest that some individuals are resistant and never show any signs of infection even after many years of follow ups. However, epidemiological methods are inconsistent and impair comparative analysis, which makes it difficult to understand why this might be (6).

Up until recently it was generally thought that TB remained latent for many years, or even decades. However, this dogma was recently challenged by Marcel Behr and colleagues, who argue that most cases of TB do not have a clear latent period, but that disease rather manifests soon after infection (7). This new insight is of great importance for prioritising research and public health strategies, as epidemiological studies brought forward by Marcel Behr and colleagues suggest that most of the TB develops from a recently transmitted infection.

Figure 1. TB in numbers.
Global numbers of individuals who are infected (grey), who develop active disease (orange) and who die (red) due to *M. tuberculosis*. Some individuals seems to be resistant to infection, even if heavily exposed, so called resisters (green). Statistics from 2022 as presented in Global Tuberculosis Report 2023 by WHO. Image created with BioRender.com.

Pathogenesis

M. tuberculosis spreads via aerosols that are coughed up by a diseased individual, travels through the air and is inhaled by a new host (3). It seems to be favourable for the bacteria to end up in the lower parts of the lungs, as the immune cells in these sterile areas are not primed/activated by commensal bacteria such as in non-sterile areas closer to the throat (8). Also, it has been observed that successful colonization results only if a few bacteria are inhaled rather than plenty (9). It is speculated that the reason for this is due to that fewer bacteria would mean a smaller aerosol, which is able to travel further into the sterile areas of the lungs, but it could also be due to that fewer bacteria increases the chance to infect incognito, *i.e.* without being detected by the immune system (3).

Upon arrival, the bacteria are phagocytosed by alveolar macrophages aiming to remove and eliminate the intruders (10,11). As a result of phagocytosis the bacteria ends up in a phagosome, in which acids and reactive oxygen species are generated to degrade the content (12). However, *M. tuberculosis* can halt the acidification of the bacteria-containing phagosome by preventing the phagosome to fuse with a low pH lysosome, and thus avoid degradation (13,14). Furthermore, after a few days of infection *M. tuberculosis* escapes the phagosome, allowing it to gain cytosolic access (15–17). From this position *M. tuberculosis* is able to manipulate the functions of the macrophage, as well as other members of the immune system (18).

As the alveolar macrophages become infected, they migrate from the alveolar space to the lung interstitium where interstitial macrophages and recruited innate immune cells, like monocyte-derived cells and neutrophils, accumulate, become infected and create an early replicative niche for the bacteria (**Fig 2**)(11,19). Over time a

granuloma, composed of different types of immune cells, will start to take form at the site of infection (1,3,18). The granuloma may remain intact and rather inactive for long periods of time, though, it is continuously renewed and several studies suggest that the bacteria drive this environmental setting (1,3,18,20). This phase of the infection is considered as latent disease, and the infected individual does not transmit mycobacteria. However, at some point latent disease can turn into active disease and bacteria will start to multiply. As the level of bacteria increases so will the level of necrotic cell death, resulting in an eruption of the granuloma leading to escalated inflammation. This phase of the infection is considered active disease, and the infected individual transmits mycobacteria by vigorous coughing, as coughing generates bacteria-containing aerosols, which can travel through the air into a new potential host (3,21).
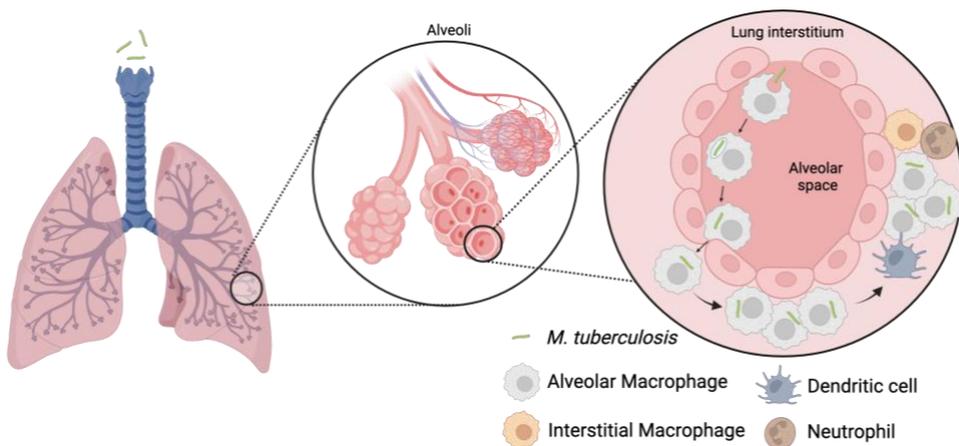
Figure 2: Pathogenesis of TB.
M. tuberculosis is inhaled into the lungs and phagocytosed by alveolar macrophages located in the alveolar space. Infected macrophages then migrate to the lung interstitium and interact with other immune cells such as interstitial macrophages, dendritic cells and neutrophils. Over time a granuloma, composed of different types of immune cells, will start to take form at the site of infection. Image created with BioRender.com.

Treatment and current vaccine

When diagnosed with tuberculosis the current treatment regimen entails a cocktail of the four antibiotics isoniazid, rifampicin, ethambutol and pyrazinamide for two months, followed by four months of treatment with isoniazid and rifampicin. If completing the treatment there is a good chance, about 85%, of recovery, however in many parts of the world the logistics and economic burden of a 6-month long treatment with four different medications is difficult (4,21). In 2006, mathematical

models predicted that if current treatment was replaced with a shorter treatment period, such as a 2-month treatment with chemotherapy, it would lead to a 20% reduction of TB-cases and 25% less TB-related deaths in the time frame of 2012-2030, only in the region of South-East Asia (22). The complicated logistics of drug-distribution, in combination with the spread of antibiotic resistant strains highlight the need for new and more effective medications.

There is however a vaccine available, the Bacille Calmette-Guérin (BCG)-vaccine developed by Albert Calmette and Camille Guérin and used in humans since 1921. The BCG is the most widely used vaccine in the world. Unfortunately, the vaccine does not offer efficient protection (23,24). Vaccination of new-borns reduces the risk of severe disseminated tuberculosis but does not effectively protect from pulmonary tuberculosis in adults and adolescents (23). Efficacy levels of BCG-vaccination in adults and adolescents range from 0-80% depending on study and geographic location (25). There is an urgent need for an improved vaccine that effectively protects adult and adolescents from developing pulmonary disease, as these patients are the main drivers of TB transmission (26).

Great efforts have been invested into developing a more efficient vaccine; either a completely new candidate or as a secondary boost to the BCG vaccine (23). One new candidate, the M72/AS01$_E$ vaccine, was brought forward by the WHO-report of 2020 as a promising future vaccine (27). This candidate, produced by GlaxoSmithKline in co-operation with the Bill & Melinda Gates Medical Research Institute, provided protection against progression to pulmonary disease for at least 3 years in individuals with latent tuberculosis (28). The efficacy after 3 years was 49,7%, which is just on the boarder of WHO's encouraged 50% efficacy of a new tuberculosis vaccine (29). However, it is still to be investigated if the vaccine has the same effect in tuberculosis and BCG naïve individuals, as all participants in the study had latent tuberculosis, and a majority had previously been vaccinated with BCG (28). Nevertheless, boosting pre-existing BCG-induced immunity with a secondary novel vaccine might be an effective way to improve immunity to prevent TB (23).

A more efficient vaccine in combination with more convenient treatment regiments could make a big difference in the eradication of tuberculosis. Together with improved diagnostic tools and better knowledge and utilization of biomarkers, humanity might finally take control of one of the world's most successful pathogens.

Mycobacterium tuberculosis

Mycobacteria

The genus *Mycobacterium* belongs to the phylum of Actinomycetota (previously Actinobacteria) and consists of more than 190 described species (30,31). *Mycobacterium* was first termed in 1896, primarily based on phenotypical characteristics such as presence of mycolic acids in the cell wall, aerobic growth and bacillary cell shape. However, modern high-throughput sequencing allows a higher resolution of informative data and comparison, resulting in continuous revisions of the taxonomy of mycobacteria (31–33).

Mycobacteria are divided into two categories: tuberculous mycobacteria, which cause TB and leprosy, and non-tuberculous mycobacteria. Tuberculous mycobacteria have evolved to host-adapted pathogens and require a host for successful progression. Non-tuberculous mycobacteria, on the other hand, mainly exist in environmental sources such as water and soil. However, some can cause disease in, primarily, immune-compromised individuals such as *Mycobacterium abscessus*, *Mycobacterium avium*, *Mycobacterium marinum*, *Mycobacterium xenopi*, *Mycobacterium gordonae* and *Mycobacterium kansasii*. It is generally thought that host-adapted tuberculous mycobacteria originate from an environmental ancestor, through a multiple step adaptation that required both gaining new genes through horizontal gene transfer, as well as genome downsizing and losing genes that are dispensable for a pathogenic lifestyle (34). The ability of mycobacteria to infect immune cells correlates with its ability to infect amoebae, suggesting that it was initially a soil-dwelling bacterium and that parasitizing on amoebae was an early step to gain its virulent features (3).

One important difference between mycobacterial species is their growth rate, if they require less than, or more than 7 days to form a colony. Based on their growth rate, they have traditionally been divided into two groups: fast-growing and slow growing-mycobacteria (32). The major mycobacterial pathogens of humans are all slow-growing. The species are then further grouped into complexes, such as the *M. tuberculosis* complex.

The *Mycobacterium tuberculosis* complex

The *M. tuberculosis* complex consists of *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. microti*, *M. caprae*, *M. pinnipedii* and *M. canetti* (35,36), which are closely related and share more than 99% nucleotide sequence identity (34). All members of the complex cause disease in humans, but to a different degree and likelihood depending on geographic location (37). The only continent that contains all lineages is Africa, suggesting that the common ancestor originated here. In the last few years new sequencing technologies have enabled identification of two new strains in eastern Africa, further strengthening this hypothesis (38,39). It is now 25 years ago since the first publication of the complete genome sequence of *M. tuberculosis* and the steadily improving sequence technologies and analysis tools have had a big impact on the phylogenetic inter- and intraspecies relationships in the *M. tuberculosis* complex, as well as in the genus (40,41). There is promising potential in modern sequencing technologies to identify even more strains, which can contribute with genetic information to fill in the gaps about the evolutionary journey made by these disease-causing strains (37,38).

Physiology

M. tuberculosis was discovered by the German scientist Robert Koch and his findings were announced at the Berlin Physiological Society in 1882 (42). It is an intracellular pathogen with the capacity to infect several animal species, however humans are its primary host. *M. tuberculosis* is a rod-shaped, non-motile, and non-spore forming bacillus that prefers highly oxygenated tissue, such as the lungs. The generation time is 15-20 hours, which is extremely long as compared to many other bacteria. It does not fit into the traditional categories of Gram-positive or Gram-negative bacteria. Instead, it is characterized as acid fast as, after staining, it retains the dye even when treated with acidified organic solvents, unlike most other bacteria (36). The cell wall consists of a multilayer of different elements with a layer of peptidoglycan beyond the cell membrane, followed by a layer of arbinogalactan that is connected to a thick layer of mycolic acids (**Fig 3**). The extensive cell wall protects the bacterium, but also contains important virulence factors that are essential for disease progression (Brennan, 2003; Gröschel et al., 2016). Due to the thickness and composition of the cell wall, there are special demands for secretion of the bacterial proteins from the cytoplasm into the external environment. To enable transport of proteins across the cell wall specialized secretion systems such as the ESX, or type VII, secretion systems are required (43).
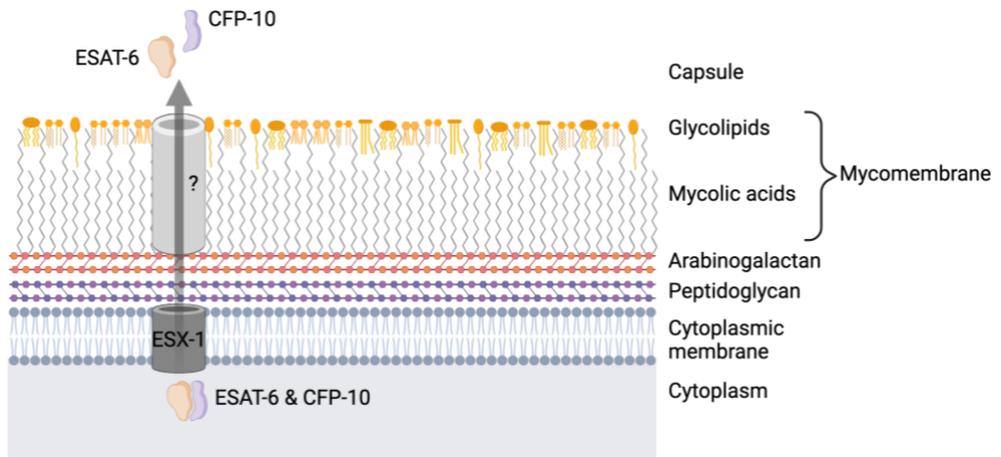
Figure 3. Membrane structure and ESX-1 localization.
Schematic representation of the ESX-1 secretion system, as described in the adjacent text. Adapted from paper (43). Image created with BioRender.com.

ESX-secretion systems

There are five different ESX-secretion systems (ESX-1 to 5) identified in *M. tuberculosis*, of which at least three (ESX-1, -3 and -5) are required for virulence. An alternative name commonly used for the ESX-secretion system is type VII secretion system. Multiple genus within the phylum Actinomycetota, such as *Streptomyces*, *Corynebacterium*, *Nocardia* and *Gordonia*, possess ESX-secretion systems, and more distantly related ESX-secretion systems are also found in the phylum of Firmicutes. All *esx* loci across the different genus contain presence of genes encoding for small (about 100 amino acids) secreted proteins with a conserved Trp-X-Gly (WXG) motif, and transmembrane proteins of the FtsK-SpoIIIE-like ATPase family. In mycobacteria, ESX-secretion systems are in particular important to enable secretion of proteins across its thick mycomembrane (43).

ESX-1 was the first ESX-secretion system to be discovered (44–47) and is known to secrete the factors ESAT-6 (6 kDA early secretory antigenic target, also known as EsxA) and CFP-10 (10 kDa culture filtrate protein, also known as EsxB). ESX-3 regulates iron-homeostasis and ESX-5 secretes a wide array of so-called Pro-Glu (PE) and Pro-Pro-Glu (PPE) proteins (43). ESX-2 and ESX-4 are less well known, but were recently suggested to be needed together with ESX-1 for phagosomal escape in *M. tuberculosis* infection of human macrophages (48). The ESX-5 secretion system of *M. tuberculosis* was recently successfully reconstituted in *M. smegmatis*, which for the first time allowed high resolution analysis of the assembly of the secretion system (49). The components of all ESX-secretion systems have a high sequence conservation, are incorporated similarly in the cell membrane and translocate their substrates into the mycobacterial periplasm-like space, located between the cytoplasmic membrane and the mycomembrane (**Fig 3** and **4**)(43,49). How the substrates then cross the cell wall is currently unknown.

The ESX-1 secretion system

The ESX-1 secretion system is required for full virulence, as indicated by the loss of virulence in the attenuated BCG vaccine strain that lacks the genetic stretch of Region of Difference 1 (RD1), encoding for several members of the ESX-1 apparatus (**Fig 4A**)(43–47,50). The ESX-1 locus encodes a core set of subunits, so called ESX-conserved components (Ecc), present in most of the five systems. It also

encodes several ESX-1 secretion-associated proteins (Esp), although some of them have homologues in the other ESX-systems. Furthermore, it encodes secreted factors such as ESAT-6 and CFP-10, as well as PE35 and PPE68, where all but PE35 express the ESX-secretion system specific WXG-motif. Additional units required for full functionality of the ESX-1 are located upstream of the RD1, such as the *espACD* operon, which have a sequence similarity to *espE*, *espF* and *espH*, respectively (43). The operon is located more than 260 kb upstream of *esx-1*, but nevertheless, secretion of ESAT-6 and CFP-10 requires EspA and EspC, and vice versa, illustrating well-orchestrated interdependence between ESX-1 substrates (51). This complicated genetic network, is required for stability and functionality of the secretion system, and has invited some challenges when studying the system using classical bacterial genetic approaches. Removing one factor, such as through a genetic mutation, might risk collapsing the whole system and experiments generated with these mutants need to be interpreted carefully (43).

ESAT-6 and CFP-10 are secreted as a 1:1 heterodimer (52), with CFP-10 being physically connected to the transport protein of EccC and translocated with ESAT-6 piggy-backing for the ride (**Fig 4B**)(53). Upon secretion the heterodimer is thought to dissociate at low pH, such as in the phagosome (54). Multiple functions have been attributed to ESAT-6 and CFP-10; suppression of pro-inflammatory cytokine signalling such as interleukin-12 (IL-12) and tumour necrosis factor α (TNFα) (47), inducing host detrimental type I interferon (IFN) signalling (55), halting acidification of the phagosome (56), inducing cell death (57,58) and maybe most famously the phagosome rupture (15–17). Many of these functions have been linked to the ability of ESAT-6 to form a pore and permeabilize host membranes (53). However, a lot of observations attributed to ESAT-6 are currently under discussion since an observation by the Ramakrishnan group suggests that some of these observations are due to a detergent contamination of the used ESAT-6 protein preparations (59). Moreover, our group recently used a phenotypic approach to demonstrate that ESAT-6 secretion, in fact, may not be needed for phagosomal escape, whereas it might be required for induction of type I IFN, as shown in **paper I** (60).
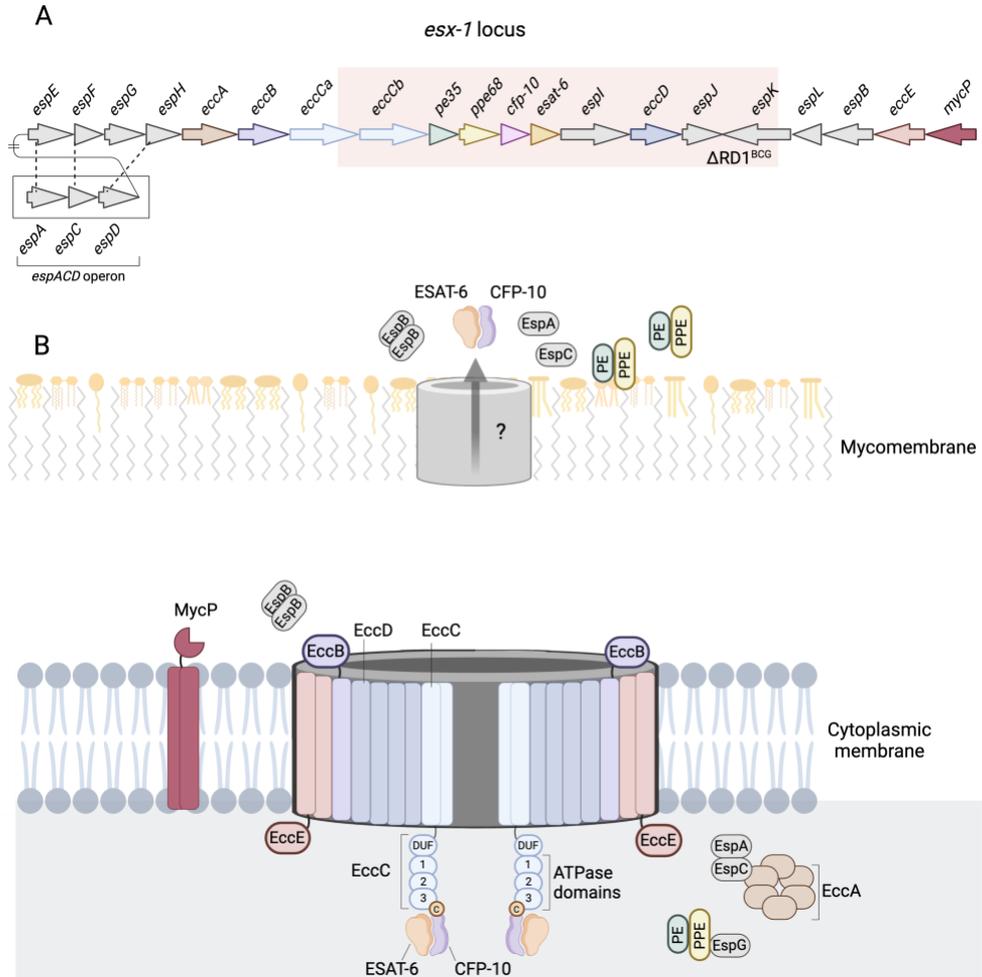
Figure 4. Components of the ESX-1 secretion apparatus.
A. The genetic region of *esx-1* including multiple genes required for the ESX-1 secretion apparatus. The Region of Difference 1 (ΔRD1) of *Mycobacterium bovis* bacille Calmette-Guérin is marked with shaded box. **B.** Model of the ESX-1 secretion system and some of its secreted factors. DUF: domain of unknown function. Adapted from paper (43). Image created with BioRender.com.

Mycobacterium marinum as a model system

Mycobacterium marinum is a close relative to *M. tuberculosis* and shares 3000 orthologs (genes that have evolved from a common ancestor by speciation) with an average amino acid identity of 85% (61). Interestingly, they both carry *esx* genes of all five ESX-systems, including the ESX-1 system. In comparison, *M. smegmatis*

and *M. ulcerans* both lack a functional ESX-1 system, and neither of them are capable to replicate intracellularly. However, the genome of *M. marinum* is 1.5-times larger (6.6 million base pairs) than the genome of *M. tuberculosis* (4.4 million base pairs), most probably due to the larger host repertoire it can infect and its ability to persist in water reservoirs (61,62). *M. marinum* causes disease in fish and amphibians but can also cause superficial infection in humans, the so-called fisher man's disease (63). Its optimal growth temperature is 25-35°C and it grows poorly at 37°C. Thus, it can only cause infection in cooler tissues, such as the skin, in humans. The granuloma formed by *M. marinum* is similar to granulomas caused by *M. tuberculosis,* indicating a similar pathogenesis (61,64,65).

Just like for *M. tuberculosis*, the ESX-1 secretion system is required for successful infection of macrophages by *M. marinum*, and for avoiding phagosome-lysosomal degradation and escaping the phagosome into the cytosol (63,66,67). Upon cytosolic access *M. marinum* can create an actin tail, described to provide motility and enable spread between adjacent cells (67,68). This is also true for several other intracellular bacteria such as *Listeria monocytogenes*, *Shigella flexneri* and *Rickettsia rickettsii* (62,69). However, there has been no sign of actin tail formation in *M. tuberculosis* infection (15). Independent of the function, actin tail formation in *M. marinum* infection has been a useful tool to detect and study phagosomal escape since actin is only located in the cytosol of host cells, a feature that we exploit in **paper I** and **II**.

All in all, *M. marinum*'s biological similarities to *M. tuberculosis* in combination with more laboratory favourable aspects, such as its shorter doubling time of 4 hours (15-20 hours for *M. tuberculosis*) and its inability to cause severe disease in humans, makes it an attractive model system to study the host-pathogen interactions and pathogenesis of mycobacterial infection (62,63).

The immune response

To combat infection a complex crosstalk involving both the innate and the adaptive immune response is often needed. The coordinated interactions of immune cells require cytokines to mediate recruitment and activation. *M. tuberculosis* has evolved several strategies to manipulate the host response to successfully establish and maintain a chronic infection. Throughout this chronic infection a continuum of infection stages is taking place, involving both the innate and adaptive immune system, rather than being a static condition. The infection may range from active to latent, to potentially active again (18,70).

The innate immune response

Upon detection of *M. tuberculosis*, the combined performance of innate immune cells has a critical role in the early anti-bacterial response, as well as for recruiting the adaptive immune system. Members of the innate immune system such as monocytes, macrophages, neutrophils, natural killer (NK) cells and dendritic cells (DCs), are early defenders at the site of a mycobacterial infection (18). Epithelial cells of the lung also contribute by secreting various inflammatory cytokines to recruit more immune cells when sensing *M. tuberculosis* (71). Macrophages, neutrophils and DCs are effective executers of phagosomal degradation as a mean to eliminate the intruder, but where DCs also have an important role as an antigen presenting cell (APC) to activate specific immunity in local lymph nodes (72). DCs may leave the site of infection and migrate to local nymph nodes to alert and activate adaptive cells by presenting bacterial antigens to members of the adaptive immune system (**Fig 5**) (73,74). DCs may also receive live *M. tuberculosis* or bacterial proteins in the lymph node, which have been transported from the infection site by inflammatory monocytes (75).

C-C chemokine receptor 2 (CCR2) binds monocyte chemoattractant proteins (MCPs) and is crucial in the early immune response during *M. tuberculosis* infection, for recruitment of myeloid cells, such as monocytes/macrophages and DCs, to the lungs (76,77). In CCR2-KO mice, fewer myeloid cells are observed at the site of infection and the mice exhibits a rapidly progressive course of disease, as compared to mice with a functional CCR2 (76,78). Interestingly, there was also reduced activation and migration of T-cells, however, this was not due to T-cell

expression of CCR2, but because of the inadequate recruitment of myeloid cells (79). Although this effect might be dose dependent, as it is not noticeable during low-dose infection (77), it highlights the importance of coordinated interactions between immune cells to establish an effective response. These findings suggest a host-protective role for monocytes in mycobacterial infection, possibly by regulating onset of specific immunity, or by inhibiting excessive neutrophilic inflammation (78).
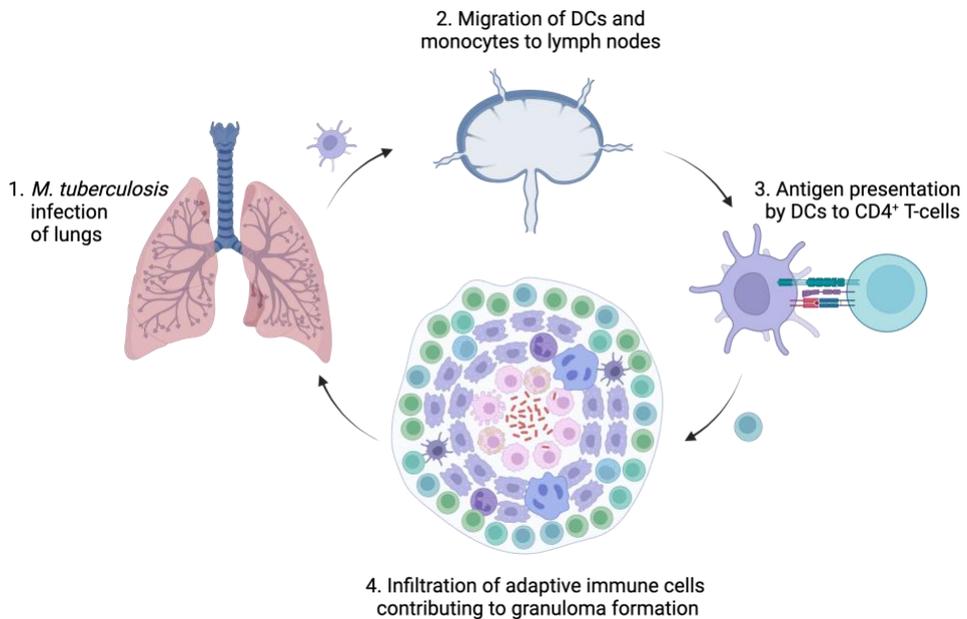
Figure 5. Extended pathogenesis of TB.
Members of the innate immune system, such as dendritic cells (purple), migrate to lymph nodes and present antigens to CD4+ T-cells. Activated T-cells then migrate to the site of infection and contributes to the immune response, and formation of the granuloma. Image created with BioRender.com.

Until recently, the macrophage was considered the major host of intracellular *M. tuberculosis*, but it is becoming evident that other cell types such as neutrophils, DCs and/or inflammatory monocytes, are also cellular reservoirs of the bacteria (11,73,78,80). However, why the bacteria infect different cell types, and how this might affect the outcome of the infection is still fairly unknown (18).

The adaptive immune response

The adaptive immune system is required for protective immunity in mycobacterial infection and consists of a cellular and a humoral arm. The main player of the cellular arm during *M. tuberculosis* infection is the CD4+ T-cell (72). The important role of CD4+ T-cells is unquestionable, as well as its defining cytokine IFNγ (81,82). IFNγ increases the microbicidal activity of macrophages, driving them into a so called M1 phenotype, by inducing the nitric oxide synthase (NOS) leading to production of reactive nitrogen intermediates (RNI) (81,83), as well as by inducing important anti-bacterial systems such as autophagy (84) and apoptosis (85). IL-1β (86) and IL-12 (87,88) are important cytokines from the innate immune response to establish a functional CD4+ T-cell response. The importance of an intact and efficient adaptive immune response in *M. tuberculosis* infection is illustrated by the high level of active disease in AIDS patients, and thus lack a healthy CD4+ T-cell compartment. A HIV-positive patient with latent TB faces a 10% annual risk of developing active disease, as compared to a 5-10% risk in a life-time in healthy individuals (18). In fact, a seventh of all TB-related deaths in 2022 was in patients co-infected with HIV (4). The important role of CD4+ T-cells is further emphasised in mice lacking these cells, as they succumb early to TB-infection (89,90). CD8+ T-cells are also important for protective immunity in TB-infection, however this cellular compartment cannot compensate for the function of CD4+ T-cells (89).

The humoral arm of the adaptive immune system may also be important as illustrated by the finding that protective antibodies have been isolated from diseased patients (91), as well as from asymptomatic healthcare workers (92). Indeed, isolated antibodies from the healthcare workers were injected into mice and shown to have a protective capacity (92). However, the importance of antibody-mediated immunity in TB is debated. B-cells have been shown to be a part of the granuloma, and mice lacking B-cells seem to have enhanced susceptibility to infection (93), although studies in B-cell deficient mice infected with *M. tuberculosis* have yielded various results (70).

The macrophage

Alveolar macrophages are the first immune cells to encounter *M. tuberculosis* in the alveolar space and phagocytose the intruder (10,11). In many cases clearance might be successful, and disease will not establish (6,94,95). However, *M. tuberculosis* exploits an array of strategies to subvert the antibacterial defences performed by the macrophage, resulting in progressed infection and a skewed outcome to the bacteria's favour. The macrophage seems particularly favoursome for mycobacteria to hijack and manipulate, allowing the bacteria to drive the immune response to a state that enables their propagation (3,18,70,94).

Subsets of macrophages

There are two major macrophage populations located in the lung: alveolar and interstitial macrophages. Alveolar macrophages line the airways within the lumen of the alveoli, interstitial macrophages on the other hand are located on the other side of the thin alveolar wall in the interalveolar space (96). Whereas alveolar macrophages continuously self-renew from progenitors derived from fetal liver during embryogenesis, interstitial macrophages arise from monocytes continuously generated in the bone marrow (96,97). The metabolic state also differs between the subsets, alveolar macrophages have a more prominent fatty acid-based metabolism, while interstitial macrophages are glycolytically active (80,98). It is becoming evident that alveolar macrophages are more permissive to mycobacterial growth, with lower bacterial stress levels and higher replication, as compared to interstitial macrophages where bacterial growth is more restrictive (19,80,98). The relative abundance of fatty acids and iron in alveolar macrophages seems to be important factors of a permissive milieu (80,98).

Detection of mycobacterial PAMPs by PPRs

Like other innate immune cells, macrophages detect pathogen associated molecular patterns (PAMPs) by an array of various evolutionary conserved pattern recognition receptors (PRRs). These can either be localized on the surface of the macrophage,

in enclosed compartments such as endosomes, or in the cytosol. Activation of a PRR by PAMPs stimulates intracellular signalling resulting in activation of an early response, as well as initiating processes leading to activation of the adaptive immune response (99,100).

Mycobacterial PAMPs may be detected by the macrophage's membrane receptors, or by cytosolic receptors due to leakage of antigens from the phagosome into the cytosol. Toll-like receptors (TLRs) are positioned in different compartments to recognize both external and internal antigens and induce important cytokines such as IL-12 and TNFα (70,101,102). The importance of each TLR during mycobacterial infection is variable (103,104), but mice lacking the TLR/IL-1R family receptor adaptor protein myeloid differentiation primary response 88 (MyD88) quickly succumb to *M. tuberculosis* infection. Although Myd88 is not exclusively involved in TNFα signalling, it indicates an important role of TNFα in controlling mycobacterial infection (104–106).

Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) sense mycobacterial infection and activate inflammasomes, such as NLR family pyrin domain containing 3 (NLRP3) or absent in melanoma 2 (AIM2), that through activation of capsase-1 converts members of the IL-1 family into their bioactive forms (**Fig 6**)(107). Furthermore, mycobacterial DNA may be detected via PRRs such as cyclic GMP-AMP (cGAMP) synthase (cGAS) and stimulator of interferon genes (STING) resulting in induction of type I IFN (**Fig 7**). Importantly, the cGAS-STING pathway similarly respond to endogenous DNA translocated into the cytosolic compartment (108).

However, there are also scenarios of PAMP-recognition that favours mycobacterial propagation. One elegant example is when *M. tuberculosis* (and *M. marinum*) uses cell-surface-associated phthiocerol dimycocerosate (PDIM) lipids to shield surface PAMPs, such as peptidoglycan and phenolic glycolipids (PGLs), and instead present related PGLs that recruit macrophages more permissive for mycobacterial growth, thus benefitting mycobacterial propagation (8).

Cytokine signalling

One important outcome of PRRs activation is cytokine production and subsequent signalling. This allows the macrophage to up-regulate self-defence (autocrine signalling), warn and alert neighbouring cells (paracrine signalling) or prepare and recruit other parts of the immune system, both innate and adaptive (109). Bellow, players of particular relevance for this thesis are described. TNFα and IL-1β are two examples of inflammatory cytokines with critical contribution to the immune response against *M. tuberculosis,* whereas the role of type I IFN may in fact be host detrimental.

TNFα

TNFα is induced by members of the TLR-family via the adaptor molecule MyD88, where TLR2 is thought to be especially important for TNFα production in macrophages (107). TNFα is produced by several immune cells and is a crucial inflammatory cytokine to control *M. tuberculosis* infection. It has several beneficial functions such as macrophage activation, induction of chemokines and granuloma formation (70). TNFα is also an important player in induction of cell death, where low levels tend to induce apoptosis, and high levels may cause necroptosis (85). CD4$^+$ T-cells are an important source of TNFα *in vivo*, and high levels of TNFα-positive CD4$^+$ T cells is a strong predictor of active disease (110). The important function of TNFα in *M. tuberculosis* infection is clearly indicated by injecting mice with neutralizing anti-TNFα or by employing TNFα-receptor deficient mice (TNFRp55$^{-/-}$), as these mice quickly succumb to the infection (111). Furthermore, the critical role of TNFα in humans with *M. tuberculosis* infection is illustrated by the increased rate of reactivation in patients with a latent TB-infection that take anti-TNFα medications for other medical reasons (112,113).

IL-1β

IL-1 has two isomers; IL-1α and IL-1β, which both signal through the common receptor IL-1R (114). IL-1R signalling is necessary for host resistance to mycobacterial infection as mice lacking the IL-1 receptor, IL-1β or the TLR/IL-1R family receptor adaptor molecule MyD88 quickly succumb to disease and have a higher bacterial load as compared to wild type (WT) mice (86,114,115). Monocytes, macrophages and DCs are main producers of IL-1α/β during the early stages of infection with *M. tuberculosis* (116). IL-1β is also described to be important for the T-cell response (and IFNγ signalling), as well as in the formation and maintenance of granulomas (86,117). However, too high signalling over time might result in a host detrimental state with inflammatory damage and cell death (118).

The induction of IL-1β requires PRR-activation from two different arms (**Fig 6**). One arm induces production of pro-IL-1β mRNA that is translated into a pro-IL-1β protein, which in the case of a *M. tuberculosis* infection, either can be activated by cell surface TLR2/6 or alternatively cytosolic NOD-containing protein 2 (NOD2) (119). In parallel, activation of a second arm is required to activate the players converting pro-IL-1β into a mature and biologically active protein product. This is executed by the assembly of inflammasomes such as AIM2 and NLRP3, upon detection of DNA or other PAMPs/damage-associated molecular patterns (DAMPs) in the cytosol. The oligomerization of inflammasomes activates the cysteine protease caspase-1 that cleaves pro-IL-1β to mature IL-1β (118). However, this

description might be over-simplified as there are more contributing factors in *M. tuberculosis* infection that influence and drive this pathway (114,118,119).
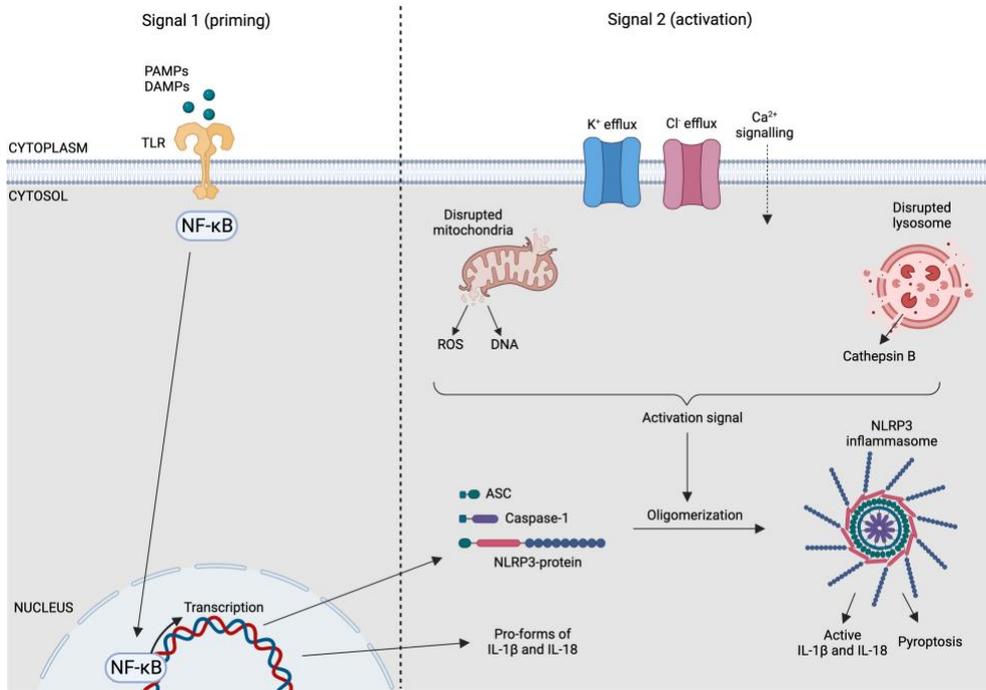
Figure 6. Activation of the NLRP3 inflammasome.
Signal 1, or arm 1, acts by inducing expression of various factors needed for inflammasome activation as well as pro-forms of IL-1β and IL-18. Signal 2, or arm 2, induces an activation signal resulting in oligomerization of the inflammasome, which cleaves the pro-forms into their mature protein form. Image created with BioRender.com.

In addition to the two IL-1 isomers, the IL-1 receptor antagonist (IL-1RA) also binds to the same receptor, thereby dampening the signalling (118). *M. tuberculosis* has been shown to induce production of the IL-1RA, indicating that it might be favourable for the bacteria to suppress this pathway (120,121). The bacteria also suppress IL-1-signalling via induction of type I IFN, which has a pro-bacterial outcome (114,122). Furthermore, there are also examples of TB-patients who have taken anti-IL1 drugs for other medical reasons, who seem to have a worsening outcome of the TB-pathology (123,124). These observations provide further confidence to that IL-1-signalling is critical for host resistance.

Type I IFN

Type I IFN can be secreted by a large repertoire of cells and is induced by several PAMP-PRR pathways, such as by detecting peptidoglycan via NOD1 and NOD2, RNA via retinoic acid-inducible gene I (RIG-I) or melanoma differentiation-associated gene 5 (MDA5), or upon recognition of DNA via DNA-dependent activator of IFN-regulatory factors (DAI), or the DNA sensor cGAS. Type I IFN may also be induced by the TLR-family, including TLR4 and the endosomal TLR3/7/8 and 9 (125,126).

In the case of *M. tuberculosis* infection, there is a consensus that cGAS is a central driver of type I IFN production, and induction is impaired in both human and mouse macrophages with deficient cGAS or STING (127–131). This is also observed within our model system with *M. marinum* infection, where type I IFN signalling completely depends on both cGAS and STING (132). As DNA enters the cytosol, cGAS senses the backbone of double stranded DNA and forms a 2:2 complex. The interaction with DNA induces a conformational change of cGAS allowing the synthesis of a cyclic dinucleotide (cGAMP) from ATP and GTP. cGAMP functions as a second messenger that binds to and activates STING at the endoplasmic reticulum, which thereafter translocates to the Golgi. Here, tank binding kinase 1 (TBK1) phosphorylates STING allowing recruitment of IFN-regulatory factor 3 (IRF3), which also is phosphorylated by TBK1, and thereafter translocate as a homodimer to the nucleus to promote transcription of type I IFNs (**Fig 7**)(133).
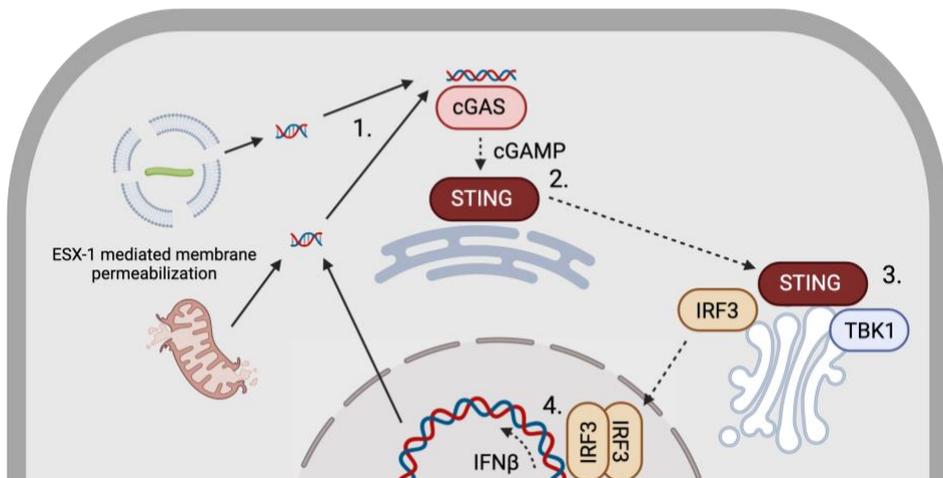
Figure 7. Induction of the type I IFN in mycobacterial infection.
1. cGAS is activated by double stranded DNA and produces cGAMP, which in turn binds to and activates STING. 2. Activated STING migrates from the endoplasmic reticulum to the Golgi and phosphorylates TBK1, resulting in phosphorylation and dimerization of IRF3. 4. IRF3 induces expression of type I IFN such as IFNβ. Image created with BioRender.com.

The source of DNA that activates cGAS-induced type I IFN-signalling in *M. tuberculosis* infection is still under debate, whether it is bacterial DNA coming from the phagosome (128–130,134,135), or if it is host DNA coming from the mitochondria (131). In **paper I**, data suggests that it is host DNA, rather than bacterial DNA inducing type I IFN signalling in our model system and that it requires a fully functional ESX-1 secretion systems to do so (60).

The type I IFN family consists of several gene products such as 13 different IFNα subtypes (14 in mice), one gene product of IFNβ as well as several poorly defined single gene products such as: IFNω, IFNε, IFNτ, IFNκ, IFNδ and IFNζ. All gene products signal through the type I IFN receptor (IFNAR), composed by the two subunits IFNAR1 and IFNAR2, which upon engagement activate the receptor-associated protein Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2). Activation of JAK1/TYK2 results in tyrosine phosphorylation of signal transducer and activator of transcription (STAT)-1 and -2, which dimerize and bind IRF9 to form a complex known as IFN-stimulated gene (ISG) factor 3 (ISGF3). The complex translocates to the nucleus and binds to the corresponding DNA site, so called IFN-stimulated response elements (ISREs), resulting in the induction of interferon stimulated genes (ISGs). There are two other IFN families; type II IFN consisting of one gene product of IFNγ mainly produced by T-cells and NK cells, and type III IFN consisting of the four, rather understudied, gene products IFNλ1, IFNλ2, IFNλ3 and IFNλ4 (125,126,136).

Generation of reactive oxygen species

One important antimicrobial activity performed by the macrophage is production of reactive oxygen species (ROS). The antimicrobial features can function to directly target and destruct invading pathogens, or through redox-regulation of inflammatory signalling. ROS is defined as a group of molecules with at least one oxygen atom and with higher reactivity than molecular oxygen (O_2), such as superoxide anion ($O_2^{\bullet-}$), peroxide ion ($O_2^{\bullet-2}$) and the hydroxyl radical ($\bullet OH$). The main producers of ROS in macrophages are the phagosomal NADPH oxidase 2 (Nox2), peroxisomes and the respiratory electron transport chain (ETC) of the mitochondria. Hydroxyl radicals are mainly produced via the Fenton reaction, where free iron (Fe^{2+}) reacts with hydrogen peroxide (H_2O_2) in the cytosol. ROS production is balanced by antioxidant defence systems, however oxidative stress will result if ROS becomes too abundant (137).

Oxidative stress causes damage to proteins, DNA and lipids, and thus has an essential antimicrobial function in pathogen-containing compartments such as the phagosome (12). Whereas damage to proteins and DNA results in potential mutations or destruction rendering the DNA or protein non-functional, damage to

lipids may result in the chain reaction of lipid peroxidation resulting in whole membrane disruption (**Fig 8**)(138). In **paper II** we are investigating the role of lipid peroxidation and resulting membrane destruction in mycobacterial infection.
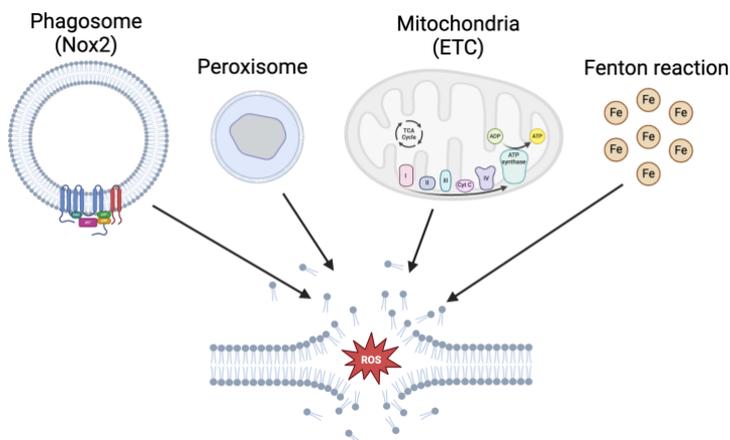
Figure 8. The main producers of reactive oxygen species (ROS) in the macrophage.
ROS is part of the antimicrobial defence, although oxygen stress may result if ROS becomes too abundant leading to lipid peroxidation and collapsed membranes. Image created with BioRender.com.

Cell death

Up until recently cell death was classified either as apoptosis or necrosis, where apoptosis occurs in a controlled manner whereas necrotic cells swell and burst in an uncontrolled manner. Today, the concept has been revised and a whole array of different types of cell death have been described such as intrinsic apoptosis, extrinsic apoptosis, necroptosis, pyroptosis, pyronecrosis, mitoptosis, NETosis, parthanotos, mitotic death, lysosome-dependent cell death, immunogenic cell death, ferroptosis, entotic cell death, autosis and autophagy-dependent cell death. Many of these types of cell death have been reported to occur in mycobacterial infection (35,85).

Cell death of macrophages might result due to intense inflammatory signalling but is also thought to occur as an active mechanism either by the macrophage to degrade the intruder, or by *M. tuberculosis* to leave its host. A general view has been that apoptosis occurs to the benefit of the host, as the controlled degradation allows a chance of targeting the bacteria by efferocytosis (clearance of apoptotic cells by phagocytes), whereas necrosis might favour the bacteria as a means to break free (18,85). However, the new array of cell death mechanisms indicate that it might be more complicated than this. Most likely, different types of cell death occur in

different situations depending on the type of mycobacteria, the bacterial load and the stage of the infection. In **paper III** we investigate one phenomenon of ESX-1 mediated cell death. During mycobacterial infection ESX-1 induces membrane permeabilization to break free from the phagosome (15,17,66,67,139,140), as well as to induce type I IFN (128,129,134,135,141). Simultaneously, the lysosomal compartment is permeabilized in an ESX-1 dependent manner, resulting in leakage of various proteases (142). One of these, cathepsin B, is active in the cytosol (143) and mediates lysosome-dependent cell death (144,145). In **paper III** we find that type I IFN signalling induce expression of two cytosolic serine protease inhibitors (serpins), *serpina3f* and *serpina3g*, which inhibit extralysosomal cathepsin B and thereby lysosome-dependent cell death. Cytosolic serpins thus act in a cytoprotective feed-back loop that limits the consequences of ESX-1 mediated membrane permeabilization.

Type I IFN in mycobacterial infection

It is clear that type I IFN play a protective role in host defence towards viral infections, whereas in bacterial infections the outcome is pathogen dependent (126,136). The role of type I IFN in mycobacterial infection is still not fully understood, although combined observations strongly suggest a host detrimental role (55,82,126,135). Whole blood gene expression analysis identifies type I IFN signalling as the most dominating signature profile of active TB, a signature that correlates with the extent of disease (146).

One indication that type I IFN signalling benefits the bacteria is the fact that ESX-1 has evolved to promote the production in infected macrophages (55,135). Also, in *M. marinum* infection, we observed that type I IFNs are induced only with an intact ESX-1 secretion system, as investigated in **paper I** (60). Furthermore, when infecting IFNAR$^{-/-}$ mice (lacking the type I IFN receptor) with *M. tuberculosis,* colony forming units (CFU) levels decreases (55) and host survival increases (147) as compared to WT mice. Vice versa, when infecting immunocompetent mice with *M. tuberculosis* and treating with additional type I IFN, a strong correlation was observed between high levels of IFN and increased pathology (82). This all together suggest that type I IFN signalling is host-detrimental and favours mycobacterial infection.

Although there is a strong consensus that type I IFN signalling has host detrimental effects during *M. tuberculosis* infection, it is not fully understood how type I IFN regulates the anti-mycobacterial immune response (148). Several studies have demonstrated various mechanisms taking place that reduce important pro-inflammatory cytokines such as IL-1β, TNFα and IL-12 (82,114,116,122,149). Furthermore, type I IFN block IFNγ-mediated activation of macrophages by abrogating the ability of IFNγ to induce antimycobacterial effects (82,149,150).

Multiple of ISGs are induced upon type I IFN signalling, many which are cell-type and context-dependent (125). Intense research is performed to investigate how each ISG contribute to the shift of the inflammatory landscape, potentially providing insights into how they could be exploited for therapeutic purposes. In **paper III** we discovered and explored the role of two novel ISGs in mycobacterial infection namely *serpina3f* and *serpina3g*.

Animal models

The complexity and heterogeneity of TB-disease makes it difficult to cover all aspects in only one type of model system. The mouse model has been of great importance to TB-research as it is susceptible to disease of the lungs and allow manipulation of cellular compartments of the immune system. Furthermore, it allows genetic manipulation of the host immune system, which in combination with genetic manipulation of bacterial strains enables informative investigations. Additionally, the mouse model system has been of great benefit to evaluate drug and vaccine candidates (18). On the other hand, pulmonary infection in mouse does not appropriately recapitulate the fully stratified and mature granulomatous structures seen in humans (151).

Infecting transparent zebrafish larvae with *M. marinum* to study granuloma formation is an example of an inventive way of overcoming investigation barriers in the mouse model. The transparency of the zebrafish larvae allows real-time observations of the early immune response as well as granuloma formation (20). Injecting *M. marinum* intravenously into mice represents another successful way of investigating mycobacterial infection as granuloma-like structures establish in the infected tissue (66,78). These granuloma-like structures are histologically similar to the *M. tuberculosis* induced granulomas found in humans (66). The *M. marinum* model offers a unique advantage by closely mimicking certain critical characteristics of human tuberculosis, which are not effectively reproduced in the traditional murine *M. tuberculosis* model. These include the formation of granulomas featuring central caseating necrosis and the spontaneous progression towards a latency-like stage (66).

The mouse model, as well as other animal model systems, have variations in the immune response as compared to humans, as well as variations of the physiological properties of the lungs. This is illustrated by the lack of translation for many results obtained in animals into the human system (152). To overcome some of these dilemmas, different types of model systems may be used in order to better understand the crosstalk between *M. tuberculosis* and the multitude of host cells involved.

Of note, we investigate a novel way of infecting *Drosophila melanogaster* as a potential candidate for genetic screening in mycobacterial infection. *D. melanogaster* has previously been successfully infected by *M. tuberculosis* (153),

however this was achieved through microinjections that is extremely time consuming and thus hampers large study groups. We hypothesised that we could infect flies orally, through food intake. This was particularly promising as *M. marinum* causes infection of fish intestines, and thus we hypothesised that we could mimic the route of infection also in flies. At this point we only have very preliminary data, but our findings suggest that we might be able to establish a model enabling large scale screenings.

Summary of papers

Paper I:

**The *Mycobacterium marinum* ESX-1 secretion system mediates phagosomal permeabilization and type I interferon production via separable mechanisms.** In **paper I** we aimed to investigate the genetic requirements of the secretion system ESX-1 during mycobacterial infection of macrophages. At the time, the dogma was that the ESX-1 secretion factor ESAT-6 (EsxA) is required for phagosome permeabilization, leading to leakage of DNA into the cytosol and a subsequent induction of type I interferons, which are key events in mycobacterial infection. However, by employing an array of *M. marinum* ESX-1 transposon insertion mutants we could show that the secretion of ESAT-6 is, in fact, not required for phagosomal escape, and that induction of type I IFN is induced by a genetically separable mechanism (**Fig 9**).

To investigate the genetic requirements of ESX-1 mediated phagosomal escape and induction of type I IFN production, we employed WT *M. marinum* (with a fully functional secretion system), the ΔRD1-mutant (with a complete lack of secretion function) and 9 isogenic transposon mutants with deficiency in different ESX-1 related genes. By analysing their ability to secrete CFP-10 and ESAT-6, we could identify three phenotypic groups that either secrete both, only CFP-10, or none. Consistent with the piggy-backing (described above), we did not observe any strain that secreted only ESAT-6. Comparing members of each phenotypic group allowed us to investigate what processes that require secretion of ESAT-6, and not. One such process of interest was phagosomal escape. To our great interest, the members of phenotypic group 2, that did not secrete ESAT-6, could nevertheless escape the phagosome. The event of phagosomal escape can be identified through microscopy of bacteria carrying an actin-tail, which only forms in the cytosol where actin is present. This finding was corroborated by CFU-analysis of the cytosol isolated from macrophages infected with members of all phenotypic groups, as well as by analysing bacterial spread between macrophages. Again, we could observe that neither cytosolic presence, nor spread, required ESAT-6 secretion.

As induction of type I IFN has been seen as an event subsequent to phagosomal escape, through leakage of bacterial DNA into the cytosol, it was of interest to analyse if the genetic requirements matched. When analysing *ifnβ* expression and

IFNβ protein secretion in macrophages infected with members from the different phenotypic groups, it was clear that type I IFN induction required a fully functional secretion system, and that the event of phagosomal escape per se, was not sufficient for induction. Instead, our data suggests that induction of type I IFN is a result of leakage of host DNA, mitochondrial and/or nuclear, into the cytosol where it is sensed by the cGAS/STING cytosolic surveillance pathway.

The observations that phagosomal escape does not require ESAT-6 secretion, and that phagosomal escape and type I IFN induction are two genetically separable events, are of great importance to further investigate to expand our basic understanding mycobacterial pathogenesis and innate immune surveillance.
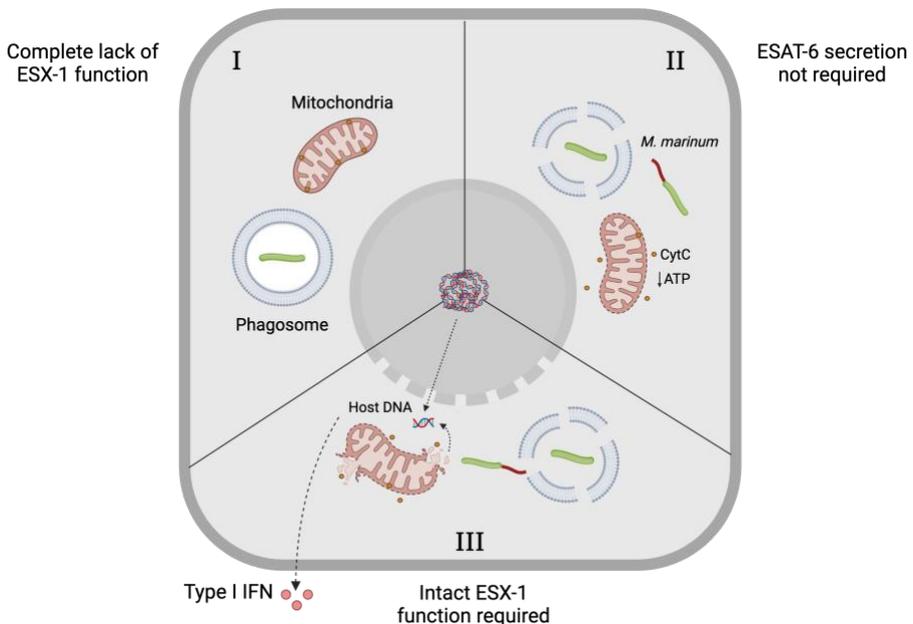
Figure 9. Working model of paper I.
By employing mycobacteria with a lack of, impaired or fully functional ESX-1 secretion system we can identify three (I - III) distinct scenarios during infection of macrophages. Phagosomal escape and induction of type I IFN requires a functional ESX-1 secretion system, however phagosomal escape does not require secretion of ESAT-6 as previously thought. Image created with BioRender.com.

Paper II:

Detection of mycobacterial infection by cytosolic surveillance pathways requires ESX-1-dependent lipid peroxidation of internal host membranes. In **paper I** we find that secretion of ESAT-6 is not required for phagosomal escape. This opens for the possibilities that membrane permeabilization of the phagosome is either caused by another, yet to be identified, bacterial factor ("factor X") and/or a host process. In **paper II** we aimed to answer this question.

ESX-1 mediated permeabilization of internal host membranes is required for detection of the infection by cytosolic surveillance pathways such as the inflammasome and the cGAS-STING pathway, resulting in secretion of IL-1β and type I IFN respectively. Upon phagosomal escape, i.e. membrane permeabilization, leaked DNA results in induction of type I IFN via the cGAS/STING pathway in an ESX-1 dependent manner (128,129,134,135,141). Therefore, we can use type I IFN secretion, as a proxy to study ESX-1 driven membrane permeabilization. As membrane permeabilization is a common character of cell death, we thus hypothesized that if testing an array of cell death inhibitors during mycobacterial infection in macrophages, we might be able to identify what systems are involved causing the permeabilization. Interestingly, we found one cell death inhibitor, ferrostatin-1, which significantly reduced the level of type I IFN secretion. Ferrostatin-1 is a common inhibitor of ferroptosis, a type of cell death where the presence of iron causes free radicals to damage lipid membranes, a process known as lipid peroxidation (154,155). Moving on we investigated the event of phagosomal escape as in **paper I** and could indeed observe that treatment with ferrostatin-1 prevented the bacteria from escaping the phagosome.

As the mode of action of ferrostatin-1 is to bind free radicals, we were curious to see if any other inhibitor of reactive oxygen species reduces the level of secreted type I IFN, and thus ESX-1 membrane permeabilization. We repeated the screening with an array of inhibitors targeting sources of reactive oxygen species. Interestingly, one inhibitor gave a similar phenotype to that of ferrostatin-1, namely apocynin, which inhibits the superoxide generating enzyme Nox2, localized in the phagosomal membrane. Furthermore, when treating macrophages infected with mycobacteria with apocynin we could not observe any phagosomal escape. Our data thus suggests that oxygen radicals generated via Nox2 are required to enable membrane permeabilization via the process of lipid peroxidation, leading to phagosomal escape. These findings suggest that ESX-1 requires a host process to permeabilize host membranes, opening novel avenues for research into fundamental aspects of mycobacterial pathogenesis, and allows us to update our working model from **paper I** (**Fig 10**).
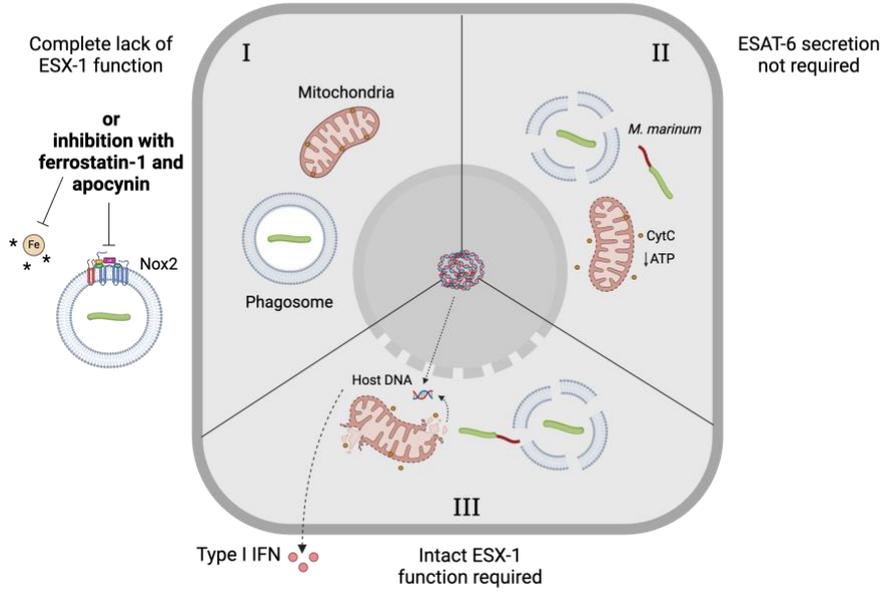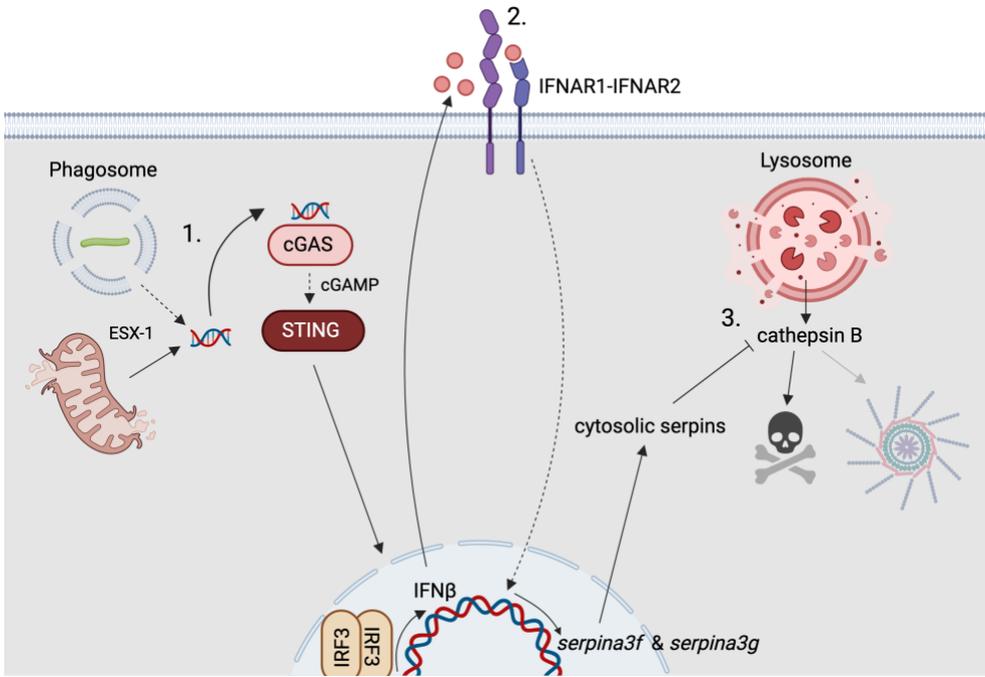
Figure 10. Working model of paper II.
Findings in **paper II** allow us to update our working model from **paper I**. Image created with BioRender.com.

Paper III:

Cytosolic serpins act in a cytoprotective feed-back loop that limits ESX-1-dependent death of *Mycobacterium marinum* infected macrophages. The aim of **paper III** was to further investigate the functional role of ESX-1-mediated induction of type I IFN signalling during mycobacterial infection of macrophages. We identified and investigated the upregulation of two novel ESX-1-mediated ISGs, namely: *seprina3f* and *serpina3g*. Serpins, or serine protease inhibitors, are the largest superfamily of protease inhibitors (156), where *serpina3f* and *serpina3g* belong to the sub-family of clade A3 serpins (157). Serpins mainly inhibit serine proteases, but there are also examples of serpins that inhibit caspases and cysteine proteases (156). Although A3 serpins have been observed to be highly upregulated in infection with *M. bovis BCG* (158) and *M. tuberculosis* (55), there are no studies providing insight into the functional role of these protease inhibitors in mycobacterial infections. Indeed, there is very little prior knowledge of the role of these factors in any type of infection.

In **paper III** we have investigated the induction of A3 serpins and found that only two of them, *serpina3f and serpina3g,* are induced in bone marrow derived macrophages infected with *M. marinum.* Furthermore, this upregulation is ESX-1 and type I IFN-dependent, suggesting that it is the bacteria driving the expression. Interestingly, these two serpins are the only members of clade A3 serpins predicted to be functional in the cytosol, as they lack secretion signal peptides, thus suggesting a functional role of inhibiting cytosolic proteases (157). Our data suggests that *serpina3f* and/or *serpina3g* inhibits cytosolic cathepsin B, a cysteine protease normally contained within the lysosome, and limit cathepsin B driven cell death *in vitro* (**Fig 11**).

Interestingly, both induction of type IFN signalling and cathepsin B leakage are dependent on ESX-1-mediated membrane permeabilization of host membranes. This suggests that upregulation of cytosolic serpins by type I IFN signalling initiates a protective feed-back loop to limit cytotoxic effects of cathepsin B leakage. Furthermore, by infecting mice lacking cytosolic serpins (*serpina3g*-KO mice) through tail vein injections, a model system previously generated by members of the group (159,160), we observed significantly reduced bacterial survival in *serpina3g*-deficient mice compared to WT animals, indicating that *M. marinum* exploits cytosolic serpins to promote growth *in vivo*.

Figure 11. Working model of paper III.
1. Double stranded DNA (bacterial or host) induces expression of type I IFN via the cGAS-STING pathway. 2. Type I IFN signalling results in expression of *serpina3f* and *serpina3g*. 3. These cytosolic serpins inhibit extralysosomal cathepsin B and cell death. Image created with BioRender.com.

Concluding remarks and future aspects

In this thesis I have explored the induction and functional role of type I IFN in mycobacterial infection. Collectively our findings can be summarized into one coherent working model (**Fig 12**).
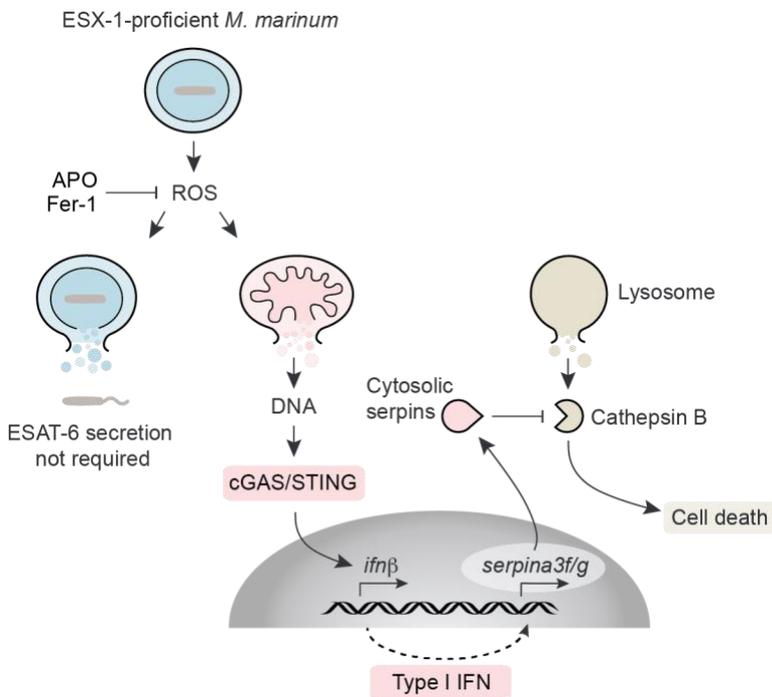
Figure 12. Summarized working model of paper I – III presented in this thesis.
Figures shaded with blue represent the event of phagosomal escape. Figures shaded with pink represent the event of host DNA leakage into the cytosol and induction of type I IFN via the cGAS/STING pathway. Figures shaded with beige represent cathepsin B leakage from the lysosome and evasion of cell death via type I IFN induced cytosolic serpins, forming a protective feed-back. ESX-1-mediated membrane permeabilization is required for all of these events to take place.

The findings presented in **paper I** left us with two open questions. First, if ESAT-6 secretion is not required for phagosomal escape then what factor, host and/or

bacterial, causes this? Second, how does host DNA end up in the cytosol leading to induction of type I IFN? In **paper II** we aimed to target the first question, and present data supporting the role of lipid peroxidation in ESX-1-mediated membrane permeabilization. However, the second question remains unanswered as we still do not know what events, in addition to measurable membrane permeabilization, that is required to induce type I IFN production. Consistent with a previous report (131), our data indicate that it is host DNA, mitochondrial or nuclear, rather than bacterial DNA (128–130,134,135) that stimulates induction. We also know that it requires a fully functional ESX-1, but how this occurs remains unknown. Further elucidating this question would be of great relevance to better understand the pathogenesis of tuberculosis.

In **paper II** we find that membrane permeabilization of the phagosome is caused by lipid peroxidation. Furthermore, data suggests a potential involvement of the superoxide generating enzyme Nox2 located in the phagosomal membrane. This finding is especially thrilling as it presents an alternative mechanism to the previously thought ESAT-6 pore formation. To further investigate this, it would be of interest to perform mycobacterial infections in macrophages from *nox2*-KO mice that lacks one subunit of the NADPH oxidase complex and is thus impaired. Furthermore, it remains to be investigated whether lipid peroxidation contributes to membrane permeabilization of other organelles, such as DNA-containing compartments and/or the lysosome. Phagosomal escape is a key event in mycobacterial infection, understanding this step is of essence to understand the early events of how mycobacteria establish successful infection and may open doors for the development of host-directed therapies.

In **paper III** we investigated the functional role of ESX-1-mediated induction of type I IFN signalling during mycobacterial infection of macrophages. There is a consensus that type I IFN is host detrimental during *M. tuberculosis* infection and it is a strong correlative factor of poor prognosis (146). We identified two novel ESX-1-mediated ISGs, the serine protease inhibitors *serpina3f* and *serpina3g* and explored their role in mycobacterial infection. It would be of interest to explore their roles during other bacterial infections, and maybe even more during viral infections where type I IFN signalling plays an important role in the immune response (126,136). Interestingly, *serpina3g* has been reported to have a host-protective role in HIV-infection (161) as well as during sepsis (162). This emphasizes the need to learn more about the role of these protease inhibitors during infection.

Understanding the host response to *M. tuberculosis* is crucial for humanity to take control of the world's most successful pathogen. Since Robert Koch discovered the causative agent of TB almost 150 years ago, extensive research has been conducted. However, it is still to this date one of the most challenging global health problems. More research is needed to understand the complexity of the disease. By studying mycobacterial regulation of macrophage responses to infection I hope to have contributed with some pieces of scientific insight.

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