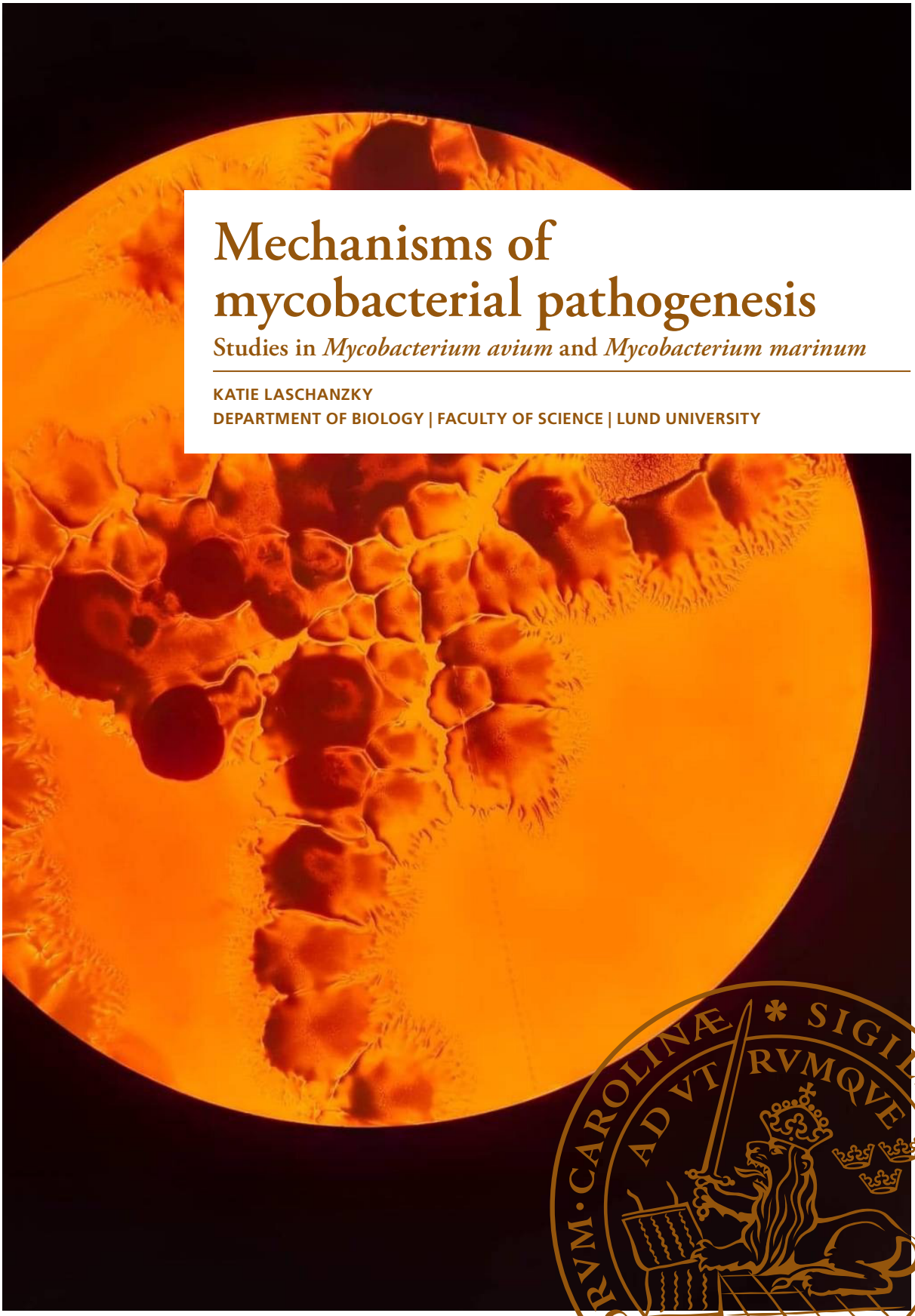
LUND UNIVERSITY

# Mechanisms of mycobacterial pathogenesis

## Studies in Mycobacterium avium and Mycobacterium marinum

Laschanzky, Katie

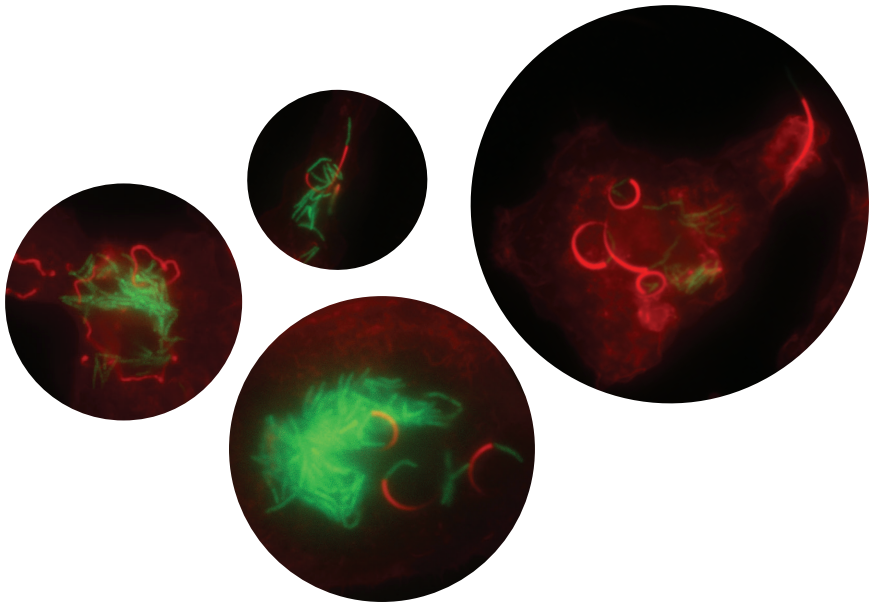
2026



# Mechanisms of mycobacterial pathogenesis

## Studies in *Mycobacterium avium* and *Mycobacterium marinum*

KATIE LASCHANZKY
DEPARTMENT OF BIOLOGY | FACULTY OF SCIENCE | LUND UNIVERSITY



# List of papers

**I.** Laschanzky, K., Ribeiro, G.M., Sequeira, R., Cancade, S., Carlsson, F. and Lienard, J., Cell wall remodeling-dependent morphotype switch in *Mycobacterium avium* differentially regulates lung colonization and tissue persistence. *In press. PNAS*.

**II.** Laschanzky, K., Nobs, E., Ahlbom, E., Valfridsson, C., Lienard, J. and Carlsson, F., Detection of mycobacterial infection by cytosolic surveillance pathways requires ESX-1-dependent lipid peroxidation of internal host membranes. *Unpublished manuscript*.

**III.** Lienard, J., Munke, K., Wulff, L., Da Silva, C., Vandamme, J., Laschanzky, K., Joeris, T., Agace, W. and Carlsson, F., Intragranuloma Accumulation and Inflammatory Differentiation of Neutrophils Underlie Mycobacterial ESX-1-Dependent Immunopathology (2023). *Mbio*. https://doi.org/10.1128/mbio.02764-22

Printed by Media-Tryck, Lund 2026 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

Faculty of Science
Department of Biology

ISBN 978-91-8104-927-5

Mechanisms of mycobacterial pathogenesis:
studies in *Mycobacterium avium* and *Mycobacterium marinum*

# Mechanisms of mycobacterial pathogenesis

## Studies in *Mycobacterium avium* and *Mycobacterium marinum*

Katie Laschanzky


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 8th of May at 13.00 in the Blue Hall, Department of Biology, Kontaktvägen 10, Lund, Sweden

*Faculty opponent*
Professor David G. Russell
Cornell University, Ithaca, NY, USA

**Organization:** LUND UNIVERSITY

**Document name:**Doctoral dissertation **Date of issue:** 2026-04-14

**Author(s):** Katie Laschanzky

**Title and subtitle:** Mechanisms of mycobacterial pathogenesis: Studies in *Mycobacterium avium* and *Mycobacterium marinum*

**Abstract:**

Understanding the basic biology of both a host and the pathogens they are afflicted by is a prerequisite for the development of effective clinical interventions. *Mycobacterium tuberculosis*, the causative agent of tuberculosis, remains one of the leading causes of disease worldwide. Despite extensive research, the appearance of highly drug-resistant bacterial strains and the prevalence of HIV co-infection in the most vulnerable populations have hindered the eradication of this devastating pathogen. Infections caused by non-tuberculous mycobacteria (NTM) such as *Mycobacterium avium* are much less common but are increasing globally. NTM infections are exceptionally difficult to diagnose and treat due to high levels of intrinsic drug resistance, and the relative paucity of knowledge surrounding NTM infections further complicates the development of more effective treatments.

Numerous observations have associated the smooth transparent (SmT) morphology of *M. avium* with human disease, however substantial gaps in knowledge remain regarding the underlying mechanisms of disease establishment and progression. Likewise, while the ESX-1 type VII secretion system is well-established to be a key driver of virulence in *M. tuberculosis* and close relatives such as *Mycobacterium marinum*, the exact mechanisms responsible for causing disease remain elusive. Further study of these two virulence determinants is thus an essential step towards understanding mycobacterial pathogenesis, facilitating the development of improved patient treatments. The work undertaken as a part of this thesis investigates how mycobacteria interact with their hosts, focusing on the bacterial and immunological processes that shape infection outcomes.

**Key words:** Infection, non-tuberculous mycobacteria, MarP, RipA, colony morphology, ESX-1, Inflammasome, neutrophil, lipid peroxidation, type I interferon

**Language**: English **Number of pages**: 94

**ISBN :** 978-91-8104-927-5 (*print*); 978-91-8104-928-2 (*electronic*)



Signature Date 2026-03-19

# Mechanisms of mycobacterial pathogenesis

## Studies in *Mycobacterium avium* and *Mycobacterium marinum*

Katie Laschanzy


LUND
UNIVERSITY

**Published by**:
Department of Biology
Faculty of Science
Lund University
Lund 2026

ISBN 978-91-8104-927-5 (*print*)
ISBN 978-91-8104-928-2 (*electronic*)

Printed in Sweden by Media-Tryck, Lund University,
Lund, 2026

Media-Tryck is a Nordic Swan Ecolabel certified provider of printed material. Read more about our environmental work at www.mediatryck.lu.se

MADE IN SWEDEN

*“The greatest research skill you can have is being a nosy bitch who wants to find out.”*

*- Teya Nicolaou, 2025*

# Table of Contents

# Abbreviations

| | |
|---|---|
| AG | Arabinogalactan |
| AIDS | Acquired immunodeficiency syndrome |
| AIM2 | Absent in melanoma 2 |
| APC | Antigen presenting cell |
| AM | Alveolar macrophage |
| ASC | Apoptosis speck-like protein with a caspase activation domain |
| BCG | Bacille Calmette-Guérin |
| CCR2 | Chemokine receptor 2 |
| CFP-10 | 10 kDa culture filtrate protein (EsxB) |
| CF | Cystic fibrosis |
| cGAS | Cyclic GMP-AMP Synthase |
| CCR2 | Chemokine receptor 2 |
| COPD | Chronic obstructive pulmonary disorder |
| Ecc | ESX-conserved component |
| Esp | ESX-specific protein |
| ESAT-6 | 6 kDa early secreted antigentic target (EsxA) |
| ESX-1 | ESAT-6 secretion system 1 |
| GPL | Glycopeptidolipid |
| HIV | Human immunodeficiency virus |
| HT | Homopolymeric tract |
| IFN | Interferon |
| IFNAR | IFN α/β receptor |
| IL | Interleukin |
| IL-1R1/2 | Interleukin 1 receptor 1/2 |
| IL-1Ra | Interleukin receptor antagonist |
| IM | Interstitial macrophage |
| LOOH | Lipid hydroperoxide |
| L(A)M | Lipo(arabino)mannan |

| | |
|---|---|
| MAC | The *Mycobacterium avium* complex |
| MAH | *Mycobacterium avium ssp. hominissuis* |
| MAP | *Mycobacterium avium ssp. paratuberculosis* |
| MGE | Mobile genetic element |
| MHC II | Major Histocompatibility Complex Class II |
| *Mtb* | *Mycobacterium tuberculosis* |
| MyD88 | Myeloid differentiation primary response 88 |
| NADP(H) | Nicotinamide Adenine Dinucleotide Phosphate |
| NK | Natural killer (cell) |
| NLR | Nod-like receptor |
| NLRP3 | NLR family pyrin domain containing protein 3 |
| NO | Nitric oxide |
| NOD | Nucleotide-binding oligomerization domain |
| NOD1/2 | NOD-containing protein 1/2 |
| NTM | Non-tuberculous mycobacteria |
| NTM-PD | Non-tuberculous mycobacterial pulmonary disease |
| PAMP | Pathogen-associated molecular patterns |
| PDIM | Phthiocerol dimycocerosate |
| PG | Peptidoglycan |
| PGL | Phenolic glycolipid |
| PRR | Pattern recognition receptor |
| RD1 | Region of difference 1 |
| RIG-I | Retinoic acid-inducible gene I |
| RIPK | Receptor-interacting serine/threonine-protein kinase |
| ROS | Reactive oxygen species |
| SmO | Smooth opaque |
| SmT | Smooth transparent |
| STAT | Signal transducer and activator of transcription |
| STING | Stimulator of IFN genes |

| | |
|---|---|
| TB | Tuberculosis |
| TCS | Two-component system |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| TMM/TDM | Trehalose mono/di mycolate |
| WT | Wild type |

# Abstract

Understanding the basic biology of both a host and the pathogens they are afflicted by is a prerequisite for the development of effective clinical interventions. *Mycobacterium tuberculosis*, the causative agent of tuberculosis, remains one of the leading causes of disease worldwide. Despite extensive research, the appearance of highly drug-resistant bacterial strains and the prevalence of HIV co-infection in the most vulnerable populations have hindered the eradication of this devastating pathogen. Infections caused by non-tuberculous mycobacteria (NTM) such as *Mycobacterium avium* are much less common but are increasing globally. NTM infections are exceptionally difficult to diagnose and treat due to high levels of intrinsic drug resistance, and the relative paucity of knowledge surrounding NTM infections further complicates the development of more effective treatments.

Numerous observations have associated the smooth transparent (SmT) morphology of *M. avium* with human disease, however substantial gaps in knowledge remain regarding the underlying mechanisms of disease establishment and progression. Likewise, while the ESX-1 type VII secretion system is well-established to be a key driver of virulence in *M. tuberculosis* and close relatives such as *Mycobacterium marinum*, the exact mechanisms responsible for causing disease remain elusive. Further study of these two virulence determinants is thus an essential step towards understanding mycobacterial pathogenesis, facilitating the development of improved patient treatments. The work undertaken as a part of this thesis investigates how mycobacteria interact with their hosts, focusing on the bacterial and immunological processes that shape infection outcomes.

# Popular science summary

Microbes make up the vast majority of all life on Earth, inhabiting even the most inhospitable environments. While most of our daily interactions with microbial life go un-noticed, infection remains the second most common cause of death globally. However, only a minority of bacterial species have been described to cause disease. For an organism to establish an infection, it must first pass through the physical barriers of our bodies and escape killing by the immune system**.**

**Some bacteria are uniquely prepared for such a monumental task.**

Mycobacteria are a remarkably diverse group of bacteria that are found in nearly every environment: from gardens and ponds to the pipes and drinking water in your home. While most mycobacteria are harmless, several species, including *Mycobacterium tuberculosis*, cause some of the world's most serious infectious diseases. In this book I present the results of three studies which investigate how two evolutionarily distinct species of mycobacteria cause disease.

The first study used a clinical isolate of the *Mycobacterium avium* ssp *hominissuis* to search for bacterial genes and host factors that are required to cause lung disease. When grown in the lab, *M. avium* colonies appear in two main forms with different characteristics: smooth transparent (SmT) colonies are associated with disease and antibiotic resistance, and smooth opaque (SmO) colonies are killed by the immune system and are more sensitive to antibiotics. *M. avium* bacteria are thought to be able to switch back and forth between these two forms, how they do this though, has remained a mystery for over 100 years. In our study we found that the quality of bacterial cell wall plays an essential role in both colonization and persistence during infection, where SmO bacteria have an advantage in establishing lung disease, but only SmT bacteria can survive over time.

The second and third studies use *Mycobacterium marinum*, a close relative to the deadly *M. tuberculosis*, as a model to study one of the most important bacterial mechanisms that allow tuberculosis to cause disease: the ESX-1 secretion system. In study two, we identified host cellular processes that result in disease-causing mycobacteria to escape containment by host cells. We further found that the ESX-1 system helps bacteria manipulate the host environment to promote bacterial escape, killing the host cell in the process. In study three, we investigated how *M. marinum* influences how immune cells respond to infection. The study revealed that rapidly recruited cells called neutrophils are pushed towards a harmful inflammatory state by ESX-1, resulting in tissue damage. In contrast, recruited monocytes play a protective role by suppressing inflammatory neutrophil accumulation.

Together, these studies add to our understanding of how mycobacteria interact with the immune system and identify bacterial traits and host responses that shape infection, highlighting potential targets for future treatments.

# Populärvetenskaplig sammanfattning

Majoriteten av livet på jorden utgörs av mikrober. De kan överleva även i de mest ogästvänliga miljöer. Trots att de flesta av våra dagliga interaktioner med dessa livsformer går obemärkt förbi, så är infektioner den näst vanligaste dödsorsaken globalt. Dock är det endast en minoritet av bakteriearter som har beskrivits som sjukdomsframkallande. För att en organism ska orsaka infektion måste den först passera de fysiska barriärerna i våra kroppar och sedan undgå immunförsvaret.

**Vissa bakterier är dock unikt förberedda för en sådan monumental uppgift.**

Mykobakterier är en oerhört varierande grupp bakterier som finns i nästan alla typer av miljöer; från trädgårdar och dammar, till rören och dricksvattnet i ditt hem. De flesta mykobakterier är harmlösa, men ett flertal av arterna, inklusive Mycobacterium tuberculosis, orsakar några av världens mest allvarliga infektioner. I denna bok presenterar jag resultatet av tre studier som undersöker hur två evolutionärt distinkta mykobakteriearter kan orsaka sjukdom.

I den första studien användes kliniskt isolat av Mycobacterium avium ssp hominissuis för att söka efter bakteriegener och värdfaktorer som krävs för att orsaka lungsjukdom. När M. avium odlas i labbet uppträder två huvudformer med olika egenskaper: släta och genomskinliga (SmT) kolonier är förknippade med sjukdom och antibiotikaresistens, medan släta och ogenomskinliga (SmO) kolonier lättare dödas av immunförsvaret, och är mer känsliga för antibiotika. M. avium-bakterier tros kunna byta fram och tillbaka mellan dessa två former, men hur de gör det har varit ett mysterium i över 100 år. I vår studie fann vi att kvaliteten på bakteriens cellvägg spelar en avgörande roll i både kolonisation och infektionens ihärdighet. SmO-bakterier har en fördel när det gäller att etablera lungsjukdom, men endast SmT-bakterier kan överleva långsiktigt.

De andra och tredje studierna fokuserade på Mycobacterium marinum, en nära släkting till den dödliga M. tuberculosis. Bakterien användes som modell för att studera en av de viktigaste bakteriella mekanismerna som tillåter M. tuberculosis att orsaka sjukdom: utsöndringssystemet ESX-1. I den andra studien identifierade vi värdcellprocesser som tillåter att sjukdomsorsakande mykobakterier bryter sig ur värdcellernas inneslutning. Vi fann dessutom att ESX-1 systemet hjälper bakterier att manipulera värdmiljön, som i sin tur främjar utbrytning av bakterier och dödar värdcellen. I den tredje studien undersökte vi hur M. marinum påverkar hur immunceller reagerar mot infektioner. Studien avslöjade att ESX-1 driver neutrofiler, en typ av snabbt rekryterade immunceller, mot ett överaktivt och skadligt inflammatoriskt tillstånd, som resulterar i vävnadsskada. Däremot har andra immunceller, som rekryterade monocyter, skyddande egenskaper, genom att hämma ansamlingen av inflammatoriska neutrofiler.

Tillsammans ökar dessa studier vår förståelse om hur mykobakterier interagerar med immunförsvaret, samt identifierar bakteriella egenskaper och värdresponser som formar infektioner. Därmed belyses potentiella terapeutiska måltavlor för framtida behandlingar

# Papers included in this thesis

I. **Laschanzky, K**., Ribeiro, G.M., Sequeira, R., Cancade, S., Carlsson, F. and Lienard, J., Cell wall remodeling-dependent morphotype switch in *Mycobacterium avium* differentially regulates lung colonization and tissue persistence. *In press. PNAS.*

II. **Laschanzky, K**., Nobs, E., Ahlbom, E., Valfridsson, C., Lienard, J. and Carlsson, F., Detection of mycobacterial infection by cytosolic surveillance pathways requires ESX-1-dependent lipid peroxidation of internal host membranes. *Unpublished manuscript.*

III. Lienard, J., Munke, K., Wulff, L., Da Silva, C., Vandamme, J., **Laschanzky, K**., Joeris, T., Agace, W. and Carlsson, F., Intragranuloma Accumulation and Inflammatory Differentiation of Neutrophils Underlie Mycobacterial ESX-1-Dependent Immunopathology (2023). *Mbio.* https://doi.org/10.1128/mbio.02764-22

*Additional papers not included in this thesis:*

IV. Nobs, E., **Laschanzky, K**., Munke, K., Movert, E., Valfridsson, C., and Carlsson. F., Cytosolic serpins act in a cytoprotective feedback loop that limits ESX-1-dependent death of *Mycobacterium marinum*-infected macrophages. (2024). *Mbio.* https://doi.org/10.1128/mbio.00384-24

# Author contributions

I. **KL** and JL conceived the study. **KL** and JL performed all the experiments with assistance of RS, SC and GMR. GMR and JL performed bioinformatic analyses. **KL**, FC, and JL wrote the manuscript with input from all authors. FC and JL supervised the project.

II. **KL** and FC conceived the study, **KL** and EN performed all the experiments with assistance of EA, JL, and CVN. **KL,** FC and EN wrote the manuscript with input from all authors. FC supervised the project.

III. FC and JL conceived the study, JL, KM, CDS, JV, TJ, and **KL** performed all experiments. KM and LW performed bioinformatic analysis. FC, JL and WA wrote the manuscript with input from all authors. FC and WA supervised the project.

**KL**- Katie Laschanzky, JL- Julia Lienard, RS- Rodrigo Sequeira, SC- Sacha Cancade, GMR- Giulia Magri Ribeiro, FC- Fredric Carlsson, EN- Esther Nobs, EA- Erik Ahlbom, CVN- Christine Valfridsson, KM- Kristina Munke, LM- Line Wulff, CDS- Clemont Da Silva, JV- Julien Vandamme, TJ- Thorsten Joeris, WA- William Agace

*"We live in the Age of Bacteria*
*(as it was in the beginning, is now,*
*and ever shall be, until the world ends…)"*

*–Stephen Jay Gould, 1993*

# I. Mycobacteria and their natural history

Mycobacteria are a diverse genus of bacteria consisting of nearly 200 described species within the phylum *Actinomycetota* (formerly *Actinobacteria*). This clade is characterized by its members' high GC content, abundant secondary metabolite production, and their presence across diverse environments (1). While most mycobacteria are considered non-pathogens, several species are associated with significant human disease. *Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis (TB), was responsible for over 10 million infections and 1.2 million deaths in 2024, making it the 10$^{th}$ most common cause of death globally (2).

Mycobacteria are often pragmatically classified into two major groups: the tuberculous mycobacteria, which cause tuberculosis and leprosy, and the atypical, or non-tuberculous, mycobacteria (NTM) representing all other species (3). Historically, NTM have been further grouped according to Runyon's classification system based on growth rate and pigmentation production- two easily identifiable traits in a clinical lab (4). This classification system, while imperfect, correctly identified that most disease-causing NTM are slow growing, requiring more than 7 days for colonies to appear on agar plates. Phylogenetic studies later found reduced growth rate to be a derived trait, with all slowly growing mycobacterial species diverging from a common, rapidly growing ancestor (**Fig. 1**) (5). It is speculated that a reduced growth rate is the consequence of adaptation to an intracellular lifestyle with the dual issues of limited access to nutrients and harsh environments within a host (6).

Tremendous genetic and phenotypic diversity exists within NTM species- so much that the splitting of the unified *Mycobacterium* genus into five new genera has been proposed, though not widely accepted (7). The majority of research conducted on mycobacteria have focused on a small number of pathogenic species. At the same time, studies of diverse and newly discovered mycobacterial species, regardless of pathogenicity, have the potential to uncover new facets of mycobacterial physiology and evolution. Each distinct species represents an organism with a unique history that has adapted to a specific ecological niche while using a conserved mycobacterial 'toolkit'. In 2008, *Mycobacterium spongiae* was discovered living within a marine sponge in the Great Barrier Reef (8, 9). Surprisingly, genetic analyses identified *M. spongiae* as the closest non-tuberculous relative of the

MTBC, with highly conserved virulence factors despite its drastically different lifestyle. This discovery of a new species 25 meters below the sea provides new opportunities to investigate the evolution of *Mtb* from a harmless microbe into the deadly pathogen it is today.

The success which the ancestral *Mtb* strain found upon transitioning from the soil to the lungs was likely not due to chance. It is widely accepted that amoebae have provided mycobacteria with an evolutionary "training ground" for adapting to multicellular hosts (10, 11). These single-cell eukaryotes are present in diverse environments and take up free-living bacteria via phagocytosis before degrading their prey in acidified vacuoles. Mycobacteria, unlike most soil-resident microbes, are able to resist degradation and can even replicate intracellularly once consumed (12). Notably, the vacuoles of amoebae and their associated cellular machinery display a high degree of similarity to those of mammalian phagocytes, the first line of defense in the immune system. The intracellular environment, while associated with its own unique challenges, also represents a space free from microbial competition and external stressors. The progressive evolution of *Mtb* to become the devastating pathogen it is today, however, represents only one successful life strategy among mycobacteria.

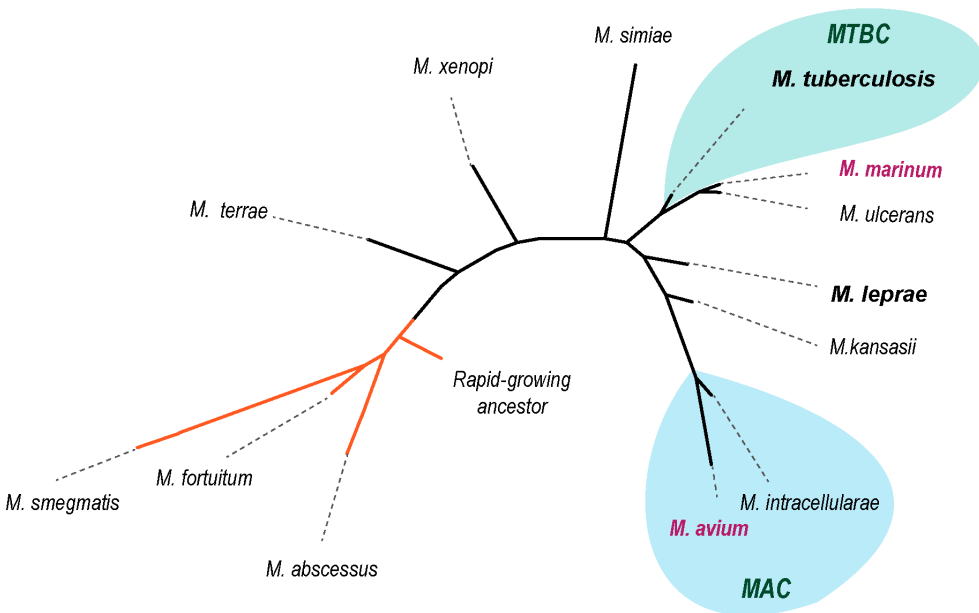



**Figure 1. Simplified illustration of genetic diversity within the genus *Mycobacterium***
Species discussed in this work are labeled. Orange branches indicate rapidly growing mycobacteria; black branches indicate slow growing mycobacteria. Unrooted tree topology and branch lengths are adapted from the maximum likelihood phylogeny obtained by Marin *et al*. (13) utilizing 16s rRNA sequences from 163 mycobacterial species. MTBC: *Mycobacterium tuberculosis* complex, MAC: *Mycobacterium avium* complex.

# *Mycobacterium tuberculosis*

Modern *Mtb* are highly specialized, host-adapted pathogens which have no environmental reservoir. Their adaptation to an intracellular life is most clearly illustrated by their reduced genome size relative to free-living mycobacteria. Where saprophytic mycobacteria have genomes of up to 8 Mb, the *Mtb* H37Rv genome has a size of only 4.4 Mb making up about 4000 genes (14). Such substantial genomic down-sizing is a characteristic step of evolution towards parasitism where optimal utilization of a host comes at the expense of extracellular persistence (15).

The oldest preserved human remains with signs of tuberculosis-like disease date back to 8,000-10,000 BCE (16). Phylogenetic studies, however, have found evidence that an early ancestor of *Mtb* diverged from other soil-dwelling mycobacteria as early 70,000 years ago, coinciding with early human migration out of Africa (17). The subsequent human migration throughout Eurasia has also been proposed to have driven the evolution of the distinct and geographically linked modern *Mtb* lineages (18). When small nomadic groups began transitioning to larger stationary settlements, the potential for person-to-person transmission increased rapidly. With the advent of agriculture and eventually the industrial revolution, human population centers became increasingly dense, accelerating infection especially among those living in poverty. During the 17$^{th}$-19$^{th}$ centuries an estimated 1 in 5 deaths were caused by tuberculosis in North America and Europe (19). Only after the introduction of the Bacille Calmette-Guérin (BCG) vaccine in 1921 and the isolation of streptomycin in 1944 by Schatz, Bugie, and Waksman, could tuberculosis become a treatable illness and not a death sentence (19). As standards of living and the quality of medical interventions continued to improve through the 20th century, tuberculosis became all but eradicated in North America and Western Europe.

TB however is not a disease of the past: in 2024, 1.7% of the global population was diagnosed with TB infection (2). The majority of these infections were concentrated in low-income countries in South-east Asia and Africa where access to basic healthcare, including TB diagnosis and treatment remains limited. Many TB-endemic regions similarly face a disproportionately high burden of HIV infection due to limited resources for treatment and prevention (20). HIV significantly increases the risk of progression to active TB through immune suppression, resulting in increased mortality (21). Further, of the 630,000 worldwide AIDS-related deaths recorded in 2022, roughly one quarter were attributable to TB coinfection, highlighting ongoing need for continued research and focused public health efforts (22).

# NTM disease and the rise of *M. avium*

> *"The clinician is confronted with problems of treatment…The bacteriologist has new problems in identification, for these acid-fast organisms exhibit unique cultural characteristics. From another point of view, here are fascinating organisms for study as to origin, relationships and control.* ***Their occurrence is not rare***"
>
> **Ernest Runyon, 1959 (4)**

Non-tuberculous mycobacteria (NTM) are ubiquitously found in soil and water environments, including drinking water reservoirs (**Fig 2**). While generally considered to be harmless saprophytes, several NTM species are capable of establishing disease in humans. NTM infections are rare globally, but are challenging both to diagnose and to treat, resulting in significant healthcare costs and poor patient outcomes (23–25). *M. avium, M. intracellularae, M. abscessus, M. xenopus,* and *M. kansasii* are commonly referred to as "pathogenic" NTM due to their more frequent association with human disease (26). As a result, these species are (comparatively) well-studied and species-specific treatment guidelines have been developed (27). Other less common strains, however, have no formal treatment guidelines due to the lack of rigorous clinical studies and low prevalence of isolation (28).

**Figure 2. Examples of common NTM reservoirs.**

(Left) Despite moving to Belgium, the student cannot escape her thesis project- NTM in the soil are everywhere. (Middle) Only the most robust bacteria can survive the toxins accumulated from student activities in Sjön Sjön. (Right) Despite exemplary cleaning (and periodic lunchbox decontamination events) there are almost certainly NTM lurking in the kitchen of the Biology C house as well.

Other important pathogenic NTM include *M. ulcerans* and *M. marinum* which are associated with soft tissue infections in humans after exposure to environmental reservoirs (29, 30). *M. ulcerans* is the causative agent of the Buruli ulcer, a treatable, but debilitating skin and soft tissue infection endemic to Western Africa that can lead to permanent disfiguration or disability (30). *M. marinum* is most associated with fish and marine mammal infections but can also cause soft tissue, bone, or joint infections of the extremities in humans (29). Despite its inability to cause pulmonary infection or survive at body temperatures above ~34°C, the lesions caused by *M. marinum* are highly homologous to the pulmonary granuloma caused by *Mtb*. Many of the mechanisms responsible for *Mtb* virulence are conserved in *M. marinum,* including the ESX-1 type VII secretion system (T7SS). Additionally, due to its conserved virulence factors, increased growth rate, and limited human pathogenicity, *M. marinum* has been utilized as a powerful model organism for studying *Mtb* in zebrafish (31) and mice (32). **Paper II** and **paper III** presented in this thesis utilize a *M. marinum* model of infection to study ESX-1 dependent virulence *in vitro* and *in vivo*.

Even among clinically relevant NTM species, however, there is spectrum of pathogenic potential (**Fig 3**). Species such as *M. kansasii* are more often associated with causing progressive disease with poor patient outcomes (33). Other species such as *M. fortuitum* only rarely establish infection under specific host or environmental conditions and are often associated with hospital-acquired infections or medical device contamination (34). Individual NTM isolates of the same species, however, can additionally vary widely in their ability to cause disease- the genetic and physiological mechanisms of which remain incompletely understood (27, 35).

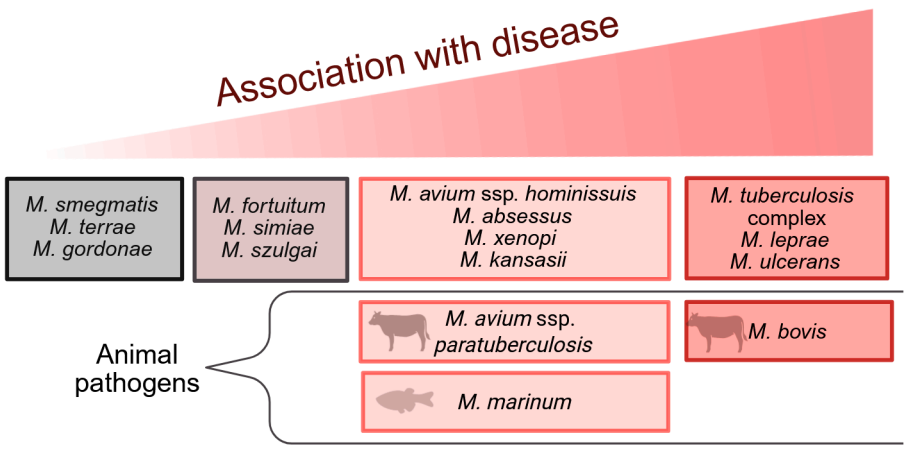



**Figure 3. Schematic representation of the spectrum of pathogenicity displayed by clinically relevent mycobacterial species.**

## The *M. avium* complex

The *M. avium* complex (MAC) is a group of ten slow growing mycobacterial species associated with human infection which cannot be easily distinguished from one another by traditional culture methods (36). *M. avium* and *M. intracellularae,* are the most commonly isolated MAC species, together representing over half of all NTM infections globally (26). *M. avium* can further be separated into four subspecies based on growth characteristics and host tropism (37).

*M. avium ssp. hominissuis* (MAH) is associated with pulmonary and disseminated infection in humans but can also cause lymphadenitis in pigs and other animals (38). MAH isolates have a high degree of genetic and phenotypic diversity. Unlike *Mtb* which is a largely clonal organism, there is evidence of a high degree of gene flow between individual MAH isolates. MAH have open "pan-genomes" of approximately 3500 core genes and large "clouds" of accessory genes which are unique to specific ecological niches or geographic regions (39).

*M. avium ssp. paratuberculosis* (MAP) causes a form of chronic-wasting disease, called Johne's disease, in ruminants and is a source of great economic burden in the agricultural industry (40). While not directly transmitted between animals, MAP is commonly isolated from farm soil and waste from infected animals can cause the rapid spread of disease in a herd. MAP has also been isolated from the GI tract of humans with Crohn's disease, a chronic inflammatory condition with unclear origin and similar intestinal pathology observed in animals Johne's disease (41). It has been speculated that MAP may contribute to the development of Crohn's disease, though the evidence in support of this theory remains limited and controversial (42).

*M. avium ssp. avium* and *ssp. silvaticum* are primarily associated with tuberculosis-like infections in birds but poorly studied compared to MAH and MAP and rarely cause infection in humans (37). Interestingly, *M. lepraemurium*, the cause of leprosy in other mammals was found, contrary to previous assumption, to share a close common ancestor with *M. avium* and not with causative agent of human leprosy, *M. leprae.* Both leprosy-causing species underwent similar genome reductions, ultimately resulting in similar disease manifestations due to convergent evolution. (43, 44).

## HIV/AIDS and disseminated MAC infection

During the rise of the HIV/AIDS epidemic in the early 1980s, a sharp increase in infections by MAC species were observed in AIDS patients with highly compromised immune systems (45, 46). MAC infection in HIV+ individuals typically presents as disseminated disease in the gastrointestinal tract and soft tissues. In these patients, the likelihood of NTM infection is tightly correlated with the reduction of protective CD4+ T cell counts (47, 48). In 1990, during the epidemic's peak, 20-40% of HIV+ patients in the United States were estimated to

have MAC infection (47, 49). During this time anti-retroviral therapies (ART) were not widely available to treat the underlying cause of hyper-susceptibility to NTM and other opportunistic infections. In vulnerable patients, even if one infection was successfully treated, another was soon to follow, resulting in only 13-29% of HIV+ patients surviving more than one year after diagnosis of MAC disease (45, 46).

The development of effective ARTs to combat HIV infection, as well as pre- and post- exposure HIV prophylaxis (PREP/PEP), has greatly decreased the incidence and mortality of AIDS in Western countries. As of 2022, of the estimated 39 million individuals living with HIV, nearly 30 million had access to antiretroviral treatments (22). However, 9 million individuals, the majority being in developing nations, still lack access to treatment, putting them at risk for NTM infection as well as severe TB disease.

## NTM Pulmonary disease

In contrast to the progress made in reducing HIV-associated NTM disease, increases in pulmonary NTM disease (NTM-PD) have been observed globally in the past decades (50). Unlike TB which spreads by direct person-to-person transmission, NTM pulmonary infections result from environmental exposure, though exposure alone is far from sufficient to cause infection.

Large-scale surveys across the United States found evidence of at least one NTM species present in over 75% of all household plumbing systems (51, 52). Contributing to their widespread isolation in household environments, NTM are highly resistant to common disinfectants that are used in drinking water treatment including chlorine (53). Exposure to contaminated water is thought to be the main route of NTM infection. Showerheads in particular have been implicated as a source infection due to the abundance of plumbing-associated biofilms (54) and the production of aerosols during showering which are then inhaled (55).

Ecological surveys of mycobacterial diversity have found that many NTM species, including potential pathogens, are enriched in humid environments with acidic soil (56) or near large bodies of water (26). Several studies have additionally reported environmental NTM “hotspots” with increased mycobacterial abundance which correlate with increased NTM isolation from patients in local hospitals (57, 58). While still not fully understood, variations in environmental conditions and bacterial community composition likely account for much of the geographic trends consistently observed in NTM-PD prevalence (**Table 1**). NTM-PD in East Asian countries and the Pacific islands is estimated to be as much as five times more prevalent when compared to the continental United States and Europe (59, 60). Due to the nearly universal presence of NTM in both natural and human-engineered environments, most epidemiological studies of NTM disease focus on identifying patient risk factors underlying susceptibility to NTM infection. Increased age, sex,

and low body weight have all been strongly associated with increased risk for NTM-PD (61, 62). Women are generally recognized to more likely to develop disease, with the term 'Lady Windermere Syndrome" being coined in 1992 for the observance of immunocompetent elderly women, with low body weight but no other comorbidities, being over-represented in NTM-PD patients (63). In contrast, NTM-PD in men is more likely to present alongside a wide range of comorbidities such as previous TB infection, poor lung function, or behaviors such as long-term cigarette smoking (61, 64). Regardless of these gender- and sex-related differences in disease manifestation, pre-existing lung disease is one of the most important factors predisposing an individual to NTM-PD (65).

**Table 1. Summary of estimated NTM-PD disease prevalence (active cases per 100,000)**
a: yearly estimate of point-prevalence 2005-2013

| Country | *(year)* | Total prevalence | Advanced age (age group) | | Reference |
|---|---|---|---|---|---|
| Germany | *(2014)* | 3.3 | 6.5 | *(>60)* | (66) |
| UK | *(2016)* | 4.7 | 7-10 | *(>55)* | (67) |
| USA | *(2015)* | 11.7 | 47.5 | *(>65)* | (68) |
| Hawaii | *(2013)* | 44 | 87 (a) | *(>65)* | (69) |
| Japan | *(2014)* | 29 | 93 | *(70-79)* | (70) |
| South Korea | *(2016)* | 39.6 | 233 | *(>70)* | (71) |

### *Cystic fibrosis*

NTM infection is of particular concern for individuals with cystic fibrosis (CF). CF is a rare hereditary disease where loss of the CFTR chloride channel function results in dysfunction in numerous organ systems, most severely impacting the lungs. CF is most prevalent in those with European ancestry, where diagnosis is made in approximately 1 in 3500 live births (72).

CF patients are particularly vulnerable to opportunistic NTM infection for numerous reasons. CFTR deficiency causes excess intracellular sodium and chlorine ions in lung epithelial cells, resulting in increased influx of extracellular water to compensate. This compensation, however, leads to thickening of lung surfactant which then cannot easily be cleared by normal mucociliary clearance (73). Excessive and thickened mucus traps bacteria and limits penetration of water-soluble antibiotics. These compounding factors make treatment of NTM infection in these patients particularly challenging to treat, resulting in greatly reduced quality of life (74, 75). Among these patients in the US between 2010-2019, just over 5% were diagnosed with NTM-PD, with isolation rates increasing 3.5% each year of the study (76).

***COPD and bronchiectasis***

Chronic lung disease is another important risk factor for developing NTM-PD. Chronic obstructive pulmonary disease (COPD) is a collective term for several airway dysfunctions including emphysema and chronic bronchitis resulting in narrowed airways and limited airflow due to inflammation. As COPD progresses, the lower airways become damaged, resulting in fibrosis and increased risk for lower respiratory infections (77). The estimated prevalence of NTM in individuals with COPD varies widely due to regional variations in diagnosis and, however as much as 22% of all COPD patients are predicted to be at least incidentally colonized by NTM (78, 79). Though colonization does not necessarily correspond to disease, the high overall prevalence of COPD likely represents an underappreciated cause of NTM-PD. In the last decades, COPD has risen to become the 4th most common cause of death globally, associated with 3,4 million deaths in 2021 (80). Both increased age and exposure to cigarette smoke or polluted air heighten the risk for COPD development— with both factors anticipated to become greater global concerns in coming decades. The combination of growing elderly populations and worsening air quality in urban areas is predicted to result in significant increases in the economic and health-related burdens of COPD (81, 82).

Bronchiectasis (BE) is another form of structural lung disease characterized by the irreversible airway widening and severe fibrosis. Chronic bronchodilation causes a decline in lung function and poor mucus clearance, increasing the risk of bacterial infection. While less common than COPD, BE prevalence in North America and Europe is similarly increasing and is responsible for considerable healthcare costs due to infections and other co-morbidities (83). A recent study found that among patients diagnosed with BE, 7.7% had NTM infections (84). Concurrent BE and NTM-PD diagnoses were also found to greatly increase all-cause mortality compared to individuals with non-complicated NTM-PD (75).

A common treatment for both COPD and BE exacerbations is inhaled corticosteroids, the long-term use of which causes mild immunosuppression (85). As immune suppression is a critical risk factor for NTM infection, these treatments have the potential to drive the progression of controlled or sub-clinical NTM infections (86).

# Antimicrobial resistance

Despite increasing knowledge surrounding mycobacterial disease, numerous challenges remain for the diagnosis and treatment of both TB and NTM disease. Great progress has been made towards the eradication of TB globally, however the COVID-19 pandemic and reductions in UNAID funding have disproportionally affected low-income countries with high TB burden in recent years (2). NTM

disease, in contrast to TB, is not systematically reported in most countries, complicating epidemiological studies (50). Additionally, the clinical and radiological presentations of NTM-PD overlap significantly with pulmonary TB (87, 88), likely resulting in significant underestimation of NTM disease in regions where TB is endemic.

One of the most significant clinical challenges for treating mycobacterial infection is antibiotic resistance, both intrinsic and acquired. “Drug sensitive” NTM isolates however have a baseline level of drug resistance comparable to “drug resistant” TB (89). Two studies of patients diagnosed with chronic, drug-resistant TB in Burkina Faso (90) and Mali (91) found that in approximately 20% of patients, NTM bacteria and not *Mtb* were in fact responsible for disease. This potential for misdiagnosis leads to further complications in both patient treatment and in the estimation of global NTM disease burden.

Current consensus guidelines for treatment of NTM-PD are administration of three antibiotics of differing biological targets until 12 months of consistent negative sputum cultures are observed (27). The standard first-line treatment for non-resistant infections is similar to that of TB, and include a macrolide, rifampin, and ethambutol. In cases where lung disease is more severe, intravenous streptomycin or amikacin is additionally recommended for shorter periods of time in order to reach damaged tissues where drugs have difficulty penetrating. Lengthy, multidrug treatment regimens are often poorly tolerated, especially in elderly patients suffering from co-morbidities (92, 93). Early cessation of treatment, however, increases the risk for incomplete eradication of metabolically inactive, but viable bacteria— a major risk for the development of drug-resistant strains (27, 94). While lengthy antimicrobial treatments are also a concern in TB treatment, significant progress has been made in implementing 4- and 6-month regimens, which improve both access and adherence to treatment (95). These shortened treatments are likely not possible for NTM disease due to more extensive intrinsic drug resistance in NTM and the difficulty in recruiting sufficiently large cohorts for clinical trials (89).

Even with access to timely and evidence-based interventions, an estimated 30-40% of first-line treatments will fail to eradicate an NTM infection (27, 96, 97). Yet, as of 2011 only an estimated 15% patients with NTM-PD globally received treatment in line with evidence-based recommendations, further reducing the rate of treatment success (98). As cases of NTM-PD are increasing globally, there is a growing need for new, more effective diagnostic and treatment strategies for NTM-PD. Ultimately, for both *Mtb* and NTM infection, development of improved treatments requires better understanding of the unique cellular biology of pathogenic mycobacteria.

# II. Mycobacterial Cell biology

## The mycobacterial cell wall

While closely related to Gram positive (or monoderm) bacteria, mycobacteria have a unique and highly specialized cell envelope architecture. The thick mycobacterial cell envelope is composed of peptidoglycan, (PG) branching arabinogalactans (AG), and an outer membrane composed of mycolic acids (99, 100). Many pathogenic species including *Mtb* and MAC additionally form an outer capsule composed of secreted lipids, polysaccharides, and proteins (101). This complex arrangement is both structurally and functionally analogous in many ways to the diderm structure of the Gram-negative envelope despite great evolutionary distance. The high lipid content of the mycobacterial cell envelope, however, gives mycobacteria a distinct 'acid-fast' staining pattern where cells are resistant to decolorization by acid alcohol- distinct from both Gram positive and negative bacteria (**Fig. 4**)

The mycobacterial cell envelope is a thick physical barrier, which is selectively permeable, excluding harmful compounds while allowing the uptake of essential nutrients. This permeability is tightly regulated and is coordinated by numerous proteins. As a result, mycobacteria are intrinsically resistant to diverse stressors including exposure to acidic stress, toxic heavy metals, and many antimicrobial compounds (100, 102). Understanding this robust structure is thus essential to understanding mycobacterial physiology.

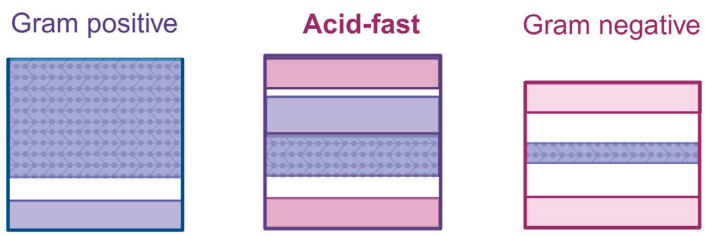



**Figure 4. Comparison of Gram postitive, Gram negative, and acid-fast mycobacterial cell wall architecture.** Cell envelople layers are simplified, but appoximate Gram staining results.

## Peptidoglycan maintenance and remodeling

Peptidoglycan (PG) is an essential component of the bacterial cell envelope, which is responsible for the maintenance of cell shape and osmoregulation (103). PG is composed of alternating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) molecules which are linked by glycosidic bonds. In mycobacteria, NAM molecules are further oxidized to *N*-glycolylmuramic acid, a modification which enhances the strength of the mesh-like structure of the PG layer, increasing resistance of PG degradation by lysozyme (99). Each NAM molecule is additionally attached to chain of five amino acids. During cell growth and division, transglycosylase enzymes add additional NAG/NAM disaccharide units to PG polymers, and transpeptidases form cross-linkages between neighboring peptide stems. Cell division requires not only the synthesis, but also the cleavage, of PG polymers in order to generate new daughter cell. Lytic transglycosylases, amidases, and endopeptidases are also essential for cleaving glycan and peptide bonds during cell growth and repairing damaged PG (104, 105) (**Fig. 5**). The endopeptidase RipA, in particular has been found to play an essential role in *Mtb* response to acid and oxidative stress, as well as during adaptation host infection (105, 106). In **Paper I** presented in this thesis, we further identify the RipA and its activator MarP as essential factors underlying intra-host persistence in virulent *M. avium*.

The PG layer of mycobacteria additionally has several unique features resulting in increased stability and resistance to cell wall-targeting antibiotics. D-D transpeptidase enzymes which form 3→4 cross-linkages between neighboring peptide stems are broadly essential and conserved among bacteria. Mycobacterial PG, however, is unusually enriched in 3→3 linkages formed by alternative L,D transpeptidases (107). During growth arrest, up to 80% of all cross-linkages are associated with increased PG stability and resistance to β-lactam antibiotics (108). Mycobacterial PG is further reinforced by interwoven arabinogalactan (AG) molecules which play a dual role in regulating both cell envelope structure and host -pathogen interactions (109). AG molecules are branching chains of primarily galactose and arabinose sugars which tether PG to the inner leaflet of the mycomembrane by covalently bonding to mycolic acids of the lipid-rich outer layer of the cell envelope (99).

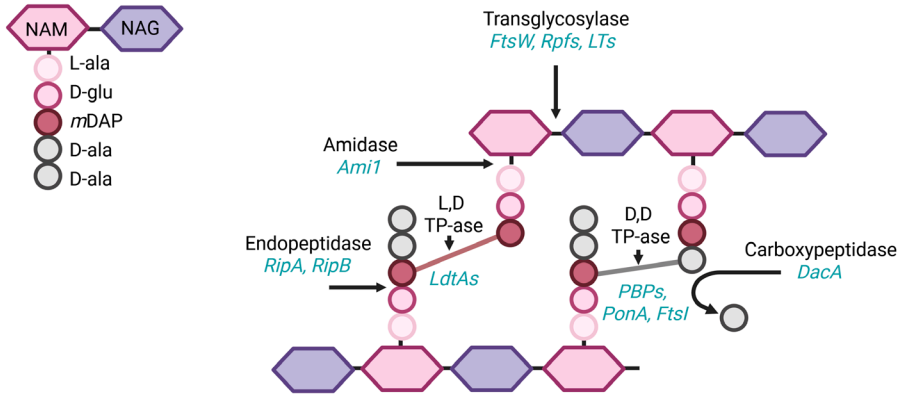



**Figure 5. Peptidoglycan structure and sites of enzymatic alterations.**
NAG: N-glucosylamine, NAM: N-acetylmuramic acid LT: lytic transclycosylas**es,** PBP: penicillin binding protein, Rpf: ressusitation promoting factor, TP-ase: transpeptidase.

## Mycobacterial membrane lipids

Biosynthesis and assembly of diverse lipids in the outer mycobacterial membrane is a complex and energetically expensive process. Both the eukaryotic-like fatty acid synthase module (FAS-I) and the prokaryotic-like module (FAS-II) as well as the polyketide synthase pks13 are required to synthesize mycolic acids with various lengths and functional modifications (110). The mycolic acid-derivatives trehalose mono- and -di mycolate (TMM/TDM) are the main components of the inner leaflet of the outer mycobacterial membrane (111). In addition to their structural role, TDM molecules are involved in modulating host cell invasion and trafficking (112, 113), as well as inhibiting and phagosome-lysosome fusion (114). The outer leaflet, in addition to mycolic acids, contains a variety of other lipid moieties, many of which have both structural and immune-modulatory properties when in contact with host cells (115). *Mtb* and related species such as *M. marinum* produce a number of specialized surface lipids such as phthiocerol dimycocerosate (PDIM) phenolic glycolipids (PGL) which are involved in modulating host cell interactions and virulence (116). During infection, PDIM has been observed to be shed from bacterial cells and become inserted into host membranes, modulating the early inflammatory response (117).

Lipomannan (LM) and lipoarabinomanan (LAM) molecules are specialized glycolipids involved in maintaining the cell envelope. LMs are linked to the plasma membrane, non-covalently bound to phosphatidylinositol (PI) heads of membrane phospholipids. LAM then contains further branching arabinan polymers terminally capped by mannose (118). In pathogenic mycobacteria, mannose-capped LAM molecules have been found to inhibit numerous host processes including phagosome

maturation, apoptosis, and inflammation (119). Conversely, LAM molecules from non-pathogenic and rapidly growing NTM, which are alternatively capped with inositol phosphate, induce secretion of pro-inflammatory cytokines such as IL-8 and TNF-α (120, 121).

Distinct from *M. tuberculosis*, most NTM produce glycopeptidolipids (GPL) which are inserted into the outer leaflet of the mycomembrane and result in a smooth colony appearance on agar (122). GPLs are structural components of the mycobacterial cell well and are present in the outer capsule. Non-polar, nonspecific GPL (nsGPL) molecules are conserved among NTM species and consist of a glycosylated tetra-peptide linked to fatty acid chain of 26 to 33 carbons in length. Core nsGPLs produced by MAC species can be further modified, resulting in polar serotype-specific GPL molecules (ssGPLs), which can be used to distinguish MAC isolates at the subspecies or serotype level (122).

Mutations or deletions in key genes involved in GPL biosynthesis and transport result in a profound change in both cell and colony morphology (123). In MAC species, GPLs are required for biofilm formation (124) and sliding motility (123, 125), two traits associated with extracellular persistence. Without surface-exposed GPLs, cells form large aggregates, or cords, resulting in rough (Rg) bacterial colonies. This change is most well-studied in *M. abscessus* where mutations in GPL loci commonly occur during infection and are associated with the development of progressive lung disease, whereas smooth colonies are primarily isolated during early infection and associated with mild disease (126, 127). *M. abscessus* GPLs have been found to interact with host toll-like receptor 2 (TLR2) resulting in a silencing of early inflammation during infection which appears to be beneficial to establishment of a lasting infection (128). Despite increased virulence and inflammation within host cells, Rg *M. abscessus* have reduced rates of survival in environmental conditions (129).

## Trans-membrane transportation

The mycobacterial cell envelope provides resistance to numerous environmental stressors, yet the thickness and complexity of this protective structure also confer physiological challenges in nutrient uptake and protein secretion. As a result, mycobacteria have acquired a multitude of trans-membrane transporters to regulate cellular import and export. An overview of the localization and function of these transporters can be found in **Fig 6.**

Environmental mycobacteria express at least one porin protein allowing the passive transport of small molecules through the outer mycobacterial membrane (130). While useful for acquisition of essential nutrients, porins also facilitate the influx of antimicrobial compounds. In *M. smegmatis,* the porin MspA is essential for cell growth (130), however many pathogenic mycobacteria have entirely lost porin function, restricting their access to essential nutrients in exchange for increased resistance to antimicrobial compounds (131). Both environmental and pathogenic mycobacteria express numerous ABC transporters which require ATP hydrolysis to selectively bind extracellular 'cargo' molecules and translocate them through the outer membrane (132). In *Mtb*, over 30 distinct ABC transporters have been identified, many of which have been implicated in aiding bacterial survival during infection (133).

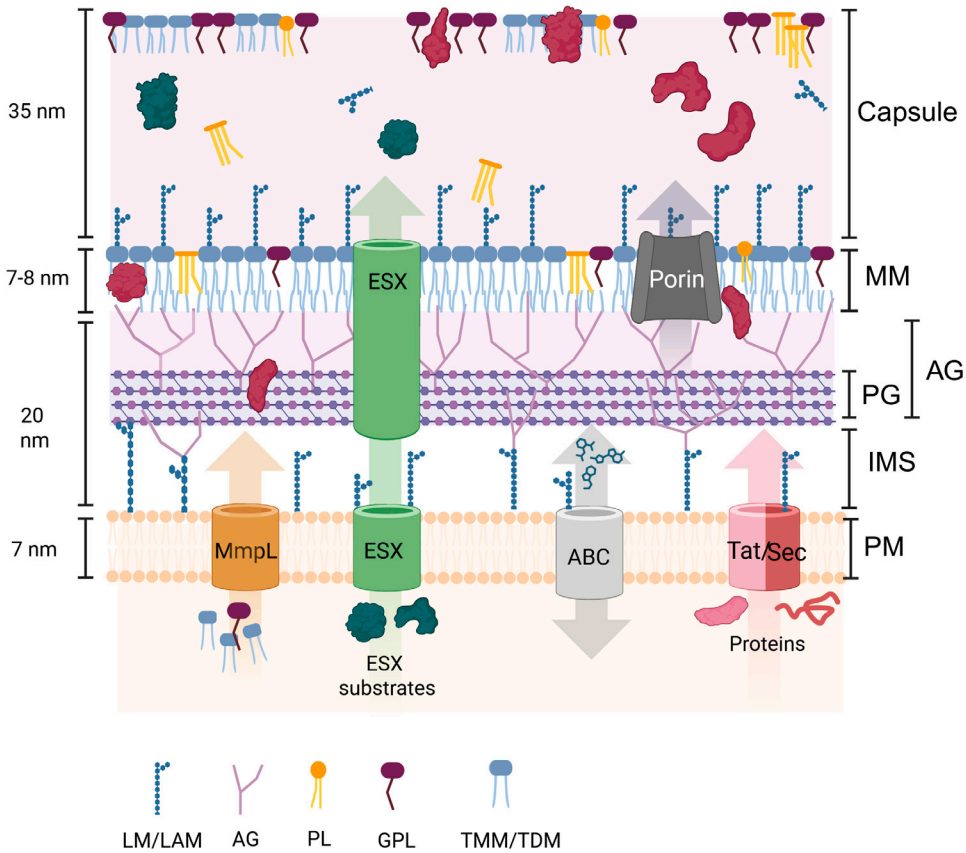



**Figure 6. Generalized illustration of the mycobacterial cell wall and major classes of transmembrane transporters.** ABC: ATP-binding cassette AG: Arabinogalactan,ESX: Early secretory antigenic target 6 (ESAT6) secretion system, GPL: glycopeptidolipid, IMS: inter-membrane space, MM: mycobacterial outer membrane, MmpL: mycobacterial membrane protein large, PG: peptidoglycan, PM: plasma membrane, TAG: triacylglycerol, TMM/TDM: trehalose mono/di mycolate. Adapted from (100, 134).

Successful maintenance of the mycobacterial cell requires the coordinated transport of numerous protein, lipid, and polysaccharide "building blocks" to their precise cellular- or extracellular- destinations. Like other bacteria, mycobacteria utilize the conserved twin arginine translocation (Tat) and general secretory (Sec) pathways to export folded and non-folded proteins, respectively to the inter-membrane space (IMS) based on recognition of specific signal peptides (135). Additionally, members of the MmpL family of proteins specific to mycobacteria are responsible for the transport of a range of cell wall lipids and other molecules essential for growth and virulence. The members of this large family vary in number and function between species, likely reflecting adaptations to diverse ecological niches. Slow-growing pathogenic mycobacteria such as *Mtb* and *M. avium* have an average of 15 MmpL transporters while rapidly growing species such as *M. abscessus* can have up to 29 MmpLs allowing them to utilize diverse resources from the environment (136). MmpL3, the only essential MmpL family, transports TMM and is required for assembling the mycolic acid- rich mycomembrane (137, 138). Other important Mmp transporters include MmpL4 which transports GPL in NTM (139), and MmpL7, which transports PDIM molecules which are essential for virulence in *Mtb* (116). In addition to their role in cell wall synthesis, several MmpL proteins, such as MmpL5, can act as drug efflux pumps, making them attractive targets for the development of new antimicrobial agents (38, 140).

## The type VII secretion system

Mycobacteria additionally rely upon specialized type VII secretion systems (T7SS) for the transport of proteins through the many layers of the cell envelope. Five paralogous T7SS systems (ESX 1-5) have been described and are thought to have arisen by duplication of an ancestral plasmid-transmitted ESX-4 system (141). While structurally analogous, individual ESX systems in mycobacteria have diverse functions and their distribution is varied throughout NTM species.

All five mycobacterial ESX systems share a set of conserved proteins (EccB/C/D/E) which assemble in the bacterial plasma membrane to form a heterodimeric channel for selective protein secretion (142). Importantly, EccC proteins have FtsK/SpoIII ATPase activity which facilitates the active transport of further T7SS structural proteins and secreted effector molecules into the inter-membrane space where they are further processed by MycP (143). Other ESX components are specific to individual secretion systems but display a high degree protein homology: Esp proteins play accessory roles in the ESX-1 secretion apparatus, and Esx proteins and PE/PPE proteins containing conserved Trp-X-Gly (WXG) motif, are primary secretion targets, many of which impact host cell processes during infection (144).

*ESX-1*

The ESX-1 system is the most well-studied T7SS due to its essential role in *Mtb* virulence. Indeed, the attenuation of the *M. bovis* BCG vaccine strain resulted from the deletion of several ESX-1-encoding genes, in the region of difference 1 (RD1) genetic locus (145). *Mtb* strains lacking the RD1 locus are profoundly impaired in their ability to cause disease or granuloma formation in animal models, and do not induce host-detrimental type I interferon (IFN) immune response (146, 147). Despite extensive investigation, the structure and secretion targets of the ESX-1 T7SS have yet to be completely determined (148). ESX-1 is under tight regulation on several levels, and its assembly occurs in a stepwise manner where disruption of one component has rippling effects. Combined genetic and proteomic analyses have begun to map the many inter-dependent proteins (149).

Once translocated to the inter-membrane space, several proteins have been proposed to consecutively assemble after MycP activation and form a second channel in the outer mycobacterial membrane (150). While the exact structure of this second channel remains elusive, a general assembly hierarchy has been described based on interdependence observed between individual ESX-1 substrates (149). ESAT6, CFP10, PE35, and PPE68 translocation is first required for the assembly of channel-like EspB heptamer (151–153). Subsequently, EspE and EspF are transported to the surface of the mycobacterial outer membrane, where proper localization and function rely additionally on PDIM membrane lipids (154, 155). Finally, the assembled ESX-1 system results in the secretion of bacterial effector proteins such as ESAT-6 (Early Secreted Antigenic Target 6 kDa; EsxA) and its "piggybacking" co-factor CFP-10 (10 kDa Culture Filtrate Protein; EsxB), PE/PPE proteins and EspA/C proteins (150, 156).

Several Esp proteins (EspD/G/H/J/K/L) have also been reported to function as chaperones, stabilizing target proteins prior to secretion (152, 157–160). The cytosolic EccA is associated with regulating chaperone-target interactions and possibly aiding in protein delivery to EccC (159). WhiB6, EspH, EspI and EspM have also been identified as regulatory proteins which further fine-tune the expression of the entire ESX-1 locus based on ATP levels, ESX-1 protein accumulation and likely other, yet-unknown, signals (159, 161–163).

**Fig. 7** presents a consolidated model of the assembly and regulation of the ESX1 T7SS based on the results of the studies summarized here. Interestingly, recent work in a preprint from the Champion lab suggests that the specific Esx/Esp proteins required for the assembly of the ESX1 apparatus vary depending upon cellular pH (164). This finding is likely to explain some of the difficulties in studying individual ESX-1 components and discrepancies in their sub-cellular localization between studies.

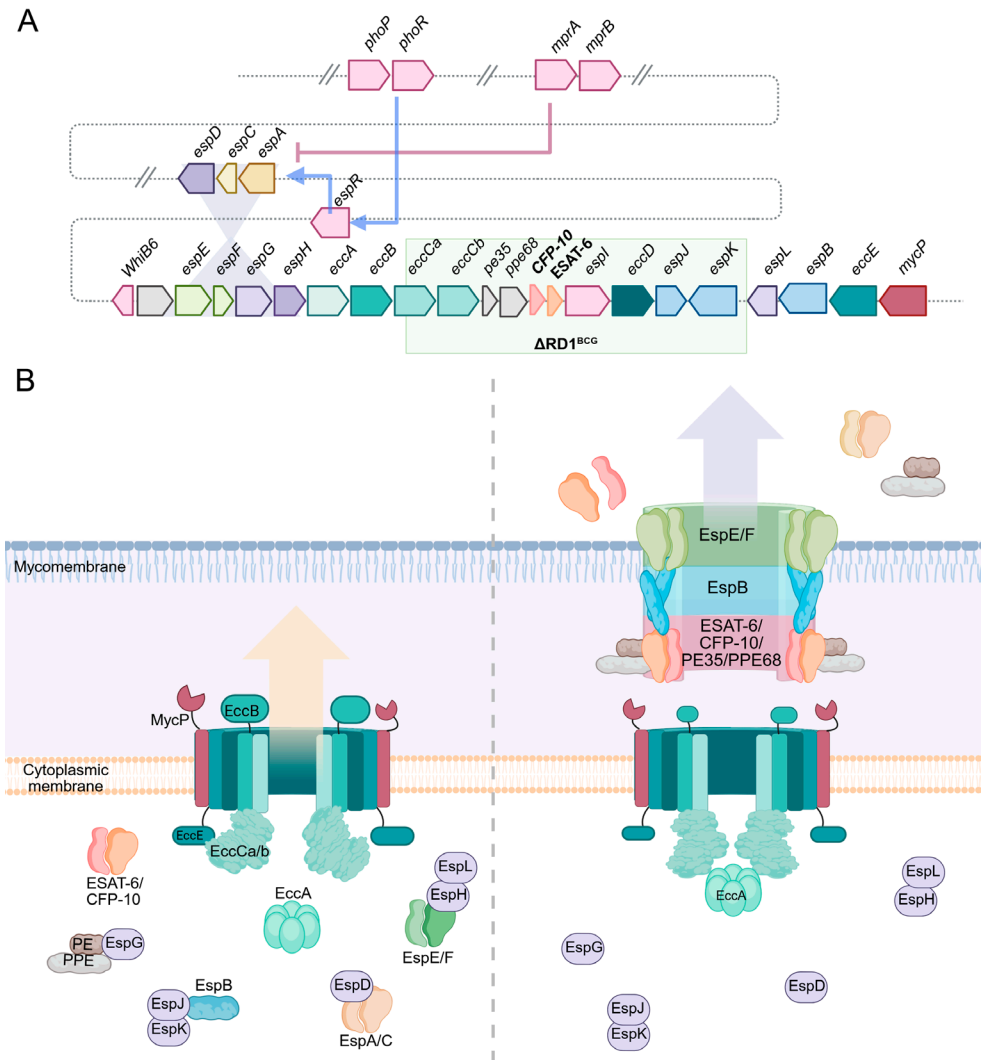



**Figure 7. Overview of the ESX-1 T7SS secretion system of *Mtb*.**
A. Schematic representation of the genes involved in the ESX-1 secretion system and some of thei important regulators. B. Overview of the structure and assembly of ESX-1. CFP-10: 10 kDa Culture Filtrate Protein, Early Secreted Antigenic Target 6 kDa.

*ESAT-6 function in virulence: the continuation of a saga*

Initial studies identified the ESAT-6 protein as a pore-forming toxin which was directly responsible for *Mtb*'s capacity for permeabilizing host membranes (156, 165). The lytic activity described in some of these studies utilizing purified ESAT-6 protein was later attributed to residual detergent from protein isolation, and not ESAT-6 itself (166, 167). Genetic studies have since found that the disruption of ESAT-6 secretion alone is not sufficient to prevent phagosomal escape in *M. marinum*, calling the exact role of ESAT-6 in causing membrane permeability into question (168). As phagosomal escape by *Mtb* requires the perturbation of host membranes for subsequent modulation of the host immune response, significant efforts have been made to understand the exact mechanisms of host membrane disruption (169, 170). Recently, several groups have proposed that the lytic activity of ESAT-6 may work in concert with secreted surface-exposed PDIM and PGL molecules to insert into, and destabilize, the host membranes (117, 171–173).

***Other ESX systems***

ESX-2 and ESX-5 are found only in slow-growing mycobacteria and are thought to be involved in adaptation to conditions of limited nutrient availability. The ESX-5 in *Mtb* secretes numerous PE/PPE genes and is involved in fatty acid transport (143). The secretion of PPE25 by the *M. avium* ESX-5 system was described to block host phagosome acidification, a process essential for intracellular growth (174). ESX-4 has recently been described to facilitate secretion of the TNT toxin in *Mtb* (175), as well as the effector proteins EsxU/T *M. abscessus*. EsxU and ESxT function analogously to *Mtb* ESAT-6 and CFP-10 and influence CD4 and CD8 T cell response during infection (176). ESX-3 is widely present in mycobacteria and is involved in siderophore-mediated iron and zinc uptake (144). In *Mtb,* ESX-3 is facilitates secretion of the effector proteins EsxG/H (177). ESX-2, however has no known function, and has been lost in the greatly reduced *M. leprae* genome indicating that it is unlikely to be involved in essential nutrient uptake or host cell interactions (143). Related, but structurally divergent, T7SSs have also been identified in to facilitate the secretion of toxins in Gram positive pathogens such as *Staphylococcus aureus* (178).

# Adaptation to an environment in flux

*"Change is the only constant in life."* – **Heraclitus ~500 B.C.**

Survival of an organism in any ecological niche requires adaptation. NTM live in diverse soil and water environments which vary greatly in terms of microbial competition, nutrient availability, as well as physical and chemical properties. The soil is home to dense communities of bacteria and fungi which produce diverse metabolites, including antibiotic compounds (179). Aquatic environments, in contrast, are more sparsely populated but have variations in oxygen and nutrient availability depending on location and depth (180). Both ecosystems are further subject to fluctuations in factors such as temperature, pH, and salinity (181).

Despite (or rather, perhaps because of!) their simplicity, bacteria are experts in adapting and responding to a wide range of environmental conditions. While lacking the genomic complexity of eukaryotes, bacteria have the advantage of small genomes and rapid generation times. In this way, the genetic 'risk' of an individual cell in acquiring new mutations is spread across the clonal population. Mechanisms for increasing mutation rate in response to cytotoxic stressors, such as the SOS response to DNA damage, have further evolved in bacteria to facilitate the appearance of new, potentially beneficial mutations to increase chances of population survival (182). While the appearance of an adaptive mutation can be advantageous for surviving an acute threat, it can also have long-term consequences for a population.

## Phenotypic heterogeneity and phase variation

An alternative strategy to reliance of repeated *de novo* mutation is the maintenance phenotypic heterogeneity within a population. Unlike fixed mutations, phenotypic heterogeneity is maintained by reversible mechanisms (183). As cells divide, phenotypes may be inherited by the daughter cells, but each new cell retains the capacity to transition to other phenotypes (**Fig. 8**). Heterogeneity can arise by numerous mechanisms- from unregulated, stochastic variation in gene expression, to variation in cellular response to diverse signals (184). Phase variable, or ON/OFF, patterns of gene expression have been recognized as a widespread strategy for pathogen adaptation to dynamic host environments (185). The term phase variation encompasses several mechanistically distinct including reversable alterations of DNA sequences, epigenetic base modifications, and altered transcriptional programs (186).

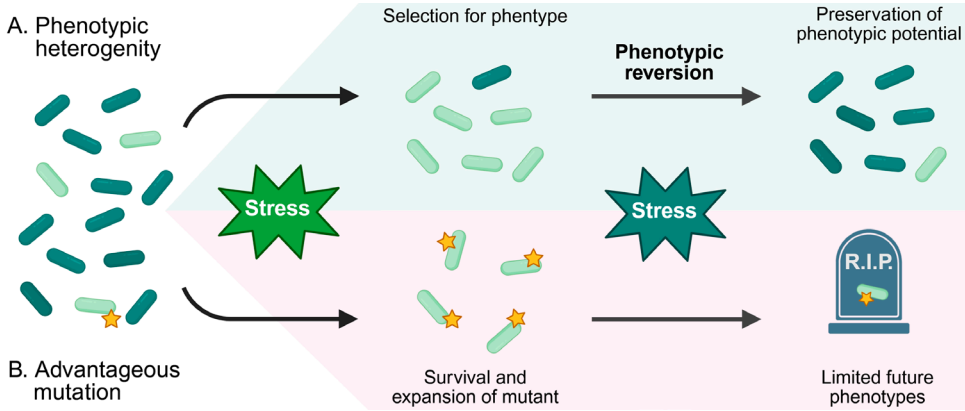



**Figure 8. Simplified Illustration of the effect of phenotypic heterogenity and mutations in a bacterial population.**

*Reversible genetic variation*

At the genomic level, two broad mechanisms of reversible phase variability have been described: microsatellite variation and genomic variation due to recombination or mobile genetic elements (MGEs). Both microsatellite regions and MGEs are common in bacterial genomes and rely upon the inherent instability of repetitive DNA sequences. The high prevalence of repetitive regions in bacterial genomes has been theorized to have arisen as a means of facilitating rapid environmental adaptation (187, 188).

Microsatellites, or short, repetitive DNA sequences, are intrinsically more unstable and subject to a higher likelihood of errors during DNA replication and repair (189). Genetic stretches with numerous simple sequence repeats (SSRs) of 2-6 bp motifs or lengths of single nucleotide homopolymeric tracts (HTs) induce genetic variation due to "slipping" of the synthesis and template strands during DNA replication or repair. When polymerase enzymes reassemble at an incorrect (but identical) site, addition or deletion of nucleotides occurs (188). Accumulation of repeats in promoter binding regions can alter or completely silence downstream gene or operon expression (190). In *Streptococcus pneumoniae* and *Hemophilus influenzae*, the expansion of HT repeats upstream of key virulence proteins over time results in decreased expression of immunogenic surface proteins facilitates rapid adaptation to the onset of the adaptive immune response (191, 192).

Microsatellites can also be present within coding sequences, where a single base alteration can result in a shift in reading frame and complete protein disruption. Indeed, HTs present in coding regions are more likely to be found in the 5' end of the gene sequence, resulting in an early frameshift or stop codon, more profoundly effecting the resulting protein (185). Rapid HT variation has been described in *Listeria monocytogenes* where a truncated host-invasion protein is associated with

spoiled food and full-length proteins were over-represented in farm animals, suggesting a role for phenotypic heterogeneity in underlying niche adaptation (193, 194).

Highly repetitive genomic regions are “hot-spots” for recombination events which can result in phase-variable gene expression (189). The genetic regions affected by rearrangements can be as small as single gene or operon (195) or larger genomic regions exceeding 200 kb (196). When a genetic locus is flanked by repetitive sequences, recombinases such as RecA can cause gene inversion, duplication, or excision. RecA is normally involved in the repair of damaged DNA through homologous recombination. However, when RecA acts on a genetic region flanked by repetitive sequences, there is an increased likelihood for the mispairings resulting in rearrangement (197). RecA activity is upregulated during the bacteria SOS response to mitigate DNA damage due to cellular stress, and as a result, RecA-mediated genetic rearrangements increase in frequency during environmental challenges.

Other recombinases such RecV found in *Clostridioides difficile* (198), or transposases associated with MGEs (199) similarly mediate rearrangements at repetitive sites but through site-specific mechanisms. Insertion sequences (IS elements) are the simplest form of MGE, consisting of a transposase gene flanked by short, inverted repeats. In contrast, transposons contain additional genes which follow during transposition. (199). Repeated cycles of insertion, recombination, and deletion contribute to long-term genome remodeling. In many bacteria, including *Mtb*, MGE proliferation is associated with reductive evolution, niche specialization, and altered pathogenicity (200, 201). The translocation of MGEs can also directly alter gene expression in a reversible manner. For example, multiple copies of IS256 are found in enterococci and staphylococci but are particularly abundant in disease-associated isolates from patients (202–204). In these isolates, insertion/excision events occur frequently in regulatory genes, leading to adaptive phenotypic heterogeneity during infection and antimicrobial treatment.

*Epigenetic variation*

DNA methylation has been well studied in the epigenetic regulation of eukaryotic genomes, however, the regulatory function of methylation in bacterial genomes is only just being uncovered. DNA restriction-modifications (R-M) systems are present in over most bacteria as phage defense systems (205). Through the constant methylation of bacterial DNA by methyltransferases, invading viral can be distinguished by its lack of methylation and degraded by endonucleases. In recent years, however, DNA methylation status has also been identified to influence bacterial gene expression (206).

In *H. influenzae* and *Neisseria* species, R-M systems have been found to control global gene expression during infection. In *S. pneumoniae,* DNA methylation regulates the appearance of 6 distant phase states and various degrees of virulence over the course of infection (192). Additionally, DNA methylation itself can be regulated on several levels. For example, the transcription of DNA methyltransferases has been found to be regulated by SSR variation in upstream promoter regions in *Nisseria* species, adding an additional level of complexity (207).

*Transcriptional regulation*

Transcriptional regulation without underlying genetic or epigenetic changes can also facilitate phase variation. Bacterial two-component systems (TCS) are one such conserved system for bacterial response and adaptation to diverse conditions. TCSs consist of a membrane-bound sensor domain and response regulator protein. Upon detection of a specific signal by the sensor, the response regulator becomes phosphorylated and binds to specific DNA regions, directing transcription (208). While bacterial TCSs are generally characterized as being discrete regulatory systems, in the 12 TCSs described in mycobacteria, significant sensor/regulator cross talk has been observed (209). In particular, the MtrB sensor proteins which has been observed interact with five response regulators in addition to its partner MtrA, a finding which may partially explain the involvement of MtrAB in diverse cellular processes (106, 210)

Other examples of transcriptional regulators include inducible TetR family regulators which at best described in regulating cellular responses to antibiotics (211). In *Acinetobacter baumannii* a temperature-sensitive TetR type transcriptional repressor has been found to drive a switch between a virulent phenotype and a proposed environmentally adapted phenotype (212). Until recently, the intra-host smooth-to-rough conversion colony switch in *M. abscessus* was thought be an evolutionary "dead-end" where mutations resulting in increased virulence limit environmental transition (129). A recent study, however, identified the possibility for reversible phenotypic switching mediated by a TetR transcription factor regulating GPL production (213).

These broad mechanisms of producing population heterogeneity (summarized in **Table 2**) are not mutually exclusive. A clonal population may in fact have a combination of a number of these mechanisms. Ongoing advances in single cell RNA sequencing (scRNAseq) technologies adapted to RNA-poor bacterial cells have great potential for elucidating the true magnitude of phenotypic heterogeneity both at a steady state as well as upon exposure to antimicrobial agents or during infection (214–216).

**Table 2. Examples of phase variation in human pathogens and their underlying mechanisms.** SSR: simple sequence repeats, HT: homopolymeric tract, IS: insertion sequence, RM: restrisction modification, TCS: two component system.

| Source of variation | Level of regulation | Mechanism | Examples |
|---|---|---|---|
| Microsatellite variation | DNA | SSR | *H. influenzae* (190) |
| | | SSR | *Neisseria spp.* (207) |
| | | HT | *L. monocytogenes* (194) |
| | | HT | *M. tuberculosis* (217) |
| Genomic rearrangement | DNA | RecV | *C. difficile* (195) |
| | | RecA | *B. thailandensis* (196) |
| | | IS256 | *S. aureus* (170) |
| | | IS256 | *E. faecalis* (202) |
| DNA methylation | Methyltransferase activity | Type III RM | *Neisseria spp.* (164) |
| | | Type I RM | *S. pneumoniae* (192) |
| Transcriptional regulation | RNA | TetR-like repressor | *A. baumanii* (212) |
| | | TetR (Gpl1) | *M. abscessus (213)* |
| | | TCS (5 RRs) | *S. pneumoniae* (218) and |
| | | TCS (PhoPR) | *S. typhimurium* (219) |

*Phenotypic heterogeneity in Mtb*

*Mtb,* have low background mutation frequency and low levels of horizontal gene transfer (220, 221). Despite this, *Mtb* are able to adapt to a wide range of conditions encountered over the course of infection. Phase variable expression of metabolic and virulence-associated genes is increasingly recognized as an underlying factor complicating complete bacterial eradication during TB treatment (184). Within the lungs of a single individual, pH, oxygen levels, and nutrient availability vary both spatially and temporally (222, 223). Bacteria within a stable granuloma are restricted by surrounding immune cells, whereas bacteria found in the caseum of necrotic lesions are more often extracellular and have increased access to nutrients from dead immune cells (224). During active TB disease, bacteria escaping unstable lesions are subsequently phagocytized by new immune cells and must once again adapt in order to survive and propagate in new conditions.

The researchers Vargas *et. al* recently identified the parallel evolution of microsatellite variation in diverse *Mtb* isolates. In this study, 45 conserved variable regions were identified across all major *Mtb* lineages, many of which were found to impact genes involved antibiotic resistance and ESX-1 activity (217). One variable HT region impacting transcription was found upstream of the *espACD* operon, which is essential for full ESX-1 secretory function. This locus is further under the control of both the two-component systems MprAB and PhoPR, as well as the DNA-binding protein EspR (143). Adding yet another layer of complexity, the transcription of *espR* itself has been found to be influenced by HT variation over the course of infection (225). The abundance of these variable phase sites in key

virulence genes suggests that *Mtb* must carefully balance ESX-1 expression during infection, similar to the cyclical expression patterns of highly antigenic virulence factors in other pathogens (190, 218).

This study, and others, additionally found numerous instances of reversible metabolic shifts impacting central metabolism (217, 226, 227). Single nucleotide insertions resulting in an inactivating frameshift in the HT within the *Mtb* glycerol kinase *glpK* have been observed to appear during both human and mice infection (226) Bacteria with non-functional *glpK* have decreased growth rates and heightened antimicrobial resistance, similar to so-called persister cells (228).

*Morphological and phenotypic variation in MAC*

In 1933, when investigating the "avian tubercule bacillus", the researchers Winn and Petrov described the bacterial colonies to have predominantly flat and spreading smooth-transparent (SmT), or larger and domed-shaped smooth-opaque (SmO) morphology (**Fig. 9**). A further minority of dry, rough (Rg) colonies, resembling *Mtb* in morphology were additionally found (229). Already then, the researchers connected this visible morphological variation to distinct virulence patterns in chickens based on crude variations in leukocyte recruitment upon infection. They, like future investigators, found that SmT bacteria were associated with more severe disease.

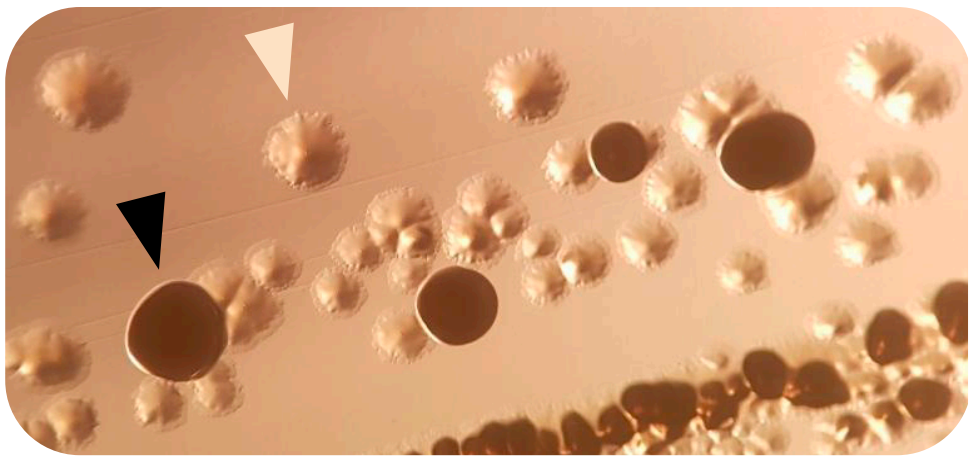


**Figure 9. Morphological variation in MAC colony apperience**
Representative SmT (black arrow) and SmO (white arrow) colony morphologies exhibited by *M. avium* ssp *hominissuis strain* LU439, described in **Paper I** of this work.

Later studies of members of the now-recognized *Mycobacterium avium* complex found that, regardless of isolation source, bacteria with SmT morphology were associated with increased virulence in cell culture and animal models of infection (230–232) and heightened antimicrobial resistance (233, 234). Unlike the appearance of rough colonies, which occur spontaneously to mutations, SmT and SmO morphotypes have been found to inter-convert (123). The researchers Kansal et. al found that upon infecting mice with SmO MAH, as early as 8 days post infection, SmT colonies were recovered from tissue. By day 35 significant bacterial proliferation in the tissue had occurred and 100% of recovered bacteria from the lungs, spleen and liver were described as having a SmT morphology (235). This observation suggests that MAH undergo phenotypic switching to a virulent and persistent infection-associated morphology during the course of infection. In contrast, under laboratory culture conditions, the spontaneous SmT-to-SmO conversion occurs several orders of magnitude more frequently than the reverse transition (236, 237). Together, these observations suggest that each MAC morphology may provide distinct advantages under different conditions, however they fail to differentiate phenotypic switching on a cell level from phenotypic selection on a population level or identify mechanisms underlying this switch. To address these open questions, **Paper I** of this thesis investigates the mechanisms underlying virulence-linked phenotypic and morphological variation found in MAH using genomic and transcriptomic approaches.

Interestingly, descriptions of reversible SmO and SmT morphologies have also been made in phylogenetically distant NTM, including the slowly growing species *M. malmoense* (238) and *M. celatum* (239), as well as the rapidly growing species *M. fortuitum* (240). These colony phenotypes remain almost entirely uncharacterized, but their further study may identify conserved cellular processes involved in environmental adaptation and pathogenesis.

# III. The innate immune system and inflammation

The innate immune system represents one of the first lines of defense against invading pathogens. Innate immune cells surveil the body and neutralize identified microbial threats or compromised cells. Macrophages and dendritic cells (DCs) are present in tissue environments at a steady-state, and enriched in mucosal barriers, positioned to phagocytize and degrade harmful microbial intruders. DCs are further specialized to migrate and present processed foreign antigens to T lymphocytes, facilitating the coordination of the adaptive immune response. Neutrophils and monocytes circulate through the bloodstream and are recruited to sites of infection by chemokines. Neutrophils in particular are involved in the destruction of foreign threats, producing an arsenal of antimicrobial compounds. Recruited monocytes can become further differentiated into macrophages and DCs depending on local stimuli to contribute to the local immune response. Natural killer (NK) cells are innate lymphocytes which, upon detection of non-self molecular patterns during infection or malignancy, eliminate compromised cells. Together these innate leukocytes drive the first arm of the immune response within minutes of pathogen recognition.

## Sensing and responding

### Immune surveillance

An essential characteristic of the innate immune system is the ability of cells to rapidly detect threats and begin orchestrating a fitting immune response. Sensing of bacterial threats by is facilitated by the binding of host pattern recognition receptors (PRRs) to conserved antigens. PRRs recognize conserved proteins which are only found in foreign or damaged cells. Pathogen-associated molecular patterns (or PAMPs) are evolutionarily conserved microbial antigens which are never produced by the host (241). It is worth noting, however, that even non-pathogenic commensal bacteria express identical antigens. As such, alternative terms such as microbial-associated molecular patterns (MAMPs) have been proposed but are less widely used (242).

Many PRRs can also recognize host-derived molecules (so-called damage-associated molecular patterns; DAMPs) found in damaged or dysfunctional cells (243, 244). During infection numerous "danger signals" of both microbial and host origin are produced, and the resulting downstream signaling response influences the outcome of infection. To initiate the right response at the right time, PRRs are distributed in both within the membranes and in the cytosol of cells, with their abundance varying between different immune cell populations as well as over the course of infection (245, 246).

Toll-like receptors (TLRs) are one important class of membrane-bound PRR with 10 receptors described in humans and 12 in mice (241). TLRs are sensitive to a wide range of stimuli and activate varied cytokine responses dependent upon the cellular location of ligand binding and downstream signal transduction (115). TLRs expressed on the cell surface and in the endosomal compartment are crucial in sensing extracellular pathogens and except for TLR3, all TLRs utilize a common MyD88 adaptor protein for signal transduction, resulting in expression of pro-inflammatory cytokines. Some TLRs such as TLR9 and endosomal TLR4, can also contribute to type I IFN production (246). Other membrane-bound PRRs include C-type lectin receptors (CLRs), such as the mannose receptor and MINCLE which are essential in triggering phagocytosis of extracellular mycobacteria (121).

Cytosolic PRRs, in contrast, detect signs of intracellular infection or cellular dysfunction. These receptors respond to molecules which are normally not present in the cytosol including nucleotides resulting from viral infection or host DNA leaking from the mitochondria or nucleus due to damage (247). The presence of cytosolic DNA activates sensors such as cGAS, which generates cGAMP to stimulate STING, leading to type I interferon production (248). Similarly, cytosolic RNA is detected by RIG-I-like receptors (247). Activation of these cytosolic nucleotide sensors results in the induction of a cellular antiviral response including the production of type I IFNs. Intracellular bacteria can also be detected by cytosolic NOD-like receptors (NLRs) such as NOD2 which recognizes muramyl dipeptides from shed PG molecules (249).

The concerted efforts of the innate immune system further rely on communication between cells via cytokine and chemokine signaling. Upon sensing PAMPs and/or DAMPs, sentinel cells secrete signaling molecules to recruit additional cells to the site of infection and coordinate an appropriate antimicrobial response. Successful immune control of TB and other mycobacterial infections requires a finely tuned inflammatory response. A summary of PRRs involved in response to mycobacterial infection can be found in **Table 3.** Two important responses will be described here in more detail: the inflammasome mediated secretion of active IL-1β and the induction of type I IFNs.

**Table 3. Summary of membrane-bound PRRs involved in mycobacterial infection.** DGR1: Dendritic Cell Natural Killer Lectin Group Receptor-1, ER: endoplasmpic reticulum, GPL: glycopeptidolipids, HMGB-1: High mobility group box 1 protein, IFN: interferon, LM/LAM: Lipo(arabino)mannan, MINCLE: Macrophage Inducible C-type Lectin, MR: mannose receptor, PG: peptidoglycan. Adapted from (243, 244, 247).

| Receptor | Bacterial Ligand | Host Ligand | Location | Downstream response |
|---|---|---|---|---|
| TLR 2 (dimerizes with TLR 1/6) | GPL, PG, Lipoprotein, LM/ LAM | β-defensin, HMGB-1 | Extracellular | Pro- inflammatory cytokines |
| TLR 4 | Mannose- capped LAM | Neutrophil elastase, Lactoferrin. HMGB-1 | Extracellular | Pro- inflammatory cytokines |
| | | | Endosome | Type I IFNs |
| TLR 9 | Microbial CpG DNA | Mitochondrial CpG.DNA | Endosome | Pro- inflammatory cytokines Type I IFNs |
| CLRs | Manose (MR), TDM (MINCLE) Glycans (Dectin-1) | Histones (Clec2d) F-actin (DGR-1) | Extracellular | Pro- or anti-inflammatory cytokines |
| NOD2 | PG | ER Stress | Cytosol | Pro-inflammatory cytokines, Type I IFNs |
| cGAS | Microbial DNA | Self DNA | Cytosol | Type I IFNs |
| RIG-I | Microbial RNA | Self RNA | Cytosol | Type I IFNs |
| AIM2 | Microbial DNA | Self DNA | Cytosol | IL-1B, IL-18, and Gasdermin D activation |
| NLRP3 | Bacterial toxins | K+ efflux, Cytosolic Ca+ , lysosome and mitochondrial damage | Cytosol | IL-1B, IL-18, and Gasdermin D activation |

## IL-1β and the inflammasome

IL-1β is a tightly regulated and pro-inflammatory cytokine, which is essential in the control of diverse bacterial infections. IL-1β is a potent pyrogen, mediating the onset of fever, vasodilation, and immune cell recruitment to the site of infection (250). In macrophages, two signals are required for the secretion of active IL-1β. The first, or priming, signal is initiated by PRR detection and signaling, resulting in the transcription of pro-IL-1β, and protein components of the inflammasome complex (251). A second activating signal, such as ion influx, cytosolic cathepsin B, ROS, or mitochondrial DNA, is required to trigger the assembly and activation of the

inflammasome. Inflammasome activation results in cleavage of pro-caspase 1 into its active form which in turn cleaves and activates pro-IL-1β and pro-IL-18. Inactive gasdermin D proteins are also cleaved by inflammasome activity. When activated, gasdermin D oligomerizes and insert into the plasma membrane, forming pores in the plasma membrane. Formation of these membrane pores, however, disrupts cellular homeostasis and results in an immunogenic and inflammatory form of cell death known as pyroptosis (252). Extracellular IL-1α and IL-1β are recognized by the IL-1 receptor (IL-1R1) present on both immune and non-immune cells, resulting in additional NF-κB- and MAPK-driven inflammatory gene expression (**Fig. 10**). The detection of IL-1α and IL-1β impacts the differentiation of nearby cells and is a signal for the recruitment of further leukocytes, such as neutrophils and CD4+ T cells (251, 253).

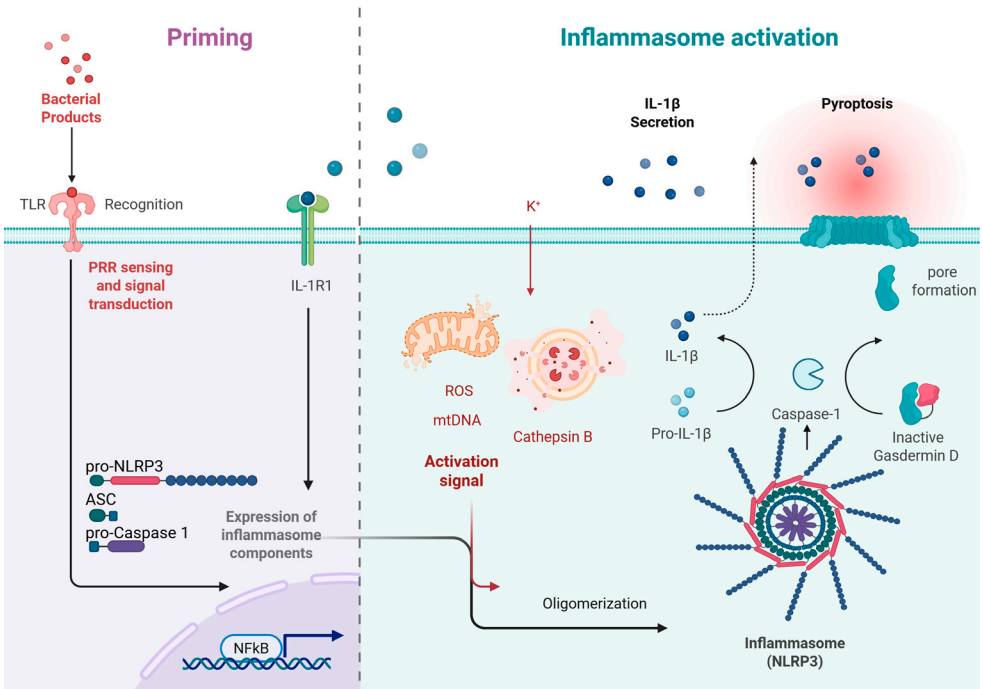



**Figure 10. Overview of the NLRP3 inflammasome assembly and secretion of active IL-1β.**
ASC: Apoptosis-associated Speck-like protein containing a CARD (caspase recruitment domain), IL-1R: IL-1 receptor, NF-κB: Nuclear factor kappa B, NLRP3: NLR family pyrin domain containing protein 3, PRR: patern recognition receptor, TLR: toll-like receptor.

At the same time, excessive IL-1β secretion during infection results in uncontrolled inflammation and pathological neutrophilic influx (254, 255). During the inflammatory response, additional IL-1 family proteins are produced to regulate inflammation including the IL-1 receptor antagonist (IL-1Ra) and a decoy IL-1 receptor (IL-1R2) proteins. These proteins bind IL-1R1 without inducing

downstream signaling and competitively bind IL-1α/IL-1β, limiting cytokine availability for detection by IL-1R, respectively, to dampen the uncontrolled propagation of inflammation (256).

During *Mtb* infection, mice unable to produce for IL-1α/IL-1β (257) or lacking the IL-1 receptor (251, 258) display greatly increased disease and mortality, indicating the essential role of this signaling pathway in mounting a protective immune response (257). While individuals deficient in IL-1 production have been found to have a higher risk for NTM-PD (259, 260), the role of IL-1β in NTM infection remains overall poorly understood. Interestingly, pro-inflammatory cytokines such as IL-1β have been described to be highly induced during infection with avirulent, but not virulent MAH, however mechanistic details of this phenomenon are lacking (261–263). It additionally remains unclear what role this differential IL-1β secretion has on the trajectory of infection *in vivo*. As such, a major aim of **Paper I** in this thesis is to describe the role of early inflammasome activation during the establishment of MAH pulmonary infection.

## Type I IFNs

Type I IFNs are another important family of cytokines which play a key role in response to microbial threats. Named for their role in interference of viral replication, Type I IFNs coordinate the expression of over 100 interferon stimulated genes (ISGs) and are required for an effective antiviral response. Though essential for responding to viral infection, the type I IFN response results in variable outcomes during bacterial infection (264). In the cases of human TB, blood IFNβ concentration is a widely recognized predictor of progression from stable to active disease (265–267). In contrast, during NTM infection, type I IFNs are generally not induced to a high degree but are associated with bacterial control (268, 269).

Despite the ability of many PRRs to induce type IFNs, the recognition of cytosolic DNA by the cGAS/STING cytosolic surveillance pathway is considered the primary driver of IFNβ production during infection with *Mtb* (270–272) and *M. marinum* (168). Upon recognition of cytosolic DNA, cGAS produces the secondary messenger cyclic GMP–AMP (cGAMP) which in turn interacts with the ER-bound STING protein. Activated STING then recruits TBK1 which phosphorylates the transcription factor IRF3, enabling its translocation to the nucleus where it triggers IFNβ production. Secreted, extracellular type I IFNs then signal through the IFNAR receptor resulting in amplification of downstream interferon-stimulated gene (ISG) expression (**Fig. 11**) (273).

During experimental infection with virulent *Mtb*, IFNAR-deficient mice exhibit decreased bacterial burden and do not succumb disease, indicating that type I IFNs drive a maladaptive immune response (265). The host-detrimental role of type I INFs is further exemplified by the hyper-susceptibility of C3HeB/Fej mice to Mtb

infection. Unlike other mouse lines, C3HeB/Fej mice develop necrotic lung granulomas similar to those seen in human TB, a trait determined by variation in a single genetic locus (274). During infection congenic B6 mice with "super susceptibility to tuberculosis 1" allele (*sst1*$^{S}$) display heightened IFN-β production, as well as increased neutrophil influx and tissue damage (275). Hyper-induction of IFN-β in these mice leads to a heightened abundance of IL-1Ra which suppresses protective IL-1β production (255, 276).

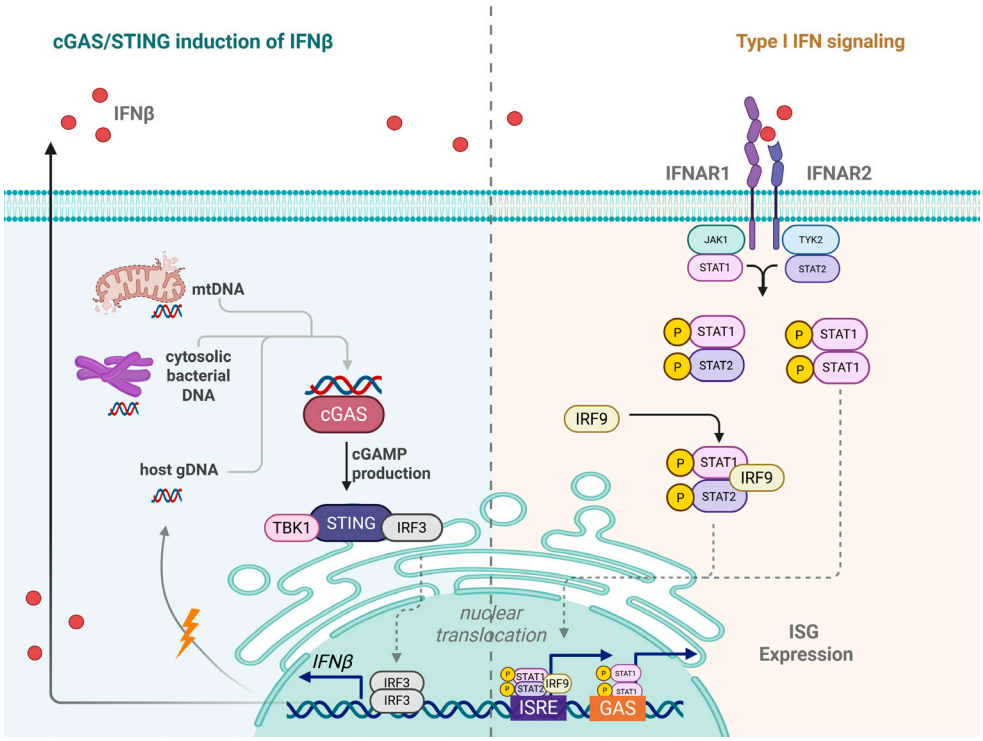



**Figure 11. Overview of cGAS/STING signalling (left) and induction of interferon stimulated genes (ISGs) after IFNβ detection by IFNAR 1/2 (right).** cGAS: cyclic GMP-AMP synthase, cGAMP: Cyclic guanosine monophosphate–adenosine monophosphate, GAS: gamma-activated site, IFNAR1/2: Interferon Alpha and Beta Receptor Subunit 1/2. IRF: interferon regulatory factor, ISRE: Interferon-sensitive response element, JAK: Janus kinase, STING: Stimulator of interferon genes, STAT: signal transducer and activator of transcription proteins, TBK1: TANK-binding kinase 1. Adapted from (264, 273).

# Innate immune cells during mycobacterial infection

> *"Disease would be a fight between the morbid agent, the microbe from outside, and the mobile cells of the organism itself. Cure would come from the victory of the cells and immunity would be the sign of their acting sufficiently to prevent the microbial onslaught."*
>
> **-Ilya Mechnikov, 1908** (277)

Much of the success of mycobacteria as human pathogens lies in their ability to subvert the immune system (10). When recognized and taken up host phagocytes, pathogenic mycobacteria are often not killed and can instead persist and utilize their host cells as a source of nutrients and as a replicative niche.

## Macrophages and monocytes

Macrophages are highly heterogenous immune cells which are abundant at mucosal surfaces. Macrophage phenotypes are influenced both by their origin and the local conditions they encounter (278). During normal conditions, the majority of macrophages in the lungs are self-renewing tissue resident alveolar macrophages (AMs) which are derived from the fetal liver. AMs act as sentinels in the normally sterile lower airways phagocytizing foreign bodies and are the initial host cells for mycobacteria (279). *Mtb*-infected AMs are permissive to bacterial growth and have been found to migrate to the lung interstitium in an ESX-1- and IL-1β- dependent manner where they serve as an initial replicative niche (218). AM metabolism relies preferentially on fatty acid oxidation, resulting in increased cellular lipids and a nutrient replete for infecting mycobacteria (280).

The relative abundance of permissive tissue resident AMs decreases over time during infection, as monocytes are recruited from the bloodstream and bone marrow (281). Sustained inflammation results in enrichment of monocytes in the affected tissue where they are a major source of effector molecules such as nitric oxide (NO) (282, 283). Recruited monocytes further differentiate into resident, interstitial macrophages (IMs). In contrast to AMs, IMs represent a more restrictive environment for intracellular pathogens due to their shift towards glycolytic metabolism. This metabolic shift is associated with increased production and inflammatory cytokines and augmented phagosomal acidification (281).

Both tissue resident macrophages and recruited monocyte-derived macrophages can be further influenced to express distinct inflammatory programs, often referred to as

M1 and M2 phenotypes. M1 (or classically activated) macrophages characterized by their increased phagocytic capacity and production of pro inflammatory cytokines via MyD88 signaling or stimulation with IFN-γ. In contrast, M2 (or alternatively activated) macrophages express anti-inflammatory cytokines and are associated with the resolution of inflammation and tissue repair functions (284). AMs are often described to exhibit an M2-like phenotype due to their essential role in recycling lung surfactant and maintaining airway homeostasis, however upon encountering pathogens, they can adopt a pro-inflammatory phenotype (285). These activation phenotypes are well-studied in *in vitro* systems but are likely to underestimate the level of cellular heterogeneity *in vivo*. Studies utilizing single cell RNA sequencing (285, 286) and cell sorting approaches (287, 288) to investigate individual cells during infection have identified both protective and permissive macrophage phenotypes based on transcriptional profiles and infection status.

## Neutrophils

Neutrophils, or polymorphonuclear monocytes (PMNs), are the most abundant human immune cells, making up 90% of the leukocytes in the bloodstream in humans. Circulating neutrophils are rapidly recruited to sites of injury or infection by recognition of chemokines and are essential in the control of many bacterial infections. Individuals with impaired neutrophil function are hyper-susceptible to bacterial infections, highlighting the essential role these cells play in host defense (289). At the same time, excessive neutrophil recruitment is associated with pathological inflammation in infections such as TB (267) and SARS-Cov-19 (290), as well as in COPD and acute lung injury (289, 291).

Like other phagocytes, neutrophils recognize and take up microbial threats and eliminate them through the induction of intraphagosomally generated reactive oxygen and nitrogen species (ROS and RNS). Neutrophils, however, also express myeloperoxidase which converts superoxide ions into hypochlorous acid, a more stable and antimicrobial molecule (292). Neutrophiles form three distinct types of intracellular granules (azurophilic, specific, and gelatinase) as well as secretory vesicles, each of which contain numerous proteins exhibiting bactericidal activities. Upon stimulation by bacterial PAMPs, neutrophils additionally produce neutrophil extracellular traps (NETs) composed of host DNA, histones and granule contents. This cellular response is also frequently referred to as NETosis, as the end result of this process results necessarily in cell death (293). These responses, however, are largely ineffective for the control of mycobacterial infections.

During pulmonary MAC infection, excessive NET production by degranulating neutrophils, has been associated with lung pathology (294). Further, several clinical studies have found overall neutrophilic content of patient bronchoalveolar lavage (BAL) fluid to correlate with NTM-PD severity and treatment outcome (295, 296). Despite the implication of neutrophils in many aspects of mycobacterial lung

disease, the exact mechanisms responsible for dysregulated inflammation and pathology have yet to be fully elucidated. Similarly to the heterogenous phenotypes found in macrophages and monocytes *in vivo*, recent studies have begun to describe the varied phenotypes displayed by neutrophils during mycobacterial infection (216, 297, 298). **Paper III** of this thesis provides further insight into the role of the ESX-1 T77S in influencing neutrophil recruitment and phenotype during *M. marinum* infection.

## The life and death of infected cells

> *"Death is not the opposite of life, but an innate part of it."*
>
> – **Haruki Murakami**, *Norwegian Wood*

To prevent the uncontrolled spread of pathogens during infection, often the best thing an infected cell can do is die. *How* cell death occurs, however, has a profound effect on the progression of infection. While numerous cell death pathways have been described they can be broadly grouped into regulated forms, such as apoptosis, and unregulated or "accidental" forms such as necrosis. Much work has been done in recent years to describe specific routes of cell death induced during mycobacterial infection in order better understand the underlying factors develop host-directed therapies (299).

Apoptosis is a type of programmed cell death where still-intact cells undergo a tightly controlled process resulting in the non-inflammatory destruction of the cell (252). Apoptosis is driven by so-called executioner caspases which are activated in response to the loss of mitochondrial membrane potential. Cells undergoing apoptosis appear to shrink and form so-called membrane "blebs" during which phosphatidylserine (PS) membrane lipids become inverted and abnormally exposed on the cell surface (252). Phagocytes recognize exposed PS and degrade damaged, but intact, cells thus minimizing further inflammation in a process known as efferocytosis (300). Efferocytosis of While this *Mtb*-infected cells has been At the same time, it has been suggested that host cell apoptosis promotes bacterial spread to new host cells (301), Similarly, apoptosis of *M. avium* infected cells appears to be host protective. Indeed, the induction of the apoptosis inhibitor of macrophages protein (AIM) produced during infection acts to prolong host cell survival and is found to contribute to chronic *M. avium* infection (302, 303).

In contrast, necrotic cell death, or necrosis, occurs when mechanisms of programmed cell death fail to be induced or are insufficient to contain cellular

damage (252). During necrosis, cellular contents are released rapidly from ruptured cell membranes, many of which are inflammatory DAMPs and triggering additional immune cells to the site of infection. Necrotic cell death can further classified into several modalities, some with a degree of regulation reflecting that not all necrosis is purely accidental. For example, necroptosis is driven by RIPK signaling, pyroptosis results from gasdermin-mediated pore formation, and ferroptosis occurs due to excessive lipid peroxidation and catastrophic membrane damage (304).

In *Mtb* infection, apoptosis is generally associated with the control of infection, while necrosis is the result of rapid bacterial growth upon failure of immune control (305, 306). Pathways such as pyroptosis (254, 307) and necroptosis (308) have been identified as important modalities of necrotic cell death occurring during *Mtb* infection *in vivo.* Other studies have observed that excess type I IFN production by *Mtb* infected cells results in a distinct mode of cell death not consistent with previously described pathways (305).

*Oxidative stress and lipid peroxidation*

During infection, host cells become damaged directly by the invading pathogens, but much of the accumulated cellular damage results from host processes. A common feature in many cell death pathways is the accumulation of reactive oxygen species (ROS). ROS are a byproduct of normal cellular metabolism but are also actively produced by phagocytes to control infection (309). The inability of a cell to resolve heightened levels of intracellular ROS results in the accumulation of damaged proteins, lipids, and DNA, ultimately resulting in cell necrosis (310).

The phagosomal NADPH peroxidase, NOX2 is an important source of antimicrobial ROS during infection (309). After phagocytosing a microbial threat, the NOX2 complex is assembled in the phagosomal membrane and produces highly reactive superoxide ions. This response, though, is inefficient for controlling infection by virulent mycobacteria which are naturally resistant to diverse stressors (311). Pathogens such as *Mtb* and *M. avium* further interfere with host processes such as phagosomal acidification and NOX2 recruitment (312, 313). Mitochondrial damage or passive leakage, peroxisomal dysfunction, and cellular enzymes are additional sources of cellular ROS under normal, as well as pathological conditions (310). The accumulation of free cellular iron acts as a further accelerator of ROS-mediated damage due to Haber-Weiss and Fenton reactions occurring in the cytosol. These reactions generate hydroxyl radicals, which are highly unstable and readily oxidize the polyunsaturated fatty acids which are abundant in cellular membranes (314). The accumulation of lipid hydroperoxides (LOOH) destabilizes the membrane structure and, when not detoxified by enzymes such as GPX4, accumulate to levels that trigger plasma membrane rupture and cell death (315) (**Fig. 12**). **Paper III** of this thesis further investigates role of host processes involved in the process of ESX-1 dependent host membrane disruption seen during *Mtb* and *M. marinum* infection.

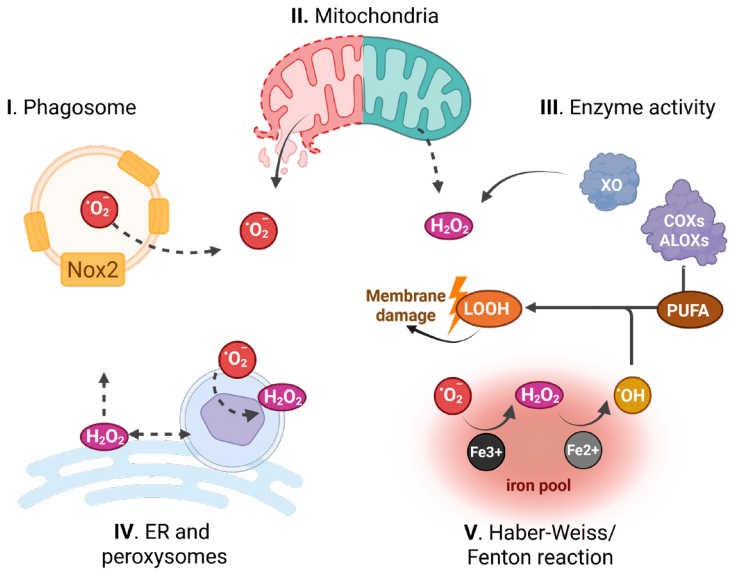



**Figure 12. Overview of cellular sources of reactive oxygen species.**
ALOX: Arachidonate lipoxygenase, COX2: cyclooxygenase, LOOH: lipid hydroperoxide, Nox2: NADPH peroxidase 2, PUFA: poly-unsaturated fatty acids, XO- Xanthine oxidase.

Recently, the term ferroptosis was introduced to describe one specific mode of ROS-mediated, necrotic cell death resulting from lipid peroxidation, and catalyzed by excessive cellular iron. In addition to being implicated in numerous pathologies such as cancer and neurodegeneration, the induction of ferroptosis has been identified as a significant factor involved in *Mtb* pathogenesis (315, 316). *Mtb* infection results in inflammation and oxidative stress as well as dysregulated immune cell metabolism (288, 317). Ferroptosis is characterized by host membrane disruption due to peroxidized lipids and high levels of cellular iron- two molecules which are present in increased amounts in granulomas during *Mtb* infection (316, 318, 319).

## The granuloma

Granuloma formation is a hallmark of both TB and NTM pulmonary disease (92, 320, 321). Granulomas arise from the sustained aggregation of immune cells which surround and attempt to contain a foreign threat. The maintenance of a stable granuloma has historically been considered a protective host response which limits bacterial growth and protects the uncontrolled spread infection. At the same time, mycobacteria have numerous mechanisms to resist immune clearance, enabling their long-term persistence within these structures (184, 223). The tuberculosis

granuloma consists of several layers with distinct immune cell populations, with a (322). The tuberculosis granuloma is organized into distinct layers. In stable granulomas, the majority of mycobacteria are contained intracellularly in the granuloma core, however disease-associated necrotic granulomas have a caseous, necrotic, core where bacterial containment has failed. Surrounding this core region are concentric zones of infected epithelioid and foamy macrophages as well as multinucleated giant cells. On the exterior of this structure is and outer cuff of T- and B-lymphocytes (**Fig. 13**).

Recent advances in single cell RNA sequencing technologies, such as spatial transcriptomics, as well as multiplex microscopy have made tremendous progress in understanding the complexity of this structure (216, 222, 322, 323). Of note, the cellular microenvironment of granulomas and solid tumors share features which complicate treatment. Both structures result in the exclusion, and exhaustion, of adaptive immune cells, poor drug penetrance, and abnormal tissue remodeling (324). The development of future TB therapies targeting pathological granuloma formation will likely need to target not only the invading mycobacteria but also the environment that supports their survival.

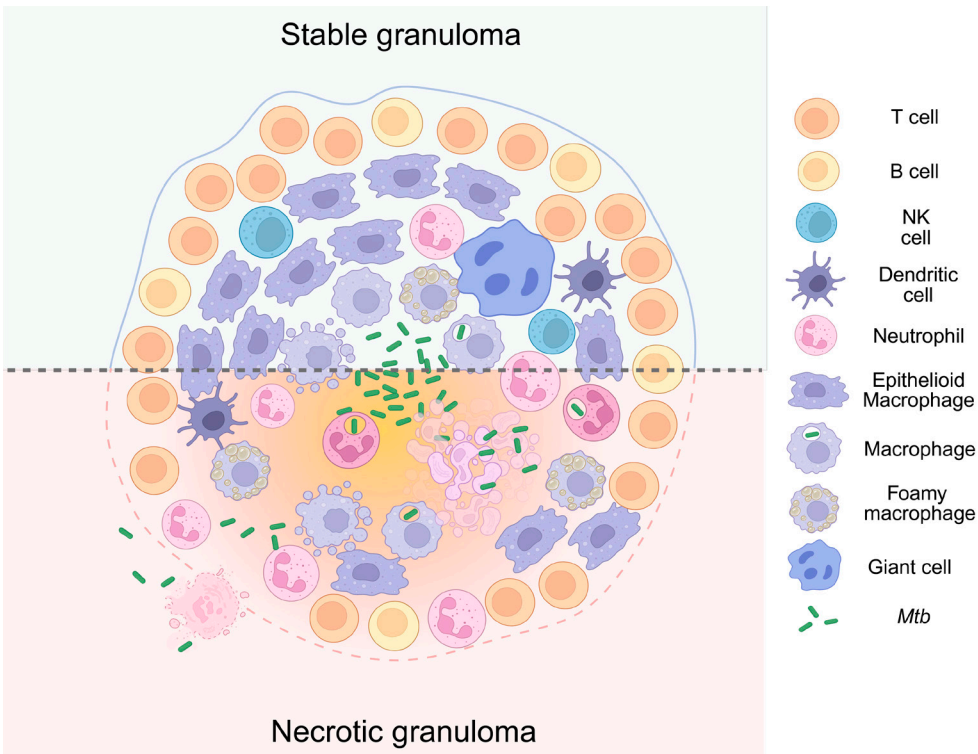



**Figure 13. Structure and cellular composition of stable (top) and necrotic granulomas (bottom).**

# A bridge to adaptive immunity

The innate arm of immune system rapidly responds to a wide range of infections; however, the adaptive immune response is needed for long-lasting, control of infectious agents (325, 326). Antigen presenting cells (APCs) such as macrophages and DCs take up invading bacteria and process microbial antigens to be presented on MHCII molecules. Activated DCs then migrate to draining lymph nodes, where they interact with naive T and B lymphocytes. DCs present processed antigens and provide costimulatory signals needed for T cell priming. At the same time, B cells recognize and bind presented antigens through the B-cell receptor and after the full activation by follicular T cells, they generate antibodies with high specificity to specific microbial threats (327).

Pathogenic mycobacteria, however, have numerous strategies to evade and inhibit the adaptive immune response. Throughout the course of infection, mycobacteria primarily reside within host cells, protected from and circulating antibodies produced by B cells (10). While inside host cells mycobacteria additionally interfere with processes required for initiation of the adaptive immune response such as DC migration and antigen presentation by APCs, resulting in a delayed onset of adaptive immunity (328).

## T Cells

T cells are responsible for facilitating cell-mediated immune responses. In both *Mtb* and NTM infections, CD4+, but not CD8+, T cells are essential for host control of infection (329). CD4+ T cells are a primary source of IFN-γ, a pro-inflammatory cytokine which supports cell-mediated control of infection through the augmentation of antimicrobial responses (330). The essentiality of the CD4+ response in controlling mycobacterial infection is best highlighted by the increased susceptibility to both active tuberculosis and systemic NTM infection seen in HIV+ individuals. During HIV and mycobacterial co-infections, the progression to active mycobacterial disease correlates diminished CD4+ cell counts and loss of immunological control (47, 330, 331). In stable granulomas, IFN-γ-producing T cells are found at the periphery, surrounding the aggregation of myeloid cells (322). The reduced infiltration of these T cells is a key factor underlying the breakdown of the granuloma structure and the progression to active disease in TB patients with suppressed immune systems (330).

## B Cells

B cells drive the humoral arm of the adaptive immune response through the production of antigen-specific antibodies. B cells, similarly, to T cells, are found in the outer cuff region of stable granulomas, but their exact role in the structure is not fully understood (332). Experimental investigations of *Mtb* infection in B cell-deficient mice have found conflicting roles for B cells during infection, indicating a less clear protective roll for these cells (333, 334), in contrast to the severe disease susceptibility observed in T cell deficiency (330) .

In many infectious diseases, the production of antibodies and the maintenance of immunological memory are required for the resolution of disease and preventing re-infection. In the case of *Mtb*, however, the role of antibody-mediated immunity is limited and relatively poorly understood (335). Although exposure to both environmental and pathogenic mycobacteria results antibody production, these antibodies generally do not correspond to protection from infection ref. As a result, identifying robust protective antibodies remains a key challenge in *Mtb* vaccine development (336).

# IV. Results

## Aims of this thesis

The over-arching goal of this PhD thesis was to investigate the molecular mechanisms of virulence in two evolutionary distinct mycobacterial pathogens.

In **Paper I**, a virulent *M. avium* ssp. *hominissuis* clinical isolate was used to identify the bacterial and host factors responsible for the observation of virulence-linked morphological variation in species of the *M. avium* complex.

**Paper II** and **Paper III** utilize an *M. marinum* model system to investigate the underlying mechanisms of ESX-1-mediated virulence on different levels.

**Paper II** evaluated the role of host processes in driving ESX-1 dependent bacterial translocation to the cytosol and subsequent type I IFN induction.

**Paper III** employed a murine model of infection to investigate the role of neutrophils in ESX-1 dependent tissue immunopathology.

# **Paper I**: A cell wall remodeling-dependent morphotype switch in *Mycobacterium avium* differentially regulates lung colonization and tissue persistence.

Numerous studies in *M. avium* and *M. intracellularae* have observed that bacterial colony appearance on agar correlates with distinct virulence profiles. Smooth transparent (SmT) bacteria are over-represented patient isolates (232, 337), display heightened antimicrobial resistance (338, 339) and replicate efficiently in host cells (262, 340). Smooth opaque (SmO) bacteria, however, are less resistant to antibiotics and fail to grow intracellularly. These virulence-linked morphotypes have been described to be reversible, where SmT colonies become enriched after macrophage or mouse infection (235, 262) and SmO bacteria arise after sustained culture in the lab (237, 341). Despite decades of study, no underlying mechanism responsible for morphological variation in smooth *M. avium* has been identified.

Thus, in **Paper I**, we set out to describe and characterize the transition of the disease-associated SmT *M. avium* morphotype to the avirulent SmO form. Specifically, we aimed to address the following questions:

**i.** What is the **genetic basis** for SmT and SmO colony morphotypes *M. avium* ssp. *hominissuis*?

**ii.** How do SmT and SmO bacteria differentially interact with the **host immune system** during infection?

Through complementary genomic and transcriptomic approaches, we demonstrated the essentiality of the MarP/RipA peptidoglycan remodeling pathway in maintaining a virulent SmT phenotype (**Fig. 14**). Long read sequencing of five representative bacterial morphotypes identified no signs of reversible genetic mechanisms associated with variation colony morphology. Instead, when isolating *de novo* SmO bacteria from SmT colonies, we found three independently arising, and non-identical, instances of mutations in the MarP-RipA peptidoglycan modification system. These mutations were associated with a concomitant loss of virulence and sensitization to diverse stressors. Importantly, while these mutations resulted in morphologically "locked" SmOs, they displayed all characteristic traits of SmO bacteria described in other studies. Transcriptomic analyses identified an enrichment in genes involved with cell envelope maintenance and trans-membrane transportation in SmT bacteria. Many of these SmT-enriched genes have been previously reported to be under the control of the MtrAB TCS in *Mtb* (210, 342–344), suggesting a possible role for this system in the regulation of SmT/SmO morphological variation *in M. avium*.

In accordance with other studies, SmO, but not SmT, bacteria induced the secretion of the pro-inflammatory cytokines IL-1β and TNF-α in macrophages. When investigating the interaction of colony morphotypes with the immune system during infection *in vivo*, we identified distinct roles for each morphotype in the establishment and persistence of infection. Surprisingly, SmO bacteria efficiently colonized the lungs at one day post-infection, though they were rapidly cleared from the lungs. SmT bacteria, in contrast, had a greatly reduced capacity for colonization, but could persist in the tissue. Colonization by SmO bacteria, however, was reduced to SmT-like levels infecting mice deficient for inflammasome activation (ASC-/-) or with ablated IL-1 signaling (*via* Anakinra treatment), indicating a requirement for early inflammation in bacterial colonization. To our knowledge, this study is the first to confirm the functional role of NLRP3 inflammasome activation in bacterial morphotype-dependent IL-1β secretion using relevant genetic knockouts.

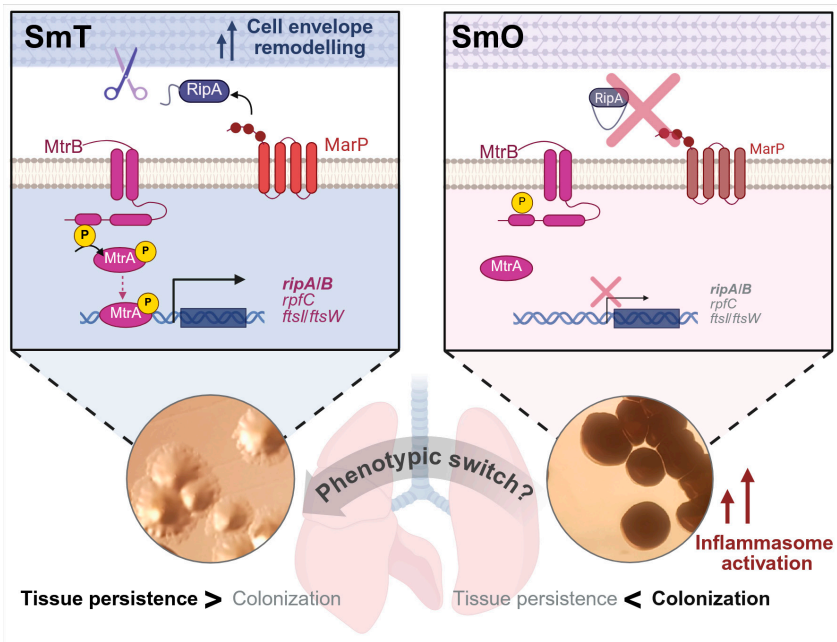



**Figure 14. Working model for Paper I.**

Based on our findings in **Paper I**, we can propose two potential scenarios for the involvement of distinct *M. avium* phenotypes during infection. First, SmO-associated traits may provide an early advantage in host colonization, but persistent infection may require the transition to a SmT phenotype. Alternatively, while SmT bacteria are poorly able to colonize the lungs of healthy individuals, the lungs of individuals with pre-existing inflammation may provide suitable conditions for SmT colonization. Future work utilizing models of chronic lung inflammation or fibrosis would be of great interest to further evaluate these hypotheses.

# **Paper II:** Detection of mycobacterial infection by cytosolic surveillance pathways requires ESX-1-dependent lipid peroxidation of internal host membranes

Since the discovery of the ESX-1 type VII secretion system, significant efforts have been made to understand its role in driving pathogenesis during mycobacterial infection. ESX-1 is a key virulence determinant in *Mtb* and *M. marinum* and is required for the induction host-detrimental type I IFNs in host macrophages (146, 265). Phagosomal escape, and the resulting detection of cytosolic DNA by the cGAS/STING surveillance pathway, is an essential step in the establishment of infection, yet the exact processes responsible remain poorly understood (168, 270). Initially, the secretion of ESAT-6 was identified as the bacterial factor responsible for host membrane disruption (169, 170). Previous work in our lab using *M. marinum* mutants carrying transposon-insertions in several ESX-1 associated genes, however, determined that this is not strictly the case (168). Several of these mutants, including those unable to secrete ESAT-6, could still translocate to the cytosol, suggesting that a yet-unknown factor is responsible for host membrane permeabilization. Thus, in **Paper II**, we investigated potential host processes involved in ESX-1 dependent membrane permeability.

Necrotic cell death results in large-scale membrane disruption and is a downstream consequence of ESX-1-dependent type I IFN induction in virulent *Mtb* and *M. marinum* infection *in vitro* (308, 345, 346). Taking advantage of this, we screened pharmacological inhibitors of cell death-related processes for an effect on ESX-1 mediated necrosis and cytokine signaling. Our pharmacological screen found no impact of the inhibition of pyroptosis, necroptosis, or DNA damage-associated cell death on type I IFN production during WT infection. Instead, we identified ferrostatin-1 (fer-1), as a potent inhibitor of ESX-1-mediated cell death as well as IL-1β and IFNβ secretion. Fer-1 treatment additionally suppressed bacterial cell-to-cell spread and actin tail formation by WT *M. marinum* (a measure of phagosomal escape) to levels observed in the ESX-1-defecient ΔRD1 mutant.

Unlike many of the compounds investigated, fer-1 is an antioxidant which has documented specificity for reducing lipid hydroperoxides and preventing cell membrane damage (347). Further investigation found that WT, but not ΔRD1, *M. marinum* infection resulted in increased accumulation of iron and MDA (a common peroxidized lipid) in host macrophages. Examination of metabolic pathways involved in iron metabolism and cellular antioxidant network by RTqPCR identified a transcriptional profile consistent with the accumulation of intracellular iron and the suppression of GPX4 activity (**Fig. 15**).

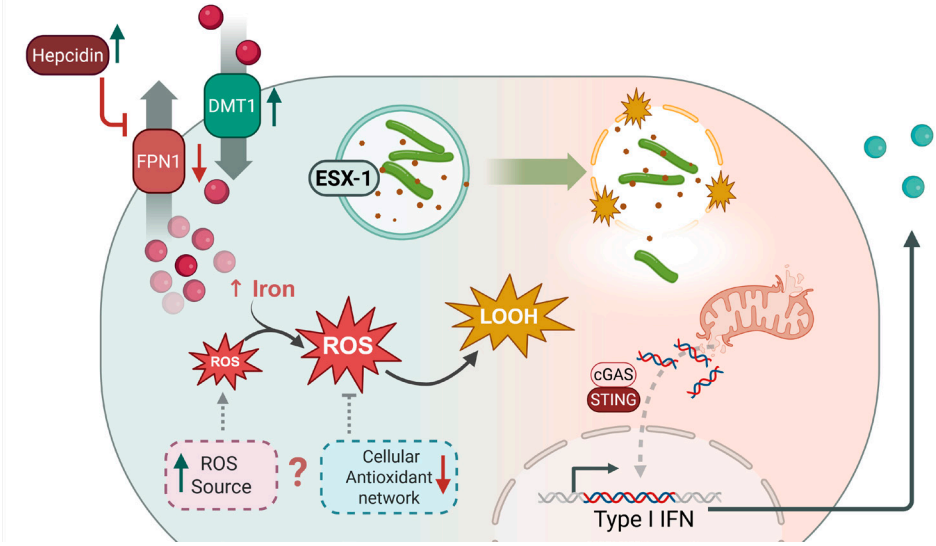



**Figure 15. Working model for Paper II**.
DMT1: Divalent Metal Transporter 1, FPN1: Ferroportin 1, LOOH: Lipid hydroperoxide.

Together, the findings described in **Paper II** suggest that ESX-1 dependent membrane disruption may be facilitated by host processes resulting in excess lipid peroxidation. These results are in line with the observation of crosstalk between type I IFN signaling and cellular antioxidant responses during infection (348), cancer (349), and autoimmune disease (350), as well as recent studies associating excessive oxidative stress with TB disease (317, 351).

## **Paper III**: Intra-granuloma accumulation and inflammatory differentiation of neutrophils underlie mycobacterial ESX-1-mediated immunopathology

In **Paper III** we utilized a *M. marinum* model of infection, to investigate the role of ESX-1 mediated inflammation in the progression of disease. *M. marinum* infection via tail vein injection results in the formation of visible lesions in the tail from day 7 post-infection, displaying similar characteristics to granulomas in human TB, allowing us to follow the progression of disease over the course of infection (352). Neutrophils are one of the first and most abundantly recruited immune cells during *Mtb* infection and their association with pathological inflammation is well-established (267, 298, 353). Recently neutrophils have also been implicated as an important replicative niche during infection (354). Still, the exact role of neutrophils in the development of ESX-1 dependent pathology remains poorly understood.

In this study, we find, in line with previous works, that WT, but not ΔRD1 bacteria, cause significant neutrophilic influx to the tissue. These recruited neutrophils, along with Ly6C+MHCII+ monocytes, represent the majority of cells harbouring bacteria during infection. To investigate the role of ESX-1 in regulating the phenotype of recruited neutrophils, we analysed the transcriptional profiles of individual infected and bystander neutrophils recruited to the site of infection by single cell RNA sequencing analysis. Trajectory analysis revealed that bacterial ESX-1 contributes to the differentiation of recruited neutrophils towards a more inflammatory phenotype.

Depletion of neutrophils during infection by administration of α-Ly6G antibodies resulted in decreased immunopathology, confirming neutrophils as an important driver of detrimental inflammation. As recruited monocytes have been previously identified as a protective population responsible for limiting excessive inflammation, we tested their role in the context of neutrophil-associated pathology. As expected, when infecting CCR2-/- mice unable to mobilize monocytes from the bone marrow, we observed a marked increase in neutrophilic influx and more severe tail lesion formation. A similar exaggerated inflammatory phenotype was found by pharmacological blockade of iNOS activity. Together, these experiments identified an antagonistic relationship between monocytes and neutrophils during infection where monocytes are required for suppressing ESX-1 dependent neutrophil recruitment and pathogenic inflammation through the production of NO (**Fig. 16**).

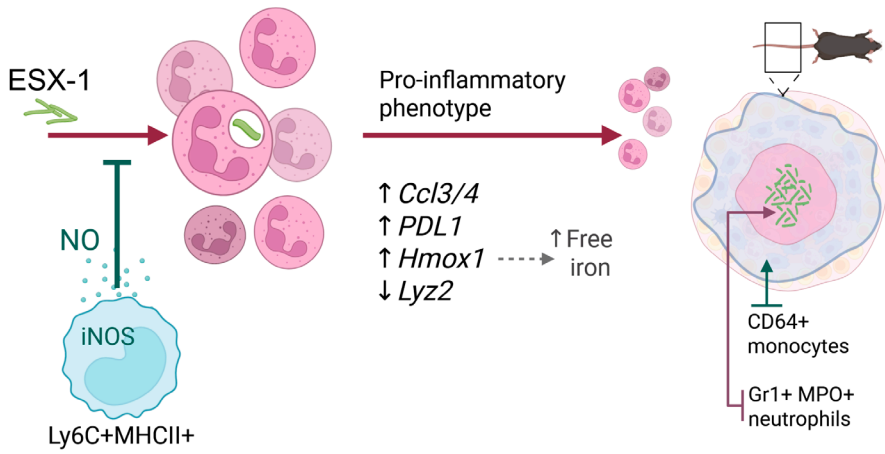



**Figure 16. Working model for Paper III**.
iNOS: inducible nitric oxide synthase, MPO: myeloperoxidase, NO: nitric oxide.

Further studies are required to fully understand the molecular mechanisms by which the ESX-1 secretion system modulates neutrophil recruitment and polarization during mycobacterial infection. This work, however, joins other recent studies in identifying dysfunctional, neutrophil-dominant inflammation as an important driver of disease. Despite utilizing different model systems, our findings of neutrophil-mediated tissue pathology closely parallel those conducted in *Mtb* infection of mice (254, 353, 354) and non-human primates (355).

# Conclusions and future directions

The work presented in this thesis provides several new insights into host–pathogen interactions during mycobacterial infection. These findings reveal previously unrecognized aspects of mycobacterial pathogenesis and suggest new directions for future research.

**Paper I** provided genetic insight into the bacterial factors required for the appearance of the disease-associated SmT *M. avium* colony morphotype. While many previous studies have investigated the phenomenon of virulence-linked morphological switching in MAC species, variations in experimental design and colony morphotype definitions and have limited the generalizability of many of these works. Identification of the MarP/RipA peptidoglycan remodeling pathway as essential for virulence and represents a significant contribution towards understanding the virulence mechanisms of pathogenic *M. avium*. This finding represents one of the first genetic insights into the morphological and phenotypic variation observed in smooth *M. avium* colony variants. While many of the SmO bacteria described in this study were phenotypically "locked" by acquired mutations, we additionally identified completely isogenic MAC 101 isolates of each colony morphotype. This indicates that morphological switching in *M. avium* appears to be driven primarily by transcriptional regulation. Our transcriptomic analyses also identified many SmT-associated genes which are likely to be regulated by the MtrAB TCS, highlighting an exciting avenue for future studies in MAC as well as other species.

In **Paper I** we further found that SmO bacteria, while otherwise avirulent, were efficient at colonizing the lungs *in vivo* in an inflammasome-dependent manner. Interestingly, several other reports in *M. avium* have linked biofilm formation, a trait normally associated with environmental persistence, to epithelial cell invasion (356, 357). While not a primary focus of our investigations, we found that colonizing SmO, but not persistent SmT, bacteria display characteristics linked to biofilm formation (e.g. sliding morality and CR binding). These findings suggest that the SmO phenotype may provide a situational advantage in both environmental and host conditions and will be of great interest for further study.

In **Paper II** we described the peroxidation of host lipids during *M. marinum* infection as a key event required for bacterial escape from the phagosome and the subsequent induction of host-detrimental type I IFNs. Both iron accumulation (318) and reduced GPX4 activity (316, 317, 351) have been observed in necrotic lesions during *Mtb* infection and implicated in the pathologic accumulation of peroxidized lipids. These studies, however, have primarily focused on characterizing the role of peroxidized lipids in advanced TB-like disease and not during the early stages of macrophage infection as we describe here.

While the exact factor responsible for inducing host lipid peroxide formation remains unknown, the identification of a host process involved in ESX-1 dependent phagosome escape represents a significant finding that may be valuable in the development of host-directed therapies. Several clinical studies have found that deficiency in vitamin E, a lipid-soluble antioxidant with similar ROS-specificity as fer-1, correlates with increased TB risk (358, 359). While vitamin E is a much weaker antioxidant than fer-1 (347), it is possible that host even minor deficiencies in host antioxidant systems play a role in resistance to TB infection. If this is the case, the supplementation of vitamin E, in at-risk populations could represent a cheap and well-tolerated adjunctive therapy- a strategy that has been proposed for other micro-nutrients such as zinc and vitamin C (360). In the same way, repurposing currently available drugs which reduce cellular LOOH accumulation, such as those used for treatment of heart disease or asthma (361, 362), is another potential strategy for improving available TB treatments.

Finally, in **Paper III** we characterized the antagonistic interaction between neutrophils and monocytes in the development of tissue immunopathology during virulent *M. marinum* infection. We found that bacterial ESX-1 is essential for the excessive and sustained recruitment of neutrophils, as well as for their polarization towards a pro-inflammatory phenotype. This polarization was seen both in infected cells, as well as in uninfected bystander cells which make up >90% of neutrophils during infection, drastically altering the inflammatory milieu. Our study joins many others in connecting dysfunctional neutrophilic inflammation with mycobacterial disease and highlights the interplay between immune cell types as an important factor in the outcome of infection. Since publication, other studies have continued to explore the role of specific neutrophils phenotypes as a source of host-mediated pathology (216, 297, 298).

# Acknowledgements

With all of the moves during my PhD (both transporting the lab and myself) I have managed to meet a truly impressive number of amazing people. This section would need to be many, MANY more pages to properly recognize everyone who has made an impact on myself and my thesis work over the years. There are surely people that are missing but please know it's not intentional, and if you are reading this I appreciate you.

**Julia**. Thank you so much for trusting me with your project. I'm proud of what our little lab managed to accomplish.

**Fredric.** You've made a better scientist. Thank you for both your constant enthusiasm and support over the years. I'm sorry the book is so long, but I wanted to have something heavy in case I need to hit you. 😇 😳 👍

**Mridula**: The Bouba to my Kiki. Thanks for loving me even though I travelled 12 hours to see you and just slept all day.

**Jaume.** What would I have done without you? I truly do not know. Thanks for quitting your well-paying job to come hang out in the lab full-time with me.

**Esther**. You taught me the basics and inspired me to keep working with the most fussy bacteria. Thank you for the tremendous amount of care you shown me, and for skipping out and taking a swim with me at Cold Spring Harbor.

To our lab group members- you've made every day more fun. **Christine** I aspire to have you vast knowledge one day **Elin**, you are an inspiration! **Lars** and **Ximena** thank you for patiently explaining stats and bioinformatics over and over**. Gunnar-** Fredric is my science dad, you're my academic grandfather! I'm excited to try working with GAS. **Giulia-** my first coworker in the Lienard lab

To my students: **Sacha**, **Arvid H, Arvid B, Nanja, Rodrigo**, **Erik** and **Olivia** thank you for being brave and testing new things along with me, these papers wouldn't have been possible without your help. **Joel**, **Elsa**, and **Sofia**, while I wasn't officially your supervisor, having you in the lab has been a pleasure.

To all of the A house- thank you for letting me hang around even years after moving. **Karla, Humberto, Dima, Vivi, Cuong, Abraham, Judith, Manuel, Juanita,**

**Heather, Ewa**, **Teresa Alberto, Irene, Nik, Courtney, Julie, Shakhira, Heloise** Thank you for the parties, karaoke, bus chats and friendship over the years.

**Nick, Nat** thank you for making life more entertaining in and out of work. And thank you **CJ, Elisa, Ana, Daniel, Andre, Ayse, Janka Abel Ola** and others in the B house for keeping us company while we're in lab limbo and welcoming us for Friday Fika (the highlight of my week!)

To everyone in the ecology house- thank you for embracing us even though we never ended up moving there before I graduated. Which is a shame- you are wonderful! Thank you **Violeta, Simon**,**Valentina, Elsie, Samantha, Robin** and many others.

The BLAM (mostly) Girls- **Karla, Sara, Daniel, Ashish, Agniezka, Susana, Ana, Emma,** and **Nima**. Organizing BLAM has been one of the most rewarding parts of my PhD time.

Thank you to my departmental representatives **Klas, Allan,** and my co-supervisor **Claes**. Thank you **Olivier** for being my mentor (even though we are both bad at planning time for coffee).

Technical and administrative staff- you are the backbone of all of the research in this department, including this thesis- Thank you **Elisabeth, Carina, Camilla, Agniezka, Lars, Nils,** and **Henrik.**

Special thanks to **Mridula**, **Dima**, and **Jaume** for proof-reading earlier drafts of my kappa. Any remaining typos are entirely my fault.

**McKenna**. I'm glad we realized we didn't hate each other after all. 5/7.

Thank you to all my friends in Malmö and elsewhere in Skåne- **Mattias, Paulina, Firas, Giancarlo, Anton** and many more thank you for keeping me sane with all of the board game nights and shows!

**Dara**. Thank you for believing in me a decade (!!) ago and putting me to work in the micro lab for the first time. **Zac**. Thank you for giving a teenager access to a golf cart and a bioreactor all those years ago- I did become a microbiologist after all!

**To my family- Dad**. I think the years of alphabetizing Pokémon cards and catching bugs on the weekends set me up for this. **Mom** and **Steve** thank you for believing in me and making a valiant effort to understand why I needed 12 years to finish college for some reason. **Brandon** next trip when? All of the **Laschanzkys,** especially **Grandpa Bob** thank you for the eternal support.

**Gailann**, **Genna**, and all **the Robinsons**. Thank you for being a second family.

**Gianni**, thank you for keeping me (mostly) sane during this PhD- you deserve an honorary degree for the constant support. I love you.

And of course: the beasties. ❤️ 🐁 ❤️

# References


1. A. Oren, G. M. Garrity, Valid publication of the names of forty-two phyla of prokaryotes. *Int. J. Syst. Evol. Microbiol.* **71**, 005056 (2021).
2. World Health Organization (WHO), Global Tuberculosis Report 2025. (2025).
3. M. D. Johansen, J.-L. Herrmann, L. Kremer, Non-tuberculous mycobacteria and the rise of *Mycobacterium abscessus*. *Nat. Rev. Microbiol.* **18**, 392–407 (2020).
4. E. H. Runyon, Anonymous Mycobacteria in Pulmonary Disease. *Med. Clin. North Am.* **43**, 273–290 (1959).
5. E. Tortoli, *et al.*, The new phylogeny of the genus Mycobacterium : The old and the news. *Infect. Genet. Evol.* **56**, 19–25 (2017).
6. N. L. Bachmann, *et al.*, Key Transitions in the Evolution of Rapid and Slow Growing Mycobacteria Identified by Comparative Genomics. *Front. Microbiol.* **10**, 495775 (2020).
7. R. S. Gupta, B. Lo, J. Son, Phylogenomics and Comparative Genomic Studies Robustly Support Division of the Genus Mycobacterium into an Emended Genus Mycobacterium and Four Novel Genera. *Front. Microbiol.* **9** (2018).
8. S. J. Pidot, *et al.*, Marine sponge microbe provides insights into evolution and virulence of the tubercle bacillus. *PLoS Pathog.* **20**, e1012440 (2024).
9. H. Izumi, *et al.*, Diversity of Mycobacterium species from marine sponges and their sensitivity to antagonism by sponge-derived rifamycin-synthesizing actinobacterium in the genus *Salinispora*. *FEMS Microbiol. Lett.* **313**, 33–40 (2010).
10. C. J. Cambier, S. Falkow, L. Ramakrishnan, Host Evasion and Exploitation Schemes of *Mycobacterium tuberculosis*. *Cell* **159**, 1497–1509 (2014).
11. Y. Shi, *et al.*, The ecology and evolution of amoeba-bacterium interactions. *Appl. Environ. Microbiol.* **87** (2021).
12. T. A. Claeys, R. T. Robinson, The many lives of nontuberculous mycobacteria. *J. Bacteriol.* **200** (2018).
13. J. Marin, F. U. Battistuzzi, A. C. Brown, S. B. Hedges, The Timetree of Prokaryotes: New Insights into Their Evolution and Speciation. *Mol. Biol. Evol.* **34**, 437–446 (2017).
14. S. T. Cole, *et al.*, Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**, 537–544 (1998).
15. L. A. Weinert, J. J. Welch, Why Might Bacterial Pathogens Have Small Genomes? *Trends Ecol. Evol.* **32**, 936–947 (2017).
16. I. Buzic, V. Giuffra, The paleopathological evidence on the origins of human tuberculosis: a review. *J. Prev. Med. Hyg.* **61** (2020).
17. G. Sapriel, R. Brosch, E. Bapteste, Shared Pathogenomic Patterns Characterize a New Phylotype, Revealing Transition toward Host-Adaptation Long before Speciation of *Mycobacterium tuberculosis*. *Genome Biol. Evol.* **11**, 2420 (2019).
18. I. Comas, *et al.*, Out-of-Africa migration and Neolithic coexpansion of *Mycobacterium tuberculosis* with modern humans. *Nat. Genet.* **45**, 1176–1182 (2013).

19. T. M. Daniel, The history of tuberculosis. *Respir. Med.* **100**, 1862–1870 (2006).
20. L. Wang, *et al.*, Global prevalence, burden and trend in HIV and drug-susceptible tuberculosis co-infection from 1990 to 2019 and prediction to 2040. *Heliyon* **10**, e23479 (2024).
21. C. K. Kwan, J. D. Ernst, HIV and Tuberculosis: a Deadly Human Syndemic. *Clin. Microbiol. Rev.* **24**, 351–376 (2011).
22. United Nations Programme on HIV/AIDS (UNAIDS), "Global HIV statistics" (2022).
23. R. Diel, *et al.*, Burden of non-tuberculous mycobacterial pulmonary disease in Germany. *Eur. Respir. J.* **49**, 1602109 (2017).
24. N. Kwak, *et al.*, Treatment Outcomes of *Mycobacterium avium* Complex Lung Disease: A Systematic Review and Meta-analysis. *Clin. Infect. Dis.* **65**, 1077–1084 (2017).
25. S. Chang, *et al.*, Medical Costs of Nontuberculous Mycobacterial Pulmonary Disease, South Korea, 2015–2019. *Emerg. Infect. Dis.* **30**, 1841–1849 (2024).
26. W. Hoefsloot, *et al.*, The geographic diversity of nontuberculous mycobacteria isolated from pulmonary samples: an NTM-NET collaborative study. *Eur. Respir. J.* **42**, 1604–1613 (2013).
27. C. L. Daley, *et al.*, Treatment of nontuberculous mycobacterial pulmonary disease: an official ATS/ERS/ESCMID/IDSA clinical practice guideline. *Eur. Respir. J.* **56**, 2000535 (2020).
28. C. Lange, *et al.*, Consensus management recommendations for less common non-tuberculous mycobacterial pulmonary diseases. *Lancet Infect. Dis.* **22**, e178–e190 (2022).
29. T.-S. Wu, *et al.*, Fish Tank Granuloma Caused by *Mycobacterium marinum*. *PLoS One* **7**, e41296 (2012).
30. R. R. Yotsu, *et al.*, Buruli Ulcer: a Review of the Current Knowledge. *Curr. Trop. Med. Rep.* **5**, 247–256 (2018).
31. D. M. Tobin, L. Ramakrishnan, Comparative pathogenesis of *Mycobacterium marinum* and *Mycobacterium tuberculosis. Cell. Microbiol.* **10**, 1027–1039 (2008).
32. J. Lienard, F. Carlsson, "Murine *Mycobacterium marinum* infection as a model for tuberculosis" in *Methods in Molecular Biology*, (Humana Press Inc., 2017), pp. 301–315.
33. J. van Ingen, *et al.*, Clinical relevance of non-tuberculous mycobacteria isolated in the Nijmegen-Arnhem region, The Netherlands. *Thorax* **64**, 502–506 (2009).
34. F. A. Romero, *et al.*, Nontuberculous Mycobacterial Infections After Silicone Breast Implant Reconstruction Emphasize a Diversity of Infecting Mycobacteria. *Open Forum Infect. Dis.* **4** (2017).
35. K. Uchiya, *et al.*, Comparative genome analyses of *Mycobacterium avium* reveal genomic features of its subspecies and strains that cause progression of pulmonary disease. *Sci. Rep.* **7**, 39750 (2017).
36. J. Van Ingen, C. Y. Turenne, E. Tortoli, R. J. Wallace, B. A. Brown-Elliott, A definition of the *Mycobacterium avium* complex for taxonomical and clinical purposes, a review. *Int. J. Syst. Evol. Microbiol.* **68**, 3666–3677 (2018).
37. M. F. Thorel, M. Krichevsky, V. V. Levy-Frebault, Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium,* and description of *Mycobacterium avium* subsp. *avium* subsp. nov., *Mycobacterium avium* subsp. *paratuberculosis* subsp. nov., and *Mycobacterium avium* subsp. *silvaticum* subsp. nov. *Int. J. Syst. Bacteriol.* **40**, 254–260 (1990).
38. K. Uchiya, T. Wajima, T. Nakagawa, T. Inagaki, K. Ogawa, Genotyping and drug susceptibility patterns of *Mycobacterium avium* subsp. *hominissuis* isolates from different hosts. *Sci. Rep.* **15**, 23121 (2025).

39. E. C. Keen, *et al.*, Comparative Genomics of *Mycobacterium avium* Complex Reveals Signatures of Environment-Specific Adaptation and Community Acquisition. *mSystems* **6**, 1194–1215 (2021).

40. J. E. Lombard, Epidemiology and economics of paratuberculosis. *Vet. Clin. North Am. Food Anim. Pract.* **27**, 525–535 (2011).

41. M. Jeyanathan, D. C. Alexander, C. Y. Turenne, C. Girard, M. A. Behr, Evaluation of In Situ Methods Used To Detect *Mycobacterium avium* subsp. *paratuberculosis* in Samples from Patients with Crohn's Disease. *J. Clin. Microbiol.* **44**, 2942–2950 (2006).

42. E. S. Pierce, Where Are All the *Mycobacterium avium* Subspecies *paratuberculosis* in Patients with Crohn's Disease? *PLoS Pathog.* **5**, e1000234 (2009).

43. E. Tortoli, *et al.*, Genome-based taxonomic revision detects a number of synonymous taxa in the genus *Mycobacterium*. *Infect. Genet. Evol.* **75**, 103983 (2019).

44. A. Benjak, *et al.*, Insights from the Genome Sequence of *Mycobacterium lepraemurium* : Massive Gene Decay and Reductive Evolution. *mBio* **8** (2017).

45. S. D. Nightingale, *et al.*, Incidence of *Mycobacterium avium-intracellulare* Complex Bacteremia in Human Immunodeficiency Virus-Positive Patients. *J. Infect. Dis.* **165**, 1082–1085 (1992).

46. M. A. Ristola, *et al.*, High rates of disseminated infection due to non-tuberculous mycobacteria among AIDS patients in Finland. *J.Infect.* **39**, 61–67 (1999).

47. R. E. Chaisson, R. D. Moore, D. D. Richman, J. Keruly, T. Creagh, Incidence and Natural History of *Mycobacterium avium* -Complex Infections in Patients with Advanced Human Immunodeficiency Virus Disease Treated with Zidovudine. *Am. Rev. Respir. Dis.* **146**, 285–289 (1992).

48. P. C. Karakousis, R. D. Moore, R. E. Chaisson, *Mycobacterium avium* complex in patients with HIV infection in the era of highly active antiretroviral therapy. *Lancet Infect. Dis.* **4**, 557–565 (2004).

49. J. A. Havlik, *et al.*, Disseminated *Mycobacterium avium* complex infection: clinical identification and epidemiologic trends. *J. Infect. Dis.* **165**, 577–80 (1992).

50. V. N. Dahl, *et al.*, Global trends of pulmonary infections with nontuberculous mycobacteria: a systematic review. *Int. J. Infect. Dis.* **125**, 120–131 (2022).

51. M. J. Gebert, *et al.*, Ecological analyses of mycobacteria in showerhead biofilms and their relevance to human health. *mBio* **9**, 1–15 (2018).

52. M. J. Donohue, *et al.*, Increased Frequency of Nontuberculous Mycobacteria Detection at Potable Water Taps within the United States. *Environ. Sci. Technol.* **49**, 6127–33 (2015).

53. E. D. Hilborn, *et al.*, Persistence of nontuberculous mycobacteria in a drinking water system after addition of filtration treatment. *Appl. Environ. Microbiol.* **72**, 5864–5869 (2006).

54. L. M. Feazel, *et al.*, Opportunistic pathogens enriched in showerhead biofilms. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 16393–16398 (2009).

55. R. Thomson, *et al.*, Isolation of nontuberculous mycobacteria (NTM) from household water and shower aerosols in patients with pulmonary disease caused by NTM. *J. Clin. Microbiol.* **51**, 3006–3011 (2013).

56. C. M. Walsh, M. J. Gebert, M. Delgado-Baquerizo, F. T. Maestre, N. Fierer, A global survey of mycobacterial diversity in soil. *Appl. Environ. Microbiol.* **85** (2019).

57. M. J. Gebert, *et al.*, Ecological analyses of mycobacteria in showerhead biofilms and their relevance to human health. *mBio* **9**, 1–15 (2018).

58. J. Adjemian, *et al.*, Spatial clusters of nontuberculous mycobacterial lung disease in the United States. *Am. J. Respir. Crit. Care Med.* **186**, 553–558 (2012).

59. J. A. Schildkraut, *et al.*, Epidemiology of nontuberculous mycobacterial pulmonary disease in Europe and Japan by Delphi estimation. *Respir. Med.* **173**, 106164 (2020).

60. K. Morimoto, *et al.*, A steady increase in nontuberculous mycobacteriosis mortality and estimated prevalence in Japan. *Ann. Am. Thorac. Soc.* **11**, 1–8 (2014).

61. Y. Park, *et al.*, Age- and sex-related characteristics of the increasing trend of nontuberculous mycobacteria pulmonary disease in a tertiary hospital in South Korea from 2006 to 2016. *Korean J. Intern. Med.* **35**, 1424–1431 (2020).

62. V. N. Dahl, *et al.*, Relationship between age, sex, geography and incidence of nontuberculous mycobacteria in Denmark from 1991 to 2022. *ERJ Open Res.* **11**, 00437–02024 (2025).

63. J. M. Reich, R. E. Johnson, Mycobacterium avium Complex Pulmonary Disease Presenting as an Isolated Lingular or Middle Lobe Pattern: The Lady Windermere Syndrome. *Chest* **101**, 1605–1609 (1992).

64. M. A. Lake, L. R. Ambrose, M. C. I. Lipman, D. M. Lowe, "Why me, why now?" Using clinical immunology and epidemiology to explain who gets nontuberculous mycobacterial infection. *BMC Med.* **14** (2016).

65. D. R. Prevots, T. K. Marras, Epidemiology of Human Pulmonary Infection with Non-Tuberculous Mycobacteria: A Review. *Clin. Chest Med.* **36**, 13 (2015).

66. F. C. Ringshausen, *et al.*, Prevalence of nontuberculous mycobacterial pulmonary disease, Germany, 2009-2014. *Emerg. Infect. Dis.* **22**, 1102–1105 (2016).

67. E. L. Axson, C. I. Bloom, J. K. Quint, Nontuberculous mycobacterial disease managed within UK primary care, 2006–2016. *Eur. J. Clin. Microbiol. Infect. Dis.* **37**, 1795–1803 (2018).

68. K. L. Winthrop, *et al.*, Incidence and prevalence of nontuberculous mycobacterial lung disease in a Large U.S. Managed Care Health Plan, 2008-2015. *Ann. Am. Thorac. Soc.* **17**, 178–185 (2020).

69. J. Adjemian, *et al.*, Epidemiology of Nontuberculous Mycobacterial Lung Disease and Tuberculosis, Hawaii, USA. *Emerg. Infect. Dis.* **23**, 439–447 (2017).

70. K. Izumi, *et al.*, Epidemiology of Adults and Children Treated for Nontuberculous Mycobacterial Pulmonary Disease in Japan. *Ann Am Thorac Soc* **16**, 341–347 (2019).

71. H. Lee, W. Myung, W. J. Koh, S. M. Moon, B. W. Jhun, Epidemiology of Nontuberculous Mycobacterial Infection, South Korea, 2007–2016. *Emerg. Infect. Dis.* **25**, 569 (2019).

72. A. Hamosh, *et al.*, Comparison of the clinical manifestations of cystic fibrosis in black and white patients. *J. Pediatr.* **132**, 255–259 (1998).

73. N. L. Turcios, Cystic Fibrosis Lung Disease: An Overview. *Respir. Care* **65**, 233–251 (2020).

74. M. S. Eikani, M. Nugent, A. Poursina, P. Simpson, H. Levy, Clinical course and significance of nontuberculous mycobacteria and its subtypes in cystic fibrosis. *BMC Infect. Dis.* **18**, 1–11 (2018).

75. C. Vinnard, *et al.*, Deaths related to nontuberculous mycobacterial infections in the United States, 1999-2014. *Ann. Am. Thorac. Soc.* **13**, 1951–1955 (2016).

76. J. E. Marshall, R. A. Mercaldo, E. M. Lipner, D. R. Prevots, Incidence of nontuberculous mycobacteria infections among persons with cystic fibrosis in the United States (2010–2019). *BMC Infect. Dis.* **23**, 1–5 (2023).

77. S. Sethi, Infection as a comorbidity of COPD. *Eur. Respir. J.* **35**, 1209–1215 (2010).

78. B. G. Quero, *et al.*, Prevalence of non-tuberculous mycobacteria (NTM) in high risk COPD patients. *Eur. Respir. J.* **58**, PA3093 (2021).

79. W. Hoefsloot, *et al.*, Prevalence of nontuberculous mycobacteria in COPD patients with exacerbations. *J. of Infect.* **66**, 542–545 (2013).

80. World Health Organization (WHO), The top 10 causes of death. (2020).

81. M. R. Loebinger, T. Aksamit, “NTM in Bronchiectasis” in *Bronchiectasis*, (Springer International Publishing, 2018), pp. 189–204.

82. E. Boers, *et al.*, Forecasting the Global Economic and Health Burden of COPD From 2025 Through 2050. *Chest* **168**, 880–889 (2025).

83. W. Guan, X. Han, D. de la Rosa-Carrillo, M. A. Martinez-Garcia, The significant global economic burden of bronchiectasis: a pending matter. *Eur. Respir. J.* **53**, 1802392 (2019).

84. Y.-N. Zhu, *et al.*, Prevalence and Clinical Characteristics of Nontuberculous Mycobacteria in Patients with Bronchiectasis: A Systematic Review and Meta-Analysis. *Respiration*. **100**, 1218–1229 (2021).

85. J. D. Chalmers, A. Shoemark, Inhaled Corticosteroids in COPD and Bronchiectasis: Use Biomarkers Rather Than Disease Labels. *Chest* **164**, 809–811 (2023).

86. V. X. Liu, *et al.*, Association between inhaled corticosteroid use and pulmonary nontuberculous mycobacterial infection. *Ann. Am. Thorac. Soc.* **15**, 1169–1176 (2018).

87. A. J. Evans, *et al.*, Pulmonary *Mycobacterium kansasii* infection: comparison of radiological appearances with pulmonary tuberculosis. *Thorax* **51**, 1243–1247 (1996).

88. E. E. Christensen, *et al.*, Initial Roentgenographic Manifestations of Pulmonary *Mycobacterium Tuberculosis, M Kansasii*, and *M Intracellularis* Infections. *Chest* **80**, 132–136 (1981).

89. V. Dartois, T. Dick, Therapeutic developments for tuberculosis and nontuberculous mycobacterial lung disease. *Nat. Rev. Drug Discov.* **23**, 381–403 (2024).

90. G. Badoum, *et al.*, Failing a re-treatment regimen does not predict MDR/XDR tuberculosis: is “blind” treatment dangerous? *Eur. Respir. J.* **37**, 1283–5 (2011).

91. M. Maiga, *et al.*, Failure to recognize nontuberculous mycobacteria leads to misdiagnosis of chronic pulmonary tuberculosis. *PLoS One* **7** (2012).

92. K. M. Pennington, *et al.*, Approach to the diagnosis and treatment of non-tuberculous mycobacterial disease. *J. Clin. Tuberc. Other Mycobact. Dis.* **24**, 100244 (2021).

93. C. S. Haworth, *et al.*, British Thoracic Society Guideline for the management of non-tuberculous mycobacterial pulmonary disease (NTM-PD). *BMJ Open Respir. Res.* **4**, e000242 (2017).

94. S. M. Moon, *et al.*, Clinical characteristics, treatment outcomes, and resistance mutations associated with macrolide-resistant *Mycobacterium avium* complex lung disease. *Antimicrob. Agents Chemother.* **60**, 6758–6765 (2016).

95. T. R. Sterling, *et al.*, Guidelines for the Treatment of Latent Tuberculosis Infection: Recommendations from the National Tuberculosis Controllers Association and CDC, 2020. *MMWR* **69**, 1–11 (2025).

96. S. M. Moon, *et al.*, Long-term natural history of non-cavitary nodular bronchiectatic nontuberculous mycobacterial pulmonary disease. *Respir. Med.* **151**, 1–7 (2019).

97. W. J. Koh, *et al.*, Outcomes of *Mycobacterium avium* complex lung disease based on clinical phenotype. *Eur. Respir. J.* **50** (2017).

98. J. Adjemian, K. N. Olivier, D. R. Prevots, Nontuberculous mycobacteria among patients with cystic fibrosis in the United States: Screening practices and environmental risk. *Am. J. Respir. Crit. Care Med.* **190**, 581–586 (2014).

99. M. Jankute, J. A. G. Cox, J. Harrison, G. S. Besra, Assembly of the Mycobacterial Cell Wall. *Annu. Rev. Microbiol.* **69**, 405–423 (2015).

100. C. L. Dulberger, E. J. Rubin, C. C. Boutte, The mycobacterial cell envelope — a moving target. *Nat. Rev. Microbiol.* **18**, 47–59 (2020).

101. R. Kalscheuer, *et al.*, The *Mycobacterium tuberculosis* capsule: a cell structure with key implications in pathogenesis. *Biochem. J.* **476**, 1995–2016 (2019).

102. S. Levitte, *et al.*, Mycobacterial Acid Tolerance Enables Phagolysosomal Survival and Establishment of Tuberculous Infection In Vivo. *Cell Host Microbe* **20**, 250–258 (2016).

103. L. J. Alderwick, J. Harrison, G. S. Lloyd, H. L. Birch, The Mycobacterial Cell Wall—Peptidoglycan and Arabinogalactan. *Cold Spring Harb. Perspect. Med.* **5**, a021113 (2015).

104. W. Vollmer, B. Joris, P. Charlier, S. Foster, Bacterial peptidoglycan (murein) hydrolases. *FEMS Microbiol. Rev.* **32**, 259–286 (2008).

105. H. Botella, *et al.*, Mycobacterium tuberculosis protease MarP activates a peptidoglycan hydrolase during acid stress. *EMBO J.* **36**, 536–548 (2017).

106. E. J. R. Peterson, *et al.*, MtrA modulates *Mycobacterium tuberculosis* cell division in host microenvironments to mediate intrinsic resistance and drug tolerance. *Cell Rep.* **42**, 112875 (2023).

107. S. R. Levine, K. E. Beatty, Investigating β-Lactam Drug Targets in *Mycobacterium tuberculosis* Using Chemical Probes. *ACS Infect. Dis.* **7**, 461–470 (2021).

108. M. Lavollay, *et al.*, The peptidoglycan of stationary-phase *Mycobacterium tuberculosis* predominantly contains cross-links generated by L,D-transpeptidation. *J. Bacteriol.* **190**, 4360–4366 (2008).

109. X. Wu, *et al.*, Sensing of mycobacterial arabinogalactan by galectin-9 exacerbates mycobacterial infection. *EMBO Rep.* **22**, e51678 (2021).

110. V. Nataraj, *et al.*, Mycolic acids: deciphering and targeting the Achilles' heel of the tubercle bacillus. *Mol. Microbiol.* **98**, 7 (2015).

111. M. Daffé, H. Marrakchi, Unraveling the Structure of the Mycobacterial Envelope. *Microbiol. Spectr.* **7** (2019).

112. M. I. Hayes, *et al.*, Mycobacteria trehalose dimycolate interactions with host Mincle remodel blood-brain barrier junctions for brain invasion. *Cell Rep.* **44**, 116661 (2025).

113. J. Indrigo, R. L. Hunter, J. K. Actor, Cord factor trehalose 6,6′-dimycolate (TDM) mediates trafficking events during mycobacterial infection of murine macrophages. *Microbiology (N. Y).* **149**, 2049–2059 (2003).

114. E. C. Patin, *et al.*, Trehalose dimycolate interferes with FcγR-mediated phagosome maturation through Mincle, SHP-1 and FcγRIIB signalling. *PLoS One* **12** (2017).

115. H. Kumar, T. Kawai, S. Akira, Toll-like receptors and innate immunity. *Biochem. Biophys. Res. Commun.* **388**, 621–625 (2009).

116. J. S. Cox, B. Chen, M. McNeil, W. R. Jacobs, Complex lipid determines tissue-specific replication of Mycobacterium tuberculosis in mice. *Nature* **402**, 79–83 (1999).

117. C. J. Cambier, S. M. Banik, J. A. Buonomo, C. R. Bertozzi, Spreading of a mycobacterial cell surface lipid into host epithelial membranes promotes infectivity. *Elife* **9**, 1–68 (2020).

118. T. Fukuda, *et al.*, Critical roles for lipomannan and lipoarabinomannan in cell wall integrity of mycobacteria and pathogenesis of tuberculosis. *mBio* **4** (2013).

119. J. Turner, J. B. Torrelles, Mannose-capped lipoarabinomannan in *Mycobacterium tuberculosis* pathogenesis. *Pathog. Dis.* **76**, fty026 (2018).

120. V. Briken, S. A. Porcelli, G. S. Besra, L. Kremer, Mycobacterial lipoarabinomannan and related lipoglycans: from biogenesis to modulation of the immune response. *Mol. Microbiol.* **53**, 391–403 (2004).

121. G. Lugo-Villarino, D. Hudrisier, A. Tanne, O. Neyrolles, C-type lectins with a sweet spot for *Mycobacterium tuberculosis. Eur. J. Microbiol. Immunol. (Bp).* **1**, 25 (2011).

122. J. S. Schorey, L. Sweet, The mycobacterial glycopeptidolipids: structure, function, and their role in pathogenesis. *Glycobiology* **18**, 832–841 (2008).

123. R. Freeman, *et al.*, Roles for cell wall glycopeptidolipid in surface adherence and planktonic dispersal of *Mycobacterium avium*. *Appl. Environ. Microbiol.* **72**, 7554–8 (2006).

124. Y. Yamazaki, L. Danelishvili, M. Wu, M. MacNab, L. E. Bermudez, *Mycobacterium avium* Genes Associated with the Ability To Form a Biofilm. *Appl. Environ. Microbiol.* **72**, 819–825 (2006).

125. A. Martínez, S. Torello, R. Kolter, Sliding motility in mycobacteria. *J. Bacteriol.* **181**, 7331–7338 (1999).

126. S. T. Howard, *et al.*, Spontaneous reversion of *Mycobacterium abscessus* from a smooth to a rough morphotype is associated with reduced expression of glycopeptidolipid and reacquisition of an invasive phenotype. *Microbiology (N. Y).* **152**, 1581–1590 (2006).

127. J. Y. Kam, *et al.*, Rough and smooth variants of *Mycobacterium abscessus* are differentially controlled by host immunity during chronic infection of adult zebrafish. *Nat. Commun.* **13**, 952 (2022).

128. L. B. Davidson, R. Nessar, P. Kempaiah, D. J. Perkins, T. F. Byrd, *Mycobacterium abscessus* Glycopeptidolipid Prevents Respiratory Epithelial TLR2 Signaling as Measured by HβD2 Gene Expression and IL-8 Release. *PLoS One* **6**, e29148 (2011).

129. J. M. Bryant, *et al.*, Stepwise pathogenic evolution of *Mycobacterium abscessus*. *Science* **372** (2021).

130. M. Niederweis, O. Danilchanka, J. Huff, C. Hoffmann, H. Engelhardt, Mycobacterial outer membranes: in search of proteins. *Trends Microbiol.* **18**, 109–116 (2010).

131. V. J. C. van Winden, E. N. G. Houben, M. Braunstein, Protein Export into and across the Atypical Diderm Cell Envelope of Mycobacteria. *Microbiol. Spectr.* **7** (2019).

132. D. C. Rees, E. Johnson, O. Lewinson, ABC transporters: the power to change. *Nat. Rev. Mol. Cell Biol.* **10**, 218–227 (2009).

133. D. K. Soni, S. K. Dubey, R. Bhatnagar, ATP-binding cassette (ABC) import systems of *Mycobacterium tuberculosis*: target for drug and vaccine development. *Emerg. Microbes Infect.* **9**, 207–220 (2020).

134. L. Chiaradia, *et al.*, Dissecting the mycobacterial cell envelope and defining the composition of the native mycomembrane. *Sci. Rep.* **7**, 12807 (2017).

135. E. R. Green, J. Mecsas, Bacterial Secretion Systems: An Overview. *Microbiol. Spectr.* **4** (2016).

136. A. Viljoen, *et al.*, The diverse family of MmpL transporters in mycobacteria: from regulation to antimicrobial developments. *Mol. Microbiol.* **104**, 889–904 (2017).

137. C. Varela, *et al.*, MmpL Genes Are Associated with Mycolic Acid Metabolism in Mycobacteria and Corynebacteria. *Chem. Biol.* **19**, 498 (2012).

138. Z. Xu, V. A. Meshcheryakov, G. Poce, S.-S. Chng, MmpL3 is the flippase for mycolic acids in mycobacteria. *Proc. Natl. Acad. Sci. U. S. A.* **114**, 7993–7998 (2017).

139. H. Medjahed, J. M. Reyrat, Construction of *Mycobacterium abscessus* defined glycopeptidolipid mutants: Comparison of genetic tools. *Appl. Environ. Microbiol.* **75**, 1331–1338 (2009).

140. G. Melly, G. E. Purdy, Mmpl proteins in physiology and pathogenesis of *M. tuberculosis*. *Microorganisms* **7**, 70 (2019).

141. M. Lagune, *et al.*, Conserved and specialized functions of type vii secretion systems in non-tuberculous mycobacteria. *Microbiology (United Kingdom)* **167**, 001054 (2021).

142. J. M. Wagner, *et al.*, Structures of EccB1 and EccD1 from the core complex of the mycobacterial ESX-1 type VII secretion system. *BMC Struct. Biol.* **16**, 5- (2016).

143. M. I. Gröschel, F. Sayes, R. Simeone, L. Majlessi, R. Brosch, ESX secretion systems: mycobacterial evolution to counter host immunity. *Nat. Rev. Microbiol.* **14**, 677–691 (2016).

144. N. Famelis, *et al.*, Architecture of the mycobacterial type VII secretion system. *Nature* **576**, 321–325 (2019).

145. G. G. Mahairas, P. J. Sabo, M. J. Hickey, D. C. Singh, C. K. Stover, Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. *J. Bacteriol.* **178**, 1274–1282 (1996).

146. S. A. Stanley, J. E. Johndrow, P. Manzanillo, J. S. Cox, The Type I IFN Response to Infection with *Mycobacterium tuberculosis* Requires ESX-1-Mediated Secretion and Contributes to Pathogenesis. *J. Immunol.* **178**, 3143–3152 (2007).

147. K. N. Lewis, *et al.*, Deletion of RD1 from *Mycobacterium tuberculosis* Mimics Bacille Calmette-Guérin Attenuation. *J. Infect. Dis.* **187**, 117–123 (2003).

148. A. Rivera-Calzada, N. Famelis, O. Llorca, S. Geibel, Type VII secretion systems: structure, functions and transport models. *Nat. Rev. Microbiol.* **19**, 567–584 (2021).

149. R. M. Cronin, M. J. Ferrell, C. W. Cahir, M. M. Champion, P. A. Champion, Proteo-genetic analysis reveals clear hierarchy of ESX-1 secretion in *Mycobacterium marinum*. *Proc. Natl. Acad. Sci. U. S. A.* **119**, e2123100119 (2022).

150. L. S. Ates, R. Brosch, Discovery of the type VII ESX-1 secretion needle? *Mol. Microbiol.* **103**, 7–12 (2017).

151. R. E. Bosserman, K. R. Nicholson, M. M. Champion, P. A. Champion, A New ESX-1 Substrate in *Mycobacterium marinum* That Is Required for Hemolysis but Not Host Cell Lysis. *J. Bacteriol.* **201** (2019).

152. A. Gijsbers, *et al.*, The crystal structure of the EspB-EspK virulence factor-chaperone complex suggests an additional type VII secretion mechanism in *Mycobacterium tuberculosis*. *J. of Biol. Chem.* **299**, 102761 (2023).

153. J. Piton, F. Pojer, S. Wakatsuki, C. Gati, S. T. Cole, High resolution CryoEM structure of the ring-shaped virulence factor EspB from *Mycobacterium tuberculosis*. *J. Struct. Biol. X* **4**, 100029 (2020).

154. A. E. Chirakos, K. R. Nicholson, A. Huffman, P. A. Champion, Conserved ESX-1 Substrates EspE and EspF Are Virulence Factors That Regulate Gene Expression. *Infect. Immun.* **88**, e00289-20 (2020).

155. B. S. Jones, *et al.*, The loss of the PDIM/PGL virulence lipids causes differential secretion of ESX-1 substrates in *Mycobacterium marinum*. *mSphere* **9** (2024).

156. P. A. D. Champion, S. A. Stanley, M. M. Champion, E. J. Brown, J. S. Cox, C-terminal signal sequence promotes virulence factor secretion in *Mycobacterium tuberculosis. Science* **313**, 1632–6 (2006).

157. C. Sala, *et al.*, EspL is essential for virulence and stabilizes EspE, EspF and EspH levels in Mycobacterium tuberculosis. *PLoS Pathog.* **14**, e1007491 (2018).

158. D. C. Ekiert, J. S. Cox, Structure of a PE–PPE–EspG complex from *Mycobacterium tuberculosis* reveals molecular specificity of ESX protein secretion. *Proc. Natl. Acad. Sci. USA.* **111**, 14758–14763 (2014).

159. T. H. Phan, *et al.*, EspH is a hypervirulence factor for *Mycobacterium marinum* and essential for the secretion of the ESX-1 substrates EspE and EspF. *PLoS Pathog.* **14**, e1007247 (2018).

160. J. M. Chen, *et al.*, EspD Is Critical for the Virulence-Mediating ESX-1 Secretion System in *Mycobacterium tuberculosis*. *J. Bacteriol.* **194**, 884–893 (2012).

161. R. E. Bosserman, *et al.*, WhiB6 regulation of ESX-1 gene expression is controlled by a negative feedback loop in *Mycobacterium marinum. Proc. Natl. Acad. Sci. U. S. A.* **114**, E10772–E10781 (2017).

162. M. Zhang, *et al.*, EspI regulates the ESX-1 secretion system in response to ATP levels in *Mycobacterium tuberculosis*. *Mol. Microbiol.* **93**, 1057–1065 (2014).

163. K. G. Sanchez, *et al.*, EspM is a conserved transcription factor that regulates gene expression in response to the ESX-1 system. *mBio* **11** (2020).

164. O. A. Collars, *et al.*, pH-responsive substrate switching in mycobacterial Type VII ESX secretion. *bioRxiv*. (2026). [Preprint]
Available at: http://biorxiv.org/lookup/doi/10.64898/2026.02.04.703728

165. M. I. De Jonge, *et al.*, ESAT-6 from Mycobacterium tuberculosis dissociates from its putative chaperone CFP-10 under acidic conditions and exhibits membrane-lysing activity. *J. Bacteriol.* **189**, 6028–6034 (2007).

166. W. H. Conrad, *et al.*, Mycobacterial ESX-1 secretion system mediates host cell lysis through bacterium contact-dependent gross membrane disruptions. *Proc. Natl. Acad. Sci. U. S. A.* **114**, 1371–1376 (2017).

167. J. Augenstreich, *et al.*, ESX-1 and phthiocerol dimycocerosates of *Mycobacterium tuberculosis* act in concert to cause phagosomal rupture and host cell apoptosis. *Cell. Microbiol.* **19**, e12726 (2017).

168. J. Lienard, *et al.*, 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**, 1160–1166 (2020).

169. N. van der Wel, *et al.*, *M. tuberculosis* and *M. leprae* translocate from the phagolysosome to the cytosol in myeloid cells. *Cell* **129**, 1287–1298 (2007).

170. R. Simeone, *et al.*, Phagosomal rupture by *Mycobacterium tuberculosis* results in toxicity and host cell death. *PLoS Pathog.* **8** (2012).

171. M. M. Osman, A. J. Pagán, J. K. Shanahan, L. Ramakrishnan, *Mycobacterium marinum* phthiocerol dimycocerosates enhance macrophage phagosomal permeabilization and membrane damage. *PLoS One* **15**, e0233252 (2020).

172. S. Ray, S. Vazquez Reyes, C. Xiao, J. Sun, Effects of membrane lipid composition on *Mycobacterium tuberculosis* EsxA membrane insertion: A dual play of fluidity and charge. *Tuberculosis* **118**, 101854 (2019).

173. A. Perret, *et al.*, Membrane microdomains are crucial for *Mycobacterium marinum* EsxA-dependent membrane damage, escape to the cytosol, and infection. *Sci. Adv.* **12**, eady0812 (2026).

174. M. McNamara, L. Danelishvili, L. E. Bermudez, The *Mycobacterium avium* ESX-5 PPE protein, PPE25-MAV, interacts with an ESAT-6 family Protein, MAV_2921, and localizes to the bacterial surface. *Microb. Pathog.* **52**, 227–238 (2012).

175. R. Ravindran Nair, *et al.*, Control of *Mycobacterium tuberculosis* protein secretion by ESX-4 and the outer membrane EsxUT-EsxEF complex. *Nat. Commun.* **16**, 10228 (2025).

176. L. Laencina, *et al.*, Identification of genes required for *Mycobacterium abscessus* growth in vivo with a prominent role of the ESX-4 locus. *Proc. Natl. Acad. Sci. U. S. A.* **115**, E1002–E1011 (2018).

177. J. M. Tufariello, *et al.*, Separable roles for *Mycobacterium tuberculosis* ESX-3 effectors in iron acquisition and virulence. *Proc. Natl. Acad. Sci. USA.* **113** (2016).

178. H. Kneuper, *et al.*, Heterogeneity in ess transcriptional organization and variable contribution of the Ess/Type VII protein secretion system to virulence across closely related *Staphylocccus aureus* strains. *Mol. Microbiol.* **93**, 928 (2014).

179. L. Philippot, C. Chenu, A. Kappler, M. C. Rillig, N. Fierer, The interplay between microbial communities and soil properties. *Nat. Rev. Microbiol.* **22**, 226–239 (2024).

180. L. Zoccarato, H. P. Grossart, "Relationship Between Lifestyle and Structure of Bacterial Communities and Their Functionality in Aquatic Systems" in *The Structure and Function of Aquatic Microbial Communities* (2019), pp. 13–52.

181. J. Nguyen, J. Lara-Gutiérrez, R. Stocker, Environmental fluctuations and their effects on microbial communities, populations and individuals. *FEMS Microbiol. Rev.* **45**, fuaa068 (2020).

182. Z. Podlesek, D. Žgur Bertok, The DNA Damage Inducible SOS Response Is a Key Player in the Generation of Bacterial Persister Cells and Population Wide Tolerance. *Front. Microbiol.* **11**, 561210 (2020).

183. L. M. R. Ruiz, C. L. Williams, R. Tamayo, Enhancing bacterial survival through phenotypic heterogeneity. *PLoS Pathog.* **16**, e1008439 (2020).

184. J. Sherry, E. H. Rego, Phenotypic Heterogeneity in Pathogens. *Annu. Rev. Genet.* **58**, 183–209 (2024).

185. R. H. Orsi, B. M. Bowen, M. Wiedmann, Homopolymeric tracts represent a general regulatory mechanism in prokaryotes. *BMC Genomics* **11**, 102 (2010).

186. M. W. van der Woude, A. J. Bäumler, Phase and Antigenic Variation in Bacteria. *Clin. Microbiol. Rev.* **17**, 581–611 (2004).

187. W. H. Lin, E. Kussell, Evolutionary pressures on simple sequence repeats in prokaryotic coding regions. *Nucleic Acids Res.* **40**, 2399–2413 (2012).

188. C. D. Bayliss, M. E. Palmer, Evolution of simple sequence repeat-mediated phase variation in bacterial genomes. *Ann. N. Y. Acad. Sci.* **1267**, 39–44 (2012).

189. E. Darmon, D. R. F. Leach, Bacterial Genome Instability. *Microbiol. Mol. Biol. Rev.* **78**, 1–39 (2014).

190. A. Fernández-Calvet, *et al.*, Phase Variation in HMW1A Controls a Phenotypic Switch in *Haemophilus influenzae* Associated with Pathoadaptation during Persistent Infection. *mBio* **12**, e0078921 (2021).

191. L. E. Winter, S. J. Barenkamp, Antibodies to the HMW1/HMW2 and Hia Adhesins of Nontypeable *Haemophilus influenzae* Mediate Broad-Based Opsonophagocytic Killing of Homologous and Heterologous Strains. *Clin. Vaccine Immunol.* **21**, 613 (2014).

192. A. S. Manso, *et al.*, A random six-phase switch regulates pneumococcal virulence via global epigenetic changes. *Nat. Commun.* **5** (2014).

193. R. H. Orsi, D. R. Ripoll, M. Yeung, K. K. Nightingale, M. Wiedmann, Recombination and positive selection contribute to evolution of *Listeria monocytogenes* inlA. *Microbiology (N. Y).* **153**, 2666–2678 (2007).

194. C. S. Manuel, A. Van Stelten, M. Wiedmann, K. K. Nightingale, R. H. Orsi, Prevalence and distribution of *Listeria monocytogenes* inlA alleles prone to phase variation and *inlA* alleles with premature stop codon mutations among human, food, animal, and environmental isolates. *Appl. Environ. Microbiol.* **81**, 8339–8345 (2015).

195. E. M. Garrett, *et al.*, Phase variation of a signal transduction system controls *Clostridioides difficile* colony morphology, motility, and virulence. *PLoS Biol.* **17**, e3000379 (2019).

196. L. C. Lowrey, L. A. Kent, B. M. Rios, A. B. Ocasio, P. A. Cotter, An IS-mediated, RecA-dependent, bet-hedging strategy in *Burkholderia thailandensis*. *Elife* **12** (2023).

197. S. L. Lusetti, M. M. Cox, The Bacterial RecA Protein and the Recombinational DNA Repair of Stalled Replication Forks. *Annu. Rev. Biochem.* **71**, 71–100 (2002).

198. O. Sekulovic, *et al.*, Genome-wide detection of conservative site-specific recombination in bacteria. *PLoS Genet.* **14** (2018).

199. P. Siguier, E. Gourbeyre, A. Varani, B. Ton-Hoang, M. Chandler, Everyman's Guide to Bacterial Insertion Sequences. *Microbiol. Spectr.* **3** (2015).

200. C. R. E. McEvoy, *et al.*, The role of IS6110 in the evolution of *Mycobacterium tuberculosis*. *Tuberculosis* **87**, 393–404 (2007).

201. J. Consuegra, *et al.*, Insertion-sequence-mediated mutations both promote and constrain evolvability during a long-term experiment with bacteria. *Nat. Commun.* **12**, 980 (2021).

202. J. M. Kirsch, *et al.*, Targeted IS-element sequencing uncovers transposition dynamics during selective pressure in enterococci. *PLoS Pathog.* **19**, e1011424 (2023).

203. J. Valle, M. Vergara-Irigaray, N. Merino, J. R. Penadés, I. Lasa, σB regulates IS256-mediated *Staphylococcus aureus* biofilm phenotypic variation. *J. Bacteriol.* **189**, 2886–2896 (2007).

204. S. Hennig, W. Ziebuhr, Characterization of the transposase encoded by IS256, the prototype of a major family of bacterial insertion sequence elements. *J. Bacteriol.* **192**, 4153–4163 (2010).

205. L. P. Shaw, E. P. C. Rocha, R. C. Maclean, Restriction-modification systems have shaped the evolution and distribution of plasmids across bacteria. *Nucleic Acids Res.* **51**, 6806 (2023).

206. A. Tourancheau, E. A. Mead, X. S. Zhang, G. Fang, Discovering multiple types of DNA methylation from bacteria and microbiome using nanopore sequencing. *Nat. Methods* **18**, 491–498 (2021).

207. J. J. Wanford, J. C. Holmes, C. D. Bayliss, L. R. Green, Meningococcal core and accessory phasomes vary by clonal complex. *Microb. Genom.* **6**, 1–15 (2020).

208. T. Parish, Two-Component Regulatory Systems of Mycobacteria. *Microbiol. Spectr.* **2**, MGM2-0010–2013 (2014).

209. B. Vemparala, A. Valiya Parambathu, D. K. Saini, N. M. Dixit, An Evolutionary Paradigm Favoring Cross Talk between Bacterial Two-Component Signaling Systems. *mSystems* **7**, e00298-22 (2022).

210. L. D. McDonough, *et al.*, An activator of a two-component system controls cell separation and intrinsic drug resistance in *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U. S. A.* **123**, e2519608123 (2026).

211. L. Cuthbertson, J. R. Nodwell, The TetR Family of Regulators. *Microbiol. Mol. Biol. Rev.* 440–475 (2013).

212. C. Y. Chin, *et al.*, A high-frequency phenotypic switch links bacterial virulence and environmental survival in *Acinetobacter baumannii*. *Nat. Microbiol.* **3**, 563–569 (2018).

213. S. S. Shell, *et al.*, GplR1, an unusual TetR-like transcription factor in *Mycobacterium abscessus*, controls the production of cell wall glycopeptidolipids, colony morphology, and virulence . *mSystems* **10** (2025).

214. P. Ma, *et al.*, Bacterial droplet-based single-cell RNA-seq reveals antibiotic-associated heterogeneous cellular states. *Cell* **186**, 877-891.e14 (2023).

215. A. W. Pountain, I. Yanai, Dissecting microbial communities with single-cell transcriptome analysis. *Science (1979).* **389** (2025).

216. G. Viswanathan, *et al.*, Granuloma dual RNA-seq reveals composite transcriptional programs driven by neutrophils and necrosis within tuberculous granulomas. *Sci. Adv.* **12**, eadw4619 (2026).

217. R. Vargas, *et al.*, Phase variation as a major mechanism of adaptation in *Mycobacterium tuberculosis* complex. *Proc. Natl. Acad. Sci. U. S. A.* **120** (2023).

218. J. Wang, *et al.*, Regulation of pneumococcal epigenetic and colony phases by multiple two-component regulatory systems. *PLoS Pathog.* **16**, e1008417 (2020).

219. R. Avraham, *et al.*, Pathogen Cell-to-Cell Variability Drives Heterogeneity in Host Immune Responses. *Cell* **162**, 1309–1321 (2015).

220. C. B. Ford, *et al.*, Use of whole genome sequencing to estimate the mutation rate of *Mycobacterium tuberculosis* during latent infection. *Nat. Genet.* **43**, 482–486 (2011).

221. F. Veyrier, D. Pletzer, C. Turenne, M. A. Behr, Phylogenetic detection of horizontal gene transfer during the step-wise genesis of *Mycobacterium tuberculosis*. *BMC Evol. Biol.* **9**, 196-(2009).

222. E. F. McCaffrey, *et al.*, The immunometabolic topography of cellular organization and bacterial control in tuberculosis granulomas. *Nat. Immunol.* 1–14 (2026). https://doi.org/10.1038/s41590-026-02431-8.

223. J. P. Sarathy, V. Dartois, Caseum: a Niche for *Mycobacterium tuberculosis* Drug-Tolerant Persisters. *Clin. Microbiol. Rev.* **33**, e00159-19 (2020).

224. R. C. Lavin, S. Tan, Spatial relationships of intra-lesion heterogeneity in *Mycobacterium tuberculosis* microenvironment, replication status, and drug efficacy. *PLoS Pathog.* **18**, e1010459 (2022).

225. M. J. Luna, *et al.*, Frequently arising ESX-1-associated phase variants influence *Mycobacterium tuberculosis* fitness in the presence of host and antibiotic pressures. *mBio* **16** (2025).

226. H. Safi, *et al.*, Phase variation in *Mycobacterium tuberculosis glpK* produces transiently heritable drug tolerance. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 19665–19674 (2019).

227. M. M. Bellerose, *et al.*, Common variants in the glycerol kinase gene reduce tuberculosis drug efficacy. *mBio* **10** (2019).

228. J. C. Kester, S. M. Fortune, Persisters and beyond: Mechanisms of phenotypic drug resistance and drug tolerance in bacteria. *Crit. Rev. Biochem. Mol. Biol.* **49**, 91–101 (2014).

229. W. A. Winn, S. A. Petroff, biological studies of the tubercle bacillus. *J. Exp. Med.* **57**, 239–264 (1933).

230. W. B. Schaefer, C. L. Davis, M. L. Cohn, Pathogenicity of transparent, opaque, and rough variants of *Mycobacterium avium* in chickens and mice. *Am. Rev. Respir. Dis.* **102**, 499–506 (1970).

231. J. Pedrosa, *et al.*, Characterization of the virulence of *Mycobacterium avium* complex (MAC) isolates in mice. *Clin. Exp. Immunol.* **98**, 210–216 (1994).

232. P. R. Meylan, D. D. Richman, R. S. Kornbluth, Characterization and growth in human macrophages of *Mycobacterium avium* complex strains isolated from the blood of patients with acquired immunodeficiency syndrome. *Infect. Immun.* **58**, 2564–2568 (1990).

233. N. Rastogi, Khye Seng Goh, E. L. Wright, W. W. Barrow, Potential drug targets for *Mycobacterium avium* defined by radiometric drug-inhibitor combination techniques. *Antimicrob. Agents Chemother.* **38**, 2287–2295 (1994).

234. C. B. Inderlied, C. A. Kemper, L. E. M. Bermudez, The *Mycobacterium avium* complex. *Clin. Microbiol. Rev.* **6**, 266–310 (1993).

235. R. G. Kansal, R. Gomez-Flores, R. T. Mehta, Change in colony morphology influences the virulence as well as the biochemical properties of the *Mycobacterium avium* complex. *Microb. Pathog.* **25**, 203–214 (1998).

236. C. McCarthy, Spontaneous and Induced Mutation in *Mycobacterium avium*. *Infect. Immun.* **2**, 223–228 (1970).

237. C. L. Woodley, H. L. David, Effect of temperature on the rate of the transparent to opaque colony type transition in *Mycobacterium avium. Antimicrob. Agents Chemother.* **9**, 113–119 (1976).

238. K. H. Schroeder, I. Juhlin, *Mycobacterium malmoense* sp. nov. *Int. J. Syst. Bacteriol.* **27**, 241–246 (1977).

239. L. Fattorini, *et al.*, Virulence and drug susceptibility of *Mycobacterium celatum*. *Microbiology (N. Y).* **146**, 2733–2742 (2000).

240. T. R. Marques da Silva, *et al.*, Virulent *Mycobacterium fortuitum* restricts NO production by a gamma interferon-activated J774 cell line and phagosome-lysosome fusion. *Infect. Immun.* **70**, 5628–5634 (2002).

241. T. Kawai, S. Akira, The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.* **11**, 373–384 (2010).

242. T. Pradeu, B. P. H. J. Thomma, S. E. Girardin, B. Lemaitre, The conceptual foundations of innate immunity: Taking stock 30 years later. *Immunity* **57**, 613–631 (2024).

243. T. Gong, L. Liu, W. Jiang, R. Zhou, DAMP-sensing receptors in sterile inflammation and inflammatory diseases. *Nat. Rev. Immunol.* **20**, 95–112 (2020).

244. M. Ma, W. Jiang, R. Zhou, DAMPs and DAMP-sensing receptors in inflammation and diseases. *Immunity* **57**, 752–771 (2024).

245. V. Hornung, *et al.*, Quantitative Expression of Toll-Like Receptor 1–10 mRNA in Cellular Subsets of Human Peripheral Blood Mononuclear Cells and Sensitivity to CpG Oligodeoxynucleotides. *J. Immunol.* **168**, 4531–4537 (2002).

246. R. Chen, *et al.*, Pattern recognition receptors: function, regulation and therapeutic potential. *Signal Transduct. Target. Ther.* **10**, 216 (2025).

247. S. M. N. K. Zihad, *et al.*, Role of pattern recognition receptors in sensing *Mycobacterium tuberculosis*. *Heliyon* **9**, e20636 (2023).

248. Q. Chen, L. Sun, Z. J. Chen, Regulation and function of the cGAS–STING pathway of cytosolic DNA sensing. *Nat. Immunol.* **17**, 1142–1149 (2016).

249. J. Y. Dubé, M. A. Behr, A nod to the bond between NOD2 and mycobacteria. *PLoS Pathog.* **19** (2023).

250. J. K. Lee, *et al.*, Differences in signaling pathways by IL-1beta and IL-18. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 8815–8820 (2004).

251. N. P. Juffermans, *et al.*, Interleukin-1 signaling is essential for host defense during murine pulmonary tuberculosis. *J. Infect. Dis.* **182**, 902–908 (2000).

252. L. Galluzzi, *et al.*, Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death Differ.* **25**, 486–541 (2018).

253. L. S. Miller, *et al.*, Inflammasome-mediated production of IL-1beta is required for neutrophil recruitment against *Staphylococcus aureus* in vivo. *J. Immunol.* **179**, 6933–6942 (2007).

254. B. B. Mishra, *et al.*, Nitric oxide controls the immunopathology of tuberculosis by inhibiting NLRP3 inflammasome–dependent processing of IL-1β. *Nat. Immunol.* **14**, 52–60 (2013).

255. V. Castiglia, *et al.*, Type I Interferon Signaling Prevents IL-1β-Driven Lethal Systemic Hyperinflammation during Invasive Bacterial Infection of Soft Tissue. *Cell Host Microbe* **19**, 375–387 (2016).

256. C. Garlanda, I. Di Ceglie, S. Jaillon, IL-1 family cytokines in inflammation and immunity. *Cell. Mol. Immunol.* **22**, 1345–1362 (2025).

257. H. Yamada, S. Mizumo, R. Horai, Y. Iwakura, I. Sugawara, Protective role of interleukin-1 in mycobacterial infection in IL-1 alpha/beta double-knockout mice. *Lab. Invest.* **80**, 759–767 (2000).

258. A. C. Bohrer, C. Tocheny, M. Assmann, V. V Ganusov, K. D. Mayer–Barber, Cutting Edge: IL-1R1 Mediates Host Resistance to *Mycobacterium tuberculosis* by Trans-Protection of Infected Cells. *J. Immunol.* **201**, 1645–1650 (2018).

259. M. F. Wu, *et al.*, NLRP3 inflammasome is attenuated in patients with *Mycobacterium avium* complex lung disease and correlated with decreased interleukin-1β response and host susceptibility. *Sci. Rep.* **9**, 1–10 (2019).

260. B.-G. Jung, *et al.*, Decreased Interleukin-1 Family Cytokine Production in Patients with Nontuberculous Mycobacterial Lung Disease. *Microbiol. Spectr.* **10** (2022).

261. L. Fattorini, *et al.*, Induction of IL-1 beta, IL-6, TNF-alpha, GM-CSF and G-CSF in human macrophages by smooth transparent and smooth opaque colonial variants of *Mycobacterium avium*. *J. Med. Microbiol.* **40**, 129–33 (1994).

262. J. B. Torrelles, *et al.*, Characterization of virulence, colony morphotype and the glycopeptidolipid of *Mycobacterium avium* strain 104. *Tuberculosis* **82**, 293–300 (2002).

263. H. Shiratsuchi, Z. Toossi, M. A. Mettler, J. J. Ellner, Colonial morphotype as a determinant of cytokine expression by human monocytes infected with *Mycobacterium avium*. *J. Immunol.* **150**, 2945–54 (1993).

264. F. McNab, K. Mayer-Barber, A. Sher, A. Wack, A. O'Garra, Type I interferons in infectious disease. *Nat. Rev. Immunol.* **15**, 87–103 (2015).

265. M. L. Donovan, T. E. Schultz, T. J. Duke, A. Blumenthal, Type I Interferons in the Pathogenesis of Tuberculosis: Molecular Drivers and Immunological Consequences. *Front. Immunol.* **8**, 1633 (2017).

266. T. J. Scriba, *et al.*, Sequential inflammatory processes define human progression from *M. tuberculosis* infection to tuberculosis disease. *PLoS Pathog.* **13**, e1006687 (2017).

267. M. P. R. Berry, *et al.*, An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. *Nature* **466**, 973–977 (2010).

268. J. A. Awuh, *et al.*, Keap1 regulates inflammatory signaling in *Mycobacterium avium*-infected human macrophages. *Proc. Natl. Acad. Sci. U. S. A.* **112**, E4272–E4280 (2015).

269. N. Ruangkiattikul, *et al.*, cGAS-STING-TBK1-IRF3/7 induced interferon-β contributes to the clearing of non tuberculous mycobacterial infection in mice. *Virulence* **8**, 1303–1315 (2017).

270. R. O. Watson, *et al.*, The Cytosolic Sensor cGAS Detects *Mycobacterium tuberculosis* DNA to Induce Type I Interferons and Activate Autophagy. *Cell Host Microbe* **17**, 811–819 (2015).

271. B. Dey, *et al.*, A bacterial cyclic dinucleotide activates the cytosolic surveillance pathway and mediates innate resistance to tuberculosis. *Nat. Med.* **21**, 401 (2015).

272. K. E. Wiens, J. D. Ernst, The Mechanism for Type I Interferon Induction by *Mycobacterium* tuberculosis is Bacterial Strain-Dependent. *PLoS Pathog.* **12** (2016).

273. L. C. Platanias, Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat. Rev. Immunol.* **5**, 375–386 (2005).

274. I. Kramnik, W. F. Dietrich, P. Demant, B. R. Bloom, Genetic control of resistance to experimental infection with virulent *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 8560–8565 (2000).

275. D. X. Ji, *et al.*, Type I interferon-driven susceptibility to *Mycobacterium tuberculosis* is mediated by IL-1Ra. *Nat. Microbiol.* **4**, 2128–2135 (2019).

276. K. D. Mayer-Barber, *et al.*, Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. *Nature* **511**, 99–103 (2014).

277. I. Mechnikov, Ilya Mechnikov – Nobel Lecture. (1908). Available at: https://www.nobelprize.org/prizes/medicine/1908/mechnikov/lecture/

278. F. Guan, *et al.*, Tissue macrophages: origin, heterogenity, biological functions, diseases and therapeutic targets. *Signal Transduct. Target. Ther.* **10**, 93 (2025).

279. S. B. Cohen, *et al.*, Alveolar Macrophages Provide an Early *Mycobacterium tuberculosis* Niche and Initiate Dissemination. *Cell Host Microbe* **24**, 439-446.e4 (2018).

280. L. Huang, E. V. Nazarova, S. Tan, Y. Liu, D. G. Russell, Growth of *Mycobacterium tuberculosis in* vivo segregates with host macrophage metabolism and ontogeny. *J. Exp. Med.* **215**, 1135 (2018).

281. M. D. Park, A. Silvin, F. Ginhoux, M. Merad, Macrophages in health and disease. *Cell* **185**, 4259–4279 (2022).

282. K. Bassler, J. Schulte-Schrepping, S. Warnat-Herresthal, A. C. Aschenbrenner, J. L. Schultze, The Myeloid Cell Compartment—Cell by Cell. *Annu. Rev. Immunol.* **37**, 269–293 (2019).

283. M. Sköld, S. M. Behar, Tuberculosis triggers a tissue-dependent program of differentiation and acquisition of effector functions by circulating monocytes. *J. Immunol.* **181**, 6349–6360 (2008).

284. S. Marino, *et al.*, Macrophage Polarization Drives Granuloma Outcome during *Mycobacterium tuberculosis* Infection. *Infect. Immun.* **83**, 324 (2015).

285. D. Pisu, *et al.*, Mycobacterium tuberculosis preferentially infects specific macrophage subsets in primate granulomas during the early stages of tuberculosis. *Mucosal Immunol.* ***4****:*S1933-0219(26)00015-2 (2026).

286. D. Pisu, L. Johnston, J. T. Mattila, D. G. Russell, The frequency of CD38+ alveolar macrophages correlates with early control of *M. tuberculosis* in the murine lung. *Nat. Commun.* **15**, 8522 (2024).

287. W. Zheng, *et al.*, *Mycobacterium tuberculosis* resides in lysosome-poor monocyte-derived lung cells during chronic infection. *PLoS Pathog.* **20**, e1012205 (2024).

288. A. C. Rothchild, *et al.*, Alveolar macrophages generate a noncanonical NRF2-driven transcriptional response to *Mycobacterium tuberculosis* in vivo. *Sci. Immunol.* **4**, 6693 (2019).

289. P. X. Liew, P. Kubes, The Neutrophil's role during health and disease. *Physiol. Rev.* **99**, 1223–1248 (2019).

290. A. J. Wilk, *et al.*, Multi-omic profiling reveals widespread dysregulation of innate immunity and hematopoiesis in COVID-19. *J. Exp. Med.* **218** (2021).

291. P. Kruger, *et al.*, Neutrophils: Between Host Defence, Immune Modulation, and Tissue Injury. *PLoS Pathog.* **11**, 1–22 (2015).

292. P. Nordenfelt, H. Tapper, Phagosome dynamics during phagocytosis by neutrophils. *J. Leukoc. Biol.* **90**, 271–284 (2011).

293. B. Amulic, C. Cazalet, G. L. Hayes, K. D. Metzler, A. Zychlinsky, Neutrophil Function: From Mechanisms to Disease. *Annu. Rev. Immunol.* **30**, 459-89 (2012).

294. K. Nakamura, H. Nakayama, S. Sasaki, K. Takahashi, K. Iwabuchi, *Mycobacterium avium-intracellulare* complex promote release of pro-inflammatory enzymes matrix metalloproteinases by inducing neutrophil extracellular trap formation. *Sci. Rep.* **12** (2022).

295. T. Inomata, S. Konno, K. Nagai, M. Suzuki, M. Nishimura, Neutrophil predominance in bronchoalveolar lavage fluid is associated with disease severity and progression of HRCT findings in pulmonary *Mycobacterium avium* infection. *PLoS One* **13**, e0190189 (2018).

296. Y. Yamazaki, K. Kubo, M. Sekiguchi, T. Honda, Analysis of BAL fluid in *M. avium-intracellulare* infection in individuals without predisposing lung disease. *Eur. Respir. J.* **11**, 1227–1231 (1998).

297. J. T. Andrews, *et al.*, Metabolically active neutrophils represent a permissive niche for *Mycobacterium tuberculosis*. *Mucosal Immunol.* **17**, 825–842 (2024).

298. P. Sankar, *et al.*, Fatty acid metabolism in neutrophils promotes lung damage and bacterial replication during tuberculosis. *PLoS Pathog.* **20**, e1012188 (2024).

299. D. M. Tobin, *et al.*, The lta4h Locus Modulates Susceptibility to Mycobacterial Infection in Zebrafish and Humans. *Cell* **140**, 717–730 (2010).

300. D. R. Green, T. H. Oguin, J. Martinez, The clearance of dying cells: table for two. *Cell Death Differ.* **23**, 915–926 (2016).

301. T. Dallenga, *et al.*, M. tuberculosis-Induced Necrosis of Infected Neutrophils Promotes Bacterial Growth Following Phagocytosis by Macrophages. *Cell Host Microbe* **22**, 519-530.e3 (2017).

302. C. Kajiwara, *et al.*, Apoptosis Inhibitor of Macrophages Contributes to the Chronicity of *Mycobacterium avium* Infection by Promoting Foamy Macrophage Formation. *The Journal of Immunology* **210**, 431–441 (2023).

303. M. Fujita, T. Matsumoto, S. Ikegame, R. On, K. Watanabe, Apoptosis plays a protective role against *Mycobacterium avium* infection in mice in *Tuberculosis*, (European Respiratory Society, 2017), p. PA2735.

304. A. Mázló, *et al.*, Types of necroinflammation, the effect of cell death modalities on sterile inflammation. *Cell Death Dis.* **13**, 423 (2022).

305. L. Zhang, X. Jiang, D. Pfau, Y. Ling, C. F. Nathan, Type I interferon signaling mediates Mycobacterium tuberculosis–induced macrophage death. *J. Exp. Med.* **218** (2021).

306. S. M. Behar, *et al.*, Apoptosis is an innate defense function of macrophages against *Mycobacterium tuberculosis*. *Mucosal Immunol.* **4**, 279–287 (2011).

307. K. S. Beckwith, *et al.*, Plasma membrane damage causes NLRP3 activation and pyroptosis during *Mycobacterium tuberculosis* infection. *Nat. Commun.* **11**, 2270 (2020).

308. F. J. Roca, L. J. Whitworth, S. Redmond, A. A. Jones, L. Ramakrishnan, TNF Induces Pathogenic Programmed Macrophage Necrosis in Tuberculosis through a Mitochondrial-Lysosomal-Endoplasmic Reticulum Circuit. *Cell* **178**, 1344-1361.e11 (2019).

309. J. Bylund, K. L. Brown, C. Movitz, C. Dahlgren, A. Karlsson, Intracellular generation of superoxide by the phagocyte NADPH oxidase: How, where, and what for? *Free Radic. Biol. Med.* **49**, 1834–1845 (2010).

310. N. Chandimali, *et al.*, Free radicals and their impact on health and antioxidant defenses: a review. *Cell Death Discov.* **11**, 19 (2025).

311. V. H. Ng, J. S. Cox, A. O. Sousa, J. D. MacMicking, J. D. McKinney, Role of KatG catalase-peroxidase in mycobacterial pathogenesis: countering the phagocyte oxidative burst. *Mol. Microbiol.* **52**, 1291–1302 (2004).

312. C. de Chastellier, The many niches and strategies used by pathogenic mycobacteria for survival within host macrophages. *Immunobiology* **214**, 526–542 (2009).

313. V. A. Kelley, J. S. Schorey, Mycobacterium's arrest of phagosome maturation in macrophages requires Rab5 activity and accessibility to iron. *Mol. Biol. Cell* **14**, 3366–3377 (2003).

314. Y. Zheng, J. Sun, Z. Luo, Y. Li, Y. Huang, Emerging mechanisms of lipid peroxidation in regulated cell death and its physiological implications. *Cell Death Dis.* **15**, 859 (2024).

315. H. Yan, *et al.*, Ferroptosis: mechanisms and links with diseases. *Signal Transduct. Target. Ther.* **6**, 49 (2021).

316. E. P. Amaral, *et al.*, A major role for ferroptosis in *Mycobacterium tuberculosis* –induced cell death and tissue necrosis. *J. Exp. Med.* **216**, 556–570 (2019).

317. E. P. Amaral, *et al.*, BACH1 promotes tissue necrosis and *Mycobacterium tuberculosis* susceptibility. *Nat. Microbiol.* **9**, 120–135 (2023).

318. D. L. Costa, *et al.*, Heme oxygenase-1 inhibition promotes IFNγ- and NOS2-mediated control of *Mycobacterium tuberculosis* infection. *Mucosal Immunol.* **14**, 253–266 (2021).

319. X. Chen, R. Kang, G. Kroemer, D. Tang, Ferroptosis in infection, inflammation, and immunity. *J. Exp. Med.* **218** (2021).

320. L. Ramakrishnan, Revisiting the role of the granuloma in tuberculosis. *Nat. Rev. Immunol.* **12**, 352–366 (2012).

321. C. N. Ratnatunga, *et al.*, The Rise of Non-Tuberculosis Mycobacterial Lung Disease. *Front. Immunol.* **11**, 1–12 (2020).

322. E. F. McCaffrey, *et al.*, The immunoregulatory landscape of human tuberculosis granulomas. *Nat. Immunol.* **23**, 318–329 (2022).

323. A. J. Sawyer, *et al.*, Spatial mapping reveals granuloma diversity and histopathological superstructure in human tuberculosis. *Journal of Experimental Medicine* **220** (2023).

324. M. Datta, *et al.*, Leveraging insights from cancer to improve tuberculosis therapy. *Trends Mol. Med.* **31**, 11–20 (2025).

325. E. Vivier, B. Malissen, Innate and adaptive immunity: specificities and signaling hierarchies revisited. *Nat. Immunol.* **6**, 17 (2004).

326. M. G. Netea, A. Schlitzer, K. Placek, L. A. B. Joosten, J. L. Schultze, Innate and Adaptive Immune Memory: an Evolutionary Continuum in the Host's Response to Pathogens. *Cell Host Microbe* **25**, 13–26 (2019).

327. F. A. Bonilla, H. C. Oettgen, Adaptive immunity. *J. Allergy Clin. Immunol.* **125**, S33-40 (2010).

328. K. B. Urdahl, Understanding and overcoming the barriers to T cell mediated immunity against tuberculosis. *Semin. Immunol.* **26**, 578 (2014).

329. P. Chandra, S. J. Grigsby, J. A. Philips, Immune evasion and provocation by *Mycobacterium tuberculosis. Nat. Rev. Microbiol.* **20**, 750–766 (2022).

330. J. Morgan, *et al.*, Classical CD4 T cells as the cornerstone of antimycobacterial immunity. *Immunol. Rev.* **301**, 10–29 (2021).

331. C. K. Kwan, J. D. Ernst, HIV and Tuberculosis: a Deadly Human Syndemic. *Clin. Microbiol. Rev.* **24**, 351–376 (2011).

332. P. J. Maglione, J. Xu, J. Chan, B cells moderate inflammatory progression and enhance bacterial containment upon pulmonary challenge with *Mycobacterium tuberculosis*. *J. Immunol.* **178**, 7222–7234 (2007).

333. J. Turner, A. A. Frank, J. V. Brooks, M. Gonzalez-Juarrero, I. M. Orme, The progression of chronic tuberculosis in the mouse does not require the participation of B lymphocytes or interleukin-4. *Exp. Gerontol.* **36**, 537–545 (2001).

334. H. M. Vordermeier, N. Venkataprasad, D. P. Harris, J. Ivanyi, Increase of tuberculous infection in the organs of B cell-deficient mice. *Clin. Exp. Immunol.* **106**, 312–316 (1996).

335. W. F. Rijnink, T. H. M. Ottenhoff, S. A. Joosten, B-Cells and Antibodies as Contributors to Effector Immune Responses in Tuberculosis. *Front. Immunol.* **12**, 640168 (2021).

336. H. McShane, Insights and challenges in tuberculosis vaccine development. *Lancet Respir. Med.* **7**, 810–819 (2019).

337. A. J. Crowle, A. Y. Tsang, A. E. Vatter, M. H. May, Comparison of 15 laboratory and patient-derived strains of *Mycobacterium avium* for ability to infect and multiply in cultured human macrophages. *J. Clin. Microbiol.* **24**, 812–21 (1986).

338. N. Rastogi, K. S. Goh, E. L. Wright, W. W. Barrow, Potential drug targets for *Mycobacterium avium* defined by radiometric drug-inhibitor combination techniques. *Antimicrob Agents Chemother*. **10**, 2287-95 (1994).

339. G. A. Cangelosi, C. O. Palermo, L. E. Bermudez, Phenotypic consequences of red–white colony type variation in *Mycobacterium avium*. *Microbiology (N. Y).* **147**, 527–533 (2001).

340. J. Pedrosa, *et al.*, Characterization of the virulence of *Mycobacterium avium* complex (MAC) isolates in mice. *Clin. Exp. Immunol.* **98**, 210–6 (1994).

341. C. McCarthy, Spontaneous and Induced Mutation in *Mycobacterium avium*. *Infect. Immun.* **2**, 223–228 (1970).

342. P. Gorla, *et al.*, MtrA Response Regulator Controls Cell Division and Cell Wall Metabolism and Affects Susceptibility of Mycobacteria to the First Line Antituberculosis Drugs. *Front. Microbiol.* **9**, 1–14 (2018).

343. K. J. Minch, *et al.*, The DNA-binding network of *Mycobacterium tuberculosis*. *Nat. Commun.* **6**, 5829 (2015).

344. A. Chatterjee, *et al.*, Global mapping of MtrA-binding sites links MtrA to regulation of its targets in *Mycobacterium tuberculosis*. *Microbiology (N. Y).* **164**, 99–110 (2018).

345. J. S. Park, M. H. Tamayo, M. Gonzalez-Juarrero, I. M. Orme, D. J. Ordway, Virulent clinical isolates of *Mycobacterium tuberculosis* grow rapidly and induce cellular necrosis but minimal apoptosis in murine macrophages. *J. Leukoc. Biol.* **79**, 80–86 (2005).

346. J. Lee, T. Repasy, K. Papavinasasundaram, C. Sassetti, H. Kornfeld, *Mycobacterium* tuberculosis Induces an Atypical Cell Death Mode to Escape from Infected Macrophages. *PLoS One* **6**, e18367 (2011).

347. O. Zilka, *et al.*, On the Mechanism of Cytoprotection by Ferrostatin-1 and Liproxstatin-1 and the Role of Lipid Peroxidation in Ferroptotic Cell Death. *ACS Cent. Sci.* **3**, 232–243 (2017).

348. S. M. Yabaji, *et al.*, Lipid peroxidation and type I interferon coupling fuels pathogenic macrophage activation causing tuberculosis susceptibility. *Elife* **14** (2025).

349. W. Chen, *et al.*, Chronic type I interferon signaling promotes lipid-peroxidation-driven terminal CD8+ T cell exhaustion and curtails anti-PD-1 efficacy. *Cell Rep.* **41**, 111647 (2022).

350. R. A. Sosa, C. Murphey, R. R. Robinson, T. G. Forsthuber, IFN-γ ameliorates autoimmune encephalomyelitis by limiting myelin lipid peroxidation. *Proc. Natl. Acad. Sci. U. S. A.* **112**, E5038–E5047 (2015).

351. E. P. Amaral, *et al.*, GPX4 regulates cellular necrosis and host resistance in *Mycobacterium tuberculosis* infection. *J. Exp. Med.* **219**, 20 (2022).

352. J. Lienard, K. Munke, F. Carlsson, A Murine *Mycobacterium marinum* Infection Model for Longitudinal Analyses of Disease Development and the Inflammatory Response. *Methods Mol. Biol.* **2674**, 313–326 (2023).

353. L. Moreira-Teixeira, *et al.*, Type I IFN exacerbates disease in tuberculosis-susceptible mice by inducing neutrophil-mediated lung inflammation and NETosis. *Nat. Commun.* **11**, 5566 (2020).

354. R. R. Lovewell, C. E. Baer, B. B. Mishra, C. M. Smith, C. M. Sassetti, Granulocytes act as a niche for *Mycobacterium tuberculosis* growth. *Mucosal Immunol.* **14**, 229–241 (2021).

355. J. T. Mattila, *et al.*, Microenvironments in tuberculous granulomas are delineated by distinct populations of macrophage subsets and expression of nitric oxide synthase and arginase isoforms microenvironments that balance anti-microbial anti-inflammatory responses to limit pathology in the lungs. *J Immunol* **191**, 773–784 (2013).

356. Y. Yamazaki, *et al.*, The ability to form biofilm influences *Mycobacterium avium* invasion and translocation of bronchial epithelial cells. *Cell. Microbiol.* **8**, 806–814 (2006).

357. A. M. Barclay, *et al.*, Mycobacteria develop biofilms on airway epithelial cells and promote mucosal barrier disruption. *iScience* **27**, 111063 (2024).

358. M. Vijayamalini, S. Manoharan, Lipid peroxidation, vitamins C, E and reduced glutathione levels in patients with pulmonary tuberculosis. *Cell Biochem. Funct.* **22**, 19–22 (2004).

359. O. Aibana, *et al.*, Vitamin E Status Is Inversely Associated with Risk of Incident Tuberculosis Disease among Household Contacts. *J. Nutr.* **148**, 56–62 (2018).

360. C. Vilchèze, J. Kim, W. R. Jacobs, Vitamin C potentiates the killing of *mycobacterium tuberculosis by* the first-line tuberculosis drugs isoniazid and rifampin in mice. *Antimicrob. Agents Chemother.* **62** (2018).

361. M. Conrad, B. Proneth, Selenium: Tracing Another Essential Element of Ferroptotic Cell Death. *Cell Chem. Biol.* **27**, 409–419 (2020).

362. J. Tschuck, *et al.*, Seratrodast inhibits ferroptosis by suppressing lipid peroxidation. *Cell Death Dis.* **15**, 853 (2024).