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Davids, Camilla

2025

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

Link to publication

Citation for published version (APA):
Davids, C. (2025). *Pathogenesis and novel treatment against tuberculosis*. [Doctoral Thesis (compilation), Department of Laboratory Medicine]. Lund University, Faculty of Medicine.

Total number of authors:
1

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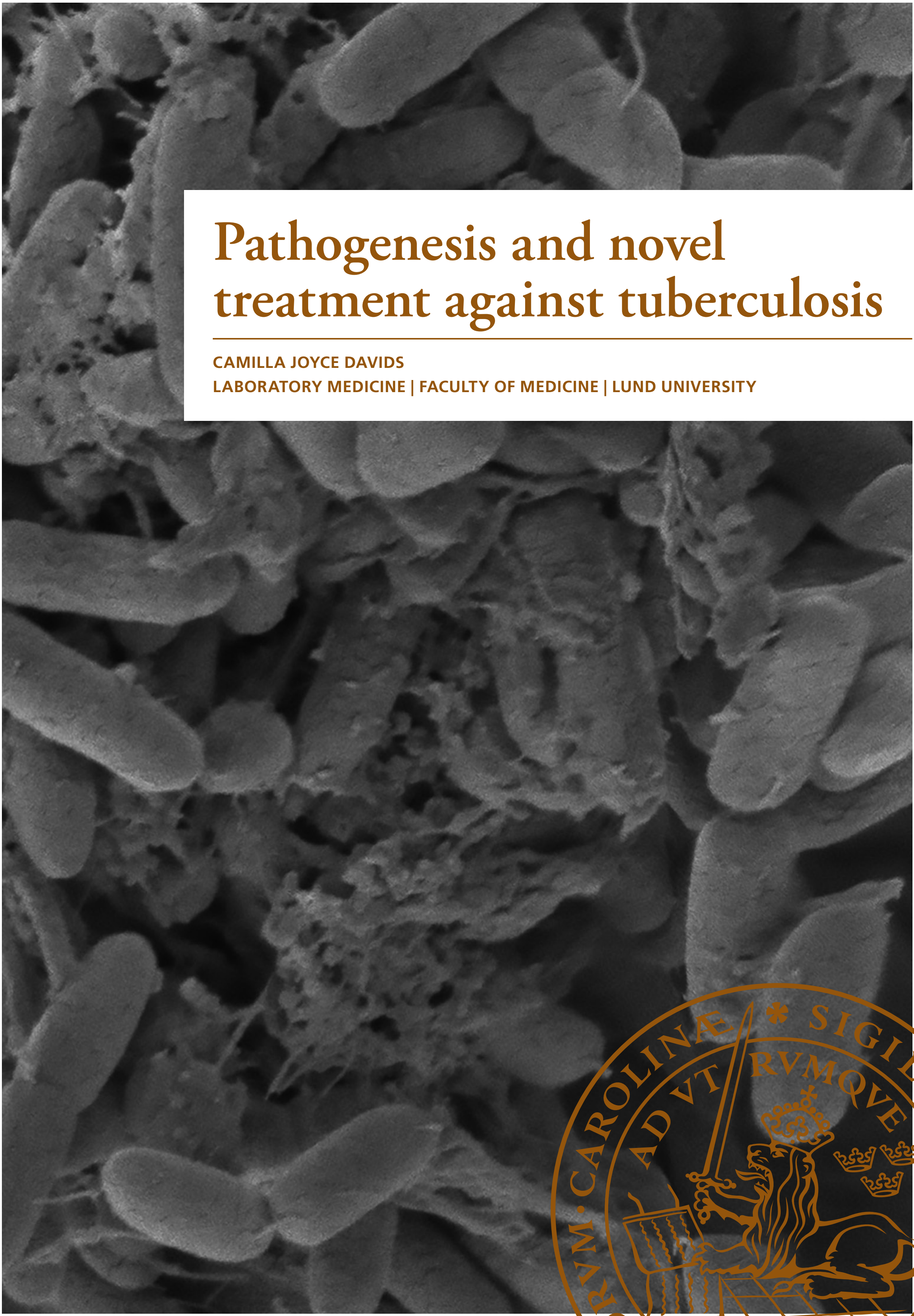

Pathogenesis and novel treatment against tuberculosis

CAMILLA JOYCE DAVIDS

LABORATORY MEDICINE | FACULTY OF MEDICINE | LUND UNIVERSITY

Printed by Media-Tryck, Lund 2025 NORDIC SWAN ECOLABEL 3041 0903

Born and raised in Windhoek, Namibia, Camilla Davids completed her Bachelor of Science (Honours) degree in Microbiology from the University of Namibia. In August 2019, she moved to Sweden to pursue a Master's degree in Molecular Biology with specialization in Microbiology at Lund University. Motivated by the high burden of tuberculosis in her home country, Camilla began a PhD in 2021, focusing on tuberculosis pathogenesis and the development of novel treatments. She is deeply passionate about infectious diseases and committed to contributing to global health research.

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MEDICINE

Department of Laboratory Medicine
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Glycobiology (MIG)

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2025:113
ISBN 978-91-8021-766-8
ISSN 1652-8220

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LUND
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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at
The Faculty of Medicine at Lund University. To be publicly defended on
the 27th November 2025 at 09:00 at Dora Jacobsohn, Biomedical Center (BMC),
Lund, Sweden

Faculty opponent
Susanna Brighenti, Stockholm, Sweden
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Organization: LUND UNIVERSITY

Document name: Doctoral Dissertation **Date of issue** 2025-10-16

Author(s): Camilla Joyce Davids

Title and subtitle: Pathogenesis and novel treatment against tuberculosis

Abstract:

Tuberculosis (TB), caused by Mycobacterium tuberculosis, remains one of the deadliest infectious diseases worldwide. In 2023, it accounted for approximately 1.25 million deaths, including 161,000 among individuals living with HIV, making it the leading cause of death from a single infectious agent. The growing prevalence of multidrug-resistant TB (MDR-TB) further complicates treatment and contributes to the global antimicrobial resistance crisis. Although the WHO-recommended 6-month BPaLM regimen has improved outcomes, novel therapeutic strategies are urgently needed.

This thesis presents three studies aimed at identifying innovative treatment options and exploring under-investigated aspects of TB pathogenesis. The first study evaluates NZ2114, a plectasin-derived antimicrobial peptide, which demonstrated strong activity against M. bovis BCG (MIC_{99}: 6.1 µM), no cytotoxicity to human cells, and retained efficacy in serum. It also showed activity against Gram-positive pathogens including MRSA and Enterococcus spp.The second study investigates the production of membrane vesicles (MVs) in mycobacteria following exposure to conventional TB antibiotics. While outer membrane vesicles are well-characterized in Gram-negative bacteria, their role in mycobacteria is less understood. Using mass spectrometry and lipidomics, vesicle-associated proteins and lipids were identified. Importantly, mycobacterial MVs induced inflammatory responses, suggesting a role in immune modulation and pathogenesis.The third study explores bacteriophage-derived lysins as alternative therapeutics. Three engineered chimeric lysins showed broad-spectrum activity against M. bovis BCG, M. abscessus, and M. avium, with no toxicity to human macrophages, retained serum activity, and effective intracellular killing.

Together, these findings enhance our understanding of TB pathogenesis and introduce promising therapeutic candidates, antimicrobial peptides and phage-derived enzymes that may help overcome current treatment limitations.

Key words: Tuberculosis, antimicrobial peptides, pathogenesis, vesicles, novel treatment, chimeric lysins

Language: English **Number of pages**: 91

ISSN and key title: 1652-8220 **ISBN:** 978-91-8021-766-8

Signature Date 2025-09-24

Pathogenesis and novel treatment against tuberculosis

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Cover photo: Effect of peptide (NZ2114) on *Mycobacterium bovis* bacillus Calmette-Guerin (BCG).
Photo by Matthias Mörgelin

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Lund, Sweden 2025

ISSN 1652-8220
ISBN 978-91-8021-766-8
Lund University, Faculty of Medicine Doctoral Dissertation Series 2025:113

Printed in Sweden by Media-Tryck, Lund University, Lund, 2025

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MADE IN SWEDEN

To my mother, Pamela

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Popular summary

Tuberculosis, caused by *Mycobacterium tuberculosis*, has impacted humans for thousands of years. Evidence of TB dates back to 2400 BC in Egyptian mummies. In the Middle Ages, a form of TB called scrofula was known as the "king's evil" and was believed to be cured by royal touch. By the 18th century TB was a major epidemic in Europe, especially affecting the youth, earning it the nickname "robber of youth." The term "tuberculosis" was coined in the mid-19th century by Johann Lukas Schönlein. A major breakthrough occurred in 1882 when Robert Koch discovered the tubercle bacillus, using staining techniques to isolate and identify the bacteria. This discovery laid the foundation for modern TB diagnosis and treatment. Around the same time, the sanatorium movement led by Hermann Brehmer introduced high altitude facilities for TB care, emphasizing rest and clean air as treatment methods.

In 2023 TB caused approximately 1.25 million deaths**,** including 161,000 among people living with HIV. Thus, making TB once again the leading cause of death from a single infectious disease, surpassing COVID-19. It remains the top killer among HIV-positive individuals and is a major contributor to deaths from antimicrobial resistance. An estimated 10.8 million people developed TB globally, affecting men (6.0 million), women (3.6 million), and children (1.3 million)**.** TB affects people of all ages and regions, however, it is preventable and curable**.** Multidrug-resistant TB (MDR-TB), however, remains a critical public health threat, with only 40% of affected individuals receiving treatment. Countries with the highest TB incidence include India, Pakistan, Bangladesh, and the Philippines**,** followed by South Africa and Nigeria**.**

After years of using long and toxic injectable-based treatments for drug-resistant tuberculosis (DR-TB), significant progress was made with the introduction of shorter regimens. Key advancements include the use of Bedaquiline (BDQ), Pretomanid, and Linezolid (LZD). The WHO now recommends the 6-month BPaLM regimen (BDQ, Pretomanid, LZD, and Moxifloxacin) for treating multidrug-resistant TB (MDR-TB). Additionally, a 9-month oral regimen is advised for patients without fluoroquinolone resistance, which involves an intensive and continuation phase using a combination of BDQ and other drugs.

This thesis is composed of three research papers. Two of which explore novel approaches to TB treatment and one paper which describes the role of antibiotic-derived mycobacterial vesicles in tuberculosis pathogenesis.

Paper *I* investigates the antimycobacterial activity of NZ2114, a derivative of the fungus *Pseudoplectania nigrella*. NZ2114 demonstrated potent activity against *M.bovis BCG* and clinical isolates of *M. abscessus*, with a minimum inhibitory concentration (MIC_{99}) of 6.1 μM. It showed no cytotoxicity to primary human cells and retained its antimicrobial efficacy in the presence of human serum. In line with previous findings, NZ2114 also exhibited antimicrobial activity against several Gram-positive clinical isolates, including *Enterococcus faecalis*, *Enterococcus faecium*, and Methicillin-Resistant *Staphylococcus aureus* (MRSA).

Paper *II* explores the production of membrane vesicles (MVs) by mycobacteria in response to conventional anti-TB drugs. Outer membrane vesicles (OMVs), which are well characterized in Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Vibrio cholerae*, are known to facilitate horizontal gene transfer and contribute to antibiotic resistance and host-pathogen interactions. However, their role in mycobacterial physiology and TB pathogenesis remains poorly understood. Using mass spectrometry and lipid profiling, our study identified key proteins and lipid components of MVs produced by mycobacteria under antibiotic stress. Additionally, toxicology analysis revealed that mycobacterial MVs induce inflammation, suggesting a role in disease progression. Lipid studies also provided insights into the possible membrane origin of these vesicles.

Paper *III* focuses on bacteriophage-derived lytic enzymes as potential therapeutics against drug-resistant TB. Although phage therapy was introduced over a century ago by scientists such as Félix d’Hérelle, it remains an evolving area of research. Mycobacteriophages produce two key lytic enzymes: Lysin A (LysA), which targets the peptidoglycan layer, and Lysin B (LysB), which cleaves the connection between the outer membrane and the arabinogalactan layer, facilitating cell lysis. In this study, four engineered chimeric lysins were evaluated for their antimicrobial activity against various mycobacterial species, including *M.bovis BCG*, *M. abscessus*, and *M. avium*. Three of the mycolysins (MCL-11, MCL-12, and MCL-17) demonstrated no cytotoxicity toward human macrophages and retained activity in human serum, indicating suitability for potential intravenous administration. In our murine studies, MCL-20 showed strong potential for treating *M. avium* infections, particularly in the lungs, where it significantly reduced bacterial counts. In contrast, MCL-12 and MCL-17 required combination with rifampicin to achieve comparable efficacy

Collectively, the work presented in this thesis contributes to a deeper understanding of mycobacterial pathogenesis and highlights promising novel therapeutic strategies for combating TB, particularly in the context of rising drug resistance.

List of Publications

Papers included in the thesis

Paper I

Davids C, Rao-Fransson K, Krishnan N, Tenland E, Mörgelin M, Robertson B and Godaly G. Antimycobacterial activity of the plectasin derivative NZ2114. Front. Microbiol.16:1613241(2025) doi: 10.3389/fmicb.2025.1613241

Paper II

Davids C, Umashankar-Rao K, Kassaliete J, Ahmadi S, Happonen L, Welinder C, et al. The role of antibiotic-derived mycobacterial vesicles in tuberculosis pathogenesis. *Sci Rep* 14, 28198 (2024). https://doi.org/10.1038/s41598-024-79215-3

Paper III

Adel Abouhmad, Jana Kassaliete, **Davids C**, Dennis Grimon, Tarek Dishisha, Karim Abdelkader, Zienab E. Eldin, Mats Clarsund, Yves Briers, Gabriela Godaly and Rajni Hatti-Kaul (2025). Engineering phage-derived enzymes: a novel approach for targeting mycobacterial (Submitted Manuscript).

Peer-reviewed papers outside this thesis

Yalovenko T, Riaz A, Kotov N, **Davids C**, Persson A, et al. Inhalable porous particles as dual micro-nano carriers demonstrating efficient lung drug delivery for treatment of tuberculosis. J Control Release. 2024;369:23150. https://doi.org/10.1016/j.jconrel.2024.03.013

Campos Pacheco JE, **Davids C**, Yalovenko T, Näsström E, Ahnlund M, Godaly G, Valetti S. Bioinspired mycobacterial lipid coating of porous particles for enhanced

antimicrobial efficacy. Eur J Pharm Sci. 2025 Oct 1;213:107225. doi: 10.1016/j.ejps.2025.107225. Epub 2025 Aug 6. PMID: 40780537.

List of Abbreviations

AFB	Acid-fast bacilli
AMP	Antimicrobial peptide
BCG	Mycobacterium bovis bacillus Calmette Guerin
BDQ	Bedaquiline
CMVs	Cytoplasmic Membrane Vesicles
DOT	Directly Observed Therapy (DOT)
DS	Drug-Sensitive (DS)
ECMVs	Explosive CMVs
EMB	Ethambutol
FQ	Fluoroquinolones
GL	Glycolipids
HBD	Human β-defensin
HDTs	Oral Host-Directed Therapies
HNP	Human Neutrophil α-defensin
IL-8	Interleukin 8
INH	Isoniazid
LAM	Lipoarabinomannan
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acids
LTBI	Latent TB Infection
LysA	Lysin A
LysB	Lysin B
LZD	Linezolid
MA	Mycolic Acids
MAP	Membrane-Associated Proteins
M.tb	*Mycobacterium tuberculosis*
MDR-TB	Multidrug-Resistant TB
MGIT	Mycobacteria Growth Indicator Tube
MIC	Minimal Inhibitory Concentration
MRSA	Methicillin-Resistant *Staphylococcus aureus*

MTBC	*Mycobacterium* Complex
NTM	Nontuberculosis Mycobacteria
OMV	Outer Membrane Vesicle
OMP	Outer Membrane Proteins
PG	Peptidoglycan
PM	Plasma Membrane
PPI	Protein-Protein Interaction
PZA	Pyrazinamide
RD1	Region of Difference 1
RIF	Rifampicin
SEM	Scanning Electron Microscopy
TA	Teichoic Acids
TB	Tuberculosis
TEM	Transmission Electron Microscopy
TMM	Trehalose Monomycolate
TNT	TB Necrotizing Toxin
TST	Tuberculin Skin Test
XDR-TB	Extensively Drug-Resistant TB
ZN	Ziehl–Neelsen

Overall aim of the thesis

- To investigate the effectiveness of NZ2114 as a potential antimycobacterial peptide both *in vitro* and *in vivo* **(*Paper I*).**
- To determine the composition and effects of mycobacterial MVs using conventional anti-TB drugs and the peptide NZX ***(Paper II).***
- To investigate the effectiveness of the novel chimeric antimycobacterial enzymes both *in vitro* and *in vivo* ***(Paper III).***

"Everything is theoretically impossible, until it's done"

- Robert A Heinlein

Background and previously published results in the field

Brief history of tuberculosis

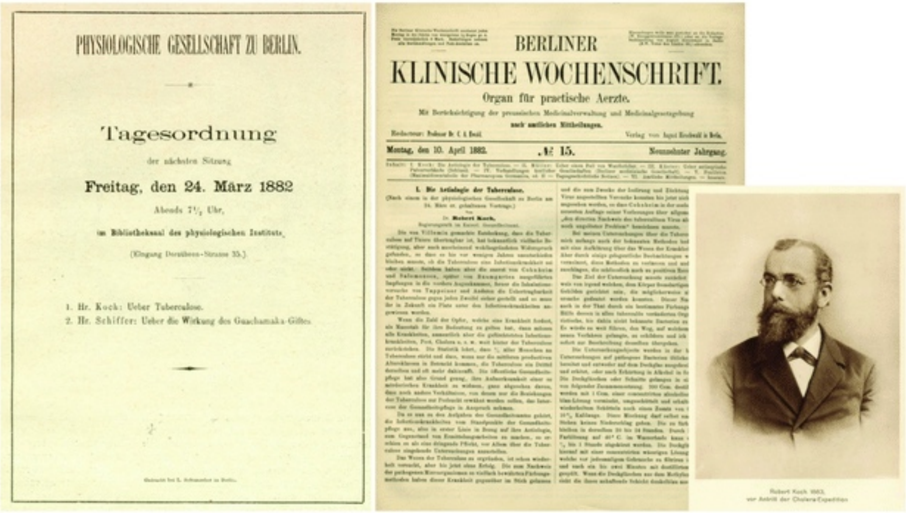
Tuberculosis (TB) is a contagious and infectious disease caused by *Mycobacterium tuberculosis,* has posed a persistent challenge throughout human history due to its significant social impact (4). It is believed that the bacteria *Mycobacterium* emerged more than 150 million years ago (5). Egyptian mummies dating back to 2400 BC reveal skeletal deformities typical of TB, including characteristic pott´s lesions and similar abnormalities clearly illustrated in Egyptian art (4, 6). During the Middle Ages, scrofula, a condition affecting the cervical lymph nodes was recognized as a new clinical form of TB (4). The illness was known in France and England as "kings evil", and it was believed that persons affected could be healed after a royal touch (4, 7). In 1720, for the first time, the infectious origin of TB was speculated by the English physician Benjamin Martin and his findings displayed a great degree of epidemiological importance (4). In the 18th century in Western Europe, TB had become an epidemic with a mortality rate as high as 900 deaths per 100,000 inhabitants per year and affecting mostly young people. For this reason, TB was called the "robber of youth." Only in the mid-19th century, Johann Lukas Schönlein invented the term "tuberculosis" (4).

After Koch´s discovery of M.tb in 1882, the sanatorium movement began to have a major influence on the care that patients with all forms of TB received (8). Hermann Brehmer, widely recognized as the founder of the sanatorium movement, established the first high-altitude sanatorium for the treatment of pulmonary tuberculosis in Görbersdorf, located in the Silesian Mountains (8-10) (fig.1).

Out on the Terrace.

Fig.1 Sun therapy at Alton Hospital (1937).
Child patients receiving sun therapy while lying in beds on the terrace of the Lord Mayor Treloar Cripples Hospital and College in Alton, Hampshire. Reproduced with permission from Wellcome Collection. (1937). *Alton 1908–1929–1937: The unconventional tribute of an outsider*. https://wellcomecollection.org/works/L0074520 Licensed under CC BY 4.0 (http://creativecommons.org/licenses/by/4.0/)

The famous scientist Robert Koch was able to isolate tubercle bacillus using the methylene blue staining method recommended by Paul Ehrlich. He identified, isolated and cultivated the bacillus in animal serum and finally reproduced the disease by infecting laboratory animals with the bacilli (11). Robert Koch presented this incredible result to the soceity of Physiology in Berlin on the 24th of March 1882 (Fig. 2), demonstrating a milestone in the fight against TB (4, 12).



PHYSIOLOGISCHE GESELLSCHAFT ZU BERLIN.

Tagesordnung
der nächsten Sitzung
Freitag, den 24. März 1882
Abends 7½ Uhr,
im Bibliotheksaal des physiologischen Instituts
(Eingang Dorotheen-Strasse 35.)

1. Hr. Koch: Ueber Tuberculose.
2. Hr. Schiffer: Ueber die Wirkung des Guachamaka-Giftes

BERLINER
KLINISCHE WOCHENSCHRIFT.
Organ für practische Aerzte.

Montag, den 10. April 1882. № 15. Neunzehnter Jahrgang.

I. Die Aetiologie der Tuberculose.

Dr. Robert Koch.

Figure 2. Robert Koch’s historic presentation and publication on the etiology of tuberculosis. Archival documents related to Robert Koch's groundbreaking discovery of *Mycobacterium tuberculosis* as the causative agent of tuberculosis. On the left is the agenda of the Physiological Society of Berlin, dated March 24, 1882, where Koch first publicly presented his findings. In the center is the first page of his original publication in *Berliner Klinische Wochenschrift*, dated April 10, 1882. On the right is a portrait of Robert Koch from 1883. These documents represent a pivotal moment in medical microbiology and the foundation of modern TB research. *Image adapted under Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0/). Sources: Koch, 1882; Kaufmann and Winau, 2005.*

Mycobacterium tuberculosis

Mycobacteria is a rod-shaped, slow-growing bacterium and belong to the diverse family of Actinobacteria (13). The main components of the mycobacterial cell wall are the PG layer, Mycolic acid (MA) and arabinogalactan (AG). The cell wall resembles both the gram-positive and gram-negative cell envelope by having a thick and waxy PG layer (Fig.3), however, it is classified as Acid-Fast (14). The two most well-known species of Mycobacterium are *Mycobacterium tuberculosis* (M.tb) and *Mycobacterium leprae,* responsible for causing tuberculosis and leprosy respectively. Both diseases have been present in human populations since ancient times (15).

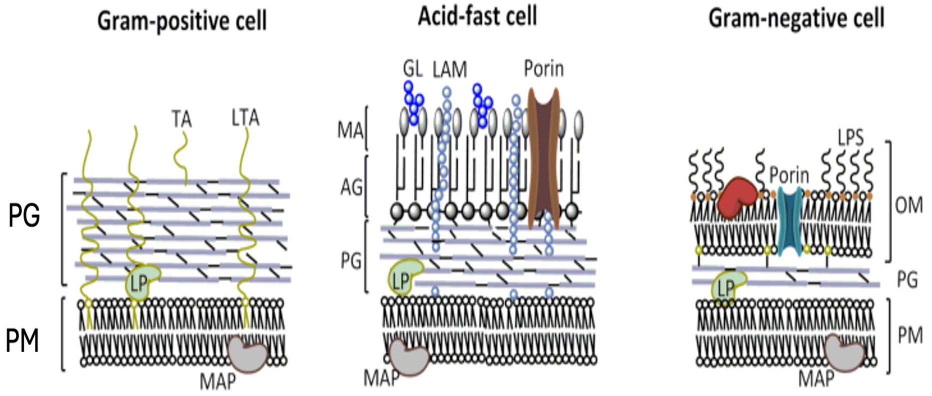


Figure 3. Comparative structure of bacterial cell envelopes in Gram-positive, acid-fast, and Gram-negative bacteria.
This figure highlights the structural differences in the cell envelopes of three major bacterial types. Gram-positive bacteria feature a thick peptidoglycan (PG) layer enriched with teichoic acids (TA) and lipoteichoic acids (LTA). Acid-fast bacteria possess a unique cell envelope composed of a thin peptidoglycan layer linked to arabinogalactan (AG) and overlaid with long-chain mycolic acids (MA), along with glycolipids (GL) and lipoarabinomannan (LAM), contributing to their hydrophobic and impermeable nature. Gram-negative bacteria are characterized by a thin peptidoglycan layer sandwiched between an inner plasma membrane (PM) and an outer membrane (OM) containing lipopolysaccharides (LPS), lipoproteins (LP), and membrane-associated proteins (MAP). This Image *is* reproduced from "Cell wall peptidoglycan in *Mycobacterium tuberculosis*: An Achilles' heel for the TB-causing pathogen."(14) https://doi.org/10.1093/femsre/fuz016.

The *Mycobacterium tuberculosis* complex (MTBC) refers to a group of species (*M. tuberculosis, M. bovis, M .canetti, M. africanum, M. microtti, M. caprae and M. pinnipedii*) that are genetically very similar, with M.tb being the most well-known member (16-22). It is believed that members of the MTBC evolved from a common ancestor through successive DNA deletions and insertions, leading to the current Mycobacterium species and their differences in pathogenicity. Genomic analysis has played a key role in these studies, identifying 14 regions known as regions of difference (RD1–14)(16). These regions are present in the reference strain M.tb *and Mycobacterium bovis* but absent in the vaccine strain BCG, providing insights into chromosomal genes associated with pathogenicity. Additionally, six regions, termed H37Rv deletions 1 to 5 (RvD1–5) and the *M. tuberculosis*-specific deletion 1 (TbD1), are missing from the *M. tuberculosis* H37Rv genome compared to other MTBC members (16, 23).

Pathogenesis

Transmission

The life cycle of M.tb depends on its ability to interact with the immune system using different approaches. Firstly, it evades the immune response and persists despite the adaptive immune response without causing symptomatic disease. Lastly, it elicits a strong inflammatory response to cause extensive tissue pathology for transmission (24, 25). Tuberculosis is transmitted through the inhalation of aerosolized droplets containing live bacteria. These droplets are expelled when an individual with active pulmonary disease coughs or sneezes. There are several factors that determine the success rate of TB transmission. Firstly, Immunocompetent persons who have been infected in the past have considerable protection against re-infection, estimated to be about 80% (26). On the other hand, immunocompromised patients may become reinfected despite having been infected and adequately treated in the past (26-28). Secondly, one or more virulence properties of M.tb may affect its ability to be transmitted. For instance, one strain may adapt better to overcome the hosts innate immune response than others (29, 30). Thirdly, environmental factors have been shown to contribute significantly to TB transmission as early as the 20th century. With rare exceptions, outdoor exposures are unlikely to result in transmission. Almost all transmission is understood to occur indoors, with proximity to the source patient being a significant determinant of transmission (27). Overcrowded settings such as prisons and hospitals lead to increased risk in the number of persons infected (31, 32).

¨The risk of infection becomes particularly high if healthy people have to sleep with the sick in the same rooms, and especially as still unfortunately happens with the poorer section of the population, in one and the same bed¨
-Robert Koch, Nobel acceptance speech, 1905.

First encounter with the host

The infectious dose of M.tb is remarkably low, estimated to be approximately three bacilli, highlighting how effective M.tb is at evading the innate immune response (24, 33). Once M.tb infected droplets enter the lungs to establish infection, M.tb recognizes macrophages, neutrophils, Dendritic cells (DCs) and other innate immune cells through pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) (34, 35). These receptors detect pathogen-associated molecular patterns (PAMPs) on *M. tb*, triggering the production of proinflammatory cytokines, including IL-12 and nitric oxide (36, 37). The bacteria are subsequently engulfed by alveolar macrophages (Fig. 4). However, M.tb can escape into the surrounding lung tissue, enabling further infection of host immune cells. Once released into the lung airspace, the bacteria can become aerosolized and transmitted to new hosts via infectious droplets (34, 38). Additionally, M.tb produces a TB necrotizing toxin (TNT), which depletes NAD^+ in infected macrophages, thereby inhibiting necrotic cell death and promoting bacterial survival (39).

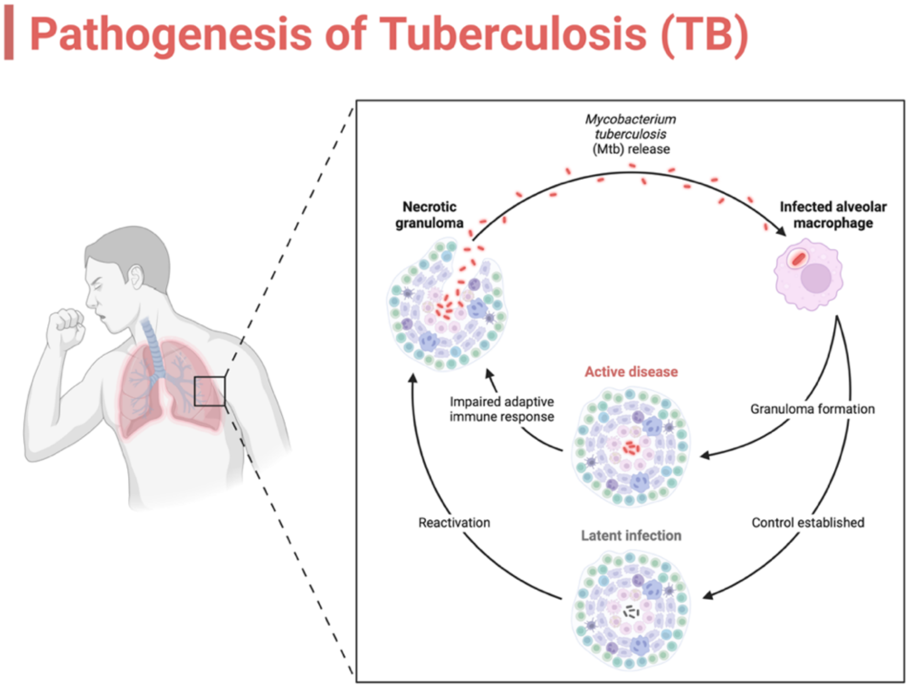


Figure 4. Pathogenesis of Tuberculosis (TB).
This illustration depicts the infection cycle of M.tb within the human host. Upon inhalation, M.tb infects alveolar macrophages in the lungs, leading to granuloma formation. The immune response may contain the bacteria, resulting in a latent TB infection. However, impaired adaptive immunity can lead to granuloma necrosis and reactivation of the disease, resulting in active TB. This figure was reproduced from Biorender with the adaptation of references (24). Source: https://app.biorender.com/biorender-templates/details/t-6632df4b45a68810b4918f5c-pathogenesis-of-tuberculosis-tb.

Evading the immune system

The adaptive immune response

Once M.tb antigens are phagocytized, they are presented to dendritic cells (DCs). The adaptive immune response is initiated when DCs present M.tb antigens in the lymph nodes, leading to the activation of CD4$^+$ T cells. These activated T cells then migrate to the lungs, where they contribute to controlling the growth of M.tb (39, 40). The adaptive cellular immune response to M.tb is only partially effective as it can limit the bacteria's progression but rarely achieves complete eradication (41). A past study suggests that during the early stages of infection *in vivo*, M.tb resides in one or more compartments that are poorly suited for antigen presentation to naïve CD4$^+$ T cells. This immune evasion permits substantial bacterial expansion within the lungs. A resulting high bacterial burden enhances resistance to adaptive immune effector mechanisms, thereby facilitating the long-term persistence of M.tb in the pulmonary environment (41).

Granulomas

One of the main features of the immune response to M. tb is the formation of an organized structure called a granuloma, whose primary role is to control the infection and protect against the spreading of M.tb by creating an immune microenvironment (42, 43). However, it also provides the mycobacterium with a niche in which it can survive by modulating the immune response to ensure its survival without damage over long periods of time (44, 45). Additionally, some M.tb bacilli can persist in the dormant state (36). A crucial factor in establishing infection is the balance between proinflammatory and anti-inflammatory cytokines, which helps regulate bacterial proliferation. TNF-α and IFN-γ play key roles in promoting granuloma formation and function, while IL-10 acts as a major negative regulator of the immune response (42, 46-48). It primarily involves the recruitment of macrophages during the infectious stage, along with highly differentiated cells such as multinucleated giant cells, epithelioid cells, and foamy cells (Fig.5) (42).

Initiation of the granuloma

Macrophages are the first to detect M.tb in the lungs, but the bacteria can also infect neutrophils and monocytes (24, 49, 50). Recognition of PAMPs and DAMPs triggers a cascade of responses in macrophages, including lysosomal uptake of M.tb, phagosomal maturation and acidification, cytokine secretion, vacuole maturation arrest, and increased intracellular nitric oxide production, all of which contribute to the elimination of M.tb (49, 51-53). However, these events do not lead to the initiation of the granuloma but their failure to eliminate M.tb does (49). A past study suggested that one of the earliest steps in granuloma formation is the migration of macrophages from the airspaces into the lung interstitium. This process requires IL-1–mediated crosstalk between M.tb-infected alveolar macrophages and the alveolar epithelium, leading to increased epithelial permeability (54).

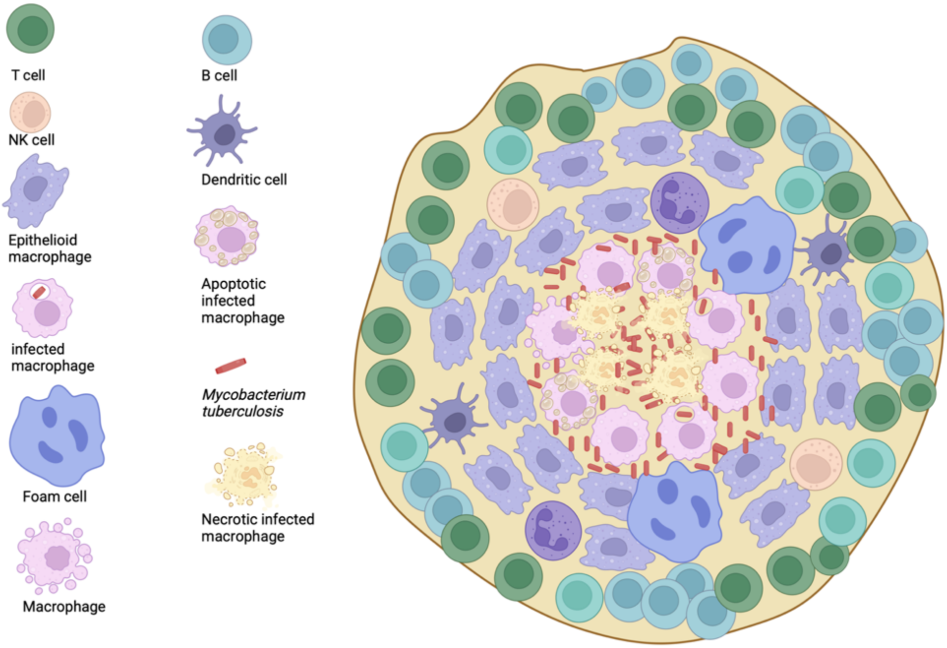


Figure 5. Cellular structure of a tuberculosis (TB) granuloma.
This diagram illustrates the complex architecture of a granuloma formed in response to *Mycobacterium tuberculosis* infection. The center consists of infected, apoptotic, and necrotic macrophages, as well as extracellular *M. tuberculosis* bacilli. Surrounding the core are foam cells, epithelioid macrophages, and dendritic cells. The outermost layer is populated by adaptive immune cells, including T cells and B cells, which help contain the infection. The structured immune cell layers highlight the host's attempt to control bacterial spread while balancing tissue preservation. This *Image was created with BioRender.com,* with the adaptation of references (42, 55-57).

Clinical manifestations

The vast majority of individuals infected with TB remain asymptomatic after the primary infection. This happens either due to effective immune control that eliminates the bacteria or because the bacteria persist in a latent state with the potential to progress to active disease later in life (58, 59). It is estimated that between 5% and 15% of those with latent TB will eventually develop active tuberculosis (60). Certain groups of individuals face a higher risk of progressing from latent to active TB, with the specific definition of these groups varying based on local epidemiology. Typically, they include people living with HIV, recent contacts of individuals with active TB, and those with fibrotic changes on chest X-rays suggestive of previous TB infection (59). In the 5-10% of TB-infected individuals, several signs and symptoms develop, which allow for clinical diagnosis. The clinical manifestations of active pulmonary TB may include pleuritic chest pain, low-grade fever, prolonged productive cough, fatigue, loss of appetite, haemoptysis, night sweats and weight loss (61, 62).

Table 1. Clinical Manifestations of Extrapulmonary Tuberculosis by Site of Disease (Soto-Martinez & Ranganathan, 2011)

Site of disease	Clinical manifestation
Lymph nodes	Typically, enlargement of cervical lymph nodes. It may affect inguinal, axillary and mediastinal lymph nodes.
Pleural TB	Pleural effusion-usually unilateral
Heart	Pericarditis
Upper airway	Laryngeal TB: hoarseness and pain. Can also affect the oral cavity, pharynx and salivary glands
Paranasal sinuses, nose and ears	Tuberculous otitis media. Granulomatous lesions with nasal discharge, nasal obstruction and septal perforation.
Ocular	Uveitis, conjunctivitis, chronic granulomatous iridocyclitis.
Central nervous system	Meningitis Cranial nerve palsies Communicating hydrocephalus Encephalopathy
Skeletal	Chronic osteomyelitis Monoarthritic (usually weight-bearing joints). Paraspinal or psoas abscesses
Gastrointestinal	Abdominal pain. Ileocecal disease: diarrhea and palpable right iliac fossa mass. Perirectal abscesses or fistulas
Genitourinary	Micturition frequency Dysuria, hematuria Flank pain/ swelling Renal calcifications, calculi, scarring, hydronephrosis
Male genital tract	Epididymitis Calcifications in vas deferens, seminal vesicles or prostate
Female genital tract	Infertility, pelvic inflammatory disease.

Pulmonary Tuberculosis

The most affected organ in an immunocompromised host during TB infection is the lungs. Lung involvement occurs in 79–87% of active TB cases and is similarly common in immunocompromised individuals, including those with HIV (70–92%). Symptoms vary depending on whether the infection is primary or reactivated, with primary TB often being asymptomatic or mildly symptomatic (59, 63).

Extrapulmonary TB

Extrapulmonary TB (EPTB), which occurs when *Mycobacterium tuberculosis* spreads beyond the lungs, presents with a wide spectrum of clinical signs depending on the organ system involved. Common forms include lymphatic TB, which is the most frequent extrapulmonary manifestation, followed by pleural, skeletal, genitourinary, and central nervous system TB (64). TB meningitis, though rare, is one of the most severe forms and can result in significant morbidity and mortality if not diagnosed promptly. EPTB is particularly common in immunocompromised individuals, including those with HIV infection. In such cases, diagnosis is often more challenging due to the nonspecific nature of symptoms and the limited sensitivity of conventional diagnostic tools (65). Furthermore, miliary TB, a disseminated form of the disease can involve multiple organs and often presents with vague systemic symptoms such as fever, hepatosplenomegaly, and pancytopenia(66). Clinical awareness and early suspicion are critical for diagnosis, particularly in high-risk populations and in regions where TB remains a cause for concern.

Epidemiology

According to WHO reports, in 2023, TB claimed the lives of approximately 1.25 million people, including 161,000 individuals living with HIV (67). After three years of being surpassed by COVID-19, TB has returned as the leading cause of death from a single infectious disease. It remains the top cause of death among people with HIV and a significant contributor to fatalities linked to antimicrobial resistance (67, 68).

An estimated 10.8 million people developed TB in 2023, comprising 6.0 million men, 3.6 million women, and 1.3 million children. TB affects individuals across all countries and age groups (67). Importantly, it is both preventable and curable. However, multidrug-resistant TB (MDR-TB) continues to pose a serious public health crisis and global health security threat. Alarmingly, only around 40% of those with drug-resistant TB received treatment in 2023. Fig.6 shows the current incidence rate of active tuberculosis disease in the world. In 2023, the countries with the highest TB incidence rate were Pakistan, India, Bangladesh and the Philippines, followed by South Africa and Nigeria (67, 69).

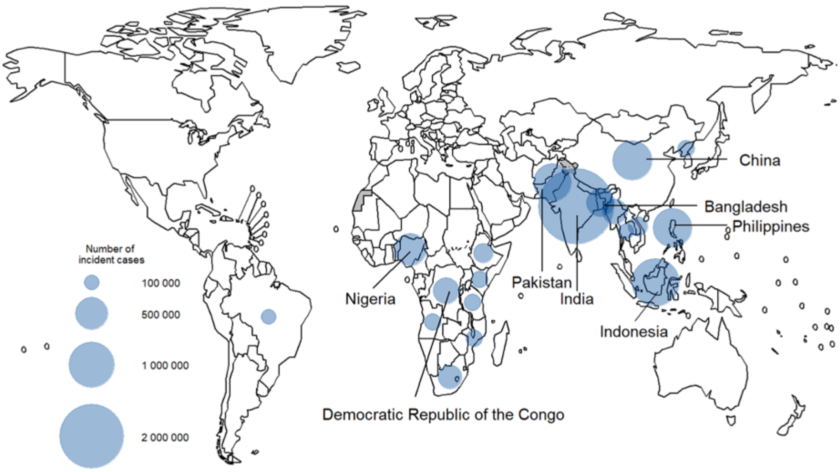


Figure 6. Estimated number of incident TB cases in 2023, for countries with at least 100 000 incident cases.
Source: WHO global Tuberculosis report 2024.

Diagnosis

There is currently no test available to directly detect live Mycobacterium tuberculosis (M.tb) infection in humans. Instead, the diagnosis of latent TB (LTB) infection is made indirectly by detecting an immune response to MT antigens, based on the assumption that this response has developed following exposure to the bacterium (70-72). The Tuberculin Skin Test (TST), aslso known as the Mantoux test, and the Interferon-Gamma Release Assays (IGRAs) are the primary diagnostic tools for LTBI. Both tests are indirect, relying on the detection of an immune response to M.tb, and do not directly detect the presence or viability of the bacteria (70).

The Tuberculin skin test (TST) is the most ancient and classical test used to detect LTBI and relies on the reaction between M.tb antigens injected in the dermis of the skin (71, 73). The intensity of the local inflammatory reaction, driven by cytokine release from sensitized lymphocytes, is measured after 48 to 72 hours (36, 59). This is also known as delayed hypersensitivity or Type IV hypersensitivity reaction (74). An alternative is the blood test relying on the *in vitro* measurement of gamma-interferon release by sensitized lymphocytes after stimulation with M.tb antigens. The test follows the same principle as the TST, assessing cytokine release by sensitized lymphocytes. It is more expensive than the TST, however, offers several advantages such as using only M.tb-specific antigens (ESAT-6 and CFP-10), absent in BCG and most environmental mycobacteria providing higher specificity (75). Additionally, the subjective bias due to the injection technique and the reading of the skin reaction is eliminated (75-77). Moreover, the test is performed with positive and negative controls, decreasing the risk of error (70, 78). However, the IGRA test lacks the ability to distinguish between latent infection and active tuberculosis disease (79, 80). Furthermore, similarly to the IGRAs test, the TST has the disadvantage that it is not specific for M.tb and does not distinguish latent infection from active disease (79). Additionally, the IGRA test may be negative or indeterminate in immunosuppressed states (81). The TST test may be positive with BCG vaccination and exposure to atypical mycobacteria and like the IGRA test, the interpretation of results may lead to false negatives. Especially in immunosuppressed patients who have extrapulmonary or miliary TB (80-82).

Sputum has long been the most used sample in TB diagnostics and has been used to identify the presence or absence of disease, the susceptibility pattern of the organism, and to measure the response to treatment (83). When TB is suspected, an acid-fast bacilli stain using the Ziehl–Neelsen staining technique should be performed to detect M.tb (84). Gram-positive bacteria possess a thick peptidoglycan layer in their cell walls, which retains the crystal violet stain. In contrast, gram-negative bacteria have a thinner peptidoglycan layer and take up the red or pink counterstain (85, 86). An AFB-positive result, such as M.tb are weakly stained due to the high content of mycolic acids, which prevents dye penetration and retention. Additionally, in contrast to gram-positive bacteria, M.tb has a waxy outer layer, resulting in resistance to typical Gram staining (87, 88). The Acid-fast staining method relies on the microscopic examination of sputum samples for acid-fast mycobacteria (89). The sputum smear microscopic examination is simple, rapid and inexpensive which is highly specific in areas with a very high prevalence of TB (90-93). Additionally, it helps identify the most infectious individuals and is broadly applicable across populations with diverse socio-economic backgrounds (94). However, sputum smear microscopy has significant limitations in its performance. The sensitivity is significantly compromised when the bacterial load is less than 10,000 organisms/mL in a sputum sample. It is also not efficient in detecting extra-pulmonary TB, paediatric TB, and in patients co-infected with HIV and TB (90, 95, 96). This is due to its low sensitivity in these populations, as they often carry a lower bacterial load in their samples, making it challenging for microscopy to detect the presence of bacteria (90, 97). In addition, sputum smear microscopy has limited sensitivity due to false positives, such as the presence of NTM in the samples, and cannot distinguish between NTM and M.tb (98, 99).

Liquid automated culture using the BACTEC Mycobacteria Growth Indicator Tube (MGIT) system (Becton Dickinson, USA) is the second step to confirm TB infection and is a sensitive confirmatory method, especially for detecting extra-pulmonary TB. Current guidelines recommend performing culture whenever possible on all initial diagnostic samples, as well as for monthly monitoring during treatment (83). Xpert (Cepheid, USA) is a molecular diagnostic tool for detecting M.tb as well as rifampicin resistance in a single automated cartridge (100). When supplemented by a second Xpert MDR/XDR test, which detects resistance to isoniazid, fluoroquinolones, amikacin, kanamycin, capreomycin, and ethionamide, it can offer a comprehensive guide for tailoring therapy in cases of drug-resistant TB (83, 101). Although ZN staining boasts high specificity, its sensitivity ranges broadly from 20 % to 80 %. Fluorescence microscopy offers greater sensitivity while maintaining similar specificity to conventional ZN methods (102).

Radiography (chest X-rays) is a screening tool used to diagnose active pulmonary TB, however, it cannot help in detecting latent TB infection (103). Additionally, the Lowenstein-Jensen medium is used to culture TB bacteria. However, it has the limitation that since M.tb is a slow-growing organism, it takes at least two weeks (sometimes 6-8 weeks) for the colonies to appear, further delaying treatment (61, 104).

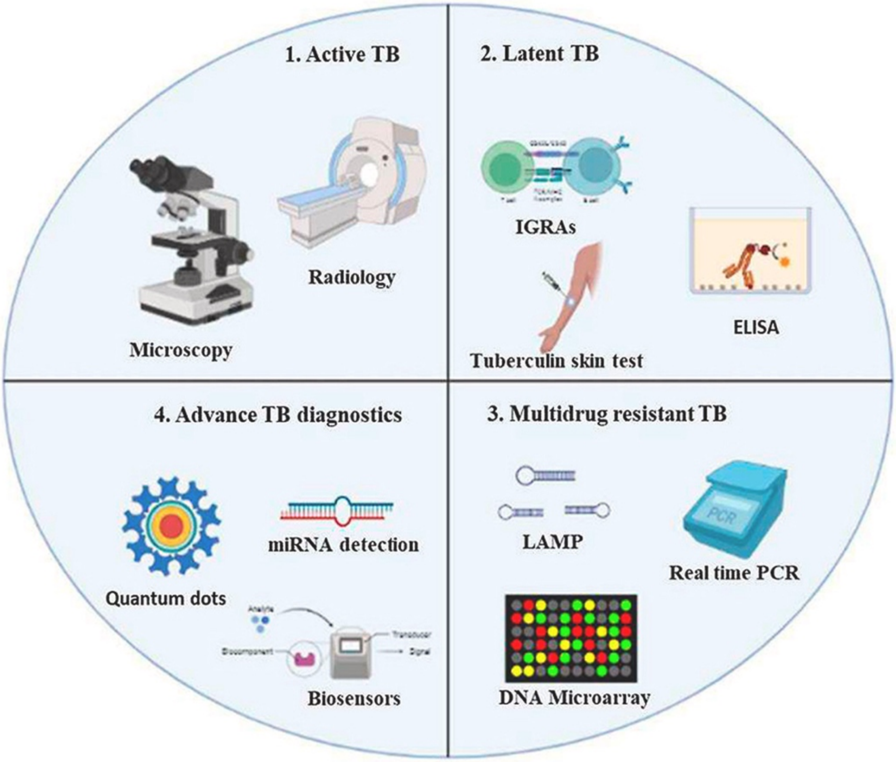


Fig. 7 Overview of diagnostic approaches for different forms of tuberculosis (TB).
The figure is divided into four quadrants representing key diagnostic categories: (1) Active TB diagnosis using microscopy and radiology to detect the presence of Mycobacterium tuberculosis and associated pulmonary damage; (2) Latent TB diagnosis using immunological methods such as Interferon-Gamma Release Assays (IGRAs), tuberculin skin test, and ELISA to identify latent infections; (3) Multidrug-Resistant TB (MDR-TB) detection employing molecular techniques including Loop-mediated Isothermal Amplification (LAMP), real-time PCR, and DNA microarrays for identifying drug resistance; (4) Advanced TB diagnostics utilizing novel technologies such as quantum dots, microRNA (miRNA) detection, and biosensors to improve sensitivity, specificity, and rapid detection of TB. These diverse diagnostic tools support comprehensive TB management and control strategies. This figure is reproduced from Srestha Mukherjee et al, Evolution of tuberculosis diagnostics: From molecular strategies to nanodiagnostics. https://doi.org/10.1016/j.tube.2023.102340.

Treatment

The current recommended treatment for Drug-Sensitive (DS) TB involves a combination of four antibiotics: isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB), which were all discovered 60 years ago (105). This four-drug regimen should be given under directly observed therapy (DOT) for a minimum of six months to maximize cure rates. It consists of two phases: an intensive phase of the four drugs for the first two months, followed by a continuation phase of isoniazid and rifampicin for the remaining four months to kill any dormant bacilli (105). Although the four first-line anti-TB drugs are effective against drug-susceptible TB, they can cause various adverse effects, including hepatic dysfunction, peripheral neuropathy, erythromelalgia, ocular toxicity, central nervous system toxicity, gastrointestinal intolerance, and skin rash (61, 106, 107). Poor patient compliance due to these unwanted side-effects, high pill count and prolonged duration of the therapy, in addition to misuse of antibiotics, contributed to the emergence of Multidrug-resistant (MDR) and extensively drug-resistant(XDR) M.tb strains (61, 106). Additionally, DR-TB transmission remains a primary mechanism whereby an individual gets infected by a resistant strain (108).

Drug-Resistant (DR) TB Crisis

MDR-TB is defined as resistance to INH and RIF, the two most powerful front-line anti-TB drugs (109, 110). The WHO has reported MDR-TB to occur in 3-4% of new TB cases, and 18-21% of previously treated cases (111, 112). After years of DR-TB patients being subjected to long and toxic injectable-based regimens of 18 to 20 months, the introduction of a short-course regimen of 9 to 12 months was a major advancement (113). Great improvement was observed with the introduction of Bedaquiline (BDQ) and all-oral short regimens such as pretomanid and linezolid (LZD) (114). The BpaLM regimen is used to treat MDR-TB and is recommended by the WHO. The 6-month all-oral regimen comprises of bedaquiline, pretomanid, linezolid and moxifloxacin (115-117) (Fig.8). In addition, the WHO recommends a 9-month all-oral treatment regimen to patients with MDR-TB who have no resistance to fluoroquinolones. This regimen includes bedaquiline administered for the first six months, combined with levofloxacin or moxifloxacin, ethionamide, ethambutol, high-dose isoniazid, pyrazinamide, and clofazimine for the initial four months. This intensive phase may be extended to six months if the patient remains sputum smear-positive at the end of the fourth month. The continuation phase which lasts five months includes levofloxacin or moxifloxacin, clofazimine, ethambutol, and pyrazinamide (115, 118).

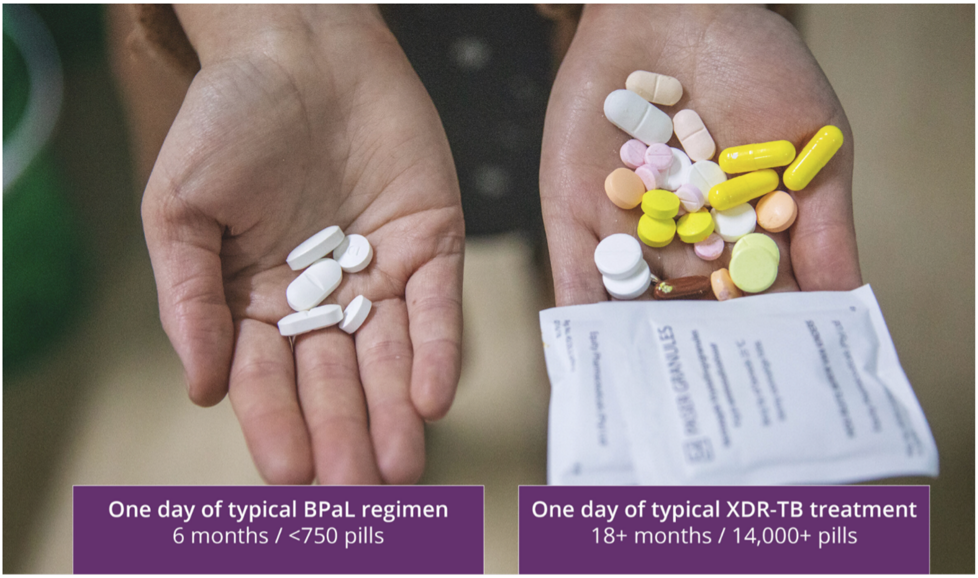


Fig.8 TB Alliance's 6-month, all oral DR-TB therapy reduces the length and number of pills compared to the previous standard of care.
Source: https://www.keepingthepromisereport.org/case-studies/pretomanid. Reference (119)

An MDR-TB strain with additional resistance to fluoroquinolones (FQs) is known as pre-extensively DR-TB , while MDR-TB strain resistant to bedaquiline(BDQ) and LZD are known as XDR-TB (113, 120). XRD-TB patients have intolerance to key components of the above-mentioned regimens and require an individually designed treatment plan based on a hierarchical grouping of second-line TB drugs, the drug-resistant profile and the patients medical history (115).

In response to the rising global burden of DR-TB, new compounds and adjunctive therapies are also being evaluated in clinical trials. Delamanid, another nitroimidazole like pretomanid, has shown promise in combination with other agents for both MDR and XDR-TB treatment (121). Studies such as the NIX-TB and ZeNIX trials have demonstrated the potential of all-oral, shorter regimens with improved tolerability and efficacy (122). Despite the advancements in new treatment options, proper diagnostic tools remain key challenges in scaling up DR-TB treatment in high-burden settings.

Immunization approaches

BCG Vaccine

The only vaccine licensed for the prevention of tuberculosis is *Mycobacterium bovis* bacillus Calmette-Guerin (BCG). BCG is based on the attenuation of bacteria naturally causing tuberculosis in cattle and occasionally in humans (123-125). It is speculated that BCG has been administered to more humans than any other vaccine for the prevention of infectious disease (123, 126). The BCG vaccine confers over 70% protection against disseminated tuberculosis and tuberculous meningitis in newborns and school-aged children, however, its effectiveness is substantially lower in adults (127).

The creation of the BCG vaccine was a meticulous, multi-year process undertaken by Léon Charles Albert Calmette and Jean-Marie Camille Guérin, who were mentored by Louis Pasteur(123, 128). Their objective was to develop a live attenuated vaccine, as attempts to inactivate the bacteria using heat or chemicals proved unsuccessful. The process began in 1906 with the treatment of *Mycobacterium bovis* with ox bile, which altered the appearance of the bacilli. When *M. bovis* was grown on potato slices infused with ox bile and glycerol, its typically dry, crumbly colonies transformed into smooth, greenish-brown masses. These cultures required 3–4 weeks of incubation before being transferred to fresh media (123). Consequently, from 1908 to 1920, Calmette and Guérin subcultured their strains every three weeks, ultimately achieving 230 successive passages by 1920 (129). This extensive serial culturing and passage process led to the attenuation of the bacterium. Crucially, during these passages, the bacterium lost a nine-gene locus known as region of difference 1 (RD1), which is present in virulent *M. bovis* and absent in all BCG strains (123, 130). Past Studies have confirmed that the RD1 locus is essential for the bacterium's full virulence (123, 131). Following these extensive attenuation efforts (fig.9), Calmette and Guérin conducted efficacy trials in animals, observing that multidose oral administration showed the most promise (123).

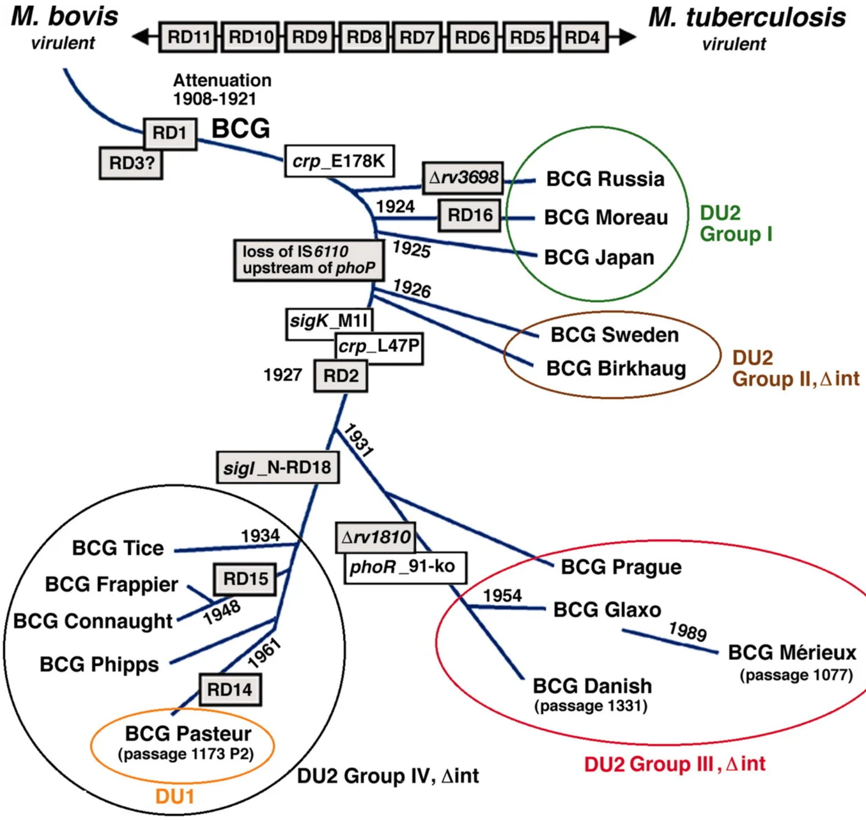


Figure 9. Evolutionary divergence and genetic modifications of BCG vaccine strains. This diagram illustrates the attenuation and divergence of *Mycobacterium bovis* BCG from its virulent ancestor between 1908 and 1921, followed by the derivation of multiple BCG vaccine strains across different regions and time points. Specific gene deletions, mutations, and region of difference (RD) losses such as RD1, RD2, RD14, RD15, RD16 are noted. Reproduced from Brosch et al (2007). https://doi.org/10.1073/pnas.0700869104

Novel Vaccination Strategies

Significant progress has been made in TB vaccine research in recent years, with the goal of licensing one vaccine in the next 5 years (132). As of August 2024, there were 15 vaccine candidates in clinical development targeting various aspects of the immune response to M.tb (Table.2) (133).

Table 2. The pipeline of new vaccines as per TB vaccine accelerator council progress update (May-October 2024) reference (132).

Phase I	Phase IIa	Phase IIb	Phase III
BNT164a1	**ChAdOx185AMVA85A** University of Oxford	**DAR-901** booster_ Dartmouth, St. Louis	**GamTBvac_** Ministry of Health, Russian Federation
BNT164b1 **BioNtech SE**	**ID93+GLA SE(QTP101)**_Quratis U.S. NIH/NIAID RUTI	RUTI_ Archivel Farma, S.L	**MIP/Immuvact_** ICMR, Cadila Pharmaceuticals
TB/FLU-05E_RIBSP	**AEC/BC02_** Anhui Zhifei Longcom		**M72/AS01E_** GSK, Gates MRI
H107e/CAF10b_SSI			**MTBVAC** Biofabri, University of Zaragoza, IAVI, TBVI

Several vaccines are currently being developed to strengthen the protection provided by BCG, especially in adults who have previously received this vaccine. Among the most promising candidates is the M72/AS01E vaccine, which demonstrated 50% efficacy in preventing TB disease in individuals with latent TB infection during phase 3 trials conducted in South Africa, as of March 2024 (132). Alongside booster vaccines, novel preventive vaccines are also being developed to provide more comprehensive protection against TB infection and disease. These next-generation vaccine candidates aim not only to reduce infection but also to prevent transmission altogether. Examples include subunit vaccines, such as the AERAS-402 tuberculosis vaccine, which is intended to enhance BCG-primed immunity. Additionally, viral vector vaccines that use platforms like adenovirus vectors to deliver TB antigens. These strategies focus on inducing robust cellular immune responses, which are critical for achieving effective TB control (134, 135).

Novel TB treatment

One of the major obstacles to developing a new vaccine is the lack of reliable TB biomarkers and a limited understanding of the complex immune response to TB (132, 136). Secondly, ensuring equitable distribution of the new TB vaccines is another critical challenge, particularly in low-and middle-income countries where the TB burden is the highest. Addressing these challenges will require coordinated global efforts, substantial investment, and innovative strategies to accelerate vaccine development and its distribution. There are clinical trials that investigate adjunctive treatment alongside standard anti-TB therapy. One of these trials, conducted across three South African sites, evaluated four different oral host-directed therapies (HDTs). They used alongside standard rifabutin-modified anti-TB treatment in adults with rifampicin-susceptible pulmonary tuberculosis. Of the four HDTs, CC-11050 and everolimus showed significant lung function improvement (137). CC-11050 downregulates pro-inflammatory cytokines such as TNF-alpha by increasing intracellular cyclic AMP levels. This helps to control excessive lung inflammation without compromising bacterial clearance (138, 139).

Furthermore, research into immune-modulating agents like auranofin and metformin are expanding treatment possibilities by targeting host pathways to improve bacterial clearance and reduce tissue damage (140, 141). The development of new generation oxazolidinones such as sutezolid and TBI-223, which may offer similar efficacy to linezolid but with fewer side effects is also underway(142). Moreover, the use of metformin**,** a commonly prescribed antidiabetic drug that has demonstrated immunomodulatory effects. Metformin enhances mitochondrial reactive oxygen species (ROS) production and promotes autophagy, contributing to better control of intracellular *M.tb* (140, 141). Clinical studies have shown that adjunctive metformin therapy can accelerate sputum conversion and reduce lung pathology (143).

Another promising candidate is statins**,** which have been shown to reduce TB-related inflammation and improve outcomes by modulating cholesterol metabolism and autophagy (144). Furthermore, although Linezolid was initially developed for gram-positive bacterial infections (145), it has gained attention for its effectiveness against drug-resistant TB strains. When used in combination regimens, it has shown high efficacy in shortening treatment duration for XDR-TB (146).

Antimicrobial peptides

Since their discovery in the 1980s, research efforts in the development of antimicrobial peptides (AMPs) to combat microbial infections, including drug-resistant bacteria, have expanded significantly (148, 149). AMPs are naturally found across a wide range of organisms, from single-celled bacteria and fungi to animals and they serve as crucial components of the innate immune system (150, 151). As part of this defense system, AMPs provide the first line of protection against bacteria, fungi, and viruses. By 2020, a total of 3,240 AMPs had been documented in the Antimicrobial Peptide Database. The database is accessible online through a web browser at: http://aps.unmc.edu/AP/main.html (152). The diversity among naturally occurring AMPs complicates their classification, however, they are currently categorized based on their biological source, functional activity, structural properties, and amino acid composition (fig. 10 and 11) (153, 154).

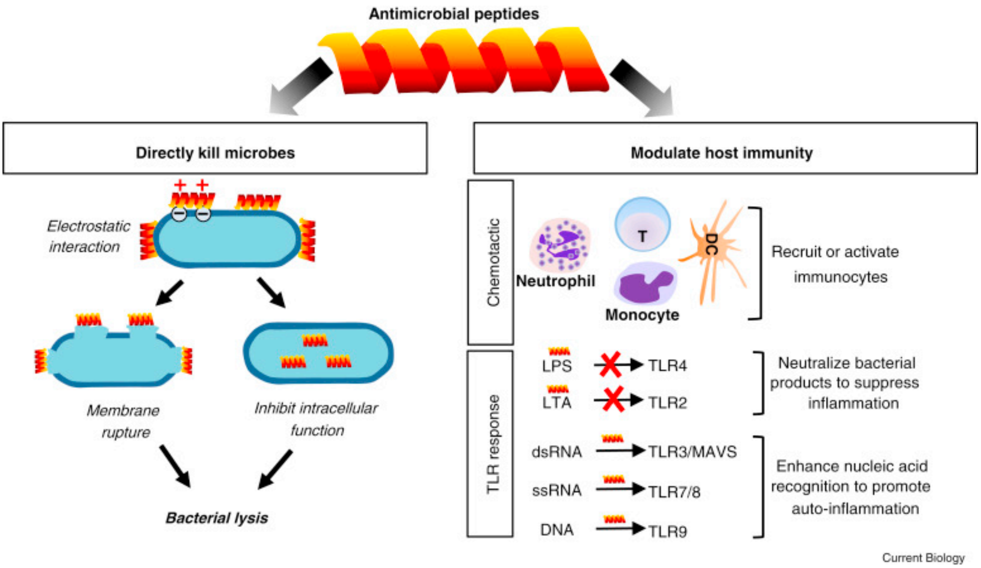


Figure 11. Dual roles of antimicrobial peptides (AMPs) in host defense.
AMPs contribute to host protection by directly killing microbes and modulating host immune responses. On the left, AMPs disrupt bacterial viability via electrostatic interaction with negatively charged membranes, leading to membrane rupture or inhibition of intracellular functions causing bacterial lysis. On the right, AMPs modulate immunity by recruiting immune cells (e.g., neutrophils, monocytes) and interacting with Toll-like receptor (TLR) pathways to neutralize bacterial components or enhance nucleic acid recognition, thereby influencing inflammatory responses. This figure is reproduced from Zhang, L.-J., Gallo, R.L. (2016). Antimicrobial peptides: multi-faceted components of the skin immune defense system. https://www.ncbi.nlm.nih.gov/pubmed/26766224 (155).

Classes of AMPs studied in Tuberculosis

Cathelicidins

Cathelicidins are initially produced as an inactive precursor protein that contains a signal peptide and a cathelin-like domain, which acts as the inactive antimicrobial region (156). From this precursor, the active C-terminal peptide known as LL-37 is generated through cleavage by the enzyme proteinase 3 (157) (158). Once released, LL-37 becomes an active antimicrobial agent. Beyond its antimicrobial properties, LL-37 also plays several other important roles, including such as supporting blood vessel formation (angiogenesis), and aiding in wound healing (159). It plays a diverse role in the innate immune cell responses including the induction of interleukin 8 (IL-8, CXCL8), and the up-regulation of cytokine receptors CXCR4, CCR2 and IL-8RB (CXCRB) and acts as a chemoattractant for neutrophils and monocytes (157). Additionally, increased binding of calcitriol to vitamin D receptors in macrophages promotes the production of LL-37, enhancing the macrophage’s ability to fight mycobacterial infections (157, 160).

Defensins

Defensins act as a bridge linking the innate and acquired immune system, largely through their chemotactic peptides. They belong to a family of small (3-5kDa) cationic cytotoxic and oxygen-independent peptides that are active against a wide variety of bacteria, viruses and fungi (161, 162). There are three major classes of defensins found in vertebrates**:** α-, β-, and θ-defensins, which are distinguished by their structure and antimicrobial properties. α-defensins are mostly identified in polymorphonuclear neutrophils and chemoattracts macrophages (163). Studies suggest that Human neutrophil α-defensin 1 and 2 (HNP-1 and HNP-2) kills both tuberculosis and non-tuberculosis mycobacteria effectively by increasing cell wall and membrane permeability. Additionally, HNP-2 reduces the therapeutic dosage of drugs (161, 163-166). Human neutrophil α-defensin 3 and 4 (HNP-3 and HNP-4) reduce tuberculosis and non-tuberculosis load *in vitro* and *in vivo*. Additionally, HNP-4 increases permeability of the cell envelope and has immune-enhancing effects such by attracting monocytes, T cells, immature dendritic cells. HNP-4 also stimulates macrophages to release pro-inflammatory cytokines such as TNF-α and IFN-γ, further enhancing the immune response (167). Moreover, HNP-4 demonstrates synergy with antituberculosis drugs (161, 163, 164). There are four families of Human β-defensin (HBD) in which all are found in the epithelial cells (161). HBD-1 is induced by mycobacterium infection and exhibits antimicrobial activity upon stimulation with mycobacteria. Furthermore, the combination of INH and HBD-1 produces a synergistic effect, significantly reducing the M.tb load (161). HBD-2 improves the capacity of macrophages to control M.tb and has the greatest antimicrobial activity (161, 168).

Lastly, a past study reported that the combination of HBD-2 with BCG at one month and 6 months post-vaccination had a higher antimicrobial effect than pre-vaccination levels in an elderly population (169) . HBD-3 and HBD-4 were found to be induced by mycobacterial infection and shows antimicrobial activity. Its stable production during latent infection is linked to long-term control of mycobacterial infection. Additionally, HBD-3can be further stimulated by L-isoleucine during TB infection (161, 170).

NZ2114 - A novel defensin to inhibit mycobacterial infection

The peptide NZ2114 is a water-soluble improved plectasin derivative, originally isolated from the fungus *Pseudoplectania nigrella* (Fig.12)*,* with amino acid sequence GFCCNGPWNEDDLRCHNHCKSIKGYKGGYCAKGGFVCKC (171).

The peptide has been widely studied for its potent antimicrobial properties. It targets bacterial cell wall synthesis by binding to lipid II, an essential precursor in peptidoglycan biosynthesis (172, 173). In earlier studies, plectasin demonstrated strong activity against Gram-positive bacteria, particularly *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Enterococcus faecalis (174, 175)*.

Past studies suggest that NZ2114 presents a promising alternative to traditional antibiotics, especially in the face of rising antibiotic resistance and may reduce the likelihood of cytotoxic effects on host cells (176). Furthermore, its high stability, low immunogenicity, and ability to retain activity in physiological environments make it an attractive candidate for therapeutic development. Ongoing research is exploring its efficacy in animal infection models and its potential synergistic effects when used in combination with other antimicrobial agents (176).

Fig. 12 *Pseudoplecatania nigrella* in their natural habitat.
These darkly pigmented cup fungi grow among moss, pine needles and leaf litter on a moist forest floor. Source: https://en.wikipedia.org/wiki/Pseudoplectania_nigrella

NZX and **NZ2114** were developed to enhance stability and broaden antimicrobial activity (177). Notably, NZ2114 has shown high efficacy against *S. aureus* in both in vitro and in vivo models (178), while NZX demonstrated promising activity against mycobacteria, including M.tb and *M. abscessus (177, 179)*.

In the first paper of the thesis ***(Paper I)***, we study the antimycobacterial activity of the plectasin NZ2114. We found that NZ2114 effectively killed mycobacteria at a minimal inhibitory concentration (MIC99) of 6.1 µM, showed no toxicity to primary human cells, and maintained its antimycobacterial activity despite exposure to serum degradation. Additionally, similarly to previous studies, NZ2114 demonstrated antimicrobial activity against various clinical isolates of Gram-positive bacteria, including *Enterococcus faecalis*, *Enterococcus faecium*, and Methicillin-Resistant *Staphylococcus aureus* (MRSA) (171).

Pulmonary TB lung damage

Although the standard six-month course of treatment with four antibiotics has success, many TB survivors experience long-term health consequences. There is increasing evidence of long-term disability and elevated mortality risks (2) (180-183). Studies have shown that even after successful tuberculosis treatment, patients may experience long-term pulmonary complications, including chronic obstructive pulmonary disease (COPD), spirometric restriction, pulmonary hypertension, bronchiectasis, and secondary lung infections (180). Lung damage in pulmonary tuberculosis (PTB) is largely driven by the host's immune response, especially through excessive inflammation and the release of matrix-degrading proteases (fig. 13) (184). Although the immune system plays a vital role in controlling the infection, it can also unintentionally cause significant tissue injury. This damage manifesting as fibrosis, cavitation, and necrosis often persists even after successful treatment (180, 185, 186). A recent meta-analysis reported a mortality ratio of 2.91 (95% CI: 2.21–3.84) among tuberculosis survivors compared to individuals with no history of TB. This previously underrecognized post-tuberculosis burden is now gaining attention, with growing calls for increased research efforts and political attention (2, 187, 188).

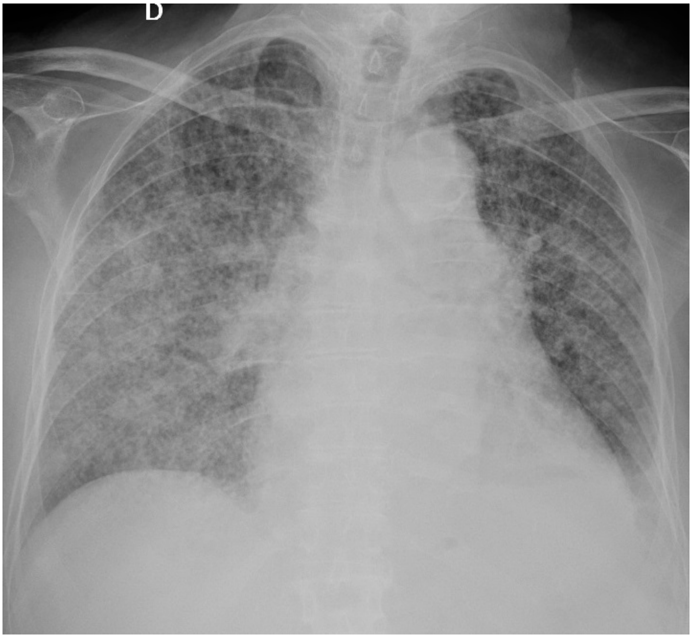


Figure 13. Chest radiograph of miliary tuberculosis in an elderly patient.
Anteroposterior chest X-ray taken 22 days after the onset of fever, chills, and general malaise in an 82-year-old immunocompetent female. The image reveals extensive bilateral reticulo-nodular infiltrates consistent with Military Tuberculosis. Adapted from: Pathogens, Zhang et al. (2018), "Miliary Tuberculosis Presenting with Hyponatremia and ARDS in an 82-Year-Old Immunocompetent Female", Pathogens, 7(3), 72. https://doi.org/10.3390/pathogens7030072. Licensed under CC BY 4.0.

"The biggest disease today is not TB or Leprosy,
but rather the feeling of being unwanted."
-Mother Theresa

Bacterial Vesicles

Bacterial vesicles can originate from either the inner or outer membrane, however, outer membrane vesicles are more extensively studied. Outer membrane vesicles (OMV) are small, spherical structures, typically ranging from 20 to 300 nm in size (189, 190). They are released during bacterial growth from the outer membranes of gram-negative bacteria into the surrounding environment through a process called blebbing or by explosive cell lysis. OMVs contain components such as phospholipids, lipopolysaccharide (LPS), outer membrane proteins (OMPs), and periplasmic proteins that become enclosed during vesicle formation (190). Due to their composition, OMVs are involved in various physiological functions, including protein transport, nutrient uptake, cell-to-cell communication, antibacterial defense, toxin delivery, and modulation of the host immune response (fig.14) (189, 191-193).

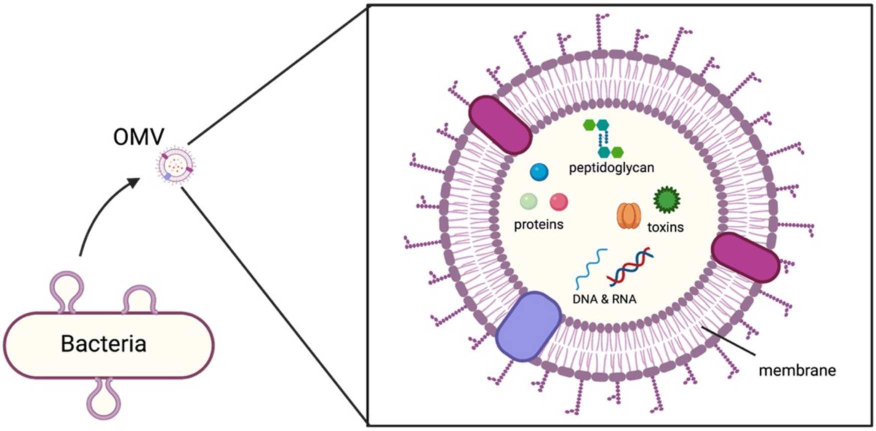


Figure 14. Structure and composition of an outer membrane vesicle (OMV) derived from Gram-negative bacteria.
This figure illustrates the formation of an outer membrane vesicle (OMV) from a bacterial outer membrane. The enlarged view shows the typical bilayer membrane of the OMV enclosing a variety of molecular cargo, including peptidoglycan fragments, proteins, toxins, and nucleic acids (DNA and RNA). These vesicles are important mediators of bacterial communication, host-pathogen interactions, and delivery of virulence factors. Source: https://misciwriters.com/2021/04/28/bacterial-outer-membrane-vesicles-little-membrane-blebs-with-big-vaccine-potential

Vesicles from gram-positive bacteria

While numerous studies have confirmed that Gram-positive bacteria produce cytoplasmic membrane vesicles (CMVs), the mechanisms behind their biogenesis have only recently been reported (194). Similar to the explosive cell lysis observed in *Pseudomonas aeruginosa*, research has shown that in *Bacillus subtilis*, the expression of a prophage-encoded endolysin triggers CMV formation (193). Although in both species these endolysins weaken the peptidoglycan layer, the outcomes differ. *P. aeruginosa* undergoes complete cell rounding and lysis, whereas *B. subtilis* extrudes cytoplasmic membrane material through localized breaches in the cell wall, releasing these protrusions as explosive CMVs (ECMVs) (193). Unlike Gram-negative cells, which completely disintegrate during explosive lysis, the thick peptidoglycan wall of *B. subtilis* remains partially intact (194, 195). However, most affected cells die due to compromised membrane integrity, as evidenced by the appearance of "ghost" cells containing internalized vesicles (194). This process, observed in other Gram-positive bacteria as well, has been termed “bubbling cell death”(195). Similar to explosive lysis in Gram-negative bacteria, bubbling cell death can be induced by DNA-damaging agents such as ciprofloxacin (196) (Fig. 15).

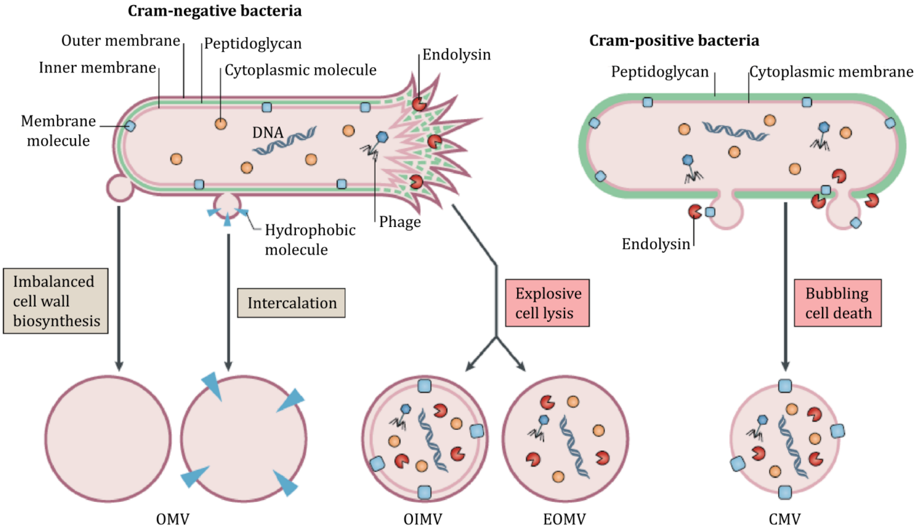


Fig. 15 Distinct pathways give rise to different types of membrane vesicles.
In Gram-negative bacteria, vesicles form via two primary mechanisms: outer membrane blebbing and explosive cell lysis. In Gram-positive bacteria, endolysin can induce a process known as "bubbling cell death," resulting in the formation of cytoplasmic membrane vesicles (CMVs), which may contain both membrane and cytoplasmic components. Other agents or enzymes that damage peptidoglycan may similarly promote CMV formation. This image is reproduced from Qing and Gong (2019) “Natural and engineered bacterial outer membrane vesicles.” Reference (197).

The clinical impact of MVs

MVs including exosomes, microvesicles, and outer membrane vesicles (OMVs), have a significant clinical impact due to their roles in diagnostics, therapeutics, immune modulation, and disease progression (198). These vesicles carry proteins, lipids, and nucleic acids from their parent cells, making them valuable as non-invasive diagnostic biomarkers in conditions such as cancer, neurodegenerative diseases, and infections. For instance, tumor-derived exosomes can reflect tumor type and stage (199), while vesicles in cerebrospinal fluid may carry pathological proteins associated with Alzheimer's disease (200). MVs also serve as natural drug delivery vehicles, capable of transporting chemotherapeutics, siRNA, or gene-editing tools like CRISPR/Cas9 across biological barriers. Additionally, they play complex roles in immune modulation. Tumor-derived vesicles can suppress immune responses to aid in immune evasion, while dendritic cell-derived exosomes can stimulate cytotoxic T cells for cancer immunotherapy (200). OMVs and engineered exosomes are being developed as vaccine platforms, with approved examples like the OMV-based meningococcal vaccine Bexsero, and ongoing research into exosome-based vaccines for COVID-19 and cancer (201).

Furthermore, MVs contribute to disease pathogenesis by facilitating processes such as cancer metastasis, neurodegenerative protein spread, and pathogen virulence (200). Despite their promise, clinical translation faces challenges, including difficulties in isolation and purification, safety concerns, and regulatory issues. Nonetheless, the growing understanding of membrane vesicles holds great potential for transforming diagnostics and therapeutics across a wide range of diseases (202-204). OMVs have been observed for all gram-negative bacterial strains studied to date, including *Pseudomonas aeruginosa, Vibro cholerae, N. meningitidis, N.gonorrhoea,* and more (192, 205). Studies have shown that OMVs contain a diverse array of biomolecules, including high levels of toxins and virulence factors (193, 206). These components allow OMVs to facilitate communication with host cells and trigger pathogenesis, even without the presence of live bacteria. Additionally, OMVs have been linked to the transfer of metabolites and essential molecules such as growth factors and anti-inflammatory agents to other bacterial species (207, 208). However, little is known about vesicle production by mycobacteria and its potential role in tuberculosis pathogenesis. Additionally, there is a knowledge gap surrounding how antibiotic induction interferes with vesiculation from mycobacteria.

In the second paper of the thesis (***Paper II*),** we investigated vesiculation by mycobacteria using different conventional anti-TB antibiotics. We previously described a mechanism for isolating vesicles from mycobacteria (209). Using mass spectrometry and lipid profiling, we identified key vesicular transport proteins and lipids that may potentially play a vital role in tubercular infection and pathogenesis. Lipid profiling analysis may provide more insight into whether mycobacterial vesicles are released from the inner or outer membrane thus providing more information about its origin. Additionally, in this study, toxicology analysis indicates that mycobacterium vesicles induce inflammation intracellularly. Extracellular vesicles have demonstrated increasing pathogenicity mechanisms in gram-negative bacteria. In our study, we have identified a potential factor contributing to the intrinsic mechanism of tuberculosis pathogenesis, shedding light on a novel aspect of mycobacterial response to conventional antibiotics. The concept of mycobacterial vesiculation deserves additional investigation as it holds promise for developing novel treatment approaches and deepening our comprehension of the pathogenesis of tuberculosis.

Phage-derived enzymes

Bacteriophages, or phages, were first introduced into medical practice just over a century ago by scientific and medical pioneers such as Félix d'Herelle(210, 211). Over the following decades, phage therapy was investigated in various countries, yielding several successful outcomes despite the fact that it remains a complex and challenging field to this day (fig.9). Phage therapy gained popularity in France, India, and the former Soviet Union. In the 1920s and 1930s, phage preparations were used to treat a variety of infections, including dysentery, cholera, skin infections, and even typhoid fever (212, 213). D'Herelle reported positive outcomes in treating plague and cholera in India, while Soviet scientists at the Eliava Institute in Tbilisi, Georgia, began a long-standing tradition of phage therapy research and clinical application, which continues to this day (212).

"A scientist is not a person who gives the right answers,
he´s one one who asks the right questions."
-Claude Levi-Strauss

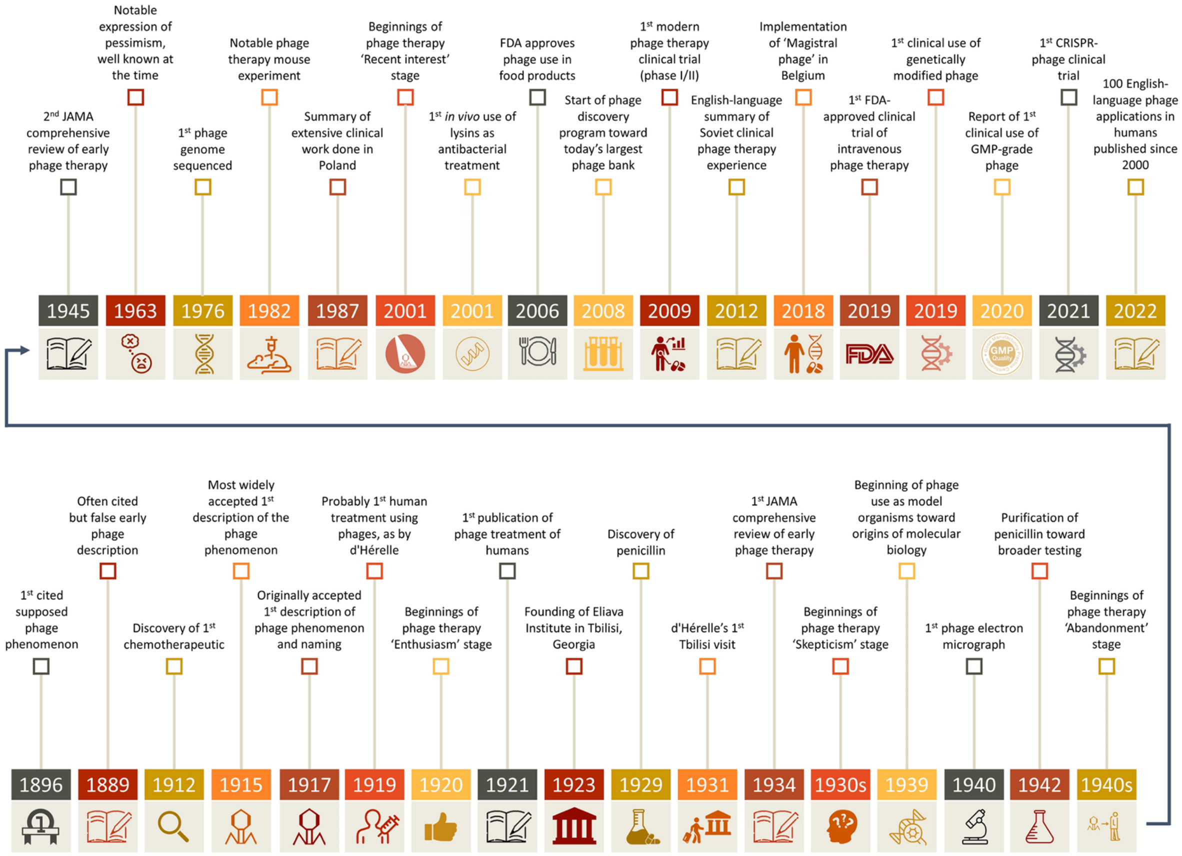


Fig. 16 Milestones in Phage therapy.
This figure is reprinted from “Translating phage therapy into the clinic: Recent accomplishments but continuing challenges”
doi: https://doi.org/10.1371/journal.pbio.3002119.g002

However, the rise of antibiotics in the 1940s, particularly penicillin, led to a decline in interest in phage therapy in most Western countries. Antibiotics were easier to mass-produce and administer, whereas phage therapy was viewed as less predictable due to the specificity of phages and the complexity of manufacturing them. As a result, much of the early Western literature on phage therapy faded and research shifted to Eastern Europe and the former Soviet Union (214). Despite early setbacks and skepticism, phage therapy demonstrated considerable potential in Soviet-era clinical settings. Phage preparations were widely used in Eastern Europe for treating infected wounds, burns and gastrointestinal infections. One study published in 1963 detailed the successful treatment of over 1,000 patients with suppurative skin infections using phage preparations, reporting significant improvement in more than 80% of cases (214).

The resurgence of interest in phage therapy in the 21st century has been driven largely by the global rise in antimicrobial resistance (AMR), which has rendered many conventional antibiotics less effective. Modern research has focused on the use of purified, well-characterized phage cocktails, genetically engineered phages, and phage-derived lytic enzymes. Clinical case reports from Western countries have documented successful use of phages in treating multidrug-resistant infections, such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* in trauma patients, and *Mycobacterium abscessus* in chronic lung disease (215).

In recent years experimental phage therapy programs have emerged in the United States and Europe. For instance, in 2019, a personalized phage therapy protocol was used to treat a 15-year-old cystic fibrosis patient infected with a drug-resistant *M. abscessus* strain following a lung transplant, resulting in significant clinical improvement (216). This case highlights the potential of phage-based therapies to address infections where antibiotics fail, particularly in immunocompromised or critically ill patients. The integration of modern molecular tools, such as whole-genome sequencing and synthetic biology, has further enhanced the precision and safety of phage therapy. These advances have enabled researchers to design phage cocktails with reduced risk of horizontal gene transfer or undesirable immune reactions, while also tailoring treatments to individual patients based on the bacterial strain profile.

Phage lytic enzymes harbour at least one domain responsible for the enzymatic cleavage of peptidoglycan, also known as murein, which is a major structural component of the bacterial cell wall (217). Mycobacteriophages and their lytic enzymes Lysin A (LysA, peptidoglycan hydrolases) and Lysin B (LysB, esterases), have attracted interest as potential therapeutic candidates against drug-resistant mycobacteria (218). The best studied lysis system of a mycobacteriophage is that of phage Ms6, a temperate phage that infects the nonpathogen *Mycobacterium smegmatis* (218, 219) The lysis cassette of phage Ms6 comprises five genes, labeled *gp1* through *gp5*. The *gp1* gene encodes a chaperone-like protein that facilitates the export of the endolysin (LysA) via the host's Sec system, functioning independently of holin (220). The *gp3* gene encodes a lysis protein, LysB, which encompasses lipolytic activity and plays a key role in disrupting the outer membrane of mycobacteria (221, 222).

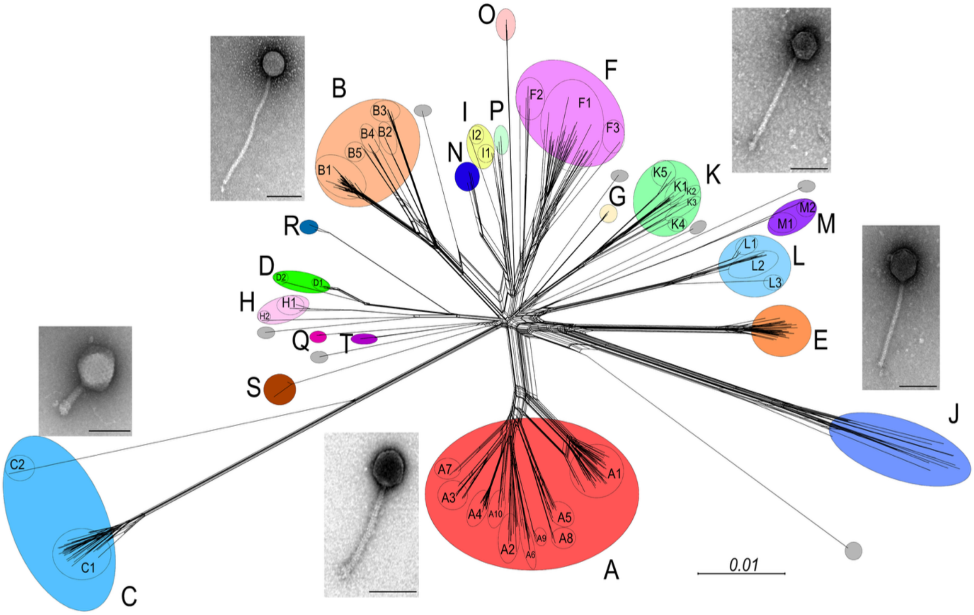


Fig.17 The diversity of mycobacteriophages was analyzed based on 471 sequenced genomes, compared by their shared gene content.
The relationships among these phages are visualized using SplitsTree. Adapted from Hatfull GF (2014) (223).

Mycobacteriophages Endolysin (LysA): A Key Mediator of Lysis

D29 mycobacteriophage encodes LysA endolysin, which mediates mycobacterial host cell lysis by targeting its peptidoglycan layer (224). Past studies report that LysA exists in two conformations of which only one is active and the protein undergoes a host peptidoglycan-dependent conformational switch to become active for carrying out endogenous host cell lysis (224). D29 maintains a pool of inactive LysA, aiding in avoiding premature host lysis. Additionally, the study indicated that the switch from active to inactive reverses after lysis, thus preventing exogenous targeting of bystanders, which otherwise negatively affects phage propagation in the environment (224).

Mycobacteriophages LysB: Essential for facilitating the final step of phage-induced host cell lysis

The LysB protein of mycobacteriophage D29 functions as a novel mycolylarabinogalactan esterase, specifically cleaving the ester bond that links the mycolic acid-rich outer membrane to the arabinogalactan layer, thereby releasing free mycolic acids. Additionally, it acts at a late stage in lysis to ensure that complete cell lysis of the host is achieved (225, 226).

The lipid hydrolase activity of a LysB protein was first demonstrated in the mycobacteriophage Ms6. Biochemical characterization revealed that Ms6 LysB can hydrolyze both esterase and lipase substrates across a broad range of carbon chain lengths (221, 225, 227).

In our third study (***Paper III***), we investigated a series of novel chimeric antimycobacterial proteins engineered by fusing LysA and LysB enzymes with outer membrane permeabilizers and cell-penetrating peptides. The resulting protein libraries were first screened against *Mycobacterium smegmatis*. This was followed by efficacy testing on *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), and two clinically relevant NTM species, *M. abscessus* and *M. avium*. The most effective candidates were selected based on their MICs values and subjected to comprehensive evaluation, including assessments of bactericidal activity, cytotoxicity, intracellular killing capacity and stability in human serum. Lastly, in vivo efficacy was assessed in murine skin and lung models infected with M. avium. This approach aimed to advance the development of alternative therapeutic strategies targeting drug-resistant mycobacterial infections.

Method description

Bacteria

The most common bacteria models used in all three papers are *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) Montreal containing the pSMT1-luxAB plasmid described by Snewin et al in 1999 (228) and the *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) Danish strain (Staten Serum Institute, Copenhagen, Denmark). The plasmid contains the *luxAB* operon, encoding a luciferase from *Vibrio harveyi* and a hygromycin resistance gene for selection (229). The incorporation of luciferase enables quantification of bacterial numbers by the addition of an aldehyde substrate, which generates bioluminescence. In ***Paper I*** and ***II,*** the mice were infected with approximately 5 ×10^3 CFU/mL *of M. tuberculosis* H37Rv. The bacteria were a kind gift from Christophe Guilhot, Institut de Pharmacologie et de Biologie Structurale (IPBS), Toulouse, France. In ***Paper I,*** *Mycobacterium bovis Bacillus Calmette–Guérin* (BCG) (ATCC) was used for checkerboard experiments. Additionally, for screening experiments, three or more clinical isolates of each of the following bacteria were used: *Mycobacterium abscessus* and the gram-positive *Enterococcus faecalis, Enterococcus faecium*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus* and *Streptococcus pneumoniae.* These isolates were obtained from Clinical Microbiology, Regional Laboratories Skåne, Lund, Sweden, and used for MIC analysis. In ***Paper III***, in addition to screening studies using BCG, two clinical NTM isolates, *M. avium* and *M. abscessus* were obtained from Clinical Microbiology, Regional Laboratories Skåne, Lund, Sweden.

Cell Culture

For cell culture experiments in ***Papers I*** to ***III***, Human macrophages were isolated following a previously published protocol in which monocytes were obtained from healthy volunteers using a lymphoprep density gradient medium as previously published(230). CD14 microbeads were added to the cell suspension, washed, and passed through a LS-column (Miltenyi Biotec) to produce pure monocytes. The monocytes were counted using the Sysmex and diluted in RPMI 1640, supplemented with 5% FCS, NEAA, 1 mM Sodium Pyruvate, 0.1 mg/mL

Gentamicin and 50 ng/mL M-CSF and finally seeded in 96-well plates for 7 days to differentiate into macrophages (230). In ***Paper II,*** the human monocyte cell line, THP-1-XBlue™-CD14 was cultured in RPMI 1640 supplemented with 10% FCS, Antibiotic-Antimycotic, Zeocin, and Geneticin.

Major Molecular techniques

Minimum inhibitory concentration

The purpose of Minimum Inhibitory Concentration (MIC) is to determine the lowest concentration of an antimicrobial that prevents visible growth of a microorganism**,** like bacteria, after overnight incubation (231). In ***Papers I*** to ***III****,* we used the resazurin microtiter assay (REMA) as previously published for MIC experiments (179). Briefly, the bacteria were seeded equally at an OD of 0.01 (~106 CFU/mL) in 96-well plates and incubated at 37°C and 5% CO2 with the antimicrobial agent. Treatment time was set according to the individual strain doubling time. Prestoblue cell viability reagent (Thermo Scientific) was added to the untreated control to monitor the growth process of the bacteria. 1:10 and 1:100 dilutions were used as additional growth controls (179). MIC was determined as the concentration where no colour change was observed (i.e., the lowest concentration that prevents visible growth of the bacteria), while the controls had turned from blue to pink indicating growth. The reported MICs are all 99% inhibition. In ***Paper I***, we utilized the luminescence of our BCG bacteria to measure growth inhibition over a 24-hour period. The intracellular growth assays used in ***Paper I*** to ***III*** used primary macrophages infected with M.tb and BCG respectively. The BCG-infected macrophages were lysed with sterile water and intracellular bacteria were quantified.

Cytotoxicity

In ***Papers I*** to ***III*** we measured the biocompatibility of the antimicrobial agents and MVs with primary human macrophages. Briefly, the primary macrophages were incubated with the antimicrobial agents and MVs overnight in fresh RPMI 1640 medium. Next, following an overnight incubation at 37°C and 5% CO2, MTT (16.5 µL, Sigma) was added to each well and incubated for 1 h at 37°C, and the absorbance was measured on a plate reader at 535 nm. For the WST-8 cytotoxicity test in ***Paper I***, NZ2114-treated primary macrophages were incubated overnight at 37°C and 5% CO2. WST-8 solution (10 µL) was then added to each well, and the cells were incubated for 2 h at 37°C, whereafter the absorbance was measured at 480 nm. In ***Paper II***, the cytotoxicity of membrane vesicles (MVs) was evaluated using the PrestoBlue assay. Primary macrophages were exposed to either 4 µM or 40 µM of MVs, or a 50 µM positive control, for 24 hours. Cell viability was then measured by comparing PrestoBlue fluorescence to untreated controls, following the manufacturer's instructions (232, 233). The Human THP-1-XBlue™-CD14 monocytic cell line was also stimulated with 4 or 40 µM MVs, or heat-killed *Listeria monocytogenes* as a positive control. To assess activation, a QUANTI-Blue™ assay was performed by adding cell supernatants to the QUANTI-Blue™ substrate for one hour, followed by absorbance measurement at 620 nm. In parallel, the ATPlite™ assay was used to confirm that the observed activation was not due to cytotoxicity.

Electron microscopy

In ***Paper I***, the effect of the peptide on BCG was evaluated using scanning electron microscopy (SEM). Bacteria were cultured to a density of 1 × 10^8 CFU and exposed to 6.3 µM of the peptide for either 0 or 24 hours. After treatment, the bacteria were pelleted by centrifugation at 3,000 × g for 7 minutes, re-suspended in a fixation solution and placed on poly-L-lysine-coated glass coverslips for 1 hour. The samples were imaged using a Philips/FEI XL30 FEG SEM at an accelerating voltage of 5 kV and a working distance of 10 mm. In ***Paper II,*** vesicle formation, morphology and the impact of the peptide on M. bovis BCG was assessed using electron microscopy as previously described (234, 235). To access vesicle formation and morphology the samples were adsorbed onto carbon-coated copper grids for 2 minutes, then briefly rinsed with two drops of distilled water. Negative staining was carried out using 0.75% uranyl formate. Imaging was performed with a Jeol JEM 1230 electron microscope at 80 kV, and high-resolution images were acquired using a Gatan Multiscan 791 CCD camera. In ***Paper III***, Clinical isolates of *M. abscessus* and *M. avium* were treated with MCL-12 or control buffer, fixed, and processed for transmission electron microscopy. Samples were stained, sectioned, and imaged using a JEOL JEM-1400 PLUS TEM, and images were analyzed with Fiji software.

Vesicle purification

Membrane vesicles (MVs) were isolated and processed based on a previously published protocol (209, 236). BCG cultures grown in the retentate were first centrifuged at 4,000 rpm, then at 15,000 rpm for 15 minutes at 4 °C. The resulting supernatant was collected and ultracentrifuged at 35,000 rpm for 2 hours. The pellet was resuspended in 1 mL of DPBS. For further purification, a density-gradient centrifugation using Optiprep was performed at 35,000 rpm for 20 hours in a swing bucket rotor. The visible band containing the vesicles was collected and quantified using a BCA protein assay. Samples were aliquoted and stored at –80 °C until further analysis.

Human Serum Stability assay

In ***Papers I*** and ***III,*** Serum stability studies were performed as previously described (179). The antimicrobial agents were incubated in human serum for 1, 2 and 3 h, at 37°C and 5% CO2. Serum was used to prepare the serial dilutions. After each time point, 10 µL of serum-incubated antimicrobial agents was added to 90 µL of BCG suspension (OD 0.01) and incubated at 37°C, 5% CO2 for 7 days. Serum-incubated rifampicin was used as a control at a concentration of 0.1 µg/mL (0.7 µM) (237). PrestoBlue was added to the cells and incubated overnight at 37°C, 5% CO2. The following day, the fluorescence intensity was measured at 620 nm.

Murine TB infection model

For the animal experiments in ***Paper I,*** all experiments were approved under a license issued by the UK Home Office and conducted in accordance with the Animals (Scientific Procedures) Act of 1986. Female BALB/c mice, aged 6–8 weeks (Charles River Ltd., UK), were housed in biosafety level 3 (BSL-3) facilities at Imperial College London, following institutional guidelines (238). In ***paper III,*** the antimycobacterial activity of mycolysins MCL-12, MCL-17, and MCL-20 was evaluated in vivo using a murine wound-infection model involving 40 male rats. Full-thickness dorsal wounds were infected with *Mycobacterium avium* and treated topically with either rifampicin, mycolysins, or combinations thereof. Treatments were applied on days 2, 3, 5, 7, and 9 post-infection, and wound swabs were collected before each application for bacterial load quantification. After the final treatment, animals were euthanized for study completion. The pulmonary antimycobacterial activity of MCL-12, MCL-17, and MCL-20 was evaluated in immunosuppressed rats using an established lung infection model. Forty-three rats were treated with cyclosporine A (25 mg/kg/day) to induce immunosuppression. A pulmonary infection was established via intratracheal instillation of *M. avium* (1×10^8 CFU/mL). One day post-infection, lungs from three animals were analyzed to confirm infection. On day three, animals were divided into eight groups (n = 5) and treated intratracheally for five consecutive days with either rifampicin, mycolysins (MCL-12, MCL-17, MCL-20), combinations, or PBS (control)**.** After treatment, lungs were harvested, homogenized, and analyzed to quantify bacterial burden.

Statistics

In ***Papers I to III***, Statistics were generated using the Prism software (version 10). One-way ANOVA for multiple comparisons followed by the post-hoc test was used to calculate significance for the serum incubation and checkerboard experiments in ***paper I***. Significance was accepted at $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$. For the *in vivo* models in ***paper III***, bacterial counts (CFU/mL) were log-transformed prior to analysis using two-way ANOVA for the wound infection model and one-way ANOVA for the pulmonary infection model, followed by Tukey's post hoc test for multiple comparisons. Statistical significance was defined as $p < 0.05$. Data are expressed as mean ± standard deviation (SD).

Ethical approval

In ***Papers I to III***, the blood used for monocyte isolation was donated by healthy volunteers. No personal data was collected from the volunteers and they were provided with verbal informed consent regarding the study's purpose, duration, potential risks, and benefits. To document their consent, the healthy volunteers also read the study information sheet before signing a document approved by the Local Ethical Review Board (Dnr 2011/403 and 2014/35). In ***Paper I***, all animal procedures were conducted under the license issued by the UK Home Office and in accordance with the Animal Scientific Procedures Act of 1986. The animal studies were approved by the Local Animal Welfare and Ethical Review Board (London, UK) (Numbers PPL 70/7160 and 70/8653). In ***Paper III***, all animal studies were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Beni-Suef University. The study complied with the ARRIVE guidelines and adhered to internationally recognized standards for the care and use of laboratory animals, as outlined in the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1985). Ethical approval for the study was obtained from the Faculty of Postgraduate for Advanced sciences (PSAS) Ethics Committee of Beni-Suef University, Egypt (Approval No. PSAS-BSU-HAREC.001).

Key results

Paper I

Title: Antimycobacterial activity of the plectasin derivative NZ2114.

Objective: To investigate the effectiveness of NZ2114 as a potential antimycobacterial peptide both *in vitro* and *in vivo.*

Results

Antimicrobial activity of NZ2114

NZ2114 was previously demonstrated to have increased inhibitory activity against Gram-positive bacteria compared to plectasin (239). In this study, we tested NZ2114 for antimicrobial activity against mycobacteria and a broad range of gram-positive pathogens. NZ2114 exhibited potent activity towards *M.bovis BCG* and clinical isolates of *M. abscessus* and several clinical isolates of gram-positive bacteria. For BCG, there was a concentration-dependent inhibition, with a MIC_{99} concentration of 6.1 µM. For *M. abscessus*, the MIC_{99} value was much higher, with a mean of 75 µM. For the Gram-positive isolates, the vancomycin-resistant *E. faecium* had the highest inhibitory concentration at 6.1 µM, while the MIC_{99} of *E. faecalis* was the same as for BCG. The methicillin-resistant *S. aureus* and methicillin-susceptible *S. aureus* had the lowest MIC_{99} value at 0.3 µM, followed by *S. pneumoniae* with an inhibitory concentration of 0.5 µM of NZ2114.

NZ2114 is not toxic to human cells

To evaluate the cytotoxicity of NZ2114, two different assays were employed the MTT assay and the WST-8 assay. The results from both assays indicated that NZ2114 did not exhibit any toxic effects on the cells at concentrations up to 25 µM.

NZ2114 induces intracellular killing of mycobacteria

The intracellular anti-mycobacterial capacity of NZ2114 was assessed after 6 days using primary human macrophages infected with BCG. NZ2114 demonstrated a concentration-dependent reduction in the intracellular bacterial load, achieving a maximum reduction of 89% at a concentration of 12.5 µM. Rifampicin, used as a positive control at a concentration of 0.1 µg/ml (0.7 µM), resulted in a similar level of intracellular bacterial killing as observed with 10 µM of NZ2114.

Serum incubation did not alter peptide efficacy

Previous studies have shown that plectasin has a terminal serum elimination half-life of 51 minutes (240). In our investigation, we analyzed the impact of serum on the function of NZ2114 by determining the MIC_{99}. Our results indicated that the MIC values remained stable, with remained antimycobacterial capacity, for up to three hours of serum incubation.

NZ2114 exhibited a synergistic effect with current TB drugs

The interactions between NZ2114 and the TB drugs rifampicin, isoniazid, ethambutol, amikacin and kanamycin were analyzed using the checkerboard assay. The FIC index analysis revealed that the interactions between NZ2114 and these TB drugs were predominantly synergistic or additive/indifferent. Importantly, none of the drug combinations demonstrated antagonistic effects. However, it was noteworthy that NZ2114 exhibited a synergistic effect when combined with ethambutol (EMB) and isoniazid (INH).

Murine evaluation of the drug additive effect

To validate our drug interaction results, we experimented using a murine model infected with the M.tb H37Rv strain. The mean bacterial implantation dose in the lungs, measured two days post-infection, was 700 CFU/ml. After 21 days, when treatment commenced, the bacterial load had increased in the untreated control group. All untreated mice survived the entire duration of the experiment. In the treated NZ2114-treated, a general reduction in CFU of 81% was observed compared to the untreated mice (p=0.0079).

> *"We cannot address TB only with vaccines and medications. We cannot address it only with comprehensive STP programs. We must also address the root cause of tuberculosis, which is injustice. In a world where everyone can eat, access healthcare, and be treated humanely, tuberculosis has no chance. Ultimately, we are the cause. We must also be the cure."*
>
> *- John Green*

Paper II

Title: The role of antibiotic-derived mycobacterial vesicles in tuberculosis pathogenesis

Objective: To determine the composition and effects of mycobacterial MVs using conventional anti-TB drugs and the peptide NZX.

Results

Anti-TB treatment induces vesicle formation

Significant variations in MV production were observed both among different batches and across various treatments, as determined by protein concentration. The membrane vesicles appeared to originate from the mycobacterial inner membrane, forming clusters as observed through transmission electron microscopy (TEM). These spherical vesicles exhibited a size range of approximately 40-50 nm.

Vesicle membrane lipid composition

In the TLC controls, polar phospholipids such as phosphatidylinositol (PI), cardiolipin (CL), and digalactosyldiacylglycerol (DGDG) remained stationary at the origin of the TLC plate. In contrast, the non-polar lipids of MVs migrated up the plate, indicating that MVs consisted mostly of apolar lipids. Additionally, MS analysis revealed the presence of apolar trehalose monomycolate (TMM) in MVs.

TB antibiotics induce diverse vesicle protein cargo

The functions of MVs released into the extracellular environment are determined by the composition of their cargos, such as their protein content. Equal concentrations of antibiotic-induced MVs were loaded onto LC-MS/MS for analysis. The results were subsequently subjected to bioinformatic protein analysis. Protein concentrations were controlled, and the variation between MV samples was found to be not significant ($p < 0.8395$). However, the number of proteins varied, with most proteins induced by EMB ($p < 0.0393$). In the identification of protein-protein interaction (PPI) enrichment networks within the upregulated proteins, differences were observed between EMB-induced MV proteins and those induced by RIF or INZ ($p < 0.05$). In comparison to RIF, EMB-induced MVs were enriched with proteins from the cytoplasmic pathway. Additionally, when compared to INZ, EMB induced a wide range of MV proteins, including those involved in the fatty acid metabolic process, organic acid metabolic process, carboxylic acid biosynthetic process, and others. Lastly, no significant differences in proteins were observed between RIF and INZ or between NZX and RIF or between NZX and INZ or between NZX and EMB.

Antibiotic-induced mycobacterial MVs induce inflammation and cell death

As mycobacterial MVs have previously demonstrated immunomodulatory effects *in vivo* (241), we conducted further investigations to assess whether MVs induced by antibiotics exhibit cytotoxic or immunomodulatory effects on human cells. Two different concentrations of MVs, 4 µg/mL and 40 µg/mL were utilized to represent high and low bacterial burdens.

Generally, higher concentrations of MVs resulted in increased cell death and inflammation. Statistically, MVs derived from INZ at both concentrations were found to be more toxic to monocytic cells compared to MVs from other stimulants (p=0.0076 and p=0.0022, respectively). The heightened toxicity was also observed in human primary macrophages but was not significant. Additionally, an analysis of NF-κB activation following exposure of antibiotic-induced MVs to the monocyte cell line revealed that the MVs at a concentration of 40 µg/mL induced approximately 50% inflammation compared to control ($p = 0.0144$), except the minimal media isolated MVs that were equally toxic as the control.

Paper III

Title: Engineering phage-derived enzymes: a novel approach for targeting mycobacterial infections.

Objective: To investigate the effectiveness of the novel chimeric antimycobacterial proteins both *in vitro* and *in vivo.*

Results

Production of pure active variants and their antimycobacterial activity

Lab-scale expression of the most active variants was performed in TB medium. The proteins were purified to homogeneity using immobilized metal ion affinity chromatography, followed by desalting. Their purity was confirmed with SDS-PAGE, and their antimycobacterial activity was verified against M. smegmatis. The minimum inhibitory concentrations (MIC) of the lead variants ranged from 3 to 440 µg/mL.

Selective activity of the chimeric lysins against different mycobacteria

MIC analysis with the in-house strain M. smegmatis identified 33 constructs effective in microbial killing. To further analyse the mycobacterial efficacy of the selected chimeric lysins, MIC99 screening was performed on BCG. In this screening, lysin complexes MCL-11, MCL-12, MCL-17, MCL-20, and MCL-22 were found to eliminate BCG at the lowest concentrations: <0.005, <0.002, 0.00128, 0.006, and 0.006 μg/mL, respectively.

Chimeric lysins induced dose-dependent toxicity

In the current investigation, none of the evaluated chimeric lysins (MCL-11, MCL-12, and MCL-17) caused significant toxicity in human primary macrophages. 17-T6 was the least toxic to human cells, while 11-T5 induced moderate cell toxicity.

Increased intracellular mycobacterial killing

Each of the three candidates (MCL-11, MCL-12, and MCL-17) demonstrated the ability to eliminate intracellular mycobacteria in a dose-dependent manner. An initial reduction of 44% in MIC 99 was observed at the lowest concentration of lysins. For the MCL-11 variant, the highest level of intracellular elimination reached 70% at a concentration of 0.03 mg/ml, for the MCL-12 variant, it was 60% at a concentration of 0.06 mg/ml, and for the MCL-17 variant, the maximum intracellular elimination was 66% at a concentration of 0.005 mg/ml.

Chimeric lysins are stable after serum incubation

All three chimeric lysins retained their activity against mycobacteria after incubation in serum for up to three hours. In general, there was a reduction in MIC99 activity ranging from 25% to 60% between one hour and three hours of incubation.

In vivo antimycobacterial activity of lysisns

Three mycolysin variants (MCL-12, MCL-17, and MCL-20) were tested in a murine wound infection model using *Mycobacterium avium*, and compared to the standard drug rifampicin. MCL-12 and MCL-17 showed similar efficacy to rifampicin, while MCL-20 demonstrated the highest potency, reducing bacterial counts by ~1.97 log-units after the first dose. Combination treatments with rifampicin enhanced the activity of all mycolysins, with MCL-20 + rifampicin achieving the greatest reduction. Over multiple doses (days 5, 7, and 9), MCL-20 remained the most effective, with final bacterial count reductions of 3.6 (MCL-12), 3.8 (MCL-17), 4.4 (MCL-20), and further improved to 5.53 log-units when MCL-20 was combined with rifampicin.

Furthermore, in a pulmonary infection model using *M. avium*, mycolysins MCL-12, MCL-17, and MCL-20 were administered intratracheally to rats. MCL-20 showed the strongest antibacterial activity, reducing bacterial counts by 1.2 ± 0.15 log-units, which improved by an additional 0.5 log-units when combined with rifampicin. In contrast, MCL-12, MCL-17, and rifampicin alone did not significantly reduce bacterial loads. However, combining rifampicin with either MCL-12 or MCL-17 resulted in effects comparable to MCL-20 alone.

General discussion

NZ2114 in the treatment of mycobacterial infections

In ***paper I***, we investigated the potential of NZ2114 in the treatment of mycobacterial infections. We found the peptide to induce mycobacterial killing at MIC and its effective elimination of M.tb in our murine TB model. Previously, we explored how NZX eliminates mycobacteria, which have a more complex outer envelope than Gram-positive bacteria (242, 243). NZX was shown to interact with the inner membrane of mycobacteria and inhibit several key enzymes involved in mycolic acid synthesis, thereby potentially affecting bacterial growth (243). In the present study, treatment with NZ2114 was found to disrupt the mycobacterial membrane, however, the exact mechanism behind this disruption requires further investigation.

The *Mycobacterium abscessus* complex consists of fast-growing, multidrug-resistant bacteria that can cause lung disease, especially in people with conditions like cystic fibrosis, bronchiectasis, or a history of tuberculosis (244, 245). When comparing the effectiveness of NZ2114 and NZX against *M. abscessus*, NZX was found to be more potent, achieving greater bacterial elimination at lower concentrations (1, 179). Moreover, NZ2114 has broad-spectrum antimicrobial activity, effectively targeting multiple bacterial pathogens, including drug-resistant (246-248). Its wide range of effectiveness highlights its potential for treating various infections.

Furthermore, we investigated the mycobacteria-eliminating activity of NZ2114 on an intracellular level and found that it maintained strong intracellular activity and remained effective after 3 hours in human serum. Notably, NZ2114 showed compatibility with existing TB drugs. It had a synergistic effect when combined with ethambutol and isoniazid, while its interactions with kanamycin, rifampicin, and amikacin were either additive or indifferent. Further supporting NZ2114's therapeutic potential, toxicology studies showed that it exhibits lower toxicity at high concentrations compared to other TB drugs (249). In vivo studies showed that NZ2114 significantly reduced M. tb levels in animal models by 81% after just three doses, highlighting its potential as an effective treatment for mycobacterial infections. These findings collectively underscore the clinical relevance of NZ2114 as a promising adjunct or alternative in TB therapy. The retention of intracellular efficacy is particularly critical given the ability of *M. tuberculosis* to persist within macrophages, where many antibiotics fail to reach effective concentrations. The fact that NZ2114 remains active in human serum suggests good pharmacokinetic stability, a key requirement for systemic administration. Its synergistic interactions with first-line drugs like isoniazid and ethambutol also present a compelling case for its integration into current therapeutic regimens, potentially allowing for dose reduction of existing drugs and, by extension, a decrease in associated toxicities and resistance development.

Moreover, the additive or indifferent interactions observed with second-line agents like kanamycin, rifampicin, and amikacin suggest that NZ2114 does not interfere with these drugs' mechanisms of action. This compatibility is important in the context of multidrug-resistant TB (MDR-TB), where therapeutic options are limited, and drug-drug interactions can severely impact treatment outcomes. Furthermore, the relatively low toxicity of NZ2114 at high concentrations further supports its potential for long-term use, which is often necessary in TB treatment protocols. This profile not only enhances its therapeutic index but also suggests a more favorable safety profile compared to conventional antibiotics. The significant bacterial load reduction observed in vivo after only a short treatment duration further strengthens its candidacy for preclinical development and warrants future clinical investigation to determine optimal dosing strategies, duration of therapy, and combination protocols.

Antibiotic-induced MVs from BCG

In our earlier study, we observed that peptide-induced bubbling caused cell death in *M. tuberculosis* and was associated with an increase in MV formation over time (243), which sparked our interest in exploring the role of MVs. In ***paper II***, we noted that BCG formed vesicles from its inner membrane, aligning with earlier studies on naturally occurring MVs (241). The majority of vesicles were 40 nm in size, spherical and formed aggregates close to the producing bacterium. This distribution may function as a protective barrier by enhancing bacterial resistance against threats such as bacteriophages or antibiotics that would otherwise damage the cell envelope (250, 251). MV production was previously thought to be regulated by a single genetic element, *virR*, as well as the Pst/SenX3-RegX3 signal transduction pathway (252, 253). Additionally, dynamin-like proteins, such as IniA and IniC, have been involved in MV biogenesis, particularly in response to isoniazid treatment (254). However, the precise regulatory mechanisms governing MV formation remain poorly understood and require further investigation.

In our study, we observed similar levels of MV production following various antibiotic treatments, though differences in the underlying molecular pathways require further investigation. Furthermore, we investigated the difference in antibiotic-induced MVs protein cargos. Previous studies have reported a wide range in the number of proteins identified in MVs from as few as one to as many as 287, depending on the isolation techniques used (255-258). Lipoproteins were commonly detected, making up approximately 8% of the MV protein content (257). In our study, we identified 1,348 proteins associated with antibiotic-induced MVs, with LprO being the only lipoprotein detected. There were distinct differences were observed in the protein composition of MVs depending on the antibiotic used, with ethambutol treatment resulting in the highest MV protein load. In comparison to RIF, EMB-induced MVs contained proteins linked to the cytoplasmic pathway. In contrast to INH, EMB showed variations in proteins involved in fatty acid metabolism, organic acid metabolism, and carboxylic acid biosynthesis.

Characterizing the lipid composition of MVs is essential, particularly for understanding their interactions with human cells. Previous studies on M. bovis BCG-derived MVs have identified several polar lipids (241). These findings support the idea that MVs originate from the bacterial outer membrane. However, the absence of mycolic acid esters, the predominant lipids of the mycobacterial outer membrane, led the authors to conclude that these MVs may originate from the inner membrane (241). Our findings show that the lipids present in vesicles stimulated by both antibiotics and minimal medium were predominantly apolar. Notably, we identified apolar trehalose monomycolate (TMM) in MVs induced by RIF and NZX. Trehalose plays an essential role in the synthesis of mycomembrane lipids in mycobacteria (259-261).

Phage-derived enzymes for targeting mycobacterial infections

In this study, we used VersaTile technology**,** a DNA assembly method that allows for the rapid and flexible creation of modular proteins. By applying this technique, we engineered lysin libraries with a variety of linkers, enabling the generation of diverse functional protein variants (262). Previous studies have shown that LysB enzymes can effectively target the non-pathogenic NTM, *Mycobacterium smegmatis* when combined with outer membrane permeabilizers such as colistin or protamine (263, 264). In our study, we identified four engineered chimeric lysins with broad-spectrum antibacterial activity against various mycobacterial strains, including the M.tb complex*, M.abscessus* and *M.avium*. By analyzing the bactericidal concentrations, a clear difference in susceptibility was noticed between slow-growing mycobacteria and the fast-growing *M. abscessus*, which required higher doses of chimeric lysins. Despite all strains being pulmonary pathogens, these differences may be related to variations in cardiolipin and phosphatidylinositol mannoside expression (265). The cytotoxicity analysis indicated that three of the chimeric lysins (MCL-11, MCL-12) and MCL-17) demonstrated exceptional non-toxicity to human macrophages. This is in agreement with other studies, where engineered lysins were created to target bacterial cell walls while remaining safe to human cells (266-269). Upon evaluating the intracellular efficacy of the lysin complexes, we found that they maintained their ability to eliminate intracellular mycobacteria. Notably, these enzymes also maintained their antimicrobial activity following serum incubation, suggesting their potential for future intravenous use in patients with severe tuberculosis infections. This is significant given the variable intracellular effectiveness of current first-line TB therapies (270). The next step will be to explore potential synergies with current TB treatment, in addition to rifampicin, and to analyze the mechanisms of microbial killing more in depth. In our murine studies, MCL-20 showed strong potential for treating *M. avium* infections, particularly in the lungs, where it significantly reduced bacterial counts. In contrast, MCL-12 and MCL-17 required combination with rifampicin to achieve comparable efficacy. Similarly, in the wound infection model, MCL-20 especially when combined with rifampicin, demonstrated the greatest antibacterial activity. These findings highlight MCL-20 as a highly promising antimycobacterial agent.

Conclusion

- NZ2114 showed strong antimycobacterial activity with an MIC99 of 6.1 µM, was non-toxic to human cells, and remained stable in serum. The peptide had a synergistic interaction with isoniazid and ethambutol and was also effective against clinical Gram-positive isolates, including MRSA. In a murine TB model, NZ2114 achieved an 81.14% (0.72 log) reduction in *M. tuberculosis* after three doses ***(Paper I).***

- Stress from sub-inhibitory concentrations of antibiotics, peptide, or starvation all increased MV formation. Additionally, electron microscopy and lipid profiling revealed that these vesicles, about 40 nm in size, were released from the bacterial inner membrane and consisted of non-polar lipids. MS analysis identified key proteins dependent on the antibiotic used, especially with ethambutol-induced MVs that contained proteins from several mycobacterial pathways. Lastly, toxicology analysis using different concentrations of MVs on primary human macrophages and the monocytic cells indicated that MVs from the different treatments were not toxic to human cells ***(Paper II).***

- Three chimeric lysin candidates effectively targeted both extracellular and intracellular mycobacteria at therapeutic levels, while also demonstrating non-toxicity and resistance to degradation. In our murine study, MCL-20 demonstrated a strong potential for treating *M. avium* lung infections, while MCL-12 and MCL-17 may require combination therapy for similar efficacy. Additionally, MCL-20, particularly in combination with rifampicin, significantly reduced bacterial counts in our murine wound infection model. Thus, indicating MCL-20 as a promising antimycobacterial agent ***(Paper III)***.

Future perspectives

The research presented in this thesis highlights the significant therapeutic potential of novel antimicrobial agents NZ2114, engineered phage-derived lysins, and the use of antibiotic-induced membrane vesicles (MVs). While these findings are promising, they also open several avenues for future investigation.

NZ2114 Mechanism of action

Although we noticed that *M.bovis BCG*-treated bacteria exhibited membranous protrusions and extensive bubbling in all cells, there is still room to investigate the molecular interactions between NZ2114 and mycobacterial cell envelope components, particularly in the context of its membrane-disrupting effects. High-resolution imaging, lipidomics, and proteomic profiling could identify the specific targets of NZ2114 and its interaction with mycolic acid synthesis pathways. Additionally, understanding how NZ2114 penetrates and persists in intracellular compartments may inform formulation strategies for improved pharmacokinetics and targeted delivery.

Clinical Development and Optimization of NZ2114

The compatibility of NZ2114 with existing TB drugs, especially the observed synergy with ethambutol and isoniazid, may lead to further exploration in combination therapy studies. Animal models of drug-resistant TB could provide deeper insight into long-term efficacy and safety. Formulation advancements such as nanoparticle encapsulation or inhalable dry powders may further enhance delivery and reduce systemic toxicity.

Furthermore, clinical trials will ultimately be needed to assess therapeutic potential, starting with phase I safety and pharmacokinetics.

Functional Role and Therapeutic Potential of Mycobacterial MVs

The observation that antibiotic treatment induces MV formation, and that these vesicles carry distinct protein and lipid cargos depending on the antibiotic used highlights the need to better understand their functional roles. Future work should investigate whether MVs contribute to drug resistance, immune evasion, or inter-bacterial communication. Moreover, the cargo composition could potentially suggest that MVs may act as biomarkers for antibiotic response or disease progression, however, more investigations are needed. A deeper molecular characterization, including transcriptomic and more in-depth lipidomic profiling, could clarify their biogenesis pathways and potential regulatory mechanisms.

From a translational perspective, MVs may serve as delivery platforms or vaccine candidates. Exploring the immunomodulatory properties of MVs in vitro and in animal models could uncover new strategies for therapeutic or prophylactic use, particularly in TB-endemic regions.

In addition, it would be valuable to examine the host immune recognition of MVs, including their interactions with pattern recognition receptors and their role in modulating cytokine responses. This is to determine whether MV production is strain-specific or influenced by host-derived stressors could help distinguish pathogenic from commensal mycobacteria.

Development of Engineered Phage Lysins

The use of engineered chimeric lysins as antimycobacterial agents represents a novel therapeutic approach, especially for targeting drug-resistant strains and intracellular bacteria. The three identified lysins with broad-spectrum activity and low cytotoxicity are strong candidates for further preclinical development. Future studies should focus on optimizing their stability, delivery, and intracellular killing. Investigating synergistic effects with existing antimycobacterial agents could pave the way for combination therapies that reduce treatment duration and improve outcomes.

Furthermore, understanding the mechanism of action of these lysins, including their interaction with specific cell wall components, will be important for the design of next-generation constructs. Additionally, efforts should also be made to engineer lysins with enhanced protease resistance and thermal stability to improve formulation stability. Advanced delivery methods, such as fusion with cell-penetrating peptides or encapsulation in liposomes, may enhance uptake by infected macrophages. Lastly, resistance surveillance through serial passaging experiments can help predict the emergence of lysin-tolerant mycobacterial strains, ensuring long-term clinical use.

“Imagination is more important than knowledge.”
-Albert Einstein

Acknowledgments

This work would not have been possible without the financial support of the Heart and Lung Foundation whose generous funding allowed me to focus fully on my research.

Completing this PhD has been a journey filled with challenges, growth, and discovery. I am deeply grateful to those who have supported me along the way. First and foremost, I would like to express my sincere gratitude to my supervisor, **Gabriela Godaly**, for her tremendous guidance, support, and encouragement throughout this journey. Gabriela, your insight, patience and grace have shaped both this project and my development as a researcher. Your empathy, compassion and understanding have helped me through difficult times and I am so grateful to have had you as my supervisor. I look up to you deeply as a mentor and as a person.

I am especially thankful to my colleague, **Komal**. Thank you for showing me the ropes of the lab when I was your master's student. We developed a friendship that I will cherish forever. Your grace, patience and compassion carried me through my masters project and my PhD. I am so grateful to have had your mentorship and support along the way. I will miss our deep and meaningful conversations in the office and look forward to witnessing all your accomplishments in life.

To all the students I supervised throughout my PhD, thank you for your dedication, curiosity, and the enthusiasm you brought to the lab. It was a privilege to learn alongside you. You challenged me to be a better mentor and reminded me why I love research and teaching.

To **Anki, Elsa**, and **Tobias,** thank you for always being willing to help us with blood donations for our experiments. Additionally, thank you for allowing us to use the Sysmex whenever needed, and for your encouragement and support whenever I had questions or needed assistance.

I would also like to thank the members of the Svalborg group: **Ines, Sharam, Arunima, Samudra**, and **Atefeh**. Thank you for always being so helpful whenever I needed support. Your patience and willingness to assist, especially when it came to the Tecan, meant a great deal to me. I’m truly grateful for your kindness throughout this journey.

To **Sabrina**, **Jesus** and **Tetiana**, I would like to express my gratitude for the success of our publications together. It was an honour to collaborate with you. Not only were you so dedicated and hardworking, but kind as well. **Jesus**, thank you for your patience and kind nature. It was a privilege to get to know you and work with you.

A big thank you to **Adel, Rajni, Jana** and **Mats**. We really appreciate the hard work, ideas and very importantly, patience and understanding to bring our manuscript together. Your support made a big difference, and it was a pleasure working together.

To my friends, thank you for keeping me grounded and reminding me of the world outside academia. To my best friends**- Zilin** and **Pari**, we met in 2019 when we first moved to Sweden to pursue our masters degrees. I could not have finished my masters or PhD without your amazing friendship and I am so thankful to God that he brought you in my life. **Frederic**, thank you for always being so kind and there for us whenever we were in need. You have a heart of gold and Ashley and I are so glad to have met you and have you in our lives. **Amankwa** and **Terry**, thank you for making Sweden a much more enjoyable place for Ashley and I. Thank you for your kind hospitality, brunchoteket dates and all the other fun adventures we had. We will treasure you in our hearts forever and look forward to many more fun experiences together. To **Aria** and **Marianne**, thank you for all the fun adventures, laughs, delicious meals but most of all for helping with our precious Mimi. We will keep you in hearts forever. To **Rassie, Diela** and **Nsozi** thank you for always checking in on me and being by my side throughout this journey. I love you all so much and appreciate having you in my life.

Finally, to my family thank you for your love, patience, and belief in me. Your support has meant everything. To my mother-in-law, **Bernadette** thank you for your support, encouragement, and prayers throughout this journey. Knowing that you were always there and cheering me on meant more to me than you may realize. I'm truly grateful to have you in my life.

To my father, **O´Brian**. Thank you for showing me what true hard work, dedication, discipline and perseverance look like. Your belief in me and your constant encouragement every step of the way has meant the world to me. Ek is baie lief vir daddy. Thank you for your sacrifices and hard work to provide the best education for us. I will forever be grateful for your hard work and thank you for motivating me to never give up,

Briny and **Barry,** I look up to you both in so many ways, more than I can ever fully express. Thank you for your wisdom, protection, and the words of encouragement you've shared with me during the moments I needed them most. Your strength and presence have been a source of comfort and inspiration throughout my life. I love you both more than words could ever truly express, even if I don't always show it. I am, and always will be, deeply grateful and proud to call you my older brothers.

To the queen of my heart, my mother, **Pamela**, I could not have accomplished this without you. You are my rock, my confidant and my best friend. Most of all, you are my biggest inspiration and role model. I dream of being even half the woman you are. You embody selflessness in its purest form, and having you as my mother is the greatest blessing of my life. Thank you for motivating me to never give up, for always being there whenever I called, and for the wonderful memories we've shared not only in Sweden but throughout my entire life. This dissertation is as much yours as it is mine, which is why I have dedicated it to you. I love you and will keep you in my heart forever.

Last but certainly not least, to my husband, **Rick-Ashley**. Thank you for your unconditional support and encouragement. I will never forget the incredible sacrifice you made in moving to Sweden so that I could pursue this journey. Thank you for always believing in me and pushing me to achieve my dreams. You have been my rock through it all, and I couldn't be more proud to be your wife. Thank you for celebrating my successes and loving me the way you do. I truly could not have done this without you, my love. I look forward to standing by your side and supporting your dreams, just as you have supported mine. I love you.

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