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Host-pathogen interactions in mycobacterial infection

Mechanisms of immunopathology and inflammation

KRISTINA MUNKE

FACULTY OF MEDICINE | LUND UNIVERSITY

Host-pathogen interactions in mycobacterial infection

Mechanisms of immunopathology and inflammation

Kristina Munke

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 3rd of October at 13.00 in Segerfalksalen, Department of Experimental Medical Science, Sölvegatan 19, Lund, Sweden

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Tuberculosis, caused by *Mycobacterium tuberculosis*, remains one of the deadliest infectious diseases globally. Despite extensive research, many aspects of *M. tuberculosis* pathogenesis and host immune responses remain unclear, complicating the development of effective treatments. Animal models are crucial for studying these interactions, and *Mycobacterium marinum* infection in mice serves a valuable surrogate system due to its genetic and pathogenic similarities to *M. tuberculosis*.

This thesis utilizes the murine *M. marinum* infection model to investigate host immune responses to mycobacterial infection, with a particular focus on the role of the ESX-1 type VII secretion system. ESX-1 is a key virulence determinant that modulates immune cell recruitment and granuloma formation, ultimately shaping disease progression.

The findings presented in this thesis provide new insight into mycobacterial persistence and pathology, particularly the role of ESX-1 in shaping immune responses. Additionally, this works highlights the functions of neutrophils and monocytes in the immune defense. By exploring their dynamic interactions and contributions to inflammation and bacterial control, this research enhances our understanding of host-pathogen interactions in mycobacterial pathogenesis.

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Mechanisms of immunopathology and inflammation

Kristina Munke

LUND UNIVERSITY

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Abstract

Tuberculosis, caused by *Mycobacterium tuberculosis*, remains one of the deadliest infectious diseases globally. Despite extensive research, many aspects of *M. tuberculosis* pathogenesis and host immune responses remain unclear, complicating the development of effective treatments. Animal models are crucial for studying these interactions, and *Mycobacterium marinum* infection in mice serves a valuable surrogate system due to its genetic and pathogenic similarities to *M. tuberculosis*.

This thesis utilizes the murine *M. marinum* infection model to investigate host immune responses to mycobacterial infection, with a particular focus on the role of the ESX-1 type VII secretion system. ESX-1 is a key virulence determinant that modulates immune cell recruitment and granuloma formation, ultimately shaping disease progression.

The findings presented in this thesis provide new insight into mycobacterial persistence and pathology, particularly the role of ESX-1 in shaping immune responses. Additionally, this works highlights the functions of neutrophils and monocytes in the immune defense. By exploring their dynamic interactions and contributions to inflammation and bacterial control, this research enhances our understanding of host-pathogen interactions in mycobacterial pathogenesis.

Populärvetenskaplig sammanfattning

Tuberkulos, även kallad TBC eller lungsot, är en infektionssjukdom som orsakas av mykobakterien *Mycobacterium tuberculosis*. Tuberkulos drabbar framför allt lungorna, men även andra organ kan bli infekterade. Bakterierna sprids via små vätskedroppar i luften, som uppkommer då sjuka personer hostar, nyser eller spottar.

Världshälsoorganisationen uppskattar att upp emot en fjärdedel av jordens befolkning idag är infekterad av tuberkulosbakterien, även om den faktiska omfattningen av latent infektion nyligen har ifrågasatts inom det vetenskapliga samfundet. Varje år resulterar detta i ca 1.6 miljoner dödsfall, vilket gör tuberkulos till en av de tio vanligaste dödsorsakerna globalt. Tuberkulos går idag att behandla med antibiotika, men problemet är att behandlingen är dyr och minst 6 månader lång, vilket sätter enorm press på både sjukvården och patienten. För att förhindra att tuberkulosbakterierna utvecklar en resistens mot antibiotikan måste behandlingen fullföljas och tas regelbundet. Utan behandling uppgår dödligheten till nästan hälften. Trots tillgängligheten av ett vaccin emot tuberkulos, är dess effektivitet tyvärr inte tillräcklig för att helt och hållet förhindra sjukdom hos vuxna. Det är därför viktigt att hitta nya och effektiva behandlingsformer. För att lyckas med detta, måste vi först förstå hur sjukdomen fungerar i detalj.

Vad händer i kroppen?

Immunförsvaret i lungorna består av makrofager och neutrofiler, som bekämpar bakterier, virus och andra farliga ämnen i inandningsluften. Makrofagerna fungerar som kroppens väktare genom att identifiera och ta bort inkräktare, samt varna andra celler i sin omgivning. Problemet med tuberkulosbakterier är att de infekterar makrofagerna och förökar sig inuti dem, vilket leder till sjukdom och kronisk infektion. Neutrofiler, som aktiveras av makrofagerna, försöker också bekämpa bakterierna, men vid långvarig tuberkulosinfektion kan de skada kroppens vävnad och bidra till inflammation.

Vår forskning

I vår forskning använder vi oss av mykobakterien *Mycobacterium marinum*, en nära släkting till tuberkulosbakterien, då den både är säkrare och enklare att arbeta med. *M. marinum* finns naturligt i söt- och saltvatten och infekterar framförallt fiskar, men kan även orsaka hudinfektioner hos människor. Sjukdomsutvecklingen och hur bakterien interagerar med immunförsvaret är väldigt likt tuberkulos, då båda bakterierna uttrycker liknande virulensfaktorer (dvs. faktorer som avgör bakteriens förmåga att orsaka sjukdom). Ett exempel på detta är det så kallade typ VII sekretionssystemet ”ESX-1” som möjliggör utsöndringen av olika faktorer genom mycobakteriernas komplexa cellmembran.

I artikel I har vi genomfört musstudier och funnit att *M. marinum* främst infekterar monocyter och neutrofiler. Monocyter är immunceller som rekryteras från blodet in

i vävnader och har förmåga att utvecklas till makrofager. Sekretionssystemet ESX-1 ökar rekryteringen av neutrofiler till vävnaden och ökar deras inflammatoriska egenskaper, vilket tillsammans bidrar till sjukdom. Monocyter verkar däremot inflammationsdämpande genom att minska rekryteringen av neutrofiler.

I artikel II har vi kartlagt genuttrycket hos monocyter från *M. marinum*-infekterad vävnad för att undersöka hur mykobakterier påverkar deras funktion. Genom att jämföra genuttrycket hos infekterade celler med icke-infekterade celler har vi kunnat se att infektionen ökar deras glykolysmetabolism och produktion av antibakteriell kväveoxid, vilket i tidigare studier kopplats till en miljö som hämmar bakteriell tillväxt. Dessutom kunde vi se att infektionen ändrar uttryck och känslighet mot en del inflammatoriska signalproteiner i monocyterna. Sekretionssystemet ESX-1 förstärkte dessa förändringar i genuttrycket framför allt i icke-infekterade monocyter.

I artikel III utforskar vi regleringen av celldöd som är förmedlad av ESX-1 hos infekterade makrofager. Tidigare studier har visat att ESX-1 kan leda till celldöd genom att skada interna cellmembran i infekterade celler. I vår studie visar vi att denna process också resulterar i en ökning av specifika proteiner, kallade serinproteashämmare, som i sin tur motverkar celldöd som uppstår till följd av ESX-1-beroende membranskada.

I vår sista artikel, artikel IV, presenterar vi olika metoder för att studera vår infektionsmodell med *M. marinum*. Genom att använda dessa metoder kan man undersöka och bidra till förståelsen hur mykobakterier påverkar immunförsvaret och bidrar till sjukdom.

Sammanfattningsvis ger vår forskning värdefull insikt i mykobakteriers komplexa natur och hur *M. marinum*-infektionsmodellen kan användas för att studeras dess patogenes. Genom att förstå hur tuberkulosbakterier och dess sekretionssystem ESX-1 manipulerar immunförsvaret och påverkar värdcellernas genuttryck, kan vi förhoppningsvis bidra till utvecklingen av nya behandlingsstrategier mot denna allvarliga sjukdom.

Papers included in this thesis

Paper I

Intragranuloma Accumulation and Inflammatory Differentiation of Neutrophils Underlie Mycobacterial ESX-1-Dependent Immunopathology.

Lienard J, **Munke K**, Wulff L, Da Silva C, Vandamme J, Laschanzky K, Joeris T, Agace W, Carlsson F. mBio. 2023 Apr 25;14(2):e0276422. doi: 10.1128/mbio.02764-22. Epub 2023 Apr 5. PMID: 37017530; PMCID: PMC10127687.

Paper II

In vivo regulation of the monocyte phenotype by *Mycobacterium marinum* and the ESX-1 type VII secretion system.

Munke K, Wulff L, Lienard J, Carlsson F, Agace WW. Sci Rep. 2025 Feb 7;15(1):4545. doi: 10.1038/s41598-025-88212-z. PMID: 39915532; PMCID: PMC11802795.

Paper III

Cytosolic serpins act in a cytoprotective feedback loop that limits ESX-1-dependent death of *Mycobacterium marinum*-infected macrophages.

Nobs E, Laschanzky K, **Munke K**, Movert E, Valfridsson C, Carlsson F. mBio. 2024 Sep 11;15(9):e0038424. doi: 10.1128/mbio.00384-24. Epub 2024 Aug 1. PMID: 39087767; PMCID: PMC11389378.

Paper IV

A murine *Mycobacterium marinum* infection model for longitudinal analyses of disease development and the inflammatory response.

Lienard J, **Munke K**, Carlsson F. Methods Mol Biol. 2023;2674:313-326. doi: 10.1007/978-1-0716-3243-7_21. PMID: 37258977.

Abbreviations

AM	Alveolar macrophage
AG	Aminoguanidine
BCG	Bacille Calmette-Guérin
CCR	C-C chemokine receptor
CFP-10	10-kDa culture filtrate protein (EsxB)
cGAS	Cyclic GMP-AMP synthase
CIITA	Class II Major Histocompatibility Complex Transactivator
CXCL	Chemokine (C-X-C motif) ligand
CXCR	CXC chemokine receptor
DC	Dendritic cell
DM	Dermal macrophage
Ecc	ESX conserved component
ESAT-6	6-kDa early secreted antigenic target (EsxA)
Esp	ESX secretion-associated proteins
ESX	ESAT-6 secretion system
GASE	IFNγ activated site element
GBP	Guanylate-binding protein
HIF	Hypoxia-inducible factor
IFN	Interferon
IFNAR	IFN-α/β receptor
IFNGR	IFNγ receptor
IL	Interleukin
IM	Interstitial macrophage
iNOS	inducible nitric oxide synthase
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
ISRE	Interferon stimulated response element
JAK	Janus kinase

Ly	Lymphocyte antigen
MDR-TB	Multidrug resistant tuberculosis
MHC	Major histocompatibility complex
MPO	Myeloperoxidase
NK cell	Natural killer cell
NO	Nitric oxide
PAMP	Pathogen-associated molecular patterns
PDIM	Phthiocerol dimycocerosate
PGL	Phenolic glycolipid
PRR	Pattern recognition receptor
RD1	Region of difference 1
RLR	RIG-I-like receptor
RNI	Reactive nitrogen species
ROS	Reactive oxygen species
scRNA-seq	single cell RNA sequencing
STAT	Signal transducer and activator of transcription
STING	Stimulator of IFN genes
TDM	Trehalose dimycolate
TLR	Toll-like receptor
TNF	Tumor necrosis factor
XDR-TB	Extensively drug-resistant tuberculosis

Introduction

Infectious diseases have shaped human history, causing devastating outbreaks while also driving the evolution of our immune system. Every day, we are exposed to a vast number of microorganisms, many of which are harmless or even beneficial. However, some pathogens have evolved sophisticated strategies to invade our bodies, evade immune defenses, and cause disease.

The immune system is a complex network of cells and molecules that work together to detect and eliminate threats. It acts as both a shield and an orchestrator - recognizing harmful invaders, mounting rapid responses, and even "remembering" past infections to prevent future illness. Yet, this is not a static defense; rather, it is engaged in a continuous evolutionary battle with pathogens, each adapting to overcome the other. While our immune system refines its ability to recognize and destroy invaders, many microbes have developed ways to persist, sometimes hiding within our own cells or manipulating immune responses to their advantage.

Tuberculosis

Mycobacterium tuberculosis, the causative agent of tuberculosis, stands as one of the oldest known human pathogens and has been responsible for more deaths than any other pathogen in history. The genus *Mycobacterium* is believed to have originated more than 150 million years ago, while the common ancestor of modern *Mycobacterium tuberculosis* strains likely emerged around 15,000–20,000 years ago (1). Evidence of skeletal deformities characteristic of tuberculosis has been found in Egyptian mummies dating back to 2400 BC (1). However, the earliest known written records describing the disease date back approximately 3,300 years (1). Although tuberculosis has existed for millennia, it was not until the 19th-century industrial revolution that it became a widespread epidemic (2). Over time, the incidence decreased due to concerted efforts, including the introduction of the Bacille Calmette-Guérin (BCG) vaccine and antimicrobial drugs in the 20th-century (2). Despite being a curable and preventable disease, tuberculosis remains a major global health problem. The WHO tuberculosis report from 2022 states that one quarter of the world's population is infected with *M. tuberculosis*, although the true extent of latent infection has recently been challenged in the scientific community (3). In 2021, this resulted in 1.6 million deaths, including 187 000 people living with HIV (4). This makes tuberculosis one of the top infectious killers in the world and the leading cause of death of people with HIV. Sadly, the COVID pandemic has reversed several years of progress in the battle against tuberculosis (4). Geographically, the areas with highest prevalence of tuberculosis are currently South-East Asia (45%), Africa (25%) and the Western Pacific (18%), while the disease is almost irradicated and forgotten in other parts of the world (4).

The vast majority of people infected with *M. tuberculosis* are asymptomatic carriers, a state referred to as latent tuberculosis (5). Latent tuberculosis is defined as a persistent and specific immune response to *M. tuberculosis* without any clinical manifestations of active disease. While these individuals are not infectious, they have a 5-10% lifetime risk of developing active disease (5).

Pathogenesis

M. tuberculosis spreads to new hosts via aerosol droplets coughed up by individuals with active tuberculosis. Interestingly, studies have observed that a smaller number of inhaled bacteria can be more effective at colonizing new hosts than larger amounts (6). One proposed explanation is that small droplets, each containing only one to three bacteria, can reach the lower part of the lung upon inhalation, making them more effective initiators of infection (7). This region of the lung is relatively sterile compared to the upper airways, which are colonised by commensal bacteria that prime resident macrophages and enhance their microbicidal activity (7). Another contributing factor could be that fewer bacteria are more likely to infect cells while evading detection by the immune system (7).

Upon arrival in the lower lung, bacteria are phagocytosed by alveolar macrophages (AMs) that patrol the airway lumen. Following *M. tuberculosis* exposure, three potential outcomes may occur: early bacterial clearance, asymptomatic latent infection, or symptomatic active disease (8). AMs employ several effector mechanisms that contribute to mycobacterial killing, including phagosome maturation, production of nitric oxide (NO) and reactive oxygen species (ROS), induction of autophagy and production of proinflammatory cytokines such as TNFα (8).

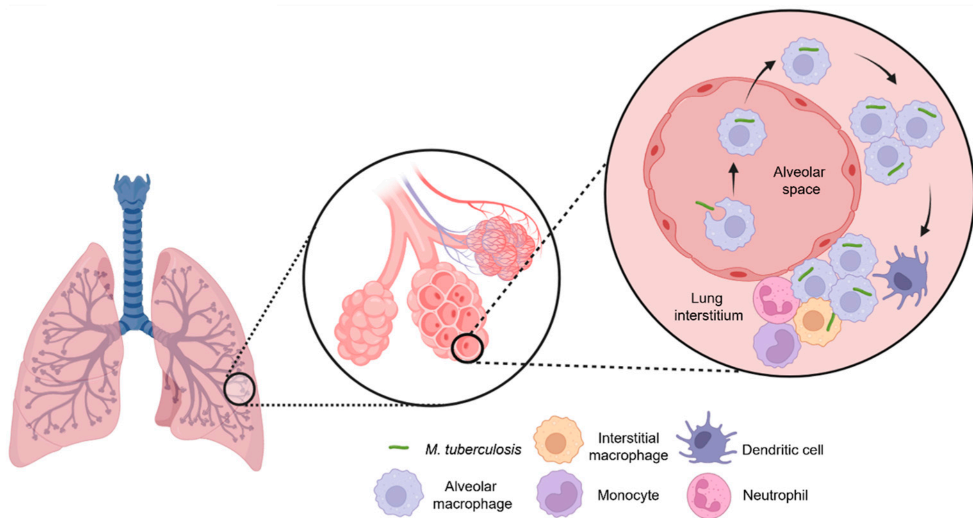

Figure 1. Pathogenesis of tuberculosis.
In the alveoli, M. tuberculosis first encounters alveolar macrophages (AMs). Infected AMs migrate to the lung intersitium, where the bacteria disseminate and infect additional innate immune cells. Adapted from reference (9). Figure created with BioRender.

M. tuberculosis has evolved several strategies to avoid intracellular killing by macrophages. The bacteria resist acidification by preventing phagosome-lysosome fusion (10, 11) and can eventually escape the phagosome into the cytosol (12-15), where they are able to modulate immune responses (16). Infected AMs migrate into the lung interstitium, where the infection is established (9) (**Fig. 1**). Innate immune cells, such as interstitial macrophages (IMs), monocytes, neutrophils and dendritic cells (DCs), are recruited to the site of infection and acquire bacteria within a few days (7, 9). The accumulation of various immune cells and bacteria trigger the formation of a granuloma, which is a pathological hallmark of tuberculosis (**Fig. 2**). For a long time, it was believed that granulomas were strictly a host defense response to keep the bacteria constrained. In recent years, several studies have argued that mycobacteria are highly involved in driving granuloma formation and exploit these structures for their survival and spread (7, 17, 18). Tuberculosis granulomas are largely composed of macrophages. As the infection advances and macrophages become activated, their membranes undergo changes and become tightly interdigitated, leading them to be called epithelioid cells (7, 19). This process, coupled with the formation of a fibrotic outer layer due to the deposition of extracellular matrix components, enhances the structural integrity of the granuloma and contributes to the containment of the bacteria (7). Nevertheless, bacteria can successfully expand within granulomas (7). Once the number of intracellular bacteria reaches a certain threshold, infected macrophages undergo cell death (7). Newly recruited cells phagocytose the dying macrophages along with the bacteria, enabling the bacteria to survive and expand within the new cells (7, 18). Ultimately, these processes lead to the development of a necrotic core within the granuloma that supports bacterial growth (7).

The adaptive immune system plays an important role in the defense against *M. tuberculosis*. Once the adaptive immune system is activated and recruited to the site of infection, the granuloma can successfully restrict bacterial growth (17). However, while the adaptive immune system is capable of controlling and containing the infection, complete eradication is often challenging (17). The infection is often maintained in a latent state, characterized by quiescent and non-replicating bacteria. Latent infections can persist for years, but certain conditions that weaken the immune system – such as HIV infection, malnutrition or immunosuppressive therapies – may trigger their reactivation, leading to active disease and spread (20).

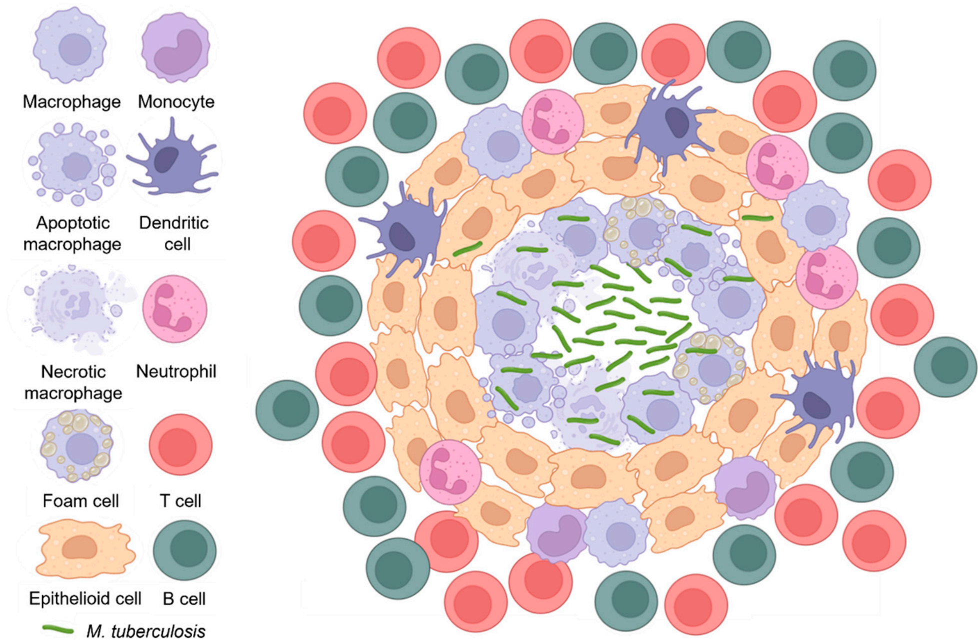

Figure 2. Illustration of the structure and cellular components of the tuberculosis granuloma. The tuberculous granuloma is a structured aggregate of immune cells, including specialized macrophages known as epithelioid cells, which form tight intercellular connections. Macrophages can also differentiate into lipid-rich foam cells, typically located at the periphery of the necrotic core. Most bacteria are found in the central necrotic region, where dying macrophages accumulate. The granuloma also contains various other immune cells, including neutrophils, dendritic cells, and T and B cells. Adapted from reference (17). Figure created with BioRender.

Several mycobacterial species have the potential to cause skin infection in humans. Although uncommon, *M. tuberculosis* infection can lead to cutaneous manifestations, giving rise to distinctive lesions and granuloma structures (21). These granulomas are pathologically identical to the dermal granulomas caused by *M. marinum* (22), often referred to as "fisherman's disease" to emphasize its association with aquatic environments. The pathogenesis of cutaneous mycobacterial infections shares similarities with pulmonary tuberculosis, involving the infection of similar immune cells. In both cases, mycobacteria infect tissue-resident macrophages constituting the first line of defense, which ultimately lead to initiation of granuloma formation (9, 18, 22, 23).

Mycobacterium tuberculosis

Physiology

M. tuberculosis has an intracellular lifestyle and prefers aerobic growth conditions, thriving in oxygen-rich tissues such as the lungs (24). It is also characterized by a slow growth rate (24).

M. tuberculosis, as all mycobacteria, are rod-shaped and possess a distinctive cell envelope which distinguishes them from most other bacteria (**Fig. 3**). The cell envelope is composed of several complex layers (25). The plasma membrane makes up the innermost layer and is surrounded by a peptidoglycan-arabinogalactan peptide layer, which in turn connects to a thick layer of mycolic acids. The outermost layer is mainly comprised of glycolipids that can interact with host cells to promote internalization of the bacterium and modulate immune responses (25). Trehalose dimycolate (TDM) glycolipids and phenolic glycolipids (PGLs) are both highly immunogenic, triggering immune responses and altering the expression of inflammatory cytokines during infection (26-28). In contrast, phthiocerol dimycocerosate (PDIM) lipids can mask bacterial structures, helping the bacteria evade detection by immune cells (29).

The composition and inherent thickness of the mycobacterial cell wall naturally reduces permeability (25). These physical properties together with the high content of mycolic acids makes mycobacteria resistant to Gram-staining (24), and they are thereby classified as acid-fast rather than Gram-positive or -negative (24).

Type VII secretion systems

Mycobacteria exhibit advanced strategies to resist the hostile macrophage environment and modulate host immune responses. They utilize specialized secretion systems to enable transport of proteins across their thick and impermeable cell wall (30). This process is vital for secreting specific molecules, such as virulence factors, and for taking up fatty acids and other nutrients. Mycobacteria express a distinct secretion system known as the type VII secretion system (T7SS), which consists of five paralogous systems denoted Early Secreted Antigenic Target 6 kDa (ESAT-6) secretion system (ESX)-1 to ESX-5 (31). While the ESX-1, ESX-

3, and ESX-5 are required for full virulence (30, 31), these systems are not uniformly co-regulated; instead, their expression is controlled by distinct regulatory mechanisms in response to specific environmental cues and bacterial needs (31). ESX-1 is a well-studied virulence determinant that is required for phagosomal rupture in macrophages, facilitating intracellular survival. ESX-3 is required for acquiring metal ions, such as iron and zinc, which are crucial for bacterial growth. ESX-5 is involved in nutrient uptake and secretion of immunomodulatory effector proteins (31). ESX-2 and ESX-4 are less studied and their function in *M. tuberculosis* is largely unknown. However, recent studies in *M. tuberculosis*-infected human macrophages suggest that these systems play a role in the phagosomal rupture in coordination with ESX-1 (32).

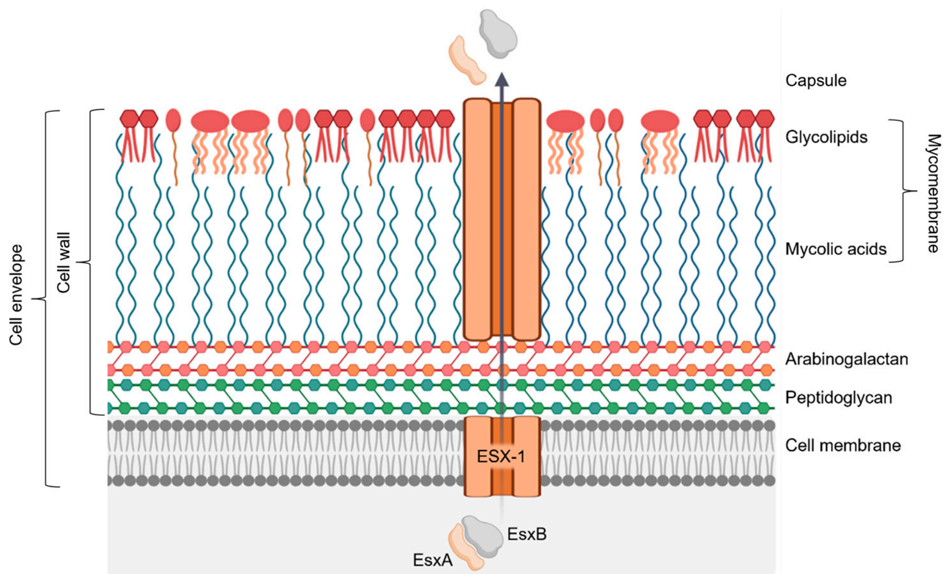

Figure 3. Overview of mycobacterial cell envelope structure.
The mycobacterial cell wall consists of multiple complex layers. The Type VII secretion system, ESX-1, is localized within the cell envelope and is responsible for the secretion of effector proteins, such as EsxA (ESAT-6) and EsxB (CFP-10), which play crucial roles in host immune modulation and pathogenesis. Adapted from reference (30). Figure created with BioRender.

The ESX-1 secretion system

ESX-1-deficient mycobacteria display attenuated virulence, diminished intracellular growth and defective granuloma formation *in vivo* (33-38), underscoring the crucial role of ESX-1 in the infection process. ESX-1 has also been implicated in phagosomal membrane permeabilization that facilitate cytosolic translocation (12-15) and modulating cytokine output in macrophages (39-42). In **Paper I**, we assess the role of the ESX-1 secretion system in driving pathology during *M. marinum* infection *in vivo*.

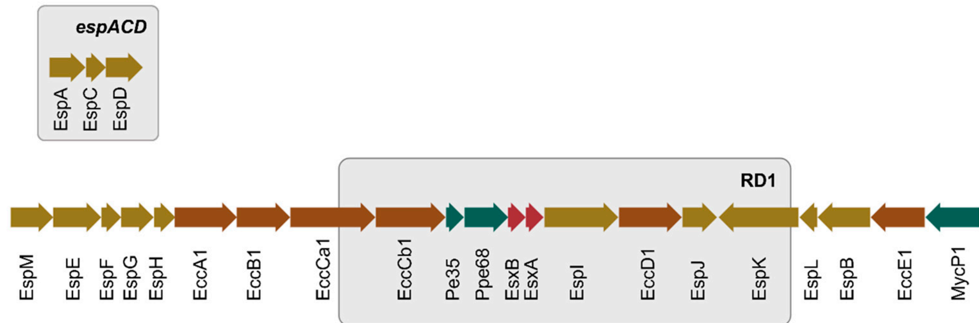

Figure 4. Schematic representation of the ESX-1 core locus and the espACD operon. Adapted from references (30, 43). Figure created with BioRender.

The ESX-1 core locus comprises both structural components and secreted proteins of the secretion system (30) (Fig. 4). The locus contains the region of difference 1 (RD1), defined by the corresponding deletion in the *M. bovis* BCG vaccine strain (44). RD1 encodes nine genes, including both structural components and secreted proteins, and is essential for ESX-1 mediated virulence (30). The components of the secretion system have been assigned a general nomenclature; Ecc proteins refer to components that are conserved across at least four of the five ESX systems and form the core structure of the ESX-1 secretion system within the inner membrane; Esp-proteins stands for ESX secretion-associated proteins; Esx-proteins encode the secreted immunogenic factors EsxA and EsxB, formerly referred to as ESAT-6 and CFP-10, respectively; MycP1 encodes Mycosin, a serine protease involved in substrate processing and regulation of ESX-1 secretion (30).

The effector proteins EsxA and EsxB are secreted as a 1:1 heterodimer that forms in the bacterial cytosol and dissociates in the phagosomal lumen, likely due to the low pH (45, 46). EsxB contains a specific signal sequence that interacts with members of the SpoIIIE/FtsK ATPase family, facilitating energy-dependent translocation of the EsxA–EsxB complex (47). Research indicates that EsxA and EsxB proteins play a role in various immunomodulatory processes during infection, such as inhibiting phagosome-lysosome fusion (48), suppressing pro-inflammatory

cytokines (37), and promoting the production of host-detrimental type I interferons (IFNs) (49). One of the most prominent functions attributed to the ESX-1 secretion system is the permeabilization of host membranes (12-15). Although this activity was previously linked to EsxA, recent findings suggest that earlier studies may have been influenced by detergent contamination in EsxA protein preparations (50). Consistently, phenotypic studies from our group using transposon mutant strains deficient in specific ESX-1-associated genes indicate that EsxA may not be essential for membrane permeabilization and cytosolic translocation, but may play a more specific role in the induction of type I IFNs (42).

A number of Esp-proteins have been described as substrates of ESX-1, including EspA, EspB, EspC, EspE and EspF, while others function as chaperones (30, 43). We and others have characterized several transposon mutant strains deficient in specific ESX-1-related genes *in vitro* (42, 51, 52), including the *espK* gene located within the RD1 locus. The EspK protein functions as a chaperone and is required for optimal EsxA secretion and crucial for EspB secretion (52-54). A previous study from our group showed that the transposon mutant *espK*::Tn retained its capacity to permeabilize host membranes and translocate to the cytosol, but was unable to induce type I IFN production in *M. marinum*-infected bone-marrow derived macrophages (42). Moreover, a recent study demonstrated that the *espK* gene is not required for bacterial growth in lung and spleen of *M. tuberculosis*-infected mice (54). In **Paper II**, we investigate the virulence and pathology of the *espK*::Tn mutant *in vivo*, as well as its transcriptional impact on monocytes (55).

While EspA, EspC and EspD are encoded by the espACD operon, located upstream of the core locus (Fig. 4), they are still essential for full ESX-1 functionality (30). Interestingly, the secretion of EsxA and EsxB is co-dependent on the secretion of EspA and EspC (56), highlighting the interdependence among ESX-1 substrates and the overall complexity of the system. Consequently, disrupting a single component through deletion or mutation can not only impair the secretion of that specific substrate but also destabilize the entire secretion system.

M. marinum as a model system for mycobacterial pathogenesis

M. marinum and *M. tuberculosis* are closely related, sharing an 85% sequence homology in orthologous regions (22). The genome of *M. marinum* is approximately 6.6 megabases, roughly 1.5 times larger than the 4.4 megabase genome of *M. tuberculosis*, which reflects its adaptation to a broader host range (22). *Mycobacterium marinum* is a natural pathogen of fish and amphibians but can also cause skin infections in humans. While *M. tuberculosis* that has an optimal growth temperature of 37˚C, reflecting the normal body temperature of humans, *M.*

marinum prefers a temperature range of 25-35°C, restricting its growth to cooler surfaces such as the skin (22). Dermal granulomas formed by *M. marinum* and *M. tuberculosis* are often pathologically similar (22). Notably, both *M. marinum*-infected mice and zebrafish are shown to develop granulomas with a central necrotic core (22, 36), which is a hallmark of tuberculosis granulomas. In contrast, mouse models infected with *M. tuberculosis* form poorly organized structures that do not caseate (57). To properly study granuloma pathology using *M. tuberculosis*, one must employ models with rabbits, guinea pigs or macaques, which entails increased financial and ethical constraints (57). Additionally, these models are more difficult to genetically manipulate compared to mouse models, making mechanistic studies aimed at understanding host responses more challenging (57).

Both *M. marinum* and *M. tuberculosis* infect macrophages and other host phagocytes at the site of infection (22). Moreover, many important virulence determinants, including the type VII ESX-1 secretion system, are highly conserved between the species (22, 43). Studies on *M. marinum* have elucidated key insights into *M. tuberculosis* virulence mechanisms, particularly regarding the role of the ESX-1 secretion system (43). In fact, it would be difficult to discuss the timeline of ESX-1 research without considering findings from studies on *M. marinum* (43). Collectively, these biological aspects make it a good alternative model system to study mycobacterial pathogenesis and host-pathogen interactions (22, 58).

In addition, *M. marinum* is both safer and easier to work with. It does not cause severe or systemic disease in humans, allowing laboratory work in a lower biosafety level compared to *M. tuberculosis* and instead of a 20 hour generation time, *M. marinum* divides and doubles in about 4 hours (22). In **Paper IV**, we present newly developed techniques for the murine *M. marinum* infection model (59), which induces localized tail lesions and recapitulates key features of tuberculosis (36, 55, 60). A key advantage of this model is its capacity to enable non-lethal, longitudinal monitoring of disease progression in live animals.

Treatment and vaccines

Antibiotic drugs

If left untreated, up to half of all individuals with tuberculosis will succumb to the disease (4), highlighting the critical importance of effective treatment strategies. Tuberculosis treatment requires a long and complicated antibiotic regimen, largely due to the slow growth rate of the bacteria, as well as the unique composition and low permeability of their cell wall. The treatment consists of a cocktail of the four different antimicrobial drugs Isoniazid, Ethambutol, Rifampicin and Pyrazinamide taken over six months (20). Isoniazid and Ethambutol target crucial components of the mycobacterial cell wall by inhibiting mycolic lipid and arabinogalactan biosynthesis, respectively (61). Rifampicin enters mycobacteria through passive diffusion and targets a RNA-polymerase to disrupt bacterial RNA synthesis and gene expression (61). The exact mechanism of Pyrazinamide is not fully understood, but recent studies suggest that it inhibits the pantothenate biosynthesis pathway, essential for the generation of vitamin B5 and coenzyme A in mycobacteria (62).

The global success rate for standard treatment in patients with drug susceptible tuberculosis is high, with about 85% chance of recovery (4). However, the treatment regimen is not only costly but also time-consuming, posing challenges for adherence. This is particularly problematic in regions heavily burdened by tuberculosis, where patients often lack adequate support (4, 20). If treatment is not completed properly, bacteria may develop drug resistance, exacerbating the spread of the disease.

The emergence of drug-resistant strains is making treatment increasingly challenging and necessitating the use of second-line drugs. These include bedaquiline, which targets the ATP synthase of mycobacteria, and fluoroquinolones, which disrupt DNA replication apparatus and generates double stranded breaks (20). Drug resistant tuberculosis is classified based on the resistance pattern to specific antibiotic drugs (4, 20). Multidrug resistant strains (MDR-TB) are characterized by resistance to at least two of the most potent first-line drugs, whereas extensively drug resistant tuberculosis (XDR-TB) is resistant to two of the most potent first-line drugs and two second-line drugs, including a fluoroquinolone and bedaquiline. In 2020, the success rate for treating MDR-TB reached 63%, marking an improvement from 50% over the past decade (4).

Vaccines

In 1921, Albert Calmette and Camille Guérin developed the BCG vaccine by repeated passaging of *M. bovis* cultures until it was no longer able to cause disease in animal models (63). It was later discovered that the attenuation of the BCG vaccine is largely associated with the loss of the RD1 region, encoding for genes essential for the functionality of the type VII ESX-1 secretion system (44). While BCG is the most widely used vaccine globally and the only licensed vaccine for tuberculosis, its efficacy is far from optimal (63). The protection against pulmonary tuberculosis in adults and adolescents ranges from 0-80% depending on vaccine strain and geographic location (64). Studies have shown that there is a positive correlation between BCG vaccine efficiency and the distance from the equator, which is speculated to relate to decreased exposure to environmental mycobacteria (64). Meanwhile, BCG vaccination in infancy has consistently provided strong protection against severe disseminated tuberculosis in children (65). However, there is still an urgent need for new vaccines that provide robust protection against pulmonary tuberculosis in adults to effectively curb disease transmission.

The inflammatory response

Innate immune responses during mycobacterial infections

Macrophages

Macrophages are innate immune cells that play a pivotal role in maintaining homeostasis and defending the body against pathogens. These versatile immune cells are involved in various functions, ranging from phagocytosis and antigen presentation to the modulation of inflammation and tissue repair (66).

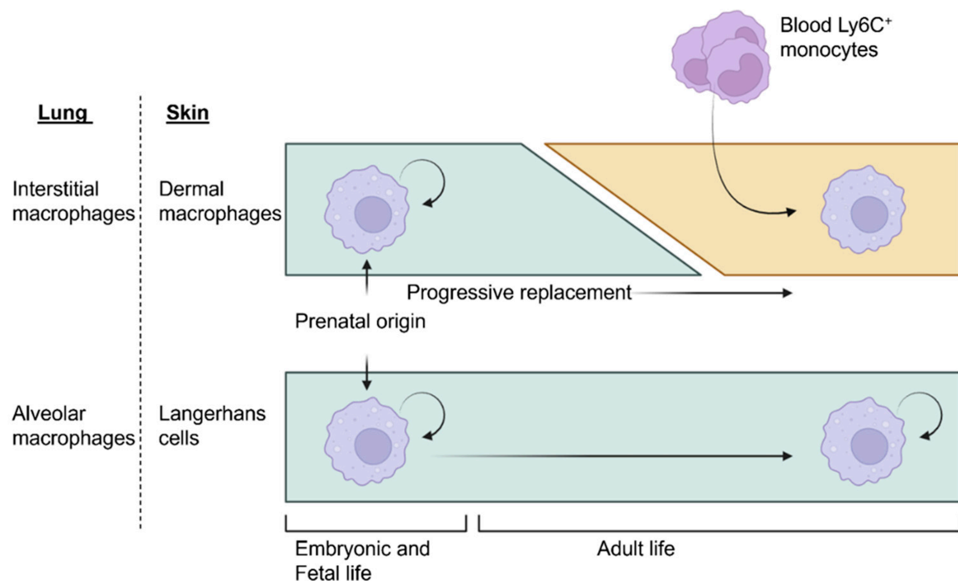

Figure 5. Origin of macrophages in the lung and skin.
Alveolar macrophages and Langerhans cells are established prenatally and maintain themselves independently from adult haematopoiesis. Interstitial and dermal macrophages consist of a pool of cells established prenatally in an CCR2-independent manner, other developing postnatally in a CCR2-dependent manner from blood Ly6C$^+$ monocytes. Adapted from reference (67). Figure created with BioRender.

Macrophages in the lung

During homeostasis, the lung harbours at least three major macrophage populations; AMs, that serve as the first line of defenders in the alveolar space, and two to three IM populations guarding the vasculature and lung interstitium (68) (**Fig. 5**). AMs develop from fetal progenitors, are long-lived and have the capacity for self-renewal in adulthood, without input from circulating monocytes. They exhibit a distinctive phenotype and function shaped by their specific environment within the lungs (68). In contrast, IMs comprise both a pool of long-lived cells derived from fetal progenitors and a pool of cells constantly replenished by circulating monocytes. IMs are distributed throughout the body, where they perform both conserved specialized functions and tissue-specific roles tailored to the unique requirements of each environment (68).

AMs are the first immune cells to encounter and phagocytose *M. tuberculosis* upon infection (9). At times, AMs are successful in killing the intracellular bacteria and avoid an infection, a process referred to as early clearance (8, 69). The outcome of infection relies on several factors, including efficient phagosome acidification, phagosome-lysosome fusion, ESX-1-dependent escape from the phagosome, inflammasome activation, the secretion of IL-1α/β and TNFα, and activation of cell death pathways (8). Additional critical determinants include upregulation of genes involved in intracellular killing mechanisms, such as NO, ROS, antimicrobial peptides, and autophagy, as well as factors favouring bacterial survival, such as type I IFNs (8). Therefore, while AMs display various effector functions to combat mycobacteria at an early stage, *M. tuberculosis* has evolved effective strategies to evade intracellular destruction by these immune cells. Unsuccessful clearance leads to the translocation of infected AMs to the lung interstitium allowing the bacteria to disseminate to other immune cells, including IMs, and establish an infection (9). In fact, depletion of AMs in mouse models of tuberculosis has been shown to decrease the bacterial load, indicating that AMs play a permissive role in facilitating early pathogenicity (70, 71). In contrast, depletion of IMs increased the bacterial load suggesting that these cells exhibit a more restrictive environment compared to AMs (70). Notably, AMs and IMs adopt different metabolic states that influence their ability to control bacterial growth. AMs rely on fatty acid oxidation, creating a metabolically favorable environment that supports *M. tuberculosis* persistence, whereas IMs are glycolytically active, leading to nutritional restriction and decreased bacterial growth. (70, 72). The restrictive phenotype in infected IMs is also associated with increased expression of inducible nitric oxide synthase (iNOS), TNFα, IL-1β and HIF1α (70, 72).

Macrophages in the skin

The skin harbours several tissue-resident macrophage populations, one being the Langerhans cells residing in the epidermis. These cells share features with both macrophages and DCs and are specialized to mediate and organize local barrier immunity (66). Their main functions include phagocytosis, antigen presentation, initiation of immune responses and maintaining tolerance (66). Langerhans cells are long-lived and primarily maintained through local proliferation during tissue homeostasis, with monocyte-derived precursors contributing to their replenishment only under conditions such as infection or injury (73, 74). In contrast, dermal macrophages (DMs) represent a distinct population in the skin that is more frequently replenished, primarily through monocyte recruitment from the bloodstream (67). Several DM subsets have been identified with distinct functions and anatomical locations (66). In addition to their well-known functions in innate immunity and phagocytosis, they are actively involved in tissue repair, wound healing, hair follicle regeneration and stress responses (67, 75).

Monocytes

Originating from the bone-marrow, monocytes circulate in the bloodstream before migrating to various tissues throughout the body. Under steady state conditions, once in the tissue, they undergo differentiation into macrophages and acquire the ability of antigen presentation among other functions. Thus, monocytes serve as a key precursor to some macrophage populations, connecting the circulatory system and the tissues where localized immune activities are needed.

Upon tissue entry and differentiation, blood Ly6C^{+} monocytes downregulate Ly6C while upregulating MHC II, a process commonly known as the "monocyte waterfall" (74, 76, 77) (**Fig. 6**). This differentiation process comprises three stages: Ly6C^{high} MHC II^{low} (P1), Ly6C^{high} MHC II^{high} (P2) and Ly6C^{low} MHC II^{high} (P3). Both P2 and P3 monocytes display a morphology that falls between that of DCs and macrophages (74, 76). In the skin, P2 and P3 monocytes are capable of migrating to the draining lymph nodes, although with less efficiency compared to DCs (74). Additionally, among the few that do reach the lymph nodes, their capacity to stimulate T cells on a per-cell basis is significantly lower than that of the predominantly abundant dermal DCs (74). Upon inflammation, there is a marked accumulation of Ly6C^{high} MHC II^{high} P2 monocytes in the tissue (74, 76), as consistently observed in our studies (**Paper I and II**) (55, 60).

Monocytes require the chemokine receptor CCR2 to emigrate from the bone-marrow into the circulation (78), rendering CCR2-knockout mice deficient in monocyte migration into tissues. Studies utilizing *M. tuberculosis*-infected CCR2-knockout mice have shown that CCR2^{+} monocytes play a protective role in immunity against mycobacteria (79-83). While the mechanisms behind this

protective role remain largely unknown, CCR2-knockout mice show decreased expression of IFNγ and iNOS (83). In addition, $CCR2^+$ monocytes have been shown to deliver *M. tuberculosis* to lymph nodes, where they transfer antigens to conventional DCs (cDCs), which induce the proliferation of antigen-specific $CD4^+$ T cells (81, 82). Our group and others have recently shown that monocytes are one of the main infected cell types in the tissues during mycobacterial infections (9, 55, 60). In **Paper I**, we assess the role of monocytes during *M. marinum* infection *in vivo*, and in **Paper II**, we evaluate their transcriptional profile.

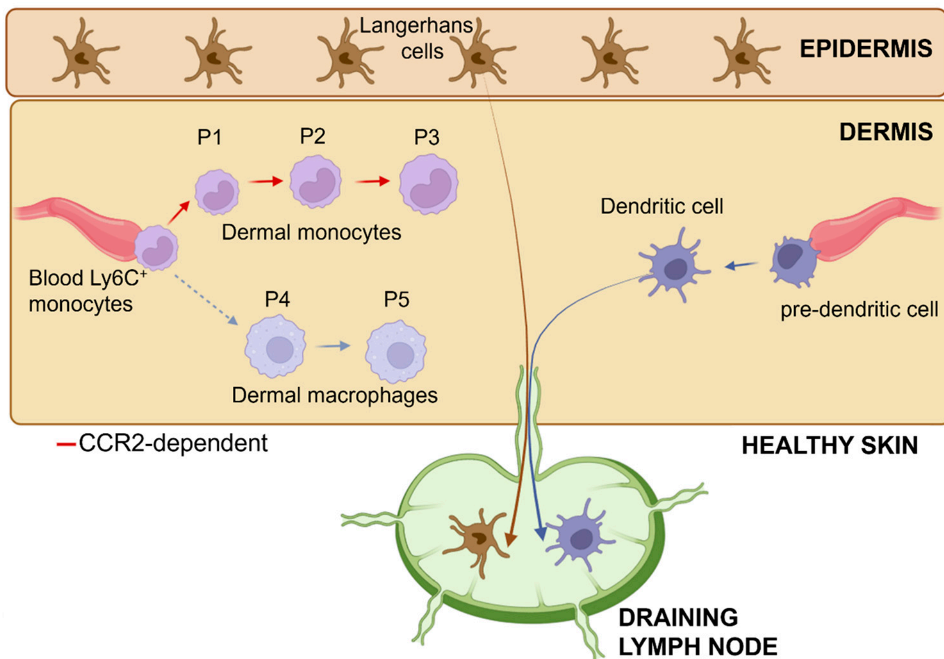

Figure 6. Illustration of the myeloid cell compartment in healthy skin.
Blood $Ly6C^+$ monocytes enter the dermis in a CCR2-dependent manner and differentiate through distinct stages: (P1) $Ly6C^{high}$ MHC II^{low}; (P2) $Ly6C^{high}$ MHC II^{high} and (P3) $Ly6C^{low}$ MHC II^{high}, collectively referred to as dermal monocytes. These cells can further mature into dermal macrophages: (P4) $Ly6C^{low}$ MHC II^{low} and (P5) $Ly6C^{low}$ MHC II^{high} cells. Langerhans cells in the epidermis and dendritic cells in the dermis take up antigens and migrate to draining lymph nodes, where they present the antigens and prime T cells. Reference Adapted from reference (74). Figure created with BioRender.

Neutrophils

Neutrophils are the most abundant immune cell in the human body and are classified as granulocytes, a category of immune cells characterized by their cytoplasmic granules, which contain proteases, antimicrobial peptides, and enzymes (84). These

granules are released during various immune responses, including those triggered by infections and allergic reactions (84). Neutrophils are crucial for the body's defense against microbial invaders and possess several potent antimicrobial functions, such as phagocytosis, intracellular killing, and the degranulation of antimicrobial effectors like myeloperoxidase (MPO) into the extracellular space (84). Their rapid recruitment to sites of infection is a hallmark of an inflammatory response and underscores their significance in initiating and amplifying immune responses. However, dysregulation or overactivation of these potent cells can be detrimental to the host, potentially contributing to tissue damage, inflammatory diseases, or autoimmunity (84).

In tuberculosis, neutrophils are rapidly recruited to the site of infection in a largely CXCR2-dependent manner (85-87), where they exert their antimicrobial functions. Neutrophils are frequently observed within granuloma structures and have recently been identified as a cellular reservoir for *M. tuberculosis*, in both humans (88) and animal models (9, 70, 89, 90). While neutrophils harbour the bacteria, *in vitro*-studies with *M. marinum* suggest that they are not a prominent niche for intracellular replication compared to macrophages [**Paper I**] (60). However, other studies have suggested that mycobacteria that grow in close association with neutrophils face a more permissive environment, rich in micronutrients such as iron and lipid carbon sources (86), indicating that neutrophils may support bacterial growth.

In human pulmonary infections, neutrophil numbers in the lung correlate with active disease and tissue destruction (91). Several studies in animal models have suggested a host-detrimental role for neutrophils in mycobacterial infection (86, 87, 90, 92, 93), while others have suggested that neutrophils play a protective role (94-97). It is possible that neutrophils have multifaceted roles in tuberculosis pathogenesis, potentially contributing to protective immunity in the initial stages of infection while inducing immunopathology as the disease progresses. Nevertheless, these conflicting results highlight the need for further research on neutrophil-pathogen interactions and mechanisms regulating neutrophil function in mycobacterial infections. In **Paper I**, we assess the role of neutrophils during *M. marinum* infection.

Effector molecules

Along with immune cells, various effector molecules are essential to the innate immune defense against mycobacteria. These molecules include cytokines, antimicrobial peptides and ROS, which work together to neutralize the pathogen and promote inflammation.

Type I IFNs

Type I IFNs consist of several subtypes, with the two primary being IFN-α and IFN-β, both of which play an important role in immune responses against bacterial and viral infections (98). The production of type I IFNs is typically regulated through the activation of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and cytosolic DNA receptors, by pathogen associated molecular patterns (PAMPs) (98). In the context of mycobacterial infections, studies in both human and mouse macrophages suggest that the induction of type I IFNs is mainly dependent on the cGAS-STING pathway (39, 40, 99-102) (**Fig 7.**). How the cGAS-STING pathway, which is associated with the sensing of double stranded DNA, is induced in mycobacterial infection remains a subject of debate. Some studies propose that bacterial DNA from the phagosome is responsible for cGAS-STING activation (40, 99, 100), while others, including studies from our group, suggest that this pathway is primarily activated via recognition of host DNA from mitochondria or the nucleus (39, 42, 101). Secreted IFNs exert their effects by binding to the type I IFNs receptor (IFNAR), triggering induction of Interferon-Stimulated Genes (ISGs) that contribute to various immune responses within the host (103).

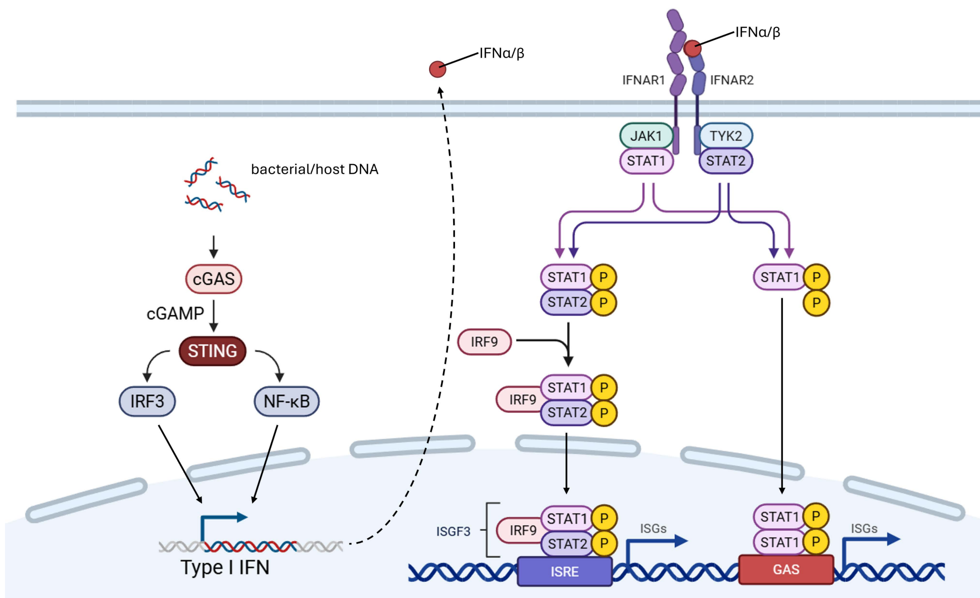

Figure 7. Illustration of the type I IFN signaling pathway.
Bacterial or host DNA triggers cGAS-STING activation, leading to the induction of type I IFNs. The type I IFN receptor is comprised of two subunits, IFNAR1 and IFNAR2, which are linked with TYK2 and JAK1 kinases, respectively. Signaling induces phosphorylation and the formation of either STAT1-STAT2-IRF9 complexes or STAT1-homodimers. These complexes translocate to the nucleus and bind to IFN-stimulated response element (ISRE) or the IFNγ activation site element (GASE) element respectively, thereby including transcription of ISGs. Adapted from references (103, 104). Figure created with BioRender.

While the contribution of type I IFNs to protective antiviral responses is wellestablished, their role in antibacterial responses is pathogen dependent (98). For instance, in infections with intracellular bacteria such as *M. tuberculosis*, type I IFNs can have detrimental effects, while in infections caused by group B Streptococcus, their effects can be protective (98). Individuals with active tuberculosis exhibit a distinct type I IFN transcriptional signature in whole blood, and this signature correlates with disease severity (105). Moreover, *M. tuberculosis*-infected IFNAR-knockout mice that are deficient in type I IFN signaling, display increased survival (106) and decreased bacterial burden (49), demonstrating a role for type I IFNs in disease development. Interestingly, the induction of type I IFNs by *M. tuberculosis* and *M. marinum* occurs in an ESX-1-dependent manner, highlighting the notion that this process is involved in the bacteria's virulence strategy (42, 49, 107).

The mechanisms underlying the adverse effects of type I IFNs remain unclear but likely involve inhibition of pro-inflammatory cytokines IL-1α, IL-1β, TNFα and IL-12 (108-112) as well as induction of anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonist (98, 110, 111). Type I IFNs also suppress IFNγ-mediated signaling in macrophages (111, 113), as well as IFNγ- production and *M. tuberculosis*-specific T-cell proliferation (108).

Serpins

Transcriptional profiling indicates that *in vivo* infection with *M. tuberculosis* and *M. bovis* BCG induces expression of clade A3 serine protein inhibitors (serpins) in macrophages and infected lung tissues (49, 114). Clade A3 serpins constitute a family of 14 genes, denoted as *Serpina3a - Serpina3n*, with 13 of them encoding functional proteins (115, 116). These proteins are expressed in various tissues and primarily function to inhibit serine proteases. Additionally, some serpins can inhibit caspases and cysteine proteases (115, 117). Despite the upregulation of clade A3 serpins during mycobacterial infection, their role remains largely unknown. Serpina3f and Serpina3g stand out within the family due to the lack of secretion signal peptides, implying a role in the cytosol where they inhibit cytosolic proteases (115). Our investigations into clade A3 serpins revealed an upregulation of Serpina3f and Serpina3g in response to ESX-1-mediated type I IFN signaling in bone-marrow derived macrophages infected with *M. marinum* (118). In **Paper III**, we assess the role of these cytosolic serpins in *M. marinum* infection.

IFNγ

IFNγ is primarily produced by T cells and NK cells in response to bacterial infections (119). It signals via the IFNGR receptor to induce transcription of genes regulated by the IFNγ activation site (GAS) (103). The antimicrobial Guanylate-Binding Proteins (GBPs), Interferon Regulatory Factor 1 (IRF1), Class II Major Histocompatibility Complex Transactivator (CIITA) and CXCR3-receptor ligands CXCL9 and CXCL10, represent a few examples of genes regulated by IFNγ (120-122).

IFNγ has been widely recognized as a critical factor in CD4$^+$ T cell-mediated protection against mycobacteria (123). In fact, humans with genetic mutants in IFNγ or its receptor have increased susceptibility to mycobacterial infections (124). IFNγ influences macrophages by enhancing their phagocytic capacity, antigen presentation, cytokine production and antimicrobial activities, including autophagy, nitric oxide production via iNOS, and induction of reactive nitrogen intermediates (RNI) (121, 122, 125).

It is well established that *M. tuberculosis* can inhibit IFNγ-mediated responses in macrophages (111-113, 126-131). Several mechanisms have been identified, including the suppression of specific IFNγ induced genes by mycobacterial lipoglycan phosphatidylinositol mannan (128), mycobacterial lipoproteins (127, 131), and the ESX-1 secretion system (130). Additionally, numerous studies have highlighted the role of type I IFN signaling in inhibiting IFNγ-mediated responses (41, 111-113). For example, research on *M. tuberculosis*-infected macrophages has shown that type I IFNs suppress IFNγ-responsiveness, IFNγ-dependent cytokine production, IFNγ-mediated killing, and bacterial growth restriction (41, 111). Collectively, these studies suggest that mycobacteria utilizes multiple mechanisms to suppress IFNγ-mediated responses.

Nitric oxide

NO exerts potent antimicrobial effects by generating RNIs through its interaction with ROS, particularly within the phagosome (132). This process contributes to the elimination of intracellular pathogens and enhances macrophage antimicrobial activity. RNIs produced in this manner directly damage pathogen cellular components, inhibit microbial replication, and disrupt essential survival processes. NO production is catalyzed by iNOS, which converts the amino acid arginine into NO using oxygen (132). Inflammatory cytokines, such as IFNγ and TNFα, induce iNOS expression and NO production in macrophages during infection (133).

M. tuberculosis-infected mice lacking iNOS or treated with aminoguanidine (AG), a potent inhibitor of inducible NO, develop exacerbated disease characterized by increased bacterial burdens and neutrophilic inflammation (85, 86, 134-136). One proposed mechanism behind this exacerbation involves NO inhibiting IL-1-dependent induction of CXCR2-mediated neutrophil recruitment, which contribute to a permissive environment for bacterial growth (**Fig. 8**) (86). In **Paper I**, we assessed iNOS expression in myeloid cells from *M. marinum*-infected tissues and explored the role of NO in the pathology of the infection (60).

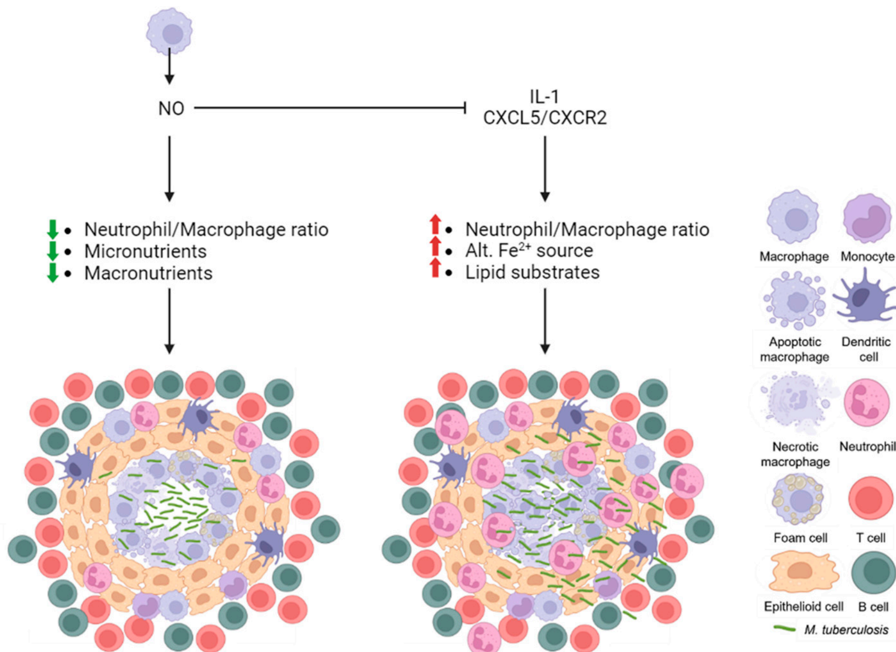

Figure 8. The role of NO in neutrophil recruitment.
M. tuberculosis infection induces IL-1 production, which in turn promotes CXCR2-dependent neutrophil recruitment to infected tissues. However, NO generated by iNOS from an unknown cellular source restricts this process by inhibiting the NLRP3 inflammasome. In iNOS$^{-/-}$ mice, increased neutrophil accumulation at the infection site correlates with higher bacterial burdens, as neutrophils contribute to an iron- and nutrient-rich environment that supports *M. tuberculosis* replication. Adapted from reference (86). Figure created with BioRender.

Adaptive immune responses during mycobacterial infections

The innate immune system plays an important role in priming and shaping the adaptive immune system to generate targeted defenses against invading pathogens when the innate immunity alone is insufficient (137). The main functions for the adaptive immune system include the recognition of self- and non-self-antigens, the generation of specific responses against pathogens and infected cells, and the development of immunological memory to protect from recurring infections (137). The main cellular components for the adaptive immune system are T cells, which recognize and eliminate infected or abnormal cells, and B cells that specialize in humoral immunity by producing antibodies that recognize and neutralize pathogens (137).

T cells

Immunity against mycobacterial infection relies on the activation of the adaptive immune system. During *M. tuberculosis* infection, antigen-presenting cells migrate to the local lymph nodes where they prime and activate antigen-specific T cells. $CD4^+$ T cells contribute to the orchestration of targeted responses by releasing cytokines and facilitating the activation of other immune cells. In contrast, $CD8^+$ T cells directly combat the infection using their cytotoxic effector functions to eliminate infected cells.

Of the adaptive immune cells, IFNγ producing $CD4^+$ T cells are crucial for constraining bacterial growth during *M. tuberculosis* infection (123, 138, 139). Studies suggest that mycobacteria manipulate host immunity, delaying the onset of adaptive immune responses through multiple mechanisms, including inhibition of apoptosis in infected cells, modulation of cytokine signaling (such as TNFα and IL-1β), regulation of chemokines (including CCL2 and CXCL10), delayed migration of antigen presenting cells to the local lymph node and modulation of regulatory T cell activity (139, 140). The delayed onset is characterized by persistent bacterial growth, lasting for about 6 weeks in humans and 3 weeks in mice , until the infiltration of antigen-specific IFNγ producing $CD4^+$ T cells (141, 142), which subsequently lead to a reduction in bacterial growth (139, 140). $CD4^+$ T cells play a crucial role not only in optimal granuloma formation and controlling mycobacterial growth, but also in enhancing $CD8^+$ T cell effector functions. This synergistic collaboration prevents cellular exhaustion and aid $CD8^+$ T cells to restrict intracellular growth more efficiently (143).

B cells

The role of B cells and humoral immunity in mycobacterial infection remains uncertain (144). Studies in B-cell-deficient mice have yielded conflicting results, showing either reduced protection or no significant impact on disease outcome (144). Despite this uncertainty, B cells are consistently found in the outer regions of the granuloma (**Fig. 2**) (17), where they can form follicular B cell aggregates (17). Additionally, protective antibodies have been identified in both patients with active tuberculosis (145) and asymptomatic healthcare workers (146), suggesting a potential role for humoral immunity in mycobacterial control.

Aims of the thesis

This study aims to investigate the cellular and molecular mechanisms underlying *Mycobacterium marinum* infection *in vivo*, with a particular focus on the role of ESX-1 in infection-induced pathology, immune cell dynamics, and transcriptional responses.

Specific Sub-Aims:

- **Pathogenic Mechanisms**: Assess the impact of ESX-1 (Paper I and II) and *espK* (Paper II) on *M. marinum*-induced pathology during infection *in vivo*.
- **Cellular Reservoirs**: Identify and characterize the primary cellular reservoirs for *M. marinum in vivo*, including their role in infection (Paper I).
- **Transcriptional Responses**: Examine the transcriptional profile of the primary cellular reservoirs and evaluate the effects of ESX-1, *espK*, and *M. marinum* infection on their gene expression (Paper II).
- **Host Defense Mechanisms**: Investigate the mechanistic role of Serpina3f/g in *M. marinum*-infected macrophages and assess its functional significance during *in vivo* infection (Paper III).
- **Model Development**: Describe and evaluate recently developed techniques for studying *M. marinum* infection using the murine *in vivo* model (Paper IV).

Summary of papers

Paper I

Intragranuloma accumulation and inflammatory differentiation of neutrophils underlie mycobacterial ESX-1-dependent immunopathology.

In this study, we investigated the cellular and molecular mechanisms underlying *M. marinum* infection *in vivo*, with a particular focus on the role of ESX-1. ESX-1 is known to mediate bacterial escape from phagosomes, modulate cytokine expression in infected cells and drive excessive inflammation, all of which contribute to mycobacterial virulence (12-14, 39-42). While ESX-1 has been extensively studied in macrophages, its effects on other immune cells in infected tissues remain less understood. Here, we identified the primary bacterial reservoirs within infected tissues and explored how ESX-1 contributes to immunopathology.

Key Findings:

- *M. marinum* drives neutrophil accumulation in an ESX-1-dependent manner.
- Neutrophils and $Ly6C^{+}MHCII^{+}$ monocytes are the primary bacterial reservoirs in infected tissues.
- Neutrophils contribute to *M. marinum*-induced pathology.
- ESX-1 promotes a pro-inflammatory transcriptional profile in neutrophils *in vivo*.
- Monocytes are host-protective because of their ability to limit neutrophilic accumulation and immunopathology via an iNOS-dependent mechanism.

Discussion:

Recent studies have identified neutrophils and monocyte-derived cells as the primary reservoirs of *M. tuberculosis* in the lung tissue of infected mice (9, 70, 90), yet the outcomes of bacterial infection in these cell types are still not fully understood. While neutrophil accumulation is linked to bacterial growth in *M. tuberculosis* infection (86), there is no clear evidence for substantial intracellular growth in neutrophils *in vivo*. In contrast to tissue-resident alveolar macrophages, monocyte-derived interstitial macrophages are more restrictive to *M. tuberculosis*

growth due to their metabolic preference for glycolysis over fatty acid oxidation (70).

Here, we extend these observations to *M. marinum* infection, identifying neutrophils and Ly6C^{+}MHCII^{+} monocytes as the predominant bacterial reservoirs. Although *M. marinum* replicates less efficiently in bone marrow-derived neutrophils than in macrophages *in vitro*, neutrophil accumulation enhances bacterial growth *in vivo.* Given that mycobacteria can replicate extracellularly in tissue (147, 148), this suggests that neutrophils may foster a pro-inflammatory microenvironment rich in iron and nutrients that support bacterial replication (86).

In animal models of mycobacterial infection, neutrophils have been shown to play dual roles, with several studies highlighting their contribution to host pathology (86, 87, 90, 92, 93), whereas others demonstrate their involvement in protective immune responses (94-96). Our findings show that ESX-1 promotes neutrophil accumulation and inflammation, thereby exacerbating tissue pathology. In addition to facilitating bacterial survival, neutrophils themselves undergo transcriptional reprogramming in response to ESX-1. Specifically, ESX-1 induces a pro-inflammatory transcriptional signature in bystander neutrophils, suggesting that these cells may amplify inflammation beyond their traditional role in microbial containment. This shift is characterized by an altered cytokine and chemokine profile, along with increased expression of Programmed Death-Ligand 1 (PDL1), a transmembrane protein that binds to its receptor PD1 on T cells to suppress activation (149). PDL1 expression on neutrophils has previously been shown to be upregulated in response to various pathogens, including *Leishmania* and HIV (150, 151), as well as in several cancer types (152, 153), where it is associated with T cell exhaustion and immune evasion. Moreover, we observe a downregulation of haptoglobin, which normally binds free hemoglobin (154), together with an upregulation of Hmox1, an enzyme that degrades heme into free iron (155). This transcriptional pattern may increase iron availability (156), further supporting the hypothesis that neutrophils contribute to the establishment of a microenvironment permissive to mycobacterial growth. Together, these findings imply that neutrophils, under the influence of ESX-1, may not only fuel inflammation but also help shape a local microenvironment favorable to bacterial persistence.

In contrast, CCR2^{+} monocytes have been previously shown to mediate host protection during *M. tuberculosis* infection (79-83), though the mechanisms underlying their protective effects remain unclear. Here, we describe an antagonistic interplay between monocytes and neutrophils, whereby Ly6C^{+}MHCII^{+} monocytes counteract ESX-1-driven neutrophilic recruitment and immunopathology through a iNOS-dependent mechanism. Previous studies have shown that NO inhibits IL-1–mediated neutrophil recruitment in *M. tuberculosis* infection (85, 86), a mechanism that appears to be conserved in our model. However, while the cellular source of iNOS was previously unresolved, we identify Ly6C^{+}MHCII^{+} monocytes as the

primary iNOS-expressing population in infected tissue, thereby providing a mechanism through which monocytes mediate their protective role during infection.

Furthermore, our findings expand the current understanding of ESX-1-mediated virulence by placing neutrophils at the center of immunopathology and positioning monocytes as regulators of inflammation rather than direct controllers of bacterial replication. This functional dichotomy not only advances our understanding of ESX-1-mediated virulence but also underscores the broader relevance of neutrophil–monocyte interactions in shaping infection outcomes. The antagonistic interplay between these cell types may represent a conserved immunoregulatory mechanism, potentially applicable to other infectious or inflammatory diseases beyond mycobacterial infections.

Paper II

***In vivo* regulation of infected and bystander monocyte transcription by the *Mycobacterium marinum* ESX-1 type VII secretion system.**

In this study, we build on the findings from **Paper I** to further investigate the role of monocytes during *M. marinum* infection, with a particular focus on how *M. marinum* and the ESX-1 virulence system influence the transcriptional profile of Ly6C$^+$MHCII$^+$ monocytes. While ESX-1 is known to regulate myeloid cell function *in vitro*, its impact on monocyte transcription during *in vivo* infection remains unclear.

To assess the role of ESX-1, we use both the wild-type *M. marinum* strain and the ΔRD1 mutant, which lacks the entire RD1 locus. In addition, we include the *espK*::Tn mutant, which carries a transposon insertion in *espK*. Previous analyses of infected bone marrow-derived macrophages have shown that while the *espK*::Tn mutant retains its ability to permeabilize phagosomal membranes, translocate to the cytosol, and promote IL-1β production, it fails to trigger type I IFN production, unlike wild-type *M. marinum*. Given that pathogenic mycobacteria are thought to exploit type I IFN responses to promote disease, we considered it important to explore the *espK*::Tn mutant *in vivo*. In this study, we combine these bacterial strains with single cell RNA sequencing (scRNA-seq) to assess how *M. marinum* infection shapes the transcriptional profile of infiltrating Ly6C$^+$MHCII$^+$ monocytes *in vivo*.

Key Findings:

- *M. marinum* infection alters the transcriptional profile of Ly6C$^+$MHCII$^+$ monocytes.
- Infection induces a metabolic shift and significant changes in chemokine/cytokine transcription in Ly6C$^+$MHCII$^+$ monocytes.
- ESX-1, but not EspK, is required for *M. marinum* virulence, bacterial growth, infectivity, and transcriptional alterations in bystander Ly6C$^+$MHCII$^+$ myeloid cells *in vivo*.

Discussion:

We demonstrate that EspK deficiency has little impact on *M. marinum* infection outcomes *in vivo*, as WT and *espK*::Tn bacteria were present in similar numbers in infected tissues, and no major differences were observed in disease pathology, cellular recruitment, or the transcriptional profiles of bystander Ly6C$^+$MHCII$^+$ cells between WT and *espK*::Tn infected mice. Given our previous findings that WT, but not the *espK::Tn* mutant, induces type I IFN production in bone marrow-derived macrophages *in vitro* (42), we were surprised to observe that EspK deficiency has only a limited impact on *M. marinum* virulence and pathology *in vivo*. One possible explanation is that the mechanisms underlying type I IFN induction differ between

infected macrophages *in vitro* and the primary target cells of infection *in vivo*. Further studies are needed to elucidate the ESX-1-dependent and cell type specific pathways that regulate type I IFN responses during infection *in vivo*.

ScRNA-seq on infected and bystander Ly6C$^+$MHCII$^+$ monocytes reveal that *M. marinum* infection alters the transcriptional profile of monocytes. We observed significant differences in chemokine and cytokine expression between infected and bystander monocytes, with bystander cells exhibiting enriched IFNγ-mediated signaling. This suggests that mycobacterial infection may actively suppress IFNγ responsiveness in infected cells. Furthermore, bystander monocytes showed higher transcriptional levels of the CCR2 ligands CCL2 and CCL7, as well as the IFNγ-inducible CXCR3 ligands CXCL9 and CXCL10. These findings indicate that bystander cells may contribute to monocyte recruitment and T cell recruitment and activation – processes known to be mediated by these chemokines (83, 157-159) – and that these functions may be diminished in infected monocytes.

M. marinum infection also induced a metabolic shift from oxidative metabolism to glycolysis, alongside upregulation of nitric oxide synthesis, iron metabolism, and hypoxia-mediated signaling. These infection-driven changes mirror those observed in *M. tuberculosis* infection and have been linked to bacterial growth restriction (70, 72, 160). However, although these factors are associated with a less permissive environment for bacterial growth, further investigation is needed to clarify the functional role of monocytes exhibiting this distinct transcriptional profile. Interestingly, infected cells also increased the expression of TNFα and several metalloproteases, which have been implicated in granuloma formation (161-164). This may indicate that infected monocytes actively contribute to tissue remodeling and granuloma organization.

While several studies have shown that ESX-1 significantly affects the function and transcriptional profile of myeloid cells *in vitro* (39-41, 165), its impact on monocyte transcription during *in vivo* infection remains less explored. A recent study found that infection of mice with either WT *M. tuberculosis* or the BCG vaccine strain induces similar epigenetic and transcriptional changes in alveolar and interstitial lung macrophages (160). Our results suggest that ESX-1 influences the transcriptional profile of bystander Ly6C$^+$MHCII$^+$ cells *in vivo*, although the differences were limited. This indicates that the transcriptional response in infected monocytes may largely be driven by conserved host-pathogen interactions, with strain-specific variations fine-tuning these responses.

Collectively, these findings offer new insights into monocyte heterogeneity during mycobacterial infection and the immune response to *M. marinum*, highlighting how infection alters monocyte function and transcriptional activity. The differential transcriptional programs between infected and bystander Ly6C$^+$MHCII$^+$ monocytes suggest distinct functional roles, potentially reflecting an adaptive host strategy to balance pathogen containment with tissue integrity. However, the partial

suppression of IFNγ responsiveness in infected monocytes raises important questions about pathogen-driven immune evasion. Further dissection of these transcriptional programs and their regulatory networks will be essential to better understand how mycobacteria modulate host immunity and may uncover novel targets for therapeutic intervention.

Paper III

Cytosolic serpins act in a cytoprotective feed-back loop that limits ESX-1-dependent death of *Mycobacterium marinum*-infected macrophages.

Previous studies from our laboratory using *M. marinum*-infected bone-marrow derived macrophages have shown that ESX-1-dependent permeabilization of host membranes, including mitochondrial membranes, leads to the release of host DNA into the cytosol (42, 101). This process subsequently triggers the production of type I IFN production via the cGAS-STING pathway (101). In this study, we demonstrate that the cytosolic clade A3 serpins Serpina3f and Serpina3g are upregulated following ESX-1-driven type I IFN signaling, and we further assess their functional role in mycobacterial infection.

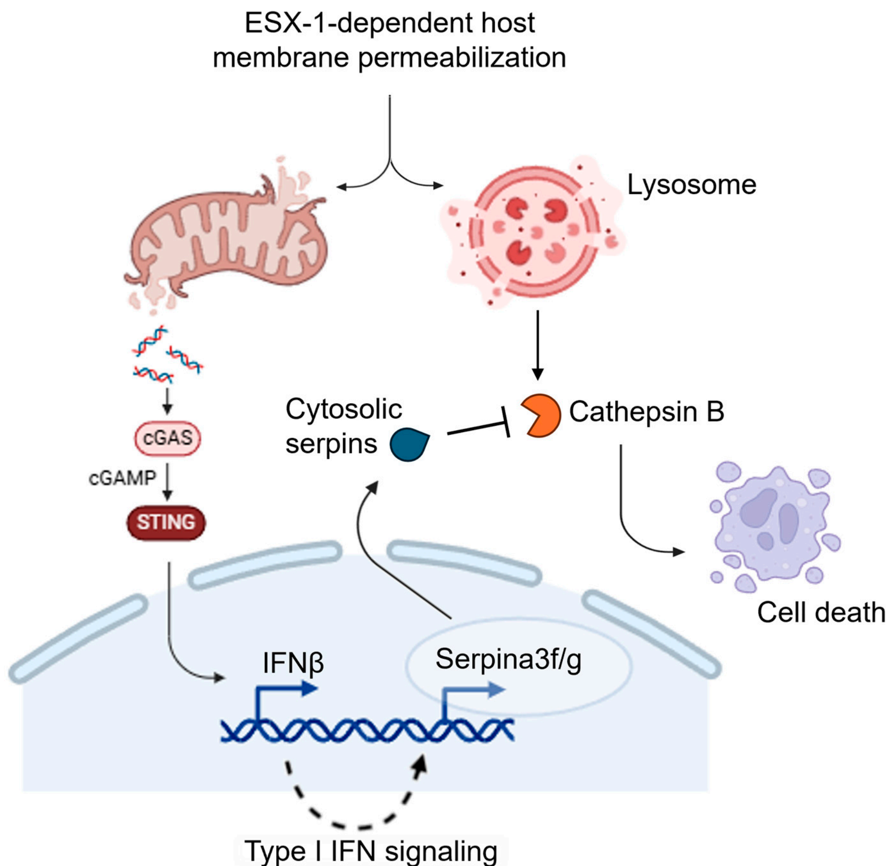

Figure 9. Working model for Paper III.
Adapted from reference (118). Figure created with BioRender.

Key findings:

- ESX-1 induces type I IFN-dependent expression of A3 serpins Serpina3f and Serpina3g.
- Serpina3f/g inhibits the activity of cytosolic cathepsin B.
- Serpina3f/g inhibit ESX-1-dependent host cell death.
- Serpina3f/g promotes *M. marinum* growth *in vivo.*

Discussion:

The detrimental impact of the type I IFN response on mycobacterial infection is well documented (49, 106, 108, 111). While studies have shown that type I IFNs suppress IFNγ and IL-1-mediated protection (41, 109-111, 113), the precise mechanism through which it regulates immune responses and facilitates bacterial growth *in vivo* remains incompletely understood. In this study, we shed light on the mechanistic link between ESX-1-dependent induction of type I IFN production and the upregulation of Serpina3f and Serpina3g, which together form a cytoprotective feedback loop that limits infection-induced cell death. Specifically, ESX-1-dependent permeabilization of lysosomes leads to the release of the cysteine protease cathepsin B into the cytosol, where it has been associated with triggering cell death (166, 167). We observed increased infection-induced cell death in bone marrow-derived macrophages from *Serpina3g*-knockout mice, where cathepsin B inhibition is absent. These findings suggest that cytosolic serpins inhibit cathepsin B in the cytosol and thereby limit infection-induced cell death.

Moreover, our findings suggest that Serpina3f/g promotes *M. marinum* growth *in vivo*, highlighting a potential link between regulated death of infected cells and bacterial proliferation. Research on cell death in *M. tuberculosis* infections has produced conflicting results. Some studies suggest that host cell death can serve as a defense mechanism by eliminating intracellular bacteria and promoting immune activation, thereby restricting bacterial growth (168, 169). In contrast, other studies indicate that certain forms of cell death, particularly necrotic or lytic death, may facilitate bacterial dissemination by releasing viable mycobacteria into surrounding tissues (168, 169). By limiting excessive cell death, Serpina3f/g-mediated cytoprotection may help preserve local tissue integrity and immune cell viability. However, this protective effect appears to come at a cost: by preventing cell death, it may also provide a stable intracellular niche that favors mycobacterial survival and persistence. These findings highlight the delicate trade-off between immune-mediated tissue protection and the risk of sustaining a permissive environment for chronic infection. Therefore, further investigation into how Serpina3f/g-mediated inhibition of cathepsin B modulates infection-induced cell death and supports *M. marinum* growth *in vivo* is needed.

Beyond determining whether a similar type I IFN-driven process occurs in human *M. tuberculosis* infection, it would also be valuable to explore the roles of cytosolic serpins in other infectious settings – particularly those where type I IFN signaling plays a central role in immune regulation. Notably, Serpina3g has been reported to exert host-protective effects during HIV infection and sepsis (170, 171). These findings highlight the broader relevance of protease inhibitors in modulating infection outcomes and underscore the need for further research to evaluate their potential as therapeutic targets across a range of infectious diseases.

Paper IV

A murine *Mycobacterium marinum* infection model for longitudinal analyses of disease development and the inflammatory response.

Understanding mycobacterial virulence and host immune responses *in vivo* is essential for developing effective prevention and treatment strategies. In this chapter, we describe our recently developed techniques for the murine *M. marinum* infection model, which mirror key aspects of human tuberculosis. This model enables localized tail tissue infections and replicates features like formation of necrotizing granulomas and latency-like stages. Importantly, it allows for non-lethal longitudinal studies of disease progression in live animals. The chapter provides detailed protocols for harvesting and preparing infected tissue samples for comprehensive and quantitative analyses of the immune response. Such analyses include flow cytometry, immunofluorescence microscopy, RT-qPCR, ELISA, Western blotting and evaluating bacterial load.

General discussion and future directions

Understanding the dynamic interactions between immune cells and mycobacteria is crucial for uncovering mechanisms of immunopathology and host defense. The studies presented in this thesis contribute to this understanding by utilizing the murine *M. marinum* infection model to dissect the role of the ESX-1 secretion system in immune modulation, bacterial persistence, and disease pathology. Our findings from **Paper I and II** are integrated into the unified working model shown in **Figure 10**.

A central finding of this work is that ESX-1 plays a pivotal role in shaping immune responses by driving neutrophil accumulation and inflammatory differentiation, which leads to exacerbated pathology. While neutrophils have long been considered key responders to mycobacterial infections, their precise contribution to disease progression remains debated. Our findings provide evidence that neutrophils act as inflammatory amplifiers in an ESX-1-dependent manner, fostering an environment that supports bacterial persistence – possibly by releasing proinflammatory cytokines, increasing iron availability, and dampening T cell activation.

In contrast, we identify monocytes as a critical counterbalance to neutrophil-driven pathology, with Ly6C^{+}MHCII^{+} monocytes suppressing neutrophil recruitment through an iNOS-dependent mechanism. Although these monocytes do not influence bacterial burden in the infected tissue, our findings demonstrate a direct immunoregulatory role for monocytes during mycobacterial infection. Notably, we demonstrate that Ly6C^{+}MHCII^{+} monocytes express iNOS at the protein level and *NOS2* (the gene encoding iNOS) at the transcriptional level. While infected cells show a higher frequency of iNOS expression and exhibit greater transcript levels of *NOS2*, bystander cells are more abundant overall and thus represent the largest population of iNOS-expressing monocytes in the tissue. Therefore, bystander monocytes are likely to contribute equally, or potentially even more, to the overall iNOS-mediated function.

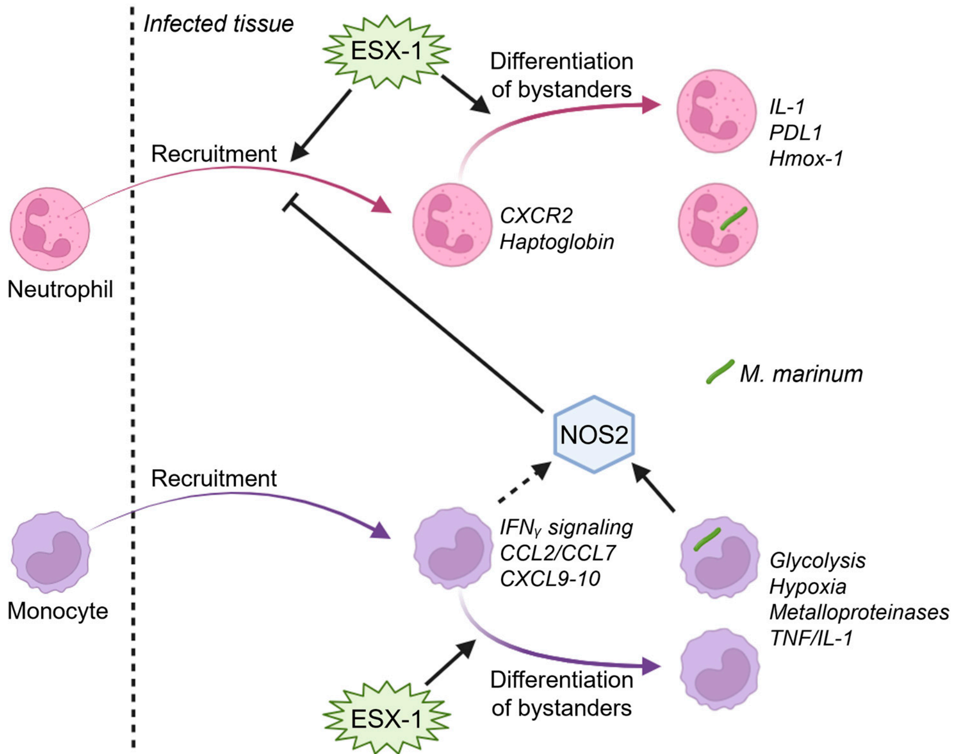

Figure 10. Working model for Paper I and II.
Figure created with BioRender.

The interplay between neutrophils and monocytes appears to be an important determinant of disease outcome in mycobacterial infection. These antagonistic interactions may reflect a finely tuned immune balance, in which excessive neutrophil activity must be restrained to limit tissue damage, while still preserving some level of antimicrobial defense. Our findings suggest that monocytes help modulate neutrophil-driven inflammation, raising the question of whether a regulatory axis between these two cell types is a broader feature of host defense. In this regard, it would be interesting to investigate whether similar monocyte–neutrophil interactions occur in other infections, particularly those characterized by strong neutrophilic inflammation. Understanding the generalizability of this mechanism could uncover conserved immune strategies and identify potential targets for therapeutic modulation in diverse inflammatory settings.

It is well established that IFNγ is a potent inducer of iNOS expression in monocytes and macrophages (133). However, since we detect iNOS/*NOS2* expression as early as 14 days post-infection, the cellular source of IFNγ at this time point remains

unclear. Although we observe an initial accumulation of $CD4^+$ T cells in the infected tissue around this stage, it is possible that innate immune cells, such as NK cells, γδ T cells, or ILC1s, contribute to the early IFNγ pool that primes monocytes. Studies of *M. tuberculosis* infection have shown that NK cells can produce IFNγ even in the absence of T cells, thereby promoting innate resistance and influencing granulocyte responses (172). Identifying the source of IFNγ during early phases of infection, and understanding how it regulates iNOS expression, would provide important mechanistic insight into the control of monocyte function in mycobacterial infection.

Furthermore, our investigation into mycobacterial-mediated transcriptional regulation in monocytes reveals that infection induces marked alterations in the expression of several chemokines and cytokines. Infected cells show reduced expression of genes associated with IFNγ-mediated signaling, monocyte recruitment, and T cell recruitment and activation, suggesting a diminished role in coordinating these immune processes. In contrast, they exhibit a metabolic shift toward glycolysis and upregulation of hypoxia-related pathways and other responses associated with bacterial growth restriction in *M. tuberculosis* infection. The distinct transcriptional profiles of infected and bystander monocytes underscore the complexity of host–pathogen interactions, reflecting both the host's immune strategies and potential immune evasion mechanisms employed by mycobacteria to modulate local immunity. These insights highlight the importance of further research into myeloid cell heterogeneity and functional specialization in tuberculosis pathogenesis.

Interestingly, we find that ESX-1 drives the differentiation of bystander cells, including both neutrophils and monocytes, suggesting that ESX-1 acts extrinsically on these cells. We define bystanders as uninfected cells, identified by the absence of wasabi-expressing bacteria. While this method distinguishes between infected and uninfected cells at the time of analysis, we cannot exclude the possibility that some bystanders were previously exposed to bacteria – either by clearing an infection or by taking up bacterial debris. Nonetheless, we believe that the transcriptional shift observed in bystander cells is driven by the ESX-1-induced inflammatory environment. This finding highlights the broader immunomodulatory capacity of ESX-1, extending beyond infected cells to shape the behavior of surrounding immune populations. Such bystander effects may be crucial in sustaining a permissive niche for bacterial survival, where uninfected immune cells are functionally reprogrammed to support, rather than clear, the infection. However, further studies need to be done to fully understand the functional role of these cells during infection.

Beyond the regulation of innate immune cell recruitment and activation, our work identifies a novel cytoprotective mechanism where cytosolic serpins regulate infection-induced cell death. Although the detrimental effects of type I IFN responses during mycobacterial infection are well established, the exact

mechanisms by which this pathway modulates immunity and promotes bacterial growth *in vivo* remain incompletely understood. Here, we show that ESX-1-driven type I IFN production induces the expression of Serpina3f/g, which inhibits cathepsin B-mediated cell death, providing a potential mechanism by which mycobacteria manipulate host cell survival. Importantly, Serpina3f/g expression correlates with increased *M. marinum* growth *in vivo*, suggesting that this cytoprotective effect may establish a stable niche that favors bacterial persistence. This feedback loop offers new insights into immune evasion in mycobacterial infection and highlights the need to further explore the role of regulated cell death in bacterial dissemination.

While this thesis advances our understanding of ESX-1-mediated immune modulation, several key questions remain. First, the functional consequences of neutrophil recruitment in mycobacterial infections remain unclear, as studies have reported conflicting results. Neutrophils may play dual roles, offering early protective immunity while driving immunopathology in later stages of infection, as observed in this thesis. What mechanisms underlie this potential shift, and how do they influence disease progression? Alternatively, could these discrepancies simply arise from variations in infection models and experimental setups? Second, although we identify monocytes as immune regulators, the long-term effects of their metabolic reprogramming during mycobacterial infection remain unclear. Additionally, the altered cytokine profiles in both infected and bystander monocyte populations raise several intriguing questions for future studies. For example, how do these altered cytokine patterns influence the balance between pro-inflammatory responses and immune regulation in the context of chronic infection? Could targeting these cytokine pathways offer therapeutic benefits in controlling mycobacterial infection and promoting immune resolution? Future studies could also explore how these transcriptional changes affect adaptive immunity and memory responses. Another intriguing avenue for future research is the role of serpins in tuberculosis immunity. Since Serpina3f/g expression is driven by type I IFN signaling, it will be important to determine whether similar regulatory pathways exist in human tuberculosis, and whether modulating this axis could provide therapeutic benefits.

In conclusion, this thesis provides novel insights into the cellular and molecular mechanisms underlying mycobacterial infections, emphasizing the critical roles of neutrophils, monocytes, and regulatory pathways in shaping disease progression. By expanding our understanding of ESX-1-dependent immunopathology, this work lays the foundation for future studies aimed at unraveling the complexities of host-pathogen interactions in mycobacterial infections.

Discussion of key experimental methods

Bacterial strains

In **Paper I** we used the WT *M. marinum* M-strain along with its isogenic mutant ΔRD1, which lacks the RD1 locus. In **Paper II**, we employed an additional strain, the espK::Tn mutant deficient for the *espK* gene encoded within the RD1 locus. All bacterial strains used in **Paper I and II** were transformed with a plasmid encoding a fluorescent protein, Wasabi, to enable identification of infected cells in vivo though both flow cytometry analysis and immuno-histochemistry. The plasmid also carried the hygromycin resistance gene, providing selective pressure to maintain the plasmid. Although we did not administrate hygromycin *in vivo*, concerns about plasmid curing were addressed. Analysis of plasmid curing in infected mice revealed that it remained intact for at least three weeks post-infection. Thus, these genetic mutant strains allow us to study the impact of ESX-1 and the *espK* gene on virulence and transcriptional profile of cells *in vivo*.

In vivo experiments in mice

Sex-dependent biological differences are well documented across numerous infectious diseases, including tuberculosis (173). Typically, males exhibit higher incidence rates and susceptibility to infectious diseases compared to females, whereas females demonstrate a predisposition toward autoimmune disorders. Although the underlying mechanisms remain incompletely elucidated, these distinctions highlight the importance of considering both sexes in immunological studies. In our research, we initially observed greater susceptibility to *M. marinum* infection in male mice (data not shown), which suggests that sex differences may influence disease progression. However, due to practical considerations such as cost, time, and the focus of our current study, we chose to use only female mice in our *in vivo* experiments. While this approach is valid for the scope of our studies, it remains important to investigate whether similar mechanisms are active in male mice. Future studies should address these differences to enhance the relevance of findings across sexes, ensuring that potential therapeutic targets are applicable to both males and females.

Neutrophil depletion

In **Paper I**, we employed the CCR2-knockout deficient mouse model to study the role of monocytes during infection, as these mice have impaired monocyte trafficking into tissues (78). Since no mouse models currently display a selective deficiency in circulating neutrophils, we utilized a new double antibody-based depletion strategy to reduce neutrophil numbers in both circulating blood and infected tissues. Traditional methods, such as anti-Ly6G or anti-GR1, have limitations in specificity and long-term effectiveness (174). Anti-GR1 targets both neutrophils (Ly6G) and monocytes (Ly6C), leading to undesired depletion of monocytes and temporary neutrophil depletion. Anti-GR1 is a rat IgG2b antibody that induces cell death through complement-mediated membrane-complex attack (174). In contrast, anti-Ly6G, a rat IgG2a antibody, specifically targets neutrophils by mediating Fc-dependent opsonization, which involves the recruitment of macrophages to clear neutrophils from circulation (174). While anti-Ly6G is more specific, it has commonly been associated with lower efficiency than anti-GR1 in depleting neutrophils (174). The new double antibody-based depletion strategy combined anti-Ly6G (rat IgG2a) with a secondary anti-rat antibody (mouse IgG2a, orthologous to rat IgG2b), utilizing the complementary strengths of both antibody isotypes to provide specific, robust, and controlled neutrophil depletion (174). This strategy enhances neutrophil depletion without significantly affecting other cell types and has been shown to be more effective compared to using anti-GR1 alone (174). Although our ethical permit did not allow for daily injections, as outlined in the original protocol, we modified the schedule to administer injections every other day and still observed a 90% reduction in neutrophils in the infected tissue (60).

Flow cytometry

In **Paper I, II and III** we use flow cytometry to identify specific cell types present in the infected tail tissue. By utilizing fluorochrome-conjugated antibodies, we designed gating panels capable of detecting a wide range of hematopoietic cells, including T cells ($CD4^+$ and $CD8^+$ T cells), B cells, conventional DCs, neutrophils, macrophages, and monocytes. While we successfully identified several monocyte and macrophage populations using Ly6C and MHC II markers, distinguishing $Ly6C^-$ MHC II^+ macrophages from $Ly6C^-$ MHC II^+ monocytes during infection proved challenging. The standard approach to differentiate these populations via MERTK or CCR2 expression was effective in uninfected mice, but the infection hindered our ability to separate them despite our efforts with varying antibody concentrations. Nonetheless, given our discovery that $Ly6C^+$ MHC II^+ monocytes were the primary infected cell type and the main producers of iNOS, we prioritized

this population for the scRNA-seq to prevent contamination of the monocyte population with macrophages.

Single cell RNA sequencing

In **Paper I and II**, we conduct extensive bioinformatic analyses on the sc-RNAseq data obtained from bystander and infected neutrophils and monocytes, respectively, isolated from infected tissue. ScRNA-seq data provides unprecedented insight into cellular heterogeneity, allowing for the analysis of gene expression at the individual cell level and characterization of cellular diversity within tissues. While scRNA-seq capture a snapshot in time, we used trajectory-based analyses to predict developmental trajectories and differentiation pathways. Trajectory-based analyses model cellular progression over time, identifying potential lineage relationships and cellular transitions, even in the absence of time-series data. This approach is particularly useful for understanding dynamic processes such as differentiation or activation in response to infection. However, there are limitations to these analyses. For instance, predicting cellular states over time relies on computational models that make assumptions about cellular behaviour, and these models can be influenced by noise in the data or inherent biological complexity. Additionally, trajectory-based analyses may miss transient or rare cellular states, which could be critical for understanding complex processes like immune response. Despite these challenges, the use of trajectory analysis opens new opportunities for understanding the progression of immune responses at a cellular level, providing valuable insights into the differentiation and activation of monocytes during *M. marinum* infection.

ScRNA-seq offers several advantages over bulk RNA-seq, primarily due to its ability to capture gene expression profiles of individual cells within heterogeneous populations. This enables identification of rare cell populations and elucidation of cellular states and transitions that may be obscured in bulk measurements. However, scRNA-seq has limitations such as the requirement for higher sequencing depth and the potentially lower sensitivity for detecting lowly expressed genes compared to bulk RNA-seq. Additionally, the data may be subjected to dropout events, where lowly expressed genes are not detected in some cells.

Another method that would be of interest to use in our mycobacterial infection model is spatial transcriptomics. While scRNA-seq provides valuable insight into gene expression at the individual cell level, it does not provide spatial information. Spatial transcriptomics, on the other hand, allows for the capturing of gene expression data directly from intact tissue sections, preserving the tissue's original physiological context. This technique involves combining high-throughput sequencing with spatially resolved tissue samples, enabling the precise mapping of gene expression onto specific tissue regions. Spatial transcriptomics offer several

possibilities, including the ability to identify how gene expression varies across different areas of infected tissues, such as within granulomas or adjacent to areas of active infection. This technique could reveal cellular interactions and molecular mechanisms that are crucial for disease progression and granuloma formation and maintenance *in vivo*. By understanding the spatial organization of immune responses and bacterial growth within tissue, spatial transcriptomics could provide insights into how cells within the microenvironment communicate and how infection influences tissue remodelling. However, like any technique, spatial transcriptomics comes with challenges, including the need for high-quality tissue preservation and the complexity of integrating spatial and transcriptomic data. Despite these challenges, the potential to study gene expression in its native spatial context would significantly enhance our understanding of the mechanisms underlying *M. marinum* infection and could be a powerful tool for future research.

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